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Coping with energy limitation, social constraints and stress in a colonial breeder, the king penguin (*Aptenodytes patagonicus*)

– Vincent A. Viblanc –

Ajustements aux contraintes énergétiques et sociales chez un reproducteur colonial, le manchot royal (*Aptenodytes patagonicus*)

Discipline : Sciences du Vivant

Spécialité : Physiologie et Biologie des Organismes – Populations – Interactions

Vincent A. VIBLANC

Thèse de Doctorat, soutenue publiquement le 9 Novembre 2011.

Devant le jury composé de:

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“There is no such uncertainty, as a sure thing.”

Robert (Rabbie) Burns, Scottish Poet (1759-1796)

Acknowledgments

They say all good times come to an end.

And so, powerlessly dictated by a rapidly drying out scholarship, it appears to be the case for the present doctoral research. Three years have now past since the endeavour started, and as it is drawing ever so close to an end, it dawns to me that we have weaved but a ridiculously small thread in our understanding of the infinite web of complexity that supports natural processes; a web which biologists endlessly try to decipher but which secrets ultimately keep them marvelling for a lifetime. Nonetheless, I do believe (and I hope you will find) that those past 3 years spent doing research as a Ph.D. candidate, have led to the paving of new avenues for future investigators to step onto, and inevitably as Nature recovers its rights over a freshly trenched road, new roots to stumble upon.

How might one end a 3–year story without recollecting the beginning?

I most certainly cannot, and I hope that you will forgive a momentary digression back into some hazy memories on how all of this started. If all else fails, just flip past the acknowledgments (they are long, be warned)! Mid-November 2008, I took a first unstable step onto the slippery deck of the French research vessel *Le Marion Dufresnes*, slowly emerging from what had been a week spent, sick as a dog (…so much for my Viking ancestry…), sailing the distant oceans of the southern hemisphere. The nausea instantaneously vanished as I first set eyes on a breath-taking sight: the king penguin colony of “La Baie du Marin”, around 22.000 breeding pairs mounting the most incredible chorus I have ever heard. I was in the Crozet Archipelago, lying at some 46° latitude, right in between the howling forties and the roaring fifties. This was my first year as a Ph.D. student, and I was about to embark for a journey that would, like for so many before me, shake the very foundations of my life. As this Ph.D. ends, I will have spent the best part of a year (two 6-months summer field seasons) working amongst those magnificent animals. And for the best part of a year, they will have endured my presence at their side. Walking upon their land, sharing their daily lives and predicaments, witnessing the determination of those animals (I confess, with a touch of anthropomorphism) that endure an incredible battle to breed, year after year, has inevitably brought me to consider the ridiculous meaning of life. Yet, it thrives and beautifully so. I am grateful for what time has been given to me, living amongst such amazing birds. And I am immensely thankful to those animals, for the moments I have spent with the king penguins of “La Baie du Marin” have taught me a great deal indeed… as much about them, as about myself.

And so,

I turn to express my deepest gratitude to the person who made all of this possible. Dr. René Groscolas. Thank you BOSS! (or as he is better known in the TAAFs, though most certainly not to his own knowledge, thank you Néné!). Not only has René been my mentor (and put up with with my skewed behavioural ecologist way of seeing things!), supervising the work presented here every step of the way; he has been a most delightful person to work with, and a great source of knowledge to learn from. I wish to thank him for teaching me rigour in the ways to think about and conduct scientific experiments, back from the drawing board on which to scrap the rationales to the final score of a manuscript. I wish to thank him for our conversations, much about science as about anything else, and for the moments (including those sipping the old, home-made, ‘nippy sweetie’ called Mirabelle) we spent together in the field. His encouragements and open-mindedness throughout those 3–years have made this experience one of the best of my lifetime yet.

Needless to say,

That it is not just the opportunities of life that ultimately matter. Rather, it is how opportunities and deceptions change our perception of life, and it is the capacity we have to learn from others which defines us. Self-made men simply do not exist. Skills and wisdom, beliefs and convictions are forged and baffled by social interactions. It is my belief that the numerous interactions I have had over the years have greatly contributed not only to my better understanding of Nature, but also to a better understanding of myself. In this regards a few special persons have done so much, and I wish to thank them especially.

Steve, one of my most valuable friends and colleague, once said to me: “*It is no longer the long crawl of evolution that is producing extinction. We are accelerating the rate.*

What jobs can we do for better and future generations? Our abilities are only as good as our knowledge. The more we know, the better we may take care". This was a plain and simple answer to the naïve questioning I had about the greater purpose of our research in animal biology. It had recently struck me in how bad a situation our environment really was. Of course people know… for some reason, they always seem to know. But some facts strike harder than others. In my case, I had recently heard of a study which, based on simulations from various climatic scenarios, had predicted the king penguin population of my study site to go extinct within the next 150 years (under the best scenario!). This is but the tip of the iceberg concerning drastic changes in our ecosystems, many of which may be attributed to ever growing man-induced disturbances. As Edward O. Wilson has put it (and many scientists now would agree), we are facing the 6th massive extinction of our planet. So why do we study animals? What is truly our role as scientists? Truth to be told, it is curiosity that drives us. But curiosity without love of our natural heritage, to my sense, is meaningless. All I can now say for sure is that I hope Steve is right. If it is not for our understanding today of what little is left to preserve of our environment, our grandchildren shall not see the world our forefathers have known. We are but the last generation who may yet do something small, and yet that is being highly optimistic – less so realistic. Whatever the stakes, let's try. So thank you Steve, for our plain and simple conversations, for your time, great knowledge, council and sound advice. From socio-biology and ecology, to religion, politics, and karma, it has been just great.

I am grateful to Dr. Stéphane Blanc for having me as a part of his lab and welcoming me within the Adaptive Ecophysiology team throughout the past three years. Thank you Stéphane for your kind support during my stay here, and help with post-doctoral applications. Working amongst distinguished and highly critical scientists has been a stimulating challenge, an honour and a real pleasure.

My dear Dr. Eichhorn, aka Göetzi, thank you mate! I couldn't possibly dream of better an office mate! From hard-core Russian nights at the 7th EOU, to the blaring trumpet symphonies of the BDM colony, our conversations and times together have been both stimulating and fun. Thank you for making time to help me and make good suggestions on this manuscript, even though you were on a tight schedule yourself. It has been great to work with you, and I sure hope it is not the end Mr. Secretary. Andrew Smith and Victorien Valette were two graduate Master students I supervised throughout the course of my PhD, and whose help in analysing data and thinking about papers has been most valuable indeed. They have been great to work with, and I hope they will have found putting up with me as a supervisor was not the worst of experiences they will have encountered. Andrew has continued to pursue his own doctoral research now and (at the time of writing) Victorien is doing some fieldwork on the Ecology of American bison. I hope their current endeavours are at least partly the reflection of a good experience they had doing their Masters' research with René and I.

I am especially grateful to a number of great people at the DEPE (and elsewhere) who have made working in this lab (and those past years) a most stimulating and challenging experience. I hope they will forgive me for not recalling specific anecdotes for them all, but from the grand designing of Napoleonic battles to the fine tasting of Beaujolais wine, from the over-hanging Vosgian cliffs to the Canadian Rockies, and for many talks and help they have provided over various subjects and matters, I would like to express some very special thanks to Drs. François Criscuolo, Yves Handrich, Jean-Patrice Robin, Jean-Yves Georges, Marion Spée, Katia Ballorain, Shirley Raveh, Thierry Raclot, Yan Ropert-Coudert, Akiko Kato, Sylvie Massemin-Challet, Ronald Noë, Odile Petit, Cédric Sueur, Marie Pelé, Valérie Dufour, Bernard Thierry, Armand Jacobs, Cédric Zimmer, Michaël Beaulieu, Céline Le Bohec and Yvon Le Maho. François, Yves, and JP especially have provided me with their good help and encouragements on several occasions. I also wish to thank François for dynamically overseeing (and sometimes managing to find the strength to oversee) our weekly clubbings, and the administrative staff of the DEPE (especially Jacqueline) for their help dealing with the crazy red tape of our world infamous French bureaucracy. Yves and François, as well as Marion Spée and Claire Saraux, also provided most valuable comments on parts of the manuscript. I wish to thank them for their time and good help in these regards. Frederic and Myriam Bertrand provided me with invaluable statistical support in a time of dire crisis. I wish to thank them deeply for their help and friendship. Dr. André Chiaradia welcomed me to his study site in Phillip Island, Australia and, having worked on the second largest penguin species, gave me the opportunity to discover the smallest of them: the little penguin. I wish to thank him for this opportunity and good times on our various meetings. My very special appreciation goes to the Entre'Etudiants team (especially Cottinnou), with whom the many discussions, laughs (and pints of beers) shared, have made those past 3–yrs. the best of my academic years yet. And of course, Nelly, Marion, Oni (aka. Onésime), Jérém', Aurél' and lapinou crétin (aka. Loïc), Benoïïït, JP and Coin-coin for our times together and your great help in the field!

Special people have made this experience unforgettable (I don't want to stop!). Thank you so much Dr. Nicolas Hanuise (aka Dr. H.), Dr. Maryline Le Vaillant and Dr. Marion Spée (YES! You guys are doctors now!) – I needn't add more… you'll know. Plottinou (aka Virgine Plot) added a delightful touch of turtle-ities into our penguin-biased world. Thanks Dzin', you're next! I also wish to express some special thanks to Dark'Thom (aka Thomas Naudin) for our Friday eve' jam sessions and for sharing the vibe when all else failed, to Victor Haumesser & Marion Michel for sharing great moments over those past years and to Maryse & Jean-Luc Saraux for their kind support and encouragements. And thank you Lise, for seeing me so far.

And above all, it is to my family: Susan, Victor, Laura-Jane, Paul, and Claire that I must express my deepest gratitude and love. For without their encouragement and support, in whatever aspects or meanings of life, this work would have not seen the light of day (at least not from my contribution!). They have laid the grounds and foundations of my curiosity, and have been my best mentors in teaching me respect and openness. I hope they will find in these pages but a small reflection of the invaluable lessons I have learned living for the past 27 years with such wonderful people. Esther, Selwyn, my grand-parents, my folk Northern and Southern, Scottish and Asian, a clashing mix of wonderful cultures have seen me through the best and worst of times. Thank you all for your ever-ending love and support.

Claire. I know you recently told me you were not sure of how appropriate it was, to go into personal acknowledgments in a PhD Thesis. Well, I do not know (nor do I care). Truth to be told, you have been my beacon of light in the darkness, and have guided me many a times to safe a shore over the course of those past years. You have helped me times over again and have enabled me to extend well beyond the scope of this thesis proper, by involving me into many exciting projects, scientific and others. You have put up with flaring tempers and sour moods. Though I do not know how you managed, or what it is I do to deserve you, thank you my love. I am so lucky to have you.

Kindly,

A number of internationally leading authorities have accepted to evaluate this work. I am ever so grateful to Professor Dr. Kurt Kotrschal and Dr. Pierre Bize for their good time and work as external examiners on this PhD thesis. I also wish to thank Professor Dr. François Gauer for accepting to evaluate this work and chair the jury – I know his position as Dean keeps him very occupied indeed. Professor Dr. F. Stephen Dobson and Dr. Etienne Challet also accepted my invitation to be members of this jury, and I wish to thank them for their time and input on the manuscript.

This Ph.D. research was conducted under the authority of the Ecole Doctorale des Sciences de la Vie (ED 414), Université de Strasbourg, and I would especially like to thank its director Dr. Catherine Florentz, as well as its secretary Fanny Hummel for being so available to help answer any glitches along the way. In order to conduct this research, I was supported by a 3-year scholarship from the French Ministry of Education, Research and Technology which should be acknowledged. I also wish to thank the CNRS-INEE, the French Polar Institute (Institut Polaire Français, Paul-Emile Victor, IPEV) and the Terre Australes et Antarctiques Françaises (TAAFs) for providing financial and logistic support in the field and in the lab.

So to conclude (finally!),

I will simply re-state the fact that the research presented here would not have seen the light of day were it not for the dedication, hard work and support of all those passionate people, and in this regards, my gratitude towards them is, of course, incommensurable. **But most importantly, this document is the reflection of collaborative work.** It is the product of many fine minds combined, much of whose efforts started long before I came along, and many of who will continue to endeavour in the years to come. I hope, even if only slightly, that I have done them justice here.

And as for my part, I hope it is just the start.

Papers

Papers presented in the Thesis

1. Viera VM, **Viblanc VA**, Filippi-Codaccioni O, Côté S, Groscolas R. 2011. Active territory defence at a low energy cost in a colonial seabird. *Animal Behaviour*, **82**: 69-76, doi: 10.1016/j.anbehav.2011.04.001.
2. **Viblanc VA**, Mathien A, Saraux C, Viera VM, Groscolas R. 2011. It costs to be clean and fit: energetics of comfort behavior in breeding-fasting penguins. *PLoS ONE*, **6(7):** e21110. doi: 10.1371/journal.pone.0021110.
3. **Viblanc VA**, Valette V, Malosse N, Groscolas R. Coping with social stress: heart rate responses to agonistic interactions in king penguins. (*submitted to Behavioral Ecology – in revision*).
4. **Viblanc VA**, Saraux C, Malosse N, Groscolas R. Breeding while fasting in a crowd: physiological and behavioural adjustments in courting and incubating male king penguins. (*manuscript*).
5. **Viblanc VA**, Smith A, Kauffmann M, Gineste B, Groscolas R. Offspring reproductive value influences heart rate responses to predation stress in a colonial seabird, the king penguin. (*manuscript*).

Boxes presented in the Thesis

1. **Viblanc VA**, Kauffmann M, Groscolas R. *In prep*. Experimental manipulation of local colony density does not affect energy expenditure in the king penguin.
2. **Viblanc VA**, Gineste B, Robin JP, Groscolas R. *In prep*. Is breeding in a dense colony associated with chronic social stress?
3. **Viblanc VA**, Gineste B, Zimmer C, Kauffmann M, Robin JP, Groscolas R. *In prep*. Is the adreno-cortical and metabolic response to acute stress modulated according to breeding status in the king penguin?

Further research papers

1. **Viblanc VA**, Arnaud CM, Dobson FS, Murie JO. 2010. Kin selection in Columbian ground squirrels (*Urocitellus columbianus*): littermate kin provide individual fitness benefits. *Proceedings of the Royal Society B – Biological Sciences*, **277:** 989-994. doi: 10.1098/rspb.2009.1960.
2. Dobson FS, **Viblanc VA**, Arnaud CM, Murie JO. 2011. Kin selection in Columbian ground squirrels : direct and indirect fitness benefits. *Molecular Ecology,* **in press** (early-view online). doi:10.1111/j.1365-294X.2011.05218.x.
3. Saraux C, **Viblanc VA**, Hanuise N, Le Maho Y, Le Bohec C. 2011. Effects of individual pre-fledging traits and environmental conditions on return patterns in juvenile king penguins. *PLoS ONE*, **6(6):** e20407. doi:10.1371/journal.pone.0020407.
4. Raveh S, Heg D, **Viblanc VA**, Dobson FS, Coltman DW, Gorrell JC, Balmer A, Neuhaus P. 2011. Male reproductive tactics to increase paternity in the polygynandrous Columbian ground squirrel (*Urocitellus columbianus*). *Behavioural Ecology and Sociobiology*, **65:** 695-706. doi: 10.1007/s00265-010-1071-4.
5. Saraux C, Le Bohec C, Durant JM, **Viblanc VA**, Gauthier-Clerc M, Beaune D, Park Y-H, Yoccoz NG, Stenseth NC, Le Maho Y. 2011. Reliability of flipper-banded penguins as indicators of climate change. *Nature*, **469:** 203-206. doi: 10.1038/nature09630.
6. Le Maho Y, Saraux C, Durant JM, **Viblanc VA**, Gauthier-Clerc M, Yoccoz NG, Stenseth NC, Le Bohec C. 2011. An ethical issue in biodiversity science: The monitoring of penguins with flipper bands. *Comptes Rendus Biologies*, **334:** 378-384. doi: 10.1016/j.crvi.2011.04.004.

Communications

Oral communications of Ph.D. results

1. **Viblanc VA**, Valette V, Saraux C, Malosse N, Kauffmann M, Groscolas R. Aggressiveness and social stress in a colonial seabird: is there an energy cost to group-living? *L'animal dans tous ses sens.* Conference organized by the French Society for Animal Behaviour (SFECA), Tours, France. May 2011.
2. **Viblanc VA**, Smith A, Valette V, Saraux C, Malosse M, Kauffmann M, Gineste B, Groscolas R. Sociality, colony density and stress: effects of social context and breeding status on the behaviour and physiology of fasting penguins. 7th Ecology and Behaviour Meeting. University of Rennes/SERL, Rennes, France. May 2011.
3. **Viblanc VA**, Saraux C, Malosse N, Groscolas R. Energetic and behavioural adjustments to fasting in king penguin. 5th Graduate School Day (ED-day), Collège Doctoral Européen, University of Strasbourg, France. December 2010. *Awarded best oral presentation*.
4. **Viblanc VA**, Saraux C, Malosse N, Groscolas R. Holding the fast: energetic and behavioural adjustments in courting and incubating male king penguins. 1st World Seabird Conference: Seabirds, linking the global oceans. Victoria Island, BC, Canada. September 2010.
5. **Viblanc VA**, Valette V, Malosse N, Groscolas R. Social stress in a colonial seabird: heart rate responses of breeding penguins to their aggressive conspecifics. 7th International Penguin Conference, Boston, MS, USA. August 2010.
6. **Viblanc VA,** Mathien A, Groscolas R. On the energetics of specific behaviours: insights from heart rate and the example of comfort behaviour in breeding king penguins. Invited talk (guest of Dr. A. Chiaradia). Phillip Island Nature Park, Victoria, Australia. December 2009.
7. **Viblanc VA**, Mathien A, Groscolas R. Comfort behaviour incurs substantial energy cost in breeding-fasting king penguins, *Aptenodytes patagonicus*. 7th European Ornithologists' Union conference. Zurich, Switzerland. August 2009.

Poster presentations of Ph.D. results

1. **Viblanc VA**, Valette V, Saraux C, Kauffmann M, Malosse N, Groscolas R. Aggressiveness and social stress in a colonial seabird: is there an energy cost to group-living? 8th European Ornithologists' Union conference. Riga, Latvia. August 2011.
2. Smith A, **Viblanc VA**, Kauffmann M, Gineste B, Groscolas R. Heart rate and behavioural responses to human disturbance in breeding king penguins (*Aptenodytes patagonicus*): does reproductive value or nutritional status matter? *L'animal dans tous ses sens.* Conference organized by the French Society for Animal Behaviour (SFECA), Tours, France. May 2011.
3. **Viblanc VA**, Mathien A, Viera VM, Groscolas R. Comfort behaviour in king penguins: what does energy tell us? 5th Graduate School Day (ED-day), Collège Doctoral Européen, University of Strasbourg, France. December 2010.
4. **Viblanc VA**, Saraux C, Malosse N, Groscolas R. Breeding in a crowded place! Physiological and behavioural adjustments to fasting in male king penguins. 7th International Penguin Conference, Boston, U.S.A. August 2010.
5. **Viblanc VA**, Mathien A, Viera VM, Groscolas R. Comfort behaviour in king penguins: what does energy tell us? 1st World Seabird Conference: Seabirds, linking the global Oceans. Victoria, Canada. September 2010. *Highly commended.*

Further communications

1. ***Invited symposia (delivered by F.S. Dobson).*** Dobson FS, **Viblanc VA**, Coline CM, Murie JO. Comparison of direct and indirect components of fitness for nepotistic philopatry. *Social systems: demographic and genetic issues*. SOCIOR conference, Paimpont, France. October 2010.
2. ***Talk (delivered by C. Saraux).*** Saraux C, Le Bohec C, Durant J, **Viblanc VA**, Gauthier-Clerc M, Beaune D, Park Y-H, Yoccoz N, Stenseth N, Le Maho Y. Using penguins as sentinels of climate change: are banding data reliable. 5th Graduate School Day (ED-day), Collège Doctoral Européen, University of Strasbourg, France. December 2010. *Awarded as the 2nd best oral presentation.*

3. ***Poster.*** Bordier C, Saraux C, Le Bohec C, **Viblanc VA**, Beaugey M, Gachot H, Le Maho Y. Is sex ratio in king penguin populations biased at hatching, or is it a result of post-zygotic mechanisms? 7th Ecology and Behaviour Meeting. University of Rennes/SERL, Rennes, France. May 2011.
4. ***Poster.*** Saraux C, Le Bohec C, **Viblanc VA**, Hanuise N, Le Maho Y. Babies come back! Environmental conditions, individual quality and juvenile survival in king penguins. 5th Doctoral School Day. Strasbourg, France. December 2010. *Awarded.*
5. ***Poster.*** Saraux C, Le Bohec C, Durant J, **Viblanc VA**, Gauthier-Clerc M, Beaune D, Park Y-H, Yoccoz N, Stenseth N, Le Maho Y. Using penguins as sentinels of climate change: are banding data reliable? 1st World Seabird Conference: Seabirds, linking the global Oceans. Victoria, Canada. Septembre 2010.
6. ***Poster*****.** Saraux C, Le Bohec C, **Viblanc VA**, Hanuise N, Le Maho Y. Babies come back! Environmental conditions, individual quality and juvenile survival in king penguins. 7th International Penguin Conference, Boston, U.S.A. August 2010.
7. ***Poster*****. Viblanc VA**, Arnaud CM, Dobson FS, Murie JO. Kin selection and individual fitness in Columbian ground squirrels. BIOSEB – 6th Summer School in Ecology and Biodiversity. Mammal Research Institute, Białowieża, Poland. May 2009.
8. ***Public understanding of science: public talk***. Studies in physiological ecology and animal behaviour. Public Talk. Marion Dufresne Scientific Research Vessel. French Polar Institute & French Austral Territories. March 2010.
9. ***Public understanding of science: public talk*****.** What does heart rate tell us in king penguins? 47th campaign, Possession Island, Crozet Archipelago, French Austral Territories. March 2010.
10. ***Public understanding of science: poster and field work presentation***. Studies on penguin behaviour and physiology. 46th campaign, Possession Island, Crozet Archipelago, French Austral Territories. February 2009.

To Laura-Jane and Esther,

With my utmost love and admiration.

Foreword

This manuscript is divided into six main parts. Following a general introduction on the study of energetics, physiology and animal behaviour set back into a natural context (**part 1**), and an overview of the species under study and the methods used (**part 2**), the thesis is further divided in four complementary sections. Those sections respectively deal with: coping with nutritional constraints (**part 3**), coping with social constraints (**part 4**), and coping with predatory-type related stress constraints (**part 5**). A final section (**part 6**) completes the thesis with a discussion on the results presented, and perspectives open for future research to grasp. In addition, preliminary results are presented in the form of three additional boxes. **Boxes 1 and 2** are concerned with the costs that may be associated with colonial breeding, both in terms of energy expenditure (Box 1) and stress (Box 2). The preliminary results presented in **Box 3** deal with the metabolic and hormonal responses of breeding king penguin to acute predator-like stressors. For each of the studies presented here, excepted for study 2, I am the main author of the manuscripts. My contribution to the work includes rationalizing on hypothesis and predictions prior to data-collection, fieldwork and data collection (two 6-month long field campaigns with the penguins), data analyses, and paper writing. The contribution of my PhD advisor, René Groscolas, has been determinant ever since the initiation of the project, to the final revision of this document. He has provided invaluable insight and work in setting up hypotheses and experimental protocols, and devoted a large amount of his time collecting data and reading over, commenting and participating in the writing of the manuscripts, as well as finding research grants. The main author of study 2 (the energy cost of aggressive behaviours) is Vanessa Viera. In this study, I contributed to the work by re-analysing data, and providing comments/re-writing on various parts of the original manuscript. In studies 4 and 5, the contribution of two Master students I supervised, Victorien Valette and Andrew Smith, should be noted. Jean-Patrice Robin, Benoit Gineste, Marion Kauffmann, Cédric Zimmer and Nelly Malosse all participated in field work at various times over those 3 years, and their substantial contribution to the work presented here should also be acknowledged. Studies 1 and 2 are published in *PLoS ONE* and *Animal Behaviour*, respectively. Studies 3 and 5 are drafted manuscripts to be submitted shortly. Study 4 has been submitted and is currently being revised for *Behavioral Ecology*.

Abbreviations

$\dot{V}O_2$	rate of oxygen consumption
ATP	adenosine-5'-triphosphate
BML	body mass loss
BMR	basal metabolic rate
CORT	corticosterone
DEE	daily energy expenditure
DLW	doubly-labelled water
EE	energy expenditure
FMR	field metabolic rate
GC	glucocorticoids
HR	heart rate
LRS	lifetime reproductive success
MR	metabolic rate
NEFA	non-esterified fatty acids
PI, PII, PIII	fasting phases 1, 2 and 3
rHR	resting heart rate
RIA	radio-immuno assay
RMR	resting metabolic rate
Ta	ambient temperature
Tb	body temperature
TBW	total body water

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PART 1.
A general introduction

I. Energy and the environment: a living creature’s dilemma

“Survival machines began as passive receptacles for the genes, providing little more than walls to protect them from the chemical warfare of their rivals and the ravages of accidental molecular bombardment. In the early days they 'fed' on organic molecules freely available in the soup. This easy life came to an end when the organic food in the soup, which had been slowly built up under the energetic influence of centuries of sunlight, was all used up (…)”.

Richard Dawkins (*The Selfish Gene*, Chapter IV – 1976).

1. Did you say Energy?

In the fourth chapter of his million copy best-seller which devises Darwin's theory of evolution by natural selection (1859) from the viewpoint of the gene, Richard Dawkins (Dawkins 1976) pinpoints the universal currency upon which all life on earth depends: ***Energy***. Energy (and its transfer) underlies the functioning of the multifarious diversity of ecosystems on the planet (Allesina and Bodini 2004, Brown et al. 2004). From biological systems as complex as the biosphere to the smallest living organism, energy is central to all biological processes (Slobodkin 1962, Hall et al. 1992). In order to survive, all living creatures must acquire energy from the environment, process it, and derive it to meet the various metabolic needs of the organism (Brown et al. 2004). From bacteria to mammals, diatoms to trees, the rule is simple: no energy, no life.

Over the course of evolution, maximizing the yield of energy substrates found in specific environments has thus led living beings to develop fascinating refinements in their anatomy, physiology and behaviour. The ultimate aim being survival of the genes, natural selection has endeavoured to maximize organisms' ***efficiency in extracting energy*** from the environment and their ***optimality in expending energy*** to fuel daily routines. Selection has thus operated on animals' shape, size, physiological functions and behaviour, to cope with an ever constraining, energy-fluctuating environment (Schmidt-Nielsen 1979, Schmidt-Nielsen 1984). As an example, let us consider one well known and long-studied phenomenon, i.e. ***the selection of optimal body surface to volume ratios (***$^{S}/_{V}$ ***ratio)***: basic geometry principles indeed have profound implications in terms of energy transfers and species biology (Schmidt-Nielsen 1984).

Animals are open energy systems. They acquire energy from the environment, store some, and use it up, eventually restoring it to their surroundings. An animal's body may thus simplistically be viewed as a volume (characterizing energy needs) delimited by a surface (where exchanges of energy occur). For very small animals that grow primarily in one direction of space (such as flatworms for instance), nutrient and gas exchanges through the integument (the body surface) are usually enough to sustain the body's (small volume) needs. However, as soon as two or three-dimensional growth occurs, the energy needs of the organism do so in a likewise fashion (**Figure 1**). If one considers that there is a strictly proportional relationship between an animal's body mass and its metabolic rate[1], then if plotted against each other on a log-log scale, one would expect the slope of the relationship to be equal to one. Similarly, if one considers metabolism to be proportional to body surface (m^2) instead of body volume (m^3), the slope of the relationship would become $^2/_3$, i.e. a slope of 0.67 (Schmidt-Nielsen 1979). However, when one looks at the actual field metabolic rates of terrestrial vertebrates, the slope lies somewhere in between those extremes (**Figure 1**, Nagy 2005). Metabolic rate is thus not solely dependent on body mass as ***it is also influenced by a size-dependent effect***. Indeed, a low (or high) body surface area to body volume ratio, enables to minimize (or maximize) the body's exposure to the environment, which in turn affects its energetics.

For animals, optimizing $^S/_V$ ratios may then (at least partly) be viewed as an evolutionary adaptation enabling them to modulate energy loss to the environment. If one considers the case of endotherms[2], for which body heat is produced by internal means (see the difference between metabolism for ectotherms and endotherms in the figure below), one easily grasps just how important scaling becomes (Schmidt-Nielsen 1979, Schmidt-Nielsen 1984). Because of their higher $^S/_V$ ratio, small animals loose body heat to the environment much more rapidly than do bigger animals. For them to maintain a more or less constant body temperature, their mass-specific metabolic rate is consequently very high (**Figure 2**). And to ***sustain a high metabolic rate, they require more energy***, and hence more food than larger animals.

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[1] **METABOLIC RATE:** Is the rate at which the organism uses energy to sustain life. Basal metabolic rate is the energy expended by an animal in thermoneutral conditions, at rest and in a post-absorptive state (no digestion processes).

[2] **ENDOTHERMS:** Are animals that produce their own body heat (thermogenesis) by internal means (e.g. by muscular work, mitochondrial uncoupling, futile metabolic cycles). They differ from ectotherms that depend on environmental heat to regulate their body temperature.

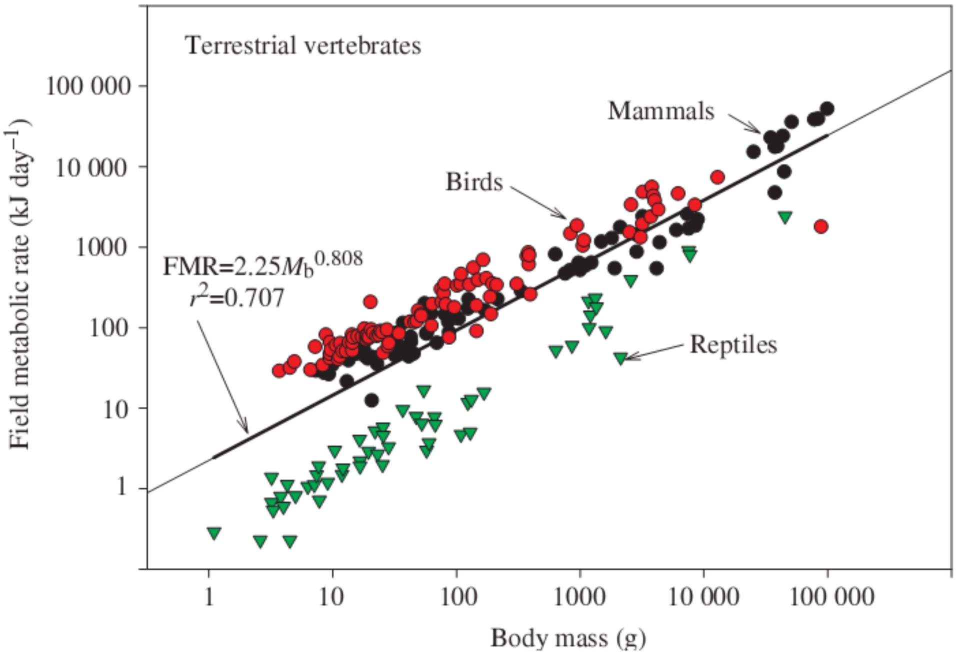

Figure 1. Relationship between field metabolic rate and body mass (log coordinates) in 229 species of terrestrial vertebrates.

The line represents the least-square regression for all the data pooled. The graph shows that greater body mass is associated with overall greater metabolic rate. Note also that for a similar body mass, endotherms (birds, mammals) pay a dearer price (8 to 10 x more) than ectotherms to sustain their metabolism (i.e. the cost of homeothermia) – (*reproduced from Nagy 2005*).

The phenomenon has led to the establishment of general ecogeographical rules[3] (Mayr 1956, Millien et al. 2006). For instance, because of a lower heat loss per unit mass, Bergmann's rule (1847) suggests that species of larger size should be found in colder environments whereas species of smaller size should be found in warmer regions. Whereas reserves should be taken about making this rule a generality (Blackburn et al. 1999), several studies have indeed shown that animal size may vary according to latitude (e.g. Ashton 2002, Blackburn and Hawkins 2004).

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[3] **ECOGEOGRAPHICAL RULES:** ''… are purely empirical generalizations describing parallelism between morphological variation and physiogeographical features.'' (Mayr 1956, p. 105).

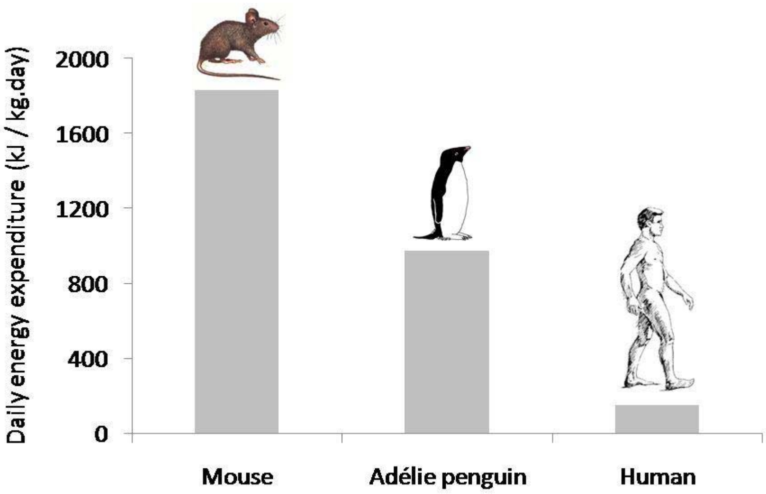

Figure 2. Daily energy expenditure of various endotherms corrected for body mass in (kJ / kg.day).

Note that as opposed to the total energy expenditure of an animal (see figure 1), the mass specific energy expenditure is much higher for small animals than for larger ones – (adapted *from Campbell and Reece 2002*).

In a similar fashion, Allen's rule (1877) suggests that one way to minimize heat loss to the environment is to reduce the surfaces that are exposed to the surroundings. A classical example is that of the morphological diversity of foxes (Klir and Heath 1992). Indeed, in this broadly distributed genus, the relative size of body surfaces exposed to the environment decreases following a South-North latitudinal gradient, from the desert fox (*Vulpes macrotis*), the red fox (*Vulpes vulpes*), to the Arctic fox (*Alopex lagopus*), thus allowing to minimize thermal heat loss the colder the environment gets (Klir and Heath 1992).

The few examples presented above give a good general idea of how questions pertaining to energy exchanges between organisms and their environments and questions relating to energy conservation have shaped animal's physiology, anatomy and behaviour. And evolution has done it so efficiently, that living organisms seem to be able to thrive using energy from the environment in just about any form – witness for instance for the incredible use of chemical energy (chemotrophy) by the abyssal communities of hydrothermal vents (Zierenberg et al. 2000). Most animals however, are ***heterotrophic*[4] *for carbon***: they derive their energy from organic matter they harvest on plants or other animals. By consuming

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[4] **HETEROTROPHS:** Are organisms that cannot fix carbon and rely on organic carbon for synthesis and growth.

readily available organic material (such as carbohydrates, fats and proteins), which is then broken down into simpler fuel compounds (such as glucose, fatty acids, glycerol and amino acids), heterotrophic animals are able to derive most of the energy from the food they consume to growth, maintenance and reproduction. However, because the ***energy they can acquire at any one time from the environment is limited***, choices have to be made about how and when to use up that energy, and besides the fundamental physiological processes required for maintaining life, to which purpose it is better suited. Crucial to an animal's life cycle are thus decisions on how to allocate available energy in order to maximize fitness, and the underlying central concept we must now discuss is that of trade-offs (Stearns 1989, Stearns 1992).

2. Trade-Offs and Bottlenecks

> "To be the best of all possible worlds is not biologically possible; to be well adapted to even one world requires compromise."
> **David Reznick** (1985).

The concept of trade-offs is central to ecology. With the understanding that resources are limited, ***the time and energy invested into one biological function is necessarily expended at the detriment of another*** (Reznick 1985, Roff 1992, Stearns 1989, 1992). Trade-offs in time/energy allocation thus shape many aspects of individuals' decisions, animal behaviour and life-history strategies[5] (Drent and Daan 1980, Van Noordwijk and De Jong 1986, Stearns 1989, Ricklefs and Wikelski 2002). As natural selection ultimately aims at maximizing genetic fitness[6], a major trade-off in life history theory[7] that has kept the attention of evolutionary biologists over many decades, is that ***between current and future reproduction***: the cost of reproduction (Williams 1966, Reznick 1985).

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[5] **LIFE HISTORY STRATEGIES:** Describe the adaptations (behavioural, physiological, and morphological) that control how individuals invest into reproduction, maintenance and survivorship in response to the environment.

[6] **FITNESS:** In evolutionary terms, fitness may be viewed as the adaptive value of a gene or a genotype. Simply, it is the ability of an individuals' set of genes to spread into the population through kin (e.g. siblings and offspring) and into the future (essentially through offspring).

[7] **LIFE HISTORY THEORY:** Provides a framework for understanding how, in the face of trade-offs, organisms should optimally allocate time and energy to traits in a way to ultimately maximize their fitness.

Indeed, increases in individual fitness occur primarily through investments either in traits affecting survival (i.e. the age-schedule of mortality) or in traits pertaining to fertility (i.e. the age-schedule of fertility) (Kaplan and Gangestad 2005). However, fitness increments in either one of those traits are usually associated with fitness decrements in the other, so that an optimal compromise between respective investments should evolve (Williams 1966, Reznick 1985). Hence, during reproduction, parents are faced with compromise, having to invest time and energy both to fuel their own metabolism, and to meet the metabolic requirements of their growing offspring (i.e. parental investment[8], Trivers 1972, Clutton-Brock 1991). When resources are limited (in time, space or global quantities), investments in one of those traits may become detrimental to the other (Clutton-Brock et al. 1983, Speakman 2008). As an example, in relatively small mammals, experimental increases in reproductive demands for the bank vole (*Clethrionomys glareolus*) have been linked to detrimental effects on mother's survival (Koivula et al. 2003). Similarly, in small birds, studies have shown that experimental delays (by clutch removal) in breeding incurred increased energy costs in terms of thermoregulation and overall adult survival costs for great tits (*Parus major*) during subsequent winters (Nilsson and Svensson 1996). In addition, adults with manipulated broods either delayed breeding or laid smaller clutches during the next breeding season (Nilsson and Svensson 1996), further illustrating the fact that potential costs may be paid during future reproductions as well. Facing limited energy stores, the question a breeding parent faces is then: ***should survival be jeopardized to the profit of raising the offspring? Or should reproduction be sacrificed to the benefit of the adult?***

Such decisions partly depend on the life-history traits[9] of the species and on the condition[10] of the individuals under consideration (McNamara and Houston 1996, Promislow and Harvey 1990). In semelparous species, where adults typically reproduce only once during their lifetime and usually die shortly afterwards, investing highly into reproduction seems the obvious choice to maximize fitness. However, in iteroparous species (which might attempt several reproductions over a life time), and especially in long-lived species, there is

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[8] PARENTAL INVESTMENT: Can be defined as any investment by parent (time, energy) into their offspring, which are beneficial to offspring fitness, at a cost to parents' ability to invest into other components of fitness.

[9] LIFE HISTORY TRAITS: May be defined as traits which characterize the life schedule of an animal (e.g. age at first reproduction, number and size of offspring, reproductive lifespan and ageing)

[10] INDIVIDUAL CONDITION: Is best viewed as a combination of traits including body condition (fat reserves, protein reserves), experience in foraging skill, territory quality, parasite loads, immune status, etc. (McNamara & Houston 1996).

room for a ***compromise between the investment into one given reproduction, and the investment into adult survival and future breeding opportunities***. Animals should then balance their current investment in young against chances of future breeding opportunities, and the compromise very much depends on the potential for survival and future breeding prospects itself (Williams 1966). For instance, when further breeding opportunities are limited (as is for instance the case for older individuals or when the species is short-lived) adults are expected to maximize their fitness by investing more into the current reproductive attempt. As an example, a comparative study on seabirds with different life-history strategies (Chastel et al. 1995a) has shown that, whereas in a long-lived species (the blue petrel, *Halobaena caerulea*) massive egg desertion and a high proportion of non-breeders was indeed associated with poor body condition early in the breeding season; shorter-lived seabirds (the thin-billed prion, *Pachyptila belcheri* and the common diving petrel, *Pelecanoides urinatrix*) invested more into the current breeding attempt by maintaining their reproductive output even during years of poor resource availability (Chastel et al. 1995a). In contrast, in long-lived species for which lifetime reproductive success[11] (LRS) is more dependent on adult survival rather than seasonal fecundity (Williams 1966), adults should behave as 'prudent parents' (Drent and Daan 1980) favouring their own survival over reproduction. In line with this, authors have predicted and shown how thresholds in body condition (and energy status) may mediate decisions on whether to breed or not (Chastel et al. 1995b, Vleck and Vleck 2002). In long-lived seabirds for instance, breeding onset has been suggested to be triggered by age and by the achievement of a threshold in body condition during the pre-breeding period (Chastel et al. 1995b, Weimerskirch 1992), whereas breeding abandonment has been linked to the critical depletion of energy stores jeopardizing adult survival (Ancel et al. 1998, Chaurand and Weimerskirch 1994, Gauthier-Clerc et al. 2001, Groscolas et al. 2000), and shown to be mediated by changes in metabolic and hormonal status (Cherel et al. 1994b, Groscolas et al. 2008, Spée et al. 2010).

It thus appears relatively clear that decisions underlying parental investment ultimately link to energy availability (Speakman 2008), and to the trade-off between current and future

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[11] **LRS:** Lifetime reproductive success (LRS) is considered as the total number of viable offspring produced by an individual, which successfully recruit into the breeding population.

breeding prospects. Nonetheless, during periods of ***especially low*** resource availability, which may be termed ***energy bottlenecks*** (where energy intake is minimal or altogether non-existent), animal survival (let alone the compromise between survival and reproduction) is then tributary to previously accumulated energy stores. In breeding individuals, the efficient management of these stores makes up for the difference between success and failure. So what adaptations have been selected for animals to face periods of drastic energy shortage? How do they survive and sometimes breed through periods where energy intake may be close to zero? In the next paragraph, I consider (non-exhaustively) some of the suggested mechanisms – physiological and behavioural – known to date.

3. Dealing with energy scarcity

Managing one's energy capital during periods of energy shortage is a problem many animals commonly have to face during their life cycles. The classical example of hibernation during periods of food scarcity is but one of the long-studied phenomena that have provided us with a good understanding of some of the adaptations animals may use to efficiently manage their limited energy stores (Lyman et al. 1982, Geiser 2004). Obviously if an animal is to survive a period of energy shortage by relying on previously accumulated energy stores, then, that energy capital is to last until the environmental conditions are propitious again. Thus, strategies used to deal with periods of energy shortage mainly aim at ***minimizing both the daily energy expended to fuel basic metabolic needs, and the energy required to come about the activities of its daily routine***.

3.1. Physiological and anatomical strategies

As previously mentioned, for endotherms, maintaining the body temperature at a (more or less) constant level (usually higher than that of the surrounding environment), comes at a significant energy cost. For instance, under natural conditions, the cost of thermoregulation may be as high as to represent 80–90% of an animal's total energy intake, and the metabolic rate of endotherms may be 20 to 30 fold higher than behaviourally thermoregulating ectotherms of similar mass (reviewed in Bennett and Ruben 1979). Thus, during periods of shortage, the energy cost of maintaining homeothermia at an elevated body temperature may become particularly high. This is even more so, as periods of decline in environmental resources are often associated with declines in environmental temperatures (at least in temperate environments) so that thermal (energy) losses of body heat to the environment during those periods are actually increased. For endothermic animals, one adaptive strategy for saving energy, which has widely been reported in mammals and birds, may then consist in ***lowering the energy cost of endothermy***. By temporarily giving up a homeothermic lifestyle, animals may conform to ambient temperature and thus save energy: (1) by cessing to invest energy into physiological mechanisms allowing to maintain a constant (relatively high) body temperature (i.e. shivering and non-shivering thermogenesis[12]), and (2) by slowing metabolic processes as their body cools. The latter occurs because the metabolic rate of an endotherm reflects the metabolic cost of all biochemical reactions occurring within the organism, and thus varies partly as a function of body temperature according to a **Q_{10} effect** (Guppy and Withers 1999, Geiser 2004): for any increase (or decrease) of 10°C, the rate of change of a biological (or chemical) system will vary in an exponential fashion (a typical range of 2 to 3 fold for chemical reactions).

Hence, when endotherms temporarily abandon regulation of their normal body temperature (i.e. resort to heterothermia) when subjected to low ambient temperatures, their metabolic rate (MR) will typically drop below their basal metabolic rate[13] (BMR), and well below what their MR would be if maintained at an elevated internal temperature (see **Figure 3**). As the animal conforms to the temperature of the surrounding environment, heat is lost to the environment and its body temperature will drop as ambient temperature drops. Considering MR, cellular and biochemical (e.g. enzymatic) reactions are such that MR should decrease when body temperature decreases from Tb_1 to Tb_2. The Q_{10} temperature coefficient then may be expressed:

$$Q_{10} = \frac{MR_2}{MR_1}^{10/_{Tb_2 - Tb_1}}$$

where the MR_2 is rate of oxygen consumption ($\dot{V}O_2$) at Tb_2, and MR_1 is $\dot{V}O_2$ at Tb_1. Lowering body temperature thus is synonym of lowering metabolic rate. However, as there is a usually

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[12] **THERMOGENESIS:** Thermogenesis corresponds to the production of metabolic heat. It might occur through muscular contraction (shivering) where ATP is converted into kenetic energy but little movement occurs, causing energy to dissipate as heat. Non-shivering pathways may also produce heat. In this process, the oxidative phosphorylation occurring in the mitochondrion is bypassed; causing energy from the proton motive force to dissipated in form of heat, rather than be used for ATP synthesis (Teulier 2010).

[13] **BASAL METABOLIC RATE:** Is the energy expended by an animal in thermoneutral conditions, at rest and in a post-absorptive state (no digestion processes).

a limit to which body temperature may drop (though some mammals have been reported to decrease their core Tb as low as - 2.9°C!, see Barnes 1989), without physiological function being irreversibly impaired, endotherms will nonetheless devote some energy to metabolic heat production, albeit far lesser energy will be required to reach a much lower set Tb (see **Figure 3**).

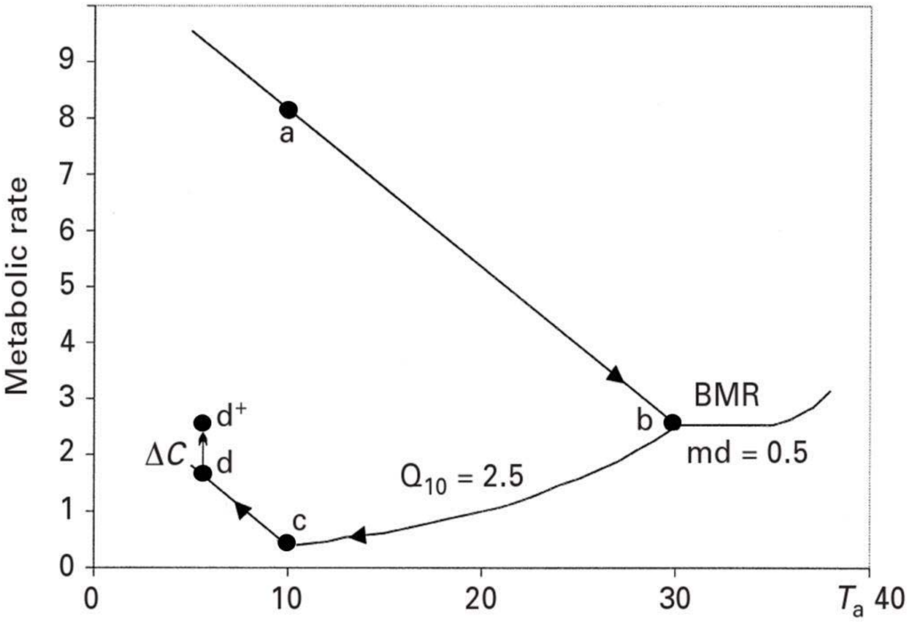

Figure 3. Theoretical model of an endothermic animal lowering its fixed set point of body temperature (Tb).

Endothermic animal can maintain their core body temperature at a high metabolic cost, even when subjected to low environmental temperatures T_a (point a). However, if T_a drops from 30°C to 10°C, some endotherms are able to save energy be resorting temporarily to heterothermia. By abandoning costly metabolic thermoregulation (through shivering and non-shivering thermogenesis), those animals are able to lower their metabolic rate below their basic metabolic rate (BMR) at 30°C (from b to c), and well below what metabolic rate would be if maintained the normal (high) body temperature at 10°C (point a). As a consequence of the animal not regulating its high body temperature anymore, and because heat is lost to the environment, the body cools down to temperatures close to T_a. Based on a Q_{10} effect (see text), the decline in body temperature causes energy expenditure ($\dot{V}O_2$) to decrease in an exponential fashion from b to c. However, because for most animals metabolic function is impaired beyond a certain limit, T_b is regulated around a new lower set point by internal heat production, and endothermia thus maintained at a lesser cost (c to d). If the body conductance changes during the process and is for instance increased, then a further energy cost is paid to compensate for the change (d to d^+) – (*adapted from Guppy and Withers 1999*).

However, for some endotherms that exhibit T_b decreases in the face of energy constraints, it is likely that in addition to temperature effects on metabolism alone, inhibition of physiological function also affects decreases in metabolic rate (Geiser 2004). Indeed, some studies having investigated the Q_{10} of species exhibiting only minor decreases in body temperatures during torpors have reported values far greater than those expected from temperature-dependant effects alone. This is for instance the case of the Eastern pygmy possum (*Cercartetus nanus*) that is able to enter torpor within its thermoneutral zone[14] and substantially depress its metabolism (to approximately 50% of BMR), with only a small decrease in body temperature (*viz.* 2.9°C) (Song et al. 1997, reviewed in Geiser 2004). When calculated, the Q_{10} of this species is of 9.7 (Geiser 2004), which is well above that expected from temperature effects alone (i.e. a Q_{10} typically close to 2). In such species depressing metabolic rate may thus also depend on processes such as the inhibition of enzymatic activity, transcriptional inhibition, or a reduction of body pH (which may lower metabolic processes) (Geiser 2004). Nonetheless, it appears clear that fuelling the necessary machinery to maintain a lower body temperature is less costly than maintaining a high body temperature (especially when heat loss to the environment is high), and that depressing MR through changes in the set point of thermoregulation appears an efficient way of sparing energy (**Figure 4**).

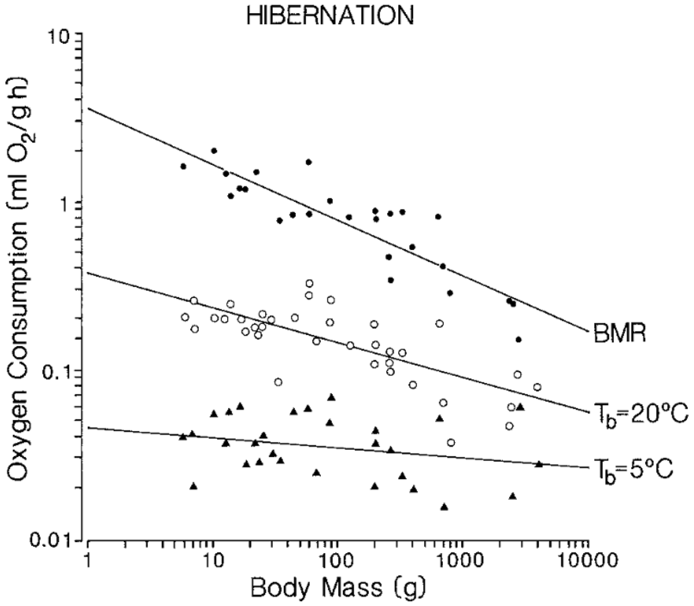

Figure 4. Rate of oxygen consumption for various mammalian hibernators at different body temperatures.

(●) Basal metabolic rate during normothermia, (○) $\dot{V}O_2$ at a body temperature of 20°C, and (▲) at a body temperature of 5°C. Note that a decrease in the set point of body temperature, results in a strong decrease in metabolic rate, well below the normal BMR of the animal at normothermia – (*reproduced from Geiser 1988*).

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[14] **THERMONEUTRAL ZONE (TNZ):** Represents an endotherm's temperature tolerance range. Within the TNZ, heat production is in equilibrium with heat loss to the environment. Endotherms then require little energy to maintain their body temperature at the point of normothermia.

This is well illustrated by a case study on the barnacle goose (*Branta leucopsis*) that probably uses hypothermia as an adaptive mechanism for preserving accumulated energy stores (Butler and Woakes 2001). In this species, starting a few days before the autumn migration and over a period of approximately 28 days, the authors found the body temperature of those animals to drop by 4.4°C. They calculated that the observed decrease would amount to decreasing metabolic rate by 34-39 % (compared to the metabolic rate at the initial body temperature) by the time the lower body temperature is reached. As migrating geese rely mainly on fat stores to overcome their long distance autumnal migration, adaptive hypothermia would allow fat stores to be substantially spared during those periods (Butler and Woakes 2001). Likewise, in king penguins, drops in body temperature have been suggested as a substantial means of energy sparing for instance contributing to explain the prolonged diving bouts of foraging adults (Handrich et al. 1997) and the long-term winter fasts of chicks (Eichhorn et al. 2011).

During periods of energy shortage, animals may also prevent excess energy loss by resorting to morphological changes. For instance, some animals rely on seasonal changes in insulating layers (such as fur or subcutaneous fat) thus minimizing heat loss to the environment. Changes in fur insulation or albedo (e.g. density, thickness, length of hairs, pigmentation) may indeed attenuate conductive heat loss or accentuate radiative inflow, thus allowing energy savings, especially when ambient temperatures are low. In the golden-mantled ground-squirrel (*Spermophilus lateralis*) for instance, an experimental removal of dorsal pelage leads to a strong increase in energy expenditure (Kauffman et al. 2004). Shaved animals increase their food consumption prior to hibernation and exhibit an accelerated rate of body mass loss (reflective of an increased metabolism and the depletion of body fat stores) during the course of hibernation (**Figure 5**). In addition rates of body re-warming during periodic arousals are reduced, and the overall time necessary to complete the arousal process is increased (Kauffman et al. 2004).

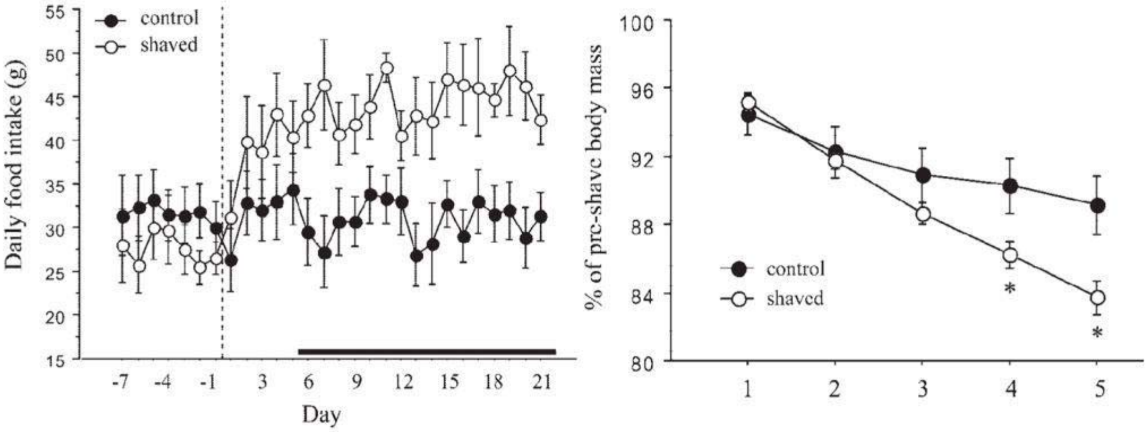

Figure 5. The isolative properties of fur in gold-mantled ground squirrels.

The left-hand panel illustrates the daily food intake (g) of two groups of ground squirrels, one in which squirrels have been stripped of their dorsal pelage. The right-hand panel shows the body mass loss (in % of pre-shaved body mass) during hibernation in control and shaved ground squirrels. Note a strong increase in pre-hibernation daily food consumption to compensate for losses in energy, and significant energy loss during hibernation due to removal of the pelage insulating effect – (*reproduced from Kauffman et al. 2004*).

This example is a good illustration of the energy savings that may be made by isolative morphological adjustments. Other strategies might aim at decreasing the gradient between the body temperature and the ambient air, such as decreasing the body temperature of the body shell whilst maintaining body core temperature high, or at reducing the normothermic core volume of the body. This is for example the case of king penguin chicks, which undergo prolonged periods of food deprivation during the winter period (from late-April to late-October) (Eichhorn et al. 2011). As may be seen on the figure below (**Figure 6**), changes in body temperatures occur both in the body shell and the internal body core, and have been suggested as effective strategies contributing to energy savings in this long-term fasting bird (Eichhorn et al. 2011).

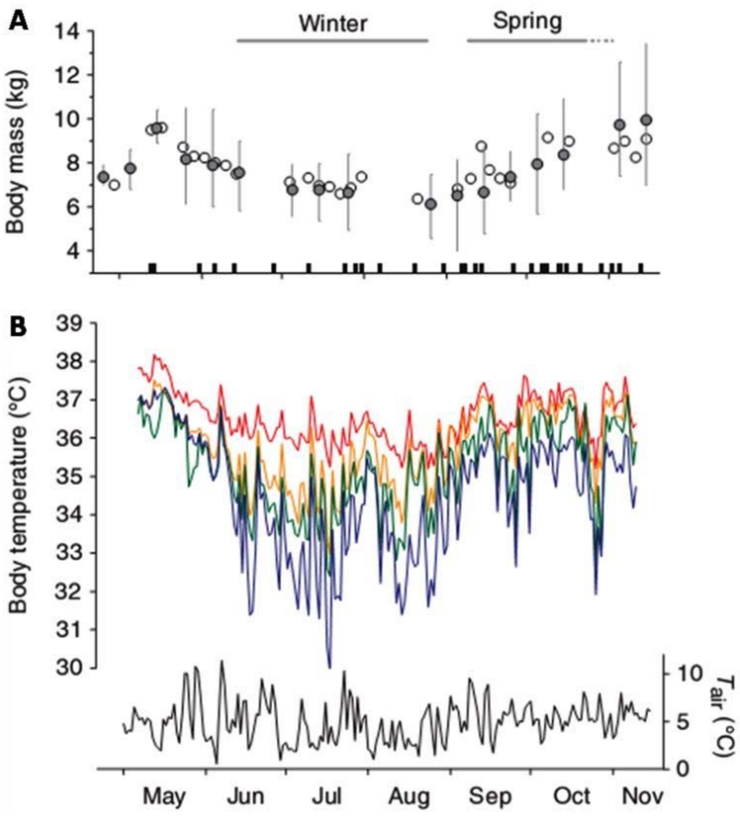

Figure 6. Seasonal changes in body mass and body temperature for fasting king penguin chicks.

(**A)** 10-day body mass means and range are represented for ten chicks. Open circles depict individual body mass for one representative chick for which feeding days are indicated by black marks at the bottom of the panel. **(B)** Daily means of thoracic (red), liver (orange), lower abdomen (green), and subcutaneous (blue) temperatures are presented for that chick. Daily mean air temperature is plotted at the bottom. Note the existence of seasonal hypothermia in all tissues from the outer-shell to the body core. Such changes allow decreasing the thermal gradient between the body and the environment, and reducing the core volume of normothermia, thus enabling substantial energy savings during the long winter fast those animals endure – (*adapted from Eichhorn et al. 2011*).

3.2. Behavioural strategies

In the face of energy shortage, some animals may simply flee from the unpropitious environment to more favourable grounds (e.g. the typical case of migration, Alerstam et al. 2003). Others may live out the unfavourable period, seeking shelters and resorting to behavioural strategies in order to manage their energy expenditure. For instance, some animals may increase their foraging effort in prevision of periods of shortage, and store food-reserves to use at a later date, i.e. caching or hoarding behaviour (e.g. Solheim 1984). As is the case during hibernation or torpor discussed above, others may save energy by reducing the amount of energy necessary to fuel costly physical activity, or by ceasing physical activity altogether.

A noteworthy behavioural strategy allowing animals to save energy is the case of social thermoregulation. Social thermoregulation is especially known to occur in cases where thermal energy loss to the environment is high and where isolated animals would normally be at a negative balance, loosing excessive heat to their surroundings. By huddling with their conspecifics, exchange of body heat and close contact limit the body surfaces exposed to the environment, and allow substantial energy savings (Gilbert et al. 2008, Gilbert et al. 2010, Willis and Brigham 2007). For instance, Willis and Brigham (2007) have shown, that by sharing roosting cavities occupied with other conspecifics, big brown bats (*Eptesicus fuscus*) are able to save as much as 40 kJ.day^{-1} (53% of their daily energy budget) by means of social thermoregulation, compared to the energy that would be expended if roosting alone (see **Figure 7**).

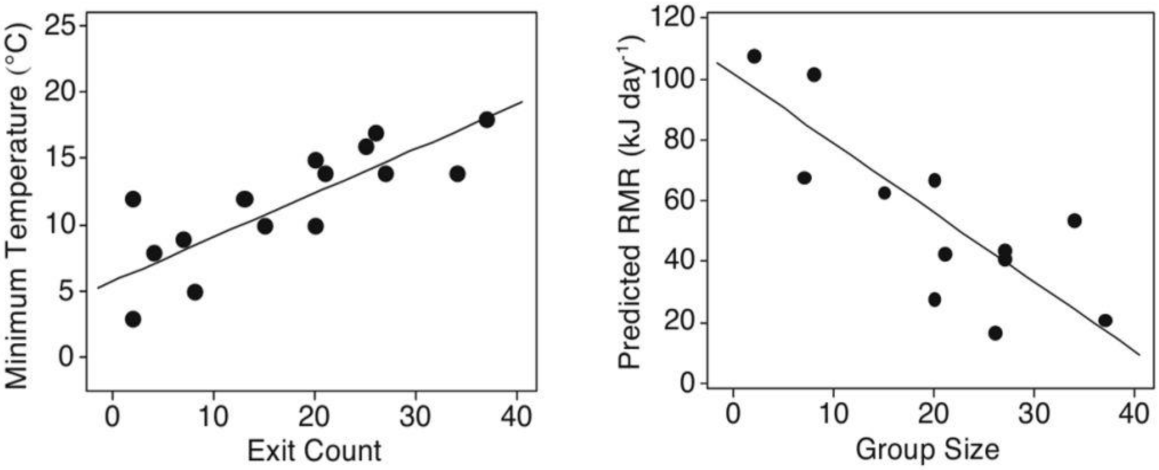

Figure 7. Social thermoregulation in the big brown bat.

The left-hand panel presents the minimum air temperature that may be found inside a roost in function of the number of bats sharing the roost (estimated by exit counts). The right-hand panel shows the estimated energy savings (resting metabolic rate, RMR) that may be achieved by roosting in large numbers – (*reproduced from Willis and Brigham 2007*).

Social thermoregulation is extremely effective. In fact, it is so effective that it is the key that allows the extreme reproductive pattern of the emperor penguin (*Aptenodytes fosteri*), which undergo a 4-month long fasting period, whilst incubating on the Antarctic ice shelf (in the midst of the Antarctic winter), at ambient temperatures averaging -17°C (Gilbert et al.

2008). Yet by huddling close together, male emperors are able to create a microclimate, temperatures during a huddling bout often rising to some 20°C, with some huddling bouts reaching as much as 37.5°C! (Gilbert et al. 2006). Social thermoregulation is thus a behavioural strategy allowing substantial reductions in energy expenditure (as much as 38% in the emperor penguin, though this figure is slightly overestimated, see Gilbert et al. 2008).

3.3. The case of long-term fasting

If behavioural and physiological strategies thus appear the key to dealing with periods of energy shortage, the first question we probably should have addressed is: ***what energy reserves exactly are animals sparing? When and how rapidly are those stores depleted?*** Answering such questions has triggered a substantial body of research over the years, and in this regards, seabirds have enlightened our understanding of the energy substrates that may be used by animals in the face of energy constraints. Seabirds are indeed, professional fasters. They may go unfed for astonishing durations as part of the natural life cycles, ranging from days to months according to species, sex and the type of fasting. For instance, in emperor penguin, males fast for a period of 90-120 days (Isenmann 1971) while resuming the whole incubation period in the midst of the Antarctic weather. King penguin (*Aptenodytes patagonicus*) chicks, are known to forestall their growth during the sub-Antarctic winter, while facing prolonged fasting periods as parents rarely resume foraging trips in the colony at that time (Barrat 1976, Weimerskirch et al. 1992, Descamps et al. 2002). Scientific research has thus thrived in studying how those animals manage their energy stores ashore, and much of our current knowledge on fasting stems from research on penguins (see Le Maho et al. 1979, Le Maho 1984, Groscolas 1982, Groscolas 1984, Groscolas 1986, Robin et al. 1986, Robin et al. 1988, Cherel 1988a, b, c, d, Cherel et al. 1993, Cherel et al. 1994a, b; see Groscolas 1990,Groscolas and Robin 2001 for reviews).

3.3.1. Metabolic changes

During long-term fasting, animals will rely on previously accumulated body energy stores to fuel their metabolism. Those come in three forms: (1) sugars or carbohydrates, i.e. glucids (principally glucose stored with water in the form of glycogen in the liver and muscles), (2) fats, i.e. lipids (which are stored in the even more compact anhydrous form of tryglicerids in the white adipose tissue), and (3) proteins (i.e. muscles, which may be used up in cases of extreme energy depletion). Because ***fats yield more than twice as much energy as carbohydrates and proteins*** (i.e. approximately 9 kcal.g^{-1} *versus* 4 kcal.g^{-1}), they are the ***preferred form of energy storage*** for most animal species (Schmidt-Nielsen 1979, Jenni and Jenni-Eiermann 1998). In addition, because fats are stored without any excess water (as opposed to glycogen or protein storage), more energy may be stored by unit of mass of body tissue reserves (e.g. the energy yield of fat per gram wet mass reaching 8 times that of protein and 8.2-10.3 times that of carbohydrates in birds; Jenni and Jenni-Eiermann 1998). For instance studies have shown how anticipatory storage of fats differs according to the duration of the fast to come (Cherel et al. 1993). In king penguins, chicks prior to the winter fast (the longest fast observed in the species) exhibit higher adiposity (34 %) than pre-molting chicks (22 %) and pre-breeding adults (18 %), underlining the fact that fats are preferred as the principal energy store when fasting is long. Nonetheless, glycogen stores present the advantage of providing readily mobilised energy (whereas the mobilisation of fat is slow) and, most importantly, may provide energy under anaerobic conditions, e.g. during intense muscular exercise. However, during long-term fasting, this reserve is rapidly depleted so that seabirds mainly rely on fat stores accumulated during anticipatory foraging trips at sea to sustain their metabolism while on land (e.g. Groscolas 1990). As an example, only a very small fraction of the total body energy content of male and female emperor penguins is made up of glycogen, i.e. 0.3-0.5% (as opposed to 77-82% of lipids and 17-23% of protein) (Groscolas 1990). Proteins on the other hand, may be broken down to amino-acids, providing substrates for gluconeogenesis. Briefly, long-term fasting may be broken up into 3 distinct phases depending on the sequential utilization of the body stores characterized above:

Phase 1 (PI) is a short period of adaptation during which carbohydrate reserves are rapidly depleted and fats progressively mobilized (Cherel et al. 1988c). It is marked by a strong reduction in protein catabolism, and lasts only a few days, so that fasting seabirds returning from a sea-trip typically arrive at the colony with little glycogen reserves left (Cherel et al. 1994a). The rapid decrease in the specific rate of body mass loss during PI (i.e. the daily mass loss per unit of body mass ${}^{\mathrm{d}m}/_{m\mathrm{d}t}$) is mainly reflective of a decrease in protein catabolism. However, the decrease in energy expenditure which occurs during PI, may not be attributed solely to body mass loss, as one may still observe a clear decrease in energy expenditure even if body mass loss is controlled for (**Figure 8**, Cherel et al. 1988c).

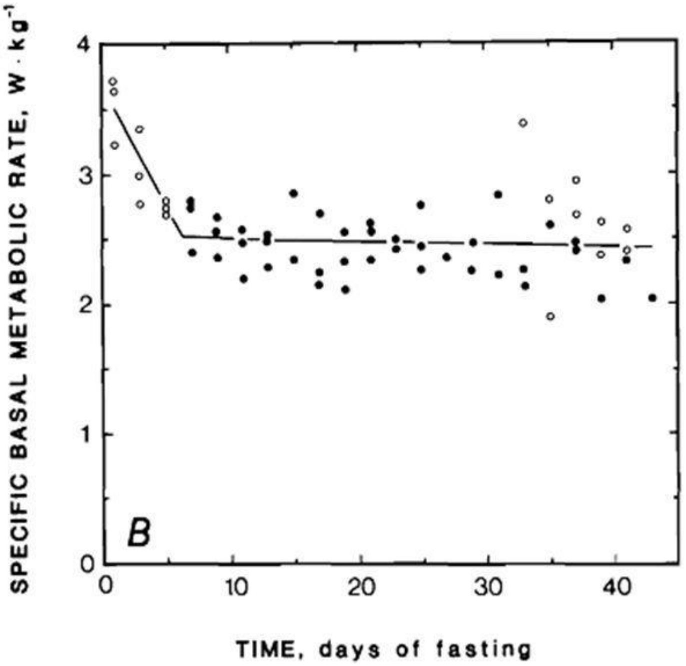

Figure 8. Basal metabolic rate corrected for body mass in fasting penguins.

