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# Stress, hormones et effort parental chez les oiseaux: une approche évolutive

Olivier Chastel

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## **Stress, hormones et effort parental chez les oiseaux: une approche évolutive**



**Olivier Chastel**

Chargé de recherche de première classe  
Centre d'Études Biologiques de Chizé UMR 7372 CNRS-Université de la Rochelle

22 octobre 2014

En vue de l'obtention de l'Habilitation à Diriger les Recherches(HDR)

Jury:

- Prof. Emmanuelle Cam (rapporteur)
- Prof. Sandrine Meylan (rapporteur)
- Dr. Philipp Heeb (rapporteur)
- Dr. Yan Ropert-Coudert (examineur)
- Prof. Paco Bustamante (examineur)

## Remerciements

Je tiens à remercier Xavier Bonnet Directeur du Centre d'Etudes Biologiques de Chizé au sein duquel j'effectue mes travaux de recherche. Un grand merci à Pierre Jouventin, ancien Directeur du laboratoire, qui m'a soutenu pendant mon doctorat et mes premières années de chercheur. Un grand merci également à mes collègues et amis de l'équipe Ecophysiologie Evolutive, Frédéric, François, Olivier, Bruno et Xavier, pour leur bonne humeur, leur passion pour ce métier et leur humanité.

Je remercie vivement Emmanuelle Cam, Sandrine Meylan, Phillip Heeb, Yan Ropert-Coudert et Paco Bustamante, d'avoir accepté de faire partie de mon jury, et ce malgré des emplois du temps très chargés.

Je remercie également l'ensemble des étudiants, volontaires, stagiaires et en particulier les thésards avec qui j'ai eu le grand plaisir de travailler: Hervé Lormée, Frédéric Angelier, Camille Bonneaud, Claire Loiseau, Adam Lendvai, Aurélie Goutte, Sabrina Tartu. Vous voir continuer dans ce métier passion est ma plus grande fierté. Au CEBC, ma recherche ne serait pas possible sans les compétences et l'efficacité du personnel du service d'analyse biologiques: André Lacroix, Charline Parenteau, Colette Trouvé et Stéphanie Ruault.

Les travaux présentés dans ce rapport sont basés sur de nombreuses collaborations nationales et internationales, avec en particulier, mes collègues norvégiens: Børge Moe, Geir Gabrielsen, Jan Ove Bustnes, Svein Åre Hansen et Claus Bech; mes collègues de IPHC, Yann Ropert-Coudert, Thierry Raclot, du LIENSs, Paco Bustamante. Un grand merci à Gabriele Sorci du laboratoire BioGéoSciences. Au CEBC, mes remerciements vont également aux collègues de l'équipe Prédateurs Marins et en particulier Christophe Barbraud.

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Enfin, je remercie mes parents pour leur soutien indéfectible, mes amis et tout particulièrement ma famille: Céline, Ondine et Cybille. Merci de votre patience quand 'Papa écrivait son petit livre de boulot'.

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## I – Curriculum Vitae

### Olivier Chastel

Date-lieu naissance : 22 novembre 1964 à Phnom Penh (Cambodge)

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#### Diplômes et cursus universitaires :

2014. **Habilitation à Diriger des Recherches (HDR)**, Université de La Rochelle)

1995. **PostDoc** au Biologisch Centrum (Université de Groningen, Pays-Bas, Department of Ecology, Professeur R. H. Drent).

1995. **Thèse de Doctorat d'Université** intitulée "Effort de reproduction chez les oiseaux longévifs: Fréquence de reproduction et condition physique chez les pétrels", Université de Tours. *Direction* : P. Jouventin.

*Jury* : E. Danchin, R. H. Drent, P. Jouventin, J. D. Lebreton, G. Periquet, H. Weimerskirch.

1989. **Diplôme d'Etudes Approfondies (DEA)** - Biologie des Populations et Eco-éthologie - Université de Rennes 1.

1988. **Maîtrise** de Biologie des Organismes et des Populations - Université de Rennes 1.

1987. **Licence** de Biologie des Organismes - Université de Bretagne Occidentale. Brest.

1986. **D.E.U.G. B.** - Université de Bretagne Occidentale. Brest.

1984. **Baccalauréat D.** Académie de Rennes.

#### Fonction et affectation

Depuis octobre 2000 : Chargé de recherche de première classe.

Depuis octobre 1996, affecté au CEBC comme chargé de recherche de seconde classe.

## II – Travaux de recherches (2005-2014)

### II.1 - Problématique générale

La compréhension du rôle de la variabilité environnementale sur la dynamique des populations nécessite l'étude des facteurs intervenant dans la régulation de l'effort de reproduction des individus. En particulier il s'agit de comprendre comment ces derniers sont en mesure de modifier leur comportement ("décisions" de reproduction) et d'allouer les ressources acquises en fonction des variations interannuelles ou à plus long terme du milieu qu'ils exploitent. **Cependant, pour comprendre comment les espèces répondent à ces changements, il est essentiel d'étudier les mécanismes physiologiques mis en jeu: en effet, la traduction des indices environnementaux (climat, ressources) en réponses écologiques (date et taille de ponte par exemple) se réalise via une cascade de processus hormonaux, qui déterminent probablement le degré d'adaptation aux modifications de l'environnement.**

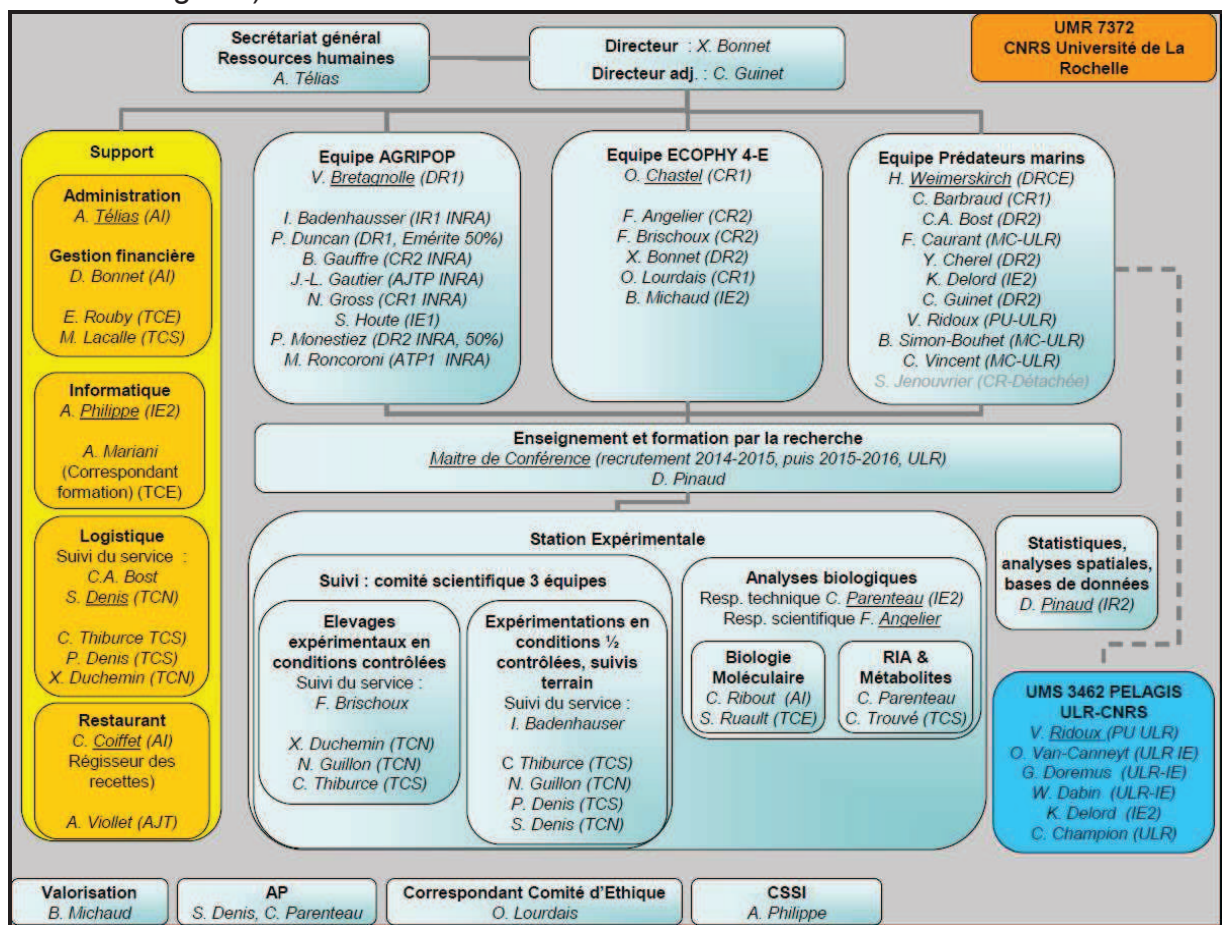
J'ai été recruté depuis octobre 1996 comme chargé de recherche pour développer un programme de recherche intitulé « **soubassement hormonal des décisions de reproduction chez les oiseaux** ». Il s'agit d'explorer les relations existant entre la date de ponte, la taille de ponte, le nombre de poussins élevés, l'effort d'approvisionnement de la nichée et les taux plasmatiques d'hormones de la reproduction. Les hormones que j'étudie principalement sont la corticostérone (stress) et la prolactine (soins parentaux et allo-parentaux). Il s'agit d'une approche principalement expérimentale permettant de suivre en parallèle l'évolution des taux hormonaux et les différentes « décisions » que les oiseaux adultes vont prendre au cours l'épisode reproducteur (se reproduire ou non, quand pondre, abandonner, réduire, poursuivre ou augmenter l'effort parental). Cette approche hormonale ouvre de grandes perspectives car il est possible de faire varier directement l'effort parental en modifiant expérimentalement les niveaux plasmatiques de certaines hormones (Ketterson et al. 1992). Enfin, depuis ces dernières années, je m'intéresse aux relations entre les contaminants (polluants organiques et métaux lourds) et les mécanismes hormonaux (perturbation endocrine) chez les oiseaux polaires.

#### Thèmes de recherche

Je développe un programme de recherche intitulé 'soubassement hormonal des traits d'histoire de vie' dans le cadre conceptuel du modèle général de l'effort de reproduction (Williams 1966). Le point central de ce modèle prévoit que l'effort consacré à un épisode reproducteur peut réduire la fécondité future ou les chances de survie ultérieure. Mes recherches visent à comprendre le rôle des hormones dans l'expression et la régulation des compromis adaptatifs («trade-off») prédits par la théorie des traits d'histoire de vie (Stearns 1992; Ricklefs & Wikelski 2002).

## Modèles étudiés et zones d'études

Mes travaux sont réalisés au CEBC-UMR 7273 dans le cadre de l'équipe 'Ecophysiologie Evolutive' dont je suis responsable depuis janvier 2014 (X. Bonnet, DR2; Olivier Chastel, CR1, Olivier Lourdais, CR1, Frédéric Angelier, CR2, François Brischoux, CR2, Bruno Michaud, IE2), voir *organigramme ci-dessous*). Les modèles étudiés (passereaux locaux, oiseaux marins polaires et tropicaux) diffèrent fortement dans leurs traits d'histoire de vie (longévité, fécondité, système d'appariement). Sur les oiseaux marins, mes recherches s'effectuent en Arctique (*Svalbard, Programme n° 330 IPEV, responsable: O. Chastel*), en zone tropicale (*Guyane, DEAL, responsable O. Chastel*), et dans les Terres Australes Françaises (programme ANR *PolarTop*, responsable O. Chastel), en collaboration avec l'équipe 'Prédateurs Marins' du CEBC, Programme n° 109 IPEV, responsable: H. Weimerskirch) et en tant que partenaire du programme IPEV n° 1091 (responsable: Y. Ropert(Coudert)). Je développe mes recherches grâce à la présence au CEBC, d'un service commun d'analyses biologiques (Charline Parenteau, IE2, Cécile Ribout, AI, Colette Trouvé, TCS, et Stéphanie Ruault, TCE), capable de doser un très grand nombre d'hormones et de métabolites d'animaux sauvages. Pendant la période 2007-2013, j'ai été responsable scientifique de ce service (fonction actuellement assurée par Frédéric Angelier).



Organigramme du CEBC-UMR 7273 (Janvier 2014)

## II.2 - Résultats obtenus

Les travaux exposés dans ce rapport couvrent la période 2005-2014 et abordent les liens entre certains mécanismes hormonaux, notamment ceux liés au stress et à

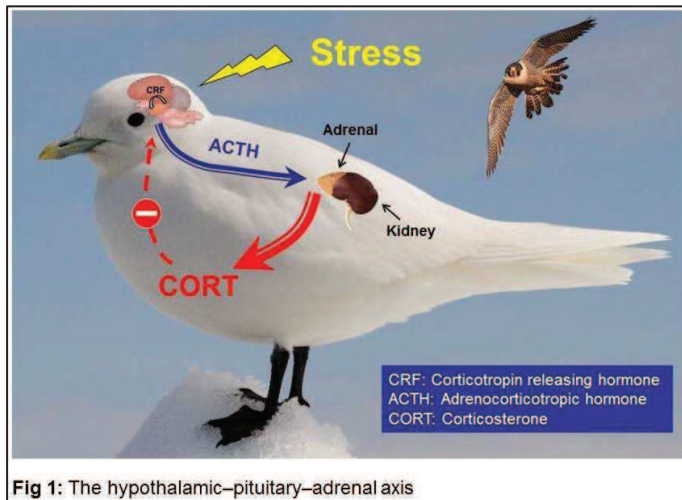


Fig 1: The hypothalamic–pituitary–adrenal axis

l'effort parental chez les oiseaux. Plus spécifiquement, il s'agit des hormones impliquées dans l'axe hypothalamo-hypophysio-surrénalien, et en particulier la corticostérone (la principale hormone de stress chez les oiseaux, Fig 1); et dans l'axe hypothalamo-pituito-gonadique, et en particulier l'hormone lutéinisante (LH) et la prolactine, respectivement liées au

démarrage de la reproduction et à l'expression des soins parentaux (Fig 2). La présente synthèse s'articule autour de 3 axes de recherche et les principaux articles s'y référant sont présentés à l'issue de chaque axe. Le premier axe (stress, hormones et valeur sélective), traite de l'interprétation possible, dans le cadre conceptuel du modèle général de l'effort de reproduction (Williams 1966), de la forte variation individuelle observée dans les réponses hormonales au stress. Le deuxième axe aborde les relations entre le stress, les hormones (corticostérone, LH et prolactine) et les décisions de reproduction (se reproduire ou pas, quand se reproduire, quel niveau d'investissement parental). Enfin, le troisième axe examine les relations entre les contaminants (polluants organiques et métaux lourds) et les mécanismes hormonaux étudiés dans le cadre des axes 1 et 2.

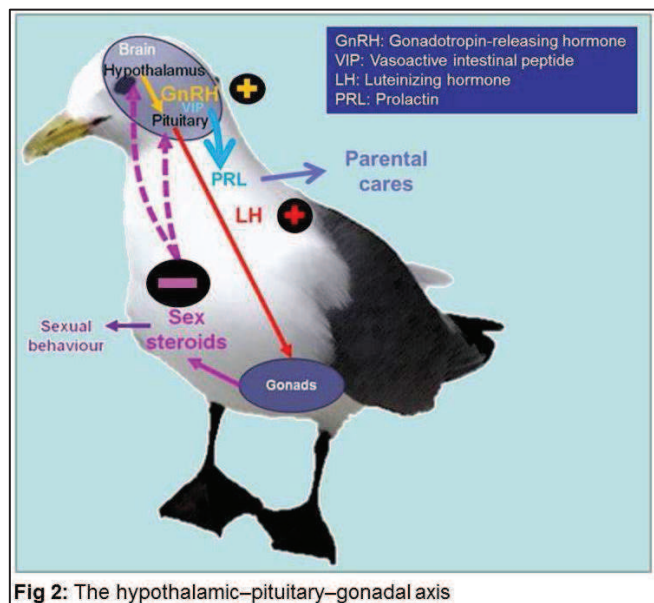
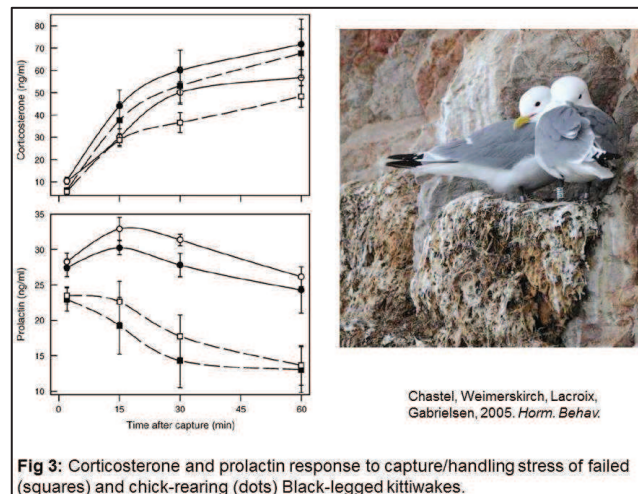


Fig 2: The hypothalamic–pituitary–gonadal axis



## 1) Stress, hormones et valeur sélective

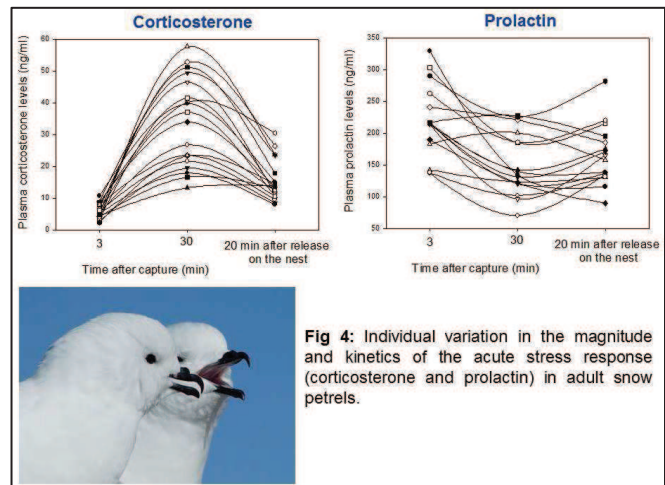
Dans ce contexte je m'intéresse principalement à la corticostérone, hormone stéroïde traditionnellement associée au stress environnemental (climat, condition trophiques, prédateurs) ou même social. En effet, lors d'un épisode de stress, les



**Fig 3:** Corticosterone and prolactin response to capture/handling stress of failed (squares) and chick-rearing (dots) Black-legged kittiwakes.

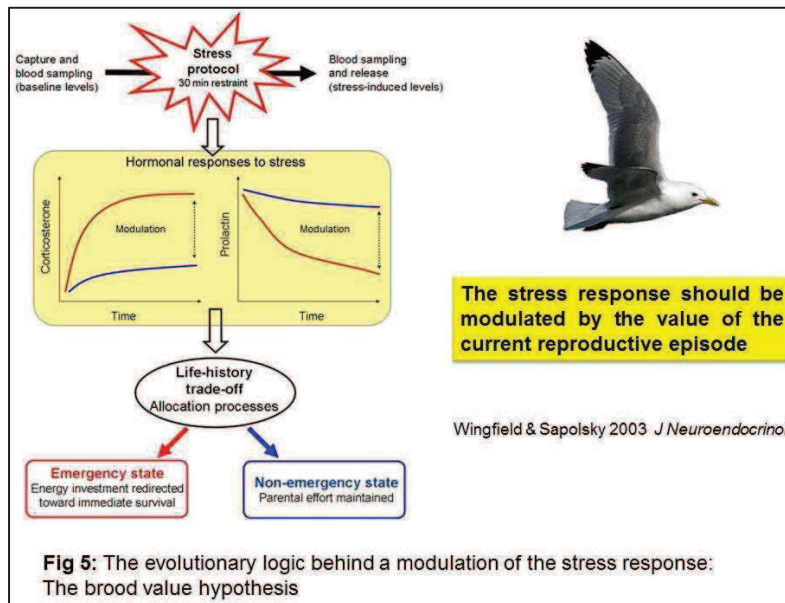
taux plasmatiques de cette hormone augmentent rapidement (réponse adrénocorticale, [1] **Fig 3**) et si la situation se dégrade d'avantage, l'oiseau abandonnera l'épisode reproducteur (Wingfield & Sapolsky 2003). Sur le terrain, cette réponse hormonale est mesurée à l'aide d'un protocole expérimental permettant de soumettre, pendant 30 minutes, l'individu à un stress transitoire de capture/contention (Wingfield 1994).

Première constatation, l'intensité de cette réponse adrénocorticale est extrêmement variable d'un individu à l'autre (**Fig 4**). Dans le cadre de la réponse hormonale au stress, j'ai également étudié l'implication d'une autre hormone dans les relations stress-effort parental: la prolactine, hormone hypophysaire qui facilite l'expression des comportements parentaux (incubation, élevage du poussin, Buntin 1996) mais également allo-parentaux. Chez les oiseaux, l'influence du stress sur les taux de prolactine était jusqu'à présent très mal connue. En utilisant le même protocole de terrain que pour l'étude de la corticostérone, j'ai pu montrer que les taux plasmatiques de prolactine déclinaient en réponse à un stress transitoire [1, 2] (**Fig 3**). De plus, tout comme pour la corticostérone, on constate une forte variabilité individuelle dans les niveaux de cette hormone et sa cinétique de réponse à un stress transitoire (**Fig 4**). Enfin, ces travaux mettent en évidence que pour ces deux hormones, l'intensité de la réponse au stress varie également en fonction des stades de reproduction. Ainsi, la réponse est maximale hors reproduction et minimale lors de la période d'élevage du ou des poussins.



**Fig 4:** Individual variation in the magnitude and kinetics of the acute stress response (corticosterone and prolactin) in adult snow petrels.

Quelle est l'origine d'une telle variation? On peut imaginer que l'intensité de cette réponse adrénocorticale pourrait être modulée par la valeur de la reproduction

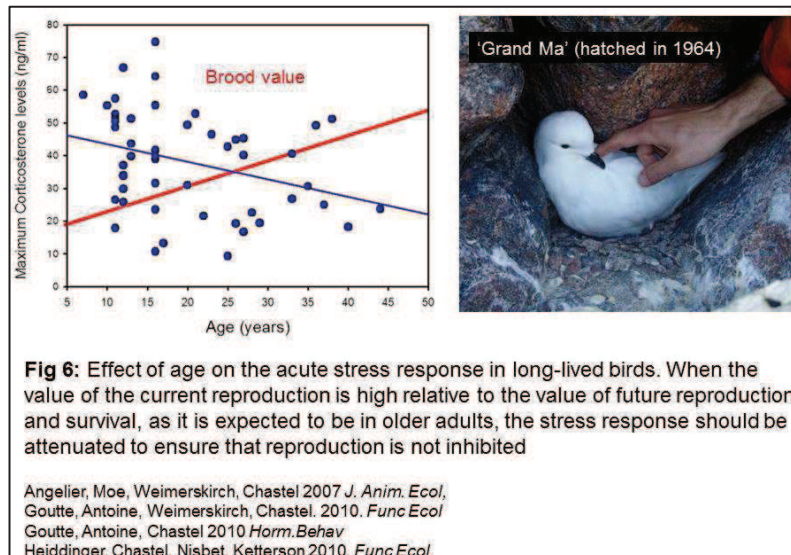


en cours (*'the brood value hypothesis'*, Wingfield & Sapolsky 2003, **Fig 5**): Lorsque la valeur sélective de cette dernière est élevée (espèces nichant dans des milieux difficiles tels que les déserts ou les zones polaires, où la 'fenêtre temporelle' de reproduction est limitée ; individus en fin de vie pour lesquels les chances de reproduction ultérieures sont réduites ; cas des

espèces sémelipares), on s'attend à une réduction ou même à une suppression de la réponse au stress, permettant de maximiser l'effort parental en cours, au dépens possible des chances de survie immédiate (**Fig 5**).

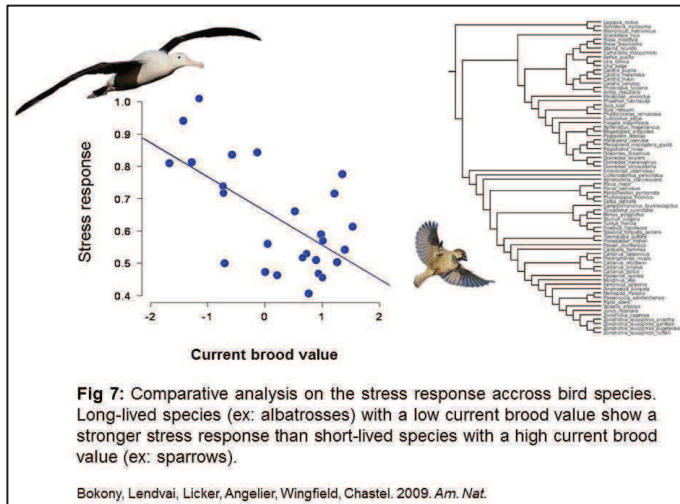
Grâce au suivi à très long terme (50 ans) des performances reproductrices chez les albatros et pétrels des Terres Australes et Antarctiques Françaises (TAAF), j'ai pu étudier le sous-bassement hormonal de l'effet de l'âge et en particulier, l'implication de la prolactine et de la corticosétrone, dans le contexte de la « *brood value hypothesis* ».

Sur le modèle pétrel des neiges, espèce très longévive (> 45 ans, **Fig 6**), j'ai exploré l'influence de l'âge sur la susceptibilité au stress. Dans le contexte de la '*brood value hypothesis*', on peut prédire que, chez cette espèce longévive, les individus les plus âgés pour lesquels les chances de reproduction ultérieures sont réduites, devraient

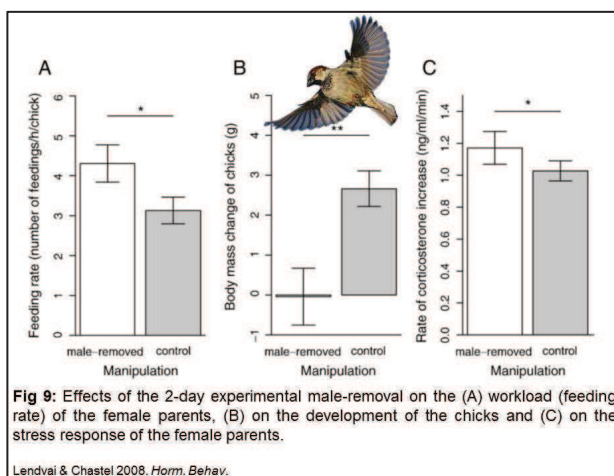
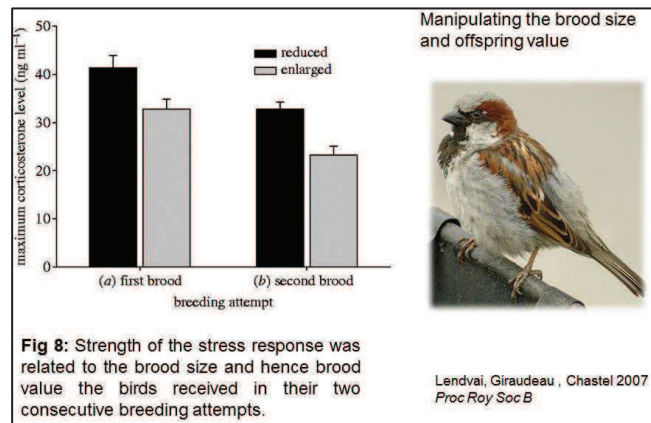


réprimer leur réponse au stress afin de maximiser la reproduction en cours, au contraire des individus jeunes (**Fig 6**). Les résultats obtenus chez le pétrel des neiges [3] et d'autres espèces mettent clairement en évidence, et en accord la '*brood value hypothesis*', une réponse au stress atténuée chez oiseaux les plus âgés ou plus expérimentés (nombre de tentatives de reproduction). A partir de mes travaux et des

données disponibles dans la littérature, il a été possible de réaliser une étude comparative de façon à tester la « brood value hypothesis » chez une large gamme d'espèces d'oiseaux (Fig 7). Cette étude, montre clairement que l'amplitude de la réponse hormonale au stress est influencée par les traits d'histoire de vie et la valeur relative de l'épisode reproducteur: Ainsi, chez les espèces longévives (comme les albatros: démographie lente, faible fécondité et donc faible valeur sélective de la nichée en cours), on observe une forte réponse au stress, au contraire des espèces peu longévives (comme les moineaux: démographie rapide, forte fécondité et donc forte valeur sélective de la nichée en cours), chez qui on observe une réponse au stress atténuée [4] (Fig 7). Cependant ces approches individuelles ou comparatives restent corrélationnelles et de nombreux facteurs peuvent contribuer à la variance observée. Ainsi, s'agissant des effets de l'âge, on ne peut pas exclure une sélection à long-terme des individus les plus tolérants au stress. Il est donc nécessaire d'adopter une approche expérimentale, en manipulant la valeur de l'épisode reproducteur. Des manipulations ont donc été conduites sur des passereaux et des oiseaux marins. Ainsi, chez le moineau domestique, qui se prête facilement à une manipulation expérimentale, lorsque l'on réduit expérimentalement la valeur sélective de la nichée (diminution du nombre de poussins ou dégradation de la croissance des poussins à la suite du retrait expérimental du mâle, Fig 8, 9), on constate une plus grande susceptibilité au stress des adultes [5,6]. A l'inverse, lorsque l'on augmente le nombre de poussin (la valeur de la nichée étant donc perçue comme plus importante), on assiste, comme prédit, à une réduction de la réponse au stress chez les adultes (Fig 8).

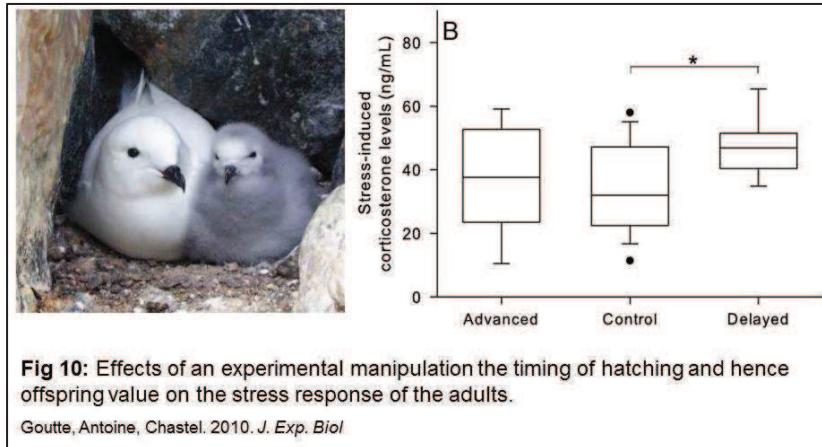


Strength of the stress response was related to the brood size and hence brood value the birds received in their two consecutive breeding attempts.



lorsque l'on augmente le nombre de poussin (la valeur de la nichée étant donc perçue comme plus importante), on assiste, comme prédit, à une réduction de la réponse au stress chez les adultes (Fig 8).

Chez le pétrel des neiges, on constate le même phénomène si l'on dégrade expérimentalement la valeur de la nichée en manipulant la date d'éclosion. Chez cette espèce antarctique, la date de ponte, doit être calée de façon précise de



manière à ce que l'éclosion de l'œuf intervienne au moment de la brève période de forte disponibilité. Le 'timing' de la ponte revêt donc un caractère majeur pour la survie du poussin. En échangeant entre les nids des œufs pondus à des dates

variables, et en créant ainsi expérimentalement des nichées tardives, et donc de moindre qualité, on constate, tout comme chez le moineau domestique, une plus forte intensité de la réponse au stress des adultes [7] (**Fig 10**). Ces résultats suggèrent que la modulation adaptative de la susceptibilité au stress est un mécanisme important pour la régulation de l'effort parental (compromis reproduction-survie, Stearns 1992).

### Articles publiés dans le cadre de l'axe 1:

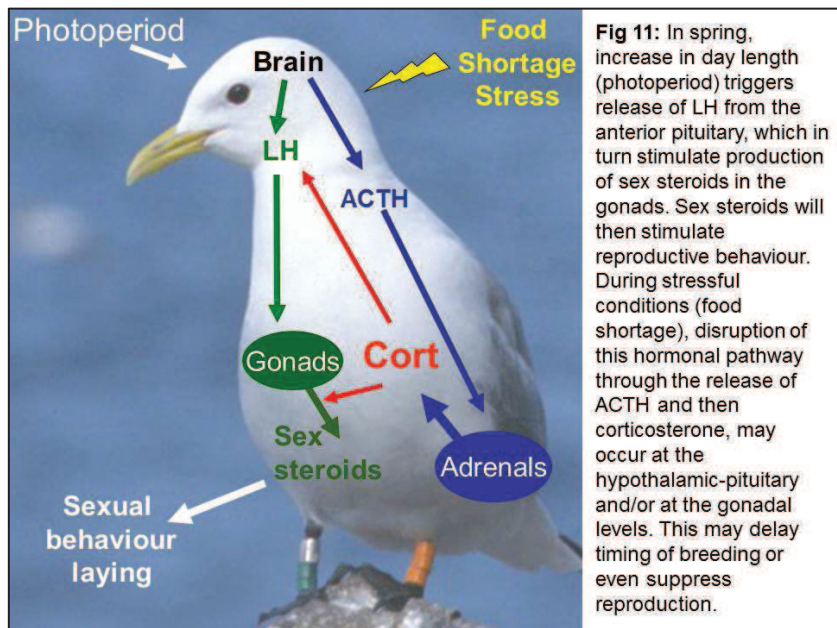
1. Chastel, O., Lacroix, A., Weimerskirch, H., Gabrielsen, G. W. 2005. Modulation of prolactin but not corticosterone responses to stress in relation to parental effort in a long-lived bird. *Horm. Behav.* 47, 459-466
2. Angelier F, Chastel O. 2009. Stress, prolactin and parental investment in birds: A review *Gen. Comp. Endocrinol.* 163, 142-148.
3. Angelier F, Moe B, Weimerskirch H, Chastel O 2007 Age-specific reproductive success in a long-lived bird: do older parents better resist stress? *J. Anim Ecol.* 76: 1181-1191
4. Bokony V, Lendvai AZ, Licker A, Angelier F, Wingfield JC. Chastel O. 2009. Stress response and the value of reproduction: Are birds prudent parents? *Am. Nat.* 173: 589-598
5. Lendvai, A. Z., Giraudeau, M., Chastel, O. 2007 Reproduction and modulation of the stress response: an experimental test in the House sparrow. *Proc. Roy. Soc. B* 274, 391-397
6. Lendvai, A. Z., Chastel, O. 2008. Experimental mate-removal increases the stress response of female house sparrows: the effects of offspring value? *Horm.Behav.* 53: 395-401
7. Goutte A, Antoine E, Chastel O. 2011. Experimentally delayed hatching triggers a magnified stress response in a long-lived bird. *Horm. Behav.* 59 167-173

## 2) Stress, hormones et décisions de reproduction

### 2-1) Se reproduire ou pas, à quel moment ?

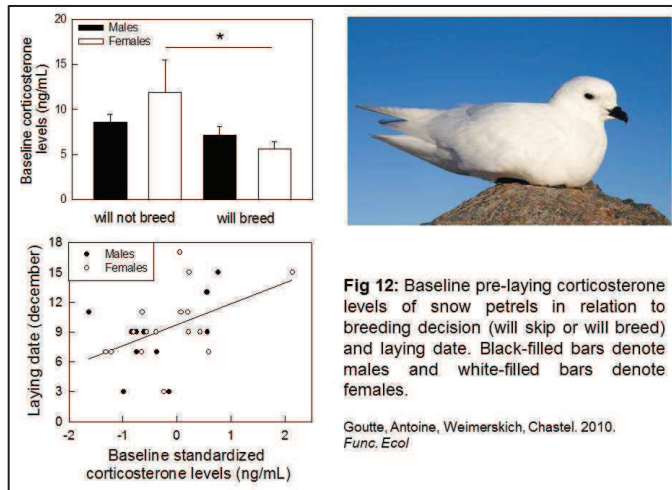
Au printemps, face à des conditions environnementales très variables d'une année à l'autre (accessibilité des sites de reproduction, disponibilité alimentaire), et en fonction de leur état corporel, les oiseaux doivent prendre un certain nombre de décisions. Celles-ci concernent par exemple le fait de s'engager ou non dans la reproduction. En effet, chez les espèces longévives comme les oiseaux marins, on observe fréquemment qu'une part non-négligeable d'adultes s'abstient de se reproduire (Chastel et al. 1993, 1995). Autre type de décision, celle relative au 'timing' de

la reproduction en fonction des conditions du milieu. En effet, la date de ponte, qui doit être calée de façon optimale de façon à ce que l'éclosion du ou des poussins intervienne au moment de la plus forte disponibilité alimentaire, revêt un caractère majeur pour le succès reproducteur. La phénologie de la reproduction constitue



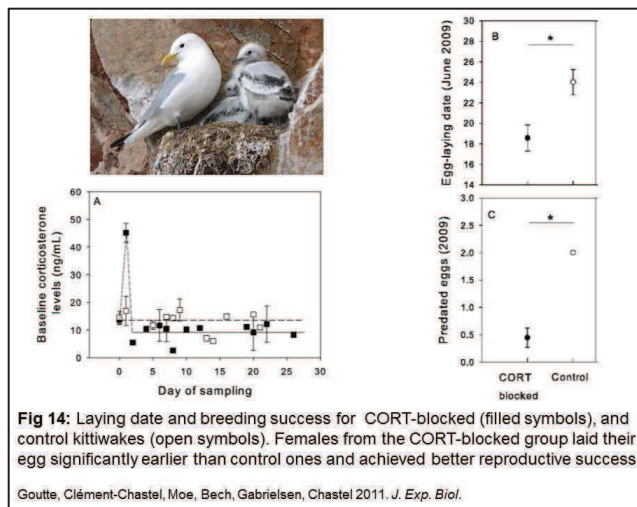
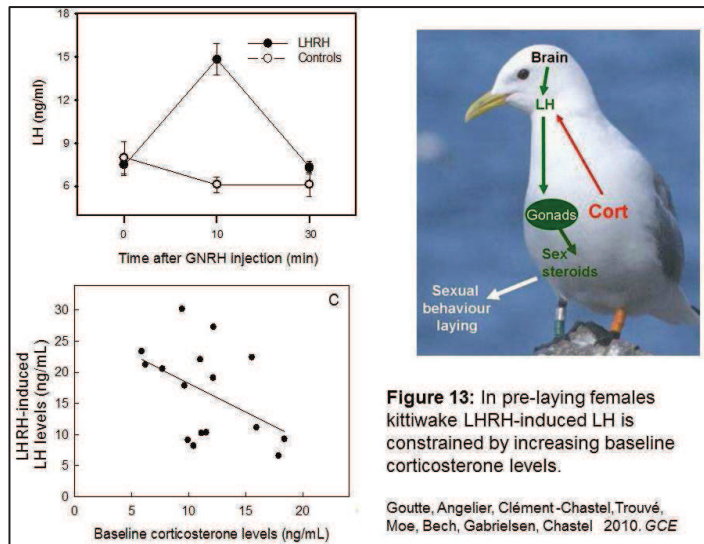
en outre un marqueur pertinent des changements climatiques et témoigne des capacités d'adaptation aux variations de l'environnement (Parmesan & Yohe 2003). Ces dernières années j'ai étudié les processus hormonaux impliqués dans les décisions de reproduction (se reproduire ou pas, quand se reproduire) et me suis ainsi intéressé aux relations entre la corticostérone (hormone de stress) et la LH (hormone lutéinisante, principalement sécrétée au printemps et déclencheur principal de la reproduction). L'hypothèse principale étant, qu'au printemps, une augmentation de la corticostéronémie, en réponse par exemple à une faible disponibilité alimentaire, pouvait empêcher ou retarder le déclenchement de la reproduction. Plus précisément, la corticostérone pourrait réprimer la sécrétion de LH et/ou de stéroïdes gonadiques (axe hypothalamo-pituitaire-gonadique, **Fig 11**).

Ces travaux ont été conduits sur des oiseaux marins polaires (mouette tridactyle au Svalbard, Pétrel des neiges en Terre Adélie). Les résultats obtenus mettent en lumière une association très claire entre des taux élevés de corticostérone, les abstentions de reproduction et une reproduction tardive [8] (Fig 12).



D'un point de vue des mécanismes, il apparaît également que la corticostérone agit au niveau hypothalamo-pituitaire, en réprimant la sécrétion de LH [9] (Fig 13). Cette exploration

a permis également de mettre en évidence le rôle prépondérant de l'âge des individus dans l'ajustement individuel des dates de ponte et sur l'abstention de la reproduction. Ainsi, sachant que les très jeunes et les très vieux oiseaux pondent tard dans la saison, voire s'abstiennent de se reproduire, nous avons décrit chez le pétrel des neiges, les effets de l'âge (7-45 ans) et de la sénescence sur la dynamique de la corticostérone et de la LH [10]. Enfin, le caractère causal des relations observées a été confirmé par une approche expérimentale: En utilisant des implants sous-cutanés contenant une faible dose de corticostérone, il a été possible de diminuer expérimentalement les taux de corticostérone (rétrocontrôle négatif) et l'intensité de la réponse au stress. En conséquence, les femelles ont pondu plus tôt et ont enregistré un meilleur succès reproducteur [11] (Fig 14). Ces travaux ont donc permis de mettre en évidence le rôle clé de la corticostérone, comme médiateur des décisions de non-reproduction et de dates de ponte, en réponse à un stress environnemental, et en fonction des caractéristiques individuelles.



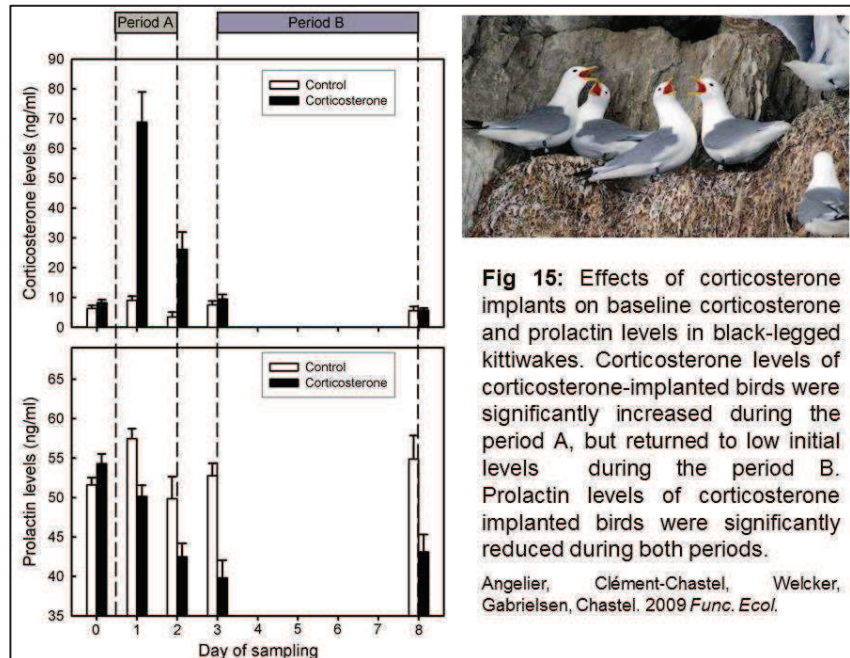
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## 2-2) Quel niveau d'investissement parental?

Au cours de la phase parentale (élevage du ou des poussins), les adultes doivent régulièrement décider, en fonction des conditions environnementales, de l'intensité des soins à prodiguer à la nichée (approvisionnement, protection thermique). En augmentant expérimentalement les taux de corticostérone (implants sous-cutanés) pendant la période d'élevage des poussins, il a été possible de mettre en évidence le rôle majeur de la prolactine, de la corticostérone et de leurs interactions, dans la régulation de l'effort parental.

Chez la mouette tridactyle, on constate en effet qu'une augmentation expérimentale et de courte durée de la corticostérone (mimant



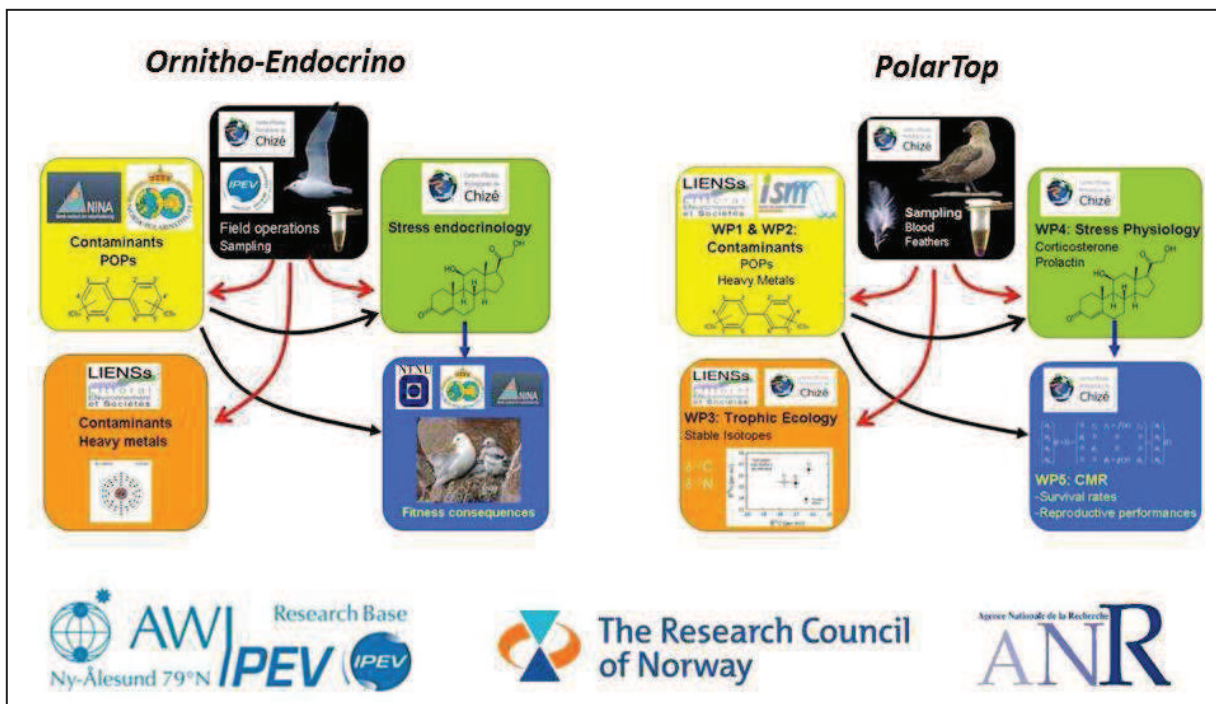
ainsi une baisse soudaine et momentanée des ressources alimentaire), entraîne une baisse concomitante des taux de prolactine (Fig 15). Le déclin de cette hormone va alors avoir pour conséquence une baisse de l'assiduité au nid, de la garde des poussins et de façon ultime du succès reproducteur [12]. On voit donc que ces deux hormones agissent de concert pour adapter l'intensité des soins parentaux aux conditions de l'environnement.