Basal metabolic rate decreases significantly during phase I (open circles) and remains unchanged thereafter (phase II, filled circles and phase III, open circles) – (*reproduced from Cherel et al. 1988c*).

Phase 2 (PII) is the longest phase of the fast, and a period of protein sparing. During this phase, fats are used as the main energy resource, and the proportion of energy derived from protein maintained at a minimum level. For instance, studies have determined that during the long-term winter fast of the emperor penguin, lipids account for 96% of energy expenditure whereas proteins only provide 4% of the metabolic fuel required (Robin et al. 1988). Mobilization of triglycerides stored in the adipose tissue results in the liberation of free fatty acids (FFAs) and glycerol into the blood stream, which then fuel metabolic needs. FFAs may be directly utilised by tissues or after partial oxidation to ketone bodies (mainly β-hydroxybutyrate, βOHB) in the liver. Fat catabolism is well reflected by the increase in plasmatic concentrations of FFAs and βOHB that occur during PII in fasting birds, whereas plasmatic uric acid (the end product of protein catabolism) concentrations are kept low (**Figure 9**). There is nonetheless a steady contribution of protein catabolism to meet energy demands, which may be explained by the need for glucose by partially or obligatory glycolitic tissues (the brain essentially). Because of the depletion of glycogen stores, production of glucose then occurs in the liver through the neoglucogenesis pathway, from glycerol and amino-acid precursors, which arise from fat and protein catabolism, respectively. The length of PII depends on the adiposity reached prior to the fast (Cherel et al. 1993), and thus determines the efficiency of protein sparing.

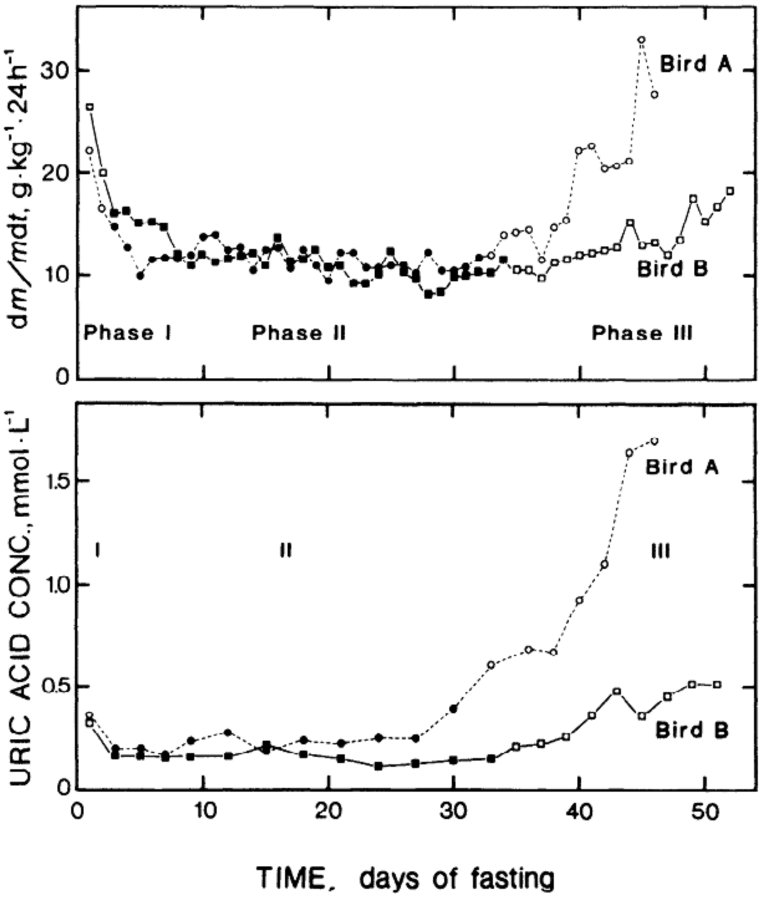

Figure 9. Changes in specific daily body mass loss (d*m*/*m*d*t*) and plasma uric acid concentrations in breeding-fasting king penguin.

Data are presented for two birds. Note that specific daily body mass loss decreases at the onset of fasting, remains low and steady during phase II (when protein is spared), and increases in phase III, as protein is catabolised. Plasmatic uric acid concentrations reflect a low contribution of proteins to energy expenditure in phase II and a sharp increase in phase III – (*reproduced from Cherel et al. 1988a*).

Phase 3 (PIII) is characterized by the attainment of a critical (yet incomplete) exhaustion of body fat stores. During PIII, protein catabolism increases rapidly and its contribution to energy expenditure gradually replaces that of fats (Cherel et al. 1994a). In fasting birds, this is reflected by an increase in plasmatic uric acid concentrations and by an increase in the specific rate of body mass loss, which had remained steady throughout PII (see **Figure 9**). The increase in the specific rate of body mass loss may be attributed to the lower energy content of proteins when compare to fats (16.7 *vs.* 39.7 $kJ.g^{-1}$; Robin et al. 1988), so that for instance, in the emperor penguin, body mass loss would be seven times greater if energy was derived exclusively from proteins, rather than exclusively from fats. In addition the high water content of protein (70% vs. 10% in fats for emperor penguins) lost when proteins are catabolised may further explain the rapid increase in body mass loss (Robin et al. 1988). PIII is reversible provided that re-feeding may occur before body protein or lipids stores are critically depleted (Handrich et al. 1993, Robin et al. 1998, 2001). In seabirds, as most individuals are relieved by their partner from their parental duties ashore before transitioning into phase 3, only a small proportion of birds are usually found at the onset of phase III under natural conditions (Groscolas and Robin 2001). Actually, in fasting seabirds, protein catabolism occurs even though substantial lipid reserves are still available (as much as 2 kg in the emperor penguin, Robin et al. 1988). This suggests that increase protein catabolism is not just a mere consequence of fat store depletion, and a safety margin in lipid stores may provide the energy necessary for seabirds to reach their foraging zones, as they are yet required to travel distances of several hundreds of km after having endured their long-term fast (Groscolas and Robin 2001).

3.3.2. Hormonal changes

Concurrently to changes in the energy substrates used during long-term fasting, hormonal changes, which contribute to the down-regulation of the animal’s metabolism and to the sparing of body protein, also occur. Indeed, circulating levels of insulin, glucagon, corticosterone (CORT) and thyroid hormones (especially T4), might all affect metabolism and the usage of energy substrates. For instance, low levels of corticosterone and insulin during phase II are thought to contribute to the sparing of body protein whereas an increase in the corticosterone/insulin ratio is likely to be a major determinant in increasing protein catabolism in PIII (Cherel et al. 1988a). Insulin (I) is indeed thought to be an anabolic regulator of protein turnover in skeletal muscle and has opposite effects to corticosterone (Goldberg et al. 1980). In addition, glucagon (G), which acts to raise blood glucose levels and is catabolic in nature, rises progressively throughout PII, and rapidly during PIII of the fast.

The corresponding G/I ratio is relatively low during PII, reflective of relatively low gluconeogenesis, whereas this ratio strongly increases during PIII (concomitantly to an increase in uric acid levels), supporting the view of an increase in gluconeogenesis from amino-acids (Cherel et al. 1988a, Le Ninan et al. 1988). Plasmatic concentrations of thyroid hormones (known to act on metabolic rate) have also been shown to decrease during PII, and suggested to be related to changes in basal metabolic rate (Cherel et al. 1988a), whereas they have been found to parallel the activation of protein catabolism in PIII. During severe starvation (i.e. well into PIII) decreases in thyroid hormones may be linked to increases in corticosterone secretion, which is known to inhibit thyroid function in birds (Groscolas and Leloup 1989). In addition, corticosterone concentrations are thought to be hormonal mediators of adult survival, as plasmatic increases in CORT concentrations at the onset of PIII have been found to stimulate locomotor activity and the feeding drive (Challet et al. 1995) and inhibit parental behaviour in association with changes in prolactine in incubating penguins (Groscolas et al. 2008, Spée et al. 2010)

4. Dealing with social constraints

In considering the physiological and behavioural adaptations animals may exhibit in the face of energy constraints, one should bear in mind that many species live in groups, and that at least in some species, times of energy bottlenecks are shared with conspecifics. Although in social species, the benefits of group living have obviously outweighed its costs over the course of evolution, one should not forget that group living nonetheless does entails costs, potentially amongst which are energy costs.

4.1. The evolution of group living

> “Social evolution is the outcome of the genetic response of populations to ecological pressure within the constraints imposed by phylogenetic inertia.”
> **Edward O. Wilson** (*Sociobiology – The New Synthesis, 1975*).

The prime determinants of social evolution may be divided in ***ecological pressures*** and ***phylogenetic inertia*** (Wilson 1975). In simple words, the evolution of social behaviour and group living may be viewed as driven by various ecological features which make it a better option for animals to aggregate, and constrained by the phylogenetic inertia of a species, i.e. its resistance to evolutionary change. Classically, the ecological pressures that have been proposed as drivers of social evolution are: defence against predators, increased competitive ability, increased foraging efficiency, increased reproductive efficiency, and increased offspring survival (all reviewed in Wilson 1975). Further drivers of sociality include penetration of new adaptive zones (i.e. sociality allows to conquer a new habitat or enter a new way of life, which would have not been possible otherwise), improved population stability (i.e. social behaviour allows to regulate population densities to a consistent, predictable level, thus avoiding risks of population extinction), and modification of the environment (i.e. sociality allows environmental changes which benefit the individual).

As an example, let us consider the case of predation: aggregating into large groups of conspecifics is indeed an effective way to reduce predation pressure on any given individual. This may be achieved through the combined effects of dilution (one individual is lost amongst a crowd), shared vigilance (which also allows individuals to invest more time in otherwise seemingly incompatible activities such as foraging), selfish-herding (i.e. one individual benefits from the shielding protection of others), and cooperation (such as mobbing or shared information). For instance, group-living individuals may be able to communicate specific information about types of predators and associated risks, and may then adapt their behaviour accordingly (Zuberbühler 2000, Zuberbühler 2001, Templeton et al. 2005). This is notably the case of Campbell's monkeys (*Cercopithecus campbelli*), which are able to communicate information on predator category (i.e. hawk eagles or leopards) to other conspecifics, thus lowering rates of predation (Zuberbüler 2001). Communication of information on predator presence and predation risk is not as obviously acquired by solitary species, unless directly subjected to the predator, asides perhaps in the case of eavesdropping (e.g. Ito and Mori 2010).

Obviously, whenever genetic benefits arise from living in groups of conspecifics, evolutionary pressures should favour sociality (Hamilton 1964a, b, Alexander 1974, Wilson 1975, Baglione et al. 2002, Viblanc et al. 2010, Dobson et al. 2011). However, sociality also comes with costs. Individual needs often conflict with conspecific needs, so that intra-specific competition is frequent. The classical example is that of intra-sexual competition for the best mating partners, which may entail the proximate costs of (sometimes lethal) physical injuries (Clutton-Brock et al. 1979, Clutton-Brock 1989). Another example of potential costs comes in the form of sharing. Whereas group living may favour the efficient localisation and acquisition of environmental food resources: the larger the group, the smaller the share, so that intra-specific competition for food may arise (Clutton-Brock and Albon 1985, Kotrschal et al. 1993). Similarly, compromises have to be reached when groups move as a collective unit (Fischhoff et al. 2007, Sueur and Petit 2008). Indeed, to benefit from group living, members should stay cohesive. However, not all individuals exhibit similar needs at the same time, hence the dilemma. Group movements may then depend on the physiological need of specific group members at specific times. In zebras for instance, lactating females (for which the access to suitable water holes is essential) appear to have a central role in leadership (Fischhoff et al. 2007). In addition, group living may incur direct physiological costs. Social stimuli are known to be potent stressors amongst group-living animals, and the presence and number of conspecifics may have dramatic effects on physiological systems. For instance, status within a social group has been shown to affect stress hormone levels and health (Kotrschal et al. 1998, Creel 2001, Goymann and Wingfield 2004, Sapolsky 2004). In greylag geese (*Anser anser*), Kotrschal and colleagues (1998) have shown that during the breeding season low-ranking ganders had higher levels of faecal corticosterone (CORT; the main stress hormone in birds) than high-ranking ganders, probably reflective of sexual conflict and lower access to females, because of their rank. However, the authors also show that high-ranking ganders display higher CORT concentrations than low-ranking birds after the breeding season, possibly as a result of parental defence and commitment to the brood, underlining the fact that the relationships between social interactions and status on one hand, and physiological correlates of stress on the other, are far from simple (Kotrschal et al. 1998). In addition, group living may entail significant energy costs. Cao and Dornhaus (2008) recently showed that in the colonial ant *Temnothorax rugatulus*, living at high densities incurred potential metabolic costs in terms of energy expenditure. By experimentally manipulating the social space available to ants within their colony (by reducing it by 50% in experimental colonies compared to the control situation), these authors show that ants under crowded conditions experience a 14.2% increase in metabolic rate compared to their non-crowded peers, which is probably due to altered worker behaviour (increased social interactions) and increased colonial activity (Cao and Dornhaus 2008). Whether changes in metabolic rate may be affected by social variables other than those pertaining to physical activity (e.g. social stress for instance), is an intriguing question that I shall later discuss, and that has yet to be properly addressed. Indeed, one limit to the evolution of group living might also be set by its cost in terms of energy and stress.

4.2. The case of colonial breeders

Colonial breeding[15] is interesting from an evolutionary stand, as individuals which aggregate in large densities apparently pay strong fitness costs (e.g. transmission of parasites and diseases, cuckoldry, increased competition for food and mates, infanticide) in order to breed (reviewed in Danchin and Wagner 1997). Whereas traditional explanations (such as lack of favourable breeding sites, increased protection from predators, information on foraging sites, etc.), have been suggested as drivers in the evolution of coloniality, more recent work has proposed that coloniality might arise though commodity selection (Danchin and Wagner 1997, Wagner et al. 2000): animals may choose their breeding habitat using cues from successful conspecifics. Danchin and Wagner thus argue that rather than being driven by the direct pressures of the *pros* and *cons* of group-living, colonies could evolve as by-products of habitat selection, whereby breeding animals would choose favourable breeding sites imitating their successful peers (Danchin and Wagner 1997). Nonetheless, if proximal mechanisms such as habitat selection may be sufficient in driving the evolution of coloniality, benefits and costs still arise from aggregating closely with conspecifics.

In seabirds, some of the noteworthy costs of colonial breeding are those associated with territorial defence. Let alone some exceptions (as that of the cooperatively breeding emperor penguin), breeding adults typically defend small territories that are essential for reproductive success. Rates of aggressiveness may be high (e.g. a mean of 100 aggressive interactions per hour in the colonial king penguin, Côté 2000), and associated risks comprise both physical injuries to the adults and chicks, as well as potential breeding failure in cases where adults may not withstand social harassment by neighbours. Whether territoriality in

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[15] **COLONIAL BREEDING:** Consists in the aggregation of densely distributed territories with no resource other than breeding sites (Danchin & Wagner 1997)

breeding seabirds may be associated with significant costs (other than physical injuries) in terms of energy or social stress are questions that will be considered in the present thesis.

5. Dealing with stress

"In times of stress, be bold and valiant".
Horace (Roman Poet, 65 BC- 8 BC).

5.1. What is stress?

The concept of stress is clearly not new to mankind. Yet defining what stress exactly is has been a challenge that has led scientific literature to use the term in a surprisingly inconsistent fashion. Romero and colleagues (2009) point out that part of the problem is that usage of the term "stress" often refers to three different, yet related concepts: (1) the stimuli (i.e. the stressor, internal or external) that cause stress to the organism, (2) the physiological, behavioural (and psychological) responses (i.e. the stress response) affected by those stimuli, and (3) the physiological consequences (sometimes pathological) associated with long-term overstimulation of emergency responses (Romero 2004, Romero et al. 2009).

Stressors may be defined as circumstances that threaten an individual's physical integrity or psychological well-being (Kemeny 2003). In response, a cascade of physiological and behavioural adaptations (the stress response) enables the organism to cope with the impending challenge it faces. Physiological systems that allow coping with the stressor are mobilised, whereas physiological systems that are not needed are concurrently inhibited. The neural and hormonal changes that occur during acute stress are summarized in **Figure 10**.

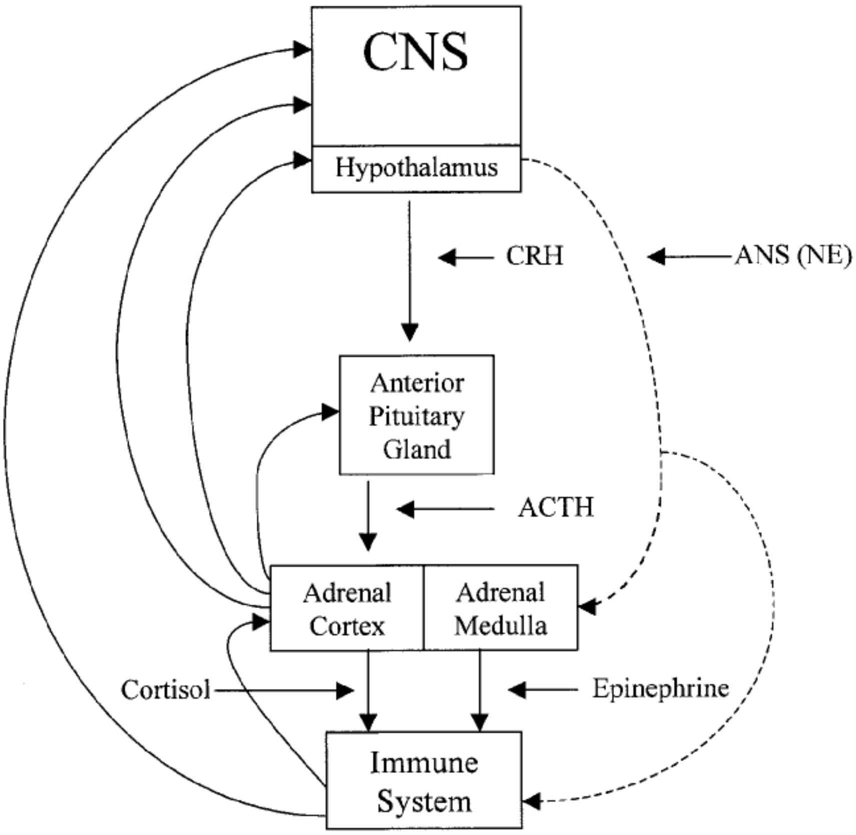

Figure 10. Schematic of the interrelationships between the central nervous system (CNS), the HPA-axis (hypothalamic-pituitary-adrenal axis), the autonomic nervous system (ANS) and the immune system.

Hormonal pathways are represented by solid lines and ANS neural pathways by dashed lines. CRH stands for corticotrophin-releasing hormone, NE for norepinephrine and ACTH for adrenocorticotropic hormone – (*reproduced from Kemeny 2003*).

Briefly, in response to a stressor, catecholamines (epinephrine) are released from the adrenal medulla under sympathetic activation of the autonomic nervous system (which releases the neurotransmitter norepinephrine at the adrenal medulla). This first rapid wave of the stress response (activated within seconds) and the adrenaline rush that then occurs causes the typical “fight-or-flight” response by causing heart rate to increase, air passages to dilate (by relaxation of the muscles), vigilance to be enhanced, and energy substrates to be mobilized (by promoting glycogenolysis in the liver and muscle, glycolysis in the muscle and lipolysis in the adipose tissue). This first rapid wave is later sustained by the activation of the hypothalamic-pituitary-adrenal (HPA) axis (within minutes rather than seconds) that results in the release of glucocorticoids (GCs) by the adrenal cortex. The main GC in birds (and in many rodents) is corticosterone (CORT) whereas it is cortisol in most other mammalian species. GCs and catecholamines act in a number of ways to deal with stressors by mobilizing energy substrates for instance, facilitating the movement of immune cells to fight an infection (Dhabhar and McEwen 1999), or acting in the brain to promote the formation of memories of events which should be considered as dangerous in the future (Roozendaal 2000, reviewed in McEwen and Wingfield 2003).

5.2. Acute and chronic effects: is stress always adaptive?

When facing an unpredictable environmental challenge, animal organisms respond by mounting a stress response. The physiological and behavioural modifications that then occur are directed at dealing with the threat, ultimately aiming at enhancing survival. However, stress mediators (namely GCs and cathecolamines) may also have adverse effects over the long run ultimately affecting health and fitness (McEwen 2000). In this regards, exposure to chronic stressors (and thus chronic stimulation of stress pathways) is known to induce dysfunctions in a number of physiological systems, increasing risk factors for cardio-vascular disease and depressing immune function, amongst others. For instance, Dhabhar (2000) has shown that whereas immune function was actually enhanced by acute stress (i.e. acute injections of low doses of CORT in rats); exposure to chronic stress (i.e. chronic injections of moderate doses of CORT) had suppressive effects on skin immune function. In addition, physiological changes in response to chronic stressors may be long-lasting, even affecting subsequent generations. For instance, in free-living European starlings (*Sturnus vulgaris*), Cyr and Romero (2007) found that chronic exposure to stressors both reduced reproductive success of adults and caused surviving nestling to sensitize to acute stressors 16-days post-hatch (Cyr and Romero 2007). In rats, chronic exposure to prenatal stress has been linked to learning deficits associated with neurogenesis inhibition in the hippocampus (Lemaire et al. 2000). Recent studies have also linked stress to ageing. For instance, Epel and colleagues (2004) have recently shown that perceived chronic stress was positively associated with higher oxidative stress, lower telomerase activity and shorter telomere length, all of which have been suggested prime determinants in the cellular process of ageing and survival (Finkel and Holbrook 2000). It thus appears clear that whereas physiological stress mediators have positive adaptive effects on the short-term, they also incur substantial costs in the long run.

Furthermore, even when subjected to acute stress, mounting a stress response may not always be adaptive. Actually, when fitness may benefit from not responding to acute stress, stress pathways may be inhibited (Wingfield and Sapolsky 2003). For instance, this has recently been suggested in accordance with the aforementioned trade-off between survival and reproduction (see chapter 2). Indeed, whereas mounting an elevated stress response may promote survival, it does so potentially at the cost of reproduction. Thus, when future breeding prospects are low and when the relative value of the current breeding attempt is high, stress pathways may be down-regulated (Bókony et al. 2009, Goutte et al. 2011).

5.3. Cognitive appraisal processes, psychological states and emotions

Challenging situations have long been thought of as eliciting general uniform physiological stress responses, whatever the nature of the stressor. However, it is now coming to light that stress responses may not be as uniform in nature as once thought. Several studies have indeed suggested how psychological assessments of specific contexts and cognitive appraisal processes (i.e. the way an individual perceives a situation in terms of well-being, Kemeny 2003) may be involved in shaping stress responses to a given situation. For instance, rats subjected to electric shocks have been shown to display lower levels of circulating GCs when they are given controllability over the noxious stimulus (Weiss 1970). In humans, studies have also shown that HPA activation is more likely to occur in laboratory conditions when those are subjected to uncontrollable stressors rather than controllable stimuli (Dickerson and Kemeny 2004). Similarly, predictability of negative stimuli has been shown to lower cardiac and startle stress responses in sheep (Greiveldinger et al. 2007). Studies have further shown that the specificity (familiarity) and suddenness of stressors affected cardiac and behavioural responses in lambs (Desire et al. 2006). Of interest is the fact that the influence of specific contexts on stress responses is not restricted to noxious stimuli. For instance, rats that have been habituated to activate a lever for 10 times in order to receive a food reward see their GCs concentrations increase if the number of lever-presses necessary to receive the reward is increased (Levine et al. 1972). In free-living animals, several studies have also reported how the evaluation of specific social contexts might influence physiological responses to stress. For instance Wascher and colleagues have shown how the evaluation of specific social contexts elicited differential heart rate responses in free-living greylag geese (Wascher et al. 2008a, Wascher et al. 2008b). Indeed, geese observing (but not participating in) aggressive interactions between conspecifics displayed higher heart rate responses when one of the participants was either the pair partner or a family member (Wascher et al. 2008b), than when those aggressions only concerned non-affiliated individuals. Similarly heart rate responses were higher when the observed individuals out-ranked the observer (Wascher et al. 2008b). In another study, the authors found that geese actively participating in aggressive interactions displayed higher heart rates both when they initiated rather than responded to a fight, and when they were confronted to an opponent winning a higher proportion of interaction than themselves (Wascher et al. 2009). It thus appears that an emotional component may play a role in modulating the physiological stress responses, and the psychological assessment of specific contexts (social stressors or others) should thus be considered in studies pertaining to stress (Kemeny 2003).

6. Motivation and scope of the thesis

This thesis investigates several aspects of the physiological and behavioural strategies that may be used by animal organisms in the face of energy constraints. Its aim is to contribute to our understanding of how, in free-living animals, the environment (*largo sensu*: social, physical, historical) may shape behaviours and physiology as to optimally manage the energy available at any given time, ultimately maximizing genetic fitness. By specifically focusing on the case study of the king penguin, a colonial breeder for which breeding is associated with periods of energy limitations (i.e. long-term fasting), the objectives addressed by the present work aim at describing the behavioural and physiological adaptations which allow king penguins to breed while facing different environmental constraints.

6.1. Dealing with nutritional constraints

First, the breeding energetics of free-living king penguin will be considered. In an attempt to answer how those professional fasters manage their time and energy budget when breeding under natural energy constraints, a first part of our studies investigates the potential trade-off between activities which may appear essential for a colonial breeder (*viz.* aggressive and self-maintenance behaviours, detailed in the following chapter) and nutritional (energy) limitations. The questions of how (whether), and at which cost those behaviours may be maintained under natural fasting conditions is thus examined. A second part of our studies considers changes in the time-course of energy expenditure in free-living birds during the longest breeding fast (i.e. the fast experienced by males throughout courtship and the first incubation shift, as detailed hereafter). Whether changes in energy expenditure, body temperature and physical activity occur in order to sustain the long-term fast is investigated. Specifically, our questions are:

- How is energy invested into physical activity during long-term fasting? Is overall physical activity maintained at a minimum level? Are the major behavioural activitites observed in breeding penguins an actual energy constraint or are they maintained during long term fasting because of their minimal energy cost?
- How is energy expenditure modulated in free-living breeders during the longest fasts? Might incubating birds as large as the penguins rely on adaptive heterothermia for substantial energy savings while breeding?

6.2. Dealing with social constraints

Second, whether the colonial environment may have an impact on the physiology of fasting birds is then examined. As previously mentioned, the social environment may have profound effects on animals' physiology, and a colonial setting may strongly influence the physiology of breeding birds. Thus, we question whether king penguins which are breeding in a crowded colonial environment, are sensitive to their social environment, and if so, whether the social environment may constitute a potential cost in terms of energy and stress.

6.3. Dealing with stress and predators

Finally, the effects of acute (predator-like) stressors on the physiology of breeding birds are examined. The stress response allows an animal to rapidly mobilize its energy substrates to adequately deal with the incoming threat. Considering nutritional limitations and the life-history trade-off between reproduction and survival, this part of the thesis investigates whether stress responses to acute stressors during reproduction may be affected by either of those factors (i.e. fasting duration and breeding status).

II. The king penguin (*Aptenodytes patagonicus*)

1. Life history of a flightless bird

Of the 18 known penguin species (**Figure 11**), the king penguin (*Aptenodytes patagonicus*, Miller 1778) is the second largest after its Antarctic cousin the Emperor penguin (*A. fosterii*) (Williams 1995). Those semi-altricial seabirds reproduce in vast colonies of several thousands of pairs on beaches of the sub-Antarctic islands (Williams 1995) (**Figure 12**).

1. **Emperor penguin – *Aptenodytes forsteri***
2. **King penguin – *A. patagonicus***
3. **Gentoo penguin – *Pygoscelis papua***
4. **Chinstrap penguin – *P. antarcticus***
5. **Adelie penguin – *P. adeliae***
6. **Yellow-eyed penguin – *Megadyptes antipodes***
7. **Royal penguin – *Eudyptes schlegeli***
8. **Macaroni penguin – *E. chrysolophus***
9. **Snares penguin – *E. robustus***
10. **Fiordland penguin – *E. pachyrhynchus***
11. **Erect-crested penguin – *E. sclateri***
12. **Southern Rockhopper penguin – *E. chrysocome***
13. **Northern Rockhopper penguin – *E. moseleyi***
14. **Humbolt penguin – *Spheniscus humboldti***
15. **Magellanic penguin – *S. magellanicus***
16. **African (Jackass) penguin – *S. demersus***
17. **Galapagos penguin – *S. mendiculus***
18. **Little blue penguin – *Eudyptula minor***

Figure 11. Schematic figure picturing the 18 currently known penguin species.

(*Photo courtesy: Yves Handrich*).

King penguins are long-lived seabirds, with an annual adult survival over 90% (Weimerskirch et al. 1992). Average age at first reproduction is reported to be around 6-years old (Weimerskirch et al. 1992), although some birds have been observed to attempt breeding at the earlier age of 3 or 4 (Barrat 1976). Those large (85-95 cm tall and 12-14 kg), deep-diving, predators forage on pelagic invertebrates (squid) and myctophid (lantern) fish (Cherel and Ridoux 1992, Olsson and North 1997, Raclot et al. 1998, Cherel et al. 2002) that are located on feeding grounds several hundreds of kilometres away from their breeding colonies (Bost et al. 1997, Pütz et al. 1998). To catch their prey, those birds repeatedly plunge to incredible depths (over 200 m, Pütz and Cherel 2005). Their foraging sites vary between seasons according to prey availability. In the austral summer, during the breeding season, birds swim upwards to forage at the polar front some 300 km from their colonies. During the winter however, as prey availability decreases, penguins are compelled to move down south reaching feeding grounds at the marginal ice zone, as far as over a 1000 km from their breeding colonies (Charrassin and Bost 2001, Pütz 2002).

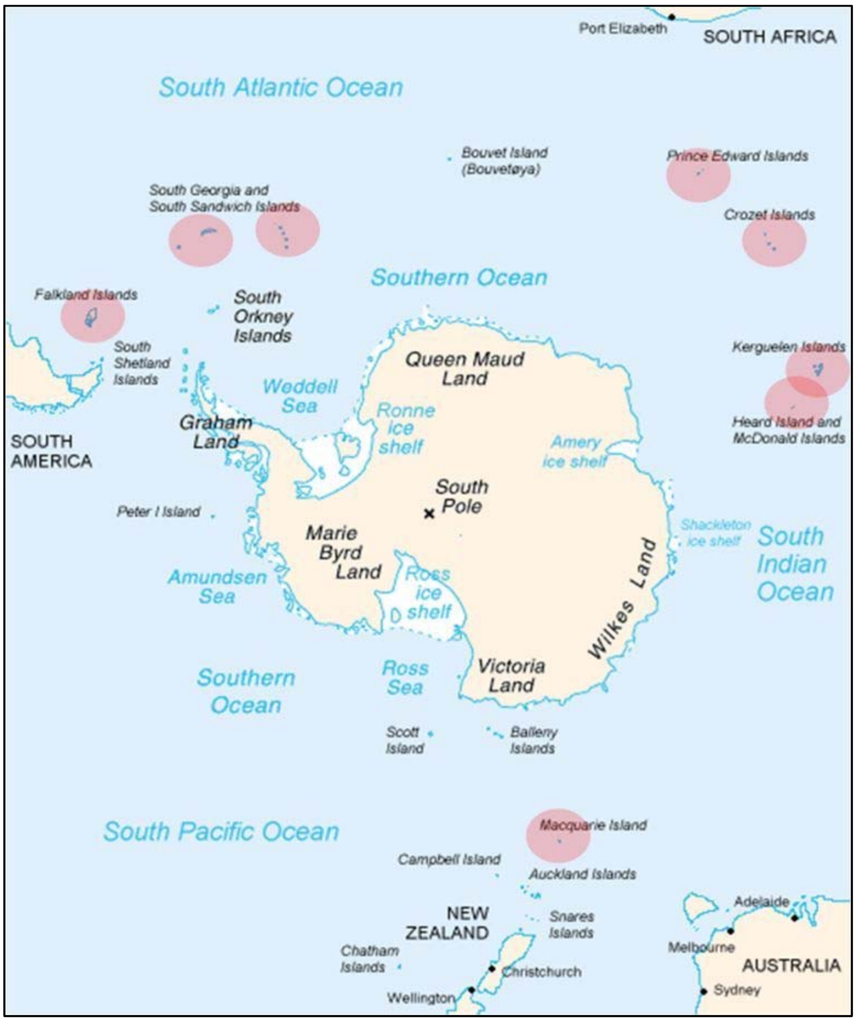

Figure 12. Map figuring the global distribution of king penguin colonies.

The species is subdivided in two subspecies, depending on their geographic localisation. *A.p. patagonicus* occur in South Georgia, the Sandwich Islands, and the Falkland Islands. *A.p. halli* occur in the Prince Edward and Marion, Crozet, Kerguelen, Heard and Macquarie Islands.

The king penguin's breeding cycle is long, complex and highly ***constraining from an energy perspective***. Indeed, of all known penguin species, the king penguin is the only species to present a breeding cycle which extends for over a year (**Figure 13**). After a moulting period of some 22 days (Descamps et al. 2002) and a subsequent period spent at sea to replenish their energy reserves, the first adults arrive at the colony to breed early November. Courtship then occurs and mating partners carefully choose each other during ritualized courtship displays, where both sexes tentatively exhibit colourful ornaments to one another (Stonehouse 1960, Jouventin et al. 2008, Nolan et al. 2010, Dobson et al. 2011a) (**Figure 14**). Once the pair has bonded, settled down on its breeding territory, and the egg has been laid, the female rapidly deserts the colony to go and feed at sea, leaving the male in charge of the first incubation shift. Then during the rest of incubation (which lasts in total some 54 days, Stonehouse 1960) and the early phases of chick rearing, parents take turns to incubate the single egg or young chick on their feet, thus undergoing prolonged periods of fasting ashore while the partner is foraging at sea (Stonehouse 1960, Groscolas 1990, Descamps et al. 2002). Hatching typically occurs shortly before mid-January, and the chick will constantly remain with one of its parents until thermally emancipated, some 5 weeks later (i.e. the chick covered in down does no longer need to remain in the parents brooding pouch).

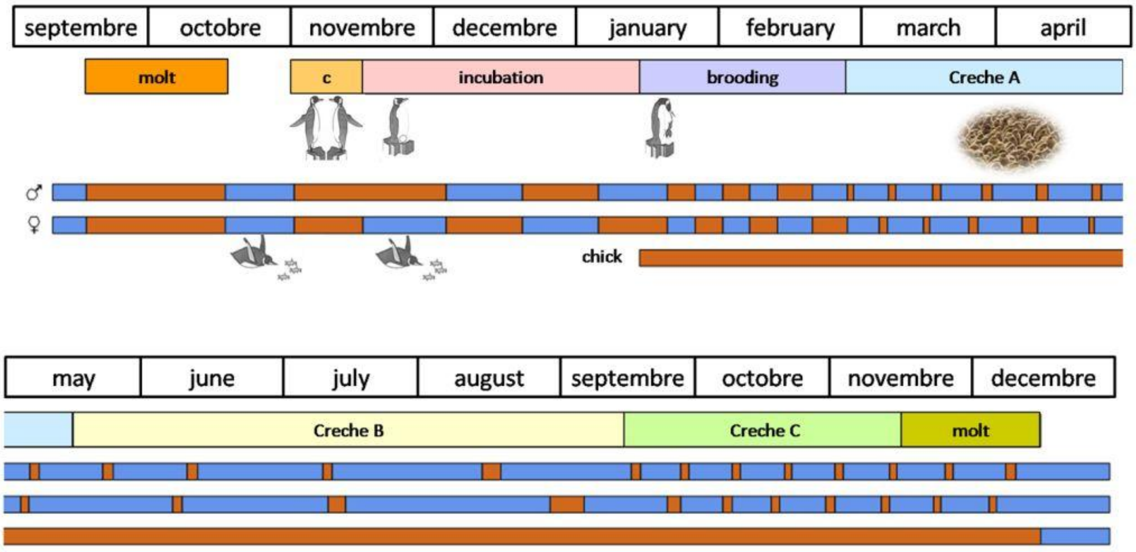

Figure 13. Schematic representation of the king penguin's breeding cycle.

The cycle presented here is for a king penguin pair that did not raise a chick the previous year (see text below). An example of periods spent foraging at sea (blue) or on land (brown) is given for both partners. The courtship period is indicated by (**c**). The egg typically hatches around mid-January. During the autumnal crèche (**A**), parents still regularly alternate sojourns at sea with chick-feeding on land. During the winter (crèche **B**) parental-feeding is scarce and king-penguin chicks undergo long periods of starvation. Feeding is resumed in the spring (crèche **C**), at the end of which the chick is finally fledged – *(adapted from Hanuise 2011).*

Chicks will then group into crèches and will be left alone in the colony, while both parents actively resume foraging trips to meet the needs of their growing offspring. By the end of the Austral summer (mid to late April), chicks will have deposited substantial fat reserves and, actually weigh more than their parents, in prevision of the shortage to come. Indeed, over the winter period (from May to September), parents will switch foraging grounds due to a sharp decline in food availability, and only seldom return to the chicks on the beach (Weimerskirch et al. 1992, Descamps et al. 2002). Chick-growth will then be interrupted as chicks are left fasting for an extend periods of time (Weimerskirch et al. 1992, Descamps et al. 2002). Starving chicks are the primary source of mortality during this period, causing ultimate breeding success to be low (around 30%, Weimerskirch et al. 1992). If the chick survives, rearing resumes in September, and the parents alternate foraging trips once more until the chick is ready to fledge in mid-November / December. Parents will then have to moult before they are ready to breed again.

Figure 14. Phases of the reproductive cycle of a king penguin pair.

The breeding cycle starts by ritualized courtship displays (picture on the left). Tentative pairs sing to each other, and mutually display their ornaments (which include the bill spot, upper breast feathers and ear patches) to each other by sky-rocketing (the typical posture adopted here by the male on the left). Once the female has laid the egg, she rapidly passes it on to the male that then resumes the first incubation shift (central picture). On the right, a brooding parent can be seen feeding its chick, which is not yet covered in isolative down and which thus, has to remain in the parent's brooding pouch to keep warm.

Because of the necessary time required to fledge a chick, ***breeding occurs asynchronously in king penguin colonies*** (Barrat 1976, Olsson 1996, Weimerskirch et al. 1992, **Figure 15**). Successfully reproducing birds in one-year can only start to breed late in the subsequent year (the year of fledging their chick), and it is extremely rare that they succeed (Weimerskirch et al. 1992). It is not until the next year that the successful pair has a good chance to successfully breed again, though not necessarily with each other (only about 20% of pairs meet again for further breeding; Olsson 1998). Thus, the most highly successful birds only produce a single chick every second year.

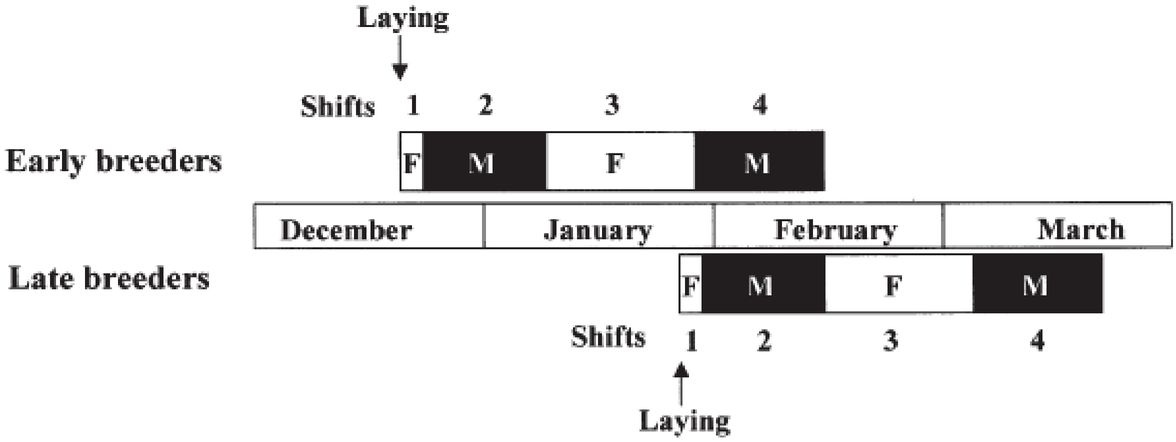

Figure 15. Schematic diagram illustrating the breeding asynchrony in king penguin colonies.

Pairs that have not fledged a chick the previous year are free to start courtship mid- to end-November and lay their egg early in the season, i.e. December (early breeders). Pairs having fledged a chick the previous year have to wait until January to start courting again – (reproduced *from Gauthier-Clerc et al. 2002a*).

For parents, the important energy constraint of such a reproductive pattern is well illustrated during the incubating and brooding shifts (when the continuous presence of one adult is required in the colony). Indeed, because of the critical depletion of their energy stores, parents may sometimes abandon the egg or young chick in order to go and re-feed at sea before their partner can return (Olsson 1997, Groscolas et al. 2000, Gauthier-Clerc et al. 2001). It is important to note that this unique breeding cycle leaves very ***little time to breeding pairs within a season***. For instance, if a pair successfully hatches its egg, but then loses the chick, it has very little chances of attempting a second reproduction within the season, and virtually no chance of successfully managing a replacement clutch. According to parental investment theory[16] (Trivers 1972), it may then be expected that the relative value

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[16] PARENTAL INVESTMENT THEORY: Parental investment theory is a branch of life history theory. It considers the time and energy invested by parents into their offspring that benefits the offspring at the detriment of other fitness components. As individuals are limited in the time and resources they can allocate to produce and raise their offspring, parental investment theory posits that evolution should favor optimal investments taking into account the current condition of the parents and offspring, future condition, survival, and future breeding opportunities.

of offspring increases with the advancing season, so ***that chicks may be considered more valuable than eggs***.

2. Behavioural repertoire of breeding king penguins

The behavioural repertoire of adult king penguins has most likely been shaped by their colonial environment and by the strong energy constraints they experience in relation to reproduction. Breeding in a dense colony poses several issues, notably those of territoriality and parasitism.

In a colony of breeding penguins, social challenges are constant and aggression rates are high (Challet et al. 1994, Côté 2000). In order to breed successfully, birds actively defend a small core territory (roughly 0.5 m², Côté 2000) by means of aggressive behaviour. Because of the possible physical costs (i.e. injuries) associated with territorial defence, animal aggressive repertoires have been shaped as to minimize risks of injuries and king penguins are no exception. They resort both to physical attacks and threat displays when defending their territory (**Figure 16**). Physical attacks are constituted of flipper blows and bill-pecks (see **Figure 16**), and may cause severe physical injuries to breeding birds. Threat displays on the other hand regroup bill-pointing (neck stretched out, bill closed) towards another individual and gapping (neck stretched out, bill opened and vocalizing) (Williams 1995). In addition, colonial species share close space with conspecifics and a high density of hosts offer suitable habitat and continuity for parasites (Arneberg et al. 1998, Arneberg 2002). King penguins are known to be infested by various ectoparasites (Gauthier-Clerc et al. 1998, Mangin et al. 2003), including ticks (*Ixodes uriae*): vectors of viruses and of the Lyme disease agent *Borrelia burgdorferi* (Gauthier-Clerc et al. 1998). Thus, one way for breeding birds (which spend prolonged periods of time in the colony) to regulate ectoparasites, is probably the long feather-preening sequences associated with comfort behaviour (**Figure 16**). Preening keeps the plumage in a good condition, thus probably also maintaining its isolative properties. In addition, comfort behaviour also includes bouts of intense activity such as head-scratching, flapping the flippers, and body-shaking. The associated muscular exercise may help penguins to deal with long periods of inactivity, preventing muscular ankylosis.

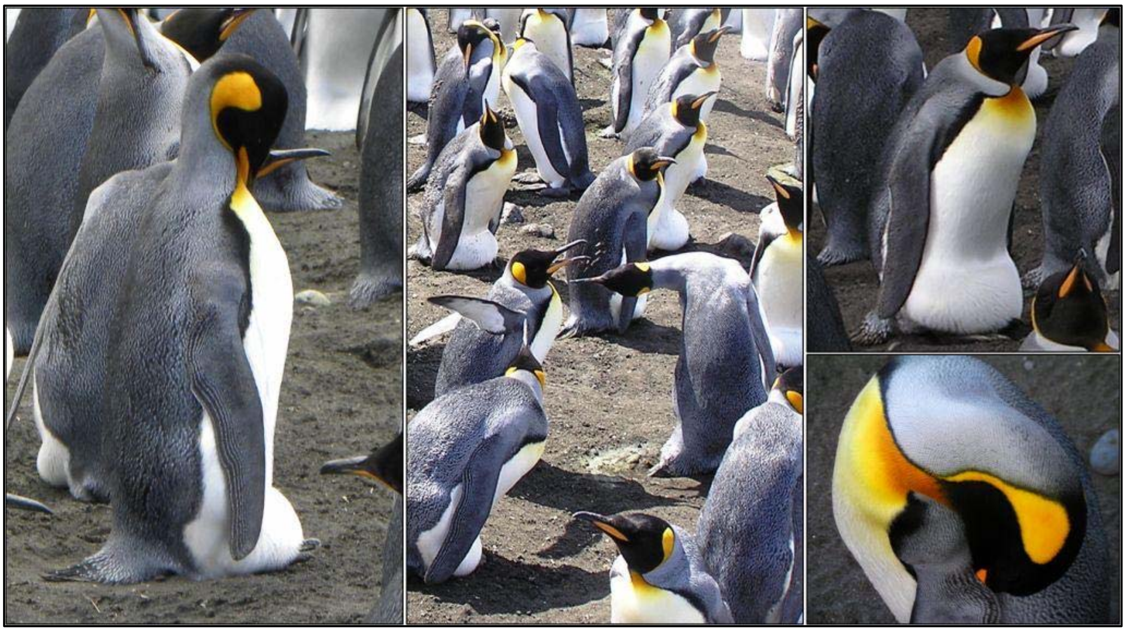

Figure 16.Various behaviours observable in breeding king penguin.

From left to right: 1) an incubating bird preening the feathers of its upper-breast, 2) three neighbouring individuals engaged in a territorial dispute (notice the individual on the left is flipper-beating, the one on the right bill-pecking, and the one in the foreground threat displaying, i.e. bill-pointing), and 3) on the high panel, an individual in a resting position, and on the lower panel a bird in a sleeping position – (*Image courtesy: Claire Saraux and Maryline Le Vaillant*).

As breeding penguins rely on their stored energy reserves to sustain their long term fast, physical activity should be kept to a minimum, and in effect, asides to the time devoted to comfort and aggressive behaviour, breeding king penguins spend the greatest proportion of the day resting and sleeping (close to 66% of their daily time budget, Challet et al. 1994, Figure 16). Chick-brooding birds obviously also spent time caring for their chick, which includes feeding and chick-preening, though this appears to be minimal in the overall time-budget of brooding birds (Challet et al. 1994).

One question that thus deserves further consideration is whether the time and energy devoted to the two major activities (i.e. comfort and aggressiveness) come at a cost that may seem prohibiting when considering long-term fasting? As explicated in **part 3**, this is indeed one of the questions we will address in this thesis.

3. Introducing our study site: the B.D.M.

The work presented in this dissertation was performed on Possession Island (some 15 km diameter), in the Crozet archipelago (46°25'S, 51°45'E), which lies at the heart of the Southern ocean, some 2860 km South from the Island of la Réunion and some 1420 km West from the Kerguelen Islands (**Figure 17**).

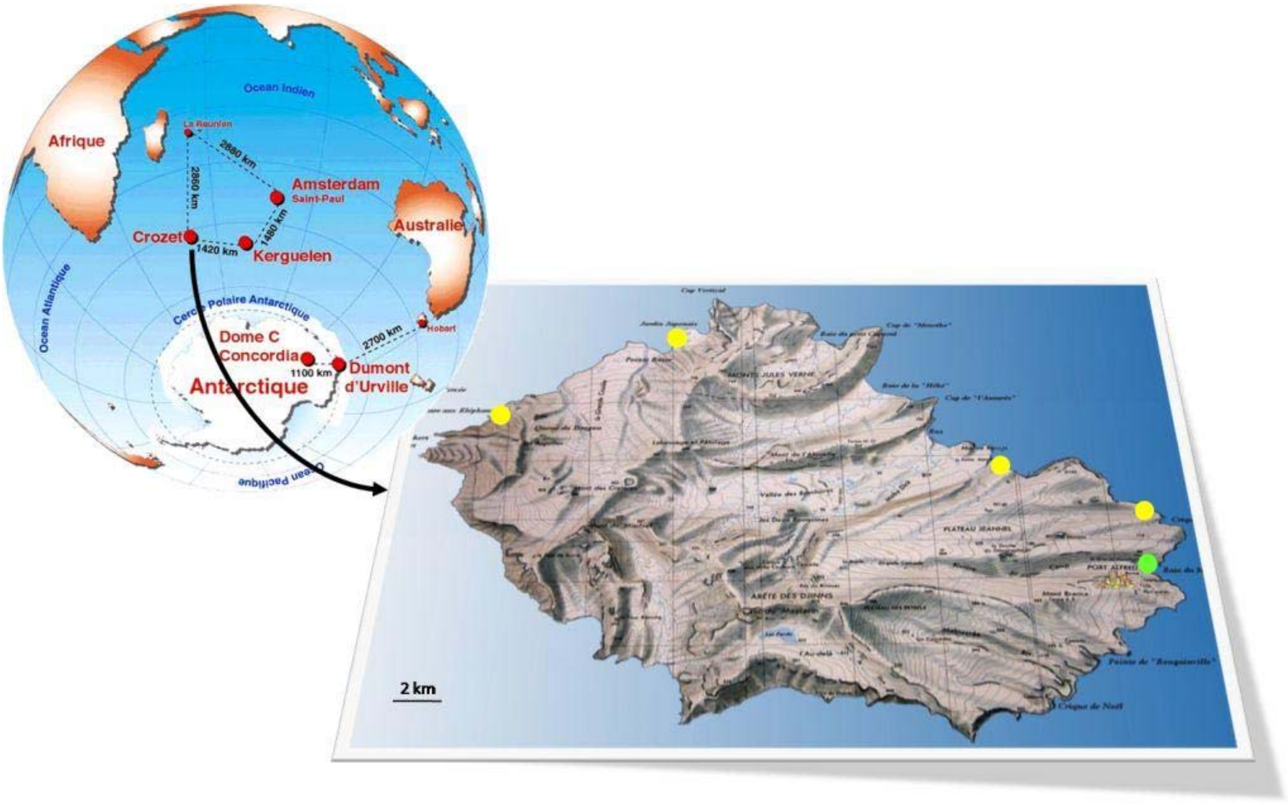

Figure 17. Location of the French Subantarctic Islands (Crozet, Kerguelen, Amsterdam & St Paul) and specific map of Possession Island (Crozet archipelago).

Note the 5 king penguin colonies situated on Possession Island. In green is our study colony "La Baie du Marin (B.D.M.)" (ca. 25,000 pairs). From east to west: la Baie du Marin, la chaloupe (ca. 1,700 pairs), la petite manchotière (ca. 14,500 pairs), le jardin japonais (ca. 34,200 pairs), la mare aux éléphants (ca. 6,000 pairs). Population estimates (except for BDM) are from Delord et al. 2004 – (*Image courtesy: IPEV*).

Most of the data was collected over 3 field seasons, from November to March, during the austral summers 2008-2009, 2009-2010 and 2010-2011. The Crozet Archipelago hosts more than half of the world population of king penguins (some 1,000,000 breeding pairs; Guinet et al. 1995). Our work was performed in the king penguin colony of "La Baie du

Marin” (the sailor’s bay; **Figure 18**), locally known as the B.D.M. (see green dot on the map presented **Figure 17**) which is one of the five king penguin colonies which may be found on the island. Recent figures estimate it at approximately 25,000 breeding pairs, which makes it the second largest colony on the island after the colony of “Le Jardin Japonais” (the Japanese garden aka. Jardin Jap’) situated to the North (some 34,200 pairs, Delord et al. 2004).

The scientific facilities, which are installed in the vicinity of the colony and shared between several different scientific programs, offer comfortable working conditions (e.g. storage and preparation of material) and welcome sheltering (and the occasional cup of coffee!) when the weather conditions meet up to the howling expectations of the roaring forties.

Figure 18. Panoramic view of the king penguin colony in Baie du Marin.

A winding path (1.2 km) leads from the beach to the main camp situated some 130 meters above the colony. Scientific shelters are situated within the colony that extends from the waterfront far up into the valley, following the riverbanks. Historical remnants (still in use today) of oil tanks can still be seen beside the scientific shelters – (*Image courtesy: Onésime Prudhomme*).

Part of the colony, located between the scientific shelters and the hill side, is dedicated to the long-term automatic monitoring of a part of the king penguin population in B.D.M. (Gendner et al. 2005) and is off-bounds to human intervention. Our studies were conducted in the other part of the colony, essentially that delimited by the path and the river on either side. Part of our studies was also conducted up in the valley (i.e. the far right side of the photo, **Figure 18**), where animals have historically little been exposed to human presence.

4. Measuring energy expenditure in the wild

For the many reasons presented above, scientists may be interested in knowing how much energy animals use for given activities in specific environments. In the specific case of king penguins, information on energetics will provide us with insight on how those animals cope with long term fasting while breeding in a dense colonial environment, where rates of aggressiveness are high, and where breeding success depends on the effective management of previously accumulated energy stores. However, in order to test the "Whys?" and "Hows?" of energy use, obviously one needs to be able to measure energy expenditure in wild animals.

4.1. The respirometry method

As most of the energy used by endotherms is derived from oxidative processes (Schmidt-Nielsen 1979), one method to estimate energy expenditure is to measure exchanges in respiratory gases. Indeed, the rates of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) by the organism are reflective of the ***oxidation of metabolic substrates, i.e. the process by which the body converts nutrients into energy***. By calculating the respiratory exchange ratio (RER = $\dot{V}CO_2$ / $\dot{V}O_2$), one can then get an idea of which type of substrate is being oxidized. As it is assumed that each type of substrate liberates a given amount of energy for each litre of oxygen consumed, and that the organism's oxygen and carbon dioxide content remain constant, energy expenditure can then be estimated from the RER and oxygen consumption. Whereas the technique is the most robust for measuring energy expenditure in captive conditions (Lighton and Halsey 2011), measuring $\dot{V}O_2$ and $\dot{V}CO_2$ in wild animals may be somewhat more delicate. Indeed, classical respirometry techniques require a complex set of various apparatus for gas analysis and exchange measurements, and are not easily transposable to field situations (but see some applications, e.g. Culik et al. 1993, 1994, 1996).

4.2. The Doubly-Labelled Water (DLW) method

An alternate and relatively easy method for estimating energy expenditure in the wild is the use of the doubly-labelled water method. The principle of this method is to get an indirect estimation of carbon dioxide production by measuring oxygen and hydrogen loss from the body in a given amount of time, which may be done using DLW (which is traceable water, e.g. $^2H_2{}^{18}O$). As opposed to classical water (H_2O), the oxygen and hydrogen atoms are replaced by stable atoms with specific isotopic signatures (Speakman 1997, Butler et al. 2004) which are often the stable isotopes deuterium (^2H) and oxygen-18 (^{18}O). The method is based on the observation that oxygen in respiratory carbon dioxide is in isotopic exchange equilibrium with that in body water, so that if DLW is provided to the animal (either orally or by injection), the oxygen present in the body water (including the marker dose of labelled ^{18}O) will rapidly equilibrate with the body's bicarbonate and dissolved carbon dioxide pool. Because oxygen (including ^{18}O) is lost from the body both through exhaled carbon dioxide and water loss (mostly urine), whereas hydrogen (including ^2H) leaves the body through water loss only, oxygen-18 (^{18}O) is lost faster than deuterium (^2H). The difference between both then yields a quantitative estimate of carbon dioxide production (**Figure 19**).

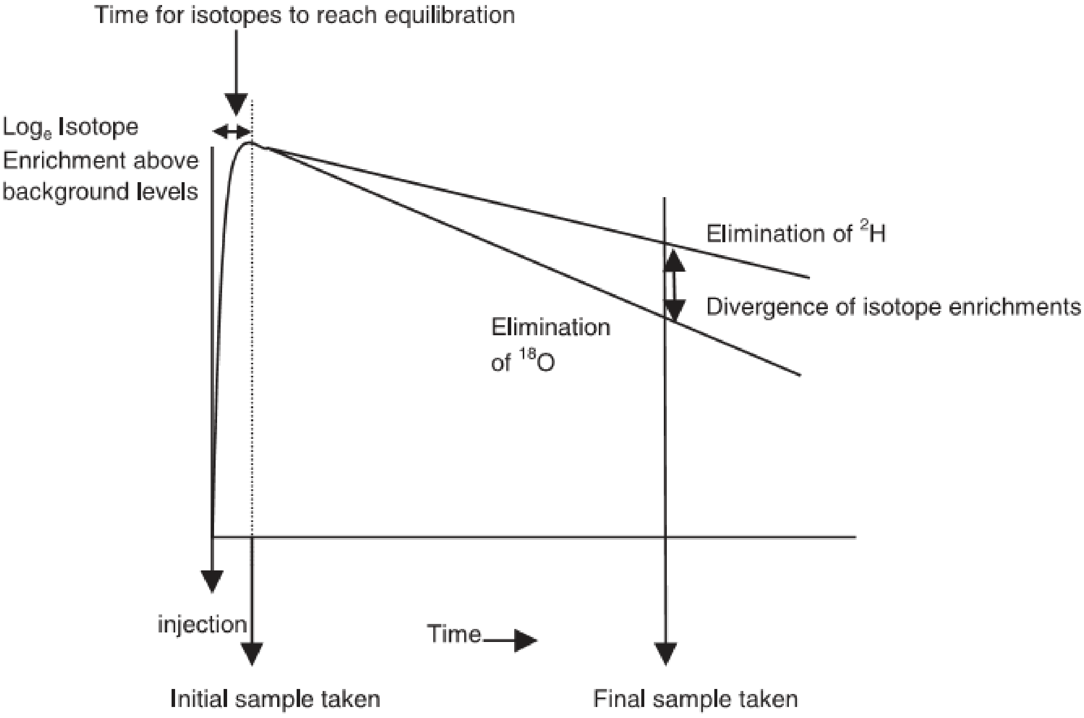

Figure 19. The Doubly-Labelled Water method.

This diagram represents the time course of changes in ^2H and ^{18}O in an animal's body, after initial injection of doubly-labelled water (DLW). Briefly, after injection (or ingestion) of DLW, the deuterium and oxygen-18 isotopes mix with the body water pool, leading to isotopic enrichment. Once equilibrium is reached, ^2H and ^{18}O are eliminated from the body trough water (in urine mostly) and carbon dioxide exhalation. Because ^{18}O is eliminated through water and CO_2, whereas ^2H is eliminated through water only, the difference in isotope elimination rates yields an estimate of CO_2 production rates – (*from Butler et al. 2004*).

As this technique only requires sampling the animal twice, it is very easy and convenient to use (given that the equilibration time is known), and several studies have successfully applied it to measure energy expenditure in the field (Shaffer 2011). However, the technique measures an animal's carbon dioxide production over the interval between the first and last body water samples, and thus only allows to obtain an average estimate of metabolic rate over that given period (Butler et al. 2004), during which a range of behaviour might occur. If one's aim is to measure precisely the energy expended during specific activities, a finer scale method is needed.

4.3. The Heart Rate (HR) method

Another alternative method that may be used in natural conditions to monitor the energy expenditure of wild animals is the heart rate method. The HR method consists in estimating the rate of oxygen consumption by measuring heart rate, and is based on Fick's convection equation for the cardiovascular system:

$$\dot{V}O_2 = f_H \text{ x } V_S \text{ x } (CaO_2 - CvO_2)$$

In which:

- $\dot{V}O_2$ is the animal's rate of oxygen consumption
- f_H is the animal's heart rate (number of heart beats per minute)
- V_S is the cardiac stroke volume (the amount of blood pumped into the circulation at each heart beat)
- $(CaO_2 - CvO_2)$ is the difference between the oxygen content of arterial blood and the oxygen content of venous blood.

The use of the HR method relies on the assumption that the amount of oxygen consumed by the animal per heartbeat (i.e. the quantity V_S x $(CaO_2 - CvO_2)$, known as the oxygen pulse) is constant. However, the $\dot{V}O_2$ – HR relationship has been shown to be affected by a number of factors including physical activity (Butler et al. 2000, Froget et al. 2002), physiological and nutritional state (Froget et al. 2001, Green et al. 2001), and gender (Green et al. 2001). Thus, for the method to yield reasonably accurate estimations of energy expenditure, it is necessary to dispose of a calibration of HR against oxygen consumption in the studied species, and in conditions similar to those of the study (Butler et al. 2004, Green

2011). In this regards, the $\dot{V}O_2$ – HR relationship has been investigated to a great length in king penguins (Fahlman et al. 2004, Froget et al. 2001, Froget et al. 2002, Halsey et al. 2007a), including in free-living conditions (Groscolas et al. 2010).

The HR method offers an interesting means to monitor the energy expenditure in free-living wild animals, with minimum disturbance. HR can be monitored using HR-loggers (cardiofrequencemeters) that are in essence portable miniaturized computers. Those data-loggers[17] are able to pick up (by electrodes) the electrical activity related to heart muscle contraction and, using pre-programmed algorithms, may directly store information in an internal memory unit. The HR may thus be monitored over several days (depending on the sampling rate and the size of the internal memory chip) without any intervention on or close to the monitored animals, which may thus resume their normal activities according to their daily routines. With advancing technological refinements, external devices are ever miniaturized and do not always require placing electrodes close to the heart muscle (and thus do not necessarily require heavy surgery).

For the purpose of our studies, we opted for the use of the HR method, which allowed us to monitor energy expenditure on finer time scales than the DLW method, and which also allowed us to monitor acute HR responses to specific situations. We thus used external HR-loggers initially developed for human athletes (Polar® Pro series, Polar Electro Oy, Kempele, Finland), and adapted for suitable use on king penguins (see Groscolas et al. 2010) (see **Figure 20**).

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[17] **DATA-LOGGERS:** Are electronic devices, which record data over time via built-in sensors that are able to measure various parameters (e.g. temperature, pressure, heart rate, acceleration, etc.) – see Ropert-Coudert and Wilson 2005.

Figure 20. Heart rate monitoring of breeding penguins in their natural environment.

The transmitter of the HR-logger is taped to the feathers in a mid-dorsal position to prevent hindering movements from the bird. It is placed in order to be out of reach of the animal. The receptor is either secured to a plastic flipper-band, which is loosely secured to the animal's flipper (left panel), or attached to a metal pole that can be placed close to the incubating/brooding bird. When visible, the digital display (central panel) allows monitoring HR (175 bpm in this case) from a distance, using binoculars.

The loggers were made of two units: (1) a transmitter, which contains a heart-rate processor filtering electric signals received by the electrodes and thus allowing to distinguish heartbeats from other electric activities (e.g. muscle or electrode noise), and (2) a receiver/logger (watch) which stores instantaneous HR values received from the transmitter by radio-wave transmission. Transmitters were attached to the feathers of the animal in the middle of the back and electrodes placed subcutaneously on the upper and lower back. The receiver was either secured to a flipper band or attached to a metal pole within a 5-m distance of the animals, depending on the study (see **Figure 20**). Methods are further detailed in the following studies. A previous study (see Groscolas et al. 2010) insured that loggers yielded accurate measurements of HR in breeding, fasting king penguin and that reasonable estimations of energy expenditure could be performed with this method.

III. Breeding energetics of the king penguin in its natural colonial environment

There has been a long-term tradition in studying the metabolic and hormonal changes related to fasting in penguins. Indeed, in relation to their life-cycle all penguin species are subjected to onshore fasts – short or long – and those animals thus appear an excellent model for adressing how energy stores are managed, particularly in terms of a trade-off between adult survival and reproduction.

In this regards, perhaps two penguin species are unrivalled in terms of fasting capacities: the emperor and king penguins. In the emperor penguin, the male takes duty for the whole incubation shift in the midst of the antarctic winter, thus coping with fasts as long as 4-months, while breeding at ambiant temperaturs well below its lower thermoneutral limit. On the other hand, whereas king penguins breed within their thermoneutral zone in the sub-Antarctic summer, males nonetheless undertake a month-long fasting shift while their partner feeds at sea. In addition for both species, whereas males undertake the longest fasting shift, both sexes face subsequent fasting periods caring for the egg or chick throughout the breeding season. Such a natural set-up has triggered a substantial field of research, and the fasting capacities of penguins have been well studied under captive and non-reproductive conditions. Surprisingly however, relatively few studies have considered how breeding birds ajust their energy expenditure in their natural colonial context. The focus of this second chapter is thus to consider the energetics of breeding king penguins, which are fasting in their natural colonial context. Its objectives are twofold:

First (**studies 1 and 2**), I consider how fasting king penguin invest into various behaviours while breeding ashore. Specifically, I address the question of whether animals subjected to prolonged periods of energy limitation may maintain specific behaviours at a minimal energy cost. Based on previous studies, we consider the two main behaviours of fasting king penguins breeding ashore: comfort and aggressive behaviour. We determine the daily time budget and suggest the very first estimates of the energy cost of those two behaviours.

Then (**study 3**), I consider the changes in energy expenditure occuring during the longest breeding fast of the species, i.e. that experienced by males during courtship and the first incubation shift at the onset of the breeding season. In this study, we ask how energy expenditure is modulated in free-living birds, whether changes in overall physical activity and body temperature may be adaptive strategies for males to limit their energy expenditure during courtship and incubation, and whether the social environment may have a potential effect on the physiology of breeding birds.

STUDY 1 – Estimating the energy costs of specific behaviours: Comfort behaviour

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It costs to be clean and fit: energetics of comfort behaviour in breeding-fasting penguins

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Abstract

Background: Birds may allocate a significant part of time to comfort behaviour (e.g. preening, stretching, shaking, etc.) in order to eliminate parasites, maintain plumage integrity, and possibly reduce muscular ankylosis. Understanding the adaptive value of comfort behaviour would benefit from knowledge on the energy costs animals are willing to pay to maintain it, particularly under situations of energy constraints, e.g. during fasting. We determined time and energy devoted to comfort activities in freely breeding king penguins (*Aptenodytes patagonicus*), seabirds known to fast for up to one month during incubation shifts ashore.

Methodology/Principal Findings: A time-budget was estimated from focal and scan- sampling field observations and the energy cost of comfort activities was calculated from the associated increase in heart rate (HR) during comfort episodes, using previously determined equations relating HR to energy expenditure. We show that incubating birds spent 22 % of their daily time budget in comfort behaviour (with no differences between day and night) mainly devoted to preening (73%) and head/body-shaking (16 %). During comfort behaviour, energy expenditure averaged 1.24 times resting metabolic rate (RMR) and the corresponding energy cost (i.e. energy expended in excess to RMR) was 58 kJ/hr. Energy expenditure varied greatly among various types of comfort behaviour, ranging from 1.03 (yawning) to 1.78 (stretching) times RMR. Comfort behaviour contributed for 8.8 – 9.3 % to total daily energy expenditure and for 69.4 – 73.5 % to energy expended daily for activity. About half of this energy was expended for caring to plumage.

Conclusion/Significance: This study is the first to estimate the contribution of comfort behaviour to overall energy budget in a free-living animal. It shows that although breeding on a tight energy budget, king penguins devote a substantial amount of time and energy to comfort behaviour. Such findings underline the importance of comfort behaviour for the fitness of colonial seabirds.

1. Introduction

Maintenance behaviours (i.e. allo- and autogrooming, allo- and autopreening, bathing, scratching, stretching, etc.) serve a variety of purposes and are widespread throughout the animal kingdom, e.g. in mammals (Eckstein and Hart 2000, Giorgi et al. 2001, Schino and Aureli 2008, in birds (Ainley 1974, McKinney 1966, Walther 2003), in fish (Bshary and Schäffer 2002), in crustaceans (Schmidt and Derby 2005), and in insects (Basibuyuk and Quicke 1999, Sumana and Starks 2004). Studies that have considered the adaptive significance of maintenance behaviours (referred to as comfort behaviour in birds; Simmons 1985) have suggested both proximate (i.e. bodily) and more ultimate (i.e. social) functions such as the maintenance of good corporeal condition (e.g. parasite control, thermal insulation or muscle condition (Ainley 1974, Giorgi et al. 2001, Booth et al. 1993, Hawlena et al. 2007, Mooring et al. 2004) or the maintenance of sexual ornaments (Walther 2003, Lenouvel et al. 2009, Walther and Clayton 2005). Maintenance behaviours have also been suggested to be facilitated by social contexts (Palestis and Burger 1998), and accredited to play a role in social relationships (Schino and Aureli 2008, Schino et al. 1988, Hart and Hart 1992).