## Articles publiés dans le cadre de l'axe 2:

- Goutte A, Antoine E, Weimerskirch H, Chastel O. 2010. Age and the timing of breeding in a long-lived bird: a role for stress hormones? *Funct. Ecol.* 24, 1007-1016
- Goutte A, Angelier F, Clément-Chastel C, Trouvé C, Moe B, Bech C, Gabrielsen GW, Chastel O. 2010. Stress and the timing of breeding: Glucocorticoid-luteinizing hormones relationships in an arctic seabird. *Gen. Comp. Endocrinol.* 169, 108-116
- Goutte A, Kriloff M, Weimerskirch H, Chastel O. 2011 b. Why do some adult birds skip breeding? A hormonal investigation in a long-lived bird. *Biol. Lett.* 7,790-792
- Goutte A, Clément-Chastel C, Moe B, Bech C, Gabrielsen GW, Chastel O. 2011 a. Experimentally reduced corticosterone release promotes early breeding in black-legged kittiwakes. *J. Exp. Biol.* 214, 2005-2013
- Angelier F, Clément-Chastel C, Weckler J, Gabrielsen GW, Chastel O. 2009 How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in Black-legged kittiwakes. *Funct. Ecol.* 23, 784-793

### 3) Stress, hormones et contaminants

Dans la continuité de mes travaux sur les soubassements hormonaux des traits d'histoire de vie chez les oiseaux, j'ai développé depuis quelques années l'étude des relations entre contaminants (polluants organiques et métaux lourds) et mécanismes hormonaux tels que ceux liés aux décisions de reproduction. Etant donné que bon nombre de contaminants sont des perturbateurs endocriniens avérés, on peut se demander si la présence de ces contaminants dans l'environnement peut altérer le fonctionnement normal de mécanismes hormonaux majeurs tels que ceux permettant d'adapter la physiologie et le comportement face aux contraintes de l'environnement. Cependant l'implication des polluants en tant que perturbateurs endocriniens sur la réponse hormonale au stress et les mécanismes sous-jacents impliqués restent très peu explorée. Depuis quelques années, je m'investis dans l'étude des effets des polluants organiques et des métaux lourds en tant que perturbateurs endocriniens chez les oiseaux marins des zones polaires, milieux très concernés par les polluants. Ces travaux sont réalisés dans deux programmes de recherche que je coordonne: en Arctique (Svalbard, programme IPEV **Ornitho-Endocrino**) et en Antarctique (Terres Australes Françaises programme ANR **PolarTop**). Ces deux programmes, qui font appel à diverses disciplines (écotoxicologie, endocrinologie, écologie, démographie) sont basés sur d'importantes collaborations nationales et internationales, notamment avec la Norvège:





### 3-1) Les contaminants en milieu polaires

Bien qu'éloigné des centres industriels et agricoles, les zones polaires et l'Arctique en particulier, connaissent depuis plusieurs décennies des apports (métaux lourds et composés organiques) d'origine anthropique (Fig 16). C'est particulièrement le cas des polluants organiques persistants (POPs) qui comprennent certains pesticides (dont le célèbre DDT) et les polychlorobiphényles (PCBs), souvent utilisés comme isolants et réfrigérants dans les transformateurs électriques. Bien que désormais interdites d'utilisation depuis plusieurs décennies, ces molécules sont cependant encore très abondantes aux pôles et font partie des "POPs d'héritage". S'y ajoute la présence croissante de "POPs émergents" tels que certains composés bromés (PBDE, retardateurs de flamme) et perfluorés (PFC, imperméabilisants, emballages et anti-adhésifs). Enfin parmi les métaux lourds, le mercure, d'origine naturelle et anthropique est également très présent en zone polaire. Ubiquistes et volatils, ces composés toxiques voyagent aisément dans l'air, par le biais de l'effet "sauterelle", phénomène d'alternance évaporation/condensation qui dirige ces contaminants vers les régions polaires. Cette forte prévalence des polluants dans les zones polaires s'explique aussi par le fait que dans les régions froides, où la dégradation est très lente, ces molécules perdurent dans l'environnement pendant des décennies. Enfin, les POPs ont tendance à s'accumuler dans les graisses (à l'exception des PFC), dont les animaux polaires sont très pourvus.

**Heavy metals**

Mercury  
**Hg**  
 $\text{H}_3\text{C}-\text{Hg}^+ \text{X}^-$   
Methylmercury

**Persistent Organic Pollutants (POPs)** Stockholm Convention on Persistent Organic Pollutants (POPs)

**Legacy POPs**

Pesticides

Polychlorinated biphenyls (PCBs)

**Emerging POPs**

Brominated flame retardant (PBDEs)

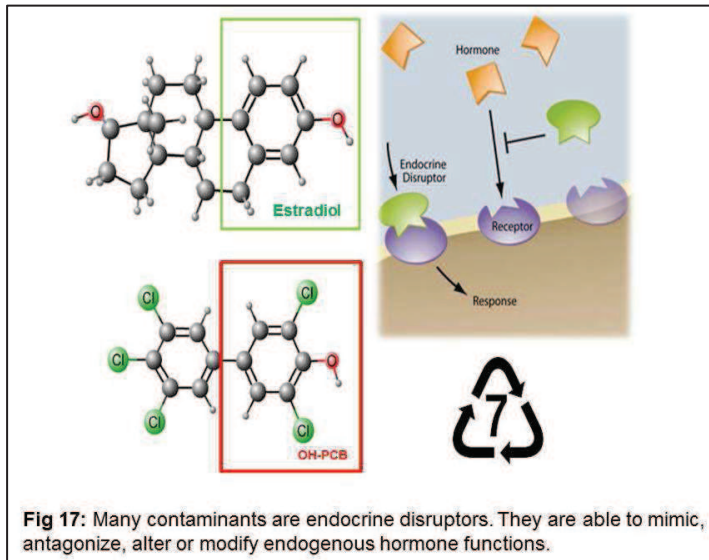
Perfluorinated Compounds (PFCs)

**Fig 16:** Polar areas are the fallout regions for long-range transport pollutants (heavy metals, persistent organic pollutants), and in some places the concentrations exceed the levels of industrialized areas.

### 3-2) Les contaminants en milieu polaires : conséquences hormonales

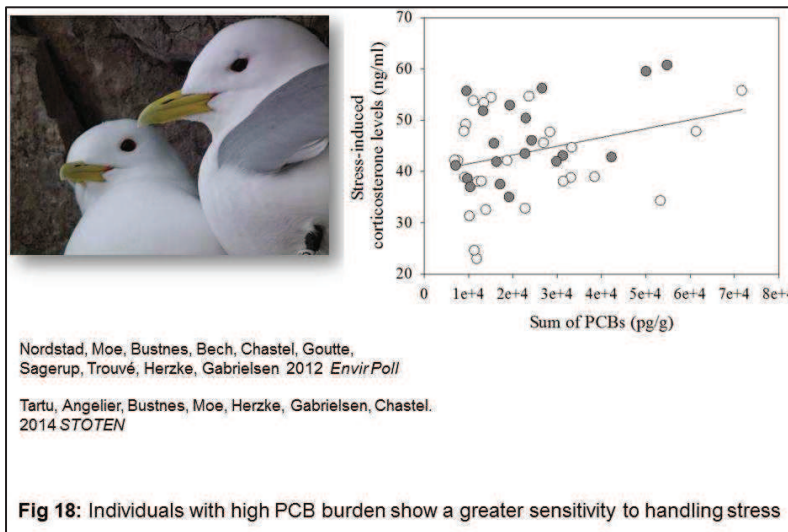
Les oiseaux marins polaires et en particulier ceux situés en haut de chaîne trophique, accumulent POPs et métaux lourds qui impactent le bon fonctionnement de leur physiologie (Gabrielsen 2007). En effet, de par leurs similarités structurales avec certaines hormones, ces polluants s'avèrent de redoutables perturbateurs endocriniens (Tan et al. 2009 ; Tyler et al. 1998, **Fig 17**).

La recherche médicale a montré que certains POPs et métaux lourds peuvent, même à faible dose, perturber significativement les mécanismes hormonaux par 1) effet mimétique en simulant l'action d'une hormone naturelle; 2) par effet antagoniste, en bloquant de l'action d'une hormone naturelle ou 3) par effet d'interférence en gênant la production, le transport, ou le métabolisme des hormones ou de leurs récepteurs.



**Fig 17:** Many contaminants are endocrine disruptors. They are able to mimic, antagonize, alter or modify endogenous hormone functions.

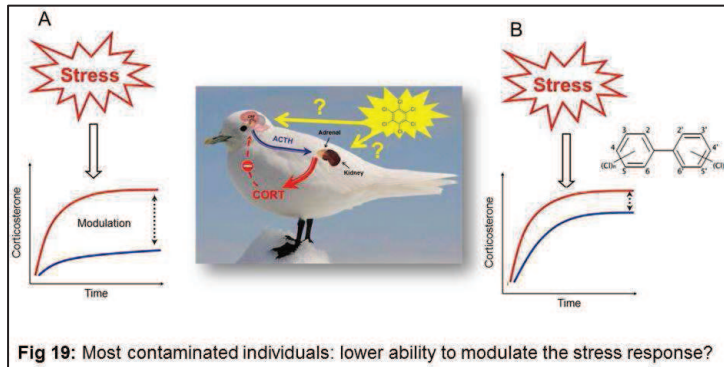
Les travaux menés dans le cadre des deux programmes que je coordonne montrent clairement que des concentrations même



**Fig 18:** Individuals with high PCB burden show a greater sensitivity to handling stress

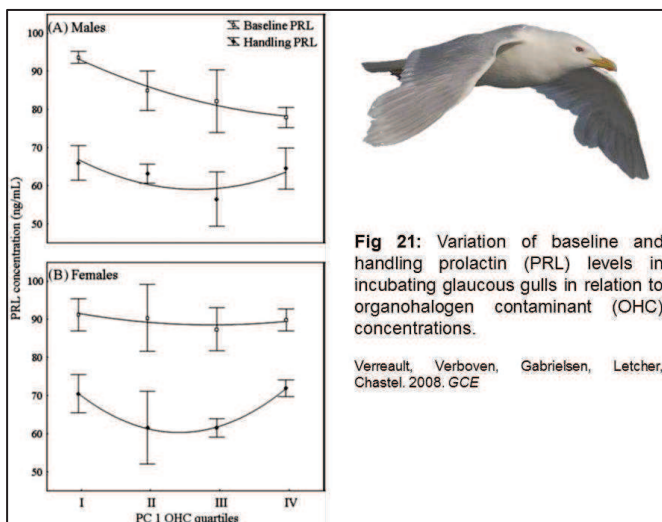
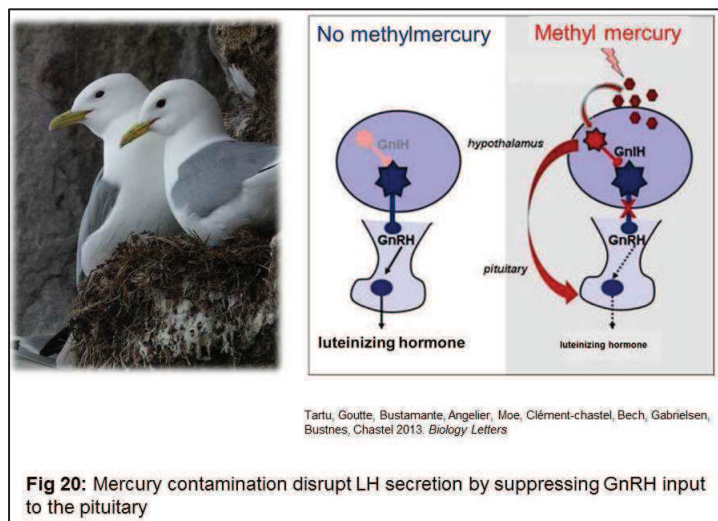
modérées de mercure ou de POPs sont assorties de modifications significatives des niveaux et des cinétiques des hormones (corticostérone, LH, prolactine) étudiées dans le cadre des axes 1 et 2 de ce rapport. Ainsi, chez les mouettes tridactyles du Svalbard, une forte contamination par les PCBs semble spécifiquement impacter l'axe hypothalamo-hypophysio-surrénalien en stimulant la production d'hormones de stress (corticostérone). Les individus les plus contaminés par les PCBs présentent alors une sensibilité accrue au stress [13, 14] (**Fig 18**).

Des tests conduits chez différentes espèces suggèrent que cette perturbation



endocrine par les PCBs opère au niveau surrénalien en stimulant la quantité de récepteurs à l'ACTH. Ainsi, la présence de PCBs pourrait restreindre les capacités de modulation de la réponse au stress (Fig 19). Au niveau de l'axe hypothalamo-pituito-

gonadique, chez la mouette tridactyle et le pétrel des neiges, la présence de méthylmercure (la forme organique et la plus toxique du mercure), entraîne une forte diminution de la sécrétion de LH, notamment en réprimant la production de GnRH, au niveau de l'hypothalamus [15] (Fig 20). C'est la première fois qu'une telle association entre le mercure et la sécrétion de LH, hormone clé pour le démarrage de la reproduction, est établie pour un animal sauvage. Les effets des contaminants sur une autre hormone hypophysaire, la prolactine ont été également examinés pour la première fois chez les goélands bourgmestres du Svalbard. Charognards, prédateurs d'œufs et de poussins et donc situés en haut de chaîne

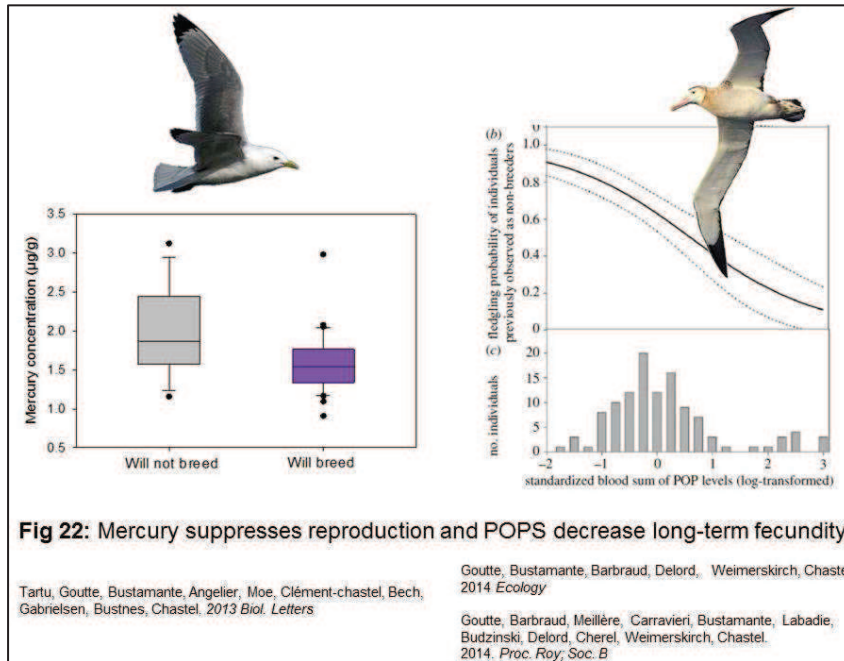


alimentaire, ces goélands présentent des taux record de contamination par les POPs et en particulier les PCBs (Bustnes et al. 2003). Chez cette espèce, la pollution par les POPs est associée, chez les individus les plus contaminés, à un déclin des taux basaux de prolactine [16] et à une modification des concentrations de cette hormone mesurées à la suite d'un protocole de stress transitoire (Fig 21). Cette perturbation

endocrine de la sécrétion de prolactine pourrait expliquer la faible assiduité à couvrir les œufs par les individus les plus contaminés.

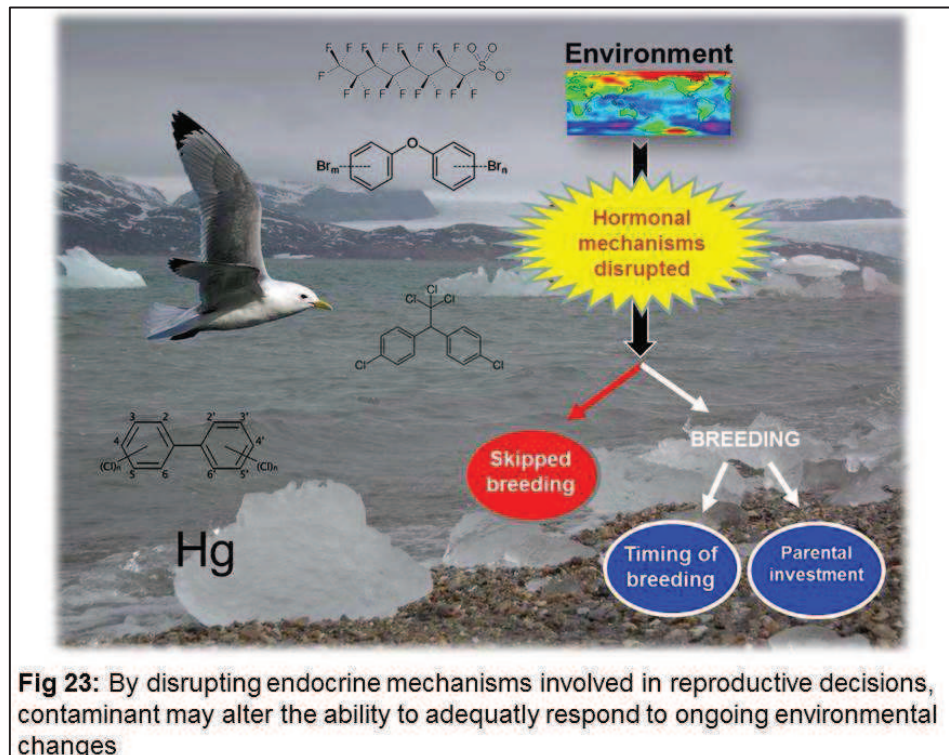
### 3-3) Les contaminants en milieu polaires: conséquences à long-terme

Chez le grand albatros, une forte contamination par le mercure et les POPs résulte en une plus forte prévalence des dommages oxydatifs [17]. Chez la mouette tridactyle, une forte exposition aux POPs est associée à une moindre intensité de certains signaux colorés présents au niveau de la langue, des commissures et du tour des yeux [18]. Ces signaux basés, sur la présence de pigments caroténoïdes, pourraient intervenir dans les processus de sélection du partenaire. Chez cette même



espèce, il a été possible de mettre en relation la fréquence des cas de non reproduction avec la contamination par le mercure (Fig 22). Ceci pourrait être la conséquence de la suppression de la sécrétion de LH par le mercure [15]. Enfin, en

croisant les données écotoxicologiques avec un suivi démographique à long-terme, on observe chez deux espèces de skuas et chez le grand albatros, un impact à long terme des POPs et en particulier du mercure qui affectent la capacité de se reproduire, le succès



reproducteur et à terme la dynamique de population [19, 20] (Fig 22). La traduction des indices environnementaux (climat, ressources) en réponses écologiques (date de ponte) se réalise via une cascade de processus hormonaux, qui déterminent probablement le degré d'adaptation aux modifications de l'environnement. Dans ce

cadre, l'exposition à certains polluants tels que les PCBs pourraient entraîner une plus grande sensibilité aux facteurs de stress (modification des habitats, baisse des ressources alimentaires, dérangement) et altérer les capacités des individus à y faire face. De façon générale, les polluants, en perturbant le bon fonctionnement des mécanismes hormonaux impliqués dans les décisions de reproduction (axes hypothalamo-hypophysio-surrénalien et hypothalamo-pituitaire-gonadotrope), pourraient théoriquement contraindre les possibilités d'adaptation des oiseaux marins aux bouleversements majeurs que connaissent aujourd'hui les pôles, tels que ceux engendrés par le changement climatique (Fig 23).

### Articles publiés dans le cadre de l'axe 3:

13. Nordstad T; Moe B; Bustnes JO; Bech C; Chastel O; Goutte A; Sagerup K; Trouvé C; Herzke D; Gabrielsen GW. 2012. Relationships between POPs and baseline corticosterone levels in black-legged kittiwakes (*Rissa tridactyla*) across its breeding cycle. *Envir. Poll.* 164, 219-226
14. Tartu S, Angelier F, Herzke D, Moe B, Bech C, Gabrielsen GW, Bustnes JO, Chastel O. 2014. The stress of being contaminated? Adrenocortical function and reproduction in relation to persistent organic pollutants in female Black-legged kittiwakes. *Sci. Total. Environ.* 476-477, 553-560
15. Tartu S, Goutte A, Bustamante P, Angelier F, Moe B, Clément-Chastel C, Bech C, Gabrielsen GW, Bustnes JO, Chastel O. 2013. To breed or not to breed: endocrine response to mercury contamination by an Arctic seabird. *Biol. Lett.* 9, (4), 20130317
16. Verreault J., Verboven N., Gabrielsen G., Letcher R.J., Chastel O. 2008. Changes in prolactin in a highly organohalogen contaminated Arctic top predator seabird, the glaucous gull. *Gen. Comp. Endocrinol.* 156: 569-576
17. Costantini D, Meillère A, Carravieri A, Lecomte V, Sorci G, Faivre B, Weimerskirch H, Bustamante P, Labadie P, Budzinski H, Chastel O. 2014, Oxidative stress in relation to reproduction, contaminants, gender and age in a long-lived seabird. *Oecologia* 175:1107-1116
18. Blévin P, Tartu S, Angelier F, Leclaire S, Bustnes JO, Moe B, Herzke D, Gabrielsen GW, Chastel O. 2014. Integument colouration in relation to persistent organic pollutants and body condition in arctic breeding black-legged kittiwakes (*Rissa tridactyla*). *Sci. Total. Environ.* 470-471, 248-254
19. Goutte A, Bustamante P, Barbraud C, Delord K, Weimerskirch H, Chastel O. 2014. Demographic responses to mercury exposure in two closely-related Antarctic top predators. *Ecology* 95, (4), 1075-1086
20. Goutte A, Barbraud C, Meillère A, Carravieri A, Bustamante P, Labadie P, Budzinski H, Delord K, Cherel Y, Weimerskirch H, Chastel O. 2014. Demographic consequences of heavy metals and persistent organic pollutants in a vulnerable long-lived bird, the wandering albatross. *Proc. Roy. Soc. B* 281, (1787), 20133313

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## Perspectives de recherches

Dans les années à venir, je désire continuer à travailler principalement sur la problématique des perturbateurs endocriniens, identifiée comme majeure par l'AMAP (Arctic Monitoring and Assessment Program, AMAP 2004), dans le cadre de collaborations internationales, en particulier pour:

1) Mieux identifier les mécanismes complexes d'interactions entre les polluants (effets cocktail) et les systèmes hormonaux liées à la reproduction, au stress. C'est le cas en particulier pour certains POPs émergents tels que les composés perfluorés (PFCs), dont la prévalence augmente chez les oiseaux marins polaires (Braune & Letcher 2012). Je travaille d'ores et déjà sur les effets des PFCs, avec le soutien du CNRS (*Initiative Structurante EC2CO, Écosphère Continentale et Côtière*, de l'INSU : *Polluants organiques émergents en milieux polaires : effets des composés perfluorés sur les prédateurs supérieurs*) et une thèse (2015-2018) que je codirigerai avec P. Bustamante de l'Université de la Rochelle, y sera consacrée.

2) Mesurer les conséquences encore peu connues de la présence des perturbateurs endocriniens sur la valeur sélective (survie, succès reproducteur) des individus et la dynamique des populations. A ce titre il est indispensable de croiser les données démographiques issues des suivis à long termes (bagueage) avec des études éco toxicologiques et hormonales.