In birds, comfort behaviour is usually referred to as a set of activities concerned with the care of the integument and the maintenance of a functional body structure, i.e. by increasing proprioceptive sensitivity and circulation in the muscles for instance (Ainley 1974, McKinney 1966). Several studies have previously shown that birds spend a substantial amount of time in comfort behaviour. Indeed, a meta-analysis over 62 different avian species, revealed that birds devoted 9.2 % of their daily time budgets to comfort activities (Cotgreave and Clayton 1994) (92.6 % of which was preening), and figures close to 15% have been reported in several species (15 % in gulls; Delius 1970, 14 % in Japanese quail; Schmid and Wechsler 1997, 14.9 % in peacocks; Walther 2003). Obviously, the time devoted to comfort behaviour must trade with that devoted to other activities, which could incur some costs, including indirect energy costs. For example, individuals allocating a higher proportion of time into comfort may face a reduction in resting time, decreased vigilance towards predators, and decreased foraging time. The temporal trade-off dilemma is well illustrated by Walther and Clayton's study (Walther and Clayton 2005) who found that, when looking for maintenance times in ornamental and non-ornamental species, wild birds only spent 8.7

% of their time on maintenance behaviours whereas captive individuals spent almost twice as much, i.e. 15.8 % (see Walther and Clayton 2005). Captive birds may indeed devote a greater amount of time to comfort behaviour, as food is usually provided *ad libitum* in a safe environment, and the amount of time spent foraging or in vigilance may be decreased.

Because of corresponding physical activity, comfort behaviour may also incur direct energy costs, which may substantially impact overall energy budget. As natural selection is thought to drive the evolution of animal behaviours whenever their benefits outweigh their costs (leading to behavioural strategies that appear differentially adaptive and that ultimately increase individual fitness; Stearns 1992), estimating the energy costs directly associated with comfort behaviour might help in understanding how adaptive strategies could evolve in regards of energy allocation and trade-offs. Such estimates would be particularly informative for species that rely on limited energy supplies for part of their life cycle, e.g. long-term fasters. Indeed, in those species the effective management of energy stores could well mean the difference between survival and death, breeding success and failure. To date, few studies have considered energy costs related to comfort behaviours (Møller et al. 1994, Giorgi et al. 2001) and those that have done so determined the animal's energy expenditure in response to parasitic infestation rather than the energy expenditure due to comfort *per se*. However, one could presume that high parasite loads may impose energy costs asides those related to grooming. One reason that could well explain the lack of data on the energy costs of specific behaviours, and on the contribution of comfort behaviour to overall energy budget, might have to do with methodological issues. Classical methods used to monitor energy expenditure (EE) such as stable isotopes or respirometry, are either not adapted to measure the energetics of specific behaviours (but see Piersma et al. 2003), nor readily transposable to field monitoring. Although the doubly labelled water technique (DLW) is relatively simple to use in the field and offers reasonably accurate measures of EE, this method only yields an average estimate over the duration of the experiment. Thus, whereas measuring EE relating to specific activities using DLW may be possible under controlled conditions (Piersma et al. 2003), obtaining those estimates in free-living field conditions and for birds alternating different types of activities of relatively short duration is not possible. On the other hand, determining the contribution of one behaviour to energy budget requires an accurate estimate of the time devoted daily to this behaviour, which is possible only for animals living in the open and thus easily observable.

In this study, we consider the energetics of comfort behaviour under a natural context using breeding-fasting king penguins (*Aptenodytes patagonicus*) as a model. King penguins are long-lived, semi-altricial seabirds that reproduce in vast colonies of several thousands of pairs on beaches of the subantarctic islands (Williams 1995). During egg-incubation, which lasts on average 53 days (Stonehouse 1960), parents take turns to incubate the single egg on their feet, undergoing prolonged periods of fasting ashore while the partner is foraging at sea. The first incubation shift is the longest observed in the species and typically lasts for approximately one month (Weimerskirch et al. 1992). Subsequent shifts last around 15 days, during which the incubating parents rely mainly on fat stores built up during the previous foraging trips to sustain their metabolism (Groscolas 1990). The important energy constraint of such a reproductive pattern is well illustrated by the fact that parents, because of the critical depletion of their energy stores, sometimes abandon the egg or young chick in order to go and re-feed at sea, before the return of their partner (Groscolas et al. 2000, Gauthier-Clerc et al. 2001). In a context where energy savings appear as such a critical issue, the previous finding that incubating male king penguins may devote a substantial part of daily time-budget to comfort behaviour (Challet et al. 1994) might seem somewhat paradoxical, even for professional fasters. Such a finding might then be explained by two alternative hypotheses. First, if comfort behaviour was not energetically costly and did not trade with other time-consuming behaviours such as foraging (given that birds are incubating and fasting), spending a substantial amount of time in comfort may be the mere consequence of penguins having actually no major time constraints while breeding ashore. Alternately, if comfort behaviour were energetically costly, this would indicate that when breeding, penguins are faced with important constraints (such as those related to parasite load or muscular ankylosis), and should pay the energy cost in order to keep in good physical condition, including in anticipation of subsequent foraging trips at sea.

To discriminate between these two hypotheses, we investigated the time and energy budget of comfort behaviour in king penguins breeding ashore using heart rate (HR) as a proxy of energy expenditure (Fahlman et al. 2004, Groscolas et al. 2010). We determined the time and energy allocated both to global (i.e. overall) and specific comfort behaviours (e.g. preening, stretching, and shaking). This allowed us to calculate the contribution of comfort behaviour to daily energy expenditure, and to suggest the very first estimates of the cost of comfort activities allocated to plumage cares *vs*. non plumage-related comfort behaviour in a colonial seabird.

2. Methods

2.1. Ethics statement

Animals in this study were cared for in accordance with the guidelines of the Ethical Committee of the French Polar Institute (Institut Paul Emile Victor – IPEV). All procedures employed during the fieldwork were approved by the committee and comply with current French laws. Authorizations to enter the breeding colony (permit N° 2006-64 issued on November 4, 2006; and permit N° 2007-148 issued on October 24, 2007) and handle birds (permit N° 2006-73 issued on November 6, 2006; and permit 2007-143 issued on October 24, 2007) were delivered by Terres Australes et Antarctiques Françaises. Copies of permits are available upon request. During field procedures, animals were hooded in order to keep them calm and reduce the disturbance to neighbouring birds. Manipulations lasted between 5 and 10 min and never resulted in egg or chick abandonment. HR logger packages weighed less than 1 % of adult body mass and were installed in a dorsal midline position to prevent hindering movements of the birds. Flipper bands were removed at the end of the study.

2.2. Field procedure

This study was carried out on Possession Island, Crozet Archipelago (46°25’S, 51°45’E), in the breeding colony of ‘La Baie du Marin’ which is host to over 16.000 pairs of king penguins (Delord et al. 2004). During two consecutive breeding seasons (November-March), from 2006 to 2008, a total of 206 incubating and brooding adults were marked using a non-permanent animal dye (Porcimark®, Kruuse, Germany) and/or flipper banded for identification during field observations. Part of the animals (N = 191) was sexed from behaviour during courtship and according to sex-specific breeding cycle chronology (males being the first to incubate upon egg-laying; Stonehouse 1960). Males (N = 102) were banded on laying-date and females (N = 89) some 15 days later, upon their return from the foraging trip at sea, to relieve their partner. Those birds were followed daily from a distance to determine their breeding status (incubation or brooding shift). A small fraction of the studied animals (N = 15) was marked when already incubating and neither sex nor shift were known.

2.3. Time-budget of global and specific comfort behaviours

2.3.1. Comfort behaviour in penguins

Based on Ainley 1974, we characterized six major types of comfort behaviours: preening, head-shaking, head-scratching, stretching, tail-wagging and yawning. Preening (re-arrangement of feathers and parasite removal on the breast, belly and flippers with the beak or head) and head shaking (brief lateral movements) were associated with moderate physical activity. Head scratching with a foot (which requires contorting and use of the tail to maintain balance) and stretching (full body stretch almost always associated with strong flipper flapping) corresponded to vigorous physical exercise. Yawning (head tilted backward, bill opened) and tail-wagging (sequences of 5-6 lateral wags in a row) required only slight physical activity. Preening and head-scratching were devoted to maintenance of the plumage (removal of dry foreign materials and ectoparasites, waterproofing, thermal insulation). Head-shaking allowed to keep the head dry under rainy weather and to eliminate excess fluid secretions from the nasal ducts of the salt-glands, whereas tail-wagging was used to remove foreign material, faeces or water from the tail and cloacal region, especially on rainy days. Stretching and to a lesser extent yawning are suggested to play a role in increasing proprioceptive sensitivity and circulation in the muscles, thus maintaining functional musculature and preventing ankylosis (Ainley 1974). In this study, the duration of episodes of these specific behaviours ranged from a few seconds (yawning, head-shaking, and tail-wagging) to several minutes (head-scratching or preening). Often, several of these behaviours were associated within the same comfort sequence, e.g. preening and head-scratching, yawning and tail-wagging, which lasted several minutes. Head-shaking often ended with head-bobbing and swallowing and could be followed by whole body shakes.

2.3.2. *Global comfort behaviour*

The time spent in global comfort behaviour was determined by instantaneous scan sampling (Altmann 1974). In 2006-2007, throughout the entire breeding season (November-March), we estimated the time budgets of 182 marked birds (90 males, 79 females and 13 unsexed birds) not equipped with HR loggers (see below). Birds were located in different parts of the colony and were early or late breeders at different stages of breeding, i.e. different incubation and brooding shifts. We recorded behavioural activities of thirty of these birds every five minutes during at least six consecutive hours. The comfort category included every type of comfort behaviour. Scans were performed at a distance of 10-50 m, using binocular and spotting scopes to avoid disturbance of the birds. Individuals observed during scans were located at least 4 m apart to maximize independence of their behaviour relative to their neighbours. We balanced observations during all hours of daylight, from 0600 to 2000 hrs. Behavioural data were obtained from a total of 2270 scans spread over 25 days and totalizing 189 hrs of observations.

2.3.3. *Specific comfort behaviours*

The contribution of the different types of comfort behaviour to the overall time spent in comfort behaviour was determined from two hundred 15-min focal observations (Altmann 1974) during which at least one episode of comfort occurred (i.e. 50 hrs in total). These focal observations were obtained from one hundred of the above-mentioned individuals. The six types of comfort behaviour characterized above were considered and the proportion of time devoted to these different behaviours was determined for each focal observation. Focal observations were obtained from 58 males (n = 136) and 42 females (n = 64) with 1 to 4 observations per bird. As for scan samplings, focal observations were spread over the breeding season and performed in birds of different incubation and brooding shifts.

2.4. Energy cost of global and specific comfort behaviours

2.4.1. *Heart rate and video monitoring*

The energy cost of comfort behaviour was estimated from the corresponding increase in heart rate (HR) above resting values (see below). The recording of HR provides a good means for estimating energy expenditure (EE) in field studies (Butler et al. 2004), as it is a relatively non-invasive technique (i.e. when using external HR loggers) that offers the possibility to monitor in situ EE with a fine time-resolution. Recent studies have investigated its use in fasting king penguins (Fahlman et al. 2004, Froget et al. 2001, Froget et al. 2002, Halsey et al. 2007a), including in freely living breeding birds (Groscolas et al. 2010). Here, we used externally mounted HR-loggers (Polar® model RS800, Polar Electro Oy, Kempele, Finland) specially adapted for suitable use on king penguins, as previously described in (Groscolas et al. 2010). Briefly, the system included two units: a sensor-transmitter (30–40 g) and a receiver/logger (30 g). After disinfection with iodine (Betadine®) and alcoholic antiseptic solutions, electrodes made from gold plated safety needles were inserted under the skin in the subcutaneous fat layer (at approximately 5 mm depth, and over a length of 1 cm). One electrode was placed at the height of the wing pit and the second one above the tail. The whole HR logger package was secured in a dorsal, midline position using Tesa® tape. We ensured that loggers and electrodes remained out of the animals' preening reach so that birds were never observed attempting to remove electrodes or HR loggers, nor did we observe any adverse effects of equipment on birds' health or behaviour. As used, the HR-logger yielded HR values highly comparable to those measured with a stethoscope (Groscolas et al. 2010). The sampling rate was set at 1 value per 5 sec, allowing for 45 hrs of continuous HR monitoring without any intervention on or close to the equipped animals. We equipped with HR-loggers a total of 24 birds (12 males, 10 females and 2 unsexed birds) at various phases of the breeding cycle (i.e. different incubation and brooding shifts), and their behaviour was monitored by continuous video recording (using IR lighting during the night) as previously described in (Groscolas et al. 2000). Equipment was performed in late afternoon and, to ensure that bird HR and behaviour was no longer affected by handling, only data obtained at least 6 hrs after equipment was considered.

2.4.2. From heart rate to energy expenditure

Energy expenditure was estimated from HR using equation 1a (obtained from a mixed-model approach) in (Groscolas et al. 2010): EE (J/min) = –387 + 36.4*HR (bpm) ($F_{1,133} = 19.33$, $R^2 = 0.85$, $P < 0.0001$). A validation test showed that EE predicted from HR using the above equation did not differ significantly from measured EE ($t = 0.54$, $n = 30$, $P = 0.60$)

(Groscolas et al. 2010). As we used a different group of individuals (selected at random) from that of (Groscolas et al. 2010) in order to estimate EE from new field HR values, it was important to account for errors associated with: (1) the scatter around the original regression line in (Groscolas et al. 2010) (i.e. EE on HR), and (2) the variability between penguins (both for the calibration group in (Groscolas et al. 2010) and for the birds in our study) (see Green et al. 2001). Thus, error terms for our estimates of EE were conservatively calculated after equation 11 in Green et al. (2001), further adapted to account for one other source of uncertainty, i.e. in the relationship between body mass and total body energy used to calculate EE from body mass loss in Groscolas et al. (2010). The advantage of such an equation is that it is obtained from freely-incubating male and female king penguins (no gender difference), i.e. for a breeding status, a level of physical activity and a situation exactly the same as in the present study. Such pre-requisites are required for validly estimating EE from HR (Butler et al. 2004, Green 2011). Moreover, as stress might affect the oxygen consumption (VO2) – HR relationship in captive animals (Groscolas et al. 2010, Green 2011), ultimately leading to an underestimation of EE (Groscolas et al. 2010), an equation obtained from free-living birds may be more appropriate for estimating energy costs in the wild (see Groscolas et al. 2010, Green 2011). Nonetheless, we are also aware that using an equation calibrated on a larger time-scale (days) than the time-scale over which behaviours are monitored (i.e. min in the case of comfort behaviours) may be subject to criticism (Green 2011). For this reason, we compared our estimates to estimates obtained using a finer time-scale calibrated equation for king penguins (equation 1 in Fahlman et al. 2004), albeit the latter was obtained from likely stressed animals, as those were held captive for respirometry purposes. Unfortunately, the errors associated to the latter estimates could not be calculated, as the original data set used to establish equation 1 (Fahlman et al. 2004) is required for their calculation but is unavailable (unpublished).

2.4.3. Energy cost of global comfort behaviour

From the simultaneous recording of HR and behaviour, we estimated the global energy cost of comfort behaviour by comparing two different methods. The first estimate (estimate 1) was based on the determination of how the proportion of time spent into comfort behaviour during 15 min focal observations explained the variability of average HR during these focal observations. Amongst the 24 equipped birds, 5 individuals (3 males and 2 females) were selected at random and their behaviour and HR were considered over a 24 hr period starting at midnight. We divided the 24 hr period into consecutive periods of 15 min during which the total time spent in comfort activity was determined (ninety-six 15-min focal observations per bird, i.e. 480 observations in total). These 480 focal observations spread over 24 hrs allowed us to search for a potential day-night pattern in comfort behaviour. Average HR and the proportion of total time spent in comfort behaviour (whatever the behaviour) were calculated for each focal. Then, the relationship between average HR and the time spent in comfort behaviour was determined, the slope of this relationship yielding the first estimate of the global energy cost of comfort behaviour after converting HR into EE. However, the error term associated with this estimate could not be validly calculated, as the reasoning made considers an increase in proportion (see Results). Indeed, whereas a 1% increase in the time spent in comfort will lead to a constant increase in HR (i.e. the slope of the relationship), the associated error itself depends on the initial and final HR values used, i.e. the last term of the calculation of the error (Green et al. 2001, Zar 1999) $\frac{(X_i - \overline{X})^2}{\sum x^2}$, depends on *Xi*, which is the heart rate at which we calculate the associated energy expenditure, see Green et al. 2001). The errors would then not be the same when increasing the time spent in comfort behaviour from 0% to 1% or from 5% to 6%, for example.

We thus established a second more conservative estimate (estimate 2), which was based on the determination of HR increase during selected episodes of continuous comfort behaviour. These episodes had to fit two criteria: (1) they had to be preceded and followed by resting periods of at least 30 sec during which HR was stabilized at basal levels; and (2) only comfort behaviour had to be performed during the considered episodes (very often comfort behaviour is transitorily interrupted by episodes of aggressiveness related to territory defence). A total of ninety-four episodes over the 24 equipped birds were characterized, with 1 to 8 episodes per individual. The selected episodes were spread over 24 hrs and selection was at random concerning the type of comfort behaviour so that data was considered representative of average comfort behaviour. Only one type of comfort behaviour was performed during half of the selected episodes whereas the other episodes were a mix of different types of comfort behaviour. When obtained from the same individual, episodes were separated by at least two hours so that each episode was considered as independent. The average (± s.e) duration of the episodes was 2.3 ± 0.5 min (from 10 sec to 28.6 min; n = 94). They were preceded and followed by resting periods averaging 2.2 ± 0.3 and 2.6 ± 0.3 min, respectively.

2.4.4. Energy cost of specific comfort behaviours

Based on the same method as for estimate 2 of the global energy cost of comfort behaviour (and using the same 24 birds), the energy cost of specific comfort behaviours was calculated from selected episodes of comfort during which only one type of comfort behaviour was performed. This was possible for five of the six types of comfort behaviour that were characterized. Tail-wagging was almost always included into sequences of various comfort behaviours so that we did not succeed in selecting episodes of that behaviour that were preceded and followed by a resting period. According to the type of behaviour, the number of selected episodes ranged from 12 (head-scratching) to 31 (stretching), the average duration of episodes ranged from 0.1 ± 0.0 (yawning, head-shaking) to 2.3 ± 0.6 min (preening) and data were obtained from 5 to 13 individuals, with 1 to 11 episodes per individual. When obtained from the same individual, episodes were separated by at least two hours so that each episode was considered as independent.

2.4.5. Calculation of EE during comfort episodes

Energy expenditure during comfort episodes (both for estimate 2 of global comfort and for specific comfort behaviours) was determined according to (Smith and Taylor 1993) and as illustrated in **Figure 21**. It was the energy spent in excess to resting metabolic rate (RMR) during comfort behaviour plus the potential recovery phase. RMR was calculated from resting HR (mean of pre- and post-comfort resting HR). Comfort HR was the mean HR during comfort behaviours and recovery HR was the mean HR during the recovery phase. Comfort behaviours ended when the bird settled back into resting posture and the recovery phase ended when HR returned to resting levels. The recovery phase lasted on average 0.5 ± 0.1 min (from 0 to 5.3 min, N = 192). Excess HR during comfort behaviours was calculated as [(comfort HR – resting HR) x comfort duration] / resting HR (Smith and Taylor 1993) and corresponded to the time that would be required for the number of heart beats in excess to occur at the resting HR level (Rovero et al. 2000). The same calculation was done for the recovery phase, using recovery HR, and the total excess due to a comfort episode was the sum of excess during comfort plus recovery. The energy cost of a comfort episode (kJ) was calculated as: excess in time (min) x RMR (kJ/hr). Dividing the cost of the episode by its duration (min) yielded an energy cost in kJ/hr.

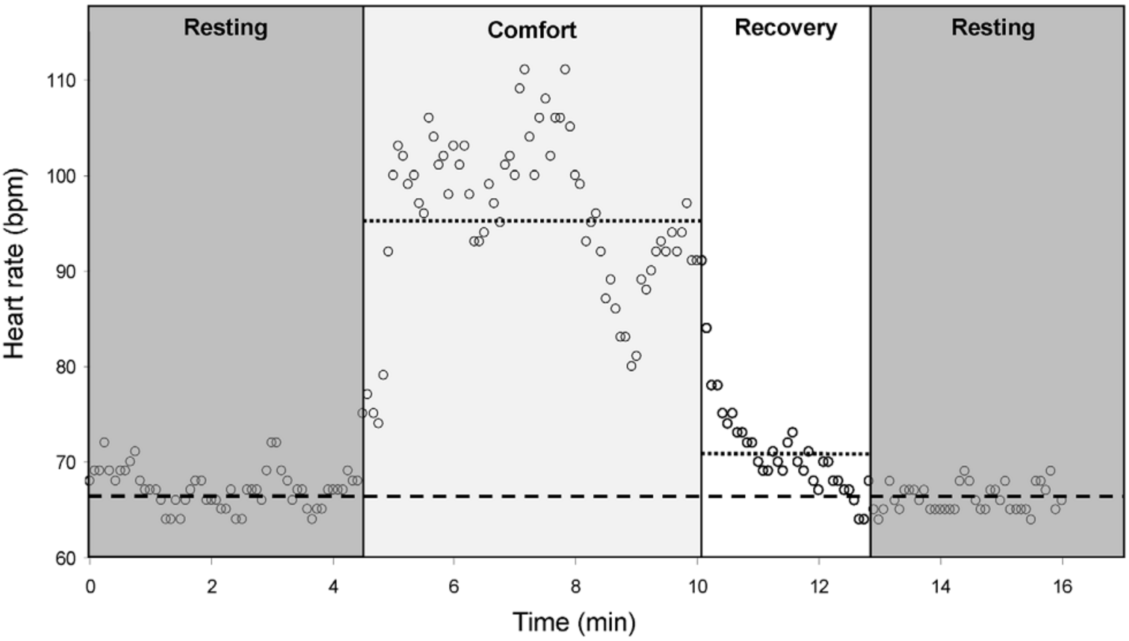

Figure 21. Heart rate increase during an episode of comfort behaviour in an incubating king penguin.

Shadowed zones delimit pre- and post-comfort resting periods and comfort behaviour, respectively, whereas the white zone delimits the recovery period. Dotted lines give average HR during comfort behaviour and recovery period, respectively, whereas the dashed line gives average HR during pre- and post-comfort resting periods. doi: 10.3171/journal.pone.0021110.g001

2.5. Contribution of global comfort behaviour to daily energy expenditure

We calculated total daily energy expenditure (DEE), RMR and energy expended daily for activity for each of the 24 birds from which the energy cost of global comfort behaviour was estimated (estimate 2). DEE and RMR were calculated from average daily and resting HR, respectively. Average daily HR of an individual was estimated from the HR measurements performed over the day (from 00h00 to 24h00) during which the episodes of comfort behaviour were selected to calculate the average cost of comfort (estimate 2). Average resting HR of an individual was calculated from HR determination during the resting periods preceding and following the different comfort episodes selected on that day (2 to 16 resting HR estimates per bird). Energy expended daily for activity was calculated as DEE – RMR. The total energy expended daily for comfort behaviour was obtained by multiplying the average energy cost of comfort by the average time spent daily into comfort behaviour, as estimated from scan sampling. The contribution of specific comfort behaviours to the total energy cost of comfort was calculated by multiplying the proportion of comfort time spent in a specific behaviour by its energy cost.

2.6. Statistics

Statistical analyses were performed using R v.2.10.1 (http://www.r-project.org/) statistical software. To determine how the proportion of time spent in comfort behaviour during 15 min focal observations explained the variability observed in average HR (estimate 1 of the cost of global comfort behaviour), we ran a Generalized Estimating Equation (GEE; Ballinger 2004) model in which the proportion of time spent in comfort behaviour was entered as a dependant variable and individual and rank of the focal observation were set as random and repeated factors (first order autoregressive structure), respectively. This allowed us to control for repeated measurements as well as for individual (and thus sex) variability in HR. Similarly, day/night patterns in comfort behaviour were checked by entering day/night as an independent factor variable in a GEE model, individuals (i.e. birds) as a random factor and the rank of focal observation as a repeated factor. Generalized estimating equations (GEE) were computed using the ‘geeglm’ function from the ‘geepack’ package in R v.2.10.1 (Højsgaard et al. 2005) and marginal R^2 was calculated according to Zheng (2000). When looking at differences between EE in different states (e.g. comfort vs. resting), errors were calculated using the method described page 682 in (Green et al. 2001). We calculated one EE value per bird and its associated error for each state (or behaviour), then the difference in the estimate of EE between states/behaviours, and finally we averaged the difference over all birds (and calculated its associated error after equation 14, in Green et al. 2001). When comparing different behaviours (e.g. preening vs. stretching), we calculated the mean differences between those behaviours. The mean associated variance was then calculated as the sum of the variance associated with the two behaviours using equations 11 and 14 in Green et al. 2001. A Z-statistic allowed us to test for significant differences. A simple approximate normal test was then used to look for differences between states. However, when looking for differences between specific behaviours, we preferred the use of a permutation test due to a lower sample size in some of our groups (i.e. n = 12 cases of head-scratching and n = 14 cases of head-shaking). The Z-statistic calculated when comparing two specific behaviours was then compared against the distribution of 1000 Z-statistics calculated from the values randomly redistributed between the two behaviours, and P-values for differences between specific behaviours were calculated accordingly. Significant results are reported for $P < 0.05$ and Bonferroni's correction was applied whenever multiple comparisons were tested (differences were thus considered significant for $P < \frac{0.05}{n}$ with n the number of comparisons done). Results are given as means ± standard error (s.e.) unless otherwise specified.

3. Results

3.1. Behavioural time-budget of comfort activities

Scan sampling data showed that king penguins breeding and fasting ashore spend on average 22.0 ± 1.1 % of time in global comfort behaviour (n = 2270 scans). Focal observations (n = 200) showed that most of this time was devoted to preening (72.5 ± 2.2 %) and head-shaking (15.9 ± 1.9 %). Time spent in stretching and head-scratching was intermediate (3.7 ± 0.4 % and 4.0 ± 0.6 %, respectively), whereas only a limited amount of time was spent in tail-wagging or yawning (2.5 ± 0.7 % and 1.4 ± 0.6 %, respectively).

When considering data obtained from the five videoed birds of which comfort behaviour was examined during consecutive periods of 15 min spread over 24 hrs, we found no apparent day/night pattern regarding the proportion of time spent in global comfort behaviour (GEE; *Wald* = 0.07, P = 0.80, n = 480 focal observations).

3.2. Energy cost of global comfort behaviour

3.2.1. Estimate 1

When included in the GEE model, the proportion of time spent in comfort behaviour during 15-min focal observations significantly explained the variability of the corresponding average HR (R^2 = 0.19, *Wald* = 167, P < 0.0001, N = 5 birds, n = 480 focal observations). HR increased with proportion of time spent in comfort behaviour (**Figure 22**). From the slope of this equation (0.28) and from equation 1a relating EE to HR (see above, Groscolas et al. 2010), we calculated that for a 1% increase in the time spent into comfort behaviour (i.e. a 0.6 sec per min increase) the associated HR increase was equivalent to a 10.2 J increase in EE (36.4 * 0.28). Thus, the average energy cost of comfort behaviour was 1.02 kJ/min, or 61.2 kJ/hr (i.e. 17 W). Applying equation 1 in Fahlman et al. 2004 to the same data, we found that a 1% increase in the time spent in comfort led to an 37.0 mL O2/min increase in oxygen consumption, equivalent to a 44.7 kJ/hr (i.e. 12.4 W) cost of comfort behaviour (assuming the energy equivalent of 1mL O2 is close to 20.112 J; Schmidt-Nielsen 1979, Bevan et al. 1995). As stated in the “Methods” section, a valid s.d. could not be calculated for these estimates.

3.2.2. Estimate 2

Resting HR averaged over the resting periods preceding and following the 94 selected comfort episodes was 67.8 ± 1.4 bpm, whereas during comfort HR averaged 81.1 ± 1.8 bpm. Applying equation 1a in Groscolas et al. 2010 to each individual HR values, we calculated that during comfort behaviour EE averaged 3871 ± (s.d.) 334 kJ/day, whereas resting EE derived from resting HR values represented 3132 ± (s.d.) 297 kJ/day (i.e. a positive mean difference between comfort and resting states of 738 ± (s.d.) 336 kJ/day; Normal Test; Z = 2.19, P = 0.01). Hence, the energy expended during comfort behaviour was 1.24 times RMR.

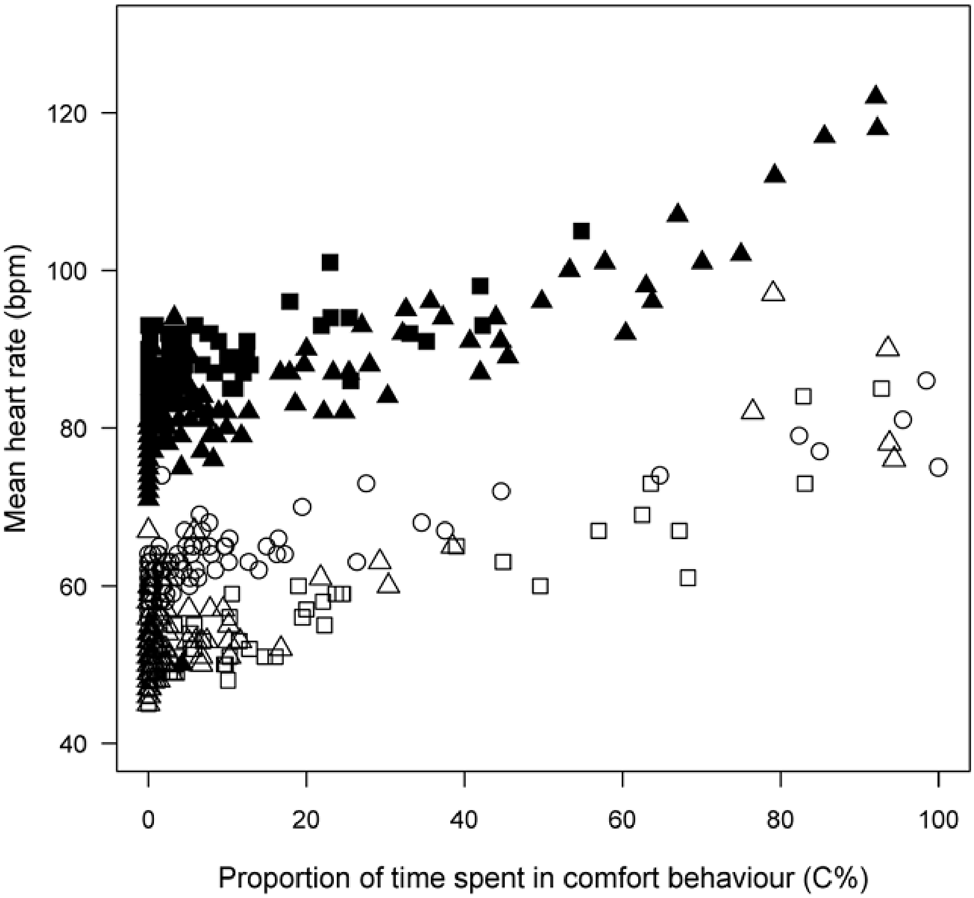

Figure 22. The energy cost of global comfort behaviour in incubating king penguins.

Relationships between the proportion of time spent in comfort behaviour during 15 min periods (C %) and corresponding mean heart rate (HR) level. Data are for 3 males (□, Δ, ○) and 2 females (■, ▲) with 96 values per individual. General equation for the mixed model is HR (bpm) = 65.89±3.95 + 0.28±0.02 * C %. (GEE, R^2=0.19, *Wald*=167, *P*< 0.001). From the slope of the relationship and the equation relating HR to energy expenditure, the energy cost of comfort behaviour was estimated at 61.2 kJ/hr (i.e. 17 W). doi: 10.3171/journal.pone.0021110.g001

From the total HR excess above resting HR, which accounts for both the increase in HR (including the recovery phase) associated to an episode of comfort and the duration of the episode, we calculated that the energy cost of global comfort averaged 58.2 ± (s.d.) 9.3 kJ/hr, or 16.2 ± (s.d.) 2.6 W (*N* = 24 birds, *n* = 94 episodes), i.e. a value differing from estimate 1 by only 5.0 %. Applying equation 1 in Fahlman et al. 2004 to these data, we found that average oxygen consumption during comfort behaviour was 1.22 higher than when the animals were resting (i.e. 102.6 mL O2/min vs. 84.8 mL O2/min, for comfort and resting states, respectively). The average cost of comfort behaviour was then 37.3 mL O2/min, corresponding to 45.0 kJ/hr (or 12.5 W), i.e. a value differing from estimate 1 by less than 1.0 %.

3.3. Energy cost of specific comfort behaviours

During stretching and head-scratching, HR markedly increased above resting values, from 63.3 ± 1.1 bpm to 106.3 ± 3.1 bpm (*n* = 31), and from 63.7 ± 2.8 bpm to 102.4 ± 8.3 bpm (*n* = 12), respectively (Wilcoxon's test, *W* = 2, *P* < 0.001 and *W* = 7, *P* < 0.001, respectively). The average HR increase associated with preening (from 60.9 ± 1.9 bpm to 75.4 ± 2.2 bpm, *n* = 21) and head-shaking (from 63.4 ± 2.3 bpm to 75.2 ± 3.8 bpm, *n* = 14) was moderate (Student's t-test, *t* = - 4.875, *P* < 0.001 and *t* = - 2.632, *P* = 0.015, respectively) whereas no significant HR increase was observed during yawning (Wilcoxon's test, *W* = 168.5, *P* = 0.40, *n* = 20). Correspondingly, energy expenditure calculated from equation 1a in Groscolas et al. 2010 ranged from 1.03 (i.e. yawning) to 1.78 (stretching) times RMR. From the total HR excess above resting HR (including the recovery phase) associated to specific behaviours, we calculated that the energy cost of stretching was 2, 6, 9 and 61 times more than that of head-scratching, preening, head-shaking and yawning, respectively (**Figure 23**). Similar results were obtained when using equation 1 in Fahlman et al. 2004. For example, cost of stretching was roughly 2, 6, 9 and 60 times more than that of head-scratching, preening, head-shaking and yawning, respectively. From these costs and from the proportion of comfort time spent in the different types of comfort behaviour, we estimated that approximately half of the energy cost of comfort behaviour was for plumage cares (preening plus head-scratching).

3.4. Contribution of comfort behaviour to daily energy expenditure

The daily energy cost of comfort behaviour calculated by multiplying the time spent each day in comfort behaviour (22 % or 5.28 hr) by its cost was 323 kJ/day (estimate 1), 307 kJ/day (estimate 2). The average daily and resting HR of the 24 birds used to determined DEE, RMR and the average cost of comfort behaviors (estimate 2) were 73.4 ± 3.0 and 65.0 ± 2.6 bpm, respectively. The corresponding DEE and RMR were 3465 ± (s.d.) 306 and 3026 ± (s.d.) 296 kJ/day, respectively. The energy cost of comfort behaviour thus represented 9.3–8.8 % of DEE (estimates 1 and 2, respectively). The cost of activity, i.e. the difference between DEE and RMR, was 440 kJ/day (or 12.7% of DEE), and most of this cost corresponded to comfort activities (viz. 73.5–69.8%; estimates 1 and 2, respectively).

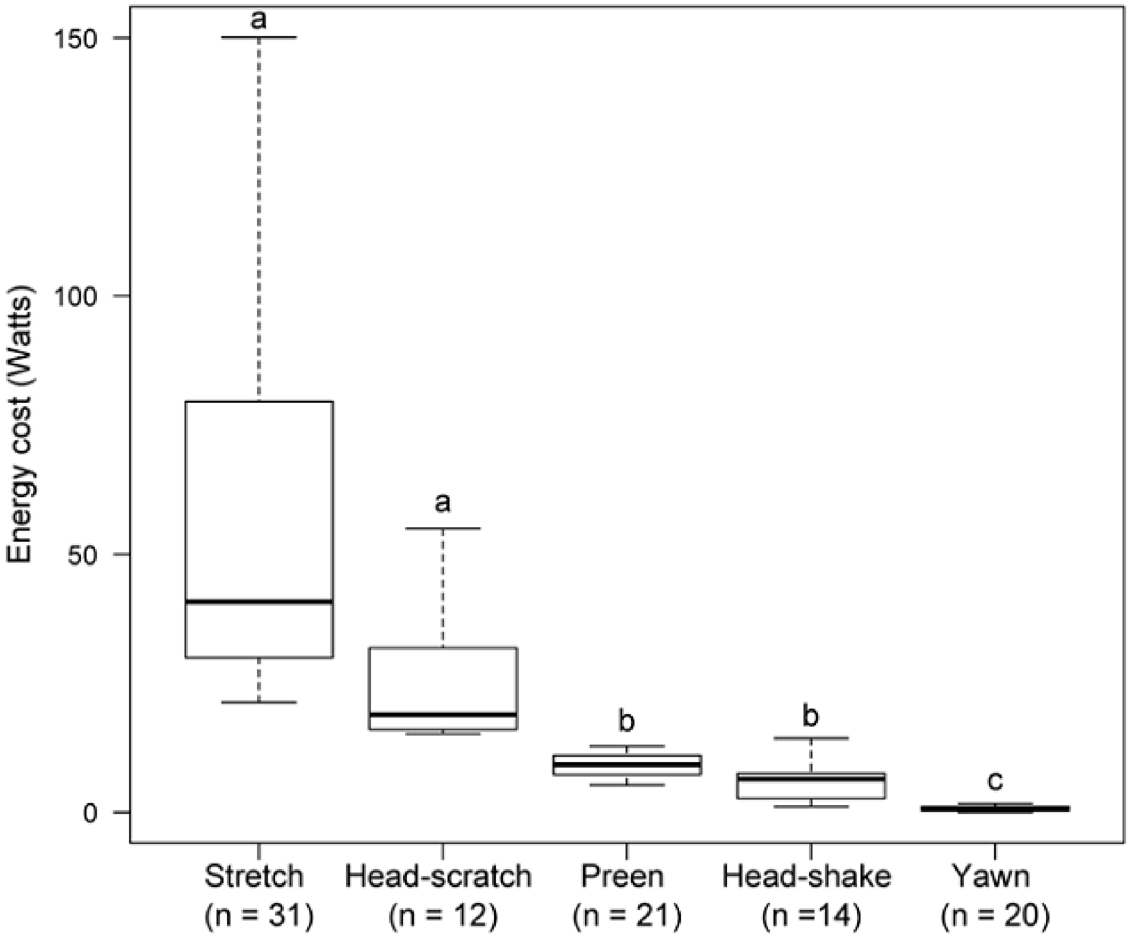

Figure 23. The energy cost of specific comfort behaviours in breeding king penguins.

Light bars and dashed lines show the range of values, heavy bars medians. Boxes give the inter-quartile values. Sample sizes are given in brackets. Values not sharing a common superscript are significantly different for $P < \frac{0.05}{10}$.

When using equation 1 from Fahlman et al. 2004, the average daily energy cost of comfort behaviour, the average DEE and RMR were estimated at 238, 2748 and 2416 kJ/day, respectively. Thus, consistently with the results presented above, the energy cost of comfort behaviour and of activity calculated using equation 1 in Fahlman et al. 2004 represented 8.6 % and 12.1% of DEE, respectively.

4. Discussion

To our knowledge, this study is the first to estimate the contribution of comfort behaviour to overall energy budget in a free-living animal. It shows that, although fasting for a prolonged duration, king penguins devote a substantial part of their daily time and energy budget to comfort behaviour when breeding ashore. Approximately half of the energy cost of comfort was allocated to plumage maintenance and half to behaviours involving a vigorous muscular activity. These results highlight the importance of comfort behaviour for self-maintenance in colonial seabirds and suggest that the energy invested into comfort activities is the necessary price to pay for animals to maintain a good body condition and proper physical fitness, potentially at the expense of immediate breeding success but to the benefit of survival and foraging efficiency. Before discussing the adaptive significance of our results, it was necessary to consider whether the methodology used yielded valid estimates of both time-budget and energy expenditure.

4.1. Comfort time and energy budget

We found that incubating penguins spend 22 % of their daily time budget in comfort behaviour. This figure was obtained using a very large number of birds and instantaneous scan sampling equally spread over the whole breeding season, including birds of both sexes and all breeding states representative of the successive incubation and brooding shifts observed in king penguins. Importantly, this figure was derived from wild birds that were not equipped with HR loggers; so that time spent preening could not have been influenced by the attachment of external devices to the animals' body (see Barron et al. 2010). Additionally, and in agreement with a previous report (Challet et al. 1994), we obtained evidence that there was no day/night pattern in comfort behaviour. Thus, we are confident that the 22% figure obtained here for the overall proportion of time in comfort behaviour is representative of king penguins breeding ashore, over 24 hrs. In a previous study, this proportion was estimated at 16% (Challet et al. 1994). However, since these data were obtained from a limited number of individuals observed for only one day, they may not be fully representative of the whole population throughout the whole breeding season.

When estimating EE from HR, one should be cautious with the calibration equation used. The method of calibration needs to match the data that are being estimated as closely as possible to avoid potential increases in the error associated to the prediction made (Green 2011). The question is then whether equations calibrated over longer time scales (i.e. equation 1a in Groscolas et al. 2010, calibrated over 4 days) are appropriate for estimating the energy cost of behaviours that last only minutes? Whereas this concern is most certainly justified, one should bear in mind the trade-off scientists must face. Establishing calibration equations relating HR to EE over the scale of seconds or minutes is simply not possible unless it is done by measuring oxygen consumption (VO2), which then requires keeping animals captive and monitored in respirometry chambers. Although this approach is undoubtedly the most thorough, it raises the issue of experienced stress (Groscolas et al. 2010, Green 2011), which may well influence the HR–VO2 relationship (as HR is then not necessarily entirely reflective of actual oxygen uptake), leading to biases in the estimation of EE (Groscolas et al. 2010). This may be the case in our study where we observe that when using equation 1 from Fahlman et al. (2004), which was calibrated over a period of time closer to the duration of comfort behaviours, we find estimates of EE for global and specific comfort behaviour to be 20 to 27 % lower than estimates of EE calculated from equation 1a in Groscolas et al. (2010), which was derived from wild birds fasting, incubating and having a moderate level of physical activity (i.e. animals in a situation similar to those used in this study). The discrepancy observed between the estimates is consistent with that previously reported in Groscolas et al. (2010). Nonetheless, as the difference is constant, it is reassuring to find that regardless of the equation used, the cost of global comfort behaviour when expressed as a proportion of RMR was almost identical (1.22 or 1.24 x RMR, for estimate 2 for instance). Also, using both equations from Fahlman et al. (2004) and Groscolas et al. (2010), we found similar relative costs of specific comfort behaviours as well as similar contributions of comfort behaviour to daily energy expenditure (e.g., 8.6 – 9.3% of DEE, respectively). Such findings support the view that EE *vs.* HR equations obtained by using two markedly different methods may yield different levels of overall energy expenditure but similar relative costs of specific activities, in this case comfort behaviour.

Further, the validity of our estimates is also supported by considering our results on the contribution of comfort behaviour to energy expended for total activity, and previous studies. Based on HR recording over 24 hrs, we calculated that total activity contributed for 12.7% to DEE. In breeding king penguins, active behaviours include comfort behaviour, aggressive behaviour and parental care provided to the egg or chick, the two later behaviours representing 8-24% and 1-2% of the time budget (Challet et al. 1994). Aggressive behaviours contribute for 2.7% to DEE (Viera et al. 2011). The contribution of comfort and aggressive behaviours (by far most frequent active behaviours) to DEE summed up to 11.3-12.0%, depending on whether equation in Groscolas et al. (2010) or Fahlman et al. (2004) is used. This sum is very close to the contribution of all daily activities to DEE (i.e. 12.1-12.7% according to which equation is used) suggesting that the cost of comfort determined in the present study is realistic. The energy cost of egg and chick caring (which involve movements with an intensity comparable to that of comfort and aggressive behaviours but only contribute to 1-2% of time-budget) would be very minor (< 0.5% of DEE), which also seems reasonable. Additionally, the finding that stretching and head-scratching are more costly than preening, head-shaking and yawning was to be expected, given that the former are associated with vigorous muscular activity whereas the latter only require discrete activity.

The proportion of time devoted to comfort behaviour by king penguins (22 %) is amongst the highest reported for birds (Cotgreave and Clayton 1994). It is also higher than the proportion of time spent in territory defence (8-10 %; Challet et al. 1994, 11.5-18.7 %; Côté 2000), a behaviour that appears highly beneficial to breeding success in this colonial species (Viera, Côté and Groscolas, unpublished data). In addition, previous data (Viera, Groscolas and Côté, unpublished data) indicated that there was no difference in the time invested into comfort according to gender or to some parameters affecting breeding success. Actually, the time spent in comfort does not differ between birds located at the periphery vs. the centre of the colony, although breeding success is suggested to be higher at the centre (Côté 2000). Similarly, early and late breeding birds devoted the same proportion of time to comfort, even though breeding success is markedly lower in late breeders (Weimerskirch et al. 1992). Lastly, Challet et al. (1994) reported that i. the time devoted to comfort behaviour was similar in incubating and brooding penguins, and ii. the same proportion of time was spent for comfort behaviour throughout an incubation shift, i.e. whatever the fasting duration and thus energy stores. Thus, it appears that king penguins spend a high proportion of time in comfort behaviour regardless of energy constraints imposed by their breeding pattern, and regardless of some components of their breeding success. If comfort behaviour were not energy costly, this high proportion of time could be the mere consequence of the fact that king penguins breeding ashore have no major time constraints (e.g. for food searching or anti-predator defence) and thus may devote a large part of time to other behaviour, e.g. body maintenance. Actually, devoting approximately 9% of its energy budget to comfort behaviour while totally depending on energy reserves for surviving must be considered as costly. Indeed, this cost is equivalent to the energy required to fuel DEE for about 1.5 of the 15-day incubation shift. When energy reserves are close to exhaustion, being able, or not, to fast for 1.5 supplementary days while waiting for the return of the partner might well mean going on incubating or abandoning the egg, i.e. being a successful breeder or not. Thus, the energy expended for comfort behaviour might be at the expense of immediate breeding success. On the other hand, the finding that king penguins are willing to pay a substantial energy cost for comfort behaviour strongly supports the view that this behaviour is adaptive and procures major benefits, including from an energy view point.

4.2. Adaptive significance of comfort behaviour

In incubating penguins, comfort behaviour likely plays an essential role in the maintenance of a functional outer shell and musculature, and in the removal of ectoparasites. Given that they are fasting and must spare energy (Groscolas 1990, Robin et al. 2001), incubating king penguins cannot afford the potential excess energy costs associated with a decrease in the insulating and waterproofing properties of their plumage. The same is of even greater importance for penguins swimming and diving into cold waters when intensively foraging between two incubation shifts ashore. Unfortunately, no data are available to estimate how much defects in plumage integrity may incur thermal costs in penguins, and thus how much maintenance of plumage through preening may allow energy saving. A doubling in body mass loss reflecting a comparable increase in metabolic rate has been observed in moulting and thus poorly insulated penguins fasting ashore (Groscolas 1978). However, how this increase partitions between thermal loss and feather synthesis is unknown. On the other hand, data on the energy cost of ectoparasite loads have been obtained both in birds and mammals. For example, a high bug (*Oeciacus hirundinis*) load imposes an about 13 % increase in mass independent DEE in house martin (*Delichon urbica*) nestlings (Møller et al. 1994). In the feral dove *(Columbia livia*), a high load in feather-feeding lice (Phthiraptera: Ischnocera) reduced feather mass, leading to an 8-12 % increase in thermal conductance and to a 10 % increase in basal metabolic rate (Booth et al. 1993). Lastly, in mouse-eared bats (*Myotis myotis*), a high mite (*Spinturnix myoti*) load induces an up to 21 % increase in O2 consumption and a 15 % body mass loss compared to non-infested individuals (Giorgi et al. 2001). Thus, a high ectoparasite load may incur significant energy costs, in addition to other negative effects such as inoculation of toxins and transmission of pathogens. King penguins are known to be infested by various species of ectoparasites, including *Ixodes uriae* ticks known to be a vector of viruses and of the Lyme disease agent *Borrelia burgdorferi* (Gauthier-Clerc et al. 1998). In the study colony, a reduced incubating success has been observed in infested areas (Mangin et al. 2003) and hyperinfestation by ticks has been suggested as a possible cause of death in adults (Gauthier-Clerc et al. 1998). Ensuring thermal insulation, by keeping the plumage in a good condition in one of the windiest and rainiest places on earth, and limiting ectoparasite load, may well be essential for king penguin survival. Thus, it is understandable that this bird devotes the greatest part of time and about half of the cost of comfort behavior (i.e. roughly 5 % of DEE) to preening activities.

Besides the need to have a perfectly insulated and waterproof plumage, ashore and at sea, and to limit ectoparasite load, king penguins would probably take advantage of being efficient foragers as soon as they return to sea to replenish their energy stores. This would allow them to limit the duration of their foraging trips at sea, and thus to limit the risk of egg desertion by their incubating partner. Given that they use feeding grounds situated several hundreds of kilometers from the colony (Bost et al. 1997, Jouventin et al. 1994), and that they have to dive repeatedly at great depths (over 200 m) to catch their prey (Charrassin et al. 1998, Pütz et al. 1998), it would be advantageous for king penguins departing to sea after an incubation shift to be as physically fit as possible. Maintaining minimum levels of muscular activity and preventing muscular ankylosis while on land (through shaking, stretching or other vigorous comfort activities), even if it costs around 5 % of DEE, might well be a necessary condition for penguins to maintain this physical fitness. We therefore suggest that the energy invested in comfort behaviour by breeding king penguins is the necessary debt to be paid ashore in order to maintain plumage insulation and waterproofing, to limit the impact of ectoparasitism, and to be optimal divers and foragers when they return at sea. Such an energy investment may contribute to improve penguin survival and foraging efficiency.

These suggestions are mostly based on the determination of the average cost of comfort behaviour over a breeding season and for penguins located in a given part of the colony. A full understanding of the adaptive significance of comfort behaviour, and, more generally, of how energy and environmental constraints shape the behavioural repertoire of colonial seabirds, will obviously require further investigations. First, it would be interesting to determine whether the time and energy devoted to preening is actually fixed or rather related to parasite load. This could be achieved by comparing the time invested into preening at different locations of the study colony known to have different parasite loads (Mangin et al. 2003), and by examining the relationship between preening time and parasite load at the individual level. Second, examining whether comfort behaviour competes with other behaviours such as territory defence will allow a better understanding of how colonial birds trade time and energy between self-maintenance and behaviours more directly related to reproductive success. Indeed, visual observations of king penguins indicate that engaging into comfort behaviour very often induces aggressiveness from neighbours such as these two behaviours seem at least partly conflicting. Lastly, whether comfort activities involving vigorous physical motions (e.g. stretching, shaking) may contribute to maintain physical fitness thus improving foraging efficiency could be tested by relating the time devoted to these specific behaviours while breeding on land, to swimming, diving and foraging performances at sea. This would help understanding how, in seabirds, behaviours ashore and at sea are energetically interrelated.

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STUDY 2 – Estimating the energy costs of specific behaviours: Aggressive behaviour

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Active territory defence at a low energy cost in a colonial seabird

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Abstract

Aggressive behaviour associated with the defence of a territory is thought to incur substantial energy costs and thus to represent a trade off with other energy demanding activities. The energy costs of aggressive behaviours, however, have rarely been estimated in the wild, and the overall contribution of territorial defence to daily energy expenditure has never been determined. We studied the activity budget of breeding king penguins (*Aptenodytes patagonicus*) equipped with heart rate data loggers to estimate the energy costs associated with territory defence in this colonial bird exhibiting very high rates of agonistic interactions. We also assessed whether threat displays incurred lower energy costs than attacks with body contact. During territorial defence (i.e., threats and physical attacks combined), energy expenditure averaged 1.27 times resting metabolic rate, aggressiveness costing 30 kJ/h. Defence accounted for 13 % of the daily time budget and contributed to 2.7 % of the total daily energy expenditure. Interactions with body contact cost 3 times more than threat displays, but accounted for only 16 % of the aggressive behaviours recorded (16 attacks/h *vs* 81 threat displays/h). Neither did body mass, body size, penguin gender nor breeding stage affect the cost of aggressiveness. Our results were consistent with previous research reporting that fighting incurs significant metabolic costs. However, we found that aggressive behaviour in king penguins was not an expensive activity compared to the total energy budget. Because while breeding ashore king penguins are fasting and sleep deprived, they may have developed behavioural strategies (e.g. lower rates of attacks with body contacts) allowing them to efficiently defend their territory at a low energy cost.

1. Introduction

In many animal species, individuals perform aggressive behaviours to access limited resources, such as food, territories, or mates, which can improve their fitness (Cutts et al. 1999, Hagelin 2002, Logue and Gammon 2004). Usually however, individuals will limit their use of aggressive behaviours because they often incur short-term costs in terms of time, energy demands, or risk of injuries (Hack 1997, Neat et al. 1998; but see Riechert 1988), and these proximate costs can result in fundamental long-term fitness costs (Hagelin 2002, Langkilde et al. 2005). They may also increase predation risk as a result of reduced vigilance during fights (Brick 1998, Jakobsson et al. 1995). Nevertheless, the benefits of defending a resource are generally higher than the costs associated with aggressive behaviours, so that agonistic interactions among individuals are common (Maynard-Smith 1982).

Based on trade-offs between expected benefits and costs of fighting, different strategies and decision rules have evolved for resolving conflicts (Enquist and Leimar 1983, Maynard-Smith and Parker 1976). As physical aggressions are associated with a greater risk of injury than threat displays (Clutton-Brock et al. 1979), most animals typically assess the fighting ability and motivation of their opponent before escalating into a fight with body contact (Jonart et al. 2007, Payne 1998). Animals may therefore assess correlates of resource holding power (RHP), a measure of the probability of winning an escalated contest (Maynard-Smith and Parker 1976, Parker 1974). Resource holding power is largely determined by the individual characteristics such as body size and condition (Huntingford and Turner 1987 for a review; Marden and Rollins 1994). In addition to RHP, fighting success may also depend on the individual’s motivation to fight (Enquist and Leimar 1987 for a review; Elwood et al. 1998), which in turn depends on its willingness to expend energy or to risk injuries (Enquist and Leimar 1987). Such motivation may be estimated by determining if the individual is the initiator or the receiver in a fight (Arnott and Elwood 2007, Briffa and Sneddon 2007).

Although aggressive behaviours are expected to incur substantial energy costs (Parker 1974; Riechert 1988), only a few empirical studies have attempted to quantify these costs (Smith and Taylor 1993), and all studies were performed in laboratory conditions (e.g. Briffa and Elwood 2004, Hack 1997, Neat et al. 1998). Previous studies estimated the energy costs of aggressive behaviours by measuring contests duration (Smith and Taylor 1993, Morrell et al. 2005) or by monitoring physiological changes, such as increases in lactate and energy expenditures or reductions in energy stores (Rovero et al. 2000, Briffa and Elwood 2004, DeCarvalho et al. 2004). An alternative and non-physiologically invasive approach to estimate the energy costs of various behaviours is the monitoring of heart rate (HR), but calibration issues still limit the application of this method (Butler et al. 2004).

In free-living animals, a direct estimate of the energy cost of specific aggressive behaviours coupled with the contribution of these costs to the overall energy budget has never been performed. This is a major gap in our understanding of the behavioural strategies used by animals for territorial defence under natural conditions because only knowledge of these costs can provide information on how energy constraints may have shaped behavioural interactions among individuals of various species. Achieving such a goal requires the simultaneous determination of the energy costs associated with the various aggressive behaviours of the study species, and of the time spent performing these behaviours. Up to now, this has proved challenging but the use of a validated indirect method to estimate the cost of activities on a short time scale, e.g. heart rate recording (Butler et al. 2004), in an easily accessible and observable species would offer such an opportunity.

King penguins (*Aptenodytes patagonicus*) are highly territorial seabirds that breed in dense colonies and exhibit high rates of aggression, as pairs vigorously defend a small territory of about 0.5 m² (average rate of 100 interactions/bird/h, Côté 2000). Given that king penguins are facing severe energy constraints while breeding ashore because they fast during extended periods (up to 30 days, Groscolas and Robin 2001) while being sleep-deprived (Dewasmes et al. 2001), our study aimed at answering the following question: how might an animal sustain high rates of aggressive behaviour in a context of energy limitation? To quantify the energy costs of aggressive behaviours in free-living breeding king penguins, we first assessed time spent in agonistic interactions using behavioural observations. Then, we used heart rate (HR) variations during agonistic interactions and equations relating energy expenditure (EE) to HR in freely breeding king penguins (Groscolas et al. 2010) to estimate the energy cost of overall and specific interactions. Notably, we evaluated the energy costs of threat displays and of attacks with physical contact, expecting physical contacts to be more costly than threat displays because of corresponding higher physical activity. We also determined whether the energy cost of aggressive behaviours differed between fight initiators and fight receivers, or was affected by gender, body mass, body size and breeding stage. Finally, we assessed the contribution of aggressive behaviours to daily energy expenditure (DEE), expecting that territory defence would contribute to a significant proportion of DEE because of the elevated rate of agonistic interactions.

2. Methods

2.1. Animals

We conducted this study on Possession Island (Crozet archipelago 46°25’ S, 51°52’ E) over three breeding seasons between 2003 and 2007. We monitored king penguins in a sub-colony of ca. 3500 pairs in the colony of La Baie du Marin. A total of 381 birds (211 males and 170 females) were flipper-banded from November to March, i.e. throughout the incubation and young chick brooding periods. During the incubation (~ 53 days) and chick-brooding stage (~ 30 days), females and males alternate between periods of fasting on land and foraging at sea (Weimerskirch et al. 1992). Incubation shifts average 15 days and brooding shifts 8 days (Weimerskirch et al. 1992). As males always perform the first incubation shift, we easily sexed birds from observations of breeding behaviour. We checked marked birds twice daily to determine the laying date as well as the onset of each incubation and brooding shift.

2.2. Behavioural observations

In king penguins, aggressive behaviours for territory defence include threat displays (no physical contact) and attacks with body contact. Threat displays consist of beak pointing (no vocalization, beak closed, body stretched out) and gaping (pointing but with bill open and vocalizing, body stretched out), whereas attacks with body contacts consist of pecking and flipper blows (Côté 2000).

2.2.1. *Scan sampling*

From November 2006 to March 2007, the average proportion of time spent in territory defence by breeding penguins was estimated using scan samplings (Altmann 1974). Scans were performed every 15 days on random unmarked (n = 88) or marked male (n = 101) and female (n = 81) king penguins. Most individuals were scanned only once but some were scanned up to three times, each time at a different incubating or brooding shift. During each scan, we considered thirty of these birds and counted the number of birds engaged into aggressive behaviours. Scans were performed every five minutes, for at least six consecutive hours. The scans totaled 63 h of observations. Individuals observed during scans were located at least 4 m apart to maximize independence of their behaviour relative to their neighbours. We balanced observations during all hours of daylight, from 6h00 a.m. to 20h00 p.m., *i.e.* approximately 7% of scans for each hour.

2.2.2. *Video recording*

Video recording was used to compare the time spent in aggressive behaviours between day and night. In January 2004, we performed video recording of 30 marked birds (18 males and 12 females). Videoed birds were located within a 6 m diameter zone under the field (8 x 20 m) of a fixed video camera and at 3 to 7 m from the edge of the colony. The video camera was set at 3 m height and coupled with infrared lighting to observe individuals 24 h/day. We recorded the behaviours of birds continuously at the frequency of three images per second using a Panasonic® (model TL750) video-recorder. A preliminary study confirmed that behavioural time-budgets from video recordings were equivalent to visual focal observations. For example, 20 focal observations (Altmann 1974, Côté 2000) performed both visually in the field and using video recordings led to estimated proportions of time spent into aggressive behaviours which were not significantly different, i.e. 18.6 ± 3.5 % (visual) versus 17.5 ± 3.3 % (paired Wilcoxon signed rank test, V = 61, *P* = 0.11). To estimate time spent into aggressive behaviours day and night, the 24h video recordings of five individuals (3 males and 2 females selected at random) were divided into 15-min consecutive focal observations (*i.e.*, 96 focals per bird and 480 focals in total) and the proportion of time spent in aggressive behaviours was calculated for diurnal and nocturnal focals. Since video recording was performed on birds previously equipped with HR loggers, a first estimate of the global energy cost of aggressive behaviours was obtained from those birds, based on HR increase during episodes of aggressive interactions (see below).

2.2.3. Visual focal observations

Visual focal observations were performed for comparing the frequency rates of threat displays *vs* attacks with body contact. Between November 2004 (laying date) and March 2005 (chick-brooding period), we performed five hundred and fifty 15-min focal observations in the field (2 to 7 focals per individual; Altmann 1974, Côté 2000) to record the behaviour of 169 marked penguins (92 males and 77 females). Body mass and body size of these birds were determined and used as indices of RHP. Birds were hand-caught in the colony and transported to a nearby shelter. They were weighed on an electrical platform scale (± 10 g), in the natural incubating position. Their flipper length was measured (with a ruler ± 1 mm) as an index of body size. Because displacing the birds was risky (i.e. potential egg loss and thus breeding failure), only birds which remained calm when handled were weighed, i.e. 100 of 169 birds. We conducted observations from 06.00 to 20.00 h at a distance of 10-250 m, using binoculars and spotting scopes when necessary to minimize disturbance. During each focal observation, aggressive interactions, but also other mutually exclusive behaviours such as resting and comfort behaviour were timed. When a bird was engaged in an aggressive interaction (which occurred for 488 of the 550 focals), we recorded the number of different aggressive behaviours to estimate frequency rates of interactions with and without body contact. Since visual focal observations were performed on penguins previously equipped with HR loggers they allowed us *i.* to obtain a second estimate of the global cost of aggressive behaviours from a large number of birds, *ii.* to determine the energy cost of threats *vs.* attacks, *iii.* to test for the potential effects of gender, breeding stage, body mass and size on these costs, and *iv.* to calculate the contribution of territory defence to the daily energy budget of breeding king penguins (see below).

2.3. Heart rate recording

When manipulated for HR logger equipment, weighing and body size measurement, incubating or brooding birds were hand-caught while standing on their territory. Equipped birds were located in a sub-colony where penguins have been habituated to human presence for the last 50 years. Indeed, the study colony is close to a permanent station and scientific installations are present in its close vicinity (*c.a.* 50 m). A current study shows that incubating penguins at this location only become vigilant (slight head movements) and initiate displacement (slight leg movements) when closely approached by humans (at 8-10 and 1-2 m, respectively; Groscolas and Viblanc, unpublished data). Thus, when approached for capture, incubating or brooding birds did not try to flee, so that their agitation and that of their neighbours was limited.

We recorded the HR of birds with an externally mounted data-logger (Polar®, model S810, Polar Electro, Finland) adapted for use on king penguins (Groscolas et al. 2010). The system included two units: a sensor-transmitter (30–40 g) and a receiver/logger (a recording watch, 30 g). The HR logger package weighed < 1 % of adult body mass. Heart rate transmitters were attached in a dorsal, midline position with Tesa® tape wrapped around several layers of feathers. This position prevented hindering movements of birds. Electrodes made from gold plated safety needles were placed subcutaneously and separated by 25 cm. One electrode was placed at the height of the wing pit and the second one just above the tail. Electrodes were disinfected with iodine (Betadine®) and alcoholic antiseptic solutions and inserted under the skin in the subcutaneous fat layer (at approximately 5 mm depth, and over a length of 1 cm). Safety needles were secured in order to prevent their retraction. We never observed birds trying to remove electrodes or HR loggers, nor did we observe any adverse effects of equipment on birds' health or behaviour. Further details on this method and how it accurately estimates HR of king penguins are described in Groscolas et al. (2010). We equipped birds with loggers one or two days after the beginning of an incubation or brooding shift, and removed loggers one or two days before the end of the average duration of the corresponding shift to avoid losing them. HR values were therefore obtained from birds fasting for one to 14 days (an average of one week). HR was recorded either at a fine scale (5-s intervals, videoed birds), but for no more than 45 h given the storage capacity of the HR logger, or at a larger scale (1-min intervals, field focal observations) allowing up to 20 days of continuous recording. The advantage of 5-s recording was the very fine resolution allowing a close matching of HR and behavioural changes, whereas the advantage of 1-min recording was to collect a large number of HR-behaviour data throughout the whole incubation and brooding shifts.

2.4. Matching aggressive behaviour with HR records

2.4.1. Videoed birds

From 7 randomly selected birds (5 males at shift 1 of incubation and 2 females at shift 2) of the 30 birds fitted with HR loggers and video-recorded, we selected 36 episodes during which birds exclusively performed continuous aggressive behaviours (3 to 10 episodes per individual, both during day or night). Selection was at random regarding the type of aggressive interactions so that data were considered representative of average aggressive behaviour. The selection of sufficiently long episodes was difficult and time consuming because aggressive behaviours were very frequently interrupted by other behaviours, e.g. comfort behaviour. In addition, episodes of aggressive behaviour had to be preceded and followed by resting periods lasting over 1 min (on average 2.4 ± 0.6 and 1.3 ± 0.2 min for the pre- and post-aggression resting durations, respectively; see **Figure 24a**), which also proved difficult to obtain. Such resting periods were required to accurately calculate the energy cost due to aggressive behaviours, i.e. energy spent in excess to resting metabolic rate (RMR; see below). These difficulties explain why the selected episodes were not equally distributed over individuals. The rate of aggressive behaviours (number/min, threats and body contacts together) was calculated for each episode.

2.4.2. Visual focal observations

For birds the HR of which was measured every min, we divided each of the 550 15-min focal observations into 15 episodes of 1 min. For each 1-min episode, we assigned the HR value measured at its end to territory defence only if this behaviour occupied at least half of the time, i.e. > 30 s, of the min preceding HR measurement. For periods of continuous territory defence lasting over one min, we assigned to this behaviour the corresponding average HR. In addition, only episodes assigned as territory defence and preceded and followed by at least one 1-min episodes assigned as resting (see **Figure 24b**) were considered. In total, we obtained 589 1-min episodes of aggressive behaviours that respected our criteria, of which 345 episodes were obtained for individuals of known morphometrics (i.e. body mass and body size). These episodes were obtained from 269 focals among the 550 focals initially performed, and from the 169 individuals used for focal observations (2 to 4 episodes per individual). The resting HR associated to each of these episodes was the average of pre- and post-aggression resting HR. We further redefined 1-min episodes associated to aggressive behaviours during 15-min focals by discriminating when birds performed only threat displays or only attacks with body contact. In addition, for these two types of behaviours we discriminated whether the focal bird was the initiator or the receiver of the interaction. We discarded all 1-min episodes when both behaviours occurred. A total of 481 episodes were thus selected, of which 127 were attacks (58 as initiator and 69 as receiver) and 354 episodes were threats (170 as initiator and 184 as receiver). Among the 481 episodes, we obtained 279 observations for which both birds' body mass and the type of behaviour were known (72 observations, 63 birds for physical attacks and 207 observations, 93 birds for threat interactions).

2.5. Estimating the energy cost of aggressive behaviours

2.5.1. Videoed birds

Energy costs of aggressive behaviours were calculated from the corresponding increase in HR (**Figure 24a**), according to the method described by Smith and Taylor (1993). This method was also used by Rovero et al. (2000) to estimate the energy cost of fighting in shore crabs (*Carcinus maenas*). All HR calculations were done using the Polar® ProTrainer software. We estimated resting HR by averaging pre- and post-aggression resting HR. Fighting HR was the mean HR during aggressive behaviours and recovery HR was the mean HR during the recovery phase, i.e. from the end of the aggressive behaviour to the time needed for HR to recover to resting levels. Excess HR during aggressive behaviours was calculated as [(fighting HR - resting HR) x fight duration] / resting HR (Smith and Taylor 1993). This excess corresponded to the time that would be required for the number of heart beats in excess during fighting to occur at the resting HR level (Rovero et al. 2000). The same calculation was performed for the recovery phase, using recovery HR, and the total excess in time due to a fighting episode was the sum of excesses during aggression and recovery. The energy cost of an aggressive episode (kJ) was calculated as: excess in time (min) x resting metabolic rate (kJ/min). Dividing the cost of the episode by its duration (min) and then multiplying by 60 yielded energy cost in kJ/h. Resting metabolic rate was estimated from resting HR using equation *1a* in Groscolas et al. (2010): EE (J/min) = –387 + 36.4*HR (bpm) ($F_{1,133} = 19.33$, $R^2 = 0.85$, $P < 0.0001$). This equation was obtained from freely incubating male and female king penguins (no gender difference) fasting for a duration comparable to that of birds in the present study and having similar breeding status and level of physical activity. Such pre-requisites are required for validly estimating EE from HR (Butler et al. 2004). A validation test performed by Groscolas et al. (2010) showed that EE predicted from HR using the above equation did not differed significantly from measured EE.

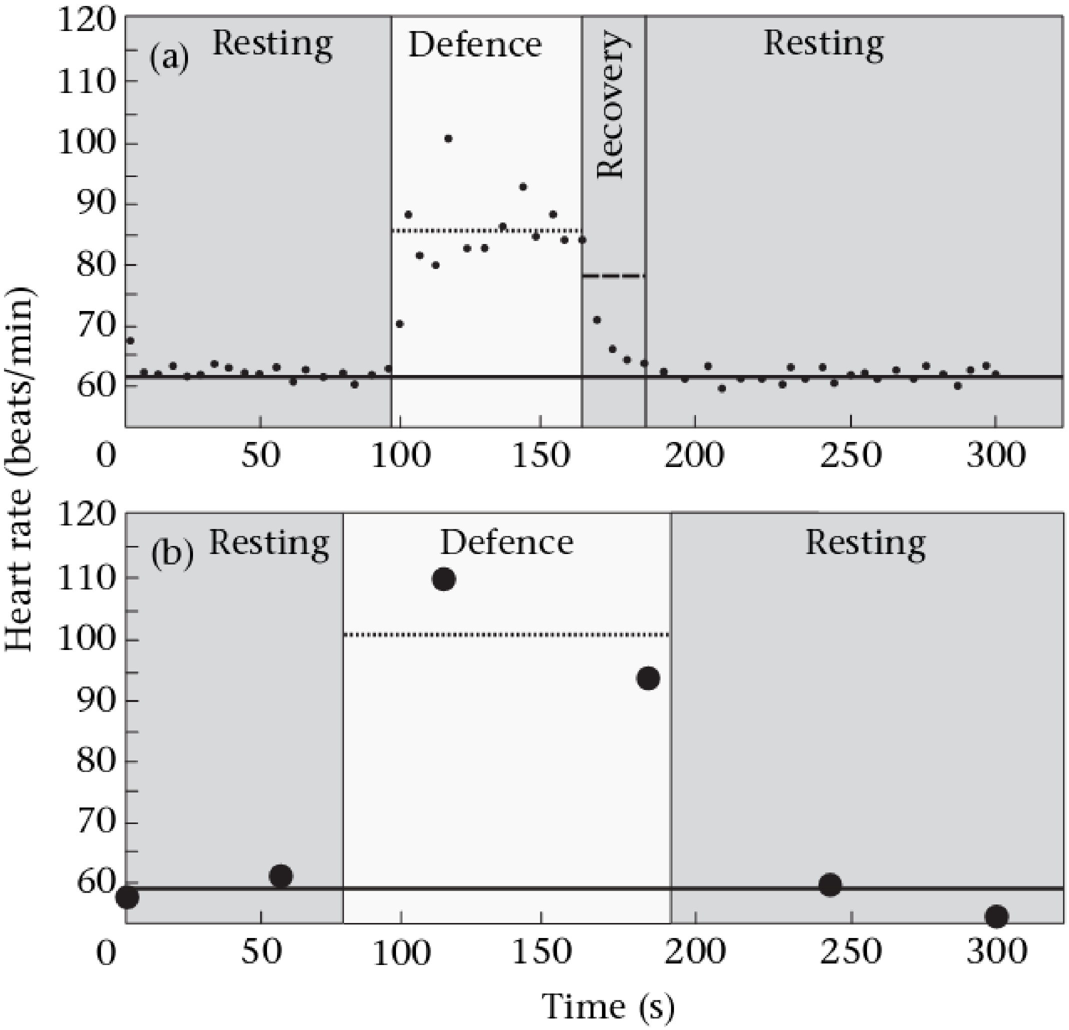

Figure 24. Example of a continuous record of heart rate (HR, beats/min) showing the increase in HR during an episode of territory defence in king penguins.

Recording was at the sampling rate of one HR value per (a) 5 s (videoed birds) or (b) per 1 min (focal observations). Shaded areas indicate the resting periods before and after the episode of defence. In (a) a recovery period is shown, which could not be accurately times in focally observed birds in (b). The continuous horizontal line shows resting HR whereas horizontal dotted and dashed lines show average HR during territory defence and recovery period, respectively. doi: 10.1016/j.anbehav.2011.04.001.

2.5.2. *Visual focal observations*

We used a similar procedure for calculating energy costs in birds subjected to visual focal observations except that we could not include the recovery phase because of the large time resolution for HR measurements (**Figure 24b**). We estimated resting HR from HR values during the resting episodes preceding and following episodes of aggressive behaviours. Fighting HR was the corresponding HR value for 'extracted' aggressive behaviour episodes. Fight duration was the number of minutes spent in defence.

The contribution of the energy cost of territory defence to total daily energy expenditure of breeding king penguins was determined from the 169 birds used for focal observations. Total DEE of each bird was calculated from its average daily HR as calculated over the whole recording period (one week on average) and using the same equation as above. The daily energy cost of territory defence was calculated as the average energy cost of aggressive behaviours (kJ/h) x average time (h) spent in aggressive behaviours daily, as determined by scan sampling.

2.6. Ethical note

We placed a hood over the bird's head immediately after capture and throughout handling to keep it calm. After weighing, we quickly returned the animal to the colony. Manipulations lasted between 5 and 10 min and during this time we placed the bird's egg or chick in an incubator. This procedure never resulted in egg or chick abandonment. Due to handling, most animals developed a tachycardia (up to 165 bpm on an average) from which they usually recovered within 15 min following release. We removed flipper bands from all banded birds before the winter, as they are known to negatively affect survival at that time of year (Gauthier-Clerc et al. 2004). Capture and tagging procedures were approved by the Ethical Committee of the Institut Polaire Français – Paul-Emile Victor. Autorizations to enter the colony and to manipulate birds were obtained from Terres Australes et Antarctiques Françaises. The experiments comply with the current laws of France.

2.7. Statistical analyses

We estimated the proportion of time spent in aggressive behaviours from scan samples. We performed all analyses using linear mixed models (LMMs with a compound symmetry structure; Littell et al. 2006), including bird identity as a random term in order to control for replicate observations on the same individual. Normality of residuals was asserted using the Shapiro-Wilk normality test and visual inspection of the residuals indicated no violation of assumptions of homoscedasticity. When gender was considered, bird identity was nested into sex as birds cannot change sex. We included body size (as indexed by flipper length) and body mass as covariates in the models, as they may affect energy costs. We performed all statistical analyses with the SAS (SAS Institute, Cary, N.C.; version 9.1) and R (http://www.r-project.org/; version 2.10.1) statistical softwares. For each LMM, the number of observations (n) used and the number of birds (N) concerned are reported. We report all values as means ± 1 SE, and the alpha level was set at 0.05 for all statistical procedures.

3. Results

3.1. Time spent in territory defence

From diurnal scan sampling, we determined that aggressive behaviours contributed to 13.1 ± 2.0 % of the time budget of breeding king penguins. From the estimates of the time spent in aggressive behaviours in the five videotaped birds that were observed for 24 h, we determined that birds devoted a similar proportion of their time budget to aggressive behaviours during the day (15.7 ± 1.0 %, n = 280) and during night (14.6 ± 1.1 %, n = 200), i.e. a day/night factor was not significant when included in a LMM controlling for repeated measurements by individual identity ($F_{1,474} = 0.75$, $P = 0.38$, $n = 480$, $N = 5$). Thus, time spent in aggressive behaviours obtained from diurnal scan sampling was used to estimate the total daily energy cost of territory defence.

3.2. The average cost of aggressive behaviours

3.2.1. Videoed birds

The average duration of the 36 selected episodes of aggressive behaviour was 1.9 ± 0.4 min (from 0.3 to 9.8 min). The average time needed for HR to recover initial levels (recovery phase) averaged 0.24 ± 0.03 min (0 to 2.6 min). HR increased during each episode of aggressive behaviour, on average from 57.5 ± 1.4 bpm at rest to 73.3 ± 1.8 bpm during aggressive behaviour (n = 36, N = 7). The corresponding RMR and EE were 1.83 ± 0.05 and 2.33 ± 0.06 kJ/min, respectively, indicating that during aggressive behaviour EE was on average 1.27 times higher than RMR. The total excess of EE due to an aggressive episode was 0.97 ± 0.04 kJ, of which 6 ± 2 % was for the recovery phase. This yielded an average energy cost of aggressive behaviour of 0.50 ± 0.04 kJ/min or 29.8 ± 2.4 kJ/h (n = 36). After controlling for the fact that this estimate was obtained from repeated measures (3 to 10 episodes) over 7 birds, i.e. by running a LMM in which only the random effect was included, we found that the average energy cost of aggressive behaviour (i.e. the estimated intercept) was highly significant (LMM; $F_{1,29}$ = 151, P < 0.001, n = 36, N = 7) and identical to that reported above. Moreover, the hourly energy cost calculated from each episode was positively related to the rate of aggressive behaviours (number/min, threats and body contacts together) during the episode (LMM; $F_{1,28}$ = 22.60, P < 0.001, n = 36, N = 7), but was not influenced by gender (males = 30.6 ± 3.1 kJ/h *vs.* females = 27.6 ± 2.8 kJ/h; LMM; $F_{1,5}$ = 0.25, P = 0.64, n = 36, N = 7) nor by the duration of the aggressive encounters (LMM; $F_{1,28}$ = 0.25, P = 0.61, n = 36, N = 7).

3.2.2. Visual focal observations

The duration of episodes associated with aggressive behaviours averaged 1.7 ± 0.2 min (n = 589). Heart rate during these episodes (71.5 ± 1.4 bpm, n = 589) was higher (LMM; $F_{1,1008}$ = 979.48, P < 0.001; n = 1178, N = 169) than during the associated resting episodes (56.7 ± 1.2 bpm, n = 589). Applying the same equations as above to these HR data and taking into account the duration of aggressive behaviours during focal observations, we estimated the average energy cost of aggressive behaviours at 27.2 ± 1.4 kJ/h (n = 589). This value was not significantly different from the 29.8 kJ/h estimate obtained from videoed birds (LMM; $F_{1,449}$ = 0.16, P = 0.69; n = 625, N = 176). In addition, neither body mass (LMM; $F_{1,244}$ = 2.07, P = 0.15; n = 345, N = 100) nor body size (LMM;$F_{1,244}$ = 0.43, P = 0. 51; n = 345, N = 100) of birds affected the hourly energy cost of aggressive behaviours. Finally, neither gender (LMM; $F_{1,167}$ = 0.66, P = 0.42; n = 589, N = 169) nor shift (LMM; $F_{6,415}$ = 0.58, P = 0.74, n = 589, N = 169) had a significant effect on the hourly energy cost of aggressive behaviours, even when we only considered individuals for which both body mass and body size were known and when those variables were considered in the model (LMM with body mass; gender: $F_{1,97} = 1.58$, $P = 0.21$; n = 345, N = 100; shift: $F_{6,239} = 0.52$, $P = 0.97$; $n = 345$, $N = 100$). Running separate LMMs with body size and the residuals of a regression between body mass and body size yielded similar results.

3.3. Cost and rate of the different types of aggressive behaviours

From data obtained during focal observations, after discriminating when birds performed only threat displays or attacks with body contact, we found that the energy cost of threat displays (19 ± 1 kJ/h, $n = 354$; Fig. 2) was more than three times lower than that of attacks with body contact (61 ± 3 kJ/h, $n = 127$; **Figure 25**; LMM; $F_{1,177} = 2978.13$, $P < 0.001$; $n = 279$, $N = 100$ when controlling for body mass as a covariate in the model). Energy expenditure during attacks with body contact averaged 171 kJ/h, i.e. 1.56 x RMR. During threat displays, it averaged 129 kJ/h, i.e. 1.17 x RMR. Threat displays, however, occurred 5 times more frequently than attacks with body contact (81 ± 4 vs. 16 ± 1 behaviours recorded/h; LMM; $F_{1,318} = 178.15$, $P < 0.001$, $n = 488$, $N = 169$). The energy cost of threat displays did not vary between initiators and receivers (LMM; $F_{1,112} = 0.16$, $P = 0.72$; $n = 207$, $N = 93$; **Figure 25**), but attacks with body contacts tended to incur higher energy costs to initiators than receivers, although the difference was not significant at the 0.05 alpha level (LMM; $F_{1,7} = 4.21$, $P = 0.07$; $n = 72$, $N = 63$). Whereas most of the birds (approximately 80 %) mainly performed threat displays (70 to 100 % of their aggressive behaviours), approximately 20 % performed a mix of aggressive behaviours including 50 % of threat displays and 50 % of physical attacks.

3.4. Contribution of territory defence to daily energy expenditure

Using the hourly energy cost of aggressive behaviours that included the recovery phase for HR, i.e. 29.8 kJ/h (95% C.I. [25.1-34.5 kJ/h] for videoed birds), and the average time spent daily in aggressive behaviour, i.e. 13.1 % or 3.14 h, we found that on average king penguins spent 94 kJ/day (95% C.I. [79.3-108.7 kJ/h]) for territory defence. This cost contributed to 2.7 % (95% C.I. [2.3-3.1 %]) of the total DEE (3454 ± 180 kJ/day, n=169).

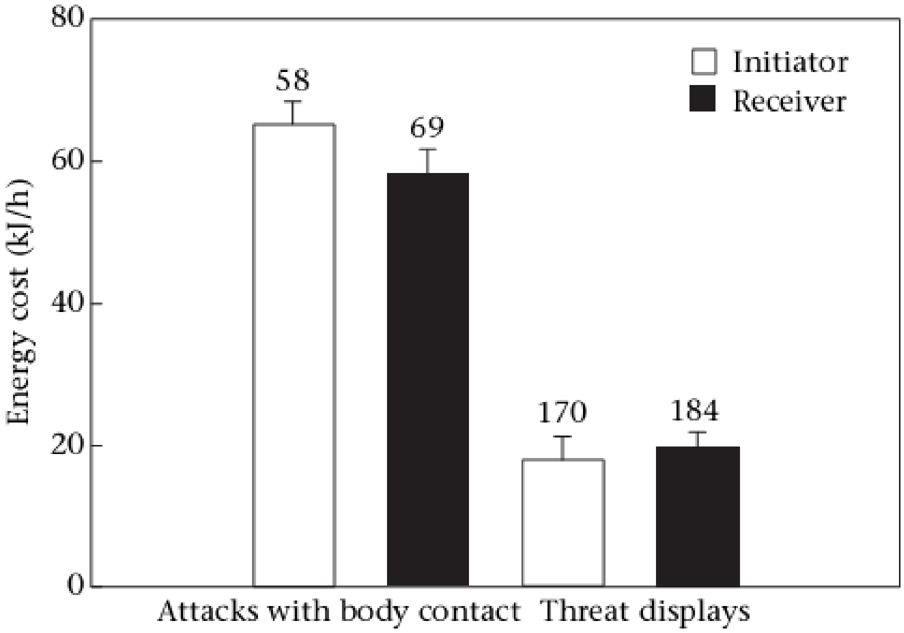

Figure 25. Energy costs of attacks with body contact (pecking and flipper blows) and threat displays (beak pointing and gaping) for receivers and initiators of agonistic encounters, in king penguin breeding on the Crozet Archipelago.

The numbers of 1 min episodes where only threats or only attacks with body contacts were observed are given above the bars.

4. Discussion

4.1. Time spent in aggressive behaviours

We determined that king penguins spent 13 % of their time in territory defence which is comparable, albeit slightly lower, than the 20 % found in previous studies (Challet et al. 1994, Côté 2000). However, we are confident that our estimation is accurate, because scan sampling used in our study is a more relevant method to estimate activity budgets of animals (Altmann 1974) as opposed to the focal samplings used in previous studies (Challet et al. 1994, Côté 2000). Moreover, we determined that vigilance accounted for 7 % of the total activity budget of king penguins (Viera, Groscolas and Côté, unpublished data), but this behaviour was not listed in the behavioural repertoire of previous studies. Vigilance may have been partly included in territory defence by previous authors (Challet et al. 1994).

The high level of aggressiveness observed in king penguins is likely related to its breeding pattern and constraints, i.e. colonial breeding, no nest building, and limited access to suitable breeding locations. To successfully reproduce, king penguins must acquire and maintain a territory, and competition for good quality territories is high (Côté 2000). Central territories offer the eggs or chicks better protection from avian predators and from frequent flooding than territories located at the edge of the colony (Côté 2000, Viera et al. 2006). During brooding, the rate of pecking and flipper blows is higher in central than in peripheral birds (Côté 2000). Thus, high aggressiveness may contribute to acquiring and maintaining a central territory, which would at least indirectly improve breeding success (Côté 2000). On the other hand, high rates of aggressive behaviours involve risks that may cause injuries and reduce future reproductive success (Descamps et al. 2009, Laidre 2007, Montgomerie and Weatherhead 1988). In king penguins, pecking is the main behaviour involving high costs in terms of risk of injury, notably when directed at the eyes. Moreover, neighbour harassment during breeding may lead to the desertion of the territory by one partner, and therefore jeopardize the success of the current reproduction. Indeed, king penguins do not build a nest but incubate their progeny on their feet so that when a parent deserts its territory, the egg or chick is lost.

4.2. Aggressive behaviour imposes an energy cost

Our estimates of energy costs were based on the measurement of daily and resting HR, on the increase of HR during episodes of territory defence, and on the use of an equation relating energy expenditure to HR in undisturbed breeding king penguins. The HR of resting birds was far below the maximum HR observed in king penguins, usually when stressed, i.e. around 250 bpm. Thus, the increase in HR during aggressive interactions could not be constrained (limited) by the fact that HR at rest was close to its maximum sustainable level, and thus could not be underestimated. On the other hand, the possibility that this increase could not be entirely the result of the increase in physical activity associated with aggressive behaviours cannot be discarded. Actually, a part of the increase could be related to an emotional or motivational component associated with aggressive interactions. This 'additional HR', i.e. HR increase beyond that associated with increased energy expenditure, has, for instance, been observed in humans and is used as an indicator of emotional activation or arousal (Stromme et al. 1978, Wilhelm and Roth 1998). If this effect occurs in king penguins, our estimates of energy cost of aggressiveness would be overestimated, suggesting that the overall energy cost of territory defence in king penguin is actually far lower than what may be expected (see below).

The two methods we used to estimate the average hourly cost of aggressive behaviours yielded similar results (29.8 kJ/h *vs* 27.2 kJ/h) and revealed that neither bird gender nor breeding season affected these costs. Thus, aggressive motivation (and therefore HR increase) did not seem to differ at different breeding stages, and defending a chick did not seem to represent a greater investment than defending an egg. The slight underestimation in birds used for focal observations likely resulted from the constraint imposed by the HR sampling rate (one value per min) that precluded us from taking into account the energy cost corresponding to the HR recovery phase. Correcting for this underestimation by considering that 6% of the energy cost of aggressive behaviours corresponded to the recovery phase (see results for videoed birds) would yield an energy cost of fighting of 28.9 kJ/h, which represents a difference of only 3% compared to the value obtained from videoed birds. Our results also showed that the average HR obtained both during resting and aggressive behaviours differed by only 2 % between videoed and focally observed birds. These findings support the view that by recording HR at a sampling rate of one value per min and by pooling information from a large number of individuals we can obtain accurate estimates of energy costs of activities in free-living animals on a long-term basis and without major disturbance.

Overall, our results were consistent with other published studies that reported EE increases during aggressive behaviours in invertebrates or fishes (DeCarvalho et al. 2004, Hack 1997, Neat et al. 1998, Rovero et al. 2000, Smith and Taylor 1993). However, the average energy cost we determined, i.e. 1.27 fold RMR, was lower than the range observed in other animal models: for instance, $\dot{V}_{O_2}$ during fights ranged from 1.3 to 4.8 (mean = 2.8) times $\dot{V}_{O_2}$ at rest in the house cricket (*Acheta domesticus* L.; Hack 1997), 3.9 times RMR in a cichlid fish (*Neolamprologus pulcher*; Grantner and Taborsky 1998), and twice as high as RMR in shore crabs (Rovero et al. 2000). These differences may have occurred because the latter costs were determined for fighting animals (attacks with body contact) whereas the average cost estimated in king penguins was for aggressive interactions involving mostly threat displays. However, even the cost of attacks with body contact seems lower in king penguin (1.57 x RMR) than in previously studied species. Nevertheless, the possibility that fights in king penguins may incur energy costs as high as for the above species should not be discarded. In the study colony, this could occasionally happen during periods of flooding when some incubating birds have to crowd on restricted un-submerged areas. In this case, birds are observed to fight intensively for prolonged periods of time, sometimes up to the moment when they have to abandon their breeding territory and egg because they are exhausted. Throughout our study, we did not have the opportunity to record HR during such periods of intense fighting.

4.3. Intensity of behaviours, energy costs, and fighting strategies

We found that the energy costs of aggressive behaviours depended on their intensity, i.e. lower costs for threats than for interactions with body contact. Because attacks with body contact are more costly, birds should perform threats to save energy, as predicted by the theory on animal conflicts (Maynard-Smith and Parker 1976). However, we found that whereas the majority of birds in our study actually devoted more time to threat displays, a significant number of penguins (20%) nonetheless performed attacks just as frequently as threats. It would be informative to further correlate each tactic to the RHP of individuals, and to their breeding success. Numerous studies have shown that in territorial defence, threat and display postures have a number of advantages over fights. Such advantages include lower energetic costs (Brown 1975, Geist 1981), reduced risk of injuries (Brown 1975, Huntingford and Turner 1987, Maynard-Smith 1982), and the opportunity to assess the opponent’s fighting abilities (Enquist and Leimar 1983, Maynard-Smith 1982). Birds in better condition should perform longer aggressive behaviours or exhibit higher rates of aggressive behaviours than birds in poor condition (Briffa and Elwood 2004, Smith and Taylor 1993). However, we did not find such a pattern in king penguins as neither body mass nor body size influenced the time spent in aggressive behaviours or the energy costs of such behaviours. Moreover, we found that the energy costs of interactions with body contact during fights tended to be higher for initiators than for receivers. The intensity of the attack by the initiator has been shown to be a key predictor of success to win the fight and is thought to act as a signal advertising the capacity to bear high costs and/or risk associated with dangerous behaviours (Briffa et al. 1998, Briffa and Elwood 2000). In line with this, Wascher et al. (2009) found a higher HR increase in individuals actively attacking conspecifics compared to individuals being attacked by others in greylag geese (*Anser anser*), although the energy equivalent was unknown. They suggested that the amount of physical activity involved in interactions was likely the same for both opponents, but the motivational states of the two individuals were different as also shown in previous studies (Sgoifo et al. 2001; Wascher et al. 2008a). The risk of injuries and/or the depletion of energy reserves could, however, also be involved in the decision of initiating or not a fight. It is difficult to explain, however, why we did not observe the same trend for threats. A possible explanation may result from limitations to detect significant differences with our 1 min-interval recordings. The individual performing a threat needs to convince the receiver of both its ability and its willingness to fight and there is an inherent risk of threatening an individual who might be willing to fight back (see Számadó 2008 for a review). Számadó (2008) suggested that proximity risk is the major factor promoting the honest use of threat displays, but in the specific context of a colony, proximity may have maintained high frequency of threats without incurring a different cost for both opponents.

4.4. Cost of territory defence and DEE

We found that aggressive behaviours contributed to 2.7 % of total DEE, i.e. incurred low energy costs in breeding king penguins. Ultimately, the influence of energy expenditure on the evolution of fighting strategies depends on their consequences for survival and reproduction. Territory defence is an important component of the reproductive allocation of the parents and involves an obvious trade-off: the time and energy devoted to protect both offspring and territory can indeed not be allocated to other activities enhancing offspring survival (Montgomerie and Weatherhead 1988). A trade-off between the energy devoted to aggressive behaviours and that allocated to parental care has been well documented in birds (Ketterson et al. 1992, Tuttle 2003), so that the more aggressive males during intra-sexual competition generally allocate less to parental care, which may negatively impact offspring survival (Stoehr and Hill 2000). During the breeding season, king penguins fast for prolonged periods and, when their body mass decreases beyond a certain critical threshold, they may abandon their egg or chick (Groscolas and Robin 2001). Expensive activities could therefore critically deplete energy stores and force an individual to abandon its breeding territory to go and forage at sea. A conflict between the energy allocated to reproduction and that allocated strictly to territory defence is thus likely to occur during critical periods of energy demand, notably at the end of an incubation or brooding shift after several days of fasting. Aggressive behaviours of low energy cost may then have been selected by natural selection, enabling king penguins to efficiently defend their territory and egg (or offspring) during reproduction and yet save energy throughout the long-term fast they face, thus delaying the critical moment of egg or offspring abandonment (Groscolas et al. 2008, Robin et al. 2001).

To conclude, we found that energy costs of aggressive behaviours represented a small proportion of the DEE of breeding king penguins. This finding supports the view that these birds manage to save energy in a context of fasting and sleep deprivation by resorting to the least energetically costly behaviour (i.e. threats) when defending their territory. Overall, we show that highly aggressive animals can devote a large proportion of time defending their territory without jeopardizing their energy status.

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STUDY 3 – Behavioural and physiological adjustments during long term fasting

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Breeding while fasting in a crowd: physiological and behavioural adjustments in courting and incubating male king penguins

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Unpublished data.

Abstract

For seabirds, which forage at sea but breed on land, successful reproduction is tributary to the effective management of energy stores, and breeding often means aggregating in dense colonies, posing the issue of social stress, which may have profound consequences on their energy budget. In king penguins, males undergo fasting periods of up to one month while courting and incubating ashore. Whereas former studies have well investigated the astonishing fasting capacities of those seabirds and their use of energy stores during long-term fasting under captive conditions, relatively little is known on the energetics of free-living birds while breeding in their colonial environment. Here, by means of bio-logging techniques, we were able to monitor heart rate (a proxy to energy expenditure), body temperature, and physical activity throughout courtship and first incubation shift in male king penguins breeding on Possession Island (Crozet Archipelago). We determined that HR decreases constantly throughout courtship but increases again shortly before egg laying (early breeders) or remained stabilized (late breeders). Changes in HR were concomitant with a constant decrease in activity, whereas body temperature remained unchanged. We suggest that decreased physical activity but no significant hypothermia may be used to sustain the long courtship-incubation fast. In early breeders, an increase in HR shortly before egg-laying may relate to changes in the social environment, namely increasing colony density, as we found a strong relationship between colony density and resting HR. Further studies are needed to test the hypothesis of the impact conspecific density may have on the physiology of colonial breeders, i.e. evidence for an energy cost of group-living.

1. Introduction

Because energy stores are finite, animals are required to trade-off a limited energy capital, allocating it to growth, survival and reproduction, with the ultimate aim of maximizing individual fitness (Stearns 1989, Stearns 1992). Energy availability, its efficient use and management, thus constrains many aspects of animal behaviour, shaping life-history strategies. For this reason, a proper understanding of the evolution of specific behaviours and life-history traits requires the study of energy budgets and of the adaptive strategies that may be used by animals in the face of energy constraints (McNamara and Houston 1996, Green et al. 2009).

An animal's energy budget is managed based on its physiological needs and on the energy it can acquire from environmental resources (Hall et al. 1992, Jodice et al. 2006). During reproduction, the efficient use of energy resources is critical as not only should parents allocate energy to fuel their own metabolism, but additionally have they to provide the energy necessary to meet the metabolic requirements of their growing offspring. The trade-off becomes challenging when resource availability is absent (i.e. periods of prolonged fasting), as observed in seabirds that forage at sea but breed on land (Lack 1968, Ricklefs 1983, Dobson and Jouventin 2007). In seabirds, successful reproduction then depends on the efficient management of energy stores ashore, and critical depletion of these stores often results in breeding failure (Olsson 1997, Gauthier-Clerc et al. 2001, Groscolas et al. 2008). ***So simply, how might those animals manage their stored capital, in case scenarios where breeding has to be conciliated with periods of virtually no energy intake?*** Obviously, metabolic and/or behavioural adaptations are required in order to limit energy expenditure (EE) to a minimum and for breeding success to ensue. However, whereas metabolic adaptations to long-term fasting in seabirds (especially penguins) have been long studied under captive and non-breeding conditions (Cherel et al. 1988a, Cherel et al. 1988b, Cherel et al. 1994a, reviewed in Groscolas and Robin 2001), relatively little is known on the energetic costs of reproduction ashore and how the EE of free-living seabirds might be modulated in their natural colonial context.

In seabirds, EE is known to be strongly modulated by physical activity, both on-land and at sea (Dewasmes et al. 1980, Chappell et al. 1993, Jodice et al. 2003, Weimerskirch et al.

2000). Thus acting either on the energy cost of physical activities (e.g. by resorting to the use of low-energy cost activities while breeding as previously seen in **study 2**; Viera et al. 2011), or on the overall quantity of physical activity allows scope for seabirds to reduce their EE when needed. One behavioural strategy fasting individuals may resort to when breeding is then to simply reduce their overall physical activity to a minimum level. Another potential energy-saving strategy, used by many small endothermic mammalian (Chruszcz and Barclay 2002, Geiser 2004) and avian species (McKechnie and Lovegrove 2002), consists in depressing metabolic rate by lowering the set point of thermoregulation (adaptive heterothermia). Such thermoregulatory control has also recently been suggested as a substantial means of decreasing EE in large seabirds (e.g. in emperor penguins; Dewasmes et al. 1980, Gilbert et al. 2007, in king penguin chicks; Eichhorn et al. 2011), though in breeding birds, the need to maintain the egg at an elevated and relatively stable temperature may constrain the potential for heterothermia (Gilbert et al. 2007).

On the other hand, when breeding, most seabirds crowd into stupendously large colonies where they share limited space with territorial conspecifics (e.g. king penguins, *Aptenodytes patagonicus*, may aggregate in colonies as large as at least 500.000 breeding pairs, Guinet et al. 1995). Social stimuli are known to be potent stressors in vertebrates (Creel et al. 1996, Creel 2001, Kotrschal et al. 1998, Wascher et al. 2008b) and asides leading to increased social interactions between conspecifics (and associated increased EE) (e.g. Cao and Dornhaus 2008), high social densities may well affect an animal’s perceived social stress and the energy expended to fuel the metabolism while resting may be consequent. Whereas Cao and Dornhaus (2008) recently showed that ants under crowded conditions indeed exhibited higher EE than their less crowded conspecifics, the authors attributed this result to increased social interactions in crowded colonies and associated increased physical activity. To our knowledge however, no study so far has considered the potential effects crowding may have on the energy expended by group-living animals, at rest.

Using colonial king penguins as a model, the objectives of the present study were thus twofold. (1) By considering changes in the time course of heart rate (HR), physical activity and body temperature in freely breeding king penguins, we wished to determine how EE was modulated in breeding seabirds naturally fasting in their colony. (2) By investigating whether changes in colony density might relate to HR, we considered whether the social environment may potentially affect EE in colonial breeders. We specifically focused our study on male king penguins, as they are subjected to the longest reproductive fast of the species, i.e. virtually a month at the beginning of the breeding season, which includes the courtship period (some 13-18 days from arrival ashore for pairing to laying) and the first incubation shift (some 17 days from laying to relief by the partner) (Descamps et al. 2002).

To investigate changes in EE related to reproduction, we continuously monitored heart rate (HR), physical activity and body temperature throughout the course of courtship and incubation. HR is a good proxy of EE in king penguins and the EE/HR relationship has been determined in that bird, both under captive (Froget et al. 2001, Froget et al. 2002, Fahlman et al. 2004), and free-living conditions (Groscolas et al. 2010). The measurement of physical activity allowed us to determine whether potential difference in HR (EE) were related to changes in physical activity, whereas the measurement of body temperature allowed us to investigate whether incubating birds (which are required to maintain their brood pouch at a high and relatively constant temperature for embryo development) were able to resort to adaptive heterothermia as a potential means to reduce their EE (Eichhorn et al. 2011).

In addition, we also investigated whether group living might affect EE in colonially breeding birds. Indeed, determining whether group living incurs an energy cost should be feasible for a species where changes in population density naturally occur over a short period of time. King penguins provide such an opportunity. These colonial seabirds breed on the remotely located sub-Antarctic islands in large groups of several thousands of breeding pairs (Guinet et al. 1995). Because of the limited number of accessible and favourable breeding zones, king penguins are compelled to crowd ashore and defend small reproductive territories (about 0.5 m², Côté 2000) during the breeding season displaying high rates of aggressive behaviours. Colony density is not high and steady from the very onset of breeding but progressively increases before more or less stabilizing for a while at high density (a process that remains to be accurately quantified and the time-course of which could slightly change from one year to another). Interestingly for this study, because of the long-lasting chick rearing (11-12 months, Descamps et al. 2002), a successful early breeder in one year is an obligate late breeder in the following year and breeding onset hence occurs asynchronously within king penguin populations (extending from mid-November to early March; Stonehouse 1960, Weimerskirch et al. 1992). Thus, individuals breeding at different densities but having the same breeding status can be compared. Therefore, king penguins provide us with an opportunity to compare breeders which start courting early (i.e. at low but rapidly increasing colony density) or late (i.e. at high and stable colony density) in the same breeding year, and to test whether the physiology of those birds may be affected by differences in their social environment (namely in colony density).

Because prolonged fasting is a strong energy constraint, king penguins should adjust their daily EE to some minimal level in order to spare energy stores and to go on fasting until being relieved from their incubation duties, i.e. before being forced to abandon the egg or chick after reaching a critical exhaustion (Olsson 1997, Gauthier-Clerc et al. 2001, Groscolas et al. 2008). Thus, demonstrating an energy cost of group living in king penguins is all the more relevant that these birds have no immediate means for compensating for this cost, by adjusting their daily energy intake for instance. In other words, paying for colonial breeding cannot be considered as a "luxury" penguins might easily cope with, but as a real and unavoidable supplementary energy constraint.

2. Methods

This study was carried out on Possession Island, Crozet Archipelago (46°25'S, 51°45'E) during the austral summer of 2008-2009. Breeding male king penguins were followed in the colony of "La grande manchotière" which is host to over 22.000 king penguins. The study area was a sub-colony located centrally in the colony and occupied by up to 5000 breeding pairs.

2.1. Study groups and field procedure

2.1.1. Captive fasting birds

To investigate changes in HR due solely to fasting, i.e. unrelated to breeding activity and changes in bird density, we monitored HR in 8 early breeding males which were caught in the colony shortly (1-3 days) after arriving ashore for courting (early December). Birds were weighed to the nearest 10 g using a platform balance and their body mass (13.5 ± 0.3 kg) was representative of males at this stage. Birds were kept in wooden pens (3 m x 3 m), in the very vicinity of the colony and under natural weather conditions. There were 3 birds per pen so that their density was 0.33 bird/m^2, i.e. the lower density observed in birds breeding in the colony in the present study (see **Figure 26**). For one of the three pens only two birds were equipped with a HR logger. Penned birds were kept fasting for a total duration (25 days) comparable to that of free-living incubating birds. At release, their body mass averaged 10.02 ± 0.2 kg, i.e more than the 9.3 kg body mass corresponding to a critical exhaustion of fat store and entrance into phase III fasting, a phase marked by a rise in locomotory activity and increased body protein catabolism (Groscolas 1990). Penned birds were therefore considered as into phase II fasting throughout the whole experiment.

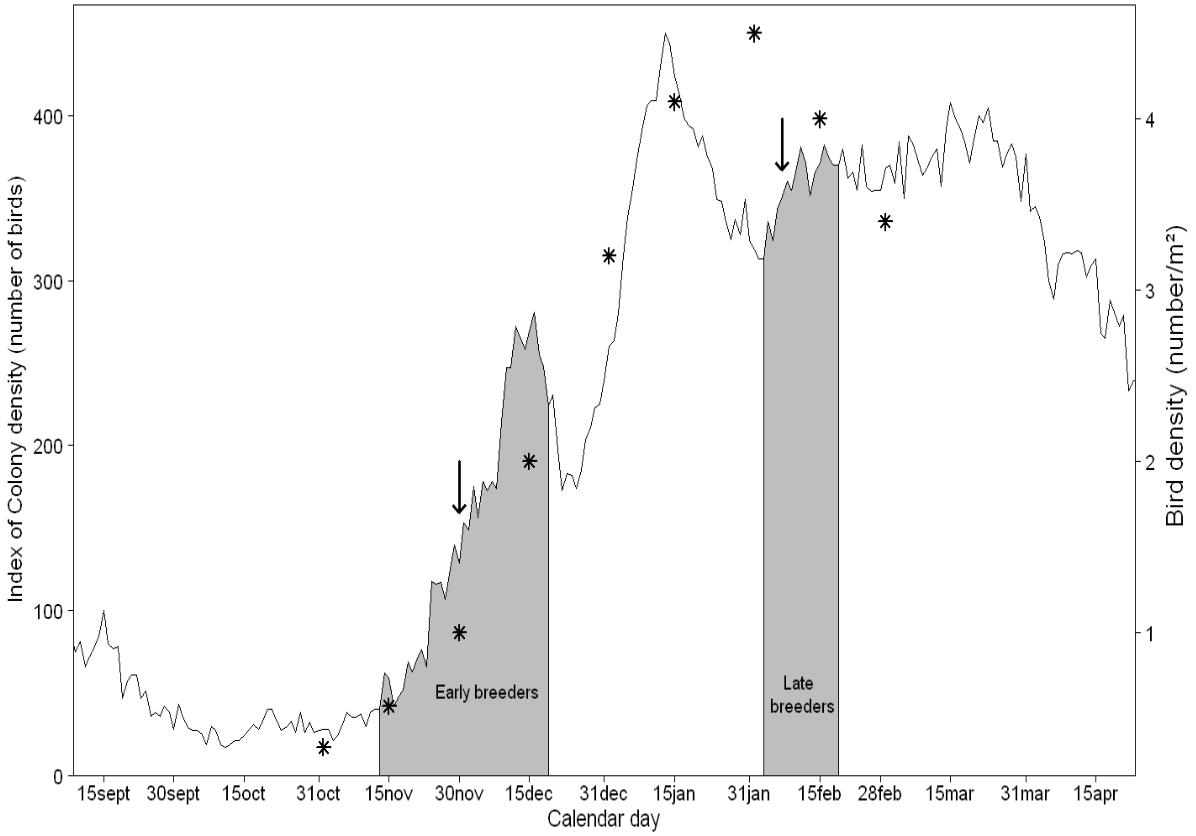

Figure 26. Changes in colony density over the 2008-2009 reproductive season for king penguins breeding in the Baie du Marin, Possession Island, Crozet Archipelago.

Bird density (stars): number of breeding birds per m^2 in the study area. Colony density index (continuous line): number of transponder-tagged breeding birds present in a nearby sub-colony of fixed area (see text). Shadowed zones under the density index curve indicate times periods during which early and late breeders were followed, with arrows showing the respective average laying dates. The date of first laying in the study area was November 10.

2.1.2. *Freely-living birds*

Birds were monitored throughout the breeding season (from November to March) and based on the date of courtship onset, we distinguished two groups: (1) birds which started breeding early in the season (i.e. courtship onset range = 11 November – 2 December 2008, 14 individuals); and (2) birds which started their reproduction later in the season (i.e. range = 27 January – 8 February 2009, 10 individuals). During these two periods, bird density in the colony was, respectively, low but increasing or high and stable (**Figure 26**). Animals were caught while courting, marked using a non-permanent animal dye (Porcimark®, Kruuse, Germany) and flipper bands for identification during field observations, and equipped with data-loggers (see below). Sexing was based on courtship behaviour, sexual size dimorphism in direct comparison with their partner (at courting, males are on average heavier and larger than females), and was later confirmed according to sex-specific breeding cycle chronology (males being the first to incubate upon egg-laying, Stonehouse 1960). Individuals were checked from a distance at least twice a day and breeding phenology (i.e. courtship, settlement on territory, egg-laying, and incubation) was established from field observations at ± 1 day. Early breeders were observed for an average duration of 27.6 ± 0.9 days (range = 21 – 33 days), i.e. for 12.5 ± 1.3 days (range = 2 – 21 days) before egg laying and during 15.1 ± 0.9 days (range = 7 – 19 days) afterwards. Late breeders were followed for an average duration of 16.5 ± 1.6 days (range = 9 – 25 days), i.e. for 4.9 ± 0.8 days (range = 1 – 9 days) before egg laying and during 11.6 ± 0.9 days (range = 8 – 16 days) afterwards (**Figure 26**). The shorter duration of pre-laying follow up in late breeders could be explained by the fact that whereas it is easy to identify early breeders very soon (1-2 days) after their arrival ashore (there are only a few birds in the colony by this time), this is harder later because of the crowding of the colony. Thus, late breeders in our study were likely marked and equipped after having been in the colony for several days. Follow up ended when birds were relieved by their partner (early breeders) or a few days before relief by the partner (late breeders) to avoid the loss of equipment, because at this time of the breeding season we have less time left to securely follow reliefs and thus avoid the loss of loggers. This, and the fact that late-breeders present accelerated phases of the breeding cycle (Gauthier-Clerc et al. 2002b) compared to early breeders, explains why the duration of follow up during the incubation shift is slightly shorter for late breeders in our study. At the end of follow up all equipment (loggers and flipper-bands) was retrieved and we checked that all birds left the colony for re-feeding at sea a few hours or days later.

2.2. Changes in HR, activity and body temperature over courtship and incubation

2.2.1. HR monitoring

We used externally mounted data-loggers (Polar® model RS800, Polar Electro Oy, Kempele, Finland) adapted for suitable use on king penguins as previously described and validated (see Groscolas et al. 2010 for details). HR was sampled continuously at a 5 second interval, yielding a total of 17280 measurements per 24-hour period. Briefly, loggers were made of two units: (i) a sensor-transmitter (30-40 g), composed of a HR processor which filtered out electrical background noise received from the electrodes (i.e. muscle activity) from heart activity; and (ii) a receiver/logger (30 g). Electrodes were composed of two stainless-steel wires attached to gold-plated safety pins, which were inserted subcutaneously on the dorsal region of the animal. Iodine (Betadine®) and alcoholic antiseptic solutions were used to disinfect the electrodes before each insertion. The transmitter was attached to the dorsal feathers of the animals using adhesive tape (Tesa®), and the receptor was secured to the flipper band. The whole apparatus weighed less than 1% of total body mass. Sensors yielded HR values highly comparable to those measured with a stethoscope ($R^2 = 0.98$, $P < 0.0001$, $N = 42$; Groscolas et al. 2010). Data-loggers did not seem to interfere with the usual routine of the birds, as equipped individuals soon resumed normal activity after handling (e.g. preening, stretching, sleeping, and fighting) and could not be distinguished in behavioural patterns from other individuals the subsequent day (V.A. Viblanc, *personal observations*). HR was monitored in all 14 early breeders and 10 late breeders, continuously for 6 to 32 days according to individuals. Because of the limitation in the logger memory, birds had to be caught for a few minutes every four days for logger re-setting.

2.2.2. Physical activity monitoring

A measure of body activity over the time course of courtship and incubation was obtained for 8 early breeders and 6 late breeders using externally attached physical activity monitoring systems (Actical®, Phillips Respironics Inc. Co., OR 97701 USA). Omnidirectional accelerometer sensors monitored the occurrence and intensity of motion. Acceleration was measured at the frequency of 32 Hz, the highest of the 32 values obtained each second being kept. Actical® devices stored the sampled information in the form of activity counts which were integrated every 30 seconds (i.e. the sum of the 30 highest values obtained each second) and allowed us to monitor the animals for a total duration of 21 days. To avoid unnecessary disturbance of the animals, devices were changed prior to memory saturation, at the same time as HR loggers were reset. Actical® devices (28 mm x 27 mm x 10 mm; 16 g) were attached beneath HR loggers, along the dorsal mid-line of the animal, halfway between the armpit and hip lines. Loggers were tightly glued on dorsal feathers, close to the skin, to detect body movements. We checked that this set up integrated movements both from the upper (head, flippers) and lower (legs) body, yielding an index of overall bird activity.

2.2.3. Body temperature monitoring

Variations in body temperature were obtained by continuous monitoring of stomach temperature (*viz.* a good overall index of deep core temperature in king penguins, Barré 1980, Handrich et al. 1997, Eichhorn et al. 2011) in 9 early breeders and 9 late breeders. Temperatures loggers (Thermochron i-buttons®, model DS1922L: 5.8 mm width, 17.35 mm diameter, Dallas Semiconductor, Dallas, Texas, 75244 USA), were coated in epoxy resin and forced fed to the birds. Loggers were later retrieved, easing them back up the oesophagus, by pulling on a string from which one of the extremities was embedded in the resin of the logger, and the other glued amongst the feathers beneath the bill. This relatively non-invasive set up allowed us to monitor stomach temperature, deploying and recovering the temperature loggers without requiring heavy procedures such as anaesthesia and surgical implantation of temperature sensors. Loggers were set as to sample stomach temperature every 10 minutes with a resolution of 0.01°C. Prior to field use, loggers were calibrated against a range of fixed temperatures using a hot water bath (range 30 – 43 °C, temperature at + or – 0.01°C, 1°C increments, step duration of 20 min., sample rate of 1/5 sec.) and measurements were shown to highly correlate with those set throughout the calibration (Spearman's rank correlation; *rho* = 0.996, *n* = 73623).

2.3. Changes in colony density

An index of colony density was obtained daily by monitoring the presence of electronically tagged individuals in a sub-colony of a fixed area located in the very vicinity of the study area. In this sub-colony where approximately 8000 pairs breed each year, a total of 6000 birds have been tagged, mostly as chicks and starting in 1998, using passive electronic chips (Gendner et al. 2005). In 2008-2009, some 650-tagged adults bred in this sub-colony. By means of antennas buried under the usual transit pathways in and out of the sub-colony, bird entrance and departure from the colony were continuously and automatically detected using RFID (system 'ANTAVIA' detailed in Gendner et al. 2005) over the whole study period, from mid-September 2008 to mid-April 2009. Birds aged more than 4 years and whose movements in and out of the sub-colony matched the reproductive cycle of king penguins were considered as breeders. Their number was used as a proxy of colony filling by reproductive birds, i.e. a quantitative index of the daily changes in colony density throughout the breeding season. This index was used to relate bird daily heart rate to density.

In addition, bird density was also estimated in the study area by determining every second week the average distance between breeding birds (incubating birds or territorial pairs), starting on 1st of November. The two observers first trained to visually estimate distances between stones spread on the ground and mimicking king penguins, until the estimate procedure was standardized (estimated distances not differing by more than 5 cm from the actual distance and between the two observers). For each determination of density, two hundred distances between breeding birds were estimated and averaged, birds being located in 10 different zones (20 randomly sampled distances per zone) regularly spread over the study area. We observed that as a general rule six neighbours surrounded a breeding bird. The area occupied by that bird plus its six neighbours, i.e. seven birds, was calculated as the surface of the circle having a radius equal to 1.5 x average distance between birds. Dividing seven by this area yielded bird density (number of birds per m^2).

2.4. Weather monitoring

Air temperature, wind speed, and relative humidity were continuously recorded (1 data per min) using a Vantage PRO 2 weather station (Davis Instruments, Hayward, CA 94545, DAVIS, USA) installed on the colony site. Daily averages during the monitoring periods for early and late breeders are illustrated **Figure 27**. Climatic conditions were relatively similar for early and late breeding birds. Wind speed and humidity did not differ significantly for those two groups, i.e. wind speed was on average 4.9 ± 0.4 m/s for early breeders *vs.* 4.7 ± 0.4 m/s for late breeders, and relative humidity was on average 80.6 ± 1.1 % for early breeders *vs*. 84.0 ± 1.2 % for late breeders; Wilcoxon tests; all $P > 0.06$). Ambient temperature was slightly higher for late breeders than for early breeders (i.e. 8.9 ± 0.3 °C *vs.* 7.6 ± 0.3 °C, t-test; $t = -3.05$, $df = 58.0$, $p = 0.003$). For late breeders, average daily ambient temperature showed a slight increase from late January to late February (0.07 ± 0.3 °C per day; linear regression, $F_{1,28} = 4.223$, $P = 0.049$), whereas for early breeders, average daily wind speed increased slightly from late November to late December (0.1 ± 0.04 m/sec per day; linear regression, $F_{1,28} = 6.841$, $P = 0.01$).

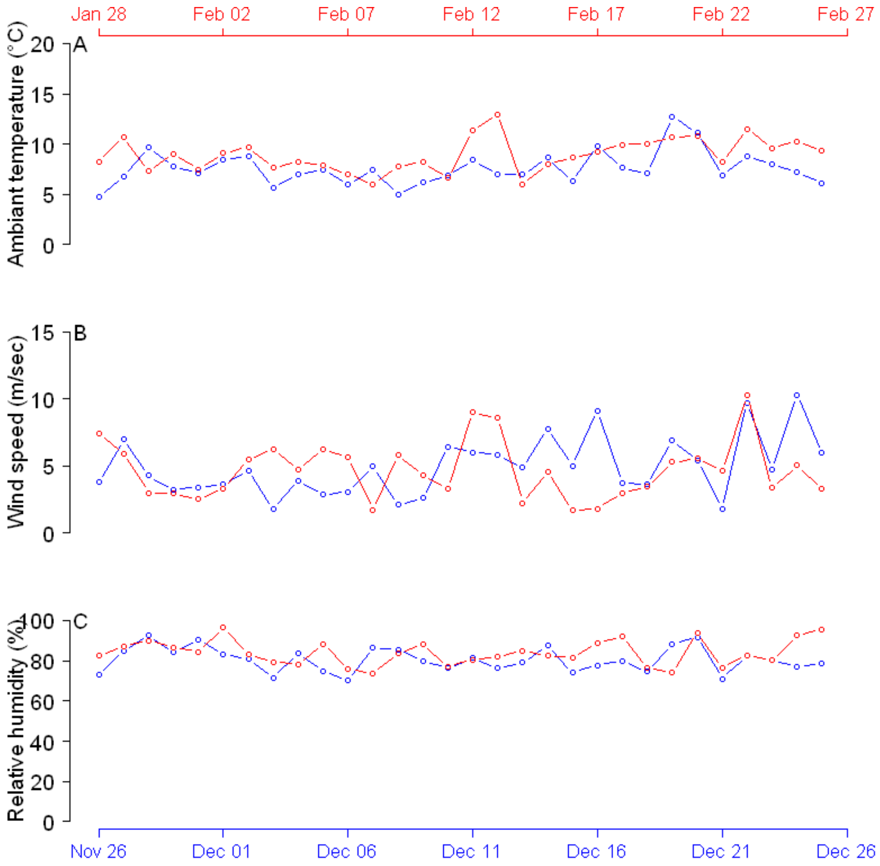

Figure 27. Changes in climate parameters over the 2008-2009 reproductive season in the king penguin colony of la Baie du Marin, Possession Island, Crozet Archipelago.

Daily averages are given for **(A)** ambient temperature (°C), **(B)** wind speed (m/sec), and **(C)** relative humidity (%). Blue lines indicate climatic variables for early breeding birds (late November – December, lower blue X-axis), red lines for late breeders (late January – February, upper red X-axis).

2.5. Calculations

Due to variations in courtship and incubation duration, technical malfunctioning during data logging and logistic reasons (i.e. to avoid loss of equipment, some loggers were retrieved a few days before individuals departed to sea), our data sample size for free-living birds is variable across the time course of courtship and incubation and details are given in the "Results" sections. In general, data sets with less than 3 individuals were not considered. All data sets collected within an hour of bird handling were systematically removed from the analyses in order to eliminate biases from animal handling.

Mean daily HR (dHR) was calculated as the average of 24-hour periods based on every 5-second measurement (i.e. a total of 17280 measurements). We calculated daily resting HR (rHR) using moving averages to determine the 10 daily consecutive minutes during which HR was stabilized at the lowest level, thus yielding daily rHR based on 120 consecutive HR measurements over a 24-hour period. Similarly, mean body temperature was averaged, and total activity counts summed, over 24 hour periods.

2.6. Statistics

Statistics were performed using R v.2.10.1 (http://www.r-project.org/). Breakpoints in the time-course of physiological parameters during courtship and incubation were identified from segmented regression analysis using the 'strucchange' and 'segmented' packages from R (Zeileis et al. 2002, Muggeo 2008). Separate Generalized Estimating Equations (GEEs) were used for pre- and post- breakpoint temporal analysis, which allowed us to include bird identity as a random factor, in order to control for various intercepts (i.e. inter-individual variation in the response). GEEs were run using the 'geepack' package (Højsgaard et al. 2005). Residuals were tested for normality, and we used appropriate distributions whenever assumptions were violated. Gamma distribution was specified to investigate daily changes in HR throughout courtship and incubation, and Poisson distribution was specified to investigate changes in daily activity, which was appropriate as working with count data. Values are reported as means ± SE and results are considered significant for $P < 0.05$.

2.7. Ethical note

All procedures described above and employed during the fieldwork were approved by the Ethical Committee acting for the French Polar Institute (Institut Polaire Français Paul Emile Victor, IPEV) and comply with current French laws. Authorization to enter the breeding colony and handle birds was delivered by Terres Australes et Antarctiques Françaises. During field procedures, animals were hooded in order to keep them calm and reduce the disturbance of neighbouring birds. Manipulations lasted between 5 and 20 min. HR logger packages weighed less than 1 % of adult body mass and were installed in a dorsal midline position to prevent hindering movements of the birds. Flipper bands were removed at the end of the study. Copies of permits are available upon request.

3. Results

3.1. Natural changes in colony density

As illustrated **Figure 26**, our two estimates of bird density demonstrated marked and consistent changes throughout the reproductive season. Colony density was very low at the start of breeding (0.3 bird/m^2 and 30 tagged birds on the surveyed area by the time of first courtships). Then, it markedly and progressively increased to reach a maximum level by the end of January (bird density), or from mid-January to the end of March (colony density index). This maximum density was approximately 15 (bird density) and 12 (colony density index) times higher than that at the start of breeding. Specifically, colony density increased rapidly during early breeder monitoring, by a 6 times factor, but was stable during the period of late breeder monitoring.

3.2. Changes in HR over fasting in captive birds

Daily (dHR) and resting HR (rHR) continuously decreased throughout fasting in males kept captive at a low density (**Figure 28**). Decreases were rapid over the first 5 days of fasting, on average by - 6.4 ± 1.5 (dHR) and - 5.9 ± 1.1 (rHR) bpm/day, and then stabilized at a steady rate of – 1.0 ± 0.3 (dHR) and - 0.7 ± 0.2 (rHR) bpm/day for the remaining of the fast. The difference between daily and resting HR significantly reduced during the fast (i.e. -0.26 ± 0.10 bpm per day, GEEs, *Wald* = 5.72, $P = 0.02$, $n = 25$, $N = 8$ birds), indicating a decrease in physical activity as the fast advanced.

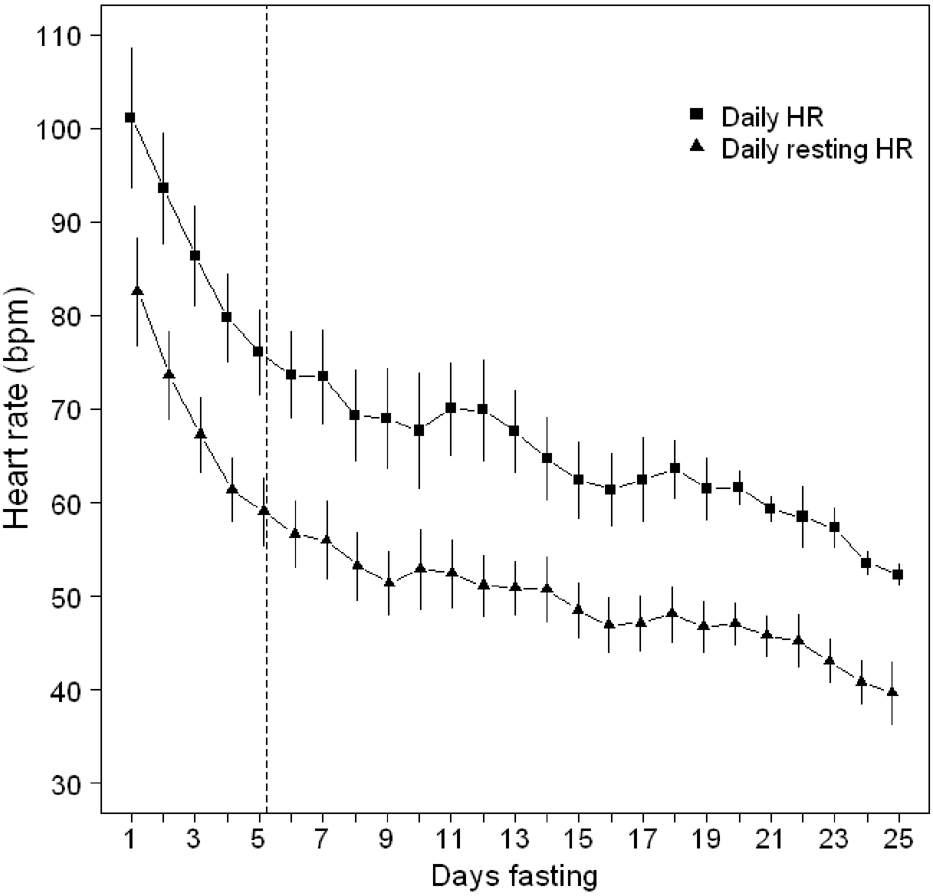

Figure 28. Changes in daily and daily resting heart rate (HR) throughout fasting in early breeding male king penguins kept captive at a 0.3 bird/m^2 density.

Values are means ± SE for 8 birds. The dotted vertical line indicates a change in the slope of HR decrease. The equations for pre- and post-breakpoint regressions in dHR are: dHR = 106.5 ± 8.3 – 6.4 ± 1.5 days and dHR = 79.0 ± 7.7 – 1.0 ± 0.3 days (GEEs; *Wald* = 17.1 and 10.9, all $P < 0.001$, $n = 5$, $N = 8$ birds), respectively. The equations for pre- and post-breakpoint regressions in rHR are: rHR = 86.6 ± 6.3 – 5.9 ± 1.1 days and rHR = 60.1 ± 5.2 – 0.7 ± 0.2 days (GEEs; *Wald* = 30.0 and 12.4, all $P < 0.001$, $n = 20$, $N = 8$ birds), respectively.

3.3. Changes in HR in freely-breeding birds

3.3.1. Early breeders

In early birds, mean dHR first decreased progressively and then surprisingly re-increased. Breakpoint analysis actually revealed that the best fitting model for dHR changes had three change points (**Figure 29A**). We thus divided subsequent analysis into four time periods, according to those breakpoints. GEE estimates for each period are reported **Table 1**.

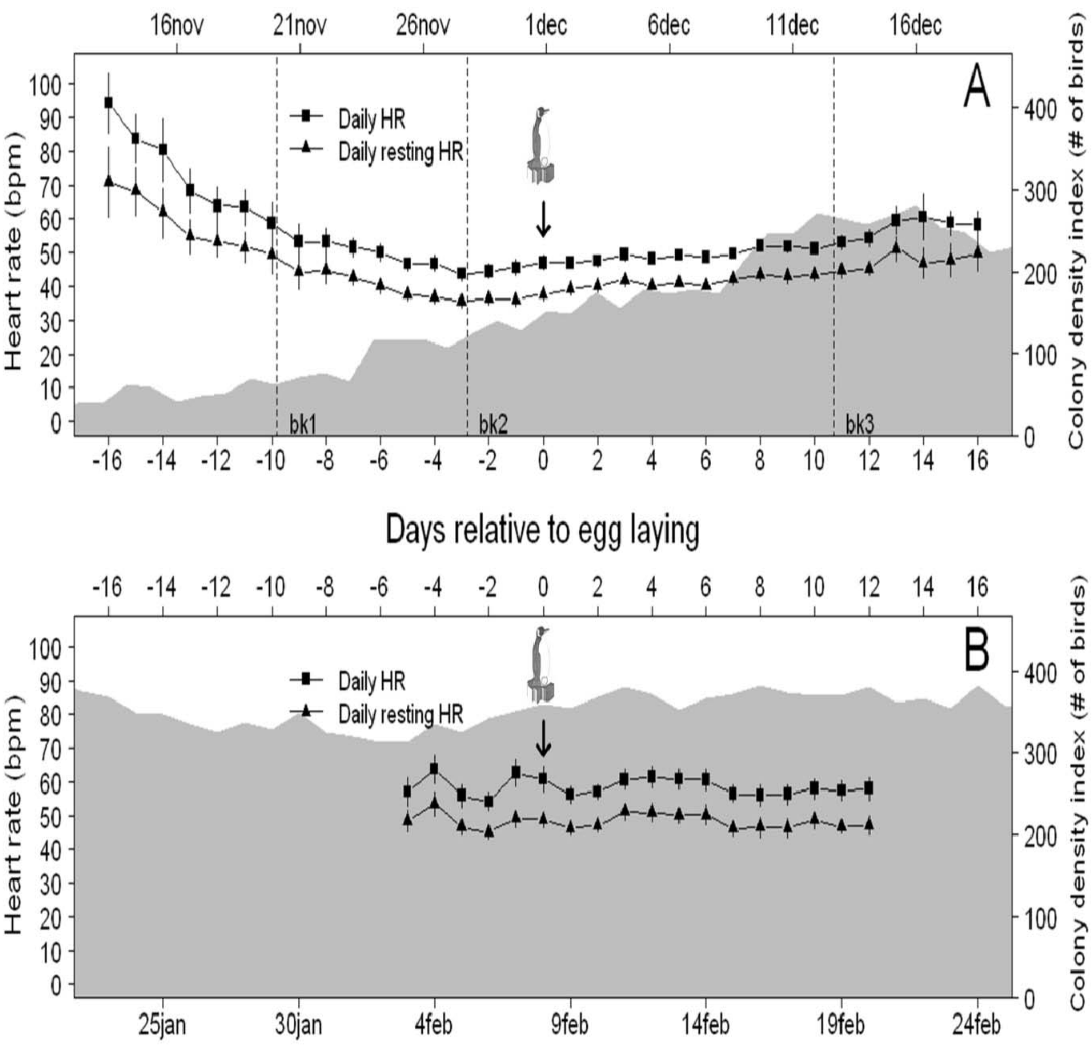

Figure 29. Changes in daily heart rate and daily resting heart rate during courtship and first incubation shift in male king penguins.

(A) Changes occurring in HR for early breeders relative to laying date. Breakpoints (bk1, bk2 and bk3) revealing changes in dHR slopes are given by black dashed lines. Values are given as means ± SE (N = 3-14 birds). **(B)** Changes occurring in HR for late breeders relative to laying date. Values are given as means ± SE (N = 3-10 birds). For both A and B, colony density index appears as the grey area and the arrow shows the laying date.

As for captive birds, dHR of courting birds first decreased rapidly, on average by - 5.3 ± 1.3 bpm per day during the first 6 days (**GEE 1**, **Table 1**). From there onwards, dHR continued to decrease at a slower rate (averaging a drop of - 1.7 ± 0.7 bpm per day) until approximately three days before egg-laying (**GEE 2**, **Table 1**). Then, starting on average on November 28 and 3 days before the onset of incubation, dHR re-increased, at the rate of 0.6 ± 0.2 bpm per day (**GEE 3**, **Table 1**).

		Estimate	S.E.	Wald	P	n (N)
GEE 1	Intercept	4.5	15.5	0.08	0.77	48 (10)
	Day	**-5.3**	**1.3**	**16.4**	**< 0.001**	
GEE 2	Intercept	39.1	3.2	146.0	< 0.001	77 (13)
	Day	**-1.7**	**0.7**	**5.8**	**0.02**	
GEE 3	Intercept	46.6	1.5	941.7	< 0.001	177 (14)
	Day	**0.6**	**0.2**	**12.0**	**0.001**	
GEE 4	Intercept	38.6	11.7	10.8	< 0.001	36 (11)
	Day	**1.3**	**0.9**	**2.2**	**0.13**	

Table 1. Generalized Estimating Equation parameters for the time course of dHR changes during courtship and incubation in early breeding male king penguins.

Parameters for the independent variable appear in bold. Sample size is given as n, and number of individual birds is given in brackets (N).

By the time dHR was at the minimum value of 44.0 ± 1.9 bpm, bird density was close to 1 bird/m^2 and the colony density index indicated that approximately 20% of tagged birds were present in the colony. Thus, daily HR started to increase when colony density reached approximately 30% of its maximum level (25% for bird density and 35% for colony density index). Eventually, dHR no longer significantly increased from day 11 of incubation onwards; **GEE 4**, **Table 1**). The stabilized dHR (56.5 ± 1.4 bpm) was reached on average on December 12, when bird density (1.8 birds per m^2) and colony density index indicated that colony density was approximately 43 % (bird density) to 65 % (colony density index) of its maximum. This stabilized dHR was 30 % higher than the minimal dHR reached three days before the onset of incubation. Changes in rHR (**Figure 29A**) were parallel to those in dHR, with the same breakpoints (statistics not shown). Notably, rHR reached a minimum value (35.5 ± 1.6 bpm) three days before laying and then progressively re-increased to stabilize at 47.1 ± 1.3 bpm (i.e. a 33 % increase) within the last days of fasting. The difference between dHR and rHR (i.e. HR excess due to activity) remained constant during the period of HR increase, i.e. the slope parameter was no different from zero (GEE; *Wald* = 0.25, *P* = 0.62, *n* = 177, *N* = 14 birds), indicating that changes in dHR were essentially modulated by changes in rHR.

3.3.2. Late breeders

In late birds, dHR remained steady throughout the whole recording period (GEE; *Wald* = 0.3, *P* = 0.60, *n* = 137, *N* = 10 birds, **Figure 29B**) and averaged 58.8 ± 0.8 bpm. Resting HR followed the exact same trend (**Figure 29B**), remaining steady (GEE; *Wald* = 0.3, *P* = 0.60, *n* = 137, *N* = 10 birds, **Figure 29B**) at the average level of 48.4 ± 0.6 bpm. These average dHR and rHR values were not significantly different from that observed at the end of fasting in early breeders (i.e. 56.5 ± 1.4 bpm and 47.1 ± 1.3 bpm, for dHR and rHR, respectively), the period during which dHR and rHR were stabilized (GEEs; *Wald* = 0.4 and 0.2, *P* = 0.51 and 0.70, *n* = 173, *N* = 21 birds, for dHR and rHR, respectively).

3.4. Changes in activity and body temperature over courtship and incubation

3.4.1. Physical activity

Consistently with our results in captive birds, total daily physical activity decreased progressively throughout the course of courtship and incubation, i.e. the slope parameter for our models was - 0.2, both for early and late breeders (**Figure 30A and Table 2: GEEs 1 and 4**). As expected, in early breeders, breakpoint analysis revealed a strong, rapid decrease in physical activity (**Table 2: GEE2;** slope = - 0.5) over the same period during which the strongest decrease HR was observed (from days -16 to days -10 prior to egg-laying). There onwards, physical activity decreased slowly (**Table 2: GEE3;** slope = - 0.1) until the end of the monitoring period (see **Figure 30A**). Breakpoint analysis did not reveal any change in slope for late breeders.

3.4.2 Body temperature

When tested over the course of fasting, we found no significant change in stomach temperature both in early and late breeding birds (**Figure 30B**). The slope parameter (- 0.01 days) for our models was similar and non significant both for early and late breeding birds (GEEs; *Wald* = 1.8 and 0.9, *n* = 224 and 144, *N* = 9 and 9 birds, *P* = 0.20 and 0.34, for early and late breeders, respectively). In addition, we found no significant differences in body temperature between early and late breeding birds (i.e. average body temperature was 38.1 ± 0.03 °C for early breeders *vs.* 38.3 ± 0.04 °C for late breeders; GEE; *Wald* = 0.57, *P* = 0.45, *n* = 368, *N* = 18 birds).

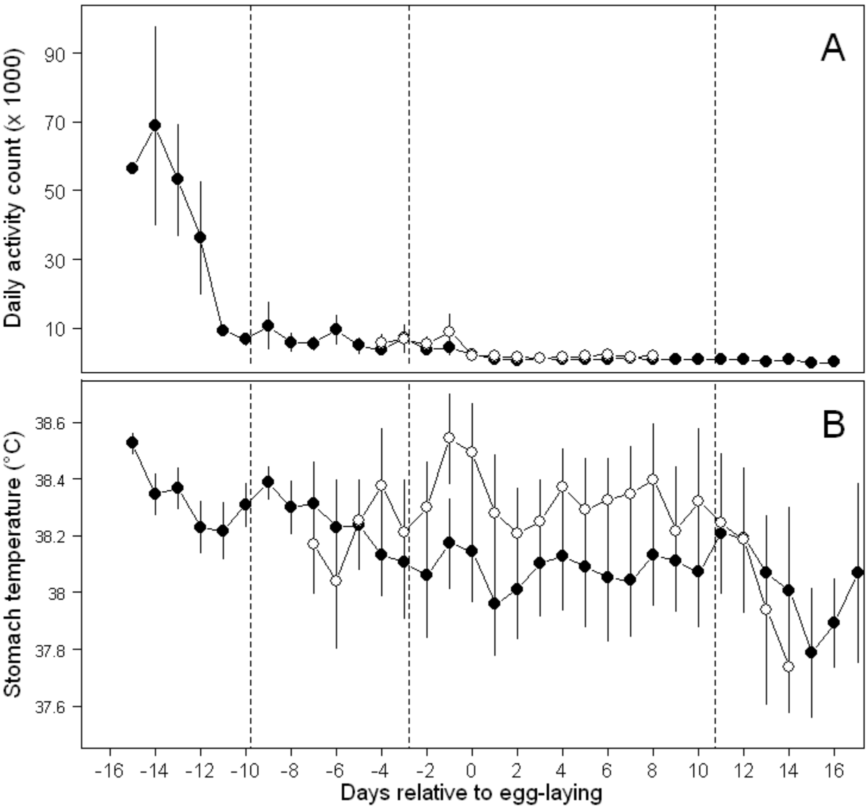

Figure 30. Daily changes in (A) activity and (B) body temperature during courtship and incubation in early (●) and late (○) breeding male king penguins.