3) Mieux appréhender la dynamique spatiale de la contamination. Grâce aux loggers miniaturisés (géolocation) on peut désormais suivre les déplacements des individus sur une année entière. En couplant ses suivis à des mesures toxicologiques dans des tissus archives (plumes), il devient possible de comprendre quand (reproduction ou hivernage) et où les oiseaux se contaminent.

## II.3 – Missions - Collaborations

### Missions de terrains

- Décembre 1989-Mars 1991: Hivernage à Kerguelen pour le Volontariat à l'Aide Technique (VAT) pour le compte du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).
- Décembre 1993-Janvier 1994: Mission en Terre Adélie dans le cadre du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).
- Juin-juillet 1998: mission dans l'arctique Norvégien. programme IPEV Franco-Norvégien n°330 (responsable : O. Chastel) Juin-Juillet 1999: mission dans l'arctique Norvégien et à l'île aux Ours (Svalbard) dans le cadre du programme IPEV Franco-Norvégien n°330 (responsable : O. Chastel).
- Juillet 2000: Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).
- Mai 2001 : Mission en Guyane Française (Ile du Grand Connétable) pour étude préliminaire de la population de Frégates superbes (*Fregata magnificens*).
- Juillet-août 2001 : Mission au Spitzberg dans le cadre du programme IPEV Franco-Norvégien n°330 (responsable : O. Chastel)
- Novembre 2001-Janvier 2002 : Mission à Kerguelen dans le cadre du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).
- Avril-Mai 2002 : Mission en Guyane française dans le cadre du programme « *Ecologie en mer et stratégie de reproduction des Frégates superbes de la Réserve naturelle du Grand Connétable, Guyane française* » (responsable : O. Chastel, Financement : DIREN Guyane).
- Juillet-Août 2002 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).
- Décembre 2002-Janvier 2003 : Mission en Terre Adélie dans le cadre du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).
- Mars 2003 : Mission en Guyane française dans le cadre du programme « *Ecologie en mer et stratégie de reproduction des Frégates superbes de la Réserve naturelle du Grand Connétable, Guyane française* » (responsable : O. Chastel, Financement : DIREN Guyane).
- Juillet-Août 2003 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).
- Juillet-Août 2004 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).
- Décembre 2004-Janvier 2005 : Mission en Terre Adélie dans le cadre du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).
- Juillet-Août 2005 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).
- Juillet-Août 2006 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).



Mai 2007 : Mission en Guyane française dans le cadre du programme DIREN « Ecologie en mer et stratégie de reproduction des Frégates superbes de la Réserve naturelle du Grand Connétable, Guyane française » (responsable : O. Chastel).

Décembre 2007-Janvier 2008 : Mission en Terre Adélie dans le cadre du programme IPEV N° 109 (Ornitho-Eco, responsable : H. Weimerskirch).

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Novembre 2012 : Mission en Guyane française dans le cadre du programme DIREN « Ecologie en mer et stratégie de reproduction des Frégates superbes de la Réserve naturelle du Grand Connétable, Guyane française » (responsable : O. Chastel).

Mai-Juin 2012 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).

Décembre-janvier 2012-2013 : Mission en Terre Adélie dans le cadre du programme ANR PolarTOP( responsable : O. Chastel).

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Juin-juillet 2013 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).

Juin-juillet 2014 : Mission au Svalbard dans le cadre du programme arctique IPEV N° 330 (Ornitho-Endocrino, responsable : O. Chastel).

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### III - Thèses dirigées et codirigées

- 1999-2001. Hervé Lormée. Paris Université Paris XIII. *Soubassement endocrinien de l'effort parental chez les oiseaux marins tropicaux.*(en codirection avec P. Jouventin). Actuellement Ingénieur d'Etudes à l'ONCFS de Chizé.
- 2001-2004 **Camille Bonneaud** Université Paris VI. *Compromis entre investissement parental et réponse immunitaire chez le moineau domestique* (en codirection avec G. Sorci, Laboratoire d'Ecologie, CNRS UMR 7625. Actuellement Lecturer in Ecology and Conservation à l'Université d'Exeter, UK
- 2003-2006 **Frédéric Angelier** Thèse Université de Poitiers (Bourse Docteur-Ingénieur CNRS/Région Poitou-Charentes). *Régulation de l'effort parental chez les oiseaux marins : une approche hormonale.* (en codirection avec H. Weimerskirch, CEBC). Actuellement Chargé de Recherches au CEBC/CNRS.
- 2005-2007 **Adam Lendvai** Thèse Université de Budapest (*Hormonal basis of social and reproductive decisions in the House sparrow* (en codirection avec A. Miklosi, Laboratoire d'Ethologie Comparative, Académie des Sciences de Hongrie, Budapest). Actuellement Maître de Conférences à : Institute of Biology, University of Nyiregyhaza, Hongrie.
- 2004-2007 **Claire Loiseau** Thèse Université Paris VI. *Mécanismes physiologiques et génétiques associés à l'impact des effets environnementaux en populations naturelles chez le moineau domestique *Passer domesticus** (en codirection avec G. Sorci, Laboratoire d'Ecologie, CNRS UMR 7625). Actuellement ATER (Université Montpellier II)
- 2007-2010. **Catherine Michel**. Thèse Université Pierre et Marie Curie. *Effets à long terme des conditions de développement sur la valeur adaptative : une étude expérimentale.* (en codirection avec X. Bonnet, O. Lourdais, CEBC). Actuellement enseignante dans l'éducation nationale
- 2007-2010 **Aurélié Goutte** Thèse Université de Poitiers (Bourse Docteur-Ingénieur CNRS/Région Poitou-Charentes). *Phénologie de la reproduction et changement climatique chez les oiseaux polaires : une approche éco-physiologique* (en codirection avec H. Weimerskirch, CEBC). Actuellement Maître de Conférences à l'EPHE, Paris.
- 2007-2010 **Vincent Leconte** Thèse Université de Poitiers (Bourse ENS). *La senescence des oiseaux longévifs:une approche pluridisciplinaires de l'influence de l'âge sur la vie et la reproduction* (en codirection avec H. Weimerskirch, CEBC). Actuellement enseignant dans l'éducation nationale
- 2011-2014 **Sabrina Tartu** Thèse Ecole doctorale Gay-Lussac, Université de La Rochelle. *Effets des polluants organiques persistants et des métaux lourds sur la physiologie du stress des oiseaux polaires.*

## IV - Publications, communications, ouvrages

### 1) Liste complète des publications scientifiques (1993 - 2014)

- Chastel, O., Weimerskirch, H. & Jouventin, P. 1993. High annual variability in reproductive success and survival of an antarctic seabird, the snow petrel *Pagodroma nivea*: a 27 year study. **Oecologia** 94: 278-285.
- Chastel, O. 1994. Maximum diving depth of common diving petrels *Pelecanoïdes urinatrix* at Kerguelen Islands. **Polar Biology** 14: 211-213.
- Genevois, F., Chastel, O. 1994. An antarctic fulmar *Fulmarus glacialis* feeding on land. **Marine Ornithology** 19: 136-137.
- Weimerskirch, H. Chastel, O., Ackermann, L., Chaurand, T., Cuenot-Chaillet, F., Hindermeier, X. & Judas, J. 1994. Alternate long and short foraging trips in seabirds parents. **Animal Behaviour** 47: 472-476.
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- Chastel, O. 1995. Influence of reproductive success on breeding frequency in four southern petrels. **Ibis** 137: 360-363.
- Chastel, O., Weimerskirch, H. & Jouventin, P. 1995. Influence of body condition on reproductive decision and reproductive success in the blue petrel. **Auk** 112: 964-972
- Chastel, O., Weimerskirch, H. & Jouventin, P. 1995. Body condition and seabird reproductive performance: a study of three petrel species. **Ecology** 76 (7): 2240-2246.
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- Lormee, H., Jouventin, P., Lacroix, A., Lallemand, J., Chastel, O. 2000. Reproductive endocrinology of tropical seabirds : sex-specific patterns in LH, steroids and Prolactin secretion in relation to parental care. **General and Comparative Endocrinology**, 117:413-426.
- Bonnet, X., Lagarde, F., Hennen, B. T., Corbin, J., Nagy, K. A., Naulleau, G., Balhoul, K., Chastel, O., Legrand, A., Cambag, R. 2001. Sexual dimorphism in steppe tortoise (*Testudo horsfieldii*) : influence of the environment and sexual selection on body shape and mobility. **Biological Journal of the Linnean Society**, 72: 357-372.
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- Cruz, C., Solis, E., Valencia, J., Chastel, O., Sorci, G. 2003. Testosterone and helping behaviour in the azure-winged magpie (*Cyanopica cyanus*) : natural covariation and an experimental test. **Behavioural Ecology and Sociobiology**

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- Alonso-Alvarez C., Bertrand S., Faivre B., Chastel O., Sorci G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. ***Proceedings of the Royal Society London B*** 274, 819 - 825
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- Moe B, Angelier F, Bech C, Chastel O 2007 Is basal metabolic rate influenced by age in a long-lived seabird? ***J Exp Biol*** 210: 3407-3414.
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## **V - ANNEXE 1**

Principaux articles illustrant les axes 1, 2 et 3



## Modulation of prolactin but not corticosterone responses to stress in relation to parental effort in a long-lived bird

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### Abstract

We tested the hypothesis that parental effort modulates the magnitude of corticosterone and prolactin responses to stress in a long-lived bird, the Black-legged kittiwake (*Rissa tridactyla*). To do so, we compared corticosterone and prolactin responses to capture/restraint stress between chick-rearing birds and failed breeders (no parental effort). We predicted that (1) the increase in plasma corticosterone levels in response to stress should be lower in chick-rearing birds, (2) the decrease in plasma prolactin levels in response to stress should be lower in chick-rearing birds, and (3) as both sexes care for the chick, there should be no sex difference in the hormonal response to stress. Baseline plasma corticosterone and prolactin levels were higher in chick-rearing birds and were not influenced by body condition. Failed breeders were in better condition than chick-rearing individuals. Corticosterone response to stress was unaffected by parental effort as both chick-rearing and failed birds exhibited a robust corticosterone increase. Prolactin response to stress was however clearly influenced by parental effort: chick-rearing birds showed a modest 9% prolactin decrease whereas in failed birds prolactin concentrations fell by 41%. Body condition did not influence hormonal responses to stress. When facing stressful condition, breeding kittiwakes attenuate their prolactin response to stress while enhancing their secretion of corticosterone. Increasing corticosterone secretion triggers foraging efforts and diminishes nest attendance whereas an attenuation of prolactin response to stress maintains parental behavior. We suggest that this hormonal mechanism facilitates a flexible time-budget that has been interpreted as a buffer against environmental variability.

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**Keywords:** Parental effort; Stress response; Corticosterone; Prolactin; Long-lived bird; Kittiwake; *Rissa tridactyla*

### Introduction

The general reproductive effort model based on the cost of reproduction (Williams, 1966) predicts the existence of an adaptive trade-off that allows reproducing individuals to allocate resources either to current reproductive episodes or to their own survival (i.e., future reproduction) (Stearns, 1992). The study of hormones can offer great insights into the mechanisms mediating life-history trade-offs (Sinervo and Sennson, 1998). For example, plasma levels of the hormone corticosterone rapidly rise in response to stressful conditions (Wingfield and Farner, 1993), and this can

ultimately lead to a cessation of territorial and reproductive behaviors (Silverin, 1986; Wingfield and Silverin, 1986). This adrenocortical response and its behavioral consequences can be considered as adaptive because it may interrupt the current reproductive episode of an individual and promote its survival and future reproduction during stressful periods (Wingfield et al., 1998).

However, in some ecological situations, individuals downregulate their adrenocortical response. In severe environments such as Arctic areas or deserts where periods of extreme weather are commonly encountered, the time-frame during which offspring can be produced is narrow and the chances of successful re-nesting after failure are low. In this context, several species show a reduced adrenocortical response during the most critical stages of parental care, thus delaying the expression of life-saving behaviors that

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might otherwise result in reproductive failure (“The short season hypothesis”, Holberton and Wingfield, 2003; Meddle et al., 2003; Reneerkens et al., 2002; Silverin et al., 1997; Wingfield et al., 1992, 1995). This facultative reduction of the adrenocortical response may also be linked to parental effort itself as suggested by Wingfield et al. (1995). They hypothesized that the magnitude of the adrenocortical response to stress is a function of the degree of parental care provided, with a lower adrenocortical response expected to be observed in the sex that provides most parental care. Likewise, it has been shown that in marine turtles, females involved in parental effort (egg laying) displayed an almost complete suppression of their corticosterone response to stress, contrary to males and non-breeding females (Jessop, 2001). Therefore, a reduction of the adrenocortical response to stress might be considered a hormonal tactic by which individuals maximize their current reproductive effort, even though under certain situations this may entail survival costs (“The parental care hypothesis”: Jessop, 2001; O’Reilly and Wingfield, 2001; Wingfield and Sapolsky, 2003; Wingfield et al., 1995).

Prolactin is another hormone that may act on parental effort through its role in the initiation and maintenance of avian parental behavior (Buntin, 1996). Plasma prolactin levels rapidly increase during stress in mammals, and this rise in plasma prolactin levels is often considered part of the classical stress response (De Vlaming, 1979). In birds, the effect of stress on plasma prolactin levels is however less clear: a study of four passerines species revealed no increase in plasma prolactin as a result of injection or restraint, suggesting that unlike mammals, prolactin is not released during capture and handling stress (Manney et al., 1999). On the contrary, there is some indications that in birds plasma prolactin levels may actually decrease during stressful events. The stress of injections, handling, and restraint has been shown to decrease plasma prolactin levels in bantam hens (*Gallus domesticus*), Florida scrub-jays (*Aphelocoma coerulescens*), Semipalmated Sandpipers (*Calidris pusilla*), and Turkey (*Meleagris gallopavo*) (Gratto-Trevor et al., 1991; Manney et al., 1999; Opel and Proudman, 1986; Sharp et al., 1989). During stressful events, a decrease in plasma prolactin levels can be considered adaptive because it may disrupt the current parental effort (via nest desertion) of an individual and promote its survival and future reproduction (Delehanty et al., 1997). Alternatively, as for the adrenocortical response, individuals involved in parental effort could decide to attenuate or even suppress their prolactin response to stress. As a consequence, attenuation of the prolactin response could be considered as a hormonal tactic permitting the maintenance of parental care behaviors and maximizing current reproductive efforts during temporary stressful conditions.

In this paper, we examined corticosterone and prolactin response to capture/restraint stress in relation to parental effort in a long-lived bird, the Black-legged kittiwake (*Rissa*

*tridactyla*). In this species, the first part of the chick-rearing period appears constraining as adults are faced with the trade-off of investing either in the chick’s needs of being regularly fed and brooded or of investing in the necessity of spending enough time at sea to restore their own body reserves and find food for the chick (Fyhn et al., 2001; Moe et al., 2002). According to the “the parental care hypothesis”, a modulation of the corticosterone and prolactin response to stress would mediate this trade-off (Jessop, 2001; O’Reilly and Wingfield, 2001; Wingfield et al., 1995). To test this hypothesis, we compared the corticosterone and prolactin response to capture/restraint stress of two categories of birds: (1) individual birds involved in parental effort (rearing a 8–12-day-old chick) and (2) individual birds not engaged in parental effort (failed birds that lost the eggs or newly hatched chick). To study the endocrine response of the two groups, we used a capture, handling, and restraint stress protocol (e.g., Wingfield, 1994). It was predicted that (1) the magnitude of the increase in plasma corticosterone levels (adrenocortical response) in response to stress would be lower in individuals involved in parental effort, (2) the magnitude of the decrease in plasma prolactin levels in response to stress would be lower in individuals involved in parental effort, and (3) as both sexes brood and provision the chick in kittiwakes, there would be no sex difference in the adrenocortical and prolactin response to capture/restraint stress.

## Methods

### *Study area and birds*

Black-legged kittiwakes are colonial, pelagic seabirds that breed on cliffs throughout the northern parts of the Pacific and the Atlantic, including the Barents Sea region up to the Svalbard Archipelago. Our study was conducted between 17 and 23 July 2000 on a colony of Black-legged kittiwakes at Kongsfjorden, Svalbard (78°54’ N, 12°13’ E), 7 km east of Ny-Ålesund. In Svalbard, continuous daylight and an average ambient temperature of 4.5°C (Bech et al., 2002) characterize the breeding period (May–September).

We studied kittiwakes in a sub-colony of ca. 60 pairs breeding on cliffs at a height of 5–10 m. Most nests were therefore accessible and birds were captured at their nest with a noose on the end of a 5 m fishing rod. In Svalbard, kittiwakes lay two eggs but rear a single chick (Gabrielsen et al., 1987; Moe et al., 2002). In order to compare hormone response to capture/restraint stress in relation to parental effort, we therefore took blood samples both from adults rearing a 8–12-day-old chick (called thereafter chick-rearing birds,  $n = 18$ , 11 females and 7 males) and from adults that had lost the eggs (called thereafter failed birds,  $n = 10$ , 6 females, 4 males). Both chick-rearing and failed birds were captured on their nest, failed attending their nest for several weeks after breeding failure (Cadiou et al., 1994). For failed

breeders, the exact timing of egg loss was not precisely known. Both chick-rearing and failed birds, only one bird was captured per nest and the data obtained with males and females were hence considered independent.

#### Blood sampling

All birds were bled according to the following standardized technique (Wingfield, 1994): immediately after capture, an initial blood sample (maximum: 300  $\mu$ l) was collected from the alar vein with a 1 ml heparinized syringe and a 25 gauge needle, and further samples (maximum: 100  $\mu$ l) were taken at 15, 30, and 60 min. To check for possible effect of handling time during initial bleeding on hormone levels, we measured the time elapsed between the capture and the end of the initial blood sampling (minimum: 2 min, maximum: 5 min and 30 s).

After each blood sample, birds were kept in an individual opaque cloth bag. Blood samples were put into ice and centrifuged as soon as possible; plasma (for corticosterone and prolactin assay) and red blood cells (for sexing, Fridolfsson and Ellegren, 1999) were subsequently stored at  $-20^{\circ}\text{C}$ . All birds were weighed to the nearest 2 g using a Pesola and their skull length (head + bill) was measured to the nearest 0.5 mm (Moe et al., 2002). Body condition was then expressed as the residual mass from a linear regression relating body mass to skull length ( $r^2 = 0.42$ ,  $n = 28$ ,  $P < 0.001$ ). Defined as such, body condition is calculated as a size-corrected body mass and is expressed in units gram. Birds were dyed on their head and breast to avoid double catching and then released in the colony. All birds returned to their nest 2–20 min after the last blood sample.

#### Hormone assays

Plasma concentrations of corticosterone were determined by radioimmunoassay at the CEBC as previously described (Lormée et al., 2003). Minimal detectable corticosterone levels were 400 pg/ml. The intra-assay variation was 8.5%. There was no significant relationship between handling time and corticosterone levels measured during the initial bleeding (chick-rearing birds:  $r = 0.06$ ,  $P = 0.80$ ,  $n = 18$ ; failed birds:  $r = 0.42$ ,  $P = 0.22$ ,  $n = 10$ ). Thus, initial blood samples were considered to reflect baseline levels of corticosterone (Wingfield et al., 1982; Kitaysky et al., 1999; Lormée et al., 2003). To check for diel variation, we plotted corticosterone values from the first bleeding against time of the day when the initial sample was collected. No significant relation was found (chick-rearing birds:  $r = 0.12$ ,  $P = 0.61$ ,  $n = 18$ ; failed birds:  $r = 0.29$ ,  $P = 0.41$ ,  $n = 10$ ).

Plasma concentrations of prolactin were determined by and heterologous radioimmunoassay (RIA) at the CEBC (Cherel et al., 1994). Pooled plasma samples of kittiwakes produced a dose response curve that paralleled chicken prolactin standard curves (“AFP 4444B”, source: Dr. Parlow, N.H.P.P. Harbor-UCLA Medical Center, Torrance,

California, USA, Fig. 1). Parallelism between the kittiwake and chicken curve indicates that the concentration-dependent binding dynamics of the kittiwake prolactin with the antibody is similar to that of the chicken prolactin with the antibody and that this RIA can be used to assess relative levels of plasma prolactin in the Black-legged kittiwake. Only one assay was performed, the intra-assay coefficient of variation being 7.3% ( $n = 4$  duplicates). There was no significant relationship between handling time and plasma prolactin levels measured during the initial bleeding (chick-rearing birds:  $r = 0.03$ ,  $P = 0.91$ ,  $n = 18$ ; failed birds:  $r = 0.24$ ,  $P = 0.51$ ,  $n = 10$ ). Thus, initial blood samples were considered to reflect baseline plasma levels of prolactin. To check for diel variation, we plotted plasma prolactin values from the first bleeding against time of the day when the initial sample was collected. No significant relation was found (chick-rearing birds:  $r = -0.06$ ,  $P = 0.82$ ,  $n = 18$ ; failed birds:  $r = -0.46$ ,  $P = 0.18$ ,  $n = 10$ ).

#### Statistics

We first analyzed the baseline plasma corticosterone and prolactin data with 2 factor ANOVAs with sex and parental effort (adults rearing chicks or failed breeder) as factors. We examined the corticosterone and prolactin responses to capture/restraint stress with two-factor repeated measures ANCOVAs with sex and parental effort as the factors and body condition as the covariate. Relationships between baseline- and stress-induced corticosterone and prolactin levels (measured after 60 min of capture and restraint) were examined using Pearson correlations. In the statistical analysis, initial data were tested for assumptions required by a statistical test according to Sokal and Rohlf (1981). If these data violated assumptions, they were  $\log_{10}$  transformed and examined again. Statistical analyses were

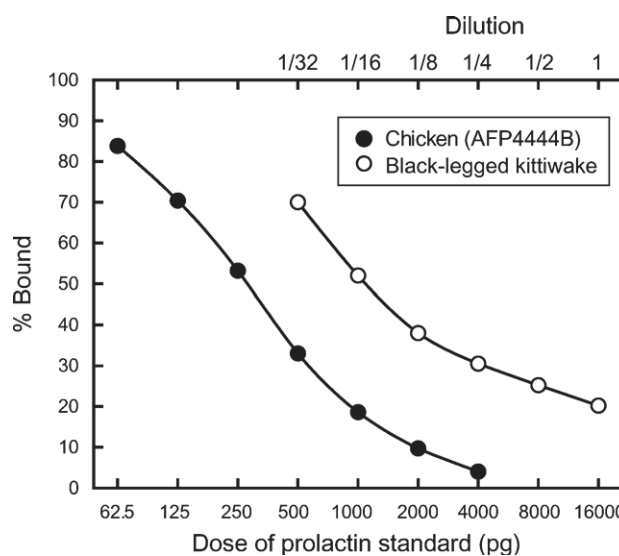


Fig. 1. Dose response curve for prolactin of chicken and Black-legged kittiwakes. Prolactin standard is expressed in pg/tube.

performed using SYSTAT 7.0 (Wilkinson, 1997). Data are presented as means  $\pm$  1 standard error of means (SEM).

## Results

### Baseline hormone levels and body condition

Baseline corticosterone levels (range: 1.94–20.35 ng/ml for chick-rearing birds; 1.39–10.51 ng/ml for failed birds) did not differ between sexes. On the other hand, parental effort had a significant effect on baseline corticosterone levels ( $F(1,24) = 7.29$ ,  $P = 0.012$ ); chick-rearing birds had higher corticosterone titers than failed breeders (Fig. 2).