Values are given as means ± SE (N = 3-14 birds for daily activity and N =3-9 birds for stomach temperature). For information, breakpoints identifying changes in the course of dHR for early breeders (see **Figure 29**) are indicated as dashed lines.

			Estimate	**S.E.**	**Wald**	**P**	**n (N)**
Early birds	GEE 1	intercept	7.7	0.4	472.6	< 0.001	171 (8)
		Day	**-0.2**	**0.03**	**45.4**	**< 0.001**	
	GEE 2	intercept	3.7	0.9	15.4	< 0.001	15 (3)
		Day	**-0.5**	**0.09**	**36.0**	**< 0.001**	
	GEE 3	intercept	7.7	0.3	509.5	< 0.001	113 (8)
		Day	**-0.1**	**0.05**	**6.8**	**0.009**	
Late birds	GEE 4	intercept	8.2	0.3	1043.7	< 0.001	60 (6)
		Day	**-0.2**	**0.04**	**18.5**	**< 0.001**	

Table 2. Generalized Estimating Equation estimates describing the changes in daily physical activity during courtship and incubation in male king penguins.

Parameters are given for early and late breeding birds. Estimates for the independent variable appear in bold. Sample size is given as n, and number of individual birds is given in brackets (N).

3.5. Changes in resting HR and colony density

We investigated whether the rise in rHR observed in early breeders as of 3 days before egg laying onwards could be attributed to an increasing density of the colony. For each day from day -3 to day 16 (**Figure 29A**), average rHR and colony density index were calculated. The same calculations were made for late breeders over the whole monitoring period. In early breeders, rHR was highly and significantly related to colony density ($R^2 = 0.88$, $F_{1, 18} = 136$, $P < 0.001$, $n = 20$; **Figure 31**). Conversely, in late breeders, colony density remained relatively constant throughout courtship and incubation and no significant change in rHR with colony density was observed ($F_{1, 16} = 0.5$, $P = 0.50$, $n = 18$; **Figure 31**).

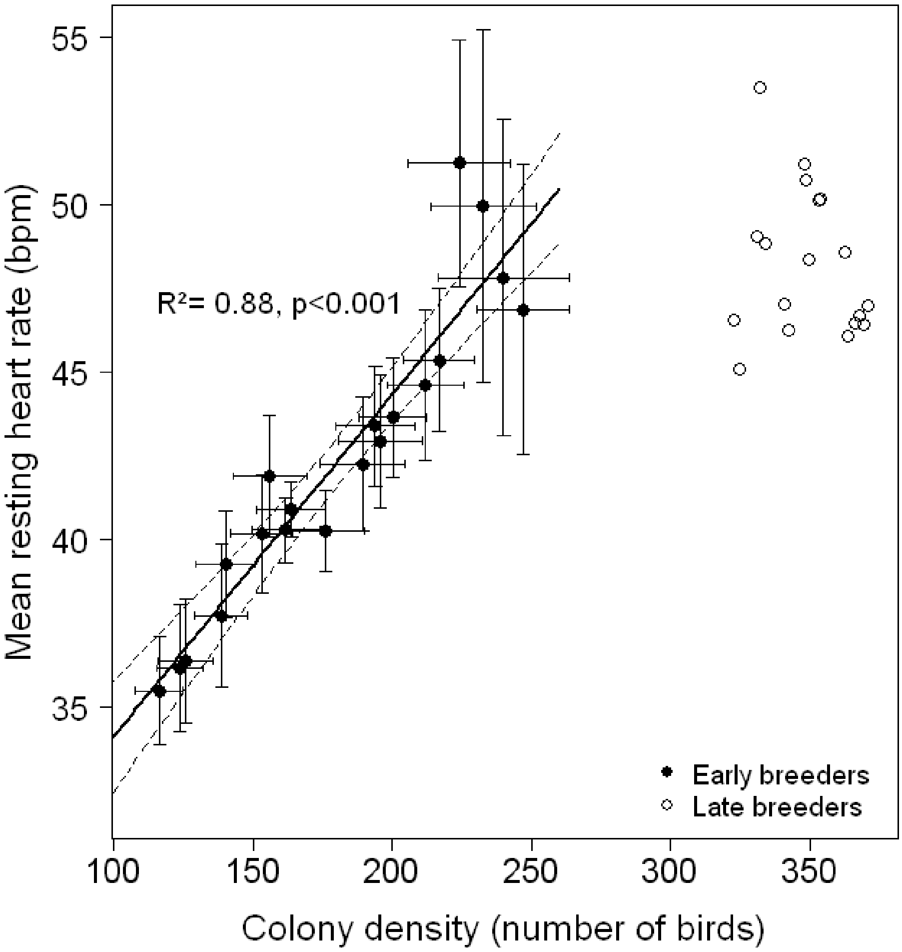

Figure 31. Influence of colony density (number of birds) on resting heart rate (rHR, bpm) in breeding male king penguins.

Significant regression line (solid line) and 95% confidence intervals (dashed lines) are given for early breeders (rHR = 24.0 ± 1.8 + 0.1 ± 0.01 x density). Values are means for 3 to 14 (early breeders) or for 3 to 10 (late breeders) birds for each day from 3 days before egg laying onwards (early breeders, *n* = 19 days) or throughout the whole monitoring period (late breeders, *n* = 18 days). For clarity, SE (bars) are presented only for early breeders.

4. Discussion

In this study, we investigated the breeding energetics of a free-living seabird for which reproduction is associated with periods of prolonged fasting in a crowded colonial environment. Specifically we considered the longest breeding fast endured by male king penguins, which includes both the courtship period and the first incubation shift. We show that whereas incubating male penguins reduce EE during long term fasting by maintaining physical activity to minimum levels, incubating adults do not seem to be able to resort to adaptive heterothermia (see Eichhorn et al. 2011) in order to depress their metabolic rate. In addition, our results suggest that colonial context (especially group density) might have an important effect on the modulation of EE in colonial seabirds. Such results might lead to considering the important nature of social contexts in conditioning important life-history variables such as breeding timing, breeding territory location, or reproductive success in colonial breeders.

4.1. Changes in body temperature: is incubation a constraint for breeding seabirds?

One of the objectives of our study was to consider whether male king penguins may adapt to the longest incubation fast by resorting to changes in body temperature to limit their energy expenditure.

Saving energy by acting on the amount of energy required to maintain a high body temperature (i.e. by modifying the set point of thermoregulation), is a strategy that has been widely reported in endotherms (Geiser 2004). However, whereas extremely effective in terms of energy savings (Geiser 2004), such a physiological adaptation appears to be mostly limited to small endotherms, given that cooling and re-warming rates of the body are mostly size-dependent (Geiser and Ruf 1995). In line with this, animals exhibiting strong torpor are usually small (Geiser 2004) and until recently, the largest torpid bird was reported to weigh around 500 g (Körtner et al. 2000, McKechnie and Lovegrove 2002). Nonetheless, recent studies have suggested that even larger endotherms may resort to transient decreases of deep core temperatures as a means of energy sparing. Such decreases in body temperature have for instance been suggested to explain the surprisingly long diving bouts of adult king penguins (Handrich et al. 1997), the extended fasting periods of king penguin chicks

(Eichhorn et al. 2011), and as a means of energy sparing during fasting in the emperor penguin (Dewasmes et al. 1980, Gilbert et al. 2007).

Here, we found that deep core temperature did not appear to change in king penguins fasting for a month ashore, while courting and incubating. Our results are partly consistent with previous finding in emperor penguins (Gilbert et al. 2007) which indicate that whereas breeding males (which take duty for the long 4-month incubation shift in this species) do experience a decrease in body temperature during courtship, the decrease ceases as soon as males start incubating the egg, whereupon body temperature actually re-increases (Gilbert et al. 2007). In breeding birds, the need to maintain an elevated temperature for egg incubation (around 36 °C in the king penguin, Groscolas et al. 2000) may thus constrain the possibility to decrease body temperature, as observed in our study. This is consistent with previous studies reporting that incubating birds usually resist torpor entry, unless incubation is interrupted or abandoned (Vehrencamp 1982, Csada and Brigham 1994, Gilbert et al. 2007) and illustrates the trade-off between the needs to save energy and incubation requirements (Gilbert et al. 2007). Nonetheless, in their study on emperor penguins, Gilbert and colleagues (2007) found that fasting males did exhibit a significant decrease in body temperature during courtship (when the birds were not yet incubating), and that in one case, a male which failed incubation exhibited an elevated body temperature while incubating, but body temperature dropped as soon as the egg was lost. How come then, did we not observe a similar decrease during courtship in male king penguins? A possible explanation may stem from the duration of fasting periods for both species (4 months for the emperor penguin *vs.* 1 month for the king penguin) highlighting the need for different energy savings. In addition, emperor penguins incubate in the midst of the Antarctic winter, well below their thermoneutral limit. This is not the case of king penguins which breed within their thermoneutral zone, with supposedly no-additional costs for thermoregulation (Barré 1980). Thus, it appears that incubation might be a factor limiting drops in core body temperature for breeding king penguins.

4.2. What factors affect HR in breeding king penguins?

Our results in captive birds kept at a low density show that both daily HR and resting HR of king penguins decrease throughout the course of a prolonged fast. This result is in agreement with previous findings in the same species (Fahlman et al. 2004) and very likely mostly reflects the decrease in daily energy expenditure associated with a decrease in physical activity and body mass, and increasingly efficient energy sparing (Fahlman et al. 2004). In addition, the rapid initial decrease in HR within the first five days in the pen could reflect both habituation to captivity, and the shift from phase I to phase II fasting, i.e. a metabolic transition during which animals adjust to a preferential utilization of lipid stores to fuel their metabolism (Groscolas 1990).

In free-living birds, we found that overall physical activity declined progressively over the course of courtship and incubation in males that were breeding either early or late in the season. Thus, a decrease in overall activity, as the birds settle down on their breeding territory, likely contributes to the decrease in HR levels (and thus DEE) observed during the courtship period in early birds. Whereas we do not observe a decrease in HR levels in late breeders during the courtship period, this is most likely due to the short length of the monitoring period prior to laying (i.e. some 5 days) preventing us from observing the decline in HR. Indeed, if we consider monitoring days which were excluded from our analyses because of low sample size (< 3) in late breeders, we observe that in a couple of cases, HR rate levels exhibited a similar decrease during courtship than early breeders (see **Figure 32**). This suggests that a larger sample size of late breeders followed over a longer courting period than in the present study would reveal a significant similar decrease in HR as observed in early breeders and captive birds.

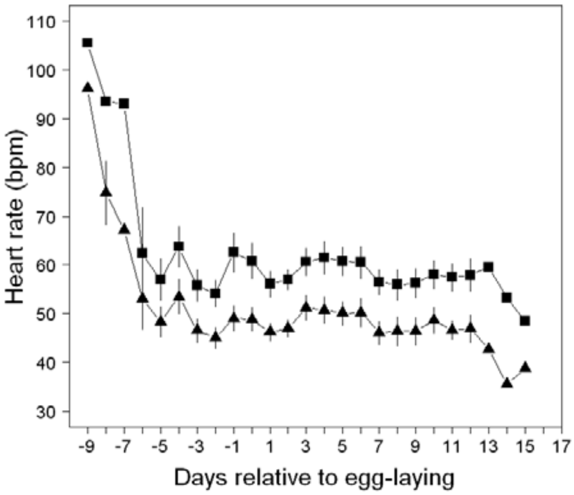

Figure 32. Heart rate levels for late breeding birds, including days for which sample size was < 3 birds.

Average daily HR values are given as squares, resting daily HR values as triangles. Values are means ± SE.

Physical activity is known to strongly increase EE in a number of species, including seabirds (Jodice et al. 2003, Weimerskirch et al. 2000). Whereas obviously, EE is at its highest during bouts of sustained physical activity such as flying or swimming (Jodice et al. 2003, Weimerskirch et al. 2000), even more discrete physical activities such as preening or walking may translate into substantial energy costs (Pinshow et al. 1977, Viblanc et al. 2011). During the breeding season, king penguins go through a phase of courtship during which breeding pairs roam the colony in search of a breeding territory. Subsequently, pairs settle down on the territory and defend it until the egg is laid. From thereon, males remain incubating, exhibiting virtually very little movement asides those related to comfort behaviour and territory defence (see **studies 1 and 2**; Viblanc et al. 2011, Viera et al. 2011).

Our results of activity patterns in free-living birds are consistent with those for captive individuals, for which we found the difference between dHR-rHR (reflective of physical activity) to decrease significantly over the course of fasting. Interestingly however, in free-living birds, when considered beyond the onset of incubation, a noticeable rise in dHR and rHR could be observed in early breeders (even though physical activity was maintained at minimum levels), whereas such an increase was not observed in late breeders. An overall decrease in physical activity throughout the fast associated with a concomitant rise in HR levels during incubation may seem somewhat paradoxical. A first explanation may lie in physiological differences between early and late breeding birds. For instance, if a change in body composition were to exist between early and late breeders, this may be reflected in metabolism and might explain the pattern observed. Typical adaptations to fasting in penguins include arriving at the colony with a high lipid to protein ratio (Cherel et al. 1993, Groscolas 1990, Robin et al. 1986). As most of the energy used during the fast is derived from fats (93% during phase II, Cherel et al. 1994a), birds arriving at the colony with a low relative content of metabolically active tissue (i.e. low lean body mass) and a high content of adipose tissue are naturally at an advantage (Cherel et al. 1993). However, variations in the build up of body reserves in anticipation of prolonged fasting are known to exist (Cherel et al. 1993) and potential changes may occur depending on breeding timing in the season. For instance, prior to a 5-month period of food scarcity during winter, king penguin chicks present twice the adiposity of pre-breeding adults (34% *vs.*18%). Similarly, during the moulting fast, adults present higher protein content than during the pre-breeding fast, as part of their protein reserves are required to fuel feather synthesis and growth at that time (Cherel et al. 1993). Such differences in body composition may also hold true (albeit to a lesser extent) when considering early and late breeding individuals. A lower lipid to protein ratio in late birds might explain why HR levels are observed to be higher in late individuals, as resting metabolic rate may generally be higher. Additionally, assuming the MR increases due to incubation (Handrich 1989, Tinbergen and Williams 2002), the rise in HR levels observed in early breeders may potentially be attributed to RMR achieving a lower level before incubation in those individuals. The rise in rHR values (and thus RMR) observed as of incubation would then be a reflection of the energy cost associated with egg-warming (**Figure 33**).

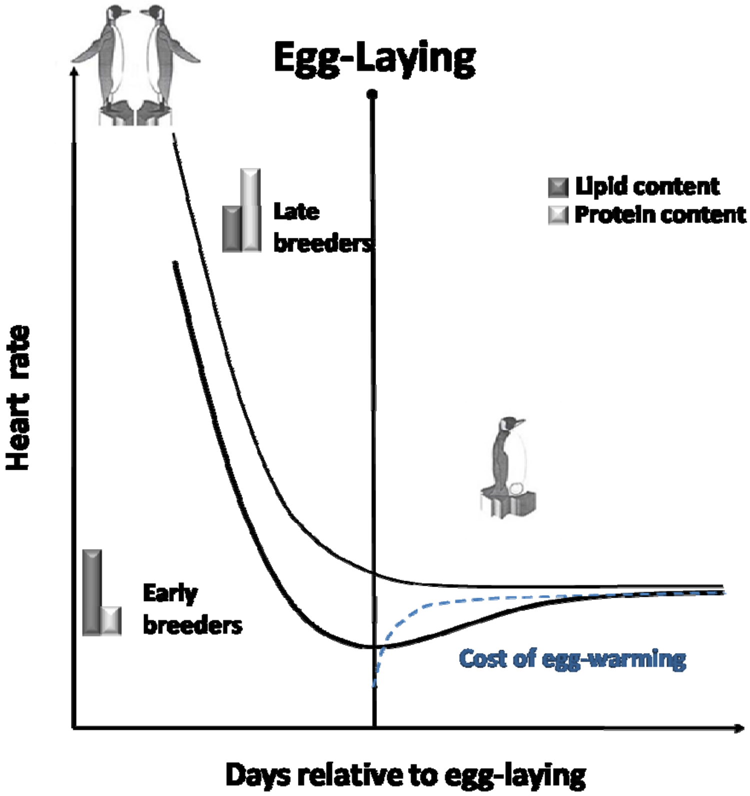

Figure 33. Theoretical figure explaining one potential mechanism for the rise in heart rate levels observed in early breeders but not in late breeders during incubation.

Under this scenario, the rise in HR levels would be a consequence of differences in body composition between early and late birds, and of the energy cost associated with egg-warming. Bold line represents HR for early breeders, normal line for late breeders. The blue dashed line represents the energy cost associated with egg-warming – (*penguin drawings courtesy of M. Gauthier-Clerc*).

However such a suggestion relies on the assumption that early and late breeding birds do indeed differ in their body compositions. Birds that arrive late for breeding at the colony are typically pressed for time to hatch and feed their chick before the arrival of the following winter. They are known to present accelerated phases of the breeding cycle (accelerated moulting and shorter pre-breeding foraging trips) when compared to early breeders (Gauthier-Clerc et al. 2002b), and actually, when comparing specific body mass (*dm/mdt*) at the onset of courtship, late breeders are found to have a significantly lower body mass gain (acquired through pre-breeding foraging trips) than early breeders (Gauthier-Clerc et al. 2002b). Such differences in body mass gain may reflect differences in body composition, and body reserves may partition differently between protein and lipid contents for early and late breeding birds. Shorter foraging trips in late birds may lead individuals to forage closer to the colony or change the ratio of preys upon which they forage, increasing the amount of protein-rich squid in their diet while decreasing the amount of oily myctophids (Cherel et al. 1996, Raclot et al. 1998, Phleger et al. 2007). Obviously, further investigations on body composition and foraging habits are required to determine whether early and late breeding birds really differ in their body composition, and whether such differences might explain differences in terms of energetics. The use of stable isotopes to determine relative contributions of various prey types in the diet and foraging grounds (Cherel et al. 2005, Cherel and Hobson 2007, Cherel et al. 2007) appears a promising, non-invasive tool for answering such questions.

Nonetheless, the fact that the increase in HR levels observed in early breeders actually occurs prior to egg laying (as soon as 3 days before on average) suggests that other mechanisms might affect the energy expended while at rest. An alternative hypothesis which may then explain the rise in HR levels (especially rHR) observed in early breeders could have to do with changes in the social environment as the season advances. This hypothesis is discussed separately below.

4.3. HR in free-living penguins: is EE influenced by the presence of conspecifics?

For one having moved from a small village to a major city, or having walked out of a peaceful garden into a crowded mall, the effects of population density on social stress may intuitively seem plain as day. The insight takes root as many empirical studies show how population density might influence the biology and ecology of species throughout the animal kingdom. Population density has for instance been shown to affect species' aggression rates (Nephew and Romero 2003), parasite loads and diversity (Arneberg et al. 1998, Arneberg et al. 2002), population sex ratio (Kruuk et al. 1999), offspring survival and adult body condition (Festa-Bianchet et al. 1998), stress hormones (Rogovin et al. 2003), and reproductive success (Festa-Bianchet et al. 1998). Experimental studies have further shown how acute changes in conspecifics density may lead to profound changes in individuals' behaviour and physiology, e.g. cardiac and hormonal stress responses, steroid concentrations (Peng et al. 1989, Chapman et al. 1998, Nephew and Romero 2003). Surprisingly however, whereas the effect of population density on Darwinian fitness have been well investigated (see references above), very few studies have considered the effect population density may have on the energy expenditure (EE) of group-living animals, nor have attempted to measure the energy cost of group living per se (but see Cao and Dornhaus 2008). However, high social densities may well affect an animal's perceived social stress and the energy expended to fuel the basal metabolism while resting may be consequent.

Here, we report for the first time a strong association ($R^2 = 0.86$) between colony density and resting HR in fasting male king penguins, and suggest that an increase in colonial density may translate into significant metabolic costs (i.e. RMR and DEE) in breeding penguins. Indeed whereas early breeders showed an increase in rHR during incubation, this was not the case for late breeders. However, as previously stated, colonial conditions were very different for those two groups. Over the study period for early breeders, our index of colony density increased 4.3 times (from 54 to 233 individuals) and did so two fold between the end of courtship and the end of the first incubation shift (i.e. from 116 to 233 individuals). In contrast, this increase was 4 and 2 times less in late breeders, respectively (i.e. from 349 to 371 individuals, and from 322 to 371 individuals, over the whole study period and from the end of courtship until the end of incubation, respectively), and such differences in the social environment may well explain the differences observed in HR levels in those two groups. Additionally, the fact that rHR in early breeders reached a plateau at a similar level to that of rHR in late breeders during incubation, suggests that rHR may increase up to a limit, fixed by a stabilization in colony density early in the breeding season. It is then of interest to consider to what extent an increase in colony density might translate in terms of increases in rHR (and thus metabolic rate) for breeding birds. A first estimate may be obtained by comparing average rHR level for early breeders at the end of the first incubation shift (i.e. 56.5 ± 1.4 bpm: as of 11 days onwards, when HR levels are stabilized and when the colony reaches a maximum density) to the minimum average rHR level achieved during courtship and incubation (i.e. 35.5 ± 1.6 bpm: three days before the onset of incubation when colony density is low but not minimum). For early breeders, this would result in a (56.5-35.5)/35.5 = 60 % increase in rHR due to the increase in colony density. In fact, this increase is probably underestimated, as the colony was not empty when rHR starts increasing, but rather not quite half of maximum density (i.e. 116/371 birds). Moreover, according to our results in captive birds, it is likely that rHR in incubating birds would have decreased under the influence of the fast, if they had been breeding out of the colonial context. Again, this could have contributed to blunt the increase in rHR due to the increase in colony density. These results suggest that the increase in rHR due to an increase in colony density is all but negligible, and that breeding animals may have to support a significant extra energy cost imposed by their social environment.

Obviously, our results are correlative in nature rather than experimental. Further studies are thus warranted in order to determine whether a substantial energy cost is actually associated with group-living and colonial density, or whether the differences observed may be the result of body composition as previously mentioned, or other environmental parameters. For instance, whereas weather conditions were similar for early and late breeding birds, the slight increase in wind speed over the monitoring period of early breeders might also explain part of the HR increase observed, as wind speed has shown to be related to HR in the Adélie penguin (*Pygoscelis adeliae*) (Culik et al. 1989). On the other hand, present weather data and previous findings on thermoregulatory processes in king penguins (Barré 1980, Le Maho and Despin 1976, Le Maho et al. 1979) suggest that breeding king penguins in our study were most likely constantly in their thermoneutral zone.

Experimental manipulations on colony density should provide further insights into the potential effects of crowding on EE. Modifying the surrounding density of incubating individuals could for instance be achieved using exclosure experiments, preventing neighbouring congeners to settle close to monitored individuals. Such experiments would allow us to consider the potential effects of high or low densities on metabolic rate and EE, throughout the various incubating and brooding shifts (and in both sexes). Additionally, it would be of interest to consider whether social crowding may relate to some form of chronic stress in breeding king penguins. This could for instance be achieved by measuring basal plasma levels of corticosterone (the main stress hormone in birds, ref), in birds breeding at different densities.

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IV. Sensitivity to the social environment and effects on physiology

From a human perspective, none of us need persuading of how strongly our social environment affects our physiological features and well being. If you have ever witnessed a bar fight or a heated discussion between working colleagues, felt anxious about giving a talk during a work seminar or at an international conference, you have most certainly experienced the adrenaline rush that got your heart pumping at an elevated rate in a fraction of seconds. If you have lived situations of prolonged social tension, such as bearing a long incomfortable situation at work or having to deal with personal problems such as family issues, you might have felt your mood go down, transitory or prolonged depression affecting your perceptions of reality and health. Those effects are those of stress, acute and chronic.

The effects of stressors (or ‘stressing situations’) have been long documented in the animal kingdom, and have lead to a florishing field of research both in the domains of medecine and ecology. Classicaly studied in terms of the ‘fight-or-flight’ response, and of the adaptive value of stress reponses for the animal’s survival, recent emphasis in ecological research has been given to the effects the social environment may have on an individual’s perceived stress and underlying physiological responses. In the following chapter, let us consider whether and how the social environment of penguins might influence the physiology of breeding birds. As previously mentionned, king penguin breed in a particulary exceptionnal social context. Surveys indicated that the humengous aggregations formed by their colonies during the austral summer may reach up to 300.000 pairs. Unlike their Antarctic cousin the emperor penguin, king penguins are extremly territorial and aggressively defend small breeding territories. As they are breeding under a situation of strong energy constraint, it is of interest to consider whether birds are consistently “on the lookout” for what is happening around them, and whether energy costly stress responses are affected by the social environment.

In the following studies, we attempt to provide some answers to the following questions: Are birds sensitive to their social environment ? Do they always react to what their neighbours are doing ? Do they acclimitize to social stressors? Is there an evaluation of the risk associated with specific situations? Does the social enviroment entail some sort of cost in terms of energy or chronic stress? And we do so by investigating heart rate and hormonal stress responses of breeding birds to their colonial context.

First (**Study 4**), we ask whether penguins are sensitive to the social environment. We consider whether focally observed breeding birds exhibit different heart rate responses to aggressive neighbours depending on whether they are actively engaged in an aggressive interaction or only remain witness to neighbouring fights. We also consider whether HR responses differ according to the risk (threats/attacks, number of participants, length of the aggression) associated with an aggressive interaction.

Then, following our previous findings (**study 3**) that high colony density (*viz*. the presence of numerous neighbours) may have a substantial effect on the energetics of breeding penguins, we further question the potential effects of colony density on individual physiology in two different studies. In **Box 1**, we experimentally manipulate colony density around breeding birds and investigate relationships between resting heart rate and experimental changes in density. In **Box 2**, we consider baseline levels of stress hormones in birds breeding at different colony densities, and search for a potential relationship between colony density and chronic stress.

STUDY 4 – Are king penguins sensitive to their social environment?

Coping with social stress: heart rate responses to agonistic interactions in king penguins.

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Submitted (in revision) – **Behavioral Ecology**.

Abstract

In colonial breeders, agonistic interactions related to territory defence may be stressing and have profound physiological implications. Animals might therefore modulate their response to social stressors according to the relevance of the latter, e.g. associated risks, and to their motivation, e.g. depending on their reproductive status. From heart rate (HR) and behaviour recordings in 20 adults at various stages of the breeding season, we investigated the sensitivity of king penguins to their highly aggressive colonial environment. A total of 589 agonistic interactions, 223 in which birds were actors and 366 in which birds remained bystanders, were characterized. We found that HR increased during agonistic interactions according to their relevance, both in actors and bystanders. The risk (threat displays or physical attacks), length, and intensity (the rate of aggressive events) of an interaction significantly influenced the HR response in actors. For bystanders, however, only the duration of interactions seemed to matter. Our results also suggest a role for individual motivation, initiators of agonistic interactions showing higher HR increases than responders and increases varying throughout the reproductive season. We conclude that individual context-assessment and motivation modulate physiological responses to social stressors in group-living animals, and suggest strong involvement of an emotional component in these responses.

1. Introduction

Reaping individual fitness benefits from clustering with conspecifics has paved the road for the evolution of group-living and animal sociality (Alexander 1974, Baglione et al. 2002, Viblanc et al. 2010, Wilson 1975, Dobson et al. 2011b). However, sociality comes with costs. Individual requirements must compromise with conspecifics needs in order for animal groups to maintain their integrity as a whole (Armitage and Schwartz 2000, Sueur et al. 2010, West-Eberhard 1979). Not surprisingly does the social world have profound effects on individuals, modulating both behavioural responses (Boissy and Le Neindre 1997) and physiological changes (Cacioppo 1994), e.g. by acting on hormone (Creel 2001, Goymann and Wingfield 2004, Oliveira et al. 2001) or neurotransmitter (Edwards and Kravitz 1997) secretion, immune function (Bartolomucci 2007), or on the regulation of the autonomous nervous system (as reflected, e.g. by changes in heart rate, Aureli et al. 1999, Berntson and Boysen 1989, Wascher et al. 2008a).

Evidence for the importance of social contexts in influencing stress responses of free-living animals is now growing (Aureli et al. 1999, Wascher et al. 2009, see Cacioppo 1994) and an 'emotional component' of individuals' physiological responses to social situations might have been frequently overlooked (Lazarus 1999, Kemeny 2003). Indeed, cognitive appraisal processes (i.e. processes enabling the categorization of situations in terms of their significance for well-being; Lazarus and Folkman 1984, Desire et al. 2004, Desire et al. 2006) might "profoundly shape the specific nature of the physiological response to stressful circumstances" and therefore, "the way the individual thinks about the situation may override the impact of the specific nature of the conditions themselves" (Kemeny 2003). For instance, in a series of studies considering social interactions in greylag geese (*Anser anser*), Wascher and colleagues (Wascher et al. 2008a, Wascher et al. 2008b, Wascher et al. 2009) recently showed that the individual's physiological response (i.e. heart rate) to stress was subject to both the nature and intensity of the social context, as well as to the identity of those involved. The authors argued that the differential physiological investment observed may reflect differences in emotional involvement depending on the social context (Wascher et al. 2009).

The case of aggressiveness and agonistic interactions is of particular relevance for social contexts. Intra-specific competition is present in a wide array of species across the animal kingdom and agonistic interactions are commonly performed when individuals defend undividable resources (such as territories, food, mates or offspring, for instance). Since the ultimate genetic benefits of defending a resource generally outweigh the costs associated with its defence, aggressive behaviours have persisted and been maintained throughout the course of evolution (Maynard-Smith 1982, Maynard-Smith et al. 1988). However costs cannot be neglected as they come in the proximate form of risk of physical injuries, time and energy invested into agonistic interactions, which may ultimately translate into affecting the individual's fitness. Of particular interest is the finding that, not only do aggressive encounters affect the physiology of those involved, they also modulate that of bystander individuals (i.e. social congeners which only remain witnesses to such interactions, Oliveira et al. 2001, Wascher et al. 2008b). There is thus little doubt that specific social contexts (e.g. such as agonistic encounters differing in risk) may elicit varying physiological responses in different individuals, and how important these responses may be in terms of physiological investment merits further consideration.

In this study, we investigated HR responses of free-living, colonial king penguins (*Aptenodytes patagonicus*) to agonistic interactions, i.e. either threat displays or physical attacks with body contact. Heart rate (HR) is a highly sensitive physiological parameter, which may be used to investigate individuals' reactivity to the social environment (Nakagawa et al. 2001, Wascher et al. 2008a) and responsiveness to stress (De Villiers et al. 2006, Nephew et al. 2003). In king penguins, reproductive pairs vigorously defend a small territory (approx. 0.5 m^2), exhibiting a very high rate of agonistic interactions while incubating their only egg or brooding their young chick in their brood pouch. Indeed, 14% of daily time-budget is devoted to territory defence (Viera et al. 2011) and a mean of 100 interactions per bird and per hour is observed by this time (Côté 2000). Thus, king penguins present an interesting model for evaluating changes in social stress responses in a strong context of aggressiveness.

We examined whether focal king penguins elicited a noticeable HR response during aggressive interactions, either when actively taking part in (actors) or remaining witness to (bystanders) the interactions. We expected the emotional component to be important in modulating HR responses and predicted that even bystanders would elicit strong physiological HR responses (albeit to a lesser extent than actors), as they may be concerned by redirected aggression. Further, we expected stress responses to show variability depending on the aggressive context and predicted HR responses to vary with the risk (i.e. threat displays or physical attacks), the duration, and intensity (rate of aggressive events within an interaction) of an agonistic interaction. Lastly, we considered the possibility that the motivation of individual birds, i.e. depending on their role as initiators versus receivers in agonistic interactions, or depending on whether they were incubating an egg or brooding a chick of potentially different reproductive value, could modulate the HR response.

2. Methods

2.1. Study population and ethical considerations

This study was conducted throughout two consecutive breeding seasons between 2008 and 2010 (from late December to late March) in the king penguin colony of La Baie du Marin (Possession Island, Crozet Archipelago, 46°25’ S, 51°45’ E) which is host to over 16.000 pairs of king penguins (Delord et al. 2004). Birds were marked using a non-permanent animal dye (Porcimark®, Kruuse, Langeskov, Denmark) and flipper banded for field identification during HR recording and behavioural observations. All procedures described in the following sections and employed during the fieldwork were approved by the Ethical Committee of the French Polar Institute (Institut Polaire Français Paul Emile Victor, IPEV) and comply with current French laws. Authorizations to enter the breeding colony and handle birds were delivered by the French Committee for Polar Environment and from the Terres Australes et Antarctiques Françaises. Flipper bands were removed at the end of the study.

2.2. Heart rate recording

We used externally mounted HR-loggers (Polar® model RS800, Polar Electro Oy, Kempele, Finland) to monitor the HR response to agonistic interactions in king penguins freely breeding in their social context. Loggers were specially adapted for suitable use on king penguins and accurately recorded HR within the HR range of free-living birds (see Groscolas et al. 2010 for details). The HR logger unit was set to store the sampled data for up to 31.5 hrs, sampling at a rate of one data point per second, which was appropriate considering the short duration of the considered interactions (e.g. threat displays lasted for 7.7 ± (s.e.) 0.3 sec. *n* = 223). Electrodes were composed of two stainless-steel wires attached to gold-plated safety pins, which were placed subcutaneously on the dorsal region of the animal. The whole apparatus was attached using adhesive tape (Tesa®) to the dorsal feathers of the animals and weighed less than 1% of total body mass. Loggers and electrodes were out of the reach of animals and did not interfere with their usual behaviour, as equipped individuals soon resumed normal activity after handling (e.g. preening, stretching, sleeping, and fighting) and could not be distinguished from other individuals in behavioural patterns during the subsequent day (personal obs.). Following equipment, birds were left to recover overnight and video recording was resumed the following day.

2.3. Behavioural observations

A total of 20 birds (12 males, 8 females), either incubating their egg or brooding a young chick, were equipped with a HR-logger and their behaviour was monitored simultaneously to HR for a period of six hours per birds (using a Sony DCR HC53 Handycam at a maximal distance of 10m to the focal individual). When incubating and brooding, male and female king penguin alternate shifts ashore for tending the egg or chick and periods at sea for foraging (Stonehouse 1960, Weimerskirch et al. 1992). Equipped birds included incubating males at shift 1 (*i.e.* at the onset of the 53-day incubation period) and 3 (i.e. close to hatching) of incubation, and at shift 5 of brooding (around 2 weeks after hatching). Females were equipped at shift 2 of incubation (central part) and at shift 6 of brooding (around 4 weeks after hatching). From the 120 hours of behaviour recording, we characterized a total of *n* = 589 agonistic encounters, which could be classified as intense or non-intense interactions. Intense interactions (*n* = 366; range = 8–48 per bird) consisted of attacks with physical contact (beak or flipper blows), whereas non-intense interactions (*n* = 223; range = 5–23 per bird) consisted of threat displays (beak pointing and gaping, i.e. vocalizing beak open towards a conspecific). Here, we refer to intense and non-intense interactions as "blows" and "threats", respectively.

2.4. Data analysis

We first investigated whether breeding king penguins showed a noticeable HR response to agonistic encounters and if so, whether this response was affected by individual involvement in, or by the nature of, encounters (*viz.* actors *vs.* bystanders, threat displays *vs.* physical blows). We compared the HR response of actors or bystanders, by calculating mean and maximum HR elevations, as well as HR excess during aggressive interactions. HR elevations were calculated as the difference between the momentary baseline HR value 2 to 3 seconds before the interaction and either the mean HR value during the interaction (until HR reached baseline levels again) or the instantaneous maximum HR value reached during the interaction (see Wascher et al. 2008b). As mean and maximum HR elevations were highly correlated (Spearman's rho test, $r = 0.95$, $p < 0.001$, $n = 589$) and yielded similar results, we chose to present only maximum HR elevations and HR excess in our results. HR excess was calculated as the mean HR value during the interaction (in beats per minute) times the duration of the HR elevation (in minutes) owing to an aggressive interaction. HR excess corresponded to the area under the HR curve and represented the number of heart beats produced in excess of what would have been produced had HR been maintained at baseline levels. As no significant gender difference was noted in HR responses (e.g. HR excess and maximum rise in HR were not significantly different between sexes; GLMMs: z = 1.9 and 1.8, p = 0.09 and 0.10, n = 589, 20 birds, respectively), both sexes were pooled in the analysis not considering specific incubation or brooding shifts. Independent variables regarding the nature of agonistic interactions included: (i) the behavioural type (BT) of considered interactions (threat displays or blows); (ii) the number of individuals involved (Ni); (iii) the duration of aggressive encounters (dAi); and (iv) their intensity (i.e. the rate of aggressive events within an interaction; number of blows or threats exchanged per unit time over the interaction). For actors, agonistic interactions were composed both of threat displays and blows directed at the focal individuals, as well as of threats displays and blows exchanged between neighbours of the focal individuals. We thus considered two types of intensities in the following analysis: intensity of interactions directed towards the focal individual (i.e. direct intensity, i1) and intensity of interactions between neighbouring individuals (i.e. indirect intensity, i2).

Additionally, we investigated whether changes in HR responses were observable throughout the course of the breeding season, depending on the incubation or chick-brooding shift. As incubation and brooding shifts are sex-specific in king penguin, we tested for (i) an effect of breeding status, by comparing all incubation and all brooding shifts together and, (ii) and effect of breeding advancement by comparing sex-specific shifts together.

2.5. Statistics

Statistical analyses were performed using the R v.2.10.1 (http://www.r-project.org/) software. We used generalized linear mixed models (GLMMs) including bird identity as a random factor to account for inter-individual variation in HR responses and repeated measurements on the same individual. As residuals were non-normal, models were fitted with negative-binomial distribution using the 'glmmADMB' package in R (Skaug et al. 2011), which was appropriate considering the distribution of our dependant variables, and working with over-dispersed data (O'Hara and Kotze 2010). Significant effects are reported for $p < 0.05$. Results are given as means ± standard error (s.e.).

3. Results

3.1. Characteristics of agonistic encounters

Overall, the duration of agonistic interactions observed lasted for 8.9 ± 0.2 sec. (range = 1 – 34 sec., $n = 589$). Blows lasted 26% longer than threat displays, i.e. 9.7 ± 0.3 sec. ($n = 366$) versus 7.7 ± 0.3 sec. ($n = 223$), respectively (GLMM; $z = -4.9$, $p < 0.001$, $n = 589$, 20 birds) and, on average, interactions with blows involved a higher number of participants than did interactions composed solely of threat displays, i.e. 2.5 ± 0.1 birds (range = 1 – 12, $n = 366$) versus 2.0 ± 0.1 birds (range = 1 – 6, $n = 223$), respectively (GLMM; $z = -3.01$, $p = 0.003$, $n = 589$, 20 birds).

3.2. HR responses during agonistic encounters

3.2.1. On the involvement of individuals birds and the nature of agonistic interactions

Overall, we found penguins to elicit strong HR increases above baseline values (on average 64 ± 1 bpm for males and 70 ± 1 bpm for females) in response to agonistic interactions, regardless of their nature (**Figure 34a, b**). The HR increases were instantaneous, i.e. 0.2 ± 0.1 sec. following the beginning of the interaction, and of a duration of 10.4 ± 0.2 sec, comparable to that of the interaction. However, the HR response of actors was greater than that of bystanders: HR excess was on average 65 % higher (i.e. 2.3 ± 0.2 beats *vs.* 0.8 ± 0.05 beats; **Figure 34a**; GLMM: $z = -11.1$, $p < 0.001$, $n = 589$, 20 birds) and maximum rise in HR at least twice as great (20.1 ± 0.9 bpm *vs.* 10.0 ± 0.4 bpm, **Figure 34b**; GLMM: z = -11.3, p < 0.001, n = 589, 20 birds) in actors than it was in bystanders. Considering the nature of agonistic interactions (*viz.* physical blows or threats; risk), birds generally appeared more responsive to physical blows (displaying higher HR responses during such interactions), albeit the difference was only significant for actors (**Figure 34a, b**).

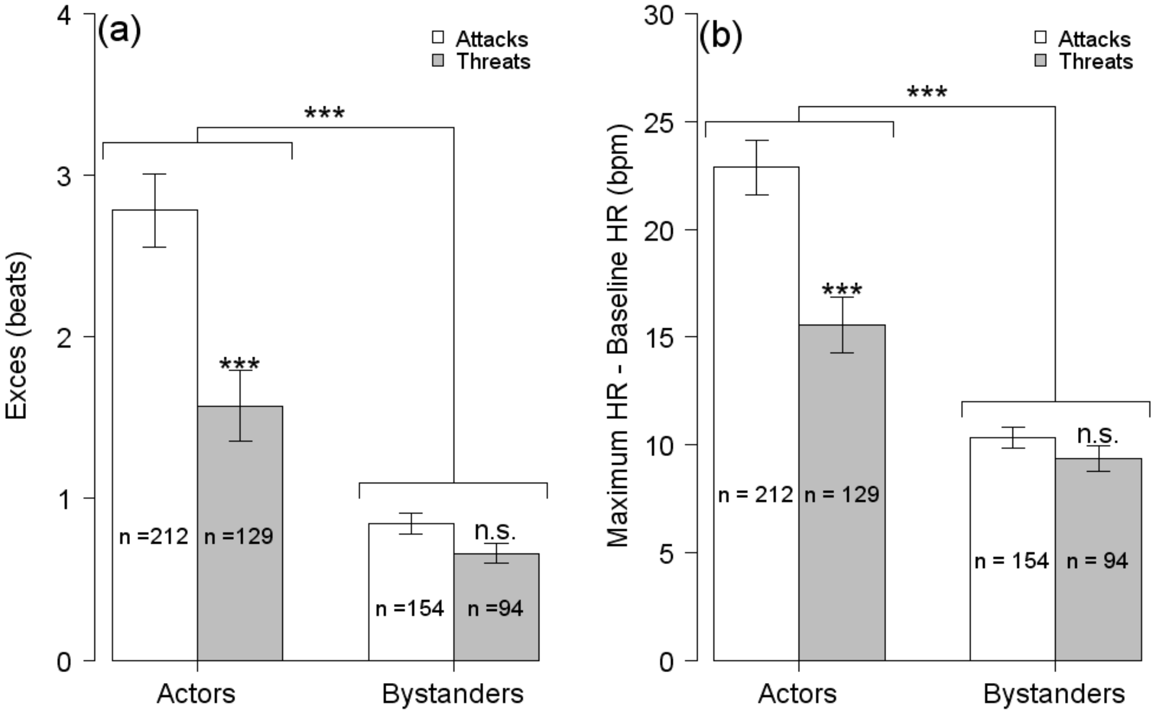

Figure 34. Heart rate responses to agonistic interactions (physical blows and threat displays) in breeding king penguins.

(a) Heart rate excess caused by the agonistic event (in beats) **(b)** Maximum rise in heart rate above pre- and post-interaction baseline values (in bpm). Values are given as means ± s.e. *** p < 0.001, $^{n.s.}$ non-significant.

3.2.2. *On individual motivations during agonistic interactions*

Considering only the case of actors, we investigated whether noticeable differences were observable when birds either initiated or responded to threats or physical blows. When included in a GLMM, HR responses were generally higher when birds were initiators rather than responders (GLMM1, **Table 3**). When considering threats and blows separately, we found that whereas actors showed higher HR responses when initiating rather than responding to blows (GLMM2, **Table 3**), the difference was not significant concerning threats (GLMM 3, **Table 3**).

N°	Model parameters	Estimate	S.E.	Z	Pval	N	Birds
GLMM 1 (Threats + Blows)	intercept	1.06	0.14	7.45	**< 0.001**	341	20
	behavior type (**BT**)	-0.68	0.13	-5.36	**< 0.001**		
	initiator/responder (**IR**)	-0.46	0.14	-3.31	**< 0.001**		
	IR x BT	0.45	0.27	1.66	0.09		
GLMM 2 (Blows)	intercept	1.03	0.14	7.34	**< 0.001**	212	20
	initiator/responder (**IR**)	-0.45	0.14	-3.22	**0.001**		
GLMM 3 (Threats)	intercept	0.40	0.16	2.37	**0.02**	129	20
	initiator/responder (**IR**)	-0.09	0.26	-0.34	0.73		

Table 3. Influence of individual motivation (initiators or responders) on HR responses of king penguins actively involved in agonistic interactions.

Parameter estimates were obtained by fitting generalized linear mixed models (GLMMs) with negative-binomial distribution to the excess HR responses during agonistic interactions. Bird identity was included as a random variable in all models. Behaviour type (BT, both blows and threats) was included as an independent variable in GLMM1, and we considered both behaviours separately in GLMM2 (blows) and GLMM3 (threats). Individual motivation (IR) was included as an independent variable in all models. In GLMM1, threats are compared to blows, and in GLMM2 and GLMM3, responders are compared to initiators. Significant values appear in bold. Fitting a similar model to maximum HR responses yielded similar results.

3.2.3. On the influence of the agonistic context

For actors, HR responses increased with the duration of the interactions (dAi) and their intensities (i1 and i2), but the number of individuals involved (Ni) had no significant effect (GLMM1, **Table 4**). Because we knew birds to elicit higher HR responses to blows than to threats, behaviour type (BT) was included in our models and we considered the interaction terms between this variable and all others. None of the interactions, however, turned out significant (all $p > 0.07$). Interestingly though, when we did consider threats and blows separately, we found that the effect of Ni on HR responses during threats was positive and almost significant ($p = 0.06$), whereas this was not the case during blows ($p = 0.91$). Additionally, the effect of dAi on HR responses was almost double during threats than during blows (i.e. higher parameter estimates for threat displays than for blows, 0.10 ± 0.02 *vs.* 0.06 ± 0.01 and 0.06 ± 0.02 *vs.* 0.03 ± 0.01 for HR excess and maximum rise in HR, respectively).

For bystander individuals, only the duration of agonistic interactions had a significant positive influence on their HR response (GLMM2, **Table 4**). As bystanders were witnesses, only intensity i2 of the interactions was considered. HR responses did not differ according to i2, behaviour type, nor was it influenced by the number of participants in the interaction (GLMM2, **Table 4**). None of the interactions between behaviour type and i2, dAi or Ni were significant (all $p > 0.98$).

N°	Model parameters	Estimate	S.E.	Z	Pval	N	Birds
GLMM 1 (Actors)	intercept	-0.28	0.21	-1.38	0.12	341	20
	intensity 1 (**i1**)	0.65	0.18	3.62	**< 0.001**		
	intensity 2 (**i2**)	0.34	0.16	2.11	**0.03**		
	number of individuals (**Ni**)	0.03	0.05	0.55	0.60		
	duration of interaction (**dAi**)	0.06	0.01	5.21	**< 0.001**		
	behavior type (**BT**)	-1.05	0.34	-3.06	**0.002**		
GLMM 2 (Bystanders)	intercept	-0.63	0.23	-2.70	**0.007**	248	20
	intensity 2 (**i2**)	0.02	0.13	0.13	0.89		
	number of individuals (**Ni**)	-0.08	0.09	-0.86	0.39		
	duration of interaction (**dAi**)	0.07	0.02	4.03	**< 0.001**		
	behavior type (**BT**)	- 0.15	0.53	-0.28	0.77		

Table 4. Influence of the agonistic context (intensity and duration of the interaction, number of individuals involved) on the HR response of king penguins.

Parameter estimates were obtained by fitting generalized linear mixed models (GLMMs) with negative-binomial distribution to the excess HR responses during agonistic interactions. Bird identity was included as a random variable in both models. Duration (dAi) and intensity (direct intensity i1 and indirect intensity i2) of agonistic interactions, as well as number of individuals involved (Ni) were included as independent variables. Behaviour type (BT), was included as an independent variable and in both GLMMs, threats are compared to blows. Models are given for actors (GLMM1) and bystander (GLMM2) individuals. Significant values appear in bold. Fitting a similar model to maximum HR responses yielded similar results.

3.3. Variations throughout the breeding season

Variations in HR responses of actors and bystanders during the breeding season are reported **Figure 35**. Overall, considering both threats and blows, we found no difference in HR responses between incubating or chick-brooding birds, (GLMM: z = 0.75 and 0.35, p = 0.45 and 0.72, n = 589, 20 birds; for excess HR and maximum rise in HR, respectively), and no difference separately in males (p = 0.48 and 0.92, for HR excess and maximum rise in HR, respectively) and females (p = 0.48 and 0.78, for HR excess and maximum rise in HR, respectively).

For males, incubating actors tended to elicit higher HR responses to physical blows during the third breeding shift (close to egg-hatching) than during the first (GLMMs: p = 0.09 and p = 0.049, n = 213, 12 birds, for excess HR and maximum rise in HR, respectively). For instance, maximum rise in HR was 60% more for breeding shift 3 (27.2 ± 2.6 bpm) than for breeding shift 1 (16.9 ± 2.0 bpm). This difference, however, was not apparent concerning threats, nor did we find any significant difference in bystanders (all p > 0.6).

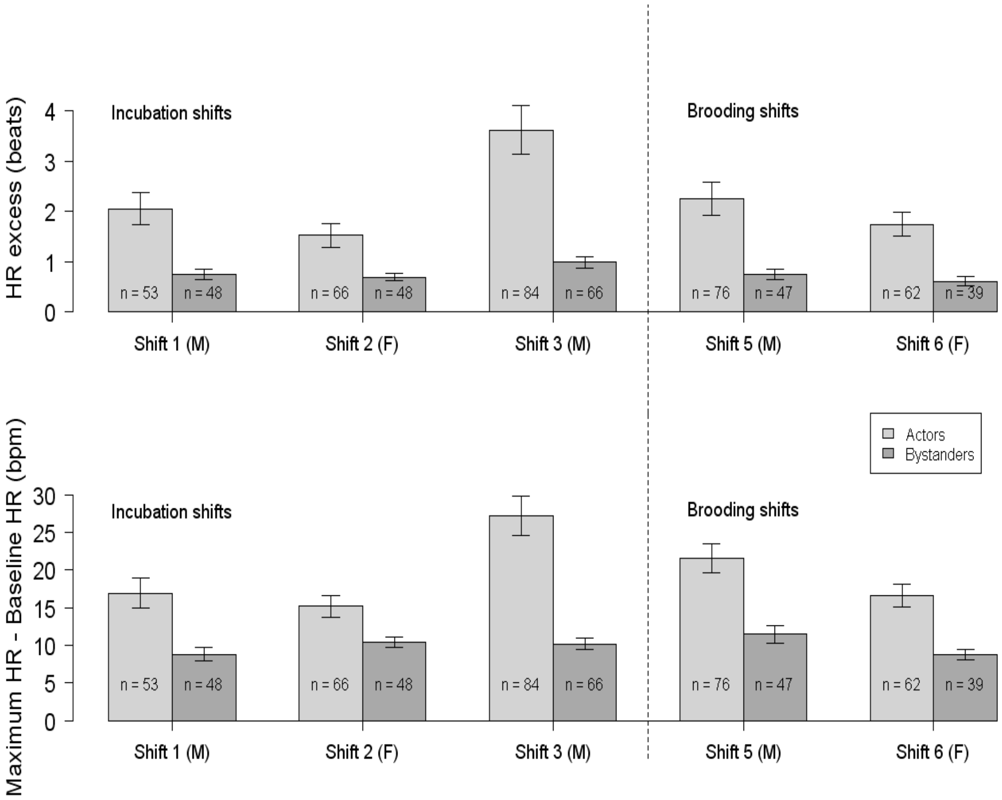

Figure 35. Variation in HR responses to agonistic interactions depending on the breeding status (incubation and brooding shifts) in male and female king penguins.

Values are given as means ± s.e. for actors and bystanders (threats and blows were pooled together).

4. Discussion

In this study, we investigated the effects of agonistic encounters (i.e. social stressors) on HR of colonial seabirds breeding in a highly aggressive social environment. We argue that breeding king penguins will elicit varying HR responses to agonistic interactions depending on their psychological assessment of the social context.

We show that HR responses to agonistic encounters were elicited both by individuals directly concerned by the social event as well as by individuals standing nearby. Agonistic encounters provoked maximum increases in HR as great as 30 % of resting values in actors and 16 % in bystanders. It may be argued that HR increases in actors were due to their physical participation in agonistic interactions. Indeed, physical exercise would cause HR to increase, due to increased energy demand and thus to the necessity to increase fuel and oxygen supply to muscles. This would be true for prolonged exercise, including prolonged bouts of agonistic interactions with blows exchanges, but seems unlikely for the specific case of very short-lived agonistic interactions, as characterized in this study. Indeed, whereas the sympathetic nervous stimulation of the heart typically shows a slow response to exercise (e.g. beginning 1-2 sec. and reaching a plateau 30 sec. after the exercise start in the domestic sow; Marchant et al. 1995), HR responses in our study were instantaneous and short-lived (i.e. a duration similar to that of the interaction). Moreover, bystanders elicited HR responses that were at least half as great as that of actors, and yet remained motionless as they witnessed encounters between neighbouring birds (personal obs.). In such cases, physical activity cannot explain the HR elevations observed. Rather, our data suggests that both in actors and bystanders very short-term HR responses mostly reflect social stress responses, and changes in the emotional state of individuals (see Wascher et al. 2008b) in response to varying social aggressiveness. The finding that HR responses in actors were higher for initiators than for responders further supports the view of an emotional component in this response, and a different assessment of agonistic contexts depending on the role the individuals holds.

Since Darwin's early book *The Expression of Emotions in Man and Animals* (Darwin 1872), several authors have documented and discussed the existence of psychological states and emotions in animals (Panksepp 1998, Bekoff 2000, Dawkins 2000, De Waal 2008). In a brief thinkpiece on animal emotions, Marc Bekoff writes: “Animals are incredibly adept social actors: they form intricate networks of relationships and live by rules of conduct that maintain social balance (…)” (Bekoff 2009). Neural circuits for emotions have been documented in several species (see Le Doux 2000) and physiological responses to social stimuli in group living animals have been shown to differ according to situation context or individual motivations (Wascher et al. 2009). In penguins, emotional arousal may play an important role in modulating HR responses during agonistic encounters. Indeed, rather than remaining constant, HR responses varied according to the relevance (i.e. risk) of aggressive contexts. For instance, physical blows provoked higher HR responses than threat displays, albeit the difference was only significant for actors. Additionally, HR responses increased both with the duration and intensity of encounters for actors, whereas only encounter duration affected HR in bystanders, suggesting a different evaluation of the aggressive context and its associated risk by those two groups. Actors are indeed faced with a greater likelihood of injuries or egg loss (thus reproductive failure) when they engage in physical disputes compared to threat displays, whereas this may be less relevant for bystanders. Moreover, longer conflicts with numerous participants might imply greater risks of redirected aggression for bystanders, giving a possible explanation of why the duration of agonistic encounters positively affected HR in bystanders. On the other hand, the finding that the number of individuals involved seemed to influence HR responses in threat interactions for actors could be explain by the fact that a greater number of individuals involved increases the likelihood for threat interactions to escalate into physical disputes with higher risks of injury. These results thus suggests that psychological evaluation of the context (i.e. whether the risk is high or low) may be important in modulating physiological responses to social stressors. In line with this, changes in physiological responses according to individual evaluation of specific contexts have been documented in several species including geese (Wascher et al. 2009), pigs (Marchant et al. 1995), fish (Oliveira et al. 2001, Oliveira et al. 2005), and humans (Steptoe 2000). In geese for instance, individuals responded with a higher HR increase when confronted with an opponent winning a higher percentage of fights than themselves (Wascher et al. 2009). Thus, motivational state of an individual might also have strong implications on modulating physiological responses. Our study shows that actors elicited different responses in HR depending on whether they initiated or responded to agonistic interactions. HR responses were indeed greater when initiating rather than when responding to physical attacks. Greater HR elevations in initiators may reflect cardiovascular changes brought about by individual preparation (both physical and emotional) of engaging in physical disputes, whereas low predictability and controllability of aggressions may constrain responses of targeted individuals.

Further, we found that HR responses of breeding penguins may vary depending on their breeding status. Indeed, we show that males relieving their partner as second time incubators (males in shift 3) elicited significantly greater HR responses to agonistic encounters than did first time incubators. Surprisingly, we found no evidence for changes in the HR responses of females. Nonetheless, the finding that males' HR responses increased with advancing incubation may suggest a change in their sensitivity to the social environment, possibly reflecting a change in parental investment as the relative reproductive value of the egg increases (Albrecht and Klvana 2004, Cézilly et al. 1994, Osiejuk and Kuczynski 2007). Indeed, according to parental investment theory (Trivers 1972), individuals may be willing to invest more into parental care as the clutch or offspring age, because of the higher probability of progeny of surviving until sexual maturity (Clutton-Brock 1991). The increased HR response of male king penguins shortly before hatching might thus reflect an increased motivation to care for the egg. It would have been of interest to test this possibility for females at shift 4 of incubation, i.e. the shift when hatching usually occurs (Stonehouse 1960), but unfortunately we were unable to obtain recordings at this stage.

To sum up, our findings in a colonial seabird breeding under a highly aggressive context advocate for the importance of an emotional component in modulating physiological response to agonistic encounters in social living species. We provided evidence that this response depends both on the perceived relevance of the encounter, and on the motivation of the animal to engage into the interaction.

Acknowledgments

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BOX 1 – Is colonial breeding costly? Social sensitivity and energy expenditure

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Experimental manipulation of local colony density does not affect energy expenditure in the king penguin (*Aptenodytes patagonicus*)

Vincent A. VIBLANC, Marion KAUFFMANN and René GROSCOLAS

Unpublished work.

In **study 3**, we found that the mean daily heart rate (dHR) of breeding males progressively increased starting a few days before the onset of incubation, even though there was no associated increase in physical activity or change in body temperature. More interestingly, the mean daily resting HR (rHR, a proxy to RMR) of breeding males also progressively followed the same trend. When testing for a relationship between this increase and changes in colony density, we found a strong association between these two parameters, and hypothesized that the HR increase observed in breeding birds at the onset of the breeding season could reflect an increase in energy expenditure associated with some form of chronic stress when the colony filled up early in the breeding season, i.e. an energy cost to group-living. Nonetheless, those results are based on a correlative approach. We thus further decided to experimentally test whether colony density affected the resting metabolic rate of breeding birds by manipulating the local density around incubating males during their first incubation shift. We hypothesized that if colony density indeed influences the mean daily resting HR (rHR) of breeding birds, then locally decreasing colony density by means of portable pens should result in a decrease in the mean daily rHR of incubating birds. On the other hand, removing the pens and allowing birds to resume incubation in their natural colonial environment should result in enhanced levels of daily rHR compared to the pen situation. In addition, we also tested whether experimental changes in local density had an influence on the rHR of males and females during later incubation shifts.

METHODS

During the 2009-2010 breeding season (from early December to late February), courting pairs were spotted in the colony, marked from a distance using a non-permanent animal dye (Porcimark®, Kruuse, Germany) and followed daily until egg laying and departure of the female. Thereupon, males were caught while incubating, flipper-banded for identification during field observations, and equipped with externally mounted HR-loggers (Polar® model RS800, Polar Electro Oy, Kempele, Finland) specially adapted for suitable use on king penguins (see Groscolas et al. 2010). Loggers were set to sample HR every 5 seconds, allowing data storage over a period of 4 days before the memory was saturated. Males were then left incubating in the colony for a period of 3 days (control period at natural colony density) before modifications of local densities began. After 3 days, we excluded neighbouring birds by use of a portable metal exclosure frame, i.e. pens of 2m x 2m (see

Figure 36) allowing us to reduce colony density in the pen to 2 birds per 4m². Neighbouring birds were gently walked away from the monitored individuals, insuring that no egg-loss occurred, until they were out of the experimental pen. HR-loggers were reset, and monitored birds left incubating at low density in the pen for a period of 4 days (**Figure 36**). Thereafter, the pen was removed, loggers reset, and birds left incubating for a second period of 3 days in the colony.

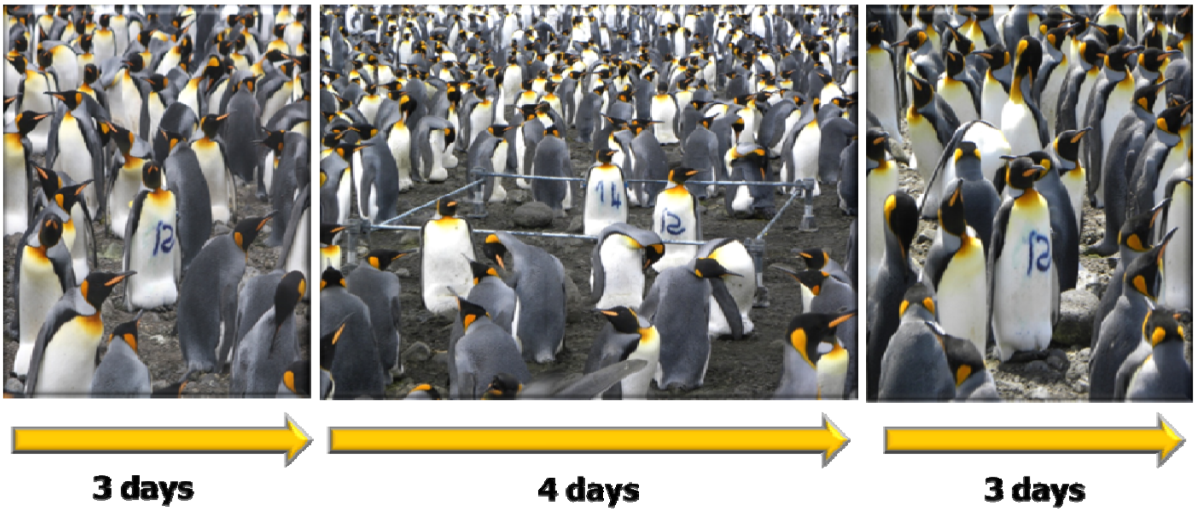

Figure 36. Illustration of the experimental protocol used to modify bird density around incubating king penguins.

Birds were equipped with HR-loggers and left to incubate in the colony for a period of 3 days. Thereafter bird density was reduced by means of portable metal exclosure frames, and monitored individuals were left incubating in the metal pens at reduced density. Four days later, pens were removed, and monitored birds could resume incubation within the colony.

The same protocol was applied to animals returning for later breeding shifts. In total, we manipulated local density around 10 males in shift one, 14 females in shift two, 15 males in shift 3 and 5 females in shift four of incubation. All interventions close to monitored birds (pen set-up and removal) were timed and lasted less than 5 minutes. We systematically discarded HR data collected 5-minutes before procedures started and one hour after interventions (including initial equipment of HR-loggers) to avoid any bias in HR resulting from stress. Mean daily HR was calculated as the average HR recorded over time-periods ≥ 20 hours (excluding periods of reset and some periods of technical malfunctioning), based on every 5-second measurement. From those periods, we calculated daily resting HR averages by using moving averages to determine the 10 daily consecutive minutes during which HR was stabilized at the lowest level, thus yielding daily rHR based on 120 consecutive HR measurements. Linear Mixed Models (LMMs) were used to investigate the effect of relative day (i.e. fasting duration) and changes in density on rHR, by including bird identity as a random factor in the models. Results are reported as means ± S.E. Significance threshold was $P < 0.05$.

RESULTS

When considered over the course of the experiment (i.e. from days 1-10), we found an overall significant daily decrease in the resting HR (rHR) of incubating birds (LMMs; all $P < 0.001$) that was likely associated with an increase in fasting duration. However, this was not the case for males during the first incubation shift, where rHR remained steady (LMM; $t = -0.21$, $P = 0.83$, $n = 89$, $N = 10$) (see **Figure 37**).

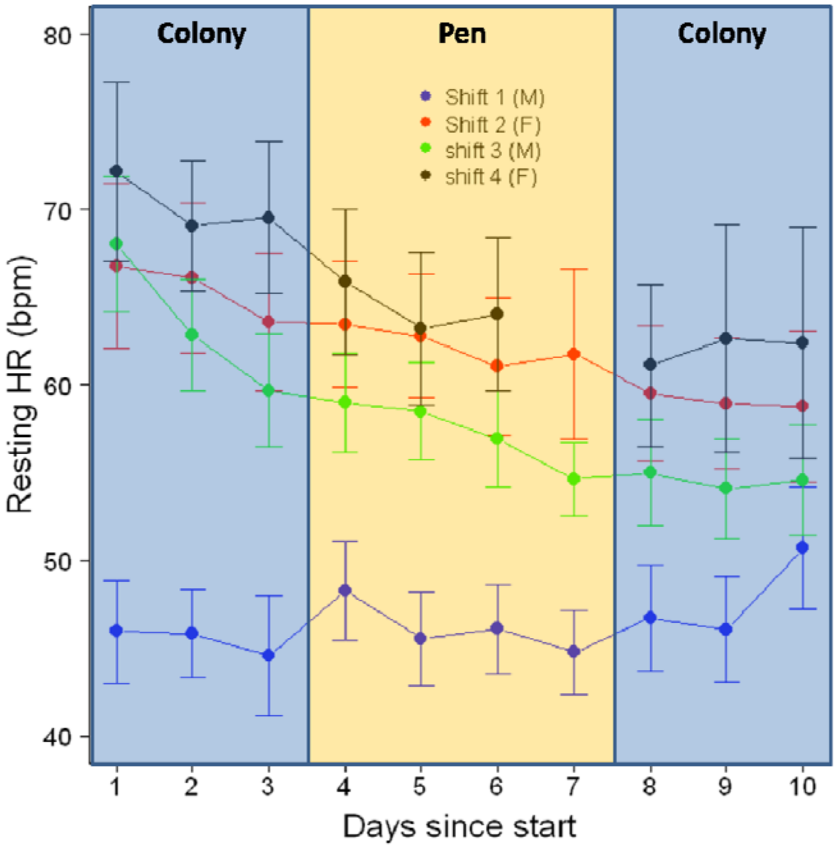

Figure 37. Time course of changes in daily resting heart rate (bpm) during experimental manipulations of colony density in incubating king penguins.

Means ± S.E. are figured for males during the 1st and 3rd incubation shift and females during the 2nd and 4th incubation shift. Birds were initially left at natural colony density (blue area; days 1-3) before being placed in a pen of reduced density (2 birds / 4m², yellow area; days 4-7). The pen was then removed (blue area; days 8-10) and thereafter, birds remained at natural colony density.

For males incubating in shift one, manipulations in local density did not seem to influence rHR (LMM; p = 0.84). Specifically, reducing local density did not result in a significant decrease in daily rHR (post-hoc; *P* = 0.83, see **Figure 38**), nor did removing the pen result in a significant increase in rHR from the pen condition (post-hoc; *P* = 0.55, see **Figure 38**). For later incubation shifts, the significant decrease in rHR over the period of study prevented us from testing the effect of our treatment, as both parameters were necessarily co-varying (i.e. confounded).

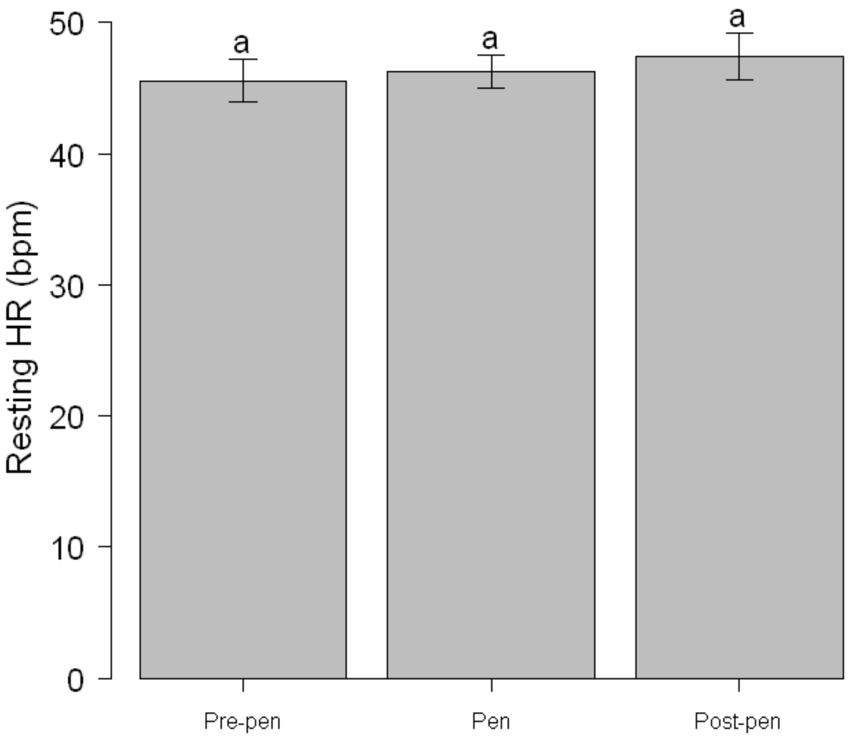

Figure 38. Effect of an experimental change in colony density on the daily resting heart rate (bpm) of males during the first incubation shift.

Birds were initially left at natural colony density for three days (pre-pen) before being placed in a pen of reduced density for four days (2 birds / 4m^2; pen). The pen was then removed (post-pen) and birds left at natural colony density for an extra three days.

CONCLUSION/DISCUSSION

Contrary to our hypothesis, experimentally manipulating the local density around incubating birds did not result in noticeable changes in average daily resting HR. Specifically, when the experimental pens were set around incubating birds (i.e. birds were transitioned from the natural colony density to an environment of much lower density), we did not find any decrease in daily rHR, suggesting that no noticeable change in RMR occurred. However, it should be noted that because for incubation shifts 2, 3 and 4, daily rHR decreased with fasting duration (i.e. relative day since the start of the experiment), we were not able to distinguish a potential effect of lowering local density from an effect due to fasting. Whereas our manipulations of local density in males in shift one do not seem to support the hypothesis that colony density may have an effect on the rHR of incubating birds, those results should be considered with caution. Indeed, for all birds, the effect of penning may actually have resulted in increased stress that might have influence rHR levels, as our manipulations likely increased predation risk. Indeed, by decreasing colony density to 2 birds per 4m² pen, we provided opportunities for predators to land in unoccupied areas within the pen. By contrast, in a colony of relaxing density, birds would be homogenously interspaced and predation risk may be more evenly distributed. This suggestion is supported by the observation that in the experimental pens, although birds had more space available to them, they tended to regroup and maintain close proximity. Moreover, as penned birds were displaced slightly from their initial breeding territory (because of the configuration of the terrain and available space to place the pen), shifting them from their initial breeding territory may also have led to increased stress. Finally, by excluding neighbours to the outskirts of the pens, we actually increased local density (leading to increased rates of fights) around the pens. This might have also resulted in an increase in experienced social stress in penned birds, resulting in overall higher rHR. Thus, although our study does not provide evidence for a decrease in rHR associated with a decrease in local density, our results are likely confounded by uncontrolled factors in our protocol, and further studies are needed to investigate the effect of colony density on social stress and energy expenditure. For instance, monitoring birds of similar fasting status (especially males in shift one) in areas of naturally varying local density (i.e. colony areas where local density is consistently higher than others throughout the breeding season) might provide a more accurate test of the potential effects of colony density on RMR.

BOX 2 – Is colonial breeding costly? Social sensitivity and chronic stress

Is breeding in a dense colony associated with chronic social stress?

Vincent A. VIBLANC, Benoit GINESTE, Jean-Patrice ROBIN and René GROSCOLAS

Unpublished work.

In a previous study (**see article 3**), we found that at the onset of breeding, the resting daily heart rate (rHR) of incubating males king penguins (*Aptenodytes patagonicus*) progressively increased paralleling an increase in the density of the colony. This HR increase could reflect the development of a chronic social stress when breeding birds have to cope with more and more neighbours, closer and closer. To test this hypothesis, we measured the baseline plasma corticosterone levels of king penguins located in colony areas of low or high density. Birds at high density were located at the centre of the colony whereas birds at low density were located at its edge or in areas where birds were separated by rocks. We considered breeding penguins at two different statuses, incubating or brooding a young chick. Baseline corticosterone is a good index of chronic stress (e.g. Ottenweller et al. 1992) and we expected birds in areas of high density to have higher baseline levels of corticosterone than those breeding at low colony density.

METHODS

From late December 2010 to early March 2011, birds were sampled at random, their sex and incubating or brooding shift being unknown. Prior to blood sampling, the status of the focal bird (incubating or brooding) was visually estimated at a distance > 20 m, only brooders of non-thermally emancipated chicks (i.e. still protected in the brood-pouch) being considered. The bird's status was checked at the end of blood sampling. In addition, the focal bird and its neighbours were observed from a distance during several minutes to ensure that they were in a resting state before blood sampling (i.e. no agonistic interactions or comfort behaviour observed). Also before sampling, we estimated from a distance the density around the focal bird. We counted the number of neighbours with which it could potentially have agonistic interactions, i.e. located at less than 1.5 m, and we estimated by eye the distance between the focal bird and each of these neighbours. An individual density index was calculated for each sampled bird as: number of neighbours / average distance to neighbours. For example, a density index of 2 might mean that the focal bird had two neighbours at an average 1 m distance whereas a density index of 10 might mean that the focal bird had six neighbours at an average distance of 0.6 m. For blood sampling (from a flipper vein), birds were approached from the back and we timed the moment at which they reacted to our approach by becoming vigilant. Since in a preliminary study we found that due to the capture stress plasma corticosterone starts to significantly increase above baseline levels when the blood sampling duration is more than 4 min, only samples obtained in less than 3.5 min after birds became alert were considered for analyses. All blood samples were taken in the afternoon, from 2.00 to 6.00 P.M., to prevent any bias due to a potential circadian rhythm in corticosterone concentration. Plasma corticosterone was measured by Radio-Immuno-Assay (RIA) using a commercial kit (MP Biomedicals, cat. No. 07-120103), as previously described and validated in Corbel et al. (2010). The plasma levels of corticosterone of birds incubating at high (average density index = 10.1, n = 49) or low (average density index = 1.6, n = 28) density was compared using a Students' t-test or its non-parametric equivalent (Wilcoxon rank sum test) after checking for normality of the data (Shapiro-Wilk test of normality). A similar comparison was performed for birds brooding at high (average density index = 10.8, n = 25) or low (average density index = 1.8, n = 17) density.

RESULTS

Baseline corticosterone levels of king penguins incubating at high density was slightly (33 %) but significantly (Wilcoxon; *W* = 302, *P* < 0.001) higher than for birds breeding at low density (**Figure 39**). Similarly, baseline corticosterone of penguins brooding at high density was 55 % higher than for penguins brooding at low density (Wilcoxon; *W* = 76, *P* < 0.001). Also, we found that overall baseline corticosterone was higher in chick brooding than in incubating birds (Wilcoxon; *W* = 517.5, *P* < 0.001, **Figure 40**). This held true when considering both birds breeding at high (+ 68%, Wilcoxon; *W* = 175.5, *P* < 0.001) as well as at low (+ 43%, t-test; *t* = -6.63, *P* < 0.001) density.

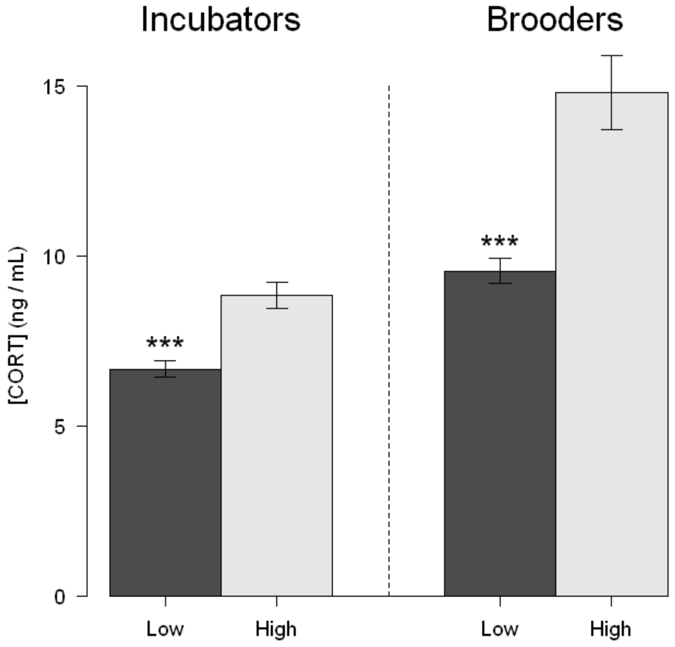

Figure 39. Effects of bird density on baseline plasmatic corticosterone concentrations in breeding king penguin.

CORT concentrations are figured for incubators and brooders at either high or low density. *** Significantly different from high density, *P* < 0.001.

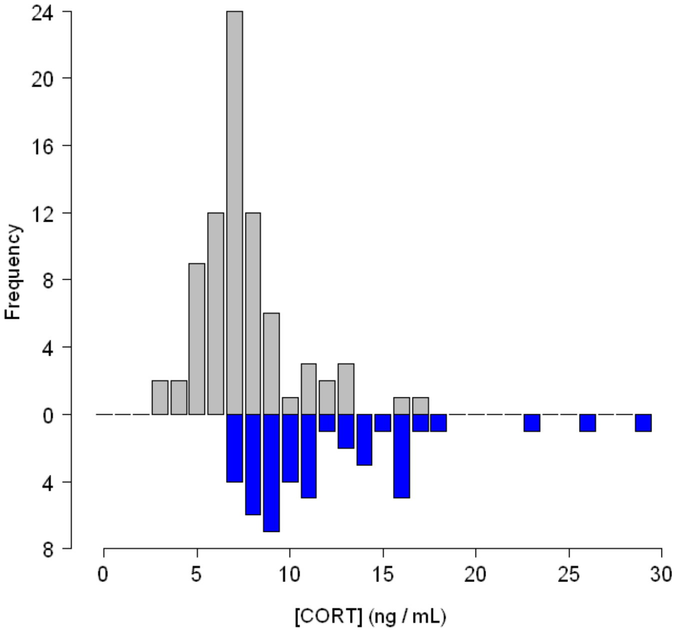

Figure 40. Frequency distribution of plasmatic corticosterone concentrations in breeding king penguin (y-axis positive both ways).

CORT concentrations are figured for incubators (grey bars) and brooders (blue bars). Note that, on average, brooding penguins display higher [CORT] than incubating penguins (Wilcoxon; P < 0.001).

CONCLUSION/DISCUSSION

Those preliminary results on basal corticosterone levels in breeding king penguin support the hypothesis that significant chronic stress may be associated with group living at high densities (Thiessen and Rodgers 1961). To our knowledge, this is one of the few studies to have investigated the effects of population density on stress hormones in a natural setting (see Rogovin et al. 2003). Former investigations in captive animals have reported similar findings. For instance, it is well established that housing conditions at high densities causes increased stress and higher plasmatic concentrations of stress hormones. In reptiles, Elsey and colleagues (1990) found that American alligators (*Alligator mississippiensis*) kept captive at high group densities exhibited higher plasmatic CORT levels than those maintained at lower group densities (Elsey et al. 1990). Interestingly, in fish, Gornati et al. (2004) have shown that rearing density may influence the expression of specific stress-related genes in the sea bass (*Dicentrarchus labrax*), causing the over-expression of those genes at high population densities (Gornati et al. 2004). The authors suggest such genes may be used as non-specific biomarkers to monitor fish stress in fish farming facilities. Potential effects of population density on individual stress have also been suggested both in mammals and birds. For instance, monitoring great gerbils (*Rhombomys opimus*) over various years of naturally fluctuating population densities, Rogovin and colleagues (2003) found males to have higher (though not significantly) concentrations of faecal CORT concentrations in years of high population density. In birds, Nephew and Romero (2003) have shown that experimental acute increases in density may have varying effects depending on bird status and conspecific density. Indeed, they showed that when resident birds were kept in captivity and their cage was crowded with intruding conspecifics, intruders exhibited higher CORT concentrations in response to increased density, whereas resident birds did not. However, resident birds did display increased heart rate with increasing density. This result is interesting as it suggests that in this case, HR may have been modulated independently of significant activation of the HPA-axis. Nonetheless, in their study, Nephew and Romero also found that intruding birds exhibited elevated CORT levels (a 3-fold increase above baseline levels), but only at high density, suggesting that the HPA-axis may be activated only after a certain threshold in density is reached. This leads to distinguishing crowding (i.e. individually perceived spatial restriction) with population density *per se* (i.e. defined solely in terms of spatial parameters) (Stokols 1972). Beyond a certain point increases in conspecific density may lead to increased competition, potential aggressiveness and associated costs. In our study, basal CORT appeared to be higher when birds were breeding at higher local density. Those results suggest that chronic stress might arise from sharing close space with breeding congeners. In king penguins, central territories of higher local densities are indeed also known to be associated with higher rates of aggressive interactions (Côté 2000). On the other hand, central territories of higher local density also offer increased protection against predators (Côté 2000), so that a trade-off might exist between predation risk and conspecific competition, shaping an optimal breeding density. In addition, it is interesting to note that in our study, basal CORT concentration appeared to be higher in chick brooding than in incubating birds. Elevated baseline CORT in brooding birds may reflect increased chronic stress associated with an increase in the reproductive value of the brood (Bókony et al. 2009).

ACKNOWLEDGMENTS

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PART 5.

Dealing with predators and stress

V. Stress responses, perceived risk, nutritional status and reproductive value

As formulated by Williams (1966), the cost of reproduction is such that parental decisions should be based on a trade-off between the fitness gains of a specific reproduction, and the fitness gains that may arise from future reproductions. As an example, older individuals may be willing to invest more than younger individuals into a given reproductive attempt, because of their relatively lower residual reproductive value (i.e. after a certain age, the older they get, the less chances they have of reproducing).

In considering decisions about parental investments, recent years have seen a surge of litterature dealing with stress responses and their adaptive value. For instance in long-lived species, it is expected that individuals should forego their reproduction when their survival is at stake, as they potentially have multiple chances of re-mating in the same or subsequent seasons. Thus have stress responses been suggested as physiologically underpinning parental decisions, and promoting the redirection of behaviour away from offspring care when survival is jeopardized. However, because stress responses are energy costly, the question arises of how their cost balances out in the trade-off between parent survival and offspring care when energy is limited.

In the following chapters, we consider the heart rate and hormonal stress responses of breeding king penguins to situations of acute predator-like stress. Specifically, we consider whether stress responses may be attenuated as a mechanism of energy sparing when the fast is long, and how stress responses are modulated throughout the breeding season (according to breeding status) in response to predators.

In **study 5**, we consider the heart rate stress responses of breeding birds to three standardized acute stressors (two of which are human approaches varying in the degree of risk for the animal, and one of which is a metal sound assumed to be novel in nature), and investigate how those responses vary according to fasting duration and breeding status. In **box 3**, we investigate the hormonal and metabolic responses of breeding king penguins during standardized capture-immobilization stresses and their variation also according to fast duration and reproductive status.

STUDY 5 – Heart rate responses to acute stress during reproduction

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Offspring reproductive value influences heart rate responses to predation stress in a colonial seabird, the king penguin

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Unpublished data.

ABSTRACT

While breeding, fasting seabirds must manage an extremely constrained energy budget. For those animals, the allocation of energy towards reproduction could be hindered by responding to unpredictable social, environmental or anthropogenic disturbances. Attenuating stress responses during prolonged fasting may then be a means to reduce energy expenditure and ensure reproductive success. In addition, attenuated stress responses may underlie increased parental commitment to the brood so that stress responses may be attenuated when the relative reproductive value of the brood is high. To investigate those hypotheses, we considered the heart rate (HR) responses of free-living king penguins breeding in the Crozet Archipelago to a series of 3 standardized human stressors, applied to birds of varying fasting and reproductive status. Over the course of the breeding season (late November-late March: 2009-2010, 2011), we performed a total of 249 stress events in which birds: (1) were either approached head on to a distance of 10 m (n = 82); (2) approached, captured and held for 3 min (n = 84); or (3) approached from behind up to 15m and exposed to a standardized series of metal sounds (n = 83). Our results do not support the hypothesis that fasting duration may be a major determinant of stress responses to acute stressors in breeding king penguin. However, we show that both males and females exhibited lower HR responses to stressors when brooding a chick rather than when incubating an egg. In addition, HR-responses where stressor-specific suggesting that the HR response to stress may be modulated by differential risk-assessment according to context. Attenuated responses may be an adaptation to continue parental care when the value of the offspring is high, reducing the chances of offspring abandonment by the parent when subjected to an acute stressor such as an approaching predator.

1. Introduction

Because parents are limited both in the time and energy they may invest to produce and raise their offspring, decision rules about parental care should be selected as to maximize individual fitness, considering a trade-off between the relative value of current reproduction *versus* that of expected future breeding opportunities (Williams 1966, Trivers 1972, Maynard-Smith 1977). According to the reproductive value hypothesis, parents may thus be willing to invest more into parental care as the clutch or offspring age, because of the higher probability of progeny surviving until sexual maturity (Andersson et al. 1980, Winkler 1987, Clutton-Brock 1991). In support of this hypothesis, various authors have predicted and shown how parental investment may change depending on the age of the offspring, i.e. the relative reproductive value of the young (Andersson et al. 1980, Winkler 1987, Redondo and Carranza 1989, Cézilly et al. 1994, Albrecht and Klvana 2004, Osiejuk and Kuczynski 2007). In birds for instance, parents have been shown to increase the amount of nest defence as offspring age, both throughout the incubation (Sjöberg 1994, Albrecht and Klvana 2004, Osiejuk and Kuczynski 2007) or chick-brooding period (Redondo and Carranza 1989).

Recently, studies have suggested how adjustments in physiological responses to stress may mechanistically underlie parental decisions relating to offspring care, with respect to the value of the current reproduction or that of the young (Wingfield and Sapolsky 2003, Lendvai et al. 2007, Bókony et al. 2009, Goutte et al. 2011). The 'brood value hypothesis' hence suggests that the magnitude of the stress response to acute challenges should be matched to the level of parental commitment to the brood (Wingfield et al. 1995, Lendvai et al. 2007 Goutte et al. 2011). Indeed, when faced with unpredictable environmental challenges (i.e. labile perturbations, reviewed in Wingfield 2003), animals will rapidly mount a series of physiological and behavioural modifications enabling them to cope with the potential threat. Entering an 'emergency life-history stage' (Wingfield et al. 1998, Wingfield 2003, Wingfield and Sapolsky 2003), parents will then redirect their energy investment towards their own survival, thereby reducing or completely giving up on parental duties altogether (Wingfield et al. 1998). The latter should especially apply to long-lived species for which lifetime reproductive success depends more on adult survival than on seasonal fecundity, so that in such species, breeding individuals should behave as 'prudent parents', favouring their own survival over the current reproductive attempt (Drent and Daan 1980, Stearns 1992, but see Erikstad et al. 1998). However, when the value of the current breeding attempt is high relative to that of future reproductions, stress responses should be attenuated to ensure that reproduction is not abandoned (Wingfield et al. 1995, Wingfield 2003). Consistently, several studies have shown how stress responses may be inhibited when breeding opportunities are limited (Wingfield et al. 1992, Wingfield et al. 1995, Romero et al. 1997, Heidinger et al. 2006), when parental care towards the offspring is increased (Meddle et al. 2003), or in the sex investing more into parental care (O'Reilly and Wingfield 2001). Further, stress responses have been shown to change according to the perceived value of the clutch or brood (Lendvai et al. 2007, Lendvai and Chastel 2008), including in long-lived species (Goutte et al. 2011).

Studies that have considered the stress response in relation to parental care have largely focused on the longer-term endocrine responses (namely glucocorticoids release) to acute stress (see references above). However, little attention so far has been given to the other (tightly linked) component of the stress response, i.e. the activation of the sympathetic nervous system. Yet, during the initial alarm phase of the stress responses (i.e. the fight-or-flight response), the sympathetic nervous system rapidly acts to promote survival, *via* the release of adrenal catecholamines (mainly epinephrine and norepinephrine), e.g. by increasing cardiac output (thus heart rate) and blood perfusion to the muscles, shunting-off circulation from non-essential organs, promoting the mobilization of energy substrates, etc. (Habib et al. 2001, Brotman et al. 2007). Whereas the sympatho-adrenal response of the body to acute stress is expected to closely reflect that of the Hypothalamic-Pituitary-Adrenal (HPA) axis, the rapid changes (over the scale of seconds, not minutes; Habib et al. 2001) mediated by catecholamine release may provide greater sensitivity in the analysis of the initial alarm phase of the stress response (see Nephew et al. 2003). Understandably however, the relatively short half-life of adrenal catecholamine (1-2 min for epinephrine, 2.5-4min for norepinephrine; Stoddard et al. 1986b) has made it hard to monitor changes in the sympatho-adrenal response to acute stress in natural conditions (Stoddard et al. 1986b). In this regards, heart rate (HR) may provide us with a useful measure of the catecholamine-mediated response to stress (Stoddard et al. 1986a, Nephew et al. 2003). Additionally, HR may inform us on the potentially fine-tuning of stress responses according to the relevance of acute stressors in terms of novelty or risk for the animal (Nephew et al. 2003, Tarlow and Blumstein 2007, Wascher et al. 2011).

In the present study, we tested whether HR responses to acute stress were modulated according to offspring age in a colonial seabird, the sub-Antarctic king penguin (*Aptenodytes patagonicus*). King penguins offer an especially interesting model for investigating HR stress responses in relation to offspring reproductive value, because they are a long-lived species for which the energy commitment to reproduction is high (Cherel et al. 1994a, Groscolas and Robin 2001). Indeed, by the time their single egg is hatched (after some 53 days of incubation, Stonehouse 1960), parents will already have devoted a substantial amount of energy to reproduction (alternating between periods of prolonged fasting while incubating on land and intense foraging at sea; Stonehouse 1960, Weimerskirch et al. 1992, Charrassin et al. 1998). Furthermore, the chick is only finally fledged the following summer (Stonehouse 1960, Weimerskirch et al. 1992). Therefore, constrained by an exceptionally long breeding cycle, successful pairs can only attempt late breeding in the subsequent season, and it is extremely rare that they succeed (Weimerskirch et al. 1992). King penguins are then expected to adjust parental effort to the relative reproductive value of the offspring, and stress responses should be decreased when the value of the brood is highest (Bókony et al. 2009, Goutte et al. 2011).

To test this hypothesis, we subjected breeding king penguin to three different stressors which varied in their degree of predation risk. Two stressors were human approaches associated with predation risk (i.e. a distant approach and a capture stress), and the third was a metal sound with no *a priori* life-history significance (but see Frid and Dill 2002) for king penguins. For all types of stressors, we expected HR stress responses to be attenuated when reproductive value was highest, i.e. in chick-brooding birds and in birds breeding early in the season. Additionally, we expected penguins to elicit highest HR responses when perceived risk was highest (i.e. during the capture stress *vs.* the distant approach or sound stress). Finally, our protocol gave us the opportunity to investigate whether HR stress responses displayed a certain degree of intra-individual consistency, possibly reflecting individual temperament along a shy-bold type continuum (Wilson et al. 1994, Réale et al. 2000).

2. Methods

2.1. Study species and data collection

King penguins (*Aptenodytes patagonicus)* were studied on Possession Island, Crozet Archipelago (46°25’ S, 51°45’ E). When breeding (late November–March), parents alternate between periods on land (incubating and brooding) and periods at sea, foraging (Stonehouse 1960, Weimerskirch et al. 1992). After the initial courtship period (some 15-days) female king penguin lay their only egg and leave the male in charge of the first incubation shift, relieving him some 16-18 days later. This alternating incubation lasts for a period of roughly 54 days (Stonehouse 1960), with the egg hatching around the transition of the 4th (female) and 5th (male) incubation shift. Parents then continue to alternate brooding shifts ashore, until the chick is thermally emancipated, around 5 weeks of age. Subsequently, both parents resume foraging trips to feed the chick until the end of the austral summer (Stonehouse 1960, Weimerskirch et al. 1992).

From 2009-2011, a total of 78 birds (44 males and 34 females) were stressed at various phases of the breeding cycle, three different stressors being applied to each bird (see below). Animals were marked at a distance while courting using a non-permanent animal dye (Porcimark® Kruuse, Langeskov, Denmark), and were later caught and flipper banded for identification during field observations, either at the very onset of the first incubation shift (males), or at relief at the end of this shift (females). Marked birds were checked twice daily from a distance to determine egg laying and hatching dates, as well as the onset of each incubation and brooding shift. Incubating birds (N = 36) were either males in shifts 1 (N = 22) and 3 (N = 11), or females in shifts 2 (N = 10) and 4 (N = 1). Chick-brooding birds (N = 49) were males in shift 5 (N = 16) and 7 (N = 8), and females in shift 4 (N = 14) and 6 (N = 13). All birds had been caught at least once (for banding) before being used for this particular study, and some birds were stressed repeatedly, at two or three different shifts throughout the breeding season (which explains the above difference in sample size when considering all incubating birds together or incubating shifts separately, for instance). We thus made sure that no habituation or sensitization to applied disturbances occurred, by investigating the effect of stress order on HR responses. The effect of stress order on HR responses was not significant when we considered the order at which the stress was applied within breeding shifts, i.e. whether the stress was the first, second or third performed after bird equipment

(LMMs; all P > 0.10), nor when we considered the overall order of the applied stress throughout the season, i.e. for birds stressed at several shifts (LMMs; all P > 0.24).

For males incubating in shift 1, we further considered two different categories of birds, i.e. early (November-December, N = 10) or late (February-March, N = 12) breeders. This allowed testing of a potential effect of breeding timing on the HR stress response, as early breeders present a far greater likelihood than late breeders of successfully fledging their chick (Weimerskirch et al. 1992). Late breeding males were only subjected to 10-m approaches and captures. Apart from some individuals incubating during shift 1 (see above), all the birds used in this study were early breeders. In addition, to test whether fasting duration might have had an effect HR stress responses, 8 of the 12 late breeding males were stressed both at the onset and towards the end of their first incubation shift. Overall, this would account for an average of 8.6 ± (s.e.) 0.3 days (range 6 – 10 days) difference in fast duration between stressors applied at the beginning or at the end of a shift.

2.2. Stress protocols

Two of our stressors (distant approaches or captures) aimed at mimicking penguin encounters with natural predators. The third stressor (purely anthropogenic) stressor was a loud metal sound that the birds had presumably never experienced before this study. Whereas predation situations are common for breeding king penguins (main on-land predators include giant petrels, *Macronectes halli* or *M. giganteus,* and sub-Antarctic skuas, *Stercorarius Antarctica*; Le Bohec et al. 2003, Descamps et al. 2005), we did not expect metal sounds to have any life-history significance for the birds.

After equipping breeding individuals with HR-loggers (see below), stressors were applied in a random order. Observations on a focal bird started several minutes before a stressor was applied to ensure that the bird was calm and in a resting state, i.e. not engaged in any form of activity (e.g. preening, territory defence), at the time of the stress. All birds in this study were located in periphery of the colony to avoid unnecessary disturbance of social congeners. During the stress, birds' behaviour, the distances between the experimenter and the focal individual, as well as the behaviour of neighbouring birds, were recorded in real time using a digital audio recorder (VN5500® Olympus Europa, Hamburg, Germany).

Behavioural observations continued several minutes after the end of the stress, until the bird reached a resting state again. Those observations allowed to evaluate the behavioural response of the subject to the stressor, and to account for the potential effect of post-stress behaviours on HR. The specific protocols for each type of stressor are described hereunder.

2.2.1. 10-m approach stress

Penguins were approached within their visual field by a walking observer (average speed of 0.5 m/sec) starting from a distance of at least 30 m, depending on the terrain and location of the test subject. The observer stopped 10 m away from the bird whereupon he remained motionless for 1 min while dictating observations on the behaviour of the subject. Subsequently, the observer retreated at a constant speed to his initial position, keeping the focal bird in sight and resuming behavioural observations. The 10-m distance was chosen because preliminary tests suggest that breeding king penguin become alert of human presence at a distance of 10 m or less (Groscolas and Viblanc, *unpublished data*), although not all approaches elicit an observable state of alertness. In addition, the distance of 10 m was too great to cause the focal bird to flee or agitate its close neighbours.

2.2.2. Capture-immobilization stress

As for the 10-m approach stress, penguins were approached at a constant speed starting from a distance ≥ 30 m. In this case however, the observer walked directly to the focal bird and gently immobilized it for 3 minutes, covering its head with a hood to keep it calm. During the approach, capture and immobilization, behaviours and the times and distances at which they occurred were recorded. The hood was rapidly removed after the 3-min immobilization and the observer retreated at a constant speed to his original position, to continue observations for several minutes until the bird had returned to a resting state.

2.2.3. Sound stress

During sound stresses, birds were discreetly approached from behind, until the observer was 15 m behind the focal individual. Care was taken to keep out of sight of the animal (both during the approach and during the stress itself), and the stress was only performed if the observer was certain that the focal individual was unaware of his presence. The stress consisted of three consecutive sounds (1 sound per second, 102.5dB on average) produced by striking two metal bars together. The bird's reaction to the sounds was recorded (as well as any reaction from neighbouring birds) and individuals were observed until they returned to a resting state. The magnitude of sound was assumed to be sufficient to be heard from the bird (as later confirmed by HR analysis), as almost all birds reacted by turning their head in direction of the sound, and many bird deterrents used in airports and around wind turbines emit noises at similar magnitudes (Dooling 2002).

2.3. Measuring the stress response: the use of external HR-loggers

In order to monitor HR responses during stress protocols, we used externally mounted HR-loggers (Polar® model RS800, Polar Electro Oy, Kempele, Finland) specially adapted for suitable use on king penguins, as previously described and validated in Groscolas *et al.* (2010). Loggers were made of two units: (i) a sensor-transmitter (30-40 g) composed of a HR processor which filtered out electrical background noise received from the electrodes (i.e. muscle activity) from heart activity; and (ii) a receiver/logger (30 g). Electrodes were composed of two stainless-steel wires attached to gold-plated safety pins, which were inserted and secured in the subcutaneous fat layer (at a depth of approximately 5 mm, and over a length of 1 cm), 25 cm apart on the back of the bird. Iodine (Betadine®) and an alcohol based antiseptic solution were used to disinfect the electrodes before each use. The transmitter was attached in the middle of the back with Tesa® tape wrapped around several layers of feathers and the receiver was either secured to the flipper band or whenever possible fixed on a metal pole within a 5-m distance of the animal. Such a set up prevented the equipment from hindering the movements of the birds. This was confirmed by the fact that we never observed birds trying to remove electrodes or HR loggers, nor did we observe any adverse effects of the equipment on the birds' health or behaviour. The whole apparatus weighed less than 1% of total body mass. The HR-logger yielded HR values highly comparable to those measured with a stethoscope (Groscolas *et al.* 2010). Further details on this method and how it accurately estimates HR of king penguins are described by Groscolas *et al.* (2010).

Penguins at the different incubating and brooding shifts were equipped with HR-loggers on their breeding territory within the colony, and left to recover for at least 6 h before applying stressors. At capture, the head of birds was hooded to keep them calm. Handling lasted between 5 and 10 min and this procedure never resulted in egg or chick abandonment. Most animals developed a tachycardia due to handling (up to 165 bpm on average), from which they usually recovered within 15-30 min following release. Birds resumed normal activity (i.e. resting, comfort behaviour or aggressive interactions with neighbours) within minutes after release. HR-loggers were set as to store the sampled data for up to 99 hrs, sampling at a rate of one data point every 5 seconds, which was appropriate considering the shortest durations of HR responses to human disturbances (e.g. HR responses to sound stress lasted for an average of 0.99 ± (s.e.) 0.10 min for instance, $n = 73$).

2.4. Heart rate analysis

HR data were plotted and analysed using Polar Pro Trainer® v.5.00.105 software. Audio recordings of each test were time-matched (by previous synchronization of the observer's digital watch with that of the HR-logger) with the corresponding HR data that allowed us to calculate a number of parameters describing the subjects' HR responses to the stressors (see **Figure 41**). The duration of HR responses was characterized as the total time that HR increased above the initial resting rates (HRi), following the application of a stressor. We defined HRi as the HR at the moment preceding a rapid constant increase in HR. The relative maximal increase in HR (%) due to the stressor was calculated as: 100 x (HRmax – HRi) / HRi. Excess HR was calculated as [(Mean HR during stress – HRi)*duration of HR elevation (in min)], and corresponded to the number of heart beats which were produced in excess of HRi due to stress. Behavioural observations were time-matched against HR profiles and used to identify and control for potential changes in HR that were independent of the stressors applied (e.g. post-stress behaviours such as redirected aggression, parental or comfort behaviour). Cases for which HR responses to stressors could not be corrected for post-stress related activity were discarded. Additionally, for the 10-m -approach and capture-immobilization stress, we used HR data and information from audio files (times at which the experimenter was at given distances from the birds) to back-calculate the distance from the approaching experimenter at which HR started to increase, i.e. the distance at which the physiological response started. We subsequently refer to this parameter as detection distance (DD).

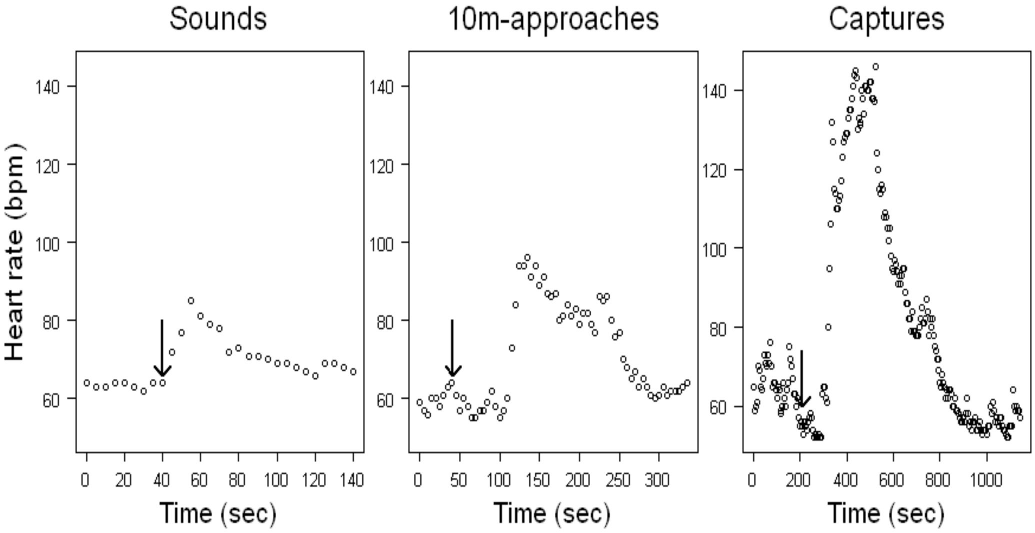

Figure 41. Example of typical heart rate (HR) responses to three different acute stressors in breeding king penguin.

The arrows specify the time stressors were applied on average. Note that for sound stresses, HR elevations were instantaneous. For 10-m and capture stresses, HR increases occurred only when the bird detected the approaching experimenter.

2.5. Statistics

All statistical analyses were performed using R (v.2.10.1 http://www.r-project.org/). Data was analyzed with linear mixed models (LMMs) fitted by restricted maximum likelihood (REML) using the 'nlme' package (Pinheiro et al. 2010). Bird identity was included as a random term in order to account for repeated measurements on the same individual. Normality of residuals was asserted using the Shapiro-Wilk normality test. Whenever necessary, data was transformed prior to analysis using Box-Cox power transformations, i.e. $x' = \frac{x^p - 1}{p}$ (where p is the power maximizing normality likelihood, obtained with the 'bcPower' function from the 'car' package in R) to ensure normality of residuals was satisfied (Peltier et al. 1998). Visual inspection of the residuals indicated no violation of assumptions of homoscedasticity. For LMMs, the number of observations used (n) and the number of birds concerned (N) are reported. Whenever covariates (e.g. stress type) were included in the models, interaction terms with other independent variables were also considered. If those were significant, we considered factorial variables separately, i.e. we ran separate models for each co-factor category (e.g. one model for sound stresses, one model for capture stress, etc.). Otherwise, most parsimonious models were selected using Akaike's

Information Criterion (AIC). Results are given as means ± SE, and significant effects reported for $p < 0.05$.

2.6. Ethical note

Animals in this study were cared for in accordance with the guidelines of the Ethical Committee of the French Polar Institute (Institut Paul Emile Victor – IPEV). Authorizations to enter the breeding colony and handle birds were delivered by Terres Australes et Antarctiques Françaises. Copies of permits are available upon request. Handling the birds, either when fitting HR-loggers or during stress protocols, was always performed within the colony on the breeding territory of the handled individual and never resulted in egg or chick abandonment. Flipper bands were removed at the end of the study because of their known detrimental long-term effects on survival and reproduction (Gauthier-Clerc et al. 2004, Saraux et al. 2011). The experiments performed in this study comply with the current laws of France.

3. Results

3.1. Changes in HR induced by stressors

Regardless of gender or breeding status, penguins exhibited marked responses in HR levels following stressful events (i.e. sounds, 10-m approaches, and captures). Indeed, the intercept was highly significant in a LMM where HR excess was specified as the dependant variable, stress type as an independent factor, and bird identity as a random variable (LMM; $t = 15.5$, $P < 0.001$, $n = 249$, $N = 78$). HR excess during captures was 10.5 times greater than during 10 m-approaches and 30 times greater than during sound stresses (**Table 5**). The relative maximal increase in HR during captures was 2.5 times greater than during 10 m-approaches and 4.5 times greater than during sound stresses (**Table 5**). When testing whether gender had an effect on HR (and controlling for stress type by adding it as a covariate in the model) we found that sex did not influence HR excess in response to stressors (LMM; $t = 0.42$, $P = 0.68$, $n = 249$, $N = 78$). However, gender did have a slight, but significant influence on the relative maximal increase in HR in response to stressors (LMM; $t = 2.10$, $P = 0.04$, $n = 249$, $N = 78$) and had a strongly significant effect on initial resting pre-stress HR (LMM; $t = -3.44$, $P < 0.001$, $n = 249$, $N = 78$). Indeed, on average females tended to have higher resting HR levels than males (i.e. 73.5 ± 1.2 *vs*. 64.0 ± 1.1, for all stress types confounded) and males tended to exhibit greater relative maximal increases in HR than females, but only during captures (i.e. 145.5 ± 9.6 % *vs*. 108.5 ± 9.1 %, for males and females, respectively; LMM; t = 2.78, P < 0.01, n = 84, N = 61). During 10-m approaches and captures, detection distances based on HR increase did not differ between stress types (i.e. 20.6 ± 1.0 m *vs.* 20.3 ± 1.1 m, for 10-m approaches and captures, respectively; LMM; t = - 0.82, P = 0.41, n = 161, N = 71), nor did they differ between genders when controlled for stress type by including it as a covariate in the model (i.e. 20.7 ± 0.9 m *vs.* 20.1 ±1.2 m, for males and females, respectively; LMM; t = - 0.28, P = 0.78, n = 161, N = 71).

HR Parameters		**Sound** **(n = 83, N = 63)**	**10-m approach** **(n= 82, N = 66)**	**Capture** **(n= 84, N = 61)**
Initial resting HR (bpm)	**Mean**	68.6^a	67.8^a	66.8^a
	S.E.	1.4	1.6	1.6
	Range	38.0-101.0	37.0-98.0	38.0-109.0
Maximum HR increase (%)	**Mean**	29.9^a	54.2^b	131.9^c
	S.E.	3.0	4.1	7.1
	Range	0.0-114.3	6.4-213.5	26.6-370.6
Excess HR (beats)	**Mean**	11.8^a	33.5^b	336.5^c
	S.E.	1.8	3.0	18.3
	Range	0.0-77.5	0.7-118.4	44.8-870.0

Table 5. Changes in heart rate of breeding king penguins subjected to three types of acute stressors.

Heart rate (HR) parameters include initial resting HR (bpm), maximum increase in HR during the stress relative to resting levels (%) and excess HR (beats) due to the stressor. Values with not sharing common superscripts are significantly different for P < 0.05. Differences were evaluated using LMMs with stressor type (i.e. sound, 10-m or capture) specified as a fixed factor and bird identity as a random variable.

3.2. Effects of breeding status and calendar day on HR responses

3.2.1. Breeding status

Overall, we found that birds caring for a young chick displayed significantly lower HR excess (LMM; t = -5.42, P < 0.001, n = 249, N = 78) and significantly lower relative maximal increases in HR (LMM; t = -4.13, P < 0.001, n = 249, N = 78) to stressors than those incubating an egg. Whereas HR excess was 5 times greater in incubators than in brooders during sound stresses (i.e. 20.3 ± 3.3 beats *vs.* 4.2 ± 0.9 beats; LMM; *t* = -3.43, *P* < 0.01, *n* = 83, *N* = 63), and 1.4 times greater in incubators than in brooders during captures (i.e. 392.7 ± 25.2 beats *vs.* 253.9 ± 18.9 beats; LMM; *t* = -3.93, *P* < 0.001, *n* = 84, *N* = 61), the trend however, was not significant for 10-m approaches (i.e. 39.8 ± 3.6 beats *vs.* 27.3 ± 4.5 beats; LMM; *t* = -1.13, *P* = 0.28, *n* = 82, *N* = 66) (**Figure 42**). Results were similar for relative maximal increases in HR: brooding birds displayed lower responses than incubating birds both during sounds (LMM; *t* = -2.64, *P* = 0.02, *n* = 83, *N* = 63) and capture stresses (LMM including sex as a covariate; *t* = -2.66, *P* = 0.01, *n* = 84, *N* = 61). Brooding birds also tended to display lower responses to 10-m approaches, however, the difference was not significant (LMM; *t* = -1.82, *P* = 0.09, *n* = 82, *N* = 66). Additionally, detection distances based on HR increase during captures were also greater for incubators than for brooders (i.e. 23.5 ± 1.3 m *vs.* 16.1 ± 1.7 m; LMM; *t* = -2.55, *P* = 0.02, *n* = 80, *N* = 60). Again however, the difference was not significant when considering 10-m approaches (LMM; *t* = -1.47, *P* = 0.16, *n* = 81, *N* = 65).

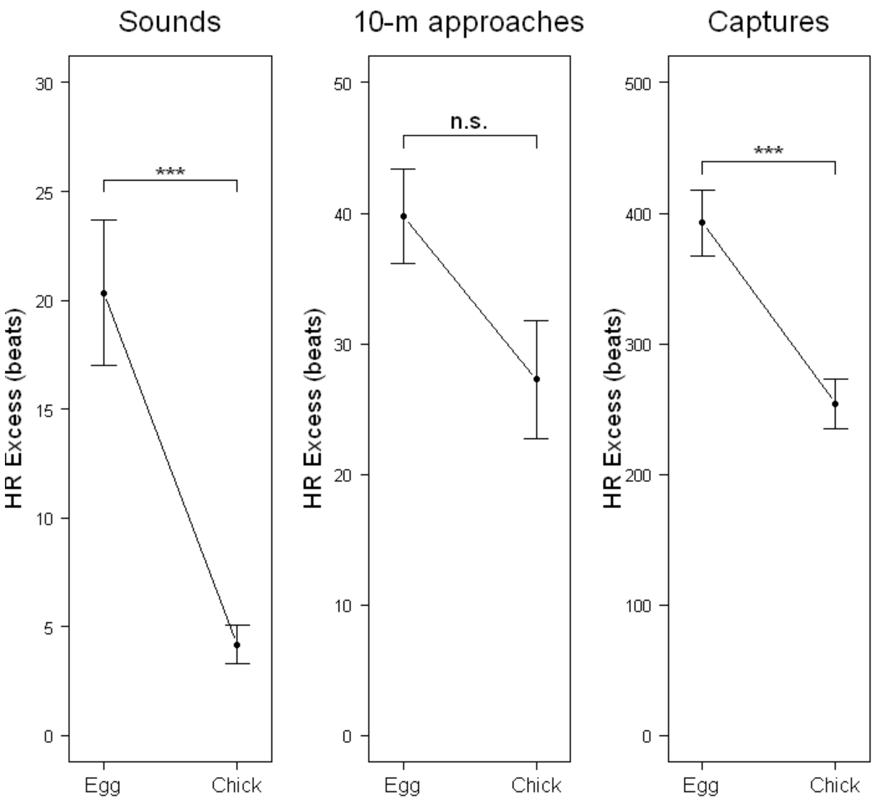

Figure 42. Heart rate (HR) excess caused by three different acute stressors in breeding king penguins either incubating an egg or brooding a young chick.

*** P < 0.001, N.S non-significant.

3.2.2. *Calendar day*

To discriminate whether HR responses may be influenced by calendar date rather than breeding status, we considered the influence of calendar date on HR responses in brooding and incubating birds separately. When controlling for stress type by including it as a covariate in the models, HR excess did not vary according to calendar date neither when birds were incubating an egg (LMM; t = -1.34, P = 0.18, n = 130, N = 36), nor when they were brooding a young chick (LMM; t = -1.17, P = 0.25, n = 119, N = 48). Results were similar when considering relative maximal increases in HR (LMMs; all P > 0.10). Detection distances based on HR during 10-m approaches and captures were not significantly influenced by calendar date in incubators (LMM; t = 1.73, P = 0.09, n = 86, N = 34), though a slight (non-significant) negative trend occurred in brooders (LMM; slope = -0.03 ± 0.02 days, t = -1.92, P = 0.06, n = 75, N = 42).

3.2.3. *Early versus late breeders*

We found no significant differences in HR responses between the 10 males that started their first incubation shift early in the season and the 12 males that started the first incubation shift late in the season (i.e. early *versus* late breeders). When controlling for stress type by including it as a covariate in the models, neither HR excess (LMM; t = -1.80, P = 0.09, n = 72, N = 22) nor relative maximal increases in HR (LMM; t = -1.25, P = 0.22, n = 72, N = 22) seemed to be affected by seasonal status (i.e. early *vs.* late). Surprisingly however, we found that late breeding birds exhibited greater detection distances than early breeders, but only during 10-m approaches (i.e. 24.3 ± 2.2 m *vs.* 15.3 ±1.9 m, for late and early breeders, respectively; LMM; t = 2.4, P = 0.03, n = 25, N = 17).

3.3. **Effect of fasting duration on HR responses**

We further tested whether fast duration might have an effect on HR responses to stress in the 8 birds that were stressed towards the beginning and the end of their first incubation shift. Overall, controlling for stress type in the models, we found that neither HR excess (LMM; t = -0.28, P = 0.78, n = 31, N = 8), nor relative maximal increases in HR (LMM; t = -0.24, P = 0.81, n = 31, N = 8), differed significantly between the beginning and the end of the incubation shift. Detection distances based on HR appeared to be influenced by nutritional status, but again, only during 10-m approaches (LMM; t = 3.36, P = 0.01, n = 15, N = 8). Birds towards the end of a shift indeed appeared to show higher detection distances (29.6 ± 2.7 m) than birds at the beginning of a shift (18.9 ± 2.5 m).

3.4. Consistency in HR responses

When investigating whether individual birds were consistent in their HR response to different stressors, and when pooling incubating and brooding birds together, we found a positive and significant (albeit weak, $R^2 = 0.15$) relationship between the HR excess displayed during capture stresses and that elicited during 10 m-approaches (**Figure 43a**; $F_{1,85} = 16.0$, $p < 0.001$, $n = 87$). HR excess during sound stresses was also significantly (again, weakly) associated to HR excess during 10-m approaches (**Figure 43b**; $R^2 = 0.09$, $F_{1,64} = 7.4$, $p < 0.01$, $n = 66$) and to HR excess during captures (**Figure 43c**; $R^2 = 0.06$, $F_{1,71} = 5.9$, $p = 0.02$, $n = 73$). However, detection distances based on HR were not correlated between 10-m approaches and captures (p = 0.51).

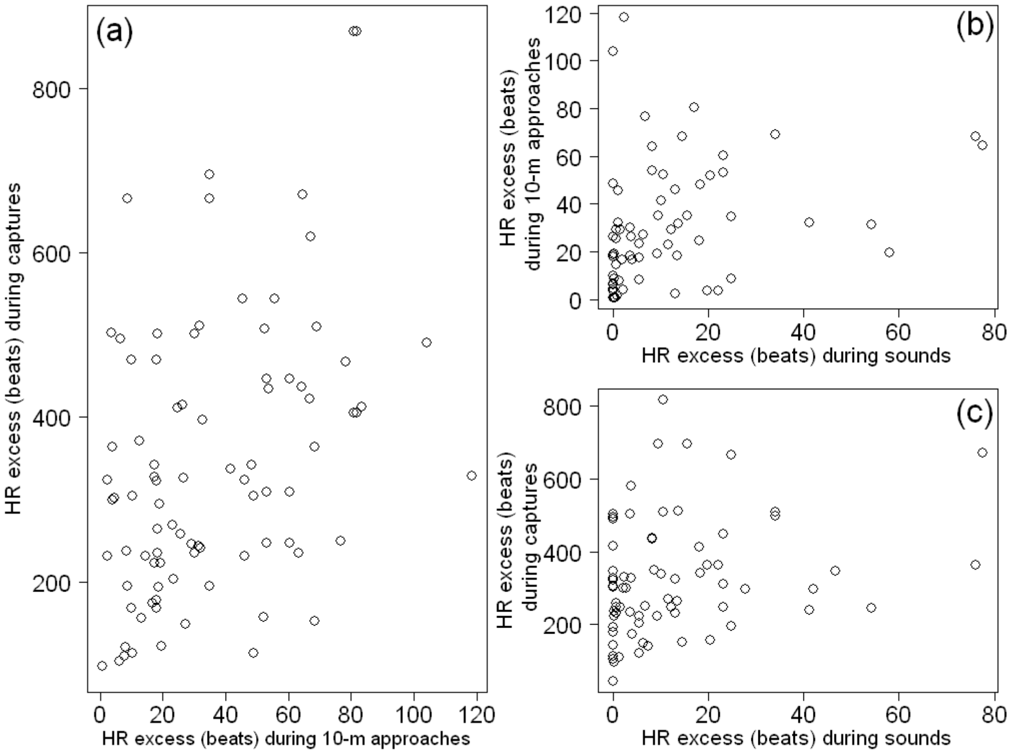

Figure 43. Relationships between individual bird heart rate (HR) responses to different acute stressors in breeding king penguin (all breeding status pooled).

Relation between **(a)** HR excess during captures and HR excess during 10-m distant approaches, **(b)** HR excess during sound stresses and HR excess during 10-m distant approaches, **(c)** HR excess during sound stresses and HR excess during captures.

4. Discussion

In the present study, we tested whether the cardiovascular component of the stress response varied according to breeding status in a long-lived species, the colonial king penguin. In support of the “brood-value hypothesis” (Bókony et al. 2009, Goutte et al. 2011, Lendvai et al. 2007), we found that penguins that were caring for young chicks elicited significantly lower HR responses during acute stress than penguins that were incubating eggs. To our knowledge, this is the first study to show that the cardiovascular component of the stress response (i.e. the initial ‘fight or flight’ stage) may be modulated by a change in breeding status, possibly reflective of a change in parental commitment to the offspring (Lendvai et al. 2007). Our results also suggest that rather than being uniform, HR responses during acute stress depend on the type of the stressor applied, and probably also on individual experience, as we found individuals to display some amount of consistency in their HR responses to different stressors.

4.1. The ‘brood value hypothesis’

Overall, our results show that HR responses to acute stressors are down regulated in birds that are breeding a young-chick. This is consistent with the ‘brood-value hypothesis’ which suggests that stress responses may mechanistically underlie parental decisions about offspring investment: when the value of the current reproduction is elevated, stress pathways should be inhibited to ensure that parents are committed to the offspring (Bókony et al. 2009, Goutte et al. 2011, Lendvai et al. 2007). Therefore, we suggest that breeding king penguins may ‘weigh’ the relative reproductive value of their offspring against the perceived risk of predation throughout the season. Parents’ commitment may be higher (and stress responses hence attenuated) when penguins are brooding chicks, because of the higher probability of chick reaching sexual maturity (Andersson et al. 1980, Winkler 1987, Clutton-Brock 1991). In line with this hypothesis, our results show that detection distances of approaching experimenters were greater (though not significantly for 10-m stresses) in incubating than in brooding birds. This suggests that incubators were less attentive to their egg, than brooders to their chicks. Several studies have shown how parental commitment may be affected by the relative reproductive value of the clutch or brood (Cézilly et al. 1994, Albrecht and Klvana 2004, Osiejuk and Kuczynski 2007). In magpies for instance, parents have been shown to increase the amount of brood defence as the offspring age (Redondo and Carranza 1989). Similarly, parental effort has also been shown to increase with offspring age in the greater flamingo (*Phoenicopterus ruber roseus*, Cézilly *et al.* 1994), and in the greylag goose (*Anser anser*, Osiejuk and Kuczynski 2007). In the snow petrel (*Pagodroma nivea*), Goutte and colleagues (2011) recently showed that increased nest desertion and increased CORT responses to acute stress could be experimentally provoked by delaying the normal hatching date of the eggs, thus decreasing their relative reproductive value (Goutte et al. 2011). The fact that a potential change in the relative reproductive value of the egg/chick (rather than external environmental factors) may influence HR responses to acute stressors in breeding penguins seems to be further supported by the lack of a relationship between calendar date and stress responses both in incubating and chick-brooding birds. For instance, both predation rates and colony density are known to increase with advancing calendar date during the breeding season (Descamps et al. 2005; Viblanc, Saraux, Malosse and Groscolas, *unpublished data*), and yet we found no significant relationship between calendar date and HR responses to stress. On the other hand, if the relative reproductive value of the egg/offspring were to explain changes in HR responses to stress, one might expect penguins breeding at different periods of the reproductive season to exhibit different HR responses to stress. Indeed, in king penguins, breeding occur in two peaks, as some birds breed early (November) and others late (January) within a given season (Stonehouse 1960). However, virtually only the former are able to successfully fledge their chick (Weimerskirch et al. 1992). Thus, the relative reproductive value of the offspring is expected to be greater in early breeders, and stress responses should be attenuated when compared to late breeders. However, we did not find any difference in HR stress responses between early and late breeders. One explanation may be that our sample size was relatively low (i.e. 10 early breeders and 12 late breeders), so that differences were not apparent. More likely is the fact that our sample selection was not fully representative of early and late breeders. Indeed, because of time constraints with fieldwork, we were only able to monitor early and late incubating males during the first incubation shift. However, if stress responses were primarily attenuated in chick-brooding birds, it would be more relevant to compare early *versus* late brooders. Future studies may consider monitoring early and late breeding pairs throughout the entire incubation and brooding periods to further test the 'brood-value hypothesis'.

4.2. Nutritional status

We did not find any relationship between birds' nutritional status and HR stress responses. Indeed, males at the end of their first incubation shift did not exhibit higher/lower responses than males at the beginning of their first breeding shift, even though they had been fasting for an average of 8.6 days more. This latter result is surprising, as one might expect body condition to have an important effect on stress responses. For instance, it is well known that increased plasmatic stress hormone (CORT) levels occur with advanced fasting (Challet et al. 1995, Cherel et al. 1988a). In penguins, those changes are believed to take part in the re-feeding signal that triggers nest/offspring abandonment and promotes adult maintenance, when prolonged fasting may jeopardize survival (Groscolas et al. 2008, Spée et al. 2010). Whereas such changes are usually linked to basal hormone concentrations, studies have also shown that hormonal responses to acute stressors are also higher during advanced fasting stages (Cockrem et al. 2006). So why was this not the case in our study? First, we cannot discard the fact that a lack of difference between HR responses to stressors at the beginning and at the end of the male's first incubation shift was, again, due to a low sample size (8 birds in each group). Alternately, it may be that the duration separating the two fasting states was not sufficiently long to affect responses to acute stressors. Indeed, all animals stressed in relation to nutritional status were males during their first incubation shift, and all birds were more than likely in phase II of fasting. If changes in stress responses occur when the metabolic and nutritional status are markedly different (e.g. fed, fasting phase I, fasting phase II, fasting phase III) (Cockrem et al. 2006), our protocol would likely not have allowed to test for such differences. Of interest however, is the finding that detection distances of the approaching experimenter during 10-m approaches and captures was greater when fasting stage was advanced, suggesting that birds were less attentive to their egg when their energy stores were more depleted.

4.3. Stress response and perceived risk

Interestingly, we found that penguins did not respond to all stressors in a similar fashion. Whereas HR responses were greatest for captures, they were intermediate for 10-m approaches and smallest for sound stresses. Obviously, as HR excess was calculated based on the duration of HR elevation, the length of the applied stress necessarily had an influence on HR excess. Thus it is not surprising that HR excess during 3-min captures appears greater than HR excess during sound stresses (with one sound produced per second over 3 seconds). On the other hand, relative maximal increases in HR were not based on HR elevation duration, and thus not likely influenced by the duration of the stress. Nonetheless, they showed the same trends as HR excess, suggesting that HR responses depended on the specific nature of the stressor. Cognitive appraisal processes, i.e. processes allowing the evaluation and categorization of specific situations in terms of well-being for the individual (Lazarus and Folkman 1984, Folkman et al. 1986, Kemeny 2003) have long been suggested as strong modulators of stress responses. Predictability and controllability over stressors for instance, have been shown to lower behavioral, hormonal, and cardiac responses during acute stress situations (Greiveldinger et al. 2007). In wild geese, HR responses are known to be influenced by stressor type, underlining the fact that individuals are able to discriminate between stressors and potentially associated risk (Wascher et al. 2011). In our study, whereas king penguins became alert to distant approaches and exhibited clear HR responses, it is conceivable that the predation-type risk associated with the presence of a standing observer was not the same than that associated with a capture stress, possibly explaining why no differences in HR occurred between incubating and chick-brooding birds. How then might we explain the fact that sound stresses (though seemingly with no life-history significance for breeding penguins) nonetheless elicited different HR responses in incubating and chick-brooding birds? Although we have no straightforward answer, a possible explanation may lie in some generalization of stress responses so that all non-lethal human disturbances (including sounds) may be considered analogous to predation risk (see Frid and Dill 2002 for a review). Differences in controllability and/or predictability of distant 10-m approaches (whereby birds are able to visually monitor the approaching experimenter) and sounds may then explain the differences observed in the HR responses to both types of stressor. For instance, HR responses may remain similar during 10-m approaches in incubators and brooders because birds able to monitor an approaching predator, with no immediate risk. Only when the risk associated with the predator becomes significantly great (i.e. captures), do we then observe differences in HR responses and attenuation of those responses in chick-brooding birds. The same may not apply to sounds, which are unpredictable and novel in nature. Similar differences in HR responses between approaches and sound stresses have been observed in Northern giant petrels (*Macronectes halli*), though not in terms of breeding status (de Villiers et al. 2006). The authors noted for instance that HR responses of giant petrels were higher when approached by an experimenter, than when disturbed by the sound of a distant helicopter.

4.4. Stress responses and personality

Finally, our results also suggest that individual penguins exhibited relatively consistent HR responses to various types of stressors. For any given bird, HR excesses during two particular stresses were correlated. Whereas the correlation was strongest between 10-m approaches and captures, it was nonetheless also significant when considering the relationships with sounds. Inter-individual variability in HR responses may reflect a certain style for coping with stressors along a shy-bold type continuum (Wilson et al. 1994, Koolhaas et al. 1999*)*. Individuals with overall lower HR responses to any particular type of stress may be at a selective advantage in a species where successful breeding is associated with the efficient management of energy stores. For those individuals lower stress responses is indeed synonym of increased energy savings. These findings underline the need to lead further investigations into the inter-individual differences that may be associated with stress responses, and the importance of reproductive trade-offs in shaping the stress responses of breeding animals.

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BOX 3 – Endocrine and metabolic responses to acute stress during reproduction

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Is the adreno-cortical and metabolic response to acute stress modulated according to breeding status in the king penguin?

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Unpublished data.

We show (**study 5**) that the heart rate response of breeding king penguins to various acute stressors changes throughout the breeding season. Notably, the stress response is blunted in brooding *vs.* incubating birds, suggesting that when the reproductive value of the current breeding is high, the stress response may be attenuated in order to favour offspring survival when parents are facing an acute predation-type stress ("brood value" hypothesis). However, the heart rate response reflects the immediate response to stress and it was thus of interest to determine whether the longer-term hormonal and metabolic component of the stress response is also modulated according to breeding status, and more specifically according to reproductive value. Indeed, several studies have suggested that stress hormones (i.e. CORT) may underlie parental commitment during reproduction, and have shown hormonal stress pathways to be down regulated when the value of the brood is high (Lendvai et al. 2007, Lendvai and Chastel 2008, Bókony et al. 2009, Goutte et al. 2011).

METHODS

During the 2010-2011 breeding season, we measured the adreno-cortical and metabolic response to an acute and standardized capture-immobilization stress in breeding king penguins at three different breeding stages characterized by a progressively increasing investment in the current breeding, and thus having a potentially increasing reproductive value: 1) courting birds at the very onset of breeding (approx. 1-3 days of fasting), 2) incubating birds at the onset of incubation (first incubation shift, approx. 15 days of fasting), and 3) birds brooding a young non-thermally emancipated chick (approx. 5 days of fasting). In addition, birds during the first incubation shift were stressed either at the beginning of the shift or at its end, i.e. 13 fasting days later, to search for a potential effect of energy/nutritional status (fasting duration) on the stress response. The adreno-cortical response was estimated from changes in the plasma concentration of corticosterone (CORT) and the metabolic response was determined from changes in the plasma concentration of non-esterified fatty acids (NEFA), a good index of metabolic response to stress in penguins (Corbel et al. 2010).

Twelve birds were stressed at each breeding stage, each bird being stressed only once. Courting birds were chosen at random and stressed while searching for a partner (songs, displays), within 1-2 days following their arrival ashore for breeding. In order to obtain birds at the onset and then at the end of the first incubation shift, both members of a pair were marked from a 1-m distance with a veterinary dye, once settled on their breeding territory. Then, we performed daily visual observation at a distance. The first time one member of the pair was observed alone and incubating an egg, it was identified as a male at day 1 of incubation (Stonehouse 1960). Some of these males were stressed the next day (day 2 of incubation) and the other birds were banded for identification and follow up until close to the end of the first incubation shift. Knowing that the average duration of the first incubation shift is 16 days (Weimerskirch et al. 1992), birds were stressed on day 15 of the shift to avoid their premature departure to sea. Brooding birds were chosen at random, when protecting a chick of approximately 10 days of age (as could be inferred from its size) in their brood-pouch.

During stress protocols, birds were approached from the back and we timed the moment of their reaction (alertness) to the experimenter, usually at a few meters distance. Then the bird was caught and rapidly hooded to keep it calm. A first blood sample (c.a. 1 mL) was taken from a flipper vein, as quickly as possible. Only initial samples obtained less than 3.5 min following alertness were considered as providing accurate baseline values for the measured plasma parameters (Romero and Reed 2005). If initial sampling was longer, the stress protocol was aborted. Following successful initial blood sampling, the bird was kept immobilized for 30 min and a second and third blood sample were taken after 10 and 30 min of immobilization, respectively. Blood was immediately centrifuged for 10 min at 3500 rpm and the plasma was kept frozen at - 80°C until analyses. Plasma concentrations of CORT were determined by radio-immuno-assay (RIA) using a commercial kit (MP Biomedicals, cat. no 07-120103), as previously described and validated for king penguins (Groscolas et al. 2008). Plasma concentration of NEFA, the product of lipolysis in adipose tissue and the major fuel in fasting penguins (Groscolas 1990), was measured by an enzyme-colorimetric method using a commercial kit (Randox, ref. FA 115).

The adreno-cortical response was calculated as the absolute increase in plasma CORT concentration from the initial baseline level to the level measured at 30 min (CORT 30 min – CORT baseline, ng/mL). The metabolic response was calculated as the area under the curve relating NEFA concentration to time, and was expressed in mmol.min.L^{-1}. Because birds were repeatedly sampled for blood (at times T_0, T_{10}, T_{30}), we used repeated-ANOVA to determine changes in CORT and NEFA over time. One-way ANOVA was used when comparing CORT and NEFA responses according to breeding stages. Normality of residuals was asserted using the Shapiro-Wilk normality test. Multiple comparisons were performed using a post-hoc Tukey test for honest significant differences (Tukey HSD). Results are given as means ± standard error (s.e.) and differences considered significant for $P < 0.05$.

RESULTS

Changes in CORT and NEFA concentrations during the stress

Whatever the breeding status, the capture-immobilization stress induced a progressive increase in plasma CORT concentrations over baseline levels (**Figure 44A**). For all birds, the increase was significant as of 10 minutes from capture and continued to increase until T_{30}. NEFA concentrations, however did not exhibit a similar trend in all birds (**Figure 44B**). In courting birds NEFA concentrations also increased gradually from T_0 to T_{30}, the T_{10} concentration however, not yet being significantly different from baseline levels (Tukey HSD; $P = 0.08$). On the other hand, both in incubating in brooding birds, NEFA concentrations significantly increased during the first 10 minutes of the capture-immobilization stress, but then subsequently decreased from T_{10} to T_{30} (**Figure 44B**). Both in males which were stressed close to the end of their first incubation shift and brooding birds, NEFA concentrations measured at T_{30} were actually not significantly different from basal NEFA concentrations measured at the beginning of the stress (T_0).

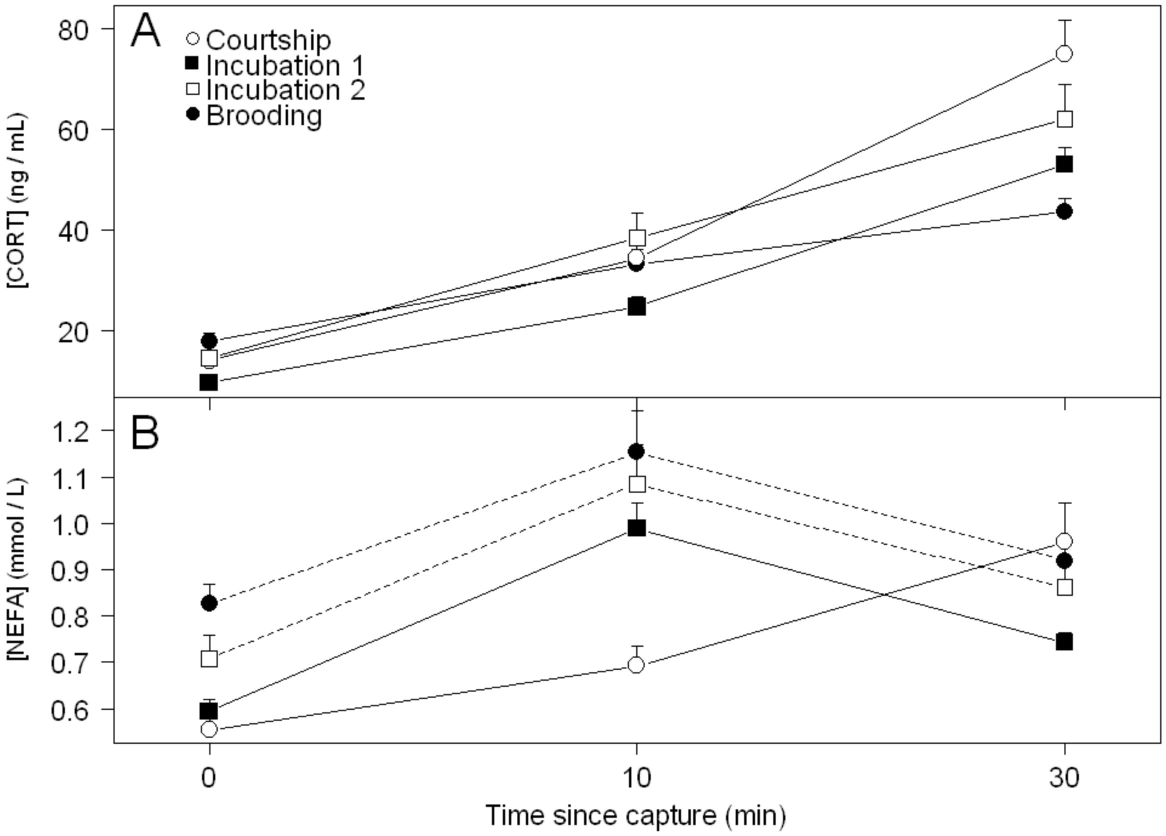

Figure 44. Changes in plasma concentrations of corticosterone (A) and NEFA (B) during a capture-immobilization stress in breeding-fasting king penguins.

Incubation 1: onset of the first incubation shift; Incubation 2: end of the first incubation shift. Full lines: values significantly different from each other at times 0, 10 and 30 minutes for $P < 0.05$ (except for NEFA concentrations in courting birds between T_0 and T_{10}, see text). Dotted lines: values at 0 and 30 minutes not significantly different. Results are presented as means ± SE. N = 12 for all values.

Effect of breeding status on CORT and NEFA concentrations

The relative increase in corticosterone concentrations (CORT 30 min – CORT baseline) differed significantly according to breeding status (ANOVA; $F_{3,44} = 8.87$, $P < 0.001$) : it was high in courting birds, intermediate during the first incubation shift and lowest (reduction by 2.4 fold *vs.* courting birds) in brooding birds (**Figure 45A**). The difference between courting and brooding birds was highly significant (Tukey HSD; $P < 0.001$). The CORT increase did not differ significantly in birds at the onset and end of the first incubation shift (**Figure 45A**, Tukey HSD; $P = 0.92$), nor did it differ significantly between courting birds (60.9 ng/mL) and incubating birds in general (45.5 ng/ml), though the difference fell short from significance between courting birds and birds at the onset of the first incubation shift (Tukey HSD; $P = 0.07$).