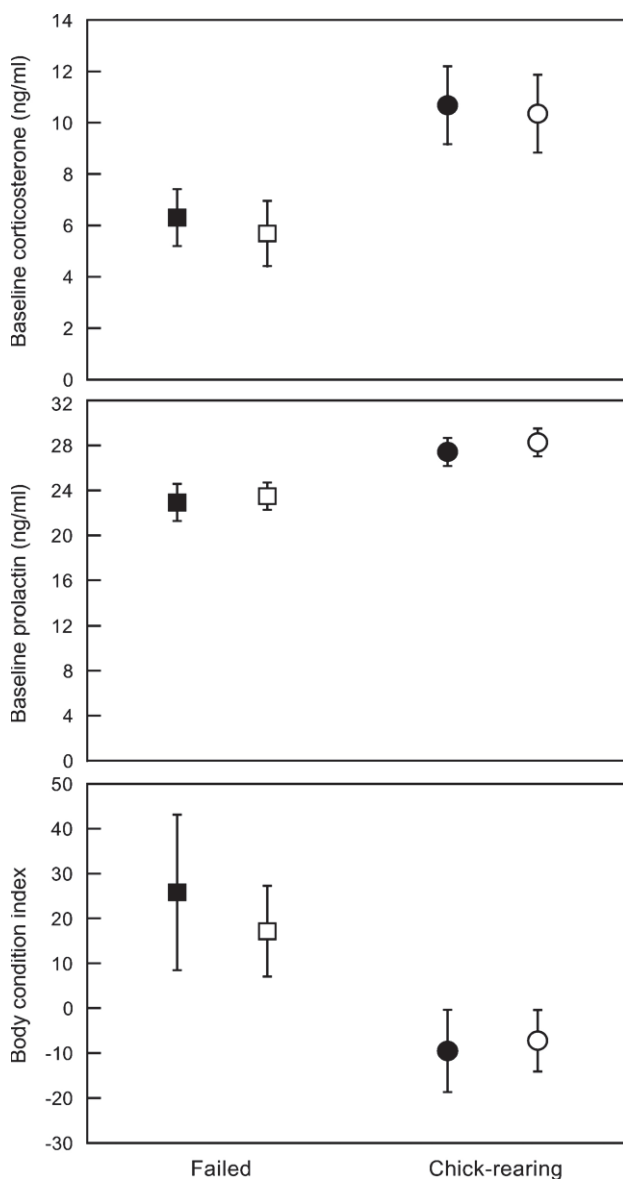


Fig. 2. Baseline corticosterone and prolactin levels and body condition index of failed (squares, 6 females, 4 males) and chick-rearing (dots, 11 females and 7 males) Black-legged kittiwakes. Open symbols denote females and filled ones males. Data are expressed as mean  $\pm$  SEM.

There was no between-factor interaction (sex  $\times$  parental effort).

Baseline plasma prolactin levels (range: 20.59–33.10 ng/ml for chick-rearing birds; 19.31–27.21 for failed birds) did not differ between sexes. On the other hand, parental effort had a significant effect on baseline plasma prolactin levels ( $F(2,24) = 19.88$ ,  $P < 0.001$ ); chick-rearing birds had slightly but significantly higher plasma prolactin titers than failed birds (Fig. 2). There was no between-factor interaction (sex  $\times$  parental effort). Baseline plasma prolactin levels were not correlated with baseline corticosterone levels in chick-rearing and failed birds.

Body condition did not differ between males and females, but failed breeders were significantly in better condition than adults rearing a chick ( $F(2,24) = 8.57$ ,  $P = 0.007$ , Fig. 2). There was no between-factor interaction (sex  $\times$  parental effort). Body condition was neither correlated with baseline corticosterone levels nor with baseline plasma prolactin levels in chick-rearing and failed birds.

### Hormones response to stress

Birds responded to acute stress by a rapid and significant response of the adrenocortical system to the stress of being captured and held (time after capture,  $F(3,69) = 110.97$ ,  $P < 0.0001$ , Fig. 3). This acute stress response was unaffected by parental effort or by body condition. On the other hand, males showed a slightly but significantly stronger response to stress than females (sex,  $F(1,23) = 4.75$ ,  $P = 0.04$ ; sex  $\times$  time after capture,  $F(3,69) = 2.90$ ,  $P = 0.044$ , Fig. 4). Maximum corticosterone levels were significantly higher in males ( $F(1,26) = 5.030$ ,  $P = 0.034$ ) and this difference was already apparent 15 min after capture ( $F(1,26) = 5.22$ ,  $P = 0.031$ ).

Birds responded to acute stress of being captured and held by a significant decrease of plasma prolactin titers (time after capture,  $F(3,63) = 14.65$ ,  $P < 0.0001$ , Fig. 3). This prolactin response to stress was slightly although not significantly more pronounced in males (Fig. 4; sex,  $F(1,21) = 3.95$ ,  $P = 0.06$ ), but was not influenced by body condition. The magnitude of the prolactin response to stress was however clearly influenced by parental effort (parental effort,  $F(1,21) = 35.82$ ,  $P < 0.001$ ; parental effort  $\times$  time after capture,  $F(3,63) = 2.86$ ,  $P = 0.04$ , Fig. 4). In chick-rearing birds, there was a slight but significant increase in plasma prolactin levels 15 min after capture ( $F(1,15) = 4.53$ ,  $P = 0.05$ ). Thereafter, plasma prolactin levels slightly declined, resulting in a modest 9% decrease in prolactin levels, after 60 min of capture and restraint. In failed birds, the effect of stress was much more pronounced and prolactin levels decreased by 41% after 60 min of capture and restraint (Fig. 3). In failed birds, there was a significant and negative relationship between stress-induced corticosterone levels and stress-induced prolactin levels ( $r = -0.71$ ,  $P = 0.02$ ,  $n = 10$ , Fig. 4). This was not observed in chick-rearing birds (Fig. 4).

**Discussion**

*Baseline hormone levels and body condition in relation to parental effort*

Baseline corticosterone levels were significantly higher in kittiwakes engaged in parental effort (chick-rearing) than in failed breeders (no parental effort). Kittiwakes lose a significant part of their body mass during the chick-rearing period (Golet and Irons, 1999; Kitaysky et al., 1999; Moe et al., 2002), and a rise in baseline corticosterone levels often mirrors a worsening of adult body condition (Kitaysky et al., 1999). In our study, there was however no significant relationship between baseline corticosterone levels and body condition at the individual level, suggesting that both failed breeders and chick-rearing birds did not have depleted protein resources (Le Ninan et al., 1988). Corticosterone is known to trigger foraging efforts (Astheimer et al., 1992; Wingfield and Silverin, 1986; Wingfield et al., 1998). The higher baseline corticosterone levels found in chick-rearing kittiwakes reflect the increased likelihood of higher foraging activity in parents that search for food both for the chicks and themselves (Kitaysky et al., 2001).

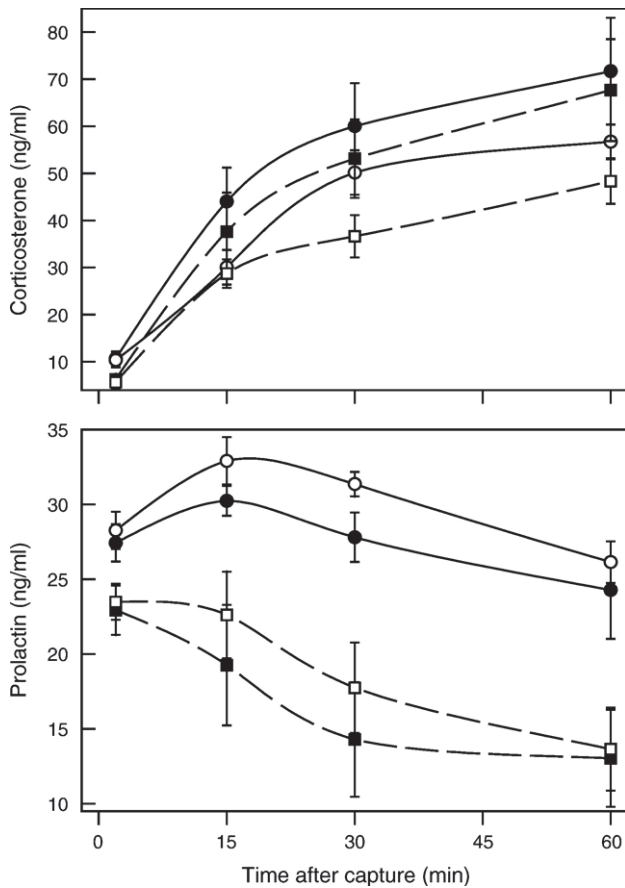


Fig. 3. Corticosterone and prolactin response to capture/handling stress of failed (squares, 6 females, 4 males) and chick-rearing (dots, 11 females and 7 males) Black-legged kittiwakes. Open symbols denote females and filled ones males. Data are expressed as mean  $\pm$  SEM.

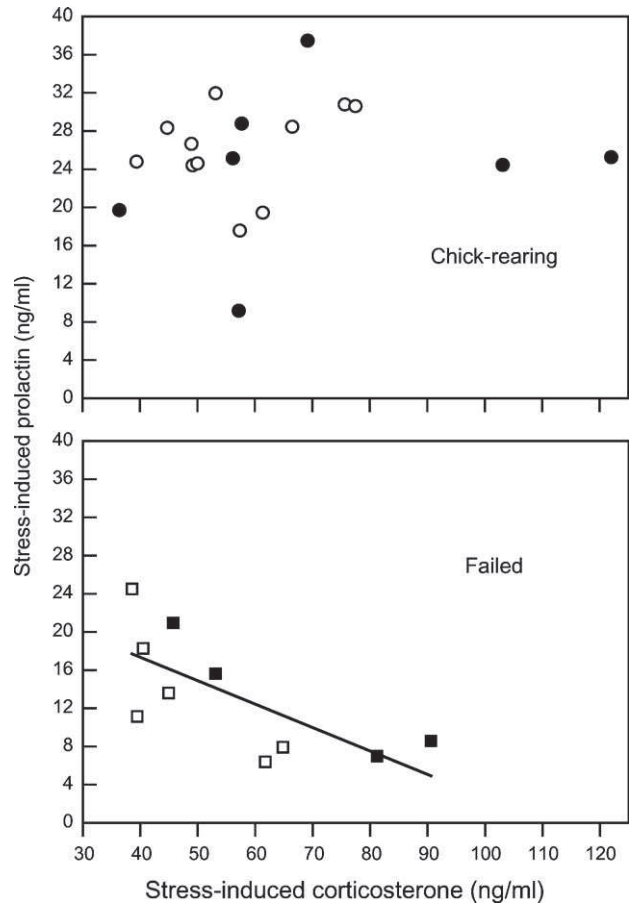


Fig. 4. Relationship between stress-induced corticosterone levels and capture/handling stress-induced prolactin levels (measured after 60 min of capture and restraint) in failed (squares, 6 females, 4 males) and chick-rearing (dots, 11 females and 7 males) Black-legged kittiwakes. Open symbols denote females and filled ones males.

In our study, plasma prolactin levels of chick-rearing birds were significantly higher than those of failed birds. This probably mirrors the parental commitment of chick-rearing kittiwakes which have to brood their young almost continuously during the first part of the chick-rearing period to protect them from cold or predator (Fyhn et al., 2001; Moe et al., 2002), a behavior that requires elevated plasma prolactin levels (Lormée et al., 2000; Sharp et al., 1988). Furthermore, elevated prolactin levels are known to trigger hyperphagia in birds (Koch et al., 2002). The higher plasma prolactin levels found in chick-rearing birds probably reflect their higher provisioning effort as they have to collect food both for the chicks and themselves. Despite being no longer engaged in parental effort, failed breeders exhibited plasma prolactin levels that were only 15% lower than levels observed in chick-rearing birds. Plasma prolactin levels in failed breeders were two times higher than levels observed in immature kittiwakes (12 ng/ml,  $n = 3$ , Chastel et al., unpublished data). Thus, as penguins (Lormée et al., 1999; Vleck et al., 2000), reproductive failure in kittiwakes does not seem to result in the same abrupt cessation of prolactin secretion that was found in other seabird species (Hall,

1986; Chastel and Lormée, 2002). Kittiwakes attend either their own nest or another nest site (squatting behavior) for several weeks after the breeding failure has occurred (Cadiou et al., 1994). It is therefore possible that such a behavior requires the maintenance of elevated plasma prolactin levels.

#### *Hormones response to stress in relation to parental effort*

Contrary to our first prediction, the magnitude of the adrenocortical response to capture/restraint stress was unaffected by parental effort as kittiwakes engaged in chick-rearing activities showed a robust 6-fold increase in corticosterone levels as did failed breeders. This result is in agreement with those found in another seabird, the Blue footed booby (*Sula nebouxi*), and which show that corticosterone response to stress was similar in breeding and non-breeding individuals (Wingfield et al., 1999). Our results might be explained by the life-history traits of the Black-legged kittiwake, which is a long-lived bird with a deferred maturity (see Golet et al., 1998 for a review). For species with a potentially long life span, the parent's lifetime reproductive interest should be to minimize the risk of mortality due to breeding to a greater extent than short-lived species (Weimerskirch, 1992). Accordingly, long-lived birds should maintain a robust stress response during breeding to ensure their post-breeding survival and the possibilities of successful future reproduction (Kitaysky et al., 1999; Wingfield et al., 1995). This is supported by data showing that in seabirds, the sensitivity of the hypothalamo–pituitary–adrenal axis increases as parental investment increases. In kittiwakes breeding in the north Pacific, the magnitude of the adrenocortical response rises steadily through the breeding cycle and peaks during the rearing of large chicks (Kitaysky et al., 1999), which corresponds to the period when parents are working the most intensely (Fyhn et al., 2001) and are in poorest condition (Kitaysky et al., 1999; Moe et al., 2002). Similarly, the strength of the adrenocortical response to handling and restraint increased throughout incubation in the Magellanic penguin (*Spheniscus magellanicus*) (Hood et al., 1998).

Male kittiwakes showed a slightly but significantly higher adrenocortical response to stress than females, independently of parental effort. In the Black-legged kittiwake, male failed breeders are more prone to squat on nests containing chicks than females (Cadiou et al., 1994) and they are also more involved in the defense of their nest against squatters (Jodice et al., 2002). The slightly more pronounced sensitivity of males to stress might be related either to the stress of challenging nest site holders or to the stress of defending the nest site against prospecting birds. This interpretation is supported by observations made on Nazca boobies (*Sula granti*) where non-breeding birds visiting unattended chicks showed an elevation of corticosterone levels (Tarlow et al., 2003).

The data presented here bring new evidence that unlike mammals, for which prolactin is released during stress (De Vlaming, 1979), birds show a decrease in circulating prolactin when exposed to acute stress (Gratto-Trevor et al., 1991; Manney et al., 1999; Sharp et al., 1989). This corroborates the findings of Delehanty et al. (1997) who showed that in a year of severe environmental stress (drought and extremely high temperatures), incubating male Wilson's phalaropes (*Phalaropus tricolor*) had lower plasma prolactin levels than in other years.

In accordance with our second prediction, the magnitude of this decrease was clearly influenced by parental effort, since kittiwakes rearing a chick only showed a modest decrease in plasma prolactin levels whereas in birds with no parental duties (failed breeders), plasma prolactin concentrations fall to levels observed in immature kittiwakes (12 ng/ml,  $N = 3$ , Chastel et al., unpublished data). Therefore, as observed for the adrenocortical response, individuals can decide to attenuate their prolactin response to stress to maintain investment in parental care and maximize their current reproductive effort during temporary stressful conditions. In kittiwakes rearing chicks, plasma levels of prolactin even slightly increased during the first 15 min of restraint, a pattern that resembles those found in mammals (De Vlaming, 1979).

When facing temporary stressful conditions, chick-rearing kittiwakes attenuate their prolactin response to stress while enhancing their secretion of corticosterone. Because high corticosterone levels are known to activate emergency behaviors associated with the ending of the current reproductive effort (Wingfield et al., 1998), our first interpretation was that the Black-legged kittiwake, as a long-lived bird, should enhance the adrenocortical response during stressful conditions to avoid any risk for post-breeding survival and future reproductive opportunities (Kitaysky et al., 1999). According to this interpretation, plasma prolactin titers should return to basal levels and parental care should cease. The relative insensitivity to stress of prolactin secretion in chick-rearing birds may provide another explanation. As pelagic seabirds, Black-legged kittiwakes exploit distant food resources (Daunt et al., 2002) and during stressful environmental conditions, such as temporary food shortage, an enhancement of corticosterone secretion would trigger foraging efforts in parents (Kitaysky et al., 2001). As a consequence, nest attendance will diminish, but due to a concomitant attenuation of the prolactin response to stress parental behavior would be maintained. In the Black-legged kittiwake, nest attendance during early chick rearing is normally 100% but can drop sharply during years of food shortage (Fyhn et al., 2001; Hamer et al., 1993; Roberts and Hatch, 1993). We suggest that this hormonal mechanism facilitates a flexible time-budget that has been interpreted as a buffer against environmental variability (Hamer et al., 1993).

On the other hand, in failed breeders, plasma prolactin levels return to basal ones as a result of capture and restraint. The standardized capture/restraint stress protocol (Wing-



field, 1994) requires repeated blood sampling and therefore loss in blood volume (a maximum of 600  $\mu$ l in our study). One way in which an individual can retain/restore blood is to dilute the blood via interstitial fluid from surrounding tissues and this may result in hemodilution (Sturkie, 1986) and lower measured plasma prolactin. One may conclude that failed breeders did not reduce their prolactin output, but rather did not increase their prolactin output in response to capture/restraint, contrary to chick-rearing birds. There was however a significant negative relationship between stress-induced corticosterone levels and stress-induced prolactin levels in failed birds. Although mechanisms linking corticosterone release and decrease in plasma prolactin levels are unclear, this correlation suggests that the more failed breeders are stressed, the more their plasma prolactin levels decrease.

Being disengaged from all parental duties, failed breeders probably have no direct fitness interests in keeping elevated plasma prolactin levels during stressful situations. The stress-induced return to basal plasma prolactin levels could lead to the desertion of the nest site and possibly of the breeding colony, whereas the concomitant increase in corticosterone levels could trigger locomotor activities and dispersal (Astheimer et al., 1992; Belthoff and Dufty, 1998). Breeding dispersal and prospection of other colonies after failure is common in kittiwakes (Cadiou et al., 1994; Danchin et al., 1998) and could be related to hormonal changes occurring during stressful events.

In conclusion, prolactin but not corticosterone responses to stress differed in relation to parental effort in the Black-legged kittiwake. Because birds engaged in parental effort were able to attenuate their prolactin response to stress, prolactin may show potential for mediating some life-history trade-offs as does the extensively studied corticosterone (Wingfield and Sapolsky, 2003). In our study, both the adrenocortical and the prolactin responses to capture/restraint stress were independent of body condition, whereas Kitaysky et al. (1999) reported that for kittiwakes breeding in the Pacific, stress-induced corticosterone levels were negatively correlated with body condition. Pelagic seabirds forage far from their breeding site and year-to-year variations in food availability affect their body reserves. Because prolactin secretion in some pelagic seabird is dependant on threshold levels of body mass (Cherel et al., 1994), future studies should focus on prolactin response to stress in situations of contrasted food supply. At the moment, few data exist on the effects of stress on avian prolactin secretion. Our results underline the need to describe the prolactin response to stress in birds in relation to different lifestyles and life-history traits.

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# Age-specific reproductive success in a long-lived bird: do older parents resist stress better?

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## Summary

1. In many vertebrates, reproductive performance increases with advancing age but mechanisms involved in such a pattern remain poorly studied. One potential mechanism may be the hormonal stress response, which shifts energy investment away from reproduction and redirects it towards survival. In birds, this stress response is achieved through a release of corticosterone and is also accompanied by a decrease in circulating prolactin, a hormone involved widely in regulating parental cares.

2. It has been predicted that, when the value of the current reproduction is high relative to the value of future reproduction and survival, as it is expected to be in older adults, the stress response should be attenuated to ensure that reproduction is not inhibited.

3. We tested this hypothesis by measuring the corticosterone and prolactin responses of known-age (8–36 years old) incubating snow petrels (*Pagodroma nivea*) to a standardized capture/handling stress protocol. We also investigated whether an attenuation of the stress responses will correlate with a lower occurrence of egg neglect, a frequently observed behaviour in snow petrels.

4. The probability of successfully fledging a chick increased from 6 years to 12 years before stabilizing after 12 years of age. Corticosterone response to stress was unaffected by age. Prolactin response to stress, however, was influenced clearly by age: in both sexes older breeders had higher stress-induced prolactin levels than younger ones. This was due to an increasing attenuation of the prolactin response to stress with advancing age in females, and in males this was due to a probably higher intrinsic capacity of older males to secrete prolactin. Moreover, higher stress-induced prolactin levels were correlated with a lower probability of neglecting the egg.

5. In young breeders, the combination of a robust corticosterone increase with a lower ability to maintain prolactin secretion during acute stress is probably one of the functional causes of their lower incubation commitment. We suggest that the ability to maintain a threshold level of prolactin during a stressful situation may be an important physiological mechanism involved in the improvement of reproductive performance with advancing age in long-lived birds.

*Key-words:* age, corticosterone, *Pagodroma nivea*, prolactin, snow petrel, stress response.

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## Introduction

Many long-term studies have described age-specific reproductive performance and the general pattern is that reproductive performance increases through the early years of breeding (Clutton-Brock 1988; Newton 1989; Weimerskirch 1992; Reid *et al.* 2003; Mauck, Huntington & Grubb 2004). Although ultimate factors

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explaining why young breeders reproduce less well than older individuals have been discussed widely (Curio 1983; Forslund & Pärt 1995), proximate factors influencing such patterns remain poorly studied (Angelier *et al.* 2006a, 2007a; Heidinger, Nisbet & Ketterson 2006). In this context physiological mechanisms deserve specific attention because they mediate life-history strategies to environmental stressors and perceived risks (Wingfield *et al.* 1998; Ricklefs & Wikelski 2002).

One potential mechanism underlying age-specific reproductive performance may be the endocrine stress response. In vertebrates, stressors such as food shortage, predators and inclement weather induce a rapid elevation of circulating glucocorticoid hormones, i.e. corticosterone or cortisol (Wingfield *et al.* 1998). This endocrine response promotes the mobilization of energy resources and the occurrence of behavioural and physiological forms that enhance the immediate survival, but also compromises the current reproductive episode by redirecting resources away from reproduction (Wingfield & Sapolsky 2003).

Although the majority of research on the stress response has focused on changes in corticosterone levels other hormones, such as prolactin, may also play a significant role (Chastel *et al.* 2005). Prolactin is a pituitary hormone that is involved widely in regulating parental behaviour and decisions in vertebrates (Buntin 1996), and has been shown to decrease in response to stressors in birds (Chastel *et al.* 2005).

According to life history theory, when the value of the current reproduction is high relative to the value of future reproduction and survival the corticosterone stress response should be attenuated to ensure that reproduction is not inhibited (Wingfield & Sapolsky 2003). Accordingly, the corticosterone stress response has been shown to be attenuated or even suppressed during the stage of the breeding cycle when adults are providing the most care for young (referred to as the 'parental care hypothesis'; Wingfield, O'Reilly & Astheimer 1995; Lendvai, Giraudeau & Chastel 2007), in the sex that provides more care for offspring (O'Reilly & Wingfield 2001) and when breeding opportunities are limited and the probability of reneating is low (referred to as the 'short season hypothesis'; Wingfield *et al.* 1995; Romero, Ramenofsky & Wingfield 1997; Silverin & Wingfield 1998). Similarly, the magnitude of the prolactin decrease has been shown to be modulated in relation to the level of parental effort (Chastel *et al.* 2005). This attenuation of the prolactin response to stress can be considered as a hormonal tactic permitting the maintenance of parental care and maximizing current reproductive efforts during temporary stressful conditions (Chastel *et al.* 2005).