Although CORT increase did not differ between birds at the onset of their first incubation shift and brooding birds, the statistic also fell short of significance (Tukey HSD; $P = 0.06$). On the other hand, we did not find any significant differences in the metabolic (NEFA) response to capture-immobilizations according to breeding status (**Figure 45B**, ANOVA; $F_{3,44} = 0.30$, $P = 0.82$).

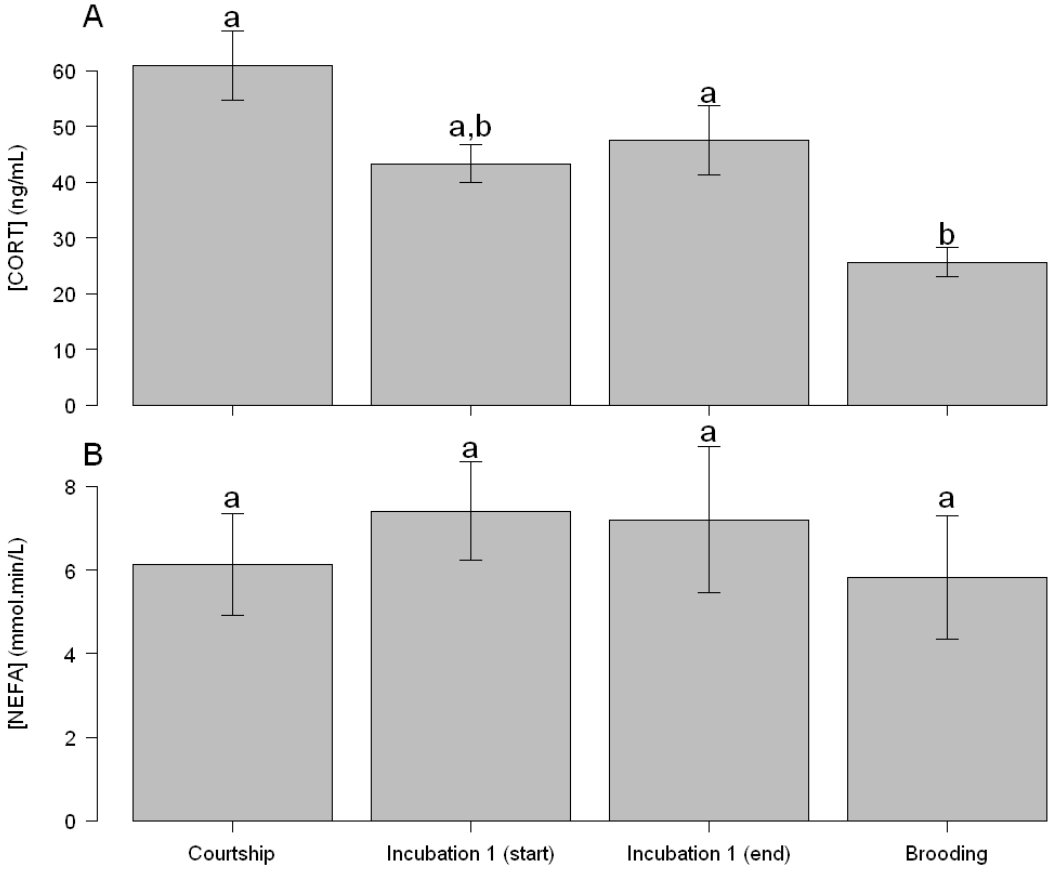

Figure 45. Hormonal and metabolic responses to a capture-immobilization stress in fasting king penguins of different breeding status.

(A) Absolute increase in plasma corticosterone concentrations ([CORT]) from the initial baseline level to the level measured at 30 min. **(B)** Area under the curve relating non-esterified fatty acids concentration ([NEFA]) to time. Values (N= 12 in all cases) are means ± SE. Values not sharing a common superscript letter are significantly different, $P < 0.05$.

CONCLUSION/DISCUSSION

In accordance with results obtained in the study of the heart rate response to various stressors (**study 5**), our results show that the adreno-cortical and metabolic response to stress may be modulated by breeding status in the king penguin. Whereas CORT responses appeared to be clearly attenuated in chick-brooding birds, this was not the case for NEFA responses. The most likely explanation to the observed difference is the overall kinetic of the NEFA response. Indeed, NEFA responses reached a maximum at 10 minutes following capture before subsequently decreasing both in incubating and brooding birds. In courting birds however, the increase in plasmatic NEFA concentrations was gradual and kept increasing after 30 minutes of capture. Unfortunately, we did not sample blood after T_{30}, so that we may not conclude on whether NEFA levels continued to rise (or not) in courting birds. Thus, whereas the NEFA response measured in incubating and brooding birds likely integrated the entire stress response, the NEFA response (i.e. the area under the curve) measured in courting birds was more than likely slightly (at best) underestimated. If we suppose (conservatively) that the NEFA response in courting birds reached a maximum at T_{30}, and that NEFA concentrations decreased in a likewise fashion in incubating, brooding and courting birds, the integrative NEFA for courting birds (the area under the curve) would be, on average, 60% greater than that reported. In this case, NEFA responses would actually be far greater in courting than in brooding birds. This would be consistent with the fact that the most significant differences observed in CORT responses were actually those between courting and brooding birds (see **Figure 45A**). The NEFA response is also interesting from another point of view. Noteworthy is the actual fact that concentrations of NEFA were still progressively increasing after 30 minutes in courting birds, whereas the maximum was reached at T_{10} in incubating and brooding birds, NEFA concentrations at T_{30} having almost rejoined baseline (T_0) values (**Figure 44B**). This supports the view that in courting birds, energy is mobilized to flee/deal with the threat even after 30 minutes, whereas the response is rapidly blunted in birds which remain on their breeding territory caring for their egg (incubators) or chick (brooders). Thus overall, stress responses appear to be attenuated in brooding birds, which agrees with the prediction of the "brood value" hypothesis. Indeed, as the parental investment required for replacing a chick is much greater than that required to replace an egg (Montgomerie and Weatherhead 1988), and as king penguins are only able to manage one chick within the same breeding season (Weimerskirch et al. 1992), they should be motivated to remain on their breeding territory (as illustrated by increased territorial defence against conspecifics; Côté 2000), and costly stress responses may be blunted (Lendvai et al. 2007, Bókony et al. 2009, Goutte et al. 2011). On the other hand, in courting birds that have not yet laid an egg, stress responses should be strong to deal with the potential threat and favour adult survival. Furthermore, our results do not support the view that the fasting duration is a major determinant of the stress response in king penguins. Indeed, CORT and NEFA responses to acute stress were not significantly different in incubating birds that were stressed some 13-days apart (onset *vs.* end of the first incubation shift). Also, courting and brooding birds had a markedly different stress response although fasting for a few days in both cases. A reduced stress response has been observed in phase II fasting *vs.* fed (or phase I fasting) king penguin chicks towards fledging (Corbel et al. 2010). In the present study, based on fasting duration and plasma CORT and NEFA levels (see Groscolas et al. 2008), all birds were likely in the phase II fasting stage. Thus, the possibility that the stress response of breeding king penguins might differ according to their nutritional status (e.g. fed vs. phase I, phase II or phase III fasting animals) should not be discarded.

ACKNOWLEDGMENTS

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VI. Coining the term: Behavioural Eco-physiology

“It has been said that the primary function of schools is to impart enough facts to make children stop asking questions. Some, with whom the schools did not succeed, become scientists”.

Knut Schmidt-Nielsen.

This thesis deals with the energetics of king penguins while breeding ashore in their natural colonial environment. By encompassing a range of different techniques and overlapping the fields of ecology, physiology and animal behaviour, its aim was to provide an integrative view of the various factors which might influence energy expenditure in breeding penguins, and on how those colonial seabirds deal with constraints of three different natures: nutritional (i.e. energy) constraints, social constraints, and stress. Before discussing the limits of our studies and the perspectives open for future research to grasp, let us proceed to a brief synthesis of the work performed.

1. An overall synthesis

As mentionned above, the aim of this work was to consider the behavioural and physiological responses of breeding king penguins to what may be considered 3 different ecological constraints. Actually, as all are various characteristics of the life history of king penguins, the critical reader may question ***whether these 3 different constraints are constraints at all?*** Nonetheless, dealing with nutritional limitations, the social environment and stress, and doing so while devoting time and energy to reproduction, necessarily means compromise. Indeed the time and energy invested into functions such as adult maintenance (or dealing with aggressive neighbours or predators) is traded-off at the detriment of current reproduction. Thus, physiological and behavioural strategies should evolve in order to maximize individual fitness, reaching an optimal compromise between adult maintenance and survival on the one hand, and offspring production on the other.

The studies I have conducted over these past 3 years thus aimed at providing some answers to the overall question of how seabirds (in general) and penguins (in particular) conciliate reproduction with long-term fasting in their natural colonial environment. Specifically I attempted to shed some light on the following questions:

- How is energy invested into physical activity during long-term fasting? Is overall physical activity maintained at a minimum level? Are the major behavioural activities observed in breeding penguins an actual energy constraint or are they maintained during long term fasting because of their minimal energy cost?

- How is energy expenditure modulated in free-living breeders during the longest fasts? Might incubating birds as large as the penguins rely on adaptive heterothermia for substantial energy savings while breeding?
- Are penguins sensitive to their social congeners? Does the social environment affect the physiology of breeding birds? Might this translate into some sort of energy cost, a supplementary unavoidable constraint for fasting penguins?
- How is the stress axis modulated throughout the breeding season in king penguins? Are costly stress responses attenuated as a means of energy saving? Are stress responses influenced by the reproductive status of the animal?

In the following pages, I will sequentially review the main findings of our studies, and discuss to what extent they have provided answers to the questions listed above.

1.1. Nutritional constraints: breeding while fasting

Long-term fasting is part of the life-history of seabirds. As a consequence, those animals have been a model of choice for a generation of scientists interested in the physiology of fasting, and studies having investigated the fasting capacities of penguins have had a long and fruitfull history (e.g. Cherel and Le Maho 1984, 1985, Cherel et al. 1986, Cherel et al. 1988a, b, c, d, Cherel et al. 1993, Cherel et al. 1994a, b, Groscolas and Clement 1976, Groscolas 1978, 1982, 1984, 1986, Groscolas and Robin 2001, Robin et al. 1988, Robin et al. 2001). In this regards, it is legitimate to question what ***our studies in particular have brought to the field*** and how they fit in to our overall understanding of the ecology of penguins. Ecology might indeed be the appropriate term. Whereas a number of studies have considered the fasting capacities of penguins, few have done so for birds breeding in their natural colonial context.

As reviewed in an early paper by Le Maho (1984), fasting seabirds provide a relatively simple study case for the field of energetics, as the energy involved in food-processing is virtually null, so that the overall DEE is limited to the components indicated **Figure 46**.

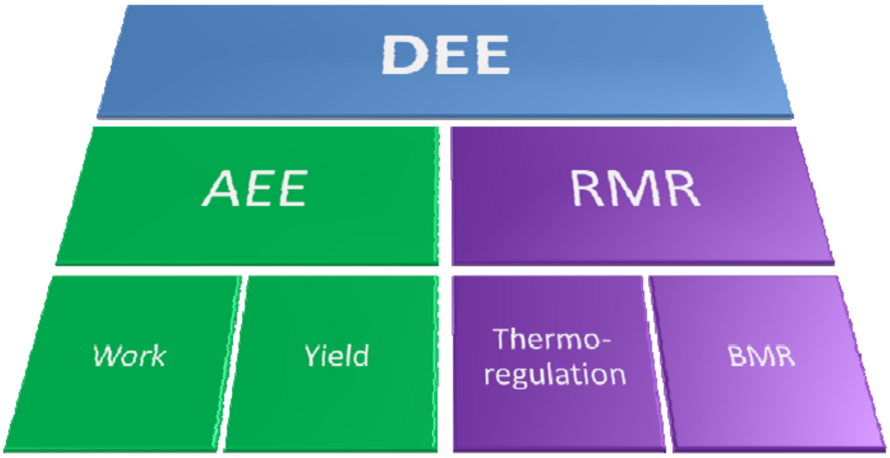

Figure 46. **Constituent elements of daily energy expenditure (DEE) for an endothermic fasting animal.** Note that the energy invested into physical activity (AEE) is influenced both by the quantity of work performed (i.e. the overall physical activity level of the animal) and by the energy yield of specific activities (i.e. their cost). On the other hand, note that in breeding king penguins, thermoregulatory costs are virtually null, so that resting metabolic rate (RMR) is very close to basal metabolic rate (BMR) – (*adapted after Le Maho 1984*).

As may be seen on the above figure, fasting seabirds might thus act on their DEE either by altering the costs of overall and specific activities (e.g. giving up coslty behaviours, decreasing overall physical activity), or by altering the costs linked to thermoregulation and BMR. Of course, the above figure is simplistic as any energy invested into physical activity will yield wastage heat which may then be recycled into thermoregulation, but those considerations are beyond the scope of this thesis. In the specific case of the king penguin, adults during the Austral summer are generally assumed to be within their thermoneutral zone (Barré 1980, Le Maho and Despin 1976, Le Maho et al. 1979), so that virtually, changes in RMR are linked to changes in BMR directly. Again, this may be a simplistic view because most studies conducted on the thermoregulatory costs in king penguin were done in still air, thus not accounting for potential wind-chill effects (but see Barré 1980). Our studies thus aimed at questionning how those components of DEE were affected in the wild (i.e. breeding fasting king penguin), an attempt which has, until yet, rarely been achieved.

1.1.1. Physical work and physical yield

In investigating the behavioural and physiological strategies free-living birds may use to conciliate breeding with long-term fasting, we questionned whether DEE may be shaped both by the overall investment into physical work (**study 3**) and the energy yield of specific physical activities (**studies 1 and 2**). Simply this ammounted to asking the question of whether fasting penguins limited their physical activity to minimum levels, and if so, which behaviours were favoured and for what particular reason were they favoured. Based on previous investigations on the behavioural time budget of breeding king penguins fasting ashore (i.e. Challet et al. 1994, see **Figure 47**), we thus considered the main behaviours into which king penguins engage while breeding ashore. Not surprinsingly, fasting penguins spend most of their time resting or sleeping, but nonetheless invest a substantial amount of time both in comfort and territorial defence behaviour (**Figure 47**).

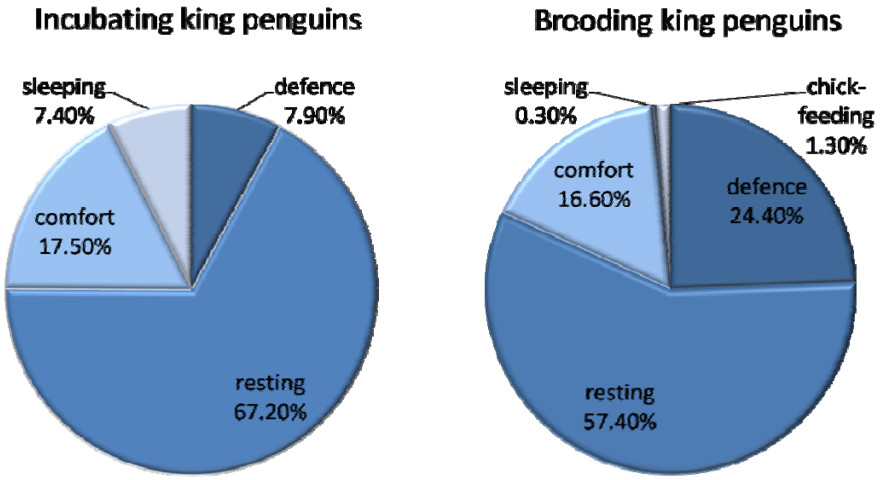

Figure 47. Behavioural time budget of breeding king penguins in our study colony.
Data are based on 24-hour focal recordings of 6 incubating and 6 chick-brooding king penguins – (*adapted from Challet et al. 1994*).

Our aim was thus to investigate the energy cost of these two behaviours and to re-evaluate the daily time-budget devoted to them using a larger sample size and the scan-sampling method (Altmann 1974). Specifically, we were interested in seeing whether those behaviours were maintained because of their particulary low energy cost (i.e. the yield component of physical activity mentionned above). Further, we also investigated whether overall physical activity (i.e. the work component) significantly decreased over the course of fasting in freely breeding birds. Indeed, whereas in captive animals one might expect physical activity to keep decreasing as the fast advances, such a trend may be constrained in colonially breeding penguins which are required to devote time to direct (egg- and chick-care) and indirect (territorial defence) breeding activities.

Our studies led to intriguing results regarding the energy cost of specific behaviours. For instance, whereas ***aggressive behaviours*** are expected to impose substantial energy costs (Parker 1974, but see Riechert 1988), and several empirical studies have reported energy costs of aggressive behaviour ranging from 1.3 to 4.8 times RMR (Hack 1997, Grantner and Taborsky 1998, Rovero et al. 2000), our study (see **study 2**) shows that in breeding king penguin, the average cost of aggressive behaviour only reaches 1.27 times RMR, i.e. a lower value than the lowest estimate of EE reported for aggressive behaviour until now. As previously discussed, the lower energy cost associated with territorial defence in this species might result from the fact that king penguins essentially resort to threat displays rather than physical contact during aggressive encounters – the latter being at least 3 times as costly as the former. In addition, it should be noted that the energy cost we suggest for territorial defence in the king penguin may actually even be overestimated. As an example, if one considers the data presented in **study 4** (see **Figure 48**), it becomes obvious that a strong emotional component is not accounted for in **study 2**.

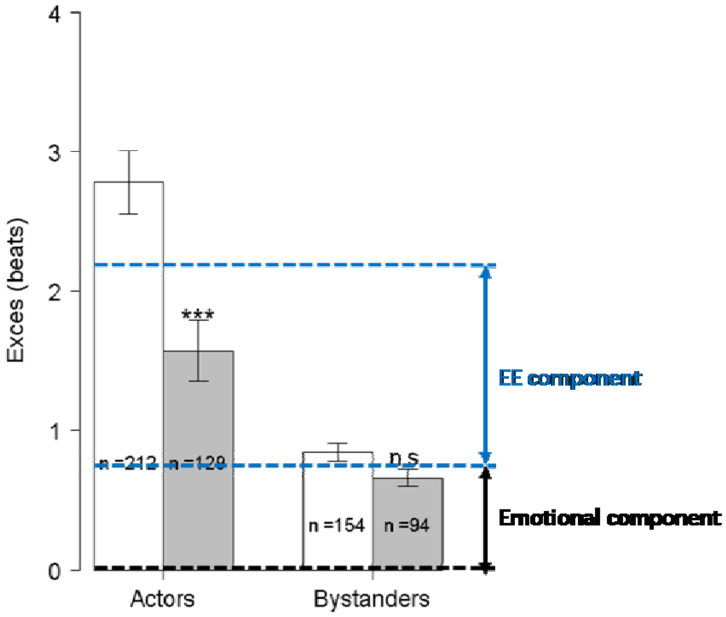

Figure 48. The emotional component involved in the HR changes of breeding penguins during aggressive interactions.

White bars are for physical attacks, grey bars for threat displays. Note that bystanders only observe neighbouring birds fight, so that no physical activity is associated with their HR increase. In contrast, actors are actively participating in conflicts so a physical (potentially energy costly) component is involved. Differentiating those two components may give us a more accurate estimate of the energy cost of aggressive behaviour.

Substracting this emotional component to estimate the energy cost of aggressive behaviour may thus yield more accurate estimates, even lower than those we have suggested. Even so, it thus appears that territorial defence is a low-cost activity for king penguins. In addition, when considering the daily time budget of aggressive behaviours (i.e. some 13 %, a value lower than previously reported; Challet et al. 1994, Côté 2000), the cost of territorial defence only accounted for some 2.7 % of DEE.

In their 1994 paper, Challet and colleagues wrote: “Considering its assumed high energy cost, breeding king penguins spend a great amount of time in territory defence, which therefore conflicts with their fasting state.” (Challet et al. 1994). The authors conclude that the apparent paradox may be explained by the fitness benefits that accrue from high aggressiveness (e.g. increased breeding success). Whereas some studies have indeed shown that higher rates of agressiveness enable penguins to maintain breeding territories in central positions in the colony (Côté 2000), and suggested those territories to be associated with higher breeding success (e.g. better protection from predators; Côté 2000, Descamps et al. 2005, Viera et al. 2006), the evidence that central territories ***do actually*** improve breeding success in the king penguin is conflicting (see Descamps et al. 2009). For instance, using an automatic monitoring system to assess the transits of freely breeding bird between the sea and the colony, Descamps and colleagues (2009) were able to reconstruct the breeding cycle and assess breeding success for individually monitored penguins. Those authors showed, that contrary to the assumption of breeding success being higher in central areas, 50 % of birds breeding in peripherial areas were successful in fledging a chick, whereas only 56 % of birds breeding in central areas managed to do so (a difference which was not significant, $P = 0.79$). Thus, whereas there may indeed be fitness advantages to maintain high rates of aggressiveness, further research is needed to evaluate the associated benefits. Rather, our results suggest a ***more conservative explanation*** to the fact that breeding king penguins are able to sustain high rates of aggressiveness while breeding on an “empty stomach”: because birds rely mainly on threat displays during territorial defence, they are able to maintain aggressive behaviour at a minimal energy cost. In addition as rates of aggressions are high (Côté 2000), resorting to threats rather than escalating into physical attacks when defending their territory allows to reduce the risk of injury associated with specific aggressive encounters, and the associated social stress (see **study 4**). This last suggestion is interesting and whether social stress associated with the colonial environment and aggressive conspecifics may translate to increased EE in the long-run remains to be thouroughly investigated (discussed below).

Further, when considering ***comfort behaviour***, the other major behaviour into which king penguins invest while breeding ashore, our studies reveal that the overall investment (both in terms of time and energy) is nothing slight of being substantial. We determined king penguins to devote some 22 % of time in comfort behaviour, and this behaviour to amount at some 8.8–9.3 % of DEE (see **study 2**). The daily time budget we report is slightly higher than that previously reported by Challet and colleagues (1994), and the difference most likely stems from the different method of sampling (i.e. scan-sampling in this study *vs.* focal observations) and the higher number of individuals used in our study. Compared against those figures, the 2.7 % DEE of aggressive behaviour appears like a marginal cost. How then might we explain that more energy is invested into comfort than aggressive behaviour, and that, ***despite its substantial cost***, king penguins do not reduce its proportion in the daily time budget while breeding? As previously discussed, the reason is probably that comfort behaviour should be considered a ***supplementary, unavoidable cost*** for a colonial breeder. In other terms, the fitness pay-offs of sustaining this relatively high-cost behaviour largely outweigh the alternate strategy, so that king penguins should continue to invest into comfort even though they are energy-constrained. A number of studies have convincingly reported how defects in plumage/pelage integrity or parasite infestation may translate into substantial fitness costs (including long-term costs; Bize et al. 2004, and developmental costs, Bize et al. 2003) and energy costs (Booth et al. 1993, Cotgreave and Clayton 1994, Møller et al. 1994, Giorgi et al. 2001). In addition of diverting energy sources from the host, parasites are also known to inocculate various pathogens, such as the Lyme disease agent (*Borrelia burgdoferi*, Gauthier-Clerc et al. 1998), which may prove lethal in some cases (Gauthier-Clerc et al. 1998). Thus, in king penguin, the costs associated with comfort behaviour obviously outweigh those associated with parasitism and defects in plumage intergrity, so that those colonial animals are willing to pay the price associated with such behaviour to maintain their integument in a good condition. In addition, as previously discussed, maintaining a functional musculature and avoiding ankylosis (or potential muscle atrophy) when remaining in an incubating position for some 15-days may be essential for those birds when they return at sea to forage in between breeding-shifts. Indeed, with maximum foraging ranges during summer ranging from 264 to 558 km (Bost et al. 1997), it seems obvious that a minimum muscle condition should be maintained while fasting onland.

Thus it appears that whereas the two main behaviours in which king penguin invest while breeding procur major fitness benefits, their energy-costs (i.e. the yield component of physical activity) differ substantially. Nonetheless those behaviours are maintained as not do so would probably result in associated fitness costs greater than those that might arise from investing energy in such activities while fasting. For instance the fact that birds do not seem to vary their investment into comfort according to timing in the breeding season or breeding status (Viera, Groscolas and Côté, unpublished data) suggests that the time invested into this behaviour is not compressible, supporting the former view.

As discussed above, animals may act either on the yield component (i.e. by resorting to the use of low-cost behaviours when energy constraints are high) or the work component of physical activity to reduce the associated EE. In **study 3**, we found that during the longest fast, the overall physical activity of breeding penguins decreased throughout courtship until a minimum level was reached at the onset of incubation. This is hardly surprising in the light of field observations. Courting penguins will initially actively look for a partner and roam around the colony engaging into physical contests with potential competitors. They will then search for a breeding territory once the pair-bond is established, and virtually remain on that territory as of a few days prior to egg-laying. Thus, it is not surprising that daily physical activity is already low a few days before the egg is laid (see **study 3**). Of interest is that, when considered during the course of incubation, physical activity does not continue to decrease (though the slope coefficient is still negative, and though the decrease we observed in dHR-rHR in captive birds – see **study 3** – suggests that physical activity decreases throughout the entire course of fasting when birds are in a non-breeding, captive condition). Thus, that the overall level of physical activity remains at a minimum but constant level in colonially breeding penguins also suggests that it is necessary for them to maintain specific activities (i.e. comfort and aggressive behaviour as previously mentionned) when incubating in their natural environment. An intriguing finding (which shall be further discussed below) was that, whereas physical activity appeared to be maintained at a minimum level, DEE (and thus

RMR) actually increased in early breeding king penguins, suggesting that environmental parameters (e.g. the social environment, climate conditions) had a strong effect on the physiology of breeding birds.

1.1.2. Adaptive heterothermia

For endothermic animals, heterothermia may be an extremly efficient energy-sparing mechanism (McKechnie and Lovegrove 2002, Geiser 2004). It was thus of interest to consider whether breeding king penguins may resort to transient decreases in body temperature to save energy during long-term fasting. This was all the more relevant that adaptive heterothermia has very recently been reported in king penguin chicks (Eichhorn et al. 2011) and has been previously shown in emperor penguins (Gilbert et al. 2007), though in the latter case, decreases in body temperature appeared to be limited to non-incubating individuals.

In our study however, the stomach temperature of male king penguins did not decrease significantly over the course of the longest incubation fast. Thus, our results do not support the view that decreasing core body temperature (i.e. the set point of thermoregulation) may be used as an adaptive strategy to limit energy expenditure in incubating birds. These findings are in agreement with the study of Gilbert and colleagues (2007), and support the idea that adaptive decreases in body temperature for incubating birds may be constrained by the need to maintain the egg at a relatively high and constant temperature, necessary for its development (Gilbert et al. 2007). Accordingly, several studies having investigated changes in body temperature in incubating birds have reported how decreases in body temperature may occur only when incubation duty ceases (Csada and Brigham 1994, Vehrencamp 1982, but see Calder and Booser 1973).

Nonetheless, the fact that we did not reveal any change in deep core (stomach) temperature in incubating king penguin does not negate the possibility that heterothermic strategies may be used as a means of energy sparing during long term fasting by adults during the breeding season. For instance, consider the following:

a. If even slight decreases in body temperature may lead to substantial energy savings (Butler and Woakes 2001, McKechnie and Lovegrove 2002), then it may be of interest to consider whether breeding penguins are able to resort to local hypothermia in non-obligatory tissues during incubation. Indeed, local heterothermies in specific tissues have previously been reported in diving king penguin (Handrich et al. 1997) and incubating emperor penguin (Gilbert et al. 2007). For instance, Gilbert and colleagues have found that when measured sub-cutaneously in the pectoral region, the body temperature of incubating emperor penguin was as low as 35°C (i.e. a drop of 2°C compared to the 37°C temperature of the body core), suggesting that whereas incubating emperor penguin are required to maintain an elevated core body temperature, they are able to resort to local heterothermia in the body shell which may already provide substantial energy savings (Gilbert et al. 2007, Gilbert et al. 2008). It may then be of interest to consider whether the temperature of the body shell or that of specific tissues might also be lowered during the fast of incubating king penguin, as a potential physiological means of energy saving.

b. If incubation is indeed a limiting factor for heterothermic strategies in incubating birds, then it would have been of interest to confirm this hypothesis by measuring the body temperature of fasting king penguins which were not bound by incubation duties. Unfortunately, we did not measure body temperature in our captive birds which were kept fasting whereas non-incubators, so that future research on the topic is needed. Moreover, it may be interesting to consider whether chick-brooding penguins are also required to continue and fuel as much heat as incubators to the brood-pouch, and whether body core temperature is required to remain as elevated as that during incubation.

1.2. Social constraints: dealing with aggressive conspecifics

One of the aims of this thesis was to address the energetics of penguins in their natural environment – colonial birds breeding in a social context. The drawback (which will be further discussed below) of such an approach is that, by stepping out of the lab into the field, one necessarily has limited control over confounding variables which might influence physiological processes, and spurrious interpretations may stem as a result. Nonetheless, for colonial seabirds, considering physiological changes during fasting without considering the potential impact of the social (colonial) environment on those changes, may similarly lead to misunderstandings in our comprehension of which factors affect energy expenditure, and how they may do so.

Over recent years, a substantial body of work has accumulated testifying just how profound the nature of social interactions may be on physiological processes (Aureli et al. 1999, Berntson and Boysen 1989, Kotrschal et al. 1998, Oliveira et al. 2001, Wascher et al. 2008a, Wascher et al. 2008b, Wascher et al. 2009). In the same way that climate might affect behavioural and physiological strategies in wild living animals, so too does the nature of the social environment for group-living species. A particular relevant example is that related to social stress (Creel et al. 1996, Creel 2001). For many social species, social relationships and hierarchies, social interactions with conspecifics, or just the mere presence of social neighbours have strong influences on stress physiology. As an example, Scott Creel (2001) reviewed the effects of dominant-subordinate relationships on stress hormones in group-living species. The outcome is that those relationships are far from simple. Whereas in some instances, studies have reported elevated glucocorticoids (GC) in subordinate animals, others have found GCs concentrations to be elevated primarily in dominant individuals (e.g. when hierarchies are unstable, reviewed in Creel 2001). Another example of effects of the social environment is the stress-alleviating mechanism of social support (active or passive) through the presence of allies. For instance, such social support has been documented to strongly influence stress levels in group-living animals in species as diverse as men (Steptoe 2000) or geese (Weiss and Kotrschal 2004, Scheiber et al. 2005a). As an example, Steptoe and colleagues (2000) submitted a large sample of full-time school teachers to 40 item questionnaires designed to assess their perceived availability of social support (e.g. availability of people to talk, practical aid, self-esteem support, etc.). The authors show, that during periods of high stress over the working day, mean levels of systolic blood pressure (adjusted for concurrent EE and body mass) were lower for individuals receiving high social support than for those receiving low social support, consistent with the view that social support may buffer the impact of perceived stress on blood pressure and HR. Similarly in geese, passive social support (i.e. the mere presence of social allies) was shown to decrease CORT levels during competitive feeding situations (Scheiber et al. 2005a), known to be stressfull situations for those animals (Scheiber et al. 2005b). Further still, studies have highlighted how the social environment experienced by mothers during pregnancy had drastic impacts on the offspring's behaviour, brain and neuroendocrine function later in life. For instance, a comprehensive review by Kaiser and Sachser (2005), underlines the effect of pre-natal social stress in mammalian species, adding a trans-generational component to the effects the social stimuli may have on the physiology of group-living animals. It is thus quite clear that the social environment is a potent source of stress for social species, and the extent to which it might affect the physiology of colonial seabirds merited consideration.

In asking whether the social environment might affect the physiology of individual animals, one makes the obvious assumption that animals ***are actually sensitive*** to their social environment. Whereas the assumption is certainly reasonable for most species, the intriguing question is ***how sensitive to their social congeners are they exactly***? Recent studies in the greylag goose (*Anser anser*) have suggested that animals may actually be more sensitive to social stimuli than non-social ones. For instance, Wascher and colleagues recently suggested that the social environment may override non-social stimuli during stress responses (Wascher et al. 2008b). Indeed, the authors found that HR responses of by-standing geese were greater when those observed social (agonistic interactions, departing or landing geese) than non-social (loud noise or passing vehicle) events. Those authors also showed that during aggressive interactions, HR stress responses in bystanders depended on the identity of the individuals involved, suggesting that geese exhibit differential stress responses when witnessing fights where an affiliated individual is or is not involved (Wascher et al. 2008b).

In our studies, we thus questionned first whether king penguins were sensitive to their social environment (see **study 4**). Because of the risk associated with aggressive behaviour, we considered whether birds displayed stress responses when aggressive interactions occurred in their neighbourhood. Our results reveal that not only do birds display strong HR responses when actively participating in aggressive interactions, they also do so when witnessing fights between neighbours. Those results are interesting in that they comfort those previously found in greylag geese by Wascher and colleagues (see Wascher et al. 2008a, Wascher et al. 2008b, Wascher et al. 2009). In addition, we found that penguins did not display similar HR stress responses depending on the nature of the stimuli. For instance, HR responses were lower during threats than during attacks, though the difference was not significant for bystanders. This suggests an ‘emotional’ component to the HR response which may be linked with risk-assessment (Kemeny 2003). This view is further supported by the finding that HR elevations of actors and bystanders were not influenced in a similar way by the specific natures of aggressive interactions. For instance, the intensity of aggressive interactions (e.g. number of threats/blows per unit time) did not influence HR in bystanders ($p = 0.89$) whereas it did so significantly in actors ($p < 0.001$). A possible explanation is that the risk associated with an increase in conflict intensity is not the same for actors (which are more likely to get injured) than for bystanders that are observing the scene. Alternately, we can not discard the possibility that physical activity may also have been involved in the observed pattern. However, for bystanders the duration of interactions had a positive significant influence on HR responses – longer lasting conflicts may be associated with greater risk of redirection towards neighbouring birds. Thus it appears that penguins are highly sensitive to their colonial environment and respond physiologically to aggressive encounters by mounting a stress response, even when they are only observing neighbouring birds. However, because aggressiveness is high in breeding penguins (mean of 100 interactions/hr/bird, Côté 2000), the question we may then ask is ***whether those stress responses translate into substantial energy costs over the long term?***

In order to answer such a question, one of the simplest methods would be to monitor EE in birds subjected to different environmental conditions, i.e. high and low rates of aggressive behaviour. Our results in **study 3**, provide such a proxy. In this study, early breeding males were monitored over a period during which substantial changes occurred in colony density. As the breeding season starts, courting birds will progressively arrive on-shore and will start actively defending their small breeding territory (Barrat 1976, Stonehouse 1960, Côté 2000). Because of their progressive arrival on-shore and courtship period, breeding birds progressively crowd the colony until it reaches its peak density in late December / early January. Breeding birds thus defend a territory whilst progressively being surrounded by more and more conspecifics, and thus the rates of aggression (i.e. and associated social stress) progressively increase as the minimum distance between birds decreases. Interestingly, our results suggest that over this period, the mean daily HR (a proxy to DEE) and mean daily resting HR (rHR, a proxy to RMR) of breeding birds actually increases, while no changes in body temperature nor increase in physical activity is recorded. We suggest such an increase may be linked to increased experienced social stress and is reflective of increased EE linked to changes in the social environment.

Finally, our results on the social environment of breeding penguins and its effects on stress responses in breeding birds has led to one intriguing finding. We have found that HR responses during aggressive interactions tend to increase as breeding status advanced (i.e. males during the third breeding shift displayed significantly higher HR responses to aggressive conspecifics than males during the 1st incubation shift). Although this result might appear marginal (indeed this increase was only significant for males during incubation), it is nonetheless of interest as it is in contrast with the HR responses we have observed when subjecting penguins to acute stressors (see **study 5**). Indeed, in the latter study, we found HR responses to be attenuated as breeding status advanced, an observation which is consistent with the brood-value hypothesis discussed in the following paragraph. ***So how come we did not observe a similar trend to HR stress responses when considering social stressors and human stressors?*** Although this may edge on the side of anthropomorphism, I suggest the explanation stems from the fact that social and human (predator-type stressors) are not the same from a penguins' point of view. HR responses are known to vary independently of CORT responses (Nephew et al. 2003) and to differ according to stressor-type (Nephew et al. 2003, **study 5**). Whereas it may be adaptive to attenuate stress pathways (also reflected in HR) to predation-like stressors when reproductive value is high (see below), strong HR responses to social interactions when reproductive value is high may be reflective of increased social (emotional) tension and hightened awarness of social conspecifics. Thus, as the egg approaches hatching, penguins may be more (emotionnally) sensitive to the social conspecifics. If such a suggestion were true, the implications would be that stress responses (as reflected by HR) may be up- or down-regualted depending on the stressor considered. This would lead to a situation where it may be useful to discriminate actual stress responses from emotional stress responses, and emphasizes the need for studies on the influence of psycho-social factors on stress responses in group-living animals.

1.3. Stress constraints: energy savings and costly chicks

Setting out on the study of acute stress responses in breeding king penguins, we hypothesized that because stress responses are costly in terms of energy – they enable the mobilization of energy stores and allow the individual to cope with the stressor (McEwen and Wingfield 2003) – they ***should be attenuated*** in fasting seabirds (for which any little energy saving may mean the difference between breeding success and failure) ***when the fast is long***. To investigate this hypothesis, we subjected breeding penguins which had been fasting for periods of different lengths to various human-induced stressors (which we assumed to be predator-like) and monitored both their heart rate and their hormonal/metabolic responses to acute stress situations. In addition, we considered whether physiological responses to accute stress situations ***were modulated differently throughout the breeding season***, and thus also subjected birds of different breeding status to the same acute stressors. The interest in monitoring both HR and endocrine (CORT) responses was two-fold: **(1)** It provided us with an integrative view of the acute stress response – i.e. both the short-term neural and the longer-term endocrine pathways – in relation to nutritional and breeding status, and **(2)** it allowed us to investigate whether stress pathways were or were not regulated independently from each other in the king penguin.

Because both stress pathways are activated when an animal is subjected to an acute stressor, one might expect HR and endocrine responses during acute stress to vary in a similar fashion. However, recent studies in birds have suggested this may not necessarely be the case. For instance, in a study on captive European starling (*Sturnus vulgaris*), Nephew and colleagues (2003) have shown that when birds were subjected to different acute stressors, whereas both HR and CORT responses were apparent in all stress trials, CORT responses were sub-maximal and similar in all treatments (regardless of the nature of the stressor), whereas HR responses depended on the type of stressor applied (Nephew et al. 2003). From those results, the authors suggested that ***HR and CORT pathways were regulated independently*** when starlings were subjected to acute stressors (Nephew et al. 2003). It was thus of interest to consider this possibility in king penguins, and though at a different time scale, to investigate whether notable differences in HR and CORT responses appeared when birds were stressed at different times during the breeding season.

Our results indicate that in breeding king penguins, both the HR (**study 5**) and the CORT/NEFA (**box 3**) responses to acute stressors varied throughout the breeding season. Interestingly both responses vary in a similar fashion, and both responses appear to be attenuated as the breeding season progresses. However, our results ***do not seem to support the hypothesis that nutritional status is a major determinant in modulating acute stress responses*** in breeding birds. Indeed, we did not find that birds which were subjected to stressors while at a later stage of their fast exhibited lower responses than when stressed at an earlier stage (see **study 5** and **box 3**). Thus, our expectation that penguins may attenuate stress responses to acute challenging situations as a means of energy sparing does not appear to be verified. This is surprising in the light of current knowledge on stress responses and fasting. As an example, by investigating the adrenocortical and metabolic responses to acute capture and handling stress during the fledging process in king penguin chicks, Corbel and colleagues (2010) found that the HPA-axis responsiveness to acute stress may be dampened in function of the nutritional status of fasting birds (Corbel et al. 2010). At the beginning of moult, chicks which were found to have a metabolic status characteristic of phase I of fasting (i.e. low plasmatic [β-hydroxibutyrate] and high plasmatic [uric acid]) also displayed high CORT responses to acute stress. Those responses were progressively dampened as the fast advanced, i.e. chick at moult end and chicks at fledging having significantly lower CORT responses, while their metabolic status was found to be characteristic of early and advanced phase II of fasting (i.e. increasing [β-hydroxibutyrate] and low [uric acid] plasmatic concentrations (see **Figure 49A**), respectively. In addition, when pooling all chicks together, those authors found that CORT responsiveness was negatively related to plasmatic concentrations of β-hydroxibutyrate (see **Figure 49B**), supporting the view that the metabolic status (i.e. fasting phase), which reflects the availability of rapidly oxidizable substrates, is an important determinant of (the adrenocortical) physiological responses to acute stress situations (Corbel et al. 2010).

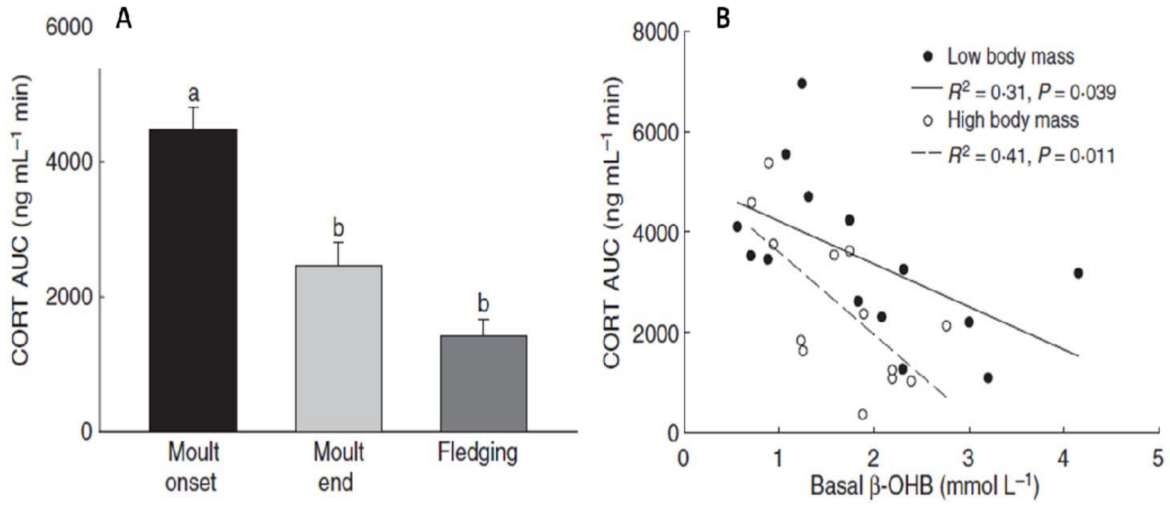

Figure 49. Integrated CORT responses (AUC) during a capture-handling stress in king penguin chicks according to their fasting status.

(A) Integrated CORT response at 3 stages of the pre-fledging period, of advancing fasting status: from moult onset (in fasting PI) to moult end and fledging (fasting PII). **(B)** Integrated CORT response to a capture-handling stress in relation to basal levels of β-hydroxibutyrate (β-OHB). Chicks with low and high body mass were considered separately. Note that stress responses are attenuated as fasting status advances.

Similar findings of dampened adrenocortical activity and stress response during nutritional challenges have been reported in other bird (Carsia and McIlroy 1998, Kitaysky et al. 2005) and mammalian species (De Boer et al. 1989, Wronska et al. 1990, Kirschbaum et al. 1997). On the other hand, some studies have reported acute CORT responses to actually increase when fasting is at an advanced stage (e.g. PIII; Cockrem et al. 2006) or under diet-restricted conditions (Kitaysky et al. 1999), an observation that may be attributed to the role for CORT in promoting protein breakdown when the fast is long, or increasing begging behaviour in chicks (Kitaysky et al. 2001) and a re-feeding signal in adults (Groscolas et al. 2008, Spée et al. 2010). ***So why did we not observe any difference in stress responses in our adult king penguins?*** The most likely explanation is that the duration of the fast did not differ sufficiently between our two groups for such a difference to occur. Birds were males that were stressed either at the onset of the first incubation shift or at its end, and were most likely all individuals which were in PII fasting. If changes in physiological responses to acute stress rather occur when drastic changes in metabolic status occur (such as a shift from PI to PII or PII to PIII), our protocole would have prevented us in observing such patterns. Whereas it would be interesting to consider whether stress responses change between fasting phases in adults, several issues would render the task complicated in the field. First, if one considers the transition between PI and PII, acquiring birds which are early in PI may prove complicated, as penguins coming back ashore may already be transitioning from PI to PII (Groscolas and Robin 2001). In addition, one would then be comparing courting and incubating birds, so that it would be hard to distinguish the nutritional from the reproductive status effect.

Nonetheless, we found stress responses (both HR and CORT) to be attenuated as the breeding season advances. This triggers the question of ***what factors that vary over the course of a breeding season might affect changes in the physiological responses of breeding penguins to acute stressors?*** Those may be of several nature, including changes in the physical environment (e.g. weather conditions), changes in the social environment (e.g. colony density, aggressiveness), changes in predation risk, or changes in reproductive status.

In **study 5**, we found HR stress responses to be influenced by the breeding status of birds, i.e. incubating birds displayed higher HR responses to acute stressors than did chick-brooding birds, but those were not influenced by calendar date when incubating and breeding birds were considered separately. This is interesting because, given that environmental changes will co-vary with calendar date (e.g. changes in colony density, **study 3**), it tends to show that it is actually a ***change in reproductive or "offspring" status*** rather than in any other parameter we could think of which mediated a change in stress responses to acute stressors. Current litterature on stress hormones and their adaptive value strongly supports the view that under specific circumstances, long-lived birds enter an emergency life history stage which is characterized by the re-direction of behaviour and physiology towards survival at the detriment of the current breeding attempt (Wingfield et al. 1995, Wingfield et al. 1998). In breeding seabirds, stress hormones are proposed to be one of the proximate mediators promoting parental decision to abandon a current breeding attempt when survival is at stake (Groscolas et al. 2008, Spée et al. 2010). However, several studies have also suggested that in some cases, it may be adaptive for stress responses to be attenuated in order for breeding success to ensue (Wingfield and Sapolsky 2003). For instance, when the breeding season is short, or future breeding opportunities low (such as in aged individuals), it may be adaptive to suppress physiological stress responses to insure that breeding is successful (Wingfield and Sapolsky 2003). In the case of long-lived species (such as the king penguin) however, the trade-off between current and future reproduction previously mentionned is such, that suppressing stress responses during breeding altogether is hardly adaptive. Thus, “valueing” the brood at a given time relative to future (and passed, discussed below) investments and “shaping” stress responses accordingly, may provide long-lived seabirds with the best compromise to maximize their fitness. The hypothesis that stress responses may be attenuated when the value of current reproduction is high relative to that of future breeding prospects, has received support from various studies (Lendvai et al. 2007, Lendvai and Chastel 2008, Bókony et al. 2009, Goutte et al. 2011) and provided a mechanistic basis underlying parental decisions in long-lived animals.

Our results on breeding penguin seem to confort the “brood-value hypothesis”, in that in addition to the traditionally studied endocrine stress response, the short-term HR response to stress also appeared to be affected by breeding status. However, rather than testing directly whether stress responses were affected by prospects of the current breeding attempt being higher than future ones, our study tested whether the reproductive value of the offspring (i.e. a proximate cue underlying parental decisions in relation to fitness), influenced physiological responses to stress (Adams et al. 2005), adding support to the thesis that stress pathways may mechanistically underly parental commitment to the offspring in long-lived seabirds (Bókony et al. 2009).

2. Limits to our studies and unanswered questions

2.1. The heart rate method for estimating energy expenditure

The challenge of accurately measuring energy expenditure (Halsey 2011) has always been central to the fields of physiology, behaviour and ecology – e.g. in 2011, the scientific journal “Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology” devoted a special issue reviewing current methods and advances in the measure of energy expenditure in captive and wild animals. In the following section, my aim is to briefly review why heart rate as a proxy to energy expenditure was a convenient method for us to use in field studies, and to point out the main limits of this method in the estimation of energy expenditure.

Beacause animals obtain energy mostly through oxidative pathways (bear in mind that this is not always the case – some animals are able to obtain energy independently of the oxidation of food), their rate of oxygen consumption is often taken to be an accurate measure of energy metabolism (Schmidt-Nielsen 1979). Whereas determining the rate of oxygen consumption for an animal is of relative ease, it has nonetheless a few drawbacks. **The first** (and most obvious) major drawback, is the assumption underlying the use of oxygen consumption to measure metabolic rate. Indeed, it is assumed that animals are in a metabolic steady state and that energy used as ATP is replenished through aerobic metabolism (Schmidt-Nielsen 1979, Green 2011). Obviously, this assumption ***ignores the energy yielded by anaerobic pathways***, hence anaerobic metabolism. To put it in the words of Knut Schmidt-Nielsen: ‘ (…) a fully anaerobic organism has zero oxygen consumption but certainly does not have a zero metabolic rate !’ (Schmidt-Nielsen 1979). However, some specific cases set apart (e.g. seasonal hypoxia in certain species, Bickler and Buck 2007), animals mainly rely on oxidative processes to fuel their metabolism so that the measure of oxygen consumption appears a valid measure of metabolic rate, at least in birds and mammals. **The second** major drawback, is the aforementionned ***relative ease*** to measure oxygen consumption accurately, especially in wild animals. Obviously, the most accurate way to measure oxygen consumption is by directly analyzing the composition of the air inhaled and exhaled by an animal either over a defined period or during specific activities, i.e. the gold standard technique of respirometry (Lighton and Halsey 2011). Whereas this technique is widely used and applicable in captive, as well as certain field conditions (e.g. Culik et al. 1994, Culik et al. 1996), it is obvious that applying it to monitor free-living animals performing their natural daily routines encounters its limits. For instance whereas face masks may be incorporated into portable repirometry systems, they are clearly not always adapted for all measures of energy expenditure nor all field conditions (e.g. obviously a bird with a face mask can not preen, nor can we directly monitor oxygen consumption in diving penguins some 100-m below the ocean’s surface).

In seeking to study animals in their natural environment, ecophysiologists have thus endeavoured to find alternative methods for estimating metabolic rates in the wild. As mentionned in the introduction, a widely used method to measure metabolic rate in the field relies on the use of stable isotopes to determine the amount of CO_2 produced by animal over a given period (Butler et al. 2004, Shaffer 2011). The method consists in injecting an animal with a known amount of doubly-labelled water (DLW) containing isotopes of oxygen (^{18}O) and hydrogen (^{2}H) and, once this bolus of water has mixed with the body water pools, examining turnover rates of both H_2O and CO_2. As oxygen will be eliminated from the body both in the form of H_2O and CO_2, but hydrogen will only be lost in the the form of H_2O, the difference in turnover rates of each isotope provides a measure of CO_2 production, when a final blood sample is collected from the animal at the time of recapture (Shaffer 2011). Because of its relative simplicity (this technique only requires capturing and sampling the animal twice) and accuracy (provided the isotopic turnover rate for the species in question is known – studies have shown this method to yield estimates of MR that fall within 8 % of values estimated with classical respirometry), this method has been used with great success over the years (reviewed in Shaffer 2011) and allowed insight into the energetics of many species in their natural environmnents. However, in addition to the fact that its relatively high cost may prevent it from being a method of choice in large animals (Speakman 1997), one of the main limitations of this method is that it does not easily allow to measure the energy expenditure associated with specific activities (but see Piersma et al. 2003). Indeed, the DLW method only allows to measure metabolic rate over a given amount of time (i.e. between initial injection of DLW and final collection of the blood sample), thereby integrating all activities occuring during the time period considered, and is thus incompatible with measuring the energy cost of short-term behaviours (e.g. comfort behaviour).

In this regards, another promising method that may be used for the study of energetics, is the HR method, which we chose to use in our studies. The HR method is convenient in that, with advancing technological refinements, HR-loggers allow to monitor animals in the wild for extended periods of time (e.g. sometimes over an entire year, Green et al. 2009) without any intervention close to the equipped animal other than during intial set-up and retrieval of HR-loggers. Some studies have also succesfully recorded HR in incubating birds via the usage of microphones set in dummy eggs (Nimon et al. 1996, Storch et al. 1999), a method which potentially allows to record heart beats (and thus infer HR) whithout even having to manipulate the animal.

In order to draw energy estimates from the measurement of HR, one needs a calibration equation relating HR to oxygen consumption. The relationship is based on Fick's equation for O_2 convection in the cardiovascular system (Dejours 1981, Butler et al. 2004) with the understanding that ATP consumption by the cells is met by the production of ATP through aerobic metabolism and thus increases oxygen consumption. An increase in HR, in parallel of an increase in ventilation rate, will increase the rate of oxygenated blood flow to the tissues, so that HR may be used as an indirect measure of oxygen consumption (Green 2011). ***The question is then which calibration equation should be used?*** The answer is not trivial. The HR-$\dot{V}O_2$ relationship is species-specific and is influenced by an array of factors such as gender (Green et al. 2001), type and level of physical activity (Butler et al. 2000, Froget et al. 2002), or physiological and nutritional state (Froget et al. 2001, Green et al. 2001). Comprehensive reviews on the subject (Butler et al. 2004, Green 2011) have highlighted the importance of calibrating HR against $\dot{V}O_2$ in groups of animals which should be matched (ideally be the same as) as closely as possible to those used for the questions under investigation (e.g. in terms of physical activity, nutritional status, etc.). In abiding by those precautions, another issue that rises is whether HR-$\dot{V}O_2$ for predicting energy expenditure may be influenced by stress experienced by the animal during the calibration process (e.g. by handling and confiment during respirometry) (McPhee et al. 2003, Groscolas et al. 2010, Green 2011), an area which still warrants further research (Green 2011). Indeed, during handling and confiment, stress may cause HR to increase at a propotionally greater rate than oxgen consumption so that EE may be underestimated (see **Figure 50**) (Brosh 2007, Groscolas 2010). Note however, that some studies have suggested an inverse relationship, as potential effects of social stress on HR may lead to overestimates in EE when calibration equations are derived from isolated individuals but studies of energetics then performed in group-living animals (Flynn and Gessaman 1979). It thus appears important to calibrate EE against HR in conditions (including physiological and social conditions) close to those experienced by the animals under study and, if possible, devoid of the effect of stress.

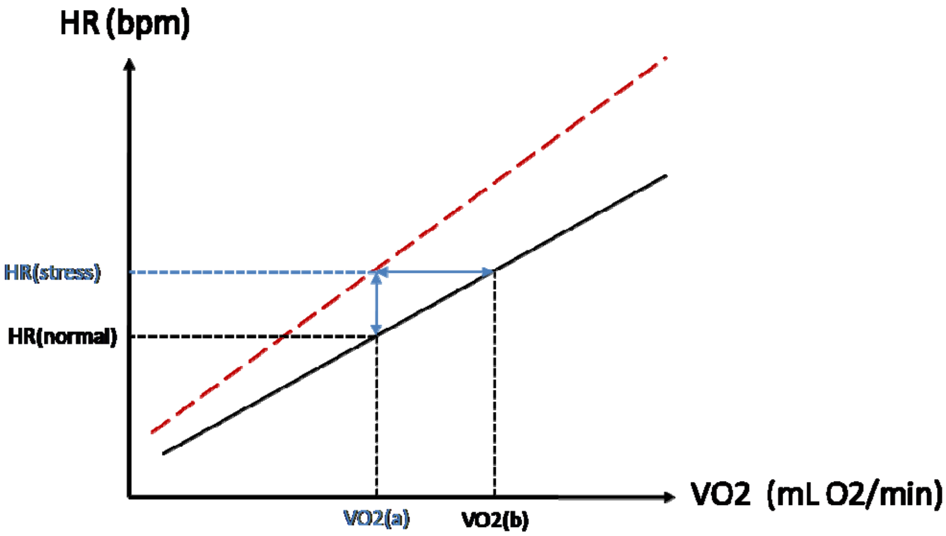

Figure 50. The potential effect of stress on the HR- O_2 relationship.

When animals are held captive and restraint in a respirometer, stress may cause HR to increase at a proportionally greater rate than oxygen consumption (red dotted line) compared to non-stressful conditions (black full line). As a consequence, using such a relationship may lead to predict lower rates of oxygen consumption (O_{2a}) than would normally be (O_{2b}) if the effect of stress was removed. If calibrated under stressful conditions,the HR- O_2 relationship may thus lead scientists to underestimate energy expenditure.

In long-term fasting animals, and especially seabirds, a potential means to estimate energy expenditure with minimal disturbance is by measuring the energy equivalent of body mass loss (BML) (Groscolas 1988, Portugal and Guillemette 2011). Thus, by monitoring concurrently HR and BML over a given period, one may derive an equation relating HR to EE calibrated in naturally, non-stressful conditions. The BML method provides an easy and non-invasive means to monitor metabolic rate, provided that the energy density of the fuels involved in BML and their respective role is known (Groscolas 1988, Portugal and Guillemette 2011). The use of this method also requires the substrate loss ratio to be constant throughout the fasting process, so that the energy contribution of substrates may be appropriately calculated. During long-term fasting however, previous studies have found compostion of BML, and thus its energy equivalent, to be constant in phase II fasting animals (Groscolas 1988, Groscolas et al. 1991), including in animals with different body compositions (Cherel and Groscolas 1999) and different levels of metabolic rate (Cherel et al. 1995). Thus measuring BML and its energy equivalent during fasting phase II should provide a good and non-invasive estimate of EE in fasting animals (Groscolas 1988, Portugal and Guillemette 2010). Therefore, in the studies presented in this thesis, we estimated EE for specific behaviours using an equation that had previously calibrated HR against EE derived from body mass loss in freely-breeding undisturbed king penguins (Groscolas et al. 2010). In doing so, our hope was to yield accurate, stress-free, estimates of EE by using, as our reference for calibration, animals which had the same level of activity and which were in the same breeding and social conditions than those under study (see Groscolas et al. 2010). Nonetheless, I must critically point to some of the drawbacks to the equation we used:

a. As stressed by Green (e.g. Green et al. 2001, Green 2011), when using calibration equations to produce estimates for animals in the field, one should make sure to account for: (*i*) the variance associated with the random distribution of intercepts in the calibration equation (i.e. the error associated with the variation between calibration animals), (*ii*) the variance associated with the scatter of points around the calibration regression line, and (*iii*) the variance for the new individuals selected in the field, which will affect the standard errors of the estimate. Increasing the number of animals used both in the calibration process, and during the study under consideration is thus essential in order to minimize the error of the estimate (Green 2001). In using an equation which derives EE from body mass loss (such as Groscolas et al. 2010), one has to control for yet another source of variability, i.e. the error associated with the regression between body mass and total body energy, used to calculate the energy equivalent of body mass loss. Whereas this is possible (see study 1; Viblanc et al. 2011), it adds to the uncertainty around the produced estimates, thus inflating the errors. Future research is thus needed to strike a balance between producing estimating equations which are virtually free from potential effects of stress, and yet methods sufficiently straightforward to avoid error propagation and inflation in the errors of the final estimates of energy expenditure in the field. Until an appropriate balance is met, recommendation (in as much as possible) is to use a ‘gold-standard’ approach, whereby animals used in the calibration protocole are the same as those used in research question adressed (Green 2011). This should allow to reduce errors around the estimates produced.

b. Another important issue when using EE-HR calibration equations is the time-scale over which the calibrations are made and the time-scale over which experiments are performed (Green 2011). Whereas calibrating equations over long-term periods (e.g. over a day or several days) increases their accuracy (any temporal mismatch between HR and EE being smoothed out as the averaging period increases, Green 2011), it does so at the cost of temporal resolution, so that those equations (such as the one established in Groscolas et al. 2010) may not be appropriate for estimating the EE associated with behaviours occuring over finer time-scales (Green 2011). Conversely, whereas decreasing the temporal resolution of calibrations will allow to derive energy estimates for shorter (time-matched) activities, it does so at the cost of accuracy. This trade-off complicates the usage of equations relating HR to EE to predict EE in the field. Indeed, if one's objective is to derive energy estimates for specific behaviours of short duration in the field, then (ideally) one should use an equation which relates HR to EE over the same scale as the behaviours under study, i.e. minutes or seconds in some cases. Although this is of course the most thorough approach, establishing such equations is hardly feasible unless the animal is kept captive in a respirometre for $\dot{V}O_2$ measurements. This leads us back to the issue of stress and its potential effects on animals' energy expenditure when keeping wild animals (such as penguins) captive out of their natural (social) context. One way around may then be to use such equations to yield relative estimates (e.g. in terms of %DEE or %RMR), as we have previously shown that EE *vs*. HR equations may lead to different levels of energy expenditure, but similar costs when relative estimates are compared (see study 1; Viblanc et al. 2011).

As a conclusion, the HR-method for estimating EE certainly appears a powerful and useful tool for the study of energetics in free-living animals. Its strengths are however, also its limits. Whereas it allows to estimations of EE for fine time-scales, it also requires fine-scale calibrations for which the effects of captivity and handling may bias the relations obtained (McPhee et al. 2003, Brosh 2007, Groscolas et al. 2010, Green 2011, Viblanc et al. 2011). Future research is thus needed to investigate the effects of stress on the HR-EE relationship and appreciate its consequences in terms of predicting EE in free-living animals. For instance, studies subjecting animals to various acute stressors while concomittantly measuring oxygen consumption and HR (e.g. Cyr et al. 2008) may provide valuable information on how stress might affect the HR-$\dot{V}O_2$ and allow to derive calibration relationships taking the effect of stress into account.

2.2. Colony density and heart rate: drawbacks of a correlative approach

Our investigations on energy expenditure in free-living male penguins (see **study 3**) revealed an unexpected and puzzling result. Namely: daily resting heart rate (a proxy to RMR) appeared to be related to changes in colony density. In study 3, our results show that whereas captive male penguins exhibit a decrease in rHR over the course of fasting, the same trend is not observed in free-living penguins. Actually in early breeders rHR decreases over most of courtship but subsequently re-increases at the end of coursthip and during most of incubation. In late breeding birds, rHR remains stable throughout coursthip and incubation.

I have previously discussed part of the observed differences between captive birds, early breeders and late breeders in **study 3**. For instance, the fact we did not observe a decrease in dHR and rHR in late breeding birds (as we did both for captive birds and early breeders at the beginning of courtship), was most likely due to the fact that our low sample size of late breeders (being monitored for a sufficiently long period during courtship) prevented the observation of such a trend. Whereas this hypothesis remains to be verified, our current data on a few late breeders monitored for a longer courting period than that presented study 3, suggests it to be realistic. Thus, ***the really intriguing result is the rHR increase we observed in early but not in late breeders***. This led us to consider environmental parameters which might affect HR in breeding penguins and may vary substantially during the course of the period over which HR was monitored.

Social interactions (and hence social dynamics) are known to be potent stressors amongst vertebrates, having a strong influence on HR (e.g. Wascher et al. 2008a). As important changes in king penguin colonies occur over the breeding season, so that early and late birds are breeding in different social environments (both in terms of density and social composition), we found it of interest to investigate ***the link between HR and colony density***, as the latter provided us with a quantative index of social change. In addition, as numerous studies have documented the effects of population density and crowding on the physiological features (and recently on energy expenditure) of group living-animals (e.g. Cao and Dornhaus 2008), the hypothesis that changes in the social environment may influence energy expenditure in colonially breeding seabirds seemed reasonable. Further still, the fact that rHR in early breeders stopped increasing after having reached similar levels to that of late breeders led us to suggest that group-living may impose an energy cost, the lower limit of which may be fixed by a threshold colony density in the king penguin (**see study 3**).

Nonetheless, it must be stressed that our findings are based ***on a correlative approach***. As such, we may not rule out other potential hypotheses (and not necessarely mutually exclusive) to explain the increase in rHR observed in early breeders (e.g. the hypothesis of different body conditions in early and late breeders previously discussed). For instance, whereas mean climatic conditions were similar in early and late breeding birds, wind speed was found to increase slightly over the monitoring period of early breeders and has been shown to affect EE in Adélie penguins (Culik et al. 1989). Thus, we may not rule out a potential effect of wind speed on the observed increase in HR of early breeders. However, current data shows that the increase in HR we observed during the 2008-2009 breeding season has also been noted in early breeders during previous breeding seasons (Viera, Côté and Groscolas, unpublished data), though the increase was not quite significant (p = 0.06), probably due to the fact that birds were only monitored during the incubation period. This suggests that the observed kinetics of HR change in early breeders may be relatively constant from one breeding season to the next, and that such changes should be associated to a relatively predictable change in environmental parameters such as colony filling-up dynamics for instance. Alternately, other (relatively) predictable changes in environmental parameters, such as an increase in predation pressure throughout the breeding season (Descamps et al. 2005) may lead to similar correlative findings. Future studies conducted over several consecutive years of varying colony dynamics and weather conditions (e.g. how rapidly the colony fills-up, when its peak density is reached, how climatic conditions and predation pressure fluctuate over time, etc.) will undoubtebly shed light on the potential relationship between colony density (crowding) and rHR.

In addition, our experimental manipulations of local colony density did not lead to conclusive results regarding changes in the HR of breeding birds (**box 1**). We did not find rHR to be lowered by a decrease in local density (i.e. birds penned at low density did not exhibit lower rHR than in control colonial situations). Thus we were not able to provide evidence for an effect of experimental changes in colony density on HR in king penguins. Nonetheless, caution should be advocated when considering those results. Indeed, on second-guess, I believe that the protocol we used was not well adapted to the question asked – i.e. the absence of an effect does not imply that there ***is actually no effect*** of colony density on rHR. Whereas our aim was to manipulate colony density in the natural environment of breeding birds (i.e. by using portable exclosures which could be placed within the very vicinity of the colony), our protocol ended up by creating ***a highly un-natural and potentially highly stressfull situation*** for breeding birds:

a. First, by reducing colony density to 2 birds per 4m², we created a situation where density was indeed low, but highly heterogeneous. Considering natural colony dynamics, whereas loosely aggregated at the beginning of the breeding season and tightly aggregated afterwards, birds appear to maintain equi-distances between breeding territories. This likely provides increased protection against predators (i.e. Hamilton's selfish heard principle, Hamilton 1971), as predators are equally likely to land in between any breeding territory, as opposed to our study where we created patches of colony clearly devoid of breeding birds (whereas areas surrounding the pens were equally crowded). Although we rarely observed arial predators landing in the pens, ***this situation is likely to have increased predation risk and associated stress***. This suggestion is somewhat confirmed by the observation that overall, although the two penned birds had greater space available to them, they tended to regroup and maintain a distance between themselves not dissimilar to that of their non-penned neighbours. Unfortunately, we did not measure distances between penned birds, so that the latter observation is qualitative rather than quantitative in nature.

b. Second, we excluded neighbouring birds from the pens by gently walking them to the outskirts. By doing so, we actually increased local density immediately around the pens (even though the penned birds themselves were incubating at low density), so that the immediate neighbours surrounding monitored birds were packed at higher density and exhibited higher aggression rates than normal (personal observation). We thus might have actually increased the potential for social stress caused by aggressive neighours, which in turn might have blurred the effect a reduction in local density on HR.

Thus overall, whereas we do find an effect of colony density on HR, our results warrant further research. The finding that stress hormones seem to be influenced by local density (**box 2**) points in the direction that breeding at high social densities might indeed influence experienced stress in colonial seabirds. In addition, the finding that king penguins are highly sensitive to their social environment (**study 4**) and appear to react to aggressive conspecifics even when they are not directly concerned by the interaction, suggests that some form of chronic stress may be associated with colonial breeding. However, whether this may translate into substantial energy costs remains to be investigated. Suggestions on futher research are given below (§3).

2.3. Reproductive value and stress: Are penguins doing fallacious economics?

Because parental fitness is subjected to a trade-off between current and future reproductions (Williams 1966), any investment into the current reproductive attempt will tend to increase the value of the offspring’s future, while simultaneously decreasing the value of the parent’s own future (Williams 1966, Sargent and Gross 1985). In performing optimal decisions in terms of parental investment, breeding adults should thus be selected as to weigh the value of their ***current reproduction*** against the value of their expected ***future breeding*** opportunities (Boucher 1976, Dawkins and Carlisle 1976), rather than against ***past investments*** (Trivers 1972). Failure to do so would lead animals into committing what is known as the concorde fallacy (Dawkins 1976, Dawkins and Carlisle 1976) – in analogy with the fallacious economic reasoning that lead to disastrous financial losses on the supersonic aircraft Concorde project, on the basis that because a great deal had already been spent, public finances should keep investing into an unstable project, when all that should have been taken into account was the expected future value of additional spendings. The past is past. Decisions should thus be based by reasoning on how to better allocate current and future ressources (be that in terms of finances or in terms of fitness alike), and not on how past ressources have been spent, which would lead to sub-optimal behaviours (Dawkins 1976, Dawkins and Carlisle 1976).