As organisms age, the value of current reproduction is predicted to increase relative to the value of future reproduction and survival because future reproductive opportunities are expected to decline (Stearns 1992). Therefore, the evolutionary logic behind a modulation

of the stress response predicts that the magnitude of the response to stressors (corticosterone and prolactin) should decrease with age (Wingfield & Sapolsky 2003). One study focusing on corticosterone supported this prediction in the common tern, *Sterna hirundo* (Heidinger *et al.* 2006), but empirical data, especially relating to prolactin, are still scarce.

In the present study, we tested the hypothesis that older parents would be better able to resist stress by attenuating their corticosterone and prolactin stress responses in a long-lived bird, the snow petrel (*Pagodroma nivea* Foster). First, we checked whether breeding success improved with age using data from a long-term survey of individual breeding success from 1963 to the present (Chastel, Weimerskirch & Jouventin 1993).

Secondly, we performed a cross-sectional study during one field season to test if older parents are better able to resist the stress response. We examined the relationships between age and corticosterone and prolactin stress responses by using a standardized capture/restraint stress protocol (Wingfield *et al.* 1995). Specifically, we predicted that (1) the rate of corticosterone increase would decrease with age and that stress-induced corticosterone levels would be lower in older birds, and that (2) the rate of prolactin decrease would decrease with age and stress-induced prolactin levels would be higher in older birds.

Finally we tested, for the first time at the individual level, the hypothesis that the strength of the stress responses can mirror parental decisions, by linking the probability of egg neglect to stress-induced corticosterone and prolactin levels. Despite the large amount of published studies on the corticosterone response to acute stress (reviewed in Romero 2002), to our knowledge there are few available data showing that individual variation in the sensibility to acute stress predicts the probability to desert reproduction (Love *et al.* 2004). Snow petrels are excellent subjects for such an investigation because, as many small-sized petrels, they frequently undergo spontaneous egg neglect which delays hatching and lowers the probability for the chick to hatch (Chaurand & Weimerskirch 1994; Olson, Vleck & Vleck 2006). We predicted that (3) elevated stress-induced corticosterone levels and low stress-induced prolactin levels would be associated with a higher probability of egg neglect during the incubation shift following the stress protocol.

## Methods

### STUDY SITE AND SPECIES

Snow petrels (250–500 g) are long-lived birds with a low annual fecundity (average adult life expectancy is 30 years and only one egg is laid per season, with no replacement clutch in case of failure; Chastel *et al.* 1993; Barbraud & Weimerskirch 2001). Sexual maturity is acquired on average at 10 years of age and birds

breed annually, although a significant proportion of birds skip breeding each year (Chastel *et al.* 1993). Although males undertake slightly longer incubation shifts than females, males and females provide roughly similar amounts of parental care in this species. The age-specific breeding performances were studied on Ile des Pétrels, Pointe Géologie Archipelago, Terre Adélie (66°40' S, 140°01' E), Antarctica. The ongoing mark–recapture programme of this species began in 1963 and has provided yearly information on breeding success of every breeding pair in the surveyed colony. Details on the monitoring methodology are provided in previous studies (Chastel *et al.* 1993; Barbraud & Weimerskirch 2001). All chicks of the monitored colony have been banded prior to fledging every year since 1963. Because some of the fledged chicks have returned to the colony to breed, a substantial proportion of the individuals are of known age (from 6 to 39 years of age). Therefore, with the long-term monitoring data, we were able to investigate the influence of age on breeding performance.

#### BLOOD SAMPLE, BODY CONDITION AND EGG NEGLECT

The field study was carried out during the austral summer 2005/2006 (16–30 December 2005). During the incubation period, 48 known-age adults (8–36 years old) were captured at their nest in an area situated outside the long-term study plots to reduce handling there, so that breeding experience of the birds was unknown. Only one bird was captured per nest so that male and female data can be considered independently. All birds were bled according to the standardized capture/restraint stress protocol described by Wingfield (1994). Immediately after capture, an initial blood sample was collected from the alar vein with a 1-mL heparinized syringe and a 25-gauge needle. After collection of the initial blood samples birds were placed into cloth bags, and subsequent samples were collected 30 min later. During handling of the adult birds, their eggs were covered with cotton and kept warm. All birds were weighed to the nearest 2 g using a spring balance (Federwaage, Germany) and their skull length (head + bill) was measured to the nearest 0.5 mm. In snow petrels, skull length appears to be a reliable measure of the overall size of a bird (unpublished data). As male and female snow petrels differ greatly in size (Barbraud & Jouventin 1998), their body condition was calculated separately. Because residuals from an ordinary least-squares linear regression of body mass against body size can lead to spurious results (Green 2001), we calculated our residual indices of condition by using the residuals from a reduced major axis regression of body mass against skull length. Petrels were then marked with spots of dye on the forehead and the breast, to distinguish them from their partner, and were released at their nests. After capture and blood sample, each nest

was monitored every 2 days until the manipulated petrel was relieved by its mate. We were therefore able to know whether a bird neglected its egg during the incubation bout following capture/restraint stress protocol. Because temporary egg neglect can last during several days in snow petrels (unpublished data), checking the nests every 2 days appears to be sufficient to monitor egg neglect.

#### MOLECULAR SEXING AND HORMONE ASSAY

Blood samples were centrifuged and plasma was decanted and stored at –20 °C until they were assayed. After centrifugation, red cells were also kept frozen for molecular sexing. The sex was determined by polymerase chain reaction (PCR) amplification of part of two highly conserved genes (CHD) genes present on the sex chromosomes (Fridolfsson & Ellegren 1999) at the Centre d'Etudes Biologiques de Chizé (CEBC), as detailed in Weimerskirch, Lallemand & Martin (2005). Plasma concentrations of corticosterone were determined first by radioimmunoassay at the CEBC, as described previously (Lormée *et al.* 2003). Because blood samples were collected within 3 min of capture, they were considered to reflect baseline levels of corticosterone (Romero & Reed 2005). Plasma concentrations of prolactin were determined with the remaining plasma by a heterologous radioimmunoassay at the CEBC as detailed in Chérel *et al.* (1994). For one petrel, we did not have enough plasma to assay both baseline and stress-induced prolactin levels. For another petrel, we did not have enough plasma to assay baseline prolactin level. Pooled plasma samples of snow petrels produced a dose–response curve that paralleled chicken prolactin standard curves ('AFP 4444B', source: Dr Parlow, NHPP Harbor-UCLA Medical Center, Torrance, CA, USA), therefore validating our prolactin radioimmunoassay for snow petrels. All samples were run in one assay for both hormones (intra-assay variation, corticosterone: 7.2%, prolactin: 8.3%,  $n = 5$  duplicates).

#### STATISTICAL ANALYSES

All analyses were performed with SAS statistical software (SAS Institute Inc., version 9.1). We used a step-down approach (backward elimination) starting from the most global model. Likelihood ratio statistics and likelihood ratio tests (LRT) were used to select the most parsimonious final model (Burnham & Anderson 2002). Models were therefore simplified by eliminating independent variables with  $P \geq 0.15$ .

#### AGE-SPECIFIC REPRODUCTIVE PERFORMANCE

Using data from the long-term study plots, we tested whether age explained a significant proportion of variation in breeding performance among birds whose age was known ( $n = 135$  birds,  $n = 673$  observations).

Because previous studies on long-lived birds (reviewed in Forslund & Pärt 1995) suggested that breeding performance could increase with age (age variable) before declining in oldest birds, we included the squared term ( $\text{age}^2$ ) in the initial model designed to explain the probability of successfully fledging a chick by age.

Mean fledging success of this population varied among years, so we included a year effect into our models to minimize confounding effects of year of observation on breeding performance patterns. As emphasized by Reid *et al.* (2003), the most rigorous approach would be to model year as a random factor. However, in some years known-age petrels are likely to have been a non-representative subset of the age-structured population and estimates of random-year effect based solely on data from known-age individuals may therefore be biased (Reid *et al.* 2003). It would have been especially true during the first years of the long-term monitoring programme, when only young known-age petrels were monitored. The mean fledging success of the whole monitored population (not only of the birds of known age) in each year was included as a covariate in a generalized linear model (GLM; year variable), as suggested by Reid *et al.* (2003). Most of the snow petrels bred many times during their life, so our fledging success data were not independent. Therefore, we used a generalized linear mixed model (GLMM) by including bird identity as a random factor in our models.

#### AGE AND HORMONES LEVELS

Because male and female body condition was calculated separately and is known to differ between sexes (Barbraud & Chastel 1999), we could not analyse female and male data together to test an effect of body condition on hormone levels and hormonal stress responses. First, we therefore tested whether baseline hormone levels and hormonal stress responses were influenced by body condition in males and in females by using linear regressions. We then used the whole data set (males and females) to test the influence of sex, age and date of sampling on (1) baseline corticosterone and prolactin levels, (2) stress-induced corticosterone and prolactin levels, (3) the rates of corticosterone increase during the restraint protocol and (4) the rates of prolactin decrease during the restraint protocol. We used GLM with a normal error distribution and an identity link function and we started our analyses from general models including the explanatory variables 'sex', 'age', 'date' and the 'age  $\times$  sex' interaction.

#### HORMONE STRESS RESPONSES AND EGG NEGLECT

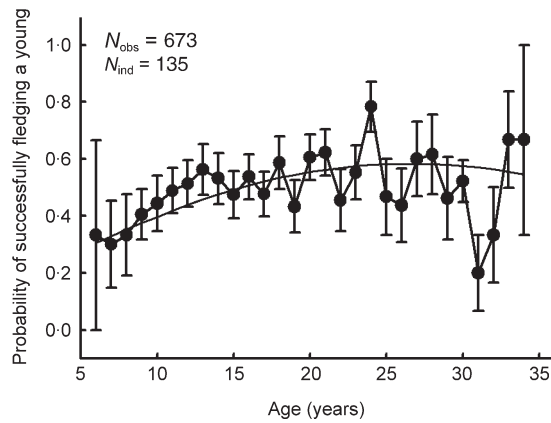
First, we tested whether the probability of neglecting the egg was influenced by body condition in males and in females by using logistic regressions. As explained in

the previous paragraph, we could not analyse female and male data together to test an effect of body condition on the probability of neglecting the egg and we therefore used one regression per sex. Then, we used the whole data set (males and females) to test the influence of age and hormone levels on the probability of neglecting the egg. Because hormone levels and age were correlated (see Results section), we had to analyse separately the influence of age and hormone levels on this probability to avoid biased results (Graham 2003). Secondly, we therefore tested whether the probability of neglecting the egg was influenced by age of individuals. We used GLM with a binomial error distribution and a logit link function and we started our analysis from the general model including the explanatory variables 'sex', 'age', 'date' and the 'age  $\times$  sex' interaction. Thirdly, we tested whether the probability of neglecting the egg during the incubation bout following the manipulation was linked to (1) baseline corticosterone levels and (2) stress-induced corticosterone levels. We started our analyses from general models including 'sex', '(baseline or stress-induced) corticosterone levels' and 'date' variables and the 'sex  $\times$  (baseline or stress-induced) corticosterone levels' interaction. Finally, we tested whether this probability was linked to (1) baseline prolactin levels and (2) stress-induced prolactin levels. Because prolactin levels were influenced by the 'sex' variable (see Results), we faced multicollinearity problems in these analyses and we could not include simultaneously the 'sex' and '(baseline or stress-induced) prolactin levels' variables in a model (Graham 2003). To solve this problem, we standardized (baseline or stress-induced) prolactin levels in each sex separately by subtracting the sexual mean prolactin levels from each observed value, and dividing by the standard deviation. This statistical approach permits avoidance of the potential confounding effects of a factor, i.e. sex, in our study, on the relationship between two parameters, i.e. prolactin levels and the probability of neglecting the egg. Because sex and standardized prolactin levels were no longer correlated, we started our analyses from the general model including the 'sex', '(baseline or stress-induced) prolactin levels' and 'date' variables and the 'sex  $\times$  (baseline or stress-induced) prolactin levels' interaction.

## Results

#### AGE AND REPRODUCTIVE PERFORMANCES

Fledging success increased with age among the snow petrels (GLMM, age,  $F_{1,534} = 7.15$ ,  $P = 0.008$ ). The probability of fledging a chick successfully increased from 6 years to 12 years before stabilizing after 12 years of age and was, however, not influenced significantly by the quadratic term ( $\text{age}^2$ ), showing that fledging success did not decline significantly in the oldest petrels of our study (Fig. 1, GLMM,  $\text{age}^2$ ,  $F_{1,534} = 2.25$ ,  $P = 0.134$ ).



**Fig. 1.** Relationship between age and the probability of successfully fledging a young (mean  $\pm$  SE) among incubating snow petrels. This probability varied significantly with individual age and slightly with individual age<sup>2</sup>. Solid lines represents the relationship described by the best model selected to explain this probability.  $N_{\text{obs}}$  and  $N_{\text{ind}}$  mean, respectively, the total number of observations and individuals.

#### AGE, BODY CONDITION AND BASELINE HORMONAL LEVELS

Baseline corticosterone levels were correlated negatively with body condition in females ( $F_{1,21} = 8.12$ ,  $P = 0.010$ ,  $r^2 = 0.279$ ), but not in males ( $F_{1,23} = 0.13$ ,  $P = 0.726$ ). Baseline prolactin levels were not correlated with body condition in both sexes ( $P > 0.60$  for both regressions). Moreover, body condition did not vary with age and

date in either sex (age:  $P > 0.35$ ; date:  $P > 0.05$ ). Baseline corticosterone levels were not influenced by date of sampling, by sex or by the 'age-sex interaction' (Table 1). The selected model that explains baseline corticosterone levels included only the 'age' variable (Table 1). However, the influence of age on baseline corticosterone levels was not significant (Table 1; Fig. 2a,b). Baseline prolactin levels were higher in females than in males and were correlated positively with date of sampling (Table 1). Moreover, there was an effect of 'age' and 'age-sex' interaction on baseline prolactin levels (Table 1). Baseline prolactin levels were correlated positively with age in males, but not in females (parameter estimates of the selected model; females:  $\chi^2 = 0.41$ ,  $P = 0.520$ ; males:  $\chi^2 = 5.88$ ,  $P = 0.015$ ; Fig. 2c,d). Baseline prolactin levels were not related to baseline corticosterone levels ( $F_{1,43} = 2.58$ ,  $P = 0.116$ ).

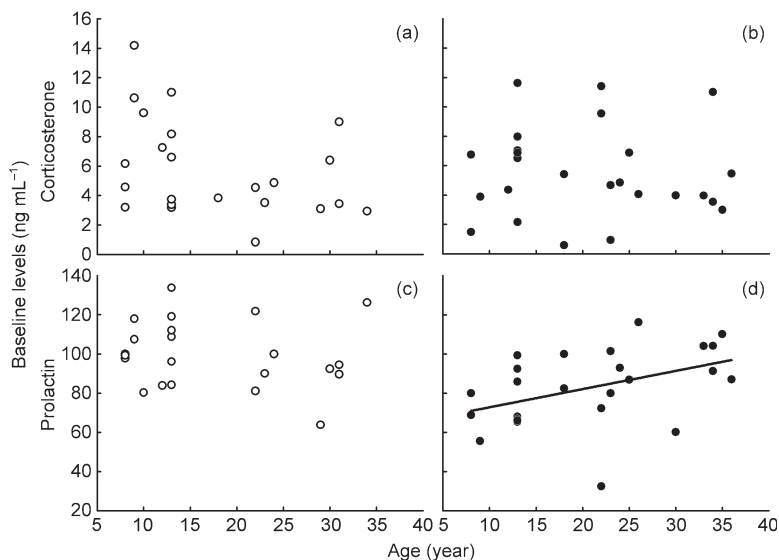
#### AGE AND HORMONAL RESPONSE TO STRESS

Males and females responded to the stress of being captured and held by a significant increase in corticosterone levels and a significant decrease in prolactin levels ( $P < 0.001$  for all the paired Student's  $t$ -tests; Fig. 3a,b). Stress-induced hormonal levels, rates of corticosterone increase and rates of prolactin decrease were not influenced by body condition in both sexes ( $P > 0.05$  for all the regressions).

Rates of corticosterone increase were not influenced by sex, date of sampling and age of individuals

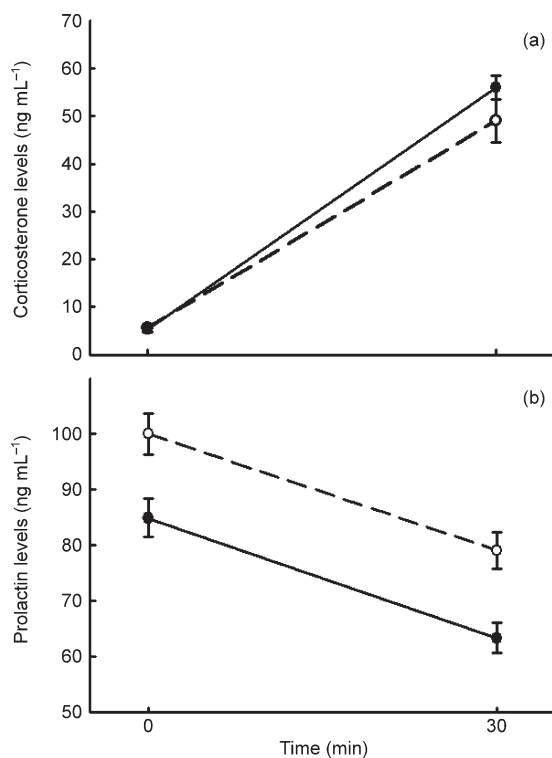
**Table 1.** Model selection and GLM (normal error distribution, identity link function) to test the influence of age on baseline corticosterone and prolactin levels, rates of corticosterone increase, rates of prolactin decrease and stress induced corticosterone and prolactin levels. The best models (in bold type) were selected by using a step-down approach starting from the most global model (models were simplified by eliminating independent variables with  $P \geq 0.15$ )

Dependent variable	Independent variables	d.f.	<i>F</i>	<i>P</i>
Baseline corticosterone levels (ng mL <sup>-1</sup> ) ( <i>n</i> = 48)	Sex	1,45	0.34	0.565
	<b>Age (years)</b>	<b>1,46</b>	<b>2.32</b>	<b>0.134</b>
	Age $\times$ sex	1,44	1.47	0.232
	Date (Julian day)	1,43	1.43	0.238
Baseline prolactin levels (ng mL <sup>-1</sup> ) ( <i>n</i> = 46)	<b>Sex</b>	<b>1,41</b>	<b>11.10</b>	<b>&lt; 0.001</b>
	<b>Age (years)</b>	<b>1,41</b>	<b>2.22</b>	<b>0.144</b>
	Age $\times$ sex	1,41	6.29	0.016
	Date (Julian day)	1,41	4.26	0.045
Rates of corticosterone increase (ng mL <sup>-1</sup> min <sup>-1</sup> ) ( <i>n</i> = 48)	<b>Sex</b>	<b>1,46</b>	<b>2.22</b>	<b>0.143</b>
	Age (years)	1,45	0.05	0.825
	Age $\times$ sex	1,44	1.93	0.172
	Date (Julian day)	1,43	1.69	0.200
Rates of prolactin decrease (ng mL <sup>-1</sup> min <sup>-1</sup> ) ( <i>n</i> = 46)	<b>Sex</b>	<b>1,42</b>	<b>&lt; 0.01</b>	<b>0.944</b>
	<b>Age (years)</b>	<b>1,42</b>	<b>1.40</b>	<b>0.244</b>
	age $\times$ sex	1,42	11.51	0.001
	Date (Julian day)	1,41	0.06	0.805
Stress-induced corticosterone levels (ng mL <sup>-1</sup> ) ( <i>n</i> = 48)	<b>Sex</b>	<b>1,44</b>	<b>1.71</b>	<b>0.198</b>
	<b>Age (years)</b>	<b>1,44</b>	<b>0.22</b>	<b>0.641</b>
	Age $\times$ sex	1,44	2.39	0.130
	Date (Julian day)	1,43	2.15	0.150
Stress-induced prolactin levels (ng mL <sup>-1</sup> ) ( <i>n</i> = 47)	<b>Sex</b>	<b>1,43</b>	<b>18.63</b>	<b>&lt; 0.001</b>
	<b>Age (years)</b>	<b>1,43</b>	<b>9.70</b>	<b>0.003</b>
	Age $\times$ sex	1,42	0.11	0.737
	Date (Julian day)	1,43	7.06	0.011



**Fig. 2.** Relationship between age and baseline corticosterone/prolactin levels among incubating snow petrels. Baseline corticosterone levels were not correlated with individual age. Baseline prolactin levels were significantly positively correlated with individual age in males (solid line), but not in females. Open symbols denote females and filled symbols denote males.

(Table 1). Stress-induced corticosterone levels were not influenced by date of sampling (Table 1). The selected model explaining stress-induced corticosterone levels included the 'sex' and 'age' variables and the 'age-sex' interaction (Table 1). However, parameter estimates of this selected model showed that stress-induced



**Fig. 3.** Changes in plasma corticosterone and prolactin levels in response to the standardized stress protocol (corticosterone:  $n = 48$ , prolactin,  $n = 46$ ). Open symbols denote females and filled symbols denote males.

corticosterone levels did not vary between sexes and did not vary with age either in females or males (Fig. 4a,b).

Rates of prolactin decrease were not influenced by sex and by date of sampling (Table 1). However, there was a highly significant effect of 'age-sex' interaction on rates of prolactin decrease (Table 1). Rates of prolactin decrease were correlated negatively with age in females but not in males (parameter estimates; females:  $\chi^2 = 10.90$ ,  $P = 0.001$ ; males:  $\chi^2 = 2.01$ ,  $P = 0.157$ ). Stress-induced prolactin levels were higher in females than in males and were correlated positively with date of sampling and age in both sexes (Table 1, Fig. 4c,d). Stress-induced prolactin levels were not related to stress-induced corticosterone levels ( $F_{1,44} = 0.40$ ,  $P = 0.531$ ).

#### HORMONAL STRESS RESPONSE AND EGG NEGLECT

The probability of neglecting the egg during the incubation bout following the stress protocol was not influenced by sex and by date of sampling (Table 2). However, young birds seem to be more likely to neglect their egg than old ones, although the effect of age was not statistically significant (Table 2). This probability of neglecting the egg was neither influenced by body condition ( $P > 0.35$  in both sexes), nor by baseline or stress-induced corticosterone levels (Table 2). However, there was a significant influence of stress-induced prolactin levels on this probability. Incubating petrels with low stress-induced prolactin levels were more likely to neglect their egg than ones with high stress-induced prolactin levels (Fig. 5a,b; Table 2).

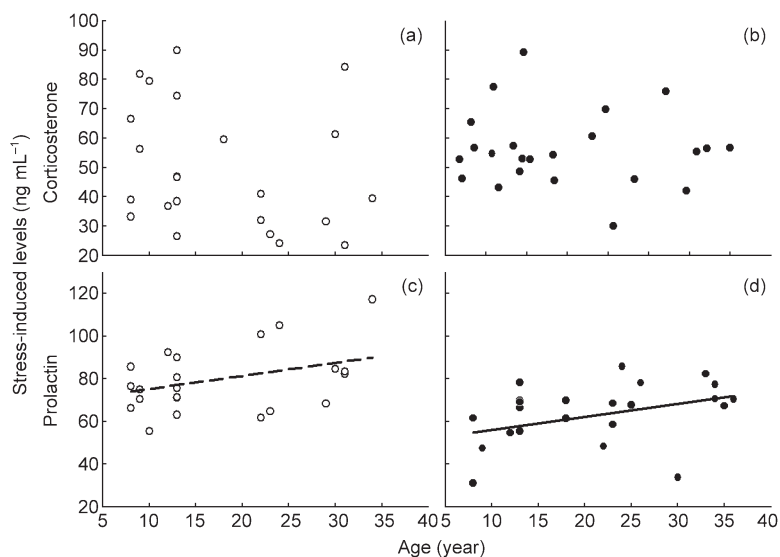
#### Discussion

In the present study we found, as in many other studies (reviewed in Forslund & Pärt 1995), that breeding success improved significantly with advancing age. Such age-specific improvement in breeding performance was not paralleled by a concomitant decrease in the magnitude of the corticosterone response to stress. Specifically, corticosterone response to stress and stress-induced corticosterone levels were not correlated with age. Prolactin response to stress was, however, influenced clearly by age: in females there was an attenuation of the prolactin response to stress with advancing age and in both sexes older breeders had higher stress-induced prolactin levels than younger ones. Moreover, elevated stress-induced prolactin levels were correlated with a lower probability of egg neglect. To our knowledge, this is the first study to show that stress-induced prolactin levels vary with age and can be linked to parental decisions.