Our studies on accute stress responses of breeding king penguin have led to the suggestion that stress responses may be attenuated in breeding penguins when the value of the current reproduction is highest, i.e. in birds brooding a chick as opposed to birds incubating an egg. In considering those results, one might come to the conclusion that penguins are comitting the so-called concorde fallacy and that stress responses are attenuated late in the breeding season ***because*** birds have already invested a great amount of time and energy into their offspring at that time. This is an interesting question, as several studies have reported what appears to be sub-optimal behaviours in parental investment considering different species (Weatherhead 1979, Dawkins and Brockmann 1980, Weatherhead 1982). For instance, in savannah sparrows (*Passerculus sandwichensis*), past investment (i.e. time since clutch initiation) was found to be a better indicator of nest defence than remaining time left in the season to lay a second clutch if the first one failed (Weatherhead 1979). In the penguins, several contentions suggest that the observed responses are economically sound in terms of parental investment. Indeed, re-emphasizing William's principle (Williams 1966), first Sargent and Gross (1985), then Curio (1987) noted that, because reproduction has a cost, both past investments and expected benefits are necessarily confounded. Heavy investments in the past will balance out with less expected future reproduction, so that seemingly sub-optimal Concordian behaviours may actually be adaptive (Sargent and Gross 1985, Curio 1987). For instance, consider the following:

a. Parents are known to increase parental defence with offspring age, because the reproductive value of offspring increases concomitantly (Andersson et al. 1980, Redondo and Carranza 1989, Osiejuk and Kuczynski 2007). If parents are able to gauge offspring age by the length of the past investment into reproduction, one would expect to find the pattern that we have. By attenuating physiological responses to stress, and preventing entry into an emergency life-history stage, parents' fitness may be maximized by not deserting the nest when the value of the offspring is highest, and offspring are older. This parallels the 'brood-value hypothesis' (Lendvai et al. 2007, Lendvai and Chastel 2008, Bókony et al. 2009, Goutte et al. 2011) which suggests that stress responses should be attenuated when reproductive value of current breeding is high relative to future prospects, and provides a mechanistic explanation for parental decisions in relation to nest-attendance.

b. Whereas replacement laying within the same breeding season has very rarely been observed in king penguin (Weimerskirch et al. 1992), it may nonetheless occur, and I have personnaly witnessed king penguin loosing their egg early in the season to successfully court and re-incubate in the same breeding season at least on a couple of occasions. Thus, whereas birds loosing an egg early in the season may attempt a second egg within the season (at least in theory), the short breeding season would prevent a bird brooding a chick to do so, which might also lead to the pattern observed. On the other hand, this pattern should not occur between late incubating and late brooding birds – as late birds have virtually no chance to lay a second egg within the same breeding season – and remains to be tested.

2.4. Human disturbance and stress: Are humans really predators ?

Our studies on the acute stress response in breeding penguins and on the trade-off between adult survival and offspring production were based on the assumption that the human-induced disturbances we applied to breeding birds were equivalent to predation situations. However, one may question whether this was indeed the case, and whether ***physiological stress responses would have been similar if birds were actually subjected to real land-based predators (e.g. giant petrels)?***

The question is not as trivial as may seem. In the Crozet Archipelago, on Possession Island, the 'Baie du Marin' colony is located in the vicinity (500 m) of a permanent station (Alfred Faure) and is adjoining a beach which has been regularly used for logistical operations over the last 50 years. Whether or not penguins may have habituated to humans and whether disturbances induced by approaching experimenters may be considered as predator-like encounters is thus worthwhile considering.

In our studies, when birds were subjected to several acute stresses, we insured that no habituation to stress occurred by investigating the effects of stressor application order on heart rate stress responses. Indeed, individuals may habituate or become more sensitive when exposed to repeated stressful events so that in such protocoles, one should insure no such effect occur (Cyr and Romero 2009). Our results are in agreement with similar studies on repeatidly stressed animals (Nephew et al. 2003), and suggest that for chronic habituation to occur, acute stressors should be applied repeatedly over short time-spans and be similar in nature (Kant et al. 1985). In contrast, the acute disturbances we used were different in nature, and applied only once during a specific breeding shift.

Nonetheless, this is not to say that an overall habituation to human presence may not have occurred over the years in the king penguins of the 'Baie du Marin' colony. For instance, it has been shown that the endocrine stress responses of animals exposed to frequent tourist visitation are lower than those inhabiting undisturbed sites (e.g. in marine iguanas; Romero and Wikelski 2002, in penguins; Walker and Boersma 2006), suggesting an effect of habituation to the presence of 'non-noxious' tourists. Actually, current data we collected show that, when subjected to acute stressors, the HR responses of penguins located in zones of high human disturbance were lower than HR responses of penguins located in zones of low human disturbance (Viblanc, Smith, Gineste, Kauffmann and Groscolas, *unpublished data*), suggesting that ***habituation to human presence may well occur in our study colony***. Although this effect was controlled for in our studies, it raises the question of whether acute human disturbances (such as the approaches, captures and sounds) trigger stress responses which are really comparable with predator-induced stress responses?

In a review on the subject, Frid and Dill (2002) argue that, from an evolutionary perspective, non-leathal disturbance stimuli caused by humans should be analogous to predations risk (Frid and Dill 2002). This is because, both disturbance stimuli and predation risk result in behavioural and physiological responses which divert time and energy from fitness-enhancing activities for the animal (Frid and Dill 2002, Beale and Monaghan 2004). In other words, the trade-off which occurs between perceived risk and energy intake indirectly affects reproductive success and survival, so that similar responses should evolve (Frid and Dill 2002, Gill and Sutherland 2000). Because animals are expected to maximize fitness by overestimating rather than underestimating risks, it is reasonable to assume that responses to non-leathal stimuli will follow a similar trend than responses to potentially leathal ones (risk-disturbance hypothesis, Frid and Dill 2002). For instance, an animal facing a trade-off between predation risk and a feeding opportunity will suffer greater fitness consequences if it underestimates the danger associated with predation risk than if it overestimates it, at the cost of a missed meal (Bouskila and Blumstein 1992, Frid and Dill 2002). Thus, whereas habituation to non-lethal stimuli (including sounds) does indeed occur, it is likely to be only partial, as may be the case for wild animals subjected to frequent human visitation (Romero and Wikelski 2002, Walker and Boersma 2006).

Nonetheless, studies investigating stress responses to human disturbances should bear in mind that those may be biased by changes in the magnitude of the responses for animals that are more or less exposed to human presence, and further research might focus on whether and how specific anti-predator responses are shaped by the continuous exposure to human presence. For instance, under the risk-disturbance hypothesis, behavioural fleeing responses should be the same when animals are approached by a potentially leathal predator and non leathal human (Frid and Dill 2002). They should increase as the incoming approacher increases in size, and as the approach increases in speed and directness, as changes in such parameters may convey information on detection and intent to capture (Frid and Dill 2002). However, whereas existing, those may be influenced by a history of close contact with non-lethal humans, and it would for instance be of interest to compare such responses with those elicited in the presence of natural predators.

3. Future directions

3.1. Energetic studies in colonial seabirds: what next ?

3.1.1. An integrative view of energetics: year-round energy expenditure in seabirds

The study of energetics "not only provides us with information about metabolic costs, but also gives us opportunities to explore the physiological, ecological, and evolutionary consequences of energy utilization in free-ranging seabirds" (Shaffer 2011). Over the years, improvements in the techniques used to measure energy expenditure have led to the blossom of quite an extensive litterature on the energetics of seabirds, both at sea and onland. For instance performing a rapid litterature search (at the time of writing) on ISI Web of Science^{SM} and typing in keywords "***seabird*" AND "metabol****" yielded a total of 247 hits, whereas "***seabird*" AND "energetic****" gave 201 hits. Refining the search criterion to "***seabird*" AND "energy expenditure*" OR "seabird*" AND "metabolic rate****" will return a total number of 150 studies (including the 2 presented in this thesis). Citation reports (see **Figure 51**) indicate that recent years have seen an increase in the number of published papers on the energetics of seabirds, and that over the past 20 years, the overall number of citations for those papers have been in almost constant increase (**Figure 51**).

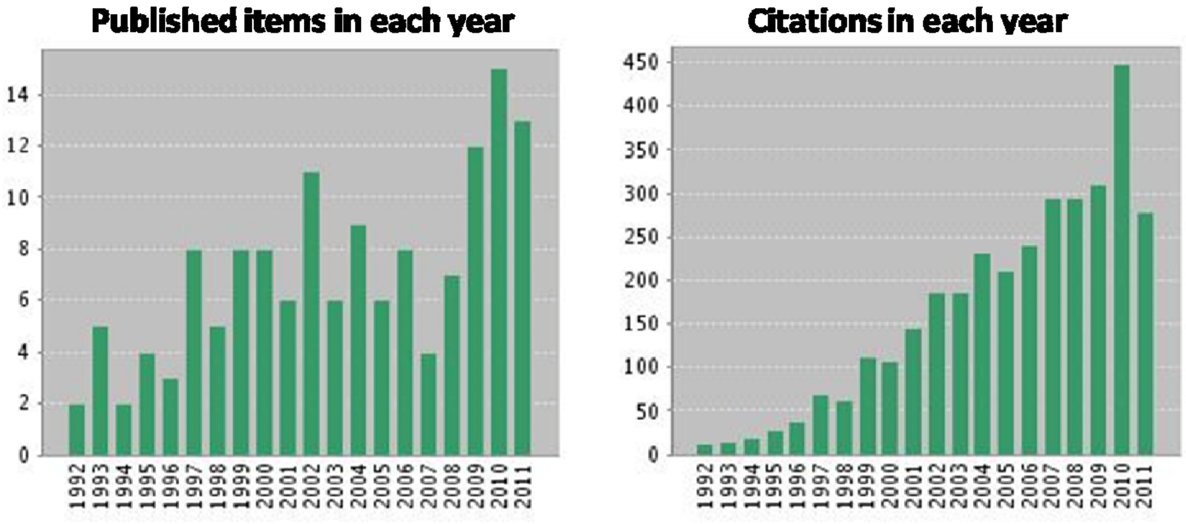

Figure 51. Trends in the number of published studies and citations for the field of seabird energetics.

Those trends are based on a literature search performed in ISI Web of Science^{SM} in August 2011. Specified key words were *"seabird*" AND "energy expenditure*" OR "seabird*" AND "metabolic rate*"*

The scientific reasons leading to this infatuation are of course linked to the life-histories of seabirds and have already been discussed at length. Nonetheless, noteworthy is the fact that whereas studies have considered the energetics of seabirds while swimming (e.g. Culik et al. 1993, Culik et al. 1996, Halsey et al. 2007b), flying (e.g. Ballance 1995, Weimerskirch et al. 2000), foraging (e.g. Chappell et al. 1993, Mullers et al. 2009, Weimerskirch et al. 2003), walking (e.g. Halsey et al. 2007c, Pinshow et al. 1977), and other on-land activities (e.g. Halsey et al. 2007d, Shaffer et al. 2001, Viblanc et al. 2011, Viera et al. 2011), further integrative research is warranted to understand how behaviours at sea and behaviours on-land are energetically interrelated.

For instance, investigating the energetics of seabirds not only over the course of acute breeding events but over the entire annual cyle of individual birds, will give us insight into how daily energy expenditure is shaped during the other phases of the life-cycle (e.g. winter, premoult, moult, etc.) yielding an integrative view of the species energetics. To date, I know of only one study which has attempted such an endeavour. Using the heart rate method, Green and colleagues (2009) monitored the energetics of macaroni penguins, (34 females and 31 males) over the course of an entire year. Amongst other results, these authors show that contrarily to the previously held assumption that energy expenditure is greatest during the chick-rearing phase, and especially late chick-rearing (Drent and Daan 1980, Ricklefs 1983), the DEE of macaroni penguin was equally high during the pre-moult and incubation foraging trips (both sexes) and the brood phase (females only). This result is interesting in that, although DEE was similar over various phases of the annual cycle of the macaroni penguins, the activities undertaken during each specific phase and the underlying physiological processes were not (Green et al. 2009). For instance, notable differences occurred in terms of behavioural activities, in the amount of time spent at sea or onshore, in the amount of time spent foraging, and in the catabolism/anabolism of body reserves related to each specific phase (Green et al. 2009). Thus it may be questionned whether ***animals are constrained by an 'energetic ceiling'*** above which costs would be prohibitive for the animal (Drent and Daan 1980, Green et al. 2009). It is also interesting to note that in their study, Green and colleagues found that the DEE expended during the various phases of the annual cycle of macaroni penguins, was roughly 3.6–4.0 times RMR, and provides strong support for the hypothesis that birds would be working at a maximal level of some 4 times BMR (Drent and Daan 1980). Alternately, the authors suggest that rather than a maximal energetical limit (set by intrinsic physiological factors), birds may rather be working at ***an 'optimal ceiling' maximizing lifetime reproductive success***, though further investigations will be needed to answer those questions. Silimar studies in the king penguin may prove informative and help in explaining how behavioural repertoires are shaped by energy constraints. For instance, it would be interesting to know whether parasitism imposes an extra-energy cost to birds onshore, both through direct effects such as decreasing plumage insulative properties (Booth et al. 1993), and indirect effects through the time and energy devoted to comfort (Viblanc et al. 2011). This could be done by investigating the comfort behaviours and energy expenditure of birds of known parasite loads, and relating such costs to the overall DEE of birds at different phases of the breeding cycle, considering the above mentionned potential optimal limit to DEE. For instance, our data in breeding king penguin suggest that during incubation birds would be working at some 1.14 – 1.31 times RMR (a value similar to the 1.0-1.5 x RMR value reported by Green et al. 2009 in macaroni penguins fasting and incubating ashore), suggesting that any increase in the costs associated with parasitism, may potentially be compensated by an increase in the energy invested into comfort behaviour. As previously mentionned, it would also be of interest to consider how ***changes in the energy expenditure devoted to activities on-land (e.g. comfort) relate to***

swimming and foraging performances at sea, and ultimately to reproductive success. Monitoring energy and diving parameters by use of time-depth recorders and accelerometers in breeding birds during their foraging trips at sea, and relating those to behaviours and energetics on-land opens further prospects for future research to grasp.

3.1.2. Energetics of group-living

In **study 3**, we have shown that the rHR of incubating penguins was strongly and positively correlated with an increase in colony density at the start of the breeding season. This finding, and the fact that the increase in HR ceased after reaching a certain threshold in colony density in early breeders, stabilizing at a level similar to the rHR of late breeders which were in a situation of high colony density, led us to the suggestion that an increase in colony density may be linked to an internal state of increased social stress, affecting the energy expenditure of breeding penguins.

As previously noted, our results were correlative in nature requiring the need for an experimental set-up. Thus, we experimentally manipulated the local density around breeding birds (**box 1**), but contrary to our expectation, found no effect of our experimental treatment on the rHR of those individuals. As previously discussed, a possible explanation may stem from the highly un-natural set-up breeding birds were subjected to, which likely increased predation-risk and associated stress. Nonetheless in order to specifically control for the variable which is believed to influence rHR (in this case density) an experimental protocole does indeed seem the appropriate approach (e.g. Cao and Dornhaus 2008). I therefore suggest 2 alternatives (one of which is not experimental) for investigating the effects of social density on HR:

a. A similar protocole to the one presented in **box 1** may be used, albeit with some modifications (see **Figure 52**). First, when colony density is highest, breeding birds should be equipped with HR-loggers, and monitored in their natural colonial environment for a set period of time (e.g. 4 days). Then an experimental pen could be placed around those individuals, however, instead of excluding neighbouring birds to the outskirts of the pen (which in our study ended up in actually increasing the density and associated conflicts immediately around the pen), those should be removed from the study site altogether and left to incubate in another colony location. Actually if one considers that the identity of immediate social neigbours may be an important factor, then birds which are situated a few rows back from the exclosure should be removed, allowing to relax the density around the exclosure and yet maintain the same composition in neighbouring birds. In addition, the number of monitored individuals inside the pen should be higher in order to balance-off potential predation risk.

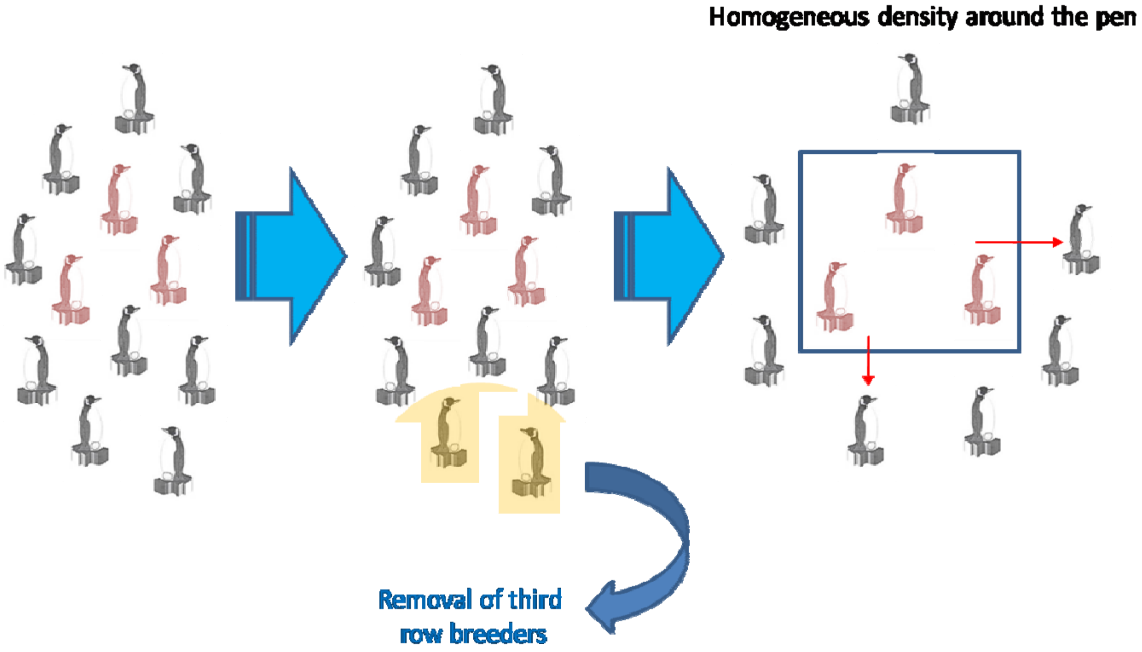

Figure 52. Proposed protocol for experimental manipulations in local density in the social environment of breeding king penguins.

Birds with HR loggers (in red) would be monitored continuously in the colony at high density. After a period of 4 days, a pen would be set up excluding neighbouring birds to reduce the density around monitored individuals. In order to avoid increasing local density around the pen, and to maintain the social composition of the environment, third-row breeders would be removed and transported to another location of the colony. Immediate neighbours of monitored birds would then be free to homogenously settle around the pen – (*penguin drawing: courtesy of M. Gauthier-Clerc*).

Alternately, rather than decreasing local density around monitored birds, one could act on acute increases in density when the colony density is relatively low. Using the same procedure as that described above, birds initially breeding at low-density could be forced to incubate at high density in a pen close to other conspecific, before relaxing the density to that of the natural colonial environment.

b. Another method would be to make use of the naturally occuring variations in colony density (as done in **box 2**) not in terms of time-fluctuations but in terms of spatial variation (see **Figure 53**). Indeed, our studies indicate that in the 'BDM' colony, variations in bird density may differ strongly between zones, even when measured on the same day. For instance, part of the colony breeds on hill sides, and, due to the rocky configuration of the terrain, those individuals are less densely packed than those which breed on the beaches (see **Figure 53**). By monitoring mean daily rHR in birds of similar breeding status and fasting duration located in areas of naturally varying density (such as hill side *vs.* beaches), one may thus make use of the natural colonial setting to investigate the potential effects of density on the metabolism of colonial breeders. Note that such a situation was used in our study presented in **box 2**, where natural variations in colony density were linked to changes in the basal CORT levels of incubating birds.

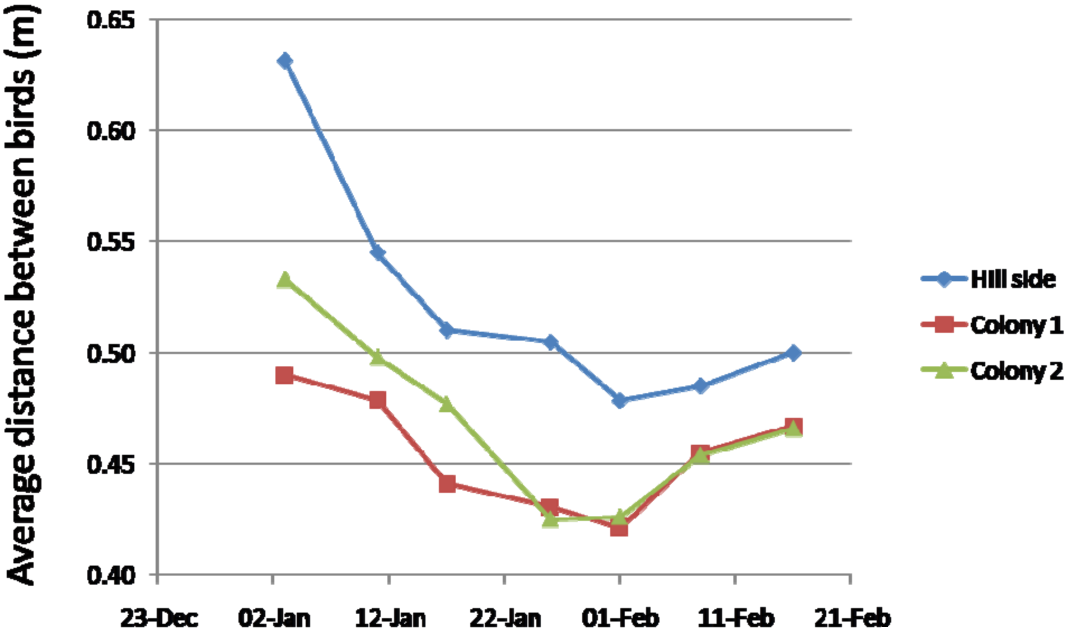

Figure 53. Natural variations in colony density (i.e. distance between birds) in breeding king penguin.

Values are obtained from the averages of 100 estimated distances between birds located in different areas (here named colony 1 and colony 2; 100 estimates per area) of the colony in 2009-2010.

3.1.3. Body composition and isotopic methods

That birds may differ in their body composition, i.e. their protein to lipid ratio, depending on the timing of their arrival for breeding (**study 3**) at the colony is certainly a puzzling suggestion which merits further investigations. If late breeding birds were indeed to arrive at the colony with a higher protein/lipid ratio, this might explain part of the differences observed in our studies. In addition, such findings would be informative as to how ***animals may differentially store body reserves in anticipation of the fast to come***. In king penguins, we have indeed observed phases of the breeding cycle to be accelerated in late breeders, consistently with results formerly reported by Gauthier-Clerc et al (2002b). Thus the fact that shorter breeding fasts may lead birds to invest differentially into energy stores, by acting for instance on the ratio of preys consumed (e.g. protein-rich squid or oily myctophids, Cherel et al. 1996, Raclot et al. 1998, Phleger et al. 2007) is a possiblity which should be considered.

Although the most accurate way to analyze body composition is a ***proximate analysis of the composition of carcasses*** of early and late breeders (Reynolds and Kunz 2001), it is certainly the least pleasant way to study body composition for ecologists, as its outcome is fatal for the animal. Nonetheless, by carcass analysis, one can precisely quantify amounts of muscle and fat stores, and thus be able to appreciate whether the protein to lipid ratio varies in a constant fashion in early and late breeders. As an alternative of having to sacrifice the animal, this could also be done by collecting naturally deceased individuals early and late in the breeding season. However, the uncertainty associated with unknown breeding status, low sampling size and fasting state, will most certainly discourage such attempts.

An altenative, non-invasive method to determine body compostion in wild animals is the ***isotope dilution method*** (Speakman et al. 2001). This method relies on the use of stable isotopes to estimate the total body water (TBW) of the animal, based on the understanding that because fat contains substantially less water than lean tissue, estimating the TBW of an animal and knowing the animal's mass will provide an estimate of the animal's fat stores (Speakman et al. 2001). Thus, by injecting a pool of labelled water (e.g. with a heavy isotope of hydrogen $^3H_20$ or $^2H_20$) to an animal and (given time for this pool of water to equilibrate with the TBW pool of the animal) by measuring isotopic enrichment of a sample of body water at a latter date, one is able to determine the ratio of fat to fat-free mass (proteins essentially), assuming that TBW is in direct proportion to the water content of fat-free mass (Groscolas et al. 1991, Speakman et al. 2001, Eichhorn and Visser 2008). However, this method nonetheless requires prior calibration as one has to determine the fractional water content of fat-free mass, which is classically done through carcass analyses.

Another interesting manner to investigate differences in body composition between early and late breeders, would be to question not directly whether their body composition differs, but rather whether the composition of their diets differ. Presumably, changes in the ratio of fat to lean prey should have direct effects on the birds' body stores. Because they enable to grasp a broad picture of species foraging ecology and, for some species (such as for marine animals), they enable to determine feeding niches that are hard to determine otherwise (i.e. the conventional methods being stomach content or faeces analysis), the use of stable isotopes has gained considerable popularity in the field of foraging ecology. They have provided many insights into the foraging habits of wild animals, allowing to determine both the trophic level on which species feed and the feeding grounds to which they travel (Hobson and Clark 1992, Hobson et al. 1994, Hobson 1999, Cherel et al. 2005, Cherel and Hobson 2007, Cherel et al. 2007). The use of stable isotopes in foraging ecology is based on the principle ***that animals are what they consume***: the isotopic composition of an animal tissues should reflect that of the diet in a predictable manner (Hobson et al. 1994, Cherel et al. 2005). Thus, once corrected for isotopic enrichment, the isotopic signature of an animals' tissue should closely match that of its preferential prey (Hobson et al. 1994, Cherel et al. 2005). In addition, because tissue turnover rates are different, stable isotopes reflect average dietary records over days to years depending on the tissue considered, which allows the investigation into nutrional variation at different temporal or spatial scales (Hobson 1999). In marine food-webs, use of the $^{15}N : ^{14}N$ ratio (expressed as $\delta^{15}N$) provides information on the trophic level at which species feed, as low values of $\delta^{15}N$ reflect a diet of bottom-level preys in the food-web. The $^{13}C : ^{12}C$ ratio ($\delta^{13}C$) on the other hand, is reflective of spatial distribution of prey ressources, as low values of $\delta^{13}C$ are found in pelagic/offshore areas, whereas high values are found in coastal/benthic areas (Beaulieu et al. 2010). In simple cases where the animals feeds preferentially on two main prey items, the isotopic signature of its tissue should thus allow to determine the relative contribution of each prey item to its diet. A nice illustration of the method is given by the study of Beaulieu and colleagues (2010), who investigated the feeding niche of Adelie penguins (*Pygoscelis adeliae*) during years of varying sea-ice retreat. Using isotopic markers they were able to show that under changing environmental conditions (early retreat of Antarctic sea-ice) birds increased the proportion of coastal fish, rather than more pelagic krill, in their diet (see **Figure 54**).

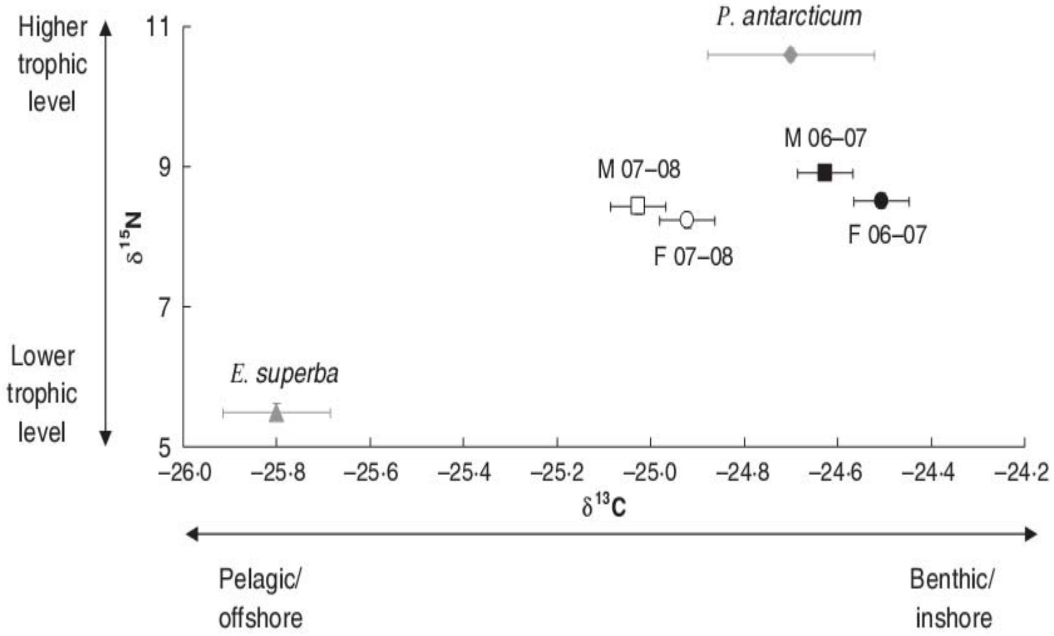

Figure 54. Feeding niches as can be inferred by blood isotopic values for Adelie penguin (*Pygoscelis adeliae*) foraging in a year of early (filled symbols) or normal (open symbols) sea-ice retreat.

Higher trophic levels are indicated by increasing values of $\delta^{15}N$ and more coastal areas indicated by higher values of $\delta^{13}C$. Note that in a year of early sea-ice retreat values for $\delta^{15}N$ and $\delta^{13}C$ were higher, indicating a increase of coastal fish (*Pleuragramma antarcticum*) in the diet – *(from Beaulieu et al. 2010)*.

In the case of king penguins, Gauthier-Clerc and colleagues (2002b) reported that late breeders had shorter post-moult foraging trips at the detriment of their body condition, suggesting that feeding strategies might indeed differ between early and late breeders. It would thus be informative to know whether time or other ecological constraints drive early and late breeding birds to forage in different areas, or to alter the ratio of preys upon which they forage. This could be done by examining isotopic signatures of penguin tissues of relatively rapid turnover rates (i.e. to integrate foraging events during the post-moult, pre-breeding sea trip) and comparing them to the isotopic signatures of their main prey item (e.g. Beaulieu et al. 2010).

Recent work has also suggested that fatty acid signature may be a robust method for evaluating trophic relationships, and thus diet composition in open ocean ecosystems (Phleger et al. 2007). Similarly to the use of stable isotopes, the idea underlying the use of fatty acid signature is that, once the lipid profiles of a particular prey group can be identified, those profiles can be used to back-trace the diet of the predator if a sample of tissue (e.g. muscle) is collected. Using this method to analyse the trophic relationship between various myctophid and cephalopod prey species and sword fish predators, Phleger and colleagues (2007) for instance showed that fatty acid profiles enabled a clear distinction between prey species (i.e. myctophid fish profiles were clearly distinguishable from squid profiles, see **Figure 55**).

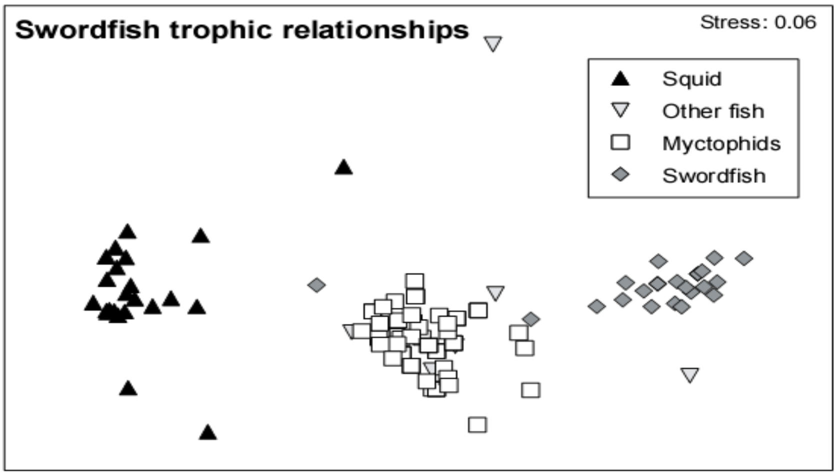

Figure 55. Signature fatty acid profiles for swordfish and potential prey species.

Data is presented as a scatter plot of multidimensional scaling for fatty acids expressed as percent of total fatty acids. Axis scales are in arbitrary units and were therefore omitted. For details, refer to Phleger et al. 2007 – (*reproduced from Phleger et al. 2007*).

In addition, the fatty acid signature of swordfish predators related more closely to that of myctophids and other fish species than that of cephalopods, suggesting a greater contribution of the former in the diet of the swordfish. In the case of early and late breeding king penguins, the comparision of fatty acid signatures from muscle tissue with that from their prey species (from collected samples or litterature) may shed further light on potential differences in their diet.

3.2. Stress and the 'brood-value hypothesis'

Our findings on HR and CORT responses to accute stress situations in breeding king penguin are also open to further research. Whereas in our studies we specifically suggest that stress responses were modulated by the relative reproductive value of the offspring, further experimental studies may allow to confirm this hypothesis. For instance, Goutte and colleagues (2011) recently performed an interesting experiment to confront this hypothesis. In long-lived snow petrels (*Pagodroma nivea*), these authors manipulated clutch value by cross-fostering eggs so that some adults would receive an egg layed 5-days previously to their own, and some adults would receive an egg layed 5-days after their own. A control group was constituted of individuals with unchanged hatching date. The rationale behind the experiment was that, if parents valued the brood relative to its expected hatching date, then they would be less comitted to delayed broods and mount stronger stress responses when subjected to acute stressors. Interestingly, the results of this study supported the authors' predictions, as they indeed found delayed parents to mount greater stress responses than controls and advanced parents (see **Figure 56A**). In addition, those authors found that CORT release during acute stress was influenced by parents' age (see **Figure 56B**), which is consistent with the hypothesis that residual reproductive value decreases with age (Williams 1966), and may be interpreted as a mechanism underlying the improvement of reproductive performance in older individuals (Angelier et al. 2007, Goutte et al. 2011).

It would thus be interesting to complement our study in king penguin by considering similar experiments to those described above. For now, our results on early and late breeding penguins do not seem to comfort the former hypothesis, as we found no difference in stress responses between those two groups, even though reproductive success is much lower for late-breeding birds (Weimerskirch et al. 1992). If parental decisions in the king penguin are shaped by the aforementioned trade-off between current *versus* expected future reproductive value, king penguin should exhibit significantly attenuated responses if the expected hatching date is delayed.

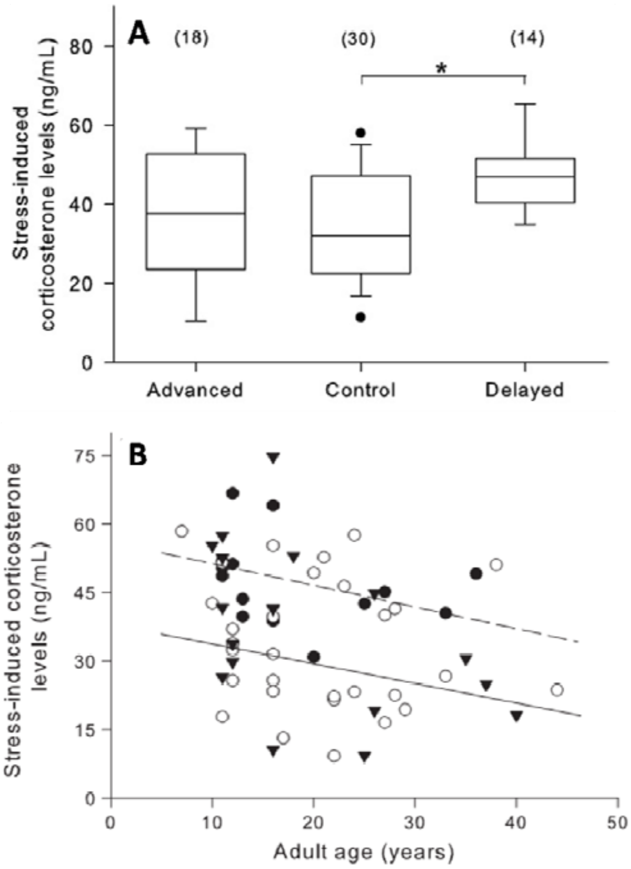

Figure 56. The CORT responses of incubating snow petrels (*Pagodroma nivea*) to situations of acute stress.

(A) CORT responses are given in birds for which hatching date was experimentally delayed, advanced or left unchanged. **(B)** The influence of the age of the parent on CORT responses is considered for delayed (●), advanced (▼), and control (○) birds. The decrease in CORT responses is represented as a solid line for controls and advanced birds, and as a dashed line for delayed birds – (*reproduced from Goutte et al. 2011*).

Cross-fostering experiments, such as those described above, would prove especially interesting in the king penguins, as it is well known from field-biologists that the incubation drive is exceptionally strong in those birds. For instance, it is common to witness king penguins that have failed their incubation to remain in the colony in the incubating position, either with their brood pouch empty, or incubating small rocks, abandoned eggs, and even mislaid cardiofrequencemeters! (*personal observation*). Whether stress responses may thus be magnified in birds for which the egg is past its hatching-date would be interesting to consider. In addition, one may also test the hypothesis that because residual reproductive value decreases with age, one also should find attenuated stress responses in older birds.

3.3. Stress and energy expenditure: a role for animal personnalities?

It is becoming widely apparent that within species, individuals exhibit some intra-individual consistency in terms of behaviour and physiology (Wilson et al. 1994). All other things being equal, some individuals are consistently more aggressive, more fearful, show greater exploratory behaviour, or are more reactive than other (Huntingford 1982, Wilson et al. 1994, Boissy 1995, Koolhaas et al. 1997, Dingemanse et al. 2002, Sih et al. 2004). Those intra-individual consistencies, termed personnalities in humans, might have been selected if such consitencies result in greater fitness pay-offs than the alternate strategy, that is to say, no consistency at all (Dall et al. 2004).

For one having manipulated king penguins in the field, it is clear as day that whereas some birds are extremely reactive and behaviourally agitated when manipulated, others are prostrate and behaviourally calm. Our results on HR responses in **study 5**, comfort those field-observations, as we have reported a certain level of individual consitency in HR stress responses when the same birds were subjected to different stressors. Some birds tended to display higher HR responses with greater consistency, whereas some birds tended to display lower HR responses with greater consistency. Several studies have reported how behavioural consistencies in personnalities are often contingent with neuroendocrine and physiological correlates (Kralj-Fišer et al. 2007, Kralj-Fišer et al. 2010). For instance, in recent studies on greylag geese, Kralj-Fišer and colleagues (2010) reported that individuals exhibiting consistency in behaviours (i.e. agressiveness) displayed consistency in physiological correlates. More aggressive geese had greater HR during aggressive interactions and CORT reactivity during restraint stress trials. It is thus likely that the physiological HR stress responses we observed were also correlated with behavioural syndroms, though we did not specifically investigate this question in our study. Future research is thus needed to understand how personnalities relate to physiological correlates and how those may be adaptive in breeding king penguin.

The question one might ask, for instance, is: ***what are the fitness pay-offs associated with being a "highly stressed" penguin or being the "zen-type" bird?*** In the context of colonial breeding and limited energy availability, one might investigate whether consistent individual differences in stress responses relate to differences in energy costs, or differences in breeding success or survival. In addition, as some studies have suggested personnalities to present a heritable component (Dingemanse et al. 2002, Van Oers et al. 2005), it would be interesting to consider whether “highly stressed” or “zen-type” phenotypes may be inheritable, and to what extent stress responses are selected and shaped by ontogeny and/or shaped by experiences of the social environment early in life.

3.4. Social stress: Is there more to physiology than meets the heart?

One of the major findings of this research is the effects the social environment may have on the physiology of colonial breeders. Our studies based on HR not only show that penguins are highly sensitive to their neighbouring conspecifics and to the very nature of what is happening around them (**study 4**), they also suggest that those colonial birds might experience some form of social stress associated with breeding at high densities (**study 3**). These first correlative findings, which have led us to consider the “social stress hypothesis”, seem to be further confirmed by the fact that birds holding breeding territories at high local density appear to exhibit higher baseline CORT concentrations than their conspecifics breeding at lower densities (**box 3**).

From a socio-biological perspective, group living is classically viewed as a balance between advantages earned and constraints suffered from aggregating with conspecifics. Its evolution, including in men, has typically been considered under the lights of predation pressure, resource competition, kin selection, and cooperation, to name but a few (Hamilton 1964a, b, Alexander 1974, Wilson 1975, Caraco and Pulliam 1984, Janson 1998, Armitage and Schwartz 2000, Oli and Armitage 2003, Silk 2007a, b, Viblanc et al. 2010, Dobson et al. 2011b). However, living in groups foremost poses the issue of experiencing stressful situations when interacting and/or competing with social congeners (e.g. Oliveira et al. 2001, Bartolomucci 2007, Wascher et al. 2008b, Wascher et al. 2009). Whether psychological stress caused by aggregating in social groups may ***set a limit to the evolution of group living*** thus presents an alternative yet complementary framework for sociobiologists to build upon.

Indeed, it has recently dawned on evolutionary biologists just how profound the effects of the social environment may be on the physiology of group-living animals. Far from modulating behaviour solely, social contexts may strongly influence individuals’ physiology, life stress and health (e.g. Bartolomucci 2007). As is the case for the results reported in this thesis, several studies in birds and mammals (including humans) have reported ***a link between social interactions and some form of social stress*** (Creel et al. 1996, Kotrschal et al. 1998, Creel 2001, Oliveira et al. 2001, Bartolomucci 2007, Wascher et al. 2008a, Wascher et al. 2008b, Wascher et al. 2009). For instance, the link between social status and stress hormones (glucocorticoids, GCs) has been well documented in social species (Creel et al. 1996, Kotrschal et al. 1998, reviewed in Creel 2001), though the strength and direction of the relationship between GC levels and social rank is highly variable between species (Creel 2001). Yet, whereas short-term stress may be adaptive, a growing number of studies in biological research (and human research in particular), have documented the ***detrimental effects of chronic st*****ress**, including risk factors for cardio-vascular disease and depressed immune function (McEwen and Stellar 1993, McEwen 1998, Stefanski 2000, 2001, Segerstrom and Miller 2004, Cyr et al. 2007). As chronically experienced stress may thus lead to pathological states, ultimately affecting survival and reproduction (i.e. fitness) in animals (e.g. Stefanski 2001, Cyr and Romero 2007), the extent to which social interactions and perceived stress might affect various components of individual’s physiology requires further consideration.

Of particular interest is the less investigated (albeit important) facet of social stress brought to light by recent research: how perceived (psychological) stress from ***the social environment may directly influence oxidative stress at the cellular level, accelerating telomere***[18] ***shortening rates*** (Epel et al. 2004), a likely cause of molecular senescence and organism ageing. Indeed, by conducting a study on women exposed to chronic social stress (i.e. women who were biological mothers to chronically ill children), Epel and colleagues (2004) recently showed that those women experienced significantly higher oxidative stress and higher telomere attrition rates (virtually cellular senescence) than women mothering healthy children (Epel et al. 2004). Furthermore, in wild mice it was recently found that telomere-shortening rates were influenced by social stress (reproductive stress and crowding), as exposing females to males (both with and without crowding) resulted in telomere attrition and subjecting males to crowding stress interfered with telomere restoration that normally occurred under non-crowded conditions (Kotrschal et al. 2007).

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[18] **TELOMERES:** Are non-coding double stranded DNA sequences located at the extremity of chromosomes

Those findings are particularly stimulating as they trigger the question as to whether in socially living species ***the nature of the social environment and rate of specific social interactions may have an effect on cellular ageing in adults and ultimately affect survival itself***.

Oxidative stress is indeed believed to be one of the most likely determinants of ageing rates in animals (Finkel and Holbrook 2000) and has been attributed to the accumulation of oxidative damages over time. In parallel, a considerable body of research has focused on cellular senescence (i.e. the "why?" and "how?" of cell death), providing explanations on how genome integrity may be maintained throughout lifespan (Finkel and Holbrook 2000). Over the past 25 years, particular attention has been given to chromosome telomeres and telomerase (an enzyme which actively rebuilds ever shortening telomeres) (Blackburn 2000) and investigated the role of the couple telomere/telomerase in cell and organism senescence and how it contributes to cancer or other diseases (Nobel Price of Blackburn and colleagues in 2009). Interestingly, rate of telomere loss is accelerated by oxidative stress. Reactive oxygen species (ROS) are known to interact with substances both at the cellular and genomic level by causing damage to lipids, proteins and to be a major source of mutations in DNA (Denver et al. 2009). In aerobic organisms, whenever an individual's anti-oxidant defences are overwhelmed by ROS production, it is said to be in a state of oxidative stress, and by interacting with DNA, ROS may cause damage to chromosomal telomeres and be implicated in the process of cellular aging and organism survival (Beckman and Ames 1998, Von Zglinicki 2002, Bize et al. 2009, Salomons et al. 2009).

From an evolutionary perspective, the effects that the social environment may have on individual's perceived stress (and potentially underlying oxidative stress and ageing) are important to consider, as they may be an important cause of phenotypic variation within and amongst group-living species. Effects of the social environment may play a key role in shaping behavioural strategies based on physiological variations during different life history stages: (1) either early in life during infant growth or (2) later in life during adulthood. Although cellular ageing occurs throughout lifespan, most of telomere loss takes place during the growth period, early in life (Hall et al. 2004). Thus it is of interest for future studies to consider ***whether and how the social environment may influence life stress***

during critical developmental stages of early life, and, given variation in environmental and genetic background, whether some individuals may have a better start than others (see **Figure 57**). A bad start in life has indeed been related to long-term changes in adult morphology, physiology, fecundity and lifespan in different species (Lindstrom 1999, Bateson et al. 2004, Weinstock 2005). For instance, human epidemiological studies have noted that human populations exhibiting catch-up growth after a bad start in life also suffered adverse long-term outcomes in terms of cardiovascular diseases, blood pressure or type 2 diabetes (Leon et al. 1996, Eriksson et al. 1999, Forsén et al. 2000).

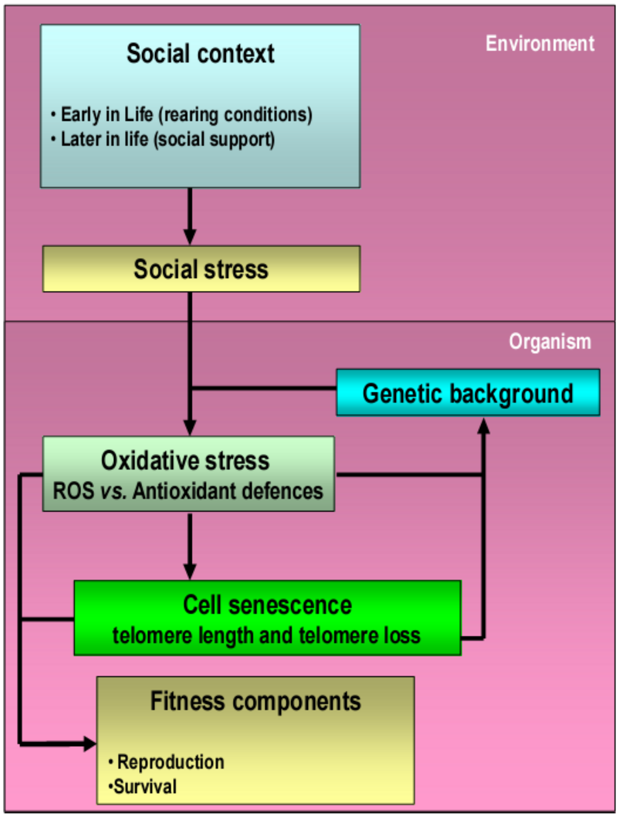


Figure 57. Conceptual framework for the study of social stress and its effects on the organism.

Environmental variation in social contexts may influence individual stress through changes early in life or at any later stage. Rearing conditions or social support, might influence social stress and how stress “get’s under the skin” in group-living individuals, including man. In turn social stress might lead to increased oxidative stress and increased cellular senescence, and both may have impacts on fitness components such as reproduction and survival. Additionally, the ability to cope with oxidative stress and/or telomere length may be inherited and influence the balance between ROS and antioxidant defences differently depending on the individual.

In the case of group-living species, it is thus of interest to question whether social stress ***early in life*** may have long term effects via precocious ageing, and whether social animals may have developed specific adaptations to cope with it. In addition, whether ***during adulthood*** the nature and composition of the social environment may have a profound influence on perceived stress (and drastically alter ageing patterns in vertebrates) remains to be investigated. This could be achieved:

a. By studying the ontogeny (*viz*. development) of stress during early life, and its influence on oxidative stress and senescence. One could for instance investigate how different social environments and individual variation may shape individual responses to stress in growing offspring, and what consequences the rearing environment may have on oxidative stress, telomere attrition rates and offspring survival. In king penguins for instance, the offspring of birds breeding at high or low local density could be studied and rates of aggressiveness of the social environment considered.

b. By studying the influence the social environment of adults might have on oxidative stress and senescence. This could be investigated in species for which variations in structures of adult social groups are known, so that some adults experience more social stress than others (e.g. in species where particular adult individuals benefit preferentially from some form of social support or greater tolerance from other individuals). In king penguins, the effect of social density on oxidative stress and telomere attrition might also be of interest to consider.

Not only would such studies provide us with a better understanding of the mechanisms driving senescence in animals, they should also shed light on some of the unexpected costs of group-living and ultimately on some of the potential limits to its evolution.

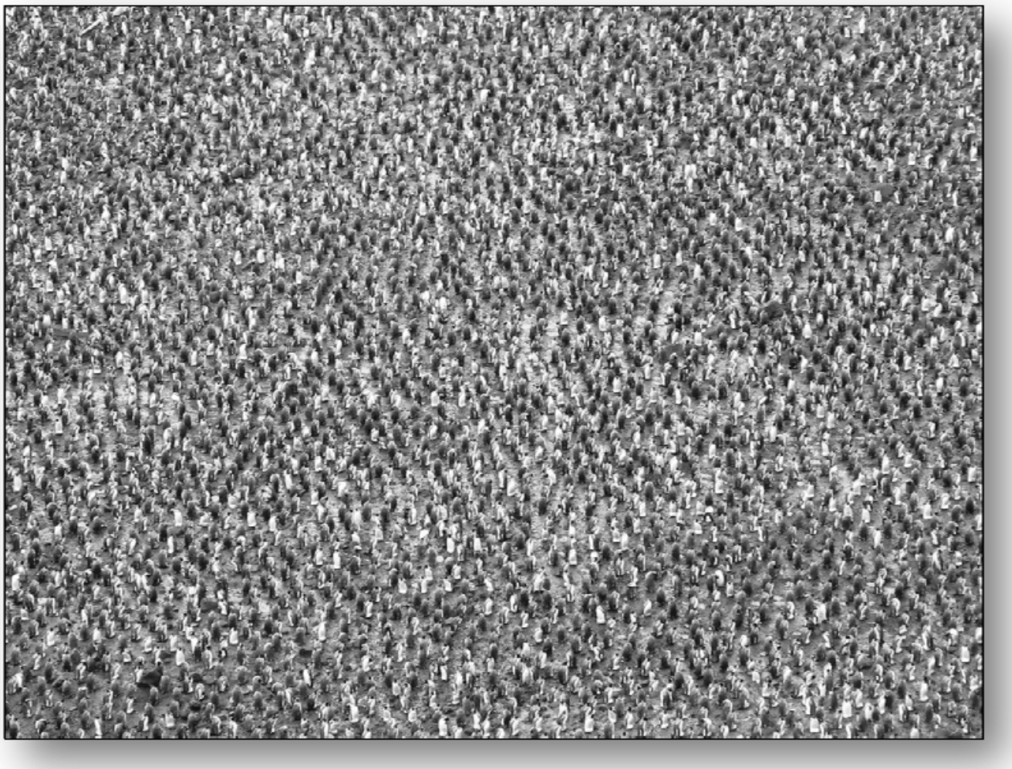
4. General conclusion

As a brief conclusion,

The work presented here has investigated how long-term fasting seabirds, the sub-antarctic king penguins (*Aptenodytes patagonicus*), deal with nutritional constraints while breeding in a potentially stressful colonial environment. Overlapping the fields of animal behaviour, physiology and ecology, our results have suggested that breeding king penguin rely on limited physical activity and resort to the use of low energy-costly activities but do not use adaptive heterothermia to limit their energy expenditure when breeding on limited body reserves. In addition, we have shown that responsiveness to acute stress may mechanistically underly trade-offs between adult maintenance and offspring production, as responses to acute predator-like stressors are attenuated when reproductive value is high. Support for a high sensitivity of breeding birds to their social environment has been provided, and our results emphasize the need for further investigations into the effects of the social environment on individual stress and energy budgets in group-living animals.

To simply finish on a quote –

> *"Darwinian social theory gives us a glimpse of an understanding symmetry and logic in social relationships which, when more fully comprehended by ourselves, should revitalize our political understanding and provide the intellectual support for a science and medicine of psychology. In the process it should also give us a deeper understanding of the many roots of our suffering".*
>
> **Robert L. Trivers** (*Harvard University* – July, 1976).



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Résumé de la Thèse

Lors de la reproduction, l’adulte doit équilibrer ses besoins énergétiques avec ceux de sa progéniture. Réaliser ce compromis devient particulièrement critique lorsque la reproduction est associée à de fortes contraintes énergétiques, comme par exemple de longues périodes de privation alimentaire. Cette situation s'observe chez certaines espèces de mammifères et d’oiseaux, tel le manchot royal (*Aptenodytes patagonicus*). Chez cet oiseau marin des régions subantarctiques, la formation des couples et de l’œuf, l’incubation et l’élevage du petit qui dure près d’un an, sont associés à d’importants épisodes de jeûne à terre pouvant atteindre plus d’un mois. Les autres contraintes que subit cet animal sont liées à la nécessité de défendre un petit territoire à l'intérieur de vastes colonies, ce qui implique un contexte social particulièrement agressif, et de faire face à une forte pression de prédation, contraintes pouvant représenter un stress. **L’objectif de cette thèse était d’étudier les adaptations énergétiques, comportementales et physiologiques permettant au manchot royal de se reproduire dans ce contexte de fortes contraintes énergétiques et sociales.**

De 2008 à 2011, mes travaux ont portés sur une colonie de manchots royaux de l’Ile de la Possession, dans l’Archipel de Crozet. La méthodologie utilisée était principalement basée sur l'utilisation de capteurs-enregistreurs permettant de suivre en continu divers paramètres physiologiques (fréquence cardiaque, température corporelle) et comportementaux (activité physique) de l’animal, ainsi que sur des observations comportementales (par vidéo ou visuelles). Ces mesures et observations ont été réalisées au cours des différentes phases du cycle reproducteur et chez les deux sexes (qui se relaient tant au cours de l'incubation que lors de l'élevage du jeune poussin). Prolongeant des travaux antérieurs ayant considéré les adaptations physiologiques au jeûne prolongé dans un contexte non naturel (animaux captifs), mon travail a consisté à 1) étudier la modulation de la dépense énergétique de manchots royaux reproducteurs dans leur milieu naturel, 2) à caractériser l’effet de l’environnement colonial sur le comportement et la physiologie de ces animaux et 3) à

étudier la modulation de la réponse physiologique à des stress aigus en fonction de leur statut nutritionnel et reproducteur.

Après une introduction générale sur l'écophysiologie et l'énergétique animale (**partie 1**), et une présentation de l'espèce étudiée (**partie 2**) cette thèse s'articule en 3 parties complémentaires regroupant 5 chapitres principaux rédigés sous la forme d'articles scientifiques. A ceux-ci viennent s'ajouter les résultats préliminaires d'études en cours présentés sous forme de trois encarts ainsi qu'une discussion générale.

Partie 3. Contraintes nutritionnelles : énergétique de la reproduction chez un oiseau marin en conditions naturelles

Investissement dans des comportements lors d'un jeûne prolongé

Dans cette partie de la thèse, je m'intéresse d'abord au compromis possible existant entre comportements et limitation énergétique. *Je cherche notamment à savoir si, chez un animal soumis à des jeûnes prolongés, certains comportements sont maintenus à un moindre coût énergétique ?* Pour ce, je considère le coût énergétique des deux comportements majeurs observables chez le manchot royal en incubation et en élevage de son jeune poussin : le comportement de confort (toilettage, étirements, etc…) et le comportement agressif (défense territoriale). En me basant à la fois sur des observations comportementales et sur le suivi en continu de la fréquence cardiaque, et en utilisant une relation établie au préalable entre fréquence cardiaque et dépense énergétique, j'ai déterminé le coût énergétique de ces deux comportements, et leurs parts respectives dans le budget énergétique et le budget temps journalier de l'animal.

Dans l'**article 1**, je montre que le comportement de confort représente une part importante du budget temps comportemental du manchot royal (22%) et contribue pour une part non négligeable à son budget énergétique journalier (9 %). La plus grosse partie du temps en confort est dévolue aux soins du plumage (73%) et aux secouements vigoureux du corps (16%), et la moitié du budget énergétique du confort est dédié aux soins du plumage. Ces résultats suggèrent que, malgré la nécessité de réduire au maximum les dépenses énergétiques pendant les jeûnes de reproduction, la dépense énergétique associée au confort est incompressible et nécessaire au manchot royal, probablement (1) afin de maintenir son intégrité tégumentaire (élimination des ectoparasites) et son isolation thermique, et (2) afin d'éviter l'ankylose musculaire et être ainsi capable d'effectuer de long voyages alimentaires en mer (plusieurs centaines de km) dès qu'il est relevé par son partenaire.

Dans l'**article 2**, dont je suis co-auteur, nous montrons que le comportement agressif, malgré une part importante dans le budget temps de l'animal (13%), ne représente qu'une part minime de sa dépense énergétique journalière (2.7%). Ce résultat peut s'expliquer par le fait que les comportements privilégiés lors de conflits territoriaux sont également les moins coûteux d'un point de vue énergétique (i.e. menaces et non pas coups). Ainsi une adaptation énergétique à la reproduction coloniale consisterait à maintenir une défense territoriale active à moindre coût énergétique.

Ajustements au jeûne prolongé dans le contexte de l'animal libre en colonie

Dans l'**article 3**, j'examine comment la dépense énergétique journalière de manchots royaux mâles est modulée au cours du jeûne de reproduction le plus long du cycle (pariade et première phase d'incubation de l'œuf). Plus particulièrement, par un suivi de la fréquence cardiaque (indice de la dépense énergétique), de la température corporelle (température stomacale), et de l'activité physique (accélérométrie), je me suis demandé si une réduction de la dépense énergétique lors du jeûne de reproduction le plus long pouvait être le reflet d'une diminution de l'activité physique de l'animal et/ou d'une hypothermie adaptative (baisse du point de consigne hypothalamique de la température corporelle). Je montre que chez le manchot royal mâle (un oiseau de grande taille), l'activité physique décroit au long du jeûne (réduisant ainsi sa dépense énergétique), mais que des animaux incubateurs ne semblent pas recourir à une hypothermie transitoire afin de limiter leur dépense énergétique ce qui pourrait permettre de retarder l'entrée en phase III de jeûne (phase critique d'activation de l'utilisation des réserves protéiques conduisant à l'abandon de la reproduction en cours). Par ailleurs, de manière surprenante, je montre que chez les reproducteurs précoces la fréquence cardiaque journalière (et donc la dépense énergétique), qui décroit au début du jeûne (pariade), se remet à croitre alors même que l'activité physique va en décroissant. Ce résultat, qui ne peut être imputé ni a une augmentation d'activité physique, ni à un changement en termes de thermorégulation, pourrait être le reflet d'un stress chronique lié à une pression sociale croissante qui serait elle-même associée à une augmentation de la densité coloniale en début de saison de reproduction. A cette époque, nous mettons en effet en évidence une forte corrélation entre la densité coloniale et la fréquence cardiaque journalière des animaux.

Partie 4. Contraintes sociales : sensibilité à l'environnement social et effets sur la physiologie des individus

Le manchot royal est-il sensible à son environnement social ?

Dans l'**article 4**, je m'intéresse à la sensibilité du manchot royal à son environnement social. Chez les espèces coloniales, les interactions agonistiques liées à la défense du territoire peuvent représenter un stress et avoir de profondes conséquences physiologiques. Il est alors concevable que lors d'un stress social, une modulation de la réponse au stress s'opère en fonction du risque perçu de l'agent stressant et de la motivation des individus (e.g. en fonction de leur statut reproducteur). En utilisant des enregistrements de fréquence cardiaque et vidéo de comportements agressifs d'adultes à différents stades de la reproduction, j'ai considéré la sensibilité des manchots royaux à leur environnement social. J'ai caractérisé un total de 589 interactions agressives : 223 cas où l'individu observé était activement impliqué et 366 cas où il demeurait observateur passif de conflits voisins. Les résultats montrent que la fréquence cardiaque augmente lors d'interactions agressives et que cette augmentation est fonction de la nature de l'interaction, que ce soit chez les acteurs ou les observateurs. Le risque lié à l'interaction (menaces *vs.* coups), sa durée et son intensité (taux d'évènements agressifs) influencent les réponses cardiaques chez les acteurs. Chez les observateurs en revanche, seule la durée des interactions semble être importante dans la modulation des réponses cardiaques. Mes résultats suggèrent également que la motivation individuelle joue un rôle dans la modulation des réponses cardiaques. En effet, les individus initiateurs d'interactions agressives présentent des augmentations de fréquence cardiaque plus importantes que les individus cibles. L'ensemble des résultats permet de conclure que l'appréciation du contexte social ainsi que la motivation des individus, modulent fortement les réponses physiologiques à des contextes de stress social chez des animaux vivant en groupe, et suggère une forte implication d'une composante émotionnelle dans ces réponses.

Se reproduire en colonie est-il coûteux?

En encart, je présente deux études en cours examinant le coût potentiel de la reproduction coloniale en termes d'énergétique et de stress chronique. Dans une première étude (**encart 1**), je teste l'effet d'un changement expérimental de densité des animaux couveurs sur leur dépense énergétique journalière (et de repos), estimée à partir de leur fréquence cardiaque. Pratiquement, à divers stades de la reproduction, des individus mâles et femelles ont été équipés d'enregistreurs permettant un suivi continu de leur fréquence cardiaque. Ce suivi a d'abord été réalisé pendant 3 jours à densité naturelle élevée (environ 2.5 individus au m^2). Puis, les individus équipés ont été maintenus pendant 4 jours, toujours au sein de la colonie, à l'intérieur d'un parc amovible (cadre métallique de 2 x 2 m et 0.5 m de haut) à deux densités différentes: la densité naturelle (lot contrôle) et une densité réduite de 5 fois (lot expérimental). Enfin, les parcs ont été retirés et la fréquence cardiaque des individus a été suivie pendant 3 jours supplémentaires, à densité naturelle. Les premiers résultats suggèrent que la réduction expérimentale de la densité n'a pas affecté significativement la fréquence cardiaque, et donc la dépense énergétique des animaux. Toutefois, plusieurs facteurs confondants sont à même d'avoir parasité cette étude (notamment la durée de jeûne des individus), ce dont nous discutons. Dans une 2ème étude (**encart 2**), la possibilité qu'une reproduction à forte densité engendre un état de stress chronique chez des animaux coloniaux est testée. Pour ce, nous utilisons un marqueur plasmatique du stress chez l'oiseau: la corticostérone (CORT; glucocorticoïde issu de la réponse adréno-corticale au stress). Des prélèvements sanguins ont été réalisés chez des individus couveurs et chez des individus éleveurs d'un jeune poussin, naturellement localisés dans des endroits de la colonie à forte (individus centraux) ou à faible (individus périphériques ou isolés) densité. La corticostéronémie basale (prise de sang d'une durée inférieure à 3 min) a été mesurée par Radioimmunoassay (RIA). Les résultats en cours d'exploitation suggèrent que la corticostéronémie basale est plus élevée (de 30 à 50%) chez les individus centraux (forte densité) que chez les individus isolés, ceci chez les couveurs comme chez les éleveurs. La vie en colonie dense serait donc associée à un léger stress chronique, ce qui peut constituer un coût de reproduction.

Partie 5. Contraintes de type prédation : effets du statut énergétique ou de la valeur reproductive

Modulation de la réponse cardiaque à un stress aigu au cours de la reproduction

Dans l'**article 5**, je m'intéresse à la réponse cardiaque de manchots royaux à un stress aigu de type prédation, et à la modulation de cette réponse au cours de la reproduction. Je cherche notamment à déterminer si des variables telles que le statut énergétique ou la valeur perçue de la reproduction en cours sont susceptibles de moduler la réponse cardiaque à un stress aigu dans un contexte de contraintes énergétiques élevées. En effet, lors d’une perturbation environnementale aigüe (ex. l’approche d’un prédateur), la réponse au stress induit la mobilisation des réserves énergétiques permettant de se soustraire (fuite) ou de faire face (lutte) au stress, au détriment d’autres fonctions non-nécessaires à la survie de l’animal (ex. reproduction). Ces réponses au stress (coûteuses en énergie) sont donc susceptibles d’être influencées par le statut nutritionnel des individus ainsi que par l’effort parental dévolu à la reproduction. A chacun des épisodes successifs de l'incubation et de l'élevage du jeune poussin, des manchots royaux ont été équipés de cardiofréquencemètres puis soumis à divers stress standardisés. Deux des stress (approche humaine à 10 m ou capture-contention) étaient assimilables à un stress de prédation. Le troisième stress consistait en un son aigu, à priori sans signification biologique pour l’animal. Les résultats montrent que dans tous les cas les manchots réagissent fortement (élévation de la fréquence cardiaque) aux stress appliqués. Toutefois, la réponse cardiaque varie en fonction du type de stress, et donc potentiellement en fonction du risque perçu. Par ailleurs, nous montrons pour la première fois que la réponse cardiaque à un stress aigu semble être modulée par la valeur reproductive de l’œuf ou du poussin. En effet, les manchots royaux élevant un jeune poussin présentent des réponses cardiaques atténuées en comparaison avec des animaux incubant un œuf. A l'inverse, le protocole employé n'a pas permis de mettre en évidence une modulation de la réponse cardiaque à un stress aigu en fonction de l’état nutritionnel des individus. Ces résultats sont en accord avec la théorie de l’investissement parental et de la valeur reproductive de la couvée qui suggère que les réponses physiologiques à des stress aigus devraient être atténuées lorsque la valeur de la reproduction est élevée. Ceci permettrait de ne pas compromettre la reproduction en cours dans le cas où les chances de reproduction dans la même saison sont limitées ou inexistantes.

Modulation de la réponse adréno-corticale et métabolique à un stress aigu au cours de la reproduction

Dans une étude en cours (**encart 3**), deux autres composantes de la réponse à un stress aigu de type prédation, à savoir la réponse adréno-corticale (sécrétion de glucocorticoïdes) et métabolique (mobilisation des réserves énergétiques), sont considérées. Alors que la réponse cardiaque reflète la phase immédiate (quelques secondes) de la réponse au stress, les réponses adréno-corticales et métaboliques reflètent la phase plus tardive (plusieurs minutes). L'objectif de l'étude était de déterminer si cette phase de réponse tardive était également modulée par le statut reproducteur et énergétique de l'animal. Pour ce, nous avons soumis des manchots royaux à des stress de capture-contention et avons prélevé des échantillons sanguins aux temps 0 (< 3 min), 10 et 30 min afin de mesurer la réponse adréno-corticale (dosage de la corticostéronémie plasmatique) et métabolique (dosage des acide gras non-estérifiés circulants). Les animaux se trouvaient à 3 stades caractéristiques de la reproduction: la pariade (formation des couples), le début de l'incubation et l'élevage du jeune poussin. Les résultats préliminaires démontrent que la réponse adréno-corticale, à l'instar de la réponse cardiaque, varie en fonction de la valeur reproductive de l'œuf ou du poussin, mais non en fonction du statut énergétique. En effet, cette réponse est très élevée lors de la pariade, intermédiaire en début d'incubation et nettement affaiblie lors de l'élevage du jeune poussin. De même, la réponse métabolique est nettement plus faible chez les éleveurs de poussins que chez les paradants. Ces résultats semblent conforter l'hypothèse qu'une modulation de la réponse au stress est l'un des mécanismes intervenant dans les décisions parentales chez un oiseau marin longévif.

Conclusions et perspectives

Ainsi, au cours de ce travail de thèse, nous avons pu mettre en évidence certaines des stratégies comportementales et physiologiques utilisées par un oiseau marin à reproduction coloniale afin de concilier jeûne et reproduction. Nous avons entre autre réussi à quantifier pour la première fois en milieu naturel le coût énergétique de comportements spécifiques (agression, confort) et mis en évidence l'influence de paramètres tels que l'appréciation du contexte social et la valeur reproductive de la progéniture dans la modulation de réponses au stress. Enfin, ce travail a permis de soulever la question d'un coût éventuel de la reproduction en colonie dense, question qui mériterait des investigations supplémentaires. Ces résultats ouvrent de nouvelles perspectives de recherche et posent notamment la question de l’influence de l’environnement social sur l'état de stress chronique qu'il peut engendrer chez les individus. De récentes études réalisées chez l’homme suggèrent en effet que le stress de nature sociale pourrait avoir des conséquences importantes sur la physiologie des organismes, notamment en termes de stress oxydatif et de dommages occasionnés sur l’ADN, et de sénescence. Une nouvelle thématique consisterait donc à rechercher l’effet potentiel de l’environnement social sur l'état de stress psychologique des individus, et à déterminer si ce stress, à travers ses conséquences physiologiques (utilisation d’énergie, dommages oxydatifs, sénescence) et adaptatives (reproduction et survie), pourrait fixer une limite à l’évolution de la vie en groupe.

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