#### AGE AND THE HORMONAL RESPONSE TO STRESS

Because a reduced sensitivity of the hypothalamo-pituitary-adrenal (HPA) system is thought to maximize





**Fig. 4.** Relationship between age and stress-induced corticosterone/prolactin levels among incubating snow petrels. Stress-induced corticosterone levels were not correlated with individual age. Stress-induced prolactin levels were significantly positively correlated with individual age in females (dashed line) and males (solid lines). Open symbols denote females and filled symbols denote males.

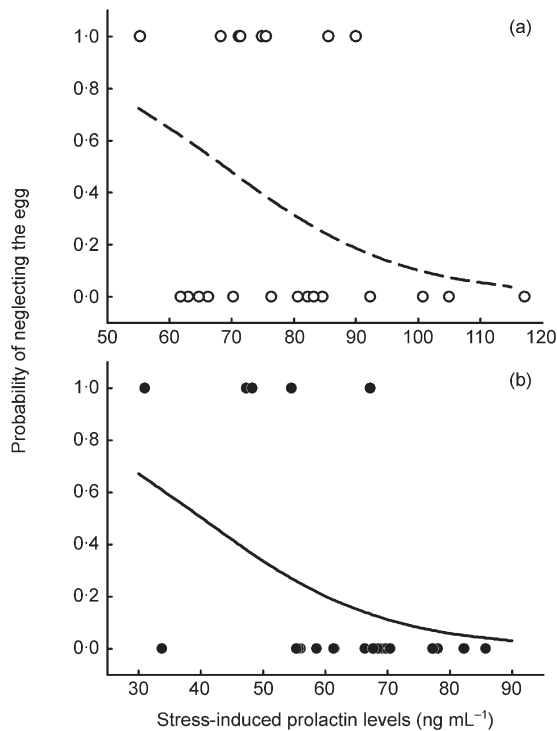
parental investment by preventing stressors from triggering an adrenocortical stress response (Wingfield *et al.* 1995; O'Reilly & Wingfield 2001; Holberton & Wingfield 2003; Moore & Jessop 2003), we predicted that the sensitivity of the HPA system would decrease with advancing age. Our data show, however, that the magnitude of the adrenocortical response to capture/restraint stress was unaffected by age, as incubating snow petrels showed a robust 14-fold increase in corticosterone levels independently of their age. Because

age did not affect the magnitude of their corticosterone response to stress it suggests, at the physiological level, that young and old snow petrels do not differ in their perception of risk and show a similar functional response of their HPA system over a 30-min capture/restraint protocol. One recent study found that corticosterone response to stress decreased with age in the common tern (Heidinger *et al.* 2006). How to explain such a difference? One possibility is that snow petrels often skip breeding opportunities and show a large variance in their age at first breeding (Chastel *et al.* 1993; Chastel 1995), so that two birds of the same age can differ greatly in their respective breeding experience and probable reproductive residual value. Two recent studies on albatrosses (Angelier *et al.* 2006a, 2007a) have shown that breeding experience was a much better descriptor of corticosterone levels than age *per se*. In our study, we sampled birds of various ages but their breeding experience was unknown. It is then possible that the magnitude of the stress response in snow petrels would be explained better by variations in breeding experience rather than age, whereas in shorter-lived common terns age and experience are probably correlated more highly (Nisbet 2001).

In our study, baseline corticosterone levels and body condition did not vary with age in incubating snow petrels. Baseline corticosterone levels are considered to reflect the energetic status of an individual (Landys, Ramenofsky & Wingfield 2006) and elevated levels have been associated with reduced food availability, low body condition and foraging success (Kitaysky, Wingfield & Piatt 1999; Adams *et al.* 2005; Angelier *et al.* 2007b). Accordingly, we found that elevated corticosterone levels were associated with low body

**Table 2.** Model selection and GLM (binomial error distribution, logit link function) to test the influence of age, baseline and stress-induced corticosterone, baseline and stress-induced prolactin levels, on the probability of neglecting the egg. The best models (in bold type) were selected by using a step-down approach starting from the most global model (models were simplified by eliminating independent variables with  $P \geq 0.15$ )

Dependent variable	Independent variables	d.f.	F	P
Probability of neglecting the egg ( $n = 48$ )	Sex	1,45	0.68	0.415
	<b>Age (year)</b>	<b>1,46</b>	<b>3.17</b>	<b>0.082</b>
	Age $\times$ sex	1,44	0.32	0.573
	Date (Julian day)	1,43	0.03	0.872
Probability of neglecting the egg ( $n = 48$ )	Sex	1,45	1.01	0.321
	<b>Baseline corticosterone levels</b>	<b>1,46</b>	<b>2.72</b>	<b>0.106</b>
	Baseline corticosterone levels $\times$ sex	1,44	1.93	0.307
Probability of neglecting the egg ( $n = 48$ )	Sex	1,45	1.18	0.284
	Stress-induced corticosterone levels	1,46	1.06	0.308
	Stress-induced corticosterone levels $\times$ sex	1,44	0.36	0.552
	Date (Julian day)	1,43	0.07	0.790
Probability of neglecting the egg ( $n = 46$ )	Sex	1,44	2.12	0.152
	Baseline prolactin levels	1,43	1.83	0.184
	Baseline prolactin levels $\times$ sex	1,42	0.04	0.839
	Date (Julian day)	1,41	0.02	0.898
Probability of neglecting the egg ( $n = 47$ )	Sex	1,44	1.75	0.193
	<b>Stress-induced prolactin levels</b>	<b>1,45</b>	<b>5.73</b>	<b>0.021</b>
	Stress-induced prolactin levels $\times$ sex	1,42	2.05	0.160
	Date (Julian day)	1,43	1.22	0.276



**Fig. 5.** Relationship between stress-induced prolactin levels and the probability of neglecting the egg during the incubation bout following the manipulation among snow petrels. GLMs with a binomial error distribution and a logit link function were used. The probability of neglecting the egg was significantly negatively correlated with stress-induced prolactin levels in females (dashed line) and males (solid line). Open symbols denote females and filled symbols denote males.

condition in females, but not in males. The absence of relationship between age and either baseline corticosterone levels or body condition suggests that young females were as able as older ones to extract energy from the environment and to maintain their body condition during the incubation period. However, it is also possible that baseline corticosterone levels and body condition varied with breeding experience rather than age in this species, as found previously in procellariiformes species (corticosterone: Angelier *et al.* 2006a, 2007a; body condition: Weimerskirch 1992; Chastel, Weimerskirch & Jouventin 1995). The absence of relationship between baseline corticosterone levels and body condition in males is difficult to interpret. It is possible that males, being significantly larger than females, may be less energetically constrained than females during incubation (Barbraud & Chastel 1999).

As reported previously in another bird species (Chastel *et al.* 2005), we found that prolactin levels decreased in response to an acute stress in incubating snow petrels. The data presented here confirm that birds show a decrease in circulating prolactin when exposed to acute stress (Sharp *et al.* 1989; Chastel *et al.* 2005).

Moreover, according to our prediction, stress-induced prolactin levels reached after 30 min of restraint were correlated positively with age. In the

snow petrel, the way age influenced the prolactin response to stress appears to be sex-dependent. In males, the magnitude of decrease in prolactin levels was similar in young and old individuals. However, older males showed higher baseline prolactin levels than young ones, as found previously in other species (Deviche, Wingfield & Sharp 2000; Pr  ault *et al.* 2005; Angelier *et al.* 2006a, 2007a), therefore explaining their higher stress-induced prolactin levels. Higher stress-induced prolactin levels found in older males may originate from their higher intrinsic capacity to secrete prolactin (Angelier *et al.* 2006a, 2007a).

In females, baseline prolactin levels were not correlated with age, but the magnitude of the decline in prolactin levels was significantly lower in older individuals, resulting therefore in a positive relationship between stress-induced prolactin levels and age. This suggests that older females were better able to maintain prolactin production during the first minutes of the stress episode. Therefore, according to our main prediction, older females seem to actually show a better hormonal resistance to acute stress than younger ones. This better hormonal resistance to stress in older females might result from an acclimation process to handling and capture with advancing age. However, it is unlikely because known-age snow petrels were sampled outside the long-term study plots and these birds have been handled very seldom prior to our study.

#### AGE, STRESS AND REPRODUCTIVE PERFORMANCES

Prolactin is a hormone involved in parental behaviour (Buntin 1996) and several studies have reported that prolactin enhances the expression of parental (Wang & Buntin 1999; Duckworth, Badyaev & Parlow 2003) and alloparental behaviours (Vleck *et al.* 1991; Schoech, Mumme & Wingfield 1996; Angelier *et al.* 2006b). Supporting this influence of prolactin levels on parental decisions, we found that parents with low stress-induced prolactin levels were more likely to neglect their egg.

Rather than a sole effect of either prolactin or corticosterone on parental decisions, corticosterone and prolactin might act jointly to mediate parental decisions in long-lived birds (Cherel *et al.* 1988, 1994; Chastel *et al.* 2005). The decrease in prolactin levels in response to an acute stress would probably reduce the motivation of young parents to continue incubation, whereas the concomitant increase in corticosterone levels would trigger escape behaviour (Wingfield *et al.* 1998), leading to temporary desertion of the nest (Silverin 1986). On the contrary, older parents may be better able to maintain a threshold level of prolactin during a stress episode, therefore retaining the willingness to incubate the egg and reducing or even suppressing the negative behavioural consequences of elevated corticosterone levels (Chastel *et al.* 2005; Criscuolo *et al.* 2005).

In conclusion, in young snow petrels, the combination of a robust corticosterone increase with a lower ability to maintain prolactin secretion during acute stress (such as inclement weather or temporary food shortage) is probably one of the functional causes of their lower incubation commitment. We suggest that the capacity to maintain a threshold level of prolactin during a stress episode may be an important physiological mechanism involved in the improvement of reproductive performance with advancing age in long-lived birds. To understand fully how modulation of the hormonal stress response governs parental investment, future studies should therefore examine simultaneously corticosterone and prolactin responses to stress in relation to different life-history strategies, contrasting environmental conditions (low/high food availability) and contrasting energetic demands (incubation/brooding).

Can such a mechanism help us to elucidate the main evolutionary theories addressing the effect of age and experience on reproductive success (Curio 1983; Forslund & Pärt 1995)? An attenuation of the stress response in reproducing vertebrates has been interpreted as a mechanism allowing parents to modify reproductive investment flexibly with age (Wingfield & Sapolsky 2003; Heidinger *et al.* 2006). Thus the observed ability of older females to attenuate the prolactin response to stress may favour the 'restraint hypothesis' (Curio 1983), which suggests that breeding investment increases with age as the residual reproductive value declines and breeding events become more valuable (Curio 1983). However, in our study, corticosterone response was unaffected by age and hence the likely perception of the stressor through the central nervous system and the HPA axis. Does this mean that corticosterone response to stress would not be involved as a mechanism permitting reproductive investment to be increased over age whereas the prolactin would? In males, lower stress-induced prolactin levels did not originate from a lower ability to resist stress but rather from a better ability to secrete prolactin, thus suggesting that young males may be more physiologically constrained (the 'constraint hypothesis' Curio 1983). It is also possible that the relationship between age and stress-induced prolactin levels could result from a selection process (Forslund & Pärt 1995; Mauck *et al.* 2004). Hence, parents with elevated stress-induced prolactin levels may be high-quality individuals and might be more likely to survive during several years and therefore to be represented in old age classes. This would imply that the magnitude of the stress response is fixed early in the reproductive career of an individual. However, it is unlikely, as Lendvai *et al.* (2007) showed experimentally in the house sparrow (*Passer domesticus*) that, in a given season, the same individual can modulate its hormonal stress response flexibly according to the value of the reproductive event. Therefore, the decreased prolactin sensitivity with advancing age may not result solely from a selection

process in snow petrels. Despite the difficulty of monitoring longitudinal hormonal data in long-lived birds, future studies should aim to compare hormonal stress responses of the same individual at different ages in order to confirm that hormonal sensitivity to stress decline with advancing age independently of selection process.

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# Stress Response and the Value of Reproduction: Are Birds Prudent Parents?

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**ABSTRACT:** In vertebrates, stressors such as starvation or predator attacks stimulate the rapid elevation of circulating glucocorticoid hormones, triggering physiological and behavioral responses that aid immediate survival but simultaneously inhibit reproduction. This stress response has been proposed to serve as a physiological mediator of life-history trade-offs: when the value of current reproduction is high relative to the value of future reproduction and survival, a mitigated stress response is expected to enable successful breeding and maximize fitness. Using phylogenetic comparative analyses, we investigated baseline and peak stress-induced plasma corticosterone levels during parental care in 64 bird species. We found that (1) species with a higher value of the current brood relative to future breeding mounted weaker corticosterone responses during acute stress, and (2) females in species with more female-biased parental care had weaker corticosterone responses. These results support the brood value hypothesis, suggesting that the stress response evolves as an adaptive basis for life-history strategies. Further, we found that (3) baseline corticosterone correlated positively with brood value and negatively with body mass, and (4) peak corticosterone was greater in species breeding at higher latitudes. The latter findings suggest that circulating corticosterone concentrations might be matched to the anticipated demands and risks during nesting.

**Keywords:** corticosterone, life history, brood value, evolution, comparative method, birds.

## Introduction

According to life-history theory, animals are faced with trade-offs in the allocation of limited resources to different functions (Stearns 1992). The general reproductive effort model based on the cost of reproduction (Williams 1966) predicts that breeding individuals trade off their resources

between current reproductive investment and their own survival, that is, future reproduction (Stearns 1992). Whether the individual should allocate more to current breeding or to survival depends on the relative importance of these two life-history components in maximizing fitness. For example, long-lived species are expected to behave as “prudent parents” and refrain from jeopardizing their own survival during reproduction (Drent and Daan 1980; Stearns 1992) because their lifetime reproductive success is primarily a function of adult survival rather than of seasonal fecundity (Williams 1966). In researching as to how animals can adopt such adaptive life-history strategies, growing attention is being paid to physiological mechanisms, as these may adjust life-history decisions to environmental conditions and perceived risks (Wingfield et al. 1998; Ricklefs and Wikelski 2002).

One potential mechanism underlying the regulation of reproductive effort may be the endocrine stress response. In vertebrates, stressors such as inclement weather, food shortage, or predator attacks activate the hypothalamic-pituitary-adrenal cortex (HPA) axis to elevate the circulating levels of glucocorticoid hormones (CORT) that promote physiological and behavioral changes to enhance immediate survival but to simultaneously inhibit reproduction (reviewed by Wingfield and Sapolsky 2003). High CORT elevations shift the animals into an “emergency life-history stage” in which they reduce or even abandon their parental activities (Wingfield et al. 1998). Thereby, the stress response redirects energy investment away from breeding, toward survival. Such a shift should be adaptive for “prudent parents” but less so when the value of current reproduction is high compared with the value of survival and future reproduction, such as in species with few breeding opportunities (Wingfield and Sapolsky 2003). Thus, the “brood value hypothesis” (Heidinger et al. 2006; Lendvai et al. 2007; Lendvai and Chastel 2008) proposes that

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the stress response should be modulated as a function of the relative importance of current reproduction; for instance, when the value of current reproduction is relatively high, the stress response should be mitigated to ensure that the current breeding attempt is not compromised.

This hypothesis has been supported by a number of correlative studies in free-living birds. For example, the stress response was found to be attenuated when the chances for rebreeding were limited, such as in northerly breeding populations with shorter breeding seasons (e.g., Silverin et al. 1997; O'Reilly and Wingfield 2001; Breuner et al. 2003; but see Wingfield et al. 1995*a*) and in older individuals (Heidinger et al. 2006; but see Angelier et al. 2007*b*). A reduced stress response was also associated with highest parental investment in comparisons across different breeding phases (Meddle et al. 2003) and in the sex that invests more into offspring care (Wingfield et al. 1995*b*; O'Reilly and Wingfield 2001; Holberton and Wingfield 2003). Moreover, recent experimental manipulations of the brood value demonstrated flexible modulation of the stress response within individuals either up- or downward according to the value of current reproductive attempt (Lendvai et al. 2007; Lendvai and Chastel 2008). Parents' stress responses were higher when they had less valuable (i.e., smaller or poor-quality) broods than when they were tending more or better-quality chicks (Lendvai et al. 2007; Lendvai and Chastel 2008). Altogether, these results corroborate that the stress response may function as a physiological mediator for the adaptive allocation between current and future reproduction. Nevertheless, we still lack large-scale interspecific studies that would help to assess the generality of this pattern (Wingfield et al. 1995*b*).

It has been suggested that hormonal mechanisms that serve as physiological bases for life-history trade-offs may be targets of selection just like other phenotypic traits (Ketterson and Nolan 1992; Hau 2007). In support of this idea, recent comparative studies of androgen levels have repeatedly shown that testosterone profiles may evolve as species-specific characteristics, differing consistently among species according to the sexual and social challenges of their breeding system and ecology (Hirschenhauser et al. 2003; Goymann et al. 2004; Garamszegi et al. 2005; Ketterson et al. 2005; Møller et al. 2005; Bókonyi et al. 2008). For example, both sexes maintain higher testosterone levels in species with stronger intrasexual competition (Garamszegi et al. 2005; Ketterson et al. 2005). Similarly, one might expect the stress response to be matched to the species's breeding environment and life history, as a recent review strongly argued for the functional significance of plasma CORT titers per se (Williams 2008). Under this scenario, the brood value hypothesis predicts that species with relatively high importance of

current reproduction should be selected to circulate lower levels of stress hormones.

In this study, we investigated the relationship between brood value and CORT level in birds at the interspecific level, using the phylogenetic comparative approach. This methodology has been successfully employed to detect evolutionary associations between various ecological and phenotypic traits, and recent studies have demonstrated the usefulness of this approach in identifying factors that influence the evolution of certain hormone levels (e.g., testosterone). Here we have compiled the most extensive data set of available CORT measures of avian species to investigate whether CORT levels may be viewed as species-specific traits and whether they may evolve in accordance with the relative importance of current reproduction. Specifically, we focused on two interspecific predictions of the brood value hypothesis. First, the stress response should be mitigated during nesting in species with higher brood value, that is, in which a single breeding attempt contributes a greater proportion of lifetime reproductive value because, for example, of limited opportunities to breed. Second, the sex differences in CORT levels should be inversely related to the sex bias in parental investment across species; that is, the stress response should be mitigated in the sex that invests more in the current brood.

## Material and Methods

### *CORT Levels*

We searched the primary literature published until May 2007 for data on circulating corticosterone concentrations of free-living birds, measured in the parental phase of breeding (incubation and/or chick rearing). We extracted two hormone levels (in ng mL<sup>-1</sup>): (1) Baseline CORT level, that is, measured from the sample taken immediately after capture within 3 min (Romero and Reed 2005) or more if the study validated that CORT level had not begun to increase during that time interval (range 0–7 min; mean + SD = 3.11 + 1.12 min,  $n = 275$ ). This measure is an approximation of the seasonal baseline level of CORT that the animals should maintain to be able to cope with the predictable demands of the current life-history stage (allostatic load; "state B," sensu Landys et al. 2006). (2) Peak CORT level, that is, the highest stress-induced level following capture and restraint for 5–70 min (mean + SD = 38.81 + 16.82 min,  $n = 170$ ). This measure corresponds to the acutely elevated levels of CORT triggered by unpredictable perturbations that cannot be prepared for and may shift the animals into the "emergency life-history stage" (allostatic overload; "state C," sensu Landys et al. 2006). For both baseline and peak CORT levels, we used the mean (weighted by sample size) value of the different estimates when more than one CORT measurement was

available for a species. Whenever the data allowed, we collected CORT levels separately for the sexes and for the incubation and chick-rearing phases. However, we also calculated both baseline and peak CORT levels as averages for the whole parental period and for the two sexes because, for many species, only such combined measurements were available. We excluded CORT data measured in individuals that were subjected to experimental manipulations (e.g., testosterone implantation, brood-size manipulation) or extreme conditions (e.g., severe storms) that are expected or known to alter CORT levels. In total, we found CORT data for 64 bird species from 104 studies (table A1 in the online edition of the *American Naturalist*). Both CORT variables were  $\log_{10}$  transformed to normalize distributions. Sex differences in CORT levels were expressed as  $\log_{10}$  (male CORT/female CORT).

To test the reliability of species-specific CORT levels, we conducted several analyses. First, we tested whether CORT measures were related to the parameters of the hormone assays (sensitivity, inter- and intra-assay coefficients of variation [CVs], recovery, and accuracy) applied by different studies. We also tested whether CORT measures differed between studies that did or did not perform chromatographic separation of steroid phases prior to assay. Second, we tested whether CORT measures were influenced by sample size, handling time (i.e., the time elapsed from capture until the collection of blood sample), or breeding phase (i.e., incubation, chick rearing, or not specified). All of these analyses included study identity as a random factor to handle the repeated nature of data (several studies measured more than one species). Third, we tested the repeatability of CORT data measured in the breeding season by different studies for the same species, following Lessells and Boag (1987). Finally, because among-species differences might arise between different labs specializing in different species, we also assessed the differences attributable to different labs.

#### *Life-History Variables*

To quantify the importance of current reproduction across species, we collected data on life-history traits related to lifetime reproductive success, specifically on (1) average clutch size, that is, number of eggs laid in a single breeding attempt; (2) number of broods raised successively per season; and (3) average reproductive life span, that is, number of years over which offspring may be produced. Because direct data on average life span were not available for most species, we extracted data on the annual rate of adult mortality, preferably from capture-recapture studies based on large samples and/or long-term observations (for further details, see Liker and Székely 2005), and we estimated average reproductive life span as  $1/\text{mortality}$ . This estimate

correlates strongly with known reproductive life spans ( $r = 0.92$ ,  $P < .001$ ,  $n = 14$ ). By multiplying the above three variables, we calculated the number of offspring that can be produced during the lifetime of an average individual of the species. We expressed the value of current reproduction relative to the lifetime reproductive output of a species (henceforth, “brood value”) as  $\log_{10}$  (clutch size/[clutch size  $\times$  broods per year  $\times$  average reproductive lifespan]).

To calculate the sex differences in parental care, we scored the contribution of each sex to incubation, chick feeding, and brooding following Liker and Székely (2005): no contribution to care (score = 0), 1%–33% of care provided (score = 1), 34%–66% of care provided (score = 2), and 67%–100% of care provided (score = 3). Parental care of each sex was calculated as the sum of their scores for the three parental activities (range, 0–9). The difference between male and female care was used as an index for the sex bias in full parental care; that is, species with more female-biased care have smaller (more negative) values, whereas species with more male-biased care have larger (more positive) values.

We also considered two potentially confounding variables. First, it has been proposed that larger birds can rely on larger fat and/or protein reserves and thus may cope with stressors by using their energy stores instead of elevating CORT levels (Wingfield et al. 1995b). Second, CORT levels are known to vary latitudinally (e.g., Breuner et al. 2003), perhaps in part because of weather conditions (Romero et al. 2000). Therefore, we collected data on body mass (the average of male and female body mass in the breeding season) and the latitude of breeding (the absolute distance from the equator, averaged for those populations in which CORT levels had been measured). The complete data set and data sources are given in appendix A.

#### *Phylogenetic Analyses*

To investigate the evolutionary relationships between CORT levels and life-history variables, we used phylogenetic generalized least squares (PGLS) models (Pagel 1997, 1999). This approach controls for the nonindependence among species by incorporating a matrix of the expected covariances among species on the basis of their phylogenetic relationships (Martins and Hansen 1997; Pagel 1997, 1999), and it also estimates the importance of phylogenetic corrections in the data (Freckleton et al. 2002). We thus conducted all analyses by setting the degree of phylogenetic dependence ( $\lambda$ ) to the most appropriate degree evaluated for each model by likelihood ratio statistics. To represent phylogenetic relationships among species, we compiled a composite tree using recent molecular phylogenies (app. B in the online edition of the *American Naturalist*). We



**Table 1:** Proportion of variance ( $\eta^2$ ) explained by species differences and lab differences in corticosterone (CORT) measures

CORT	<i>F</i>	df	<i>P</i>	$\eta^2$
Baseline:				
Species	15.99	(61, 177)	<.001	.85
Lab	7.81	(14, 177)	<.001	.38
Peak:				
Species	6.83	(48, 89)	<.001	.79
Lab	5.79	(9, 89)	<.001	.37

set branch lengths proportional to the number of nodes, that is, gradual branch lengths, according to Nee's method (Maddison and Maddison 2006).

First, we tested the relationships of CORT levels with brood value, body mass, and breeding latitude in bivariate analyses. Then, we investigated the relative importance of these three predictors in multivariate analyses, comparing candidate models using the information theoretic approach (Burnham and Anderson 2002) based on the second-order Akaike's Information Criterion corrected for small sample size ( $AIC_c$ ). We evaluated the candidate models by their relative Akaike weights ( $\omega_i$ ) and the predictors by the sums of their Akaike weights across all models that contain the given predictor ( $\Sigma$ ); thus, inference was based on the entire set of plausible models. To test the effect of brood value on the magnitude of stress response across species, we used peak CORT level as a dependent variable and controlled for baseline CORT level as a covariate, and we also included any confounding variable identified for peak CORT level by the previous  $AIC_c$  analyses. We used this approach because differences in baseline CORT levels might reflect species differences in various components of the "CORT machinery" such as receptor affinities and binding proteins (Romero 2004). Thus, a given concentration of peak CORT level might have different impacts in species with very different baseline CORT concentrations.

To investigate the effects of sex differences in parental care, first we tested its relationship with sex differences in CORT levels. Then, to assess the contribution of male and female CORT in these relationships, we analyzed both sexes' CORT levels in relation to sex differences in parental care. Finally, we tested the effect of parental role differences on the magnitude of the stress response across species by using peak CORT level as a dependent variable and controlling for baseline CORT level (both for the two sexes separately and for the sex differences in CORT levels).

For all analyses, we used the R statistical computing environment (R Development Core Team 2006). All statistical tests were two-tailed, with a 95% confidence level. We followed the recent recommendations of Nakagawa (2004) and provided effect size estimates, the correlation effect size  $r$  sensu Cohen (1988), and the associated 95%

confidence intervals (CIs) to evaluate the strength and direction of each relationship in our multiple tests. Sample sizes differ across analyses because data are incomplete for various species. Because the PGLS method does not allow the graphical presentation of phylogenetically corrected data, we present figures that are based on raw species data.

## Results

### *Reliability of CORT Measurements*

The amount of measured CORT was not related to any of the assay parameters, that is, assay sensitivity (baseline:  $F_{1,45} = 2.33$ ,  $P = .134$ ; peak:  $F_{1,13} = 0.51$ ,  $P = .487$ ), intra-assay CV (baseline:  $F_{1,105} = 0.52$ ,  $P = .475$ ; peak:  $F_{1,66} < 0.01$ ,  $P = .952$ ), interassay CV (baseline:  $F_{1,79} = 0.93$ ,  $P = .338$ ; peak:  $F_{1,51} = 1.49$ ,  $P = .227$ ), and assay recovery (baseline:  $F_{1,28} = 0.01$ ,  $P = .912$ ; peak:  $F_{1,22} = 0.81$ ,  $P = .378$ ). Studies with and without chromatographic separation of steroid phases did not differ in their measured CORT concentrations (baseline:  $F_{1,12} = 2.18$ ,  $P = .166$ ; peak:  $F_{1,5} = 0.15$ ,  $P = .715$ ). There was no correlation between sample size and CORT measures (baseline:  $F_{1,198} = 1.49$ ,  $P = .224$ ; peak:  $F_{1,108} = 0.10$ ,  $P = .755$ ). Baseline CORT level was unrelated to handling time ( $F_{1,168} = 2.64$ ,  $P = .106$ ); although peak CORT level tended to increase with the time required to reach the

**Table 2:** Bivariate relationships of corticosterone (CORT) levels with brood value, body mass, and breeding latitude, and the magnitude of stress response in relation to brood value, controlled for breeding latitude

Model, dependent, predictor	<i>r</i>	Confidence limits of <i>r</i>		<i>P</i>	$\lambda$	<i>n</i>
		Lower	Upper			
Bivariate models:						
Baseline CORT:						
Brood value	.32	.01	.57	.041	<.01	42
Body mass	-.28	-.50	-.02	.038	<.01	57
Breeding latitude	.13	-.14	.38	.337	<.01	57
Peak CORT:						
Brood value	.14	-.21	.45	.444	<.01	34
Body mass	.05	-.24	.33	.734	<.01	47
Breeding latitude	.38	.10	.60	.009	<.01	47
Multivariate model of stress response:						
Peak CORT:						
Baseline CORT	.75	.55	.87	<.001		33
Breeding latitude	.42	.08	.66	.020		
Brood value	-.37	-.63	-.03	.043		

Note: Brood value is defined as the relative importance of current reproduction. Stress response is expressed as the peak CORT level controlled for the baseline CORT level. Correlation effect sizes ( $r$ ) with 95% confidence intervals have been adjusted by the degree of phylogenetic dependence ( $\lambda$ ) in each phylogenetic generalized least squares model.

**Table 3:** Comparison of multivariate phylogenetic generalized least squares models for corticosterone (CORT) levels

CORT level, predictor	<i>k</i>	AIC <sub>c</sub>	Δ <sub>i</sub>	ω <sub>i</sub>
Baseline ( <i>n</i> = 42):				
Body mass	2	24.76	.00	.32
Brood value	2	25.00	.24	.28
Body mass + latitude	3	26.51	1.75	.13
Brood value + latitude	3	27.39	2.64	.09
Brood value + body mass	3	27.48	2.72	.08
Latitude	2	27.81	3.05	.07
Brood value + latitude + body mass	4	29.71	4.95	.03
Peak ( <i>n</i> = 34):				
Latitude	2	-13.41	.00	.70
Brood value + latitude	3	-9.95	3.46	.12
Body mass + latitude	3	-9.94	3.47	.12
Brood value + latitude + body mass	4	-6.26	7.15	.02
Brood value	2	-6.17	7.24	.02
Body mass	2	-5.62	7.79	.01
Brood value + body mass	3	-2.80	10.61	<.01

Note: Akaike's Information Criterion corrected for small sample size (AIC<sub>c</sub>), number of estimated parameters (*k*), AIC<sub>c</sub> difference between the best model and each candidate model (Δ<sub>i</sub>), and Akaike weight (a relative estimate of the probability that a given model is actually the best model in the model set; ω<sub>i</sub>) are given for each candidate model. Models with Δ<sub>i</sub> < 2 are considered to have substantial support.

hormone peak ( $F_{1,102} = 3.54$ ,  $P = .063$ ), this tendency was largely dependent on one extremely high CORT value at 60 min (excluding this data point:  $F_{1,101} = 2.23$ ,  $P = .139$ ). There was no difference between incubating, chick-rearing, and overall breeding season CORT levels (baseline:  $F_{1,174} = 0.64$ ,  $P = .531$ ; peak:  $F_{1,110} = 0.64$ ,  $P = .530$ ). Repeatability was high for both baseline CORT ( $R = 0.81$ ,  $F_{31,47} = 11.39$ ,  $P < .001$ ) and peak CORT ( $R = 0.75$ ,  $F_{16,25} = 8.19$ ,  $P < .001$ ) levels, implying that variation within species is small compared with variation among species. The variance attributable to differences among species remained significant when we controlled for lab differences (table 1), with the effect of species being twice as great as the lab effect (table 1).

#### CORT and the Value of Reproduction

Baseline CORT level correlated positively with brood value (table 2; fig. 1A) and negatively with body mass (table 2). Breeding latitude was not related to baseline CORT level (table 2). In the multivariate model comparison (table 3), brood value ( $\Sigma = 0.48$ ) and body mass ( $\Sigma = 0.56$ ) received approximately equal support, whereas breeding latitude ( $\Sigma = 0.32$ ) had a smaller impact on baseline CORT level.

Peak CORT level in itself was unrelated to brood value (table 2; fig. 1A) and body mass (table 2), but it correlated positively with breeding latitude (table 2). In the multivariate model comparison (table 3), peak CORT level was

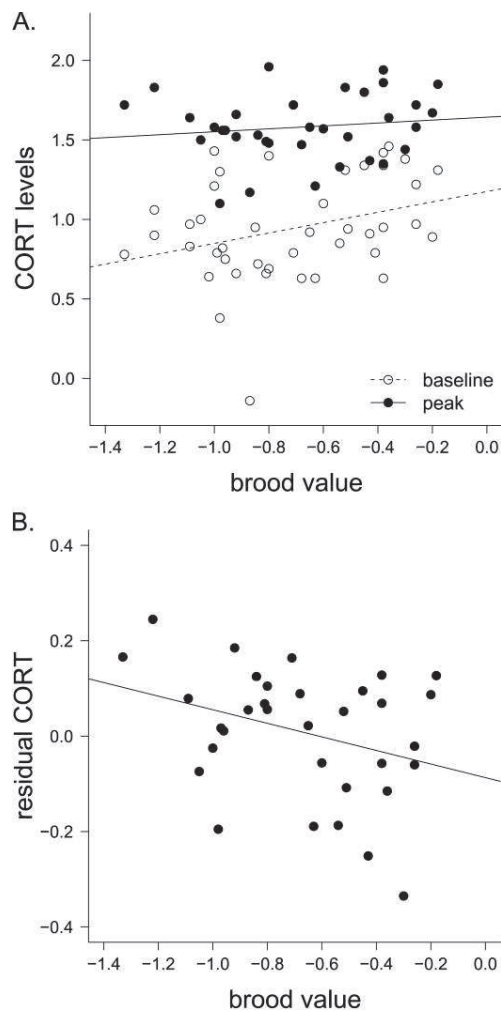
best explained by breeding latitude ( $\Sigma = 0.96$ ), whereas brood value ( $\Sigma = 0.17$ ) and body mass ( $\Sigma = 0.16$ ) were each of far less importance.

To investigate the magnitude of stress response, we built a multivariate model for peak CORT level that included baseline CORT level (see "Material and Methods"), breeding latitude (see tables 2, 3), and brood value. After controlling for the potentially confounding effects of baseline CORT level and breeding latitude, peak CORT level correlated negatively with brood value (table 2; fig. 1B). The variance inflation factor was less than 1.3 for all variables in the latter model.

The sex differences in parental care correlated negatively with the sex differences in CORT level, such that the more caring sex tended to have a lower baseline CORT level (table 4; fig. 2A) and a lower peak CORT level (table 4; fig. 2A). The latter relationship remained significant when we controlled for the sex differences in baseline CORT level (table 4B). Neither male nor female baseline CORT level was related to sex differences in parental care (table 4; fig. 2B, 2C). Female but not male peak CORT level was greater in species with more male-biased care (table 4; fig. 2B, 2C). When we controlled for baseline CORT level, both male and female peak CORT levels correlated positively with the sex differences in parental care (table 4).

#### Discussion

Here we carried out the first large-scale study of avian stress hormones using modern phylogenetic comparative



**Figure 1:** A, Baseline and peak corticosterone (CORT) levels ( $\text{ng mL}^{-1}$ ;  $\log_{10}$  transformed) in relation to brood value. B, Stress response, that is, peak CORT level controlled for baseline CORT level and breeding latitude (absolute distance from the equator), in relation to brood value. Brood value expresses the putative importance of current reproduction as  $\log_{10}(\text{clutch size}/[\text{clutch size} \times \text{broods per year} \times \text{average reproductive life span}])$ .

analyses to test whether the species's CORT levels may be adaptively selected traits and whether these levels are related to the relative importance of current reproduction as predicted by life-history theory. We found that the species means of both baseline and peak CORT levels were highly repeatable across different studies that sampled different populations or applied different sampling and assay procedures. Furthermore, the influence of phylogeny ( $\lambda$ ) was estimated to be close to 0 in most of our analyses. High repeatability and low phylogenetic dependence are both indicative of selection acting on CORT levels. Our

results are in accordance with previous comparative studies of other hormones (androgens; Garamszegi et al. 2005; Bókonyi et al. 2008) and imply that hormone levels are not phylogenetically conservative traits but rather evolve as species-specific attributes and may readily adapt to current conditions. Because CORT levels show high individual variation (e.g., Cockrem and Silverin 2002) that is at least partly heritable (Evans et al. 2006) and affects survival and reproduction (Romero and Wikelski 2001; Wingfield and Sapolsky 2003; Brown et al. 2005), they are likely to be subjects of selection. Our findings support the conclusion of artificial selection studies that plasma CORT levels themselves are functionally significant, adaptive characteristics of a species (reviewed by Williams 2008).

To investigate the relationship between the importance of current reproductive attempt and the magnitude of stress response, we focused on the stress-induced peak CORT levels while controlling for the interspecific differences in baseline levels that may reflect differences in other endocrine factors influencing the effect of hormone titers (Romero 2004). In doing so, we found two correlations that accord with the predictions of the brood value hypothesis at the interspecific level. First, higher brood value was associated with lower residual CORT level, suggesting that the stress response is mitigated in species with a life-history strategy that is characterized by fewer breeding opportunities and greater annual fecundity. This finding implies that the "CORT machinery" may evolve in response to the value of current reproduction, such that stress resistance may be a part of adaptation to a lifestyle with few breeding opportunities and robust responses to stress may be an adaptation for "prudent parents" with many breeding opportunities. In a previous comparative study, Wingfield et al. (1995b) found no association between life span and CORT response; however, CORT levels were then available for a smaller number of species, most of which were relatively short lived (two to three breeding seasons, on average). More than a decade after the seminal article of Wingfield et al. (1995b), the increasing number of published CORT data allowed us to include a greater diversity of species with a wider range of life spans (up to 44 years).

Second, sex differences in parental effort were inversely related to sex differences in residual CORT, such that the greater share the male takes in parental care, the higher the female's peak CORT level. Surprisingly, although we expected a similar inverse relationship in males, we found the opposite: male residual CORT level was higher in species with more male-biased care. The pattern observed in females is in accordance with the brood value hypothesis, and it corroborates former interspecific comparisons (Wingfield et al. 1995b; O'Reilly and Wingfield 2001; Holberton and Wingfield 2003) in a considerably larger sample

**Table 4:** Sex differences (SD) and sex-specific values of corticosterone (CORT) levels in relation to sex differences in parental care

Model, dependent, predictor	<i>r</i>	Confidence limits of <i>r</i>		<i>P</i>	$\lambda$	<i>n</i>
		Lower	Upper			
Bivariate models: <sup>a</sup>						
SD in baseline CORT	-.25	-.49	.02	.066	.02	54
SD in peak CORT	-.46	-.67	-.19	.002	<.01	43
Male baseline CORT	-.20	-.44	.07	.142	<.01	57
Male peak CORT	.05	-.24	.34	.721	<.01	46
Female baseline CORT	-.02	-.29	.24	.874	<.01	55
Female peak CORT	.37	.09	.60	.012	<.01	45
Multivariate models of stress response:						
SD in peak CORT:					<.01	42
SD in baseline CORT	.23	-.08	.50	.147		
SD in parental care	-.39	-.62	-.10	.011		
Male peak CORT:					<.01	45
Male baseline CORT	.79	.65	.88	<.001		
SD in parental care	.34	.05	.57	.025		
Female peak CORT:					<.01	44
Female baseline CORT	.79	.65	.88	<.001		
SD in parental care	.56	.32	.74	<.001		

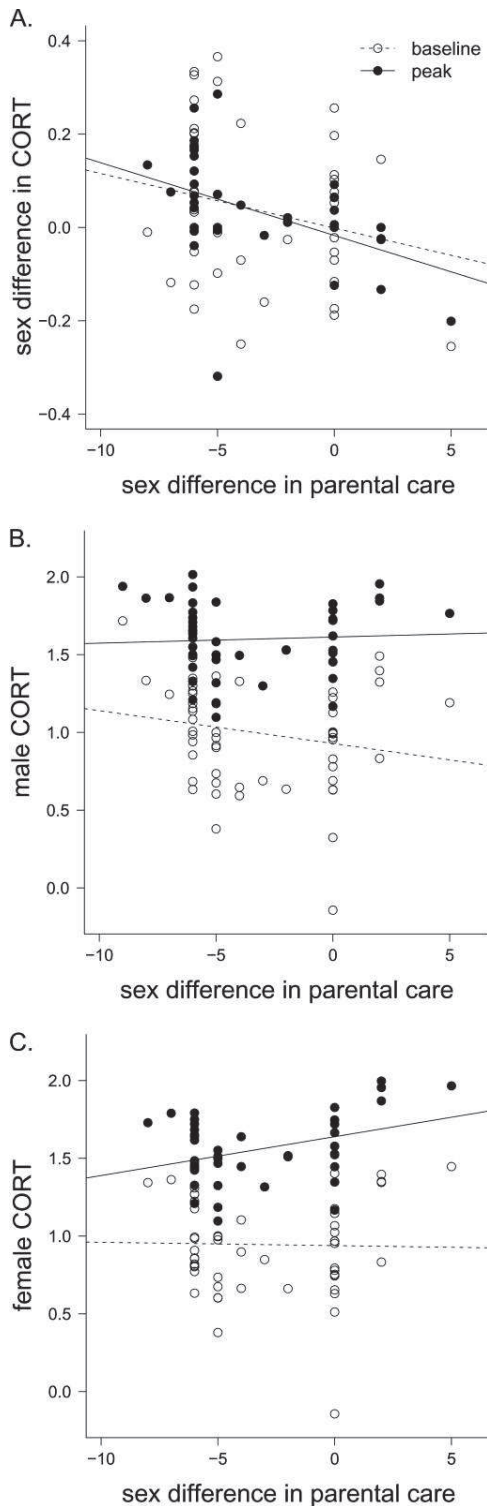
Note: Greater values of parental care indicate more male-biased care. Correlation effect sizes (*r*) with 95% confidence intervals have been adjusted by the degree of phylogenetic dependence ( $\lambda$ ) in each phylogenetic generalized least squares model.

<sup>a</sup> For all bivariate models, the predictor is SD in parental care.

of species ( $n = 42$  vs.  $n = 13, 4,$  and  $3,$  respectively). Why males show a pattern that is so different from that of females is puzzling, as intraspecific studies demonstrated that CORT reduces parental behavior in both sexes (reviewed by Wingfield et al. 1998), and increased paternal investment is associated with reduced CORT levels in males (Meddle et al. 2003). However, male care is not always essential; that is, females of several biparental species can compensate for the loss of the male parent, which seems to be an important determinant of male hormonal responsiveness in breeding birds (Gill et al. 2008).

Our finding, that baseline CORT levels were higher in species with presumably higher importance placed on current reproduction, is unexpected in light of the brood value hypothesis. We suggest that this result might be explained by a recently proposed idea, the "preparative hypothesis" (Romero 2002), which is based on the permissive effects of CORT; that is, CORT prepares the organism to perform better under subsequent stress (Sapolsky et al. 2000). Therefore, it may be adaptive to circulate higher baseline CORT levels whenever the risk to be stressed is predictably high (Romero 2002). Although this hypothesis has not yet been tested directly, it appears to well explain the seasonal changes in vertebrate CORT levels, especially baseline levels (Romero 2002). We suggest that several lines of our results provide further indirect support for this idea. First, the positive correlation between baseline CORT level and

brood value might have arisen because parents of more valuable broods may be willing (and prepared) to make greater efforts and take more risks for their offspring (e.g., Hegner and Wingfield 1987; Montgomerie and Weatherhead 1988). Second, we found that larger species have lower baseline CORT levels, which might indicate that birds with more body reserves prepare for stressors by storing more energy instead of circulating more CORT. Although Wingfield et al. (1995b) found no interspecific relationship between body mass and peak CORT level, they did not investigate baseline CORT level, for which the preparative hypothesis is more relevant. Third, the reduced CORT response of less caring males may be due to the tendency for increased baseline CORT level in these males (see fig. 2B). It is possible that males have to face a higher risk of unpredictable stressors (such as social challenges, injuries, or infections) if they invest more in sexual competition (such as territory defense or the pursuit of extrapair copulations) than in parental care, and thus they are better prepared for these risks by having more elevated baseline CORT levels. In contrast, female baseline CORT level shows no hint of such a relationship (see fig. 2C), perhaps because polyandrous females still have to produce the eggs, and thus they might not be able to afford as high of elevations of baseline CORT level as males can if they are to reproduce successfully. Finally, we found higher peak CORT levels in species breeding at higher latitudes. Be-



**Figure 2:** Sex differences in parental care (greater values indicate more male-biased care) in relation to sex differences in corticosterone (CORT) levels ( $\log_{10}$  [male CORT/female CORT]; A), to male CORT level (B), and to female CORT level ( $\text{ng mL}^{-1}$ ;  $\log_{10}$  transformed; C).

cause the preparative hypothesis predicts higher peak CORT levels when multiple stressors are more likely (Romero 2002), our result might reflect the effect of extreme weather conditions toward the poles (Romero et al. 2000). Alternatively, the latter result may be due to the higher energetic costs demanded by a colder climate (Romero 2002). These intriguing possibilities are all open to testing in future comparative studies and experiments.

As with all studies of plasma hormone levels, it should be borne in mind that the effect of circulating CORT may be either enhanced or attenuated by various internal factors such as the concentration and affinity of corticosterone-binding globulins (CBG) and CORT receptors in the target tissues (Romero 2004). Because of these complexities, hormone levels have been considered to be difficult to interpret as species-specific traits, and, up until now, phylogenetically controlled interspecific comparisons have used only some rank measures of CORT levels such as their qualitative differences across seasons (Romero 2002) or between high- and low-ranking individuals (Goymann and Wingfield 2004). In our study, we used plasma CORT concentrations per se as a proxy for the biological impact of CORT; therefore, the validity of our results and conclusions depend on the extent to which circulating CORT predicts biological impact. While the above-mentioned regulators of stress response may well differ across species (Breuner and Orchinik 2002), several lines of evidence suggest that hormone titers probably coevolve with downstream endocrine components, such that plasma levels may be viewed as a relatively easily measurable manifestation of the phenotypic variation in the underlying endocrine machinery that also determines responsiveness (reviewed by Williams 2008). Our findings provide further support for the functional significance of circulating CORT per se. First, despite the tremendous differences in technical details across studies and potentially in CBG or receptor levels across species, both baseline and peak measures of circulating CORT proved to be highly repeatable and reliable measures of the species, evolving almost without phylogenetic inertia. Second, these species-specific CORT levels show significant evolutionary relationships with relevant life-history traits that are known to influence intraspecific variation in CORT levels. Finally, the species levels of baseline and peak CORT are correlated with different ecological and life-history variables, conforming to the recent idea that baseline and peak CORT may be considered to be two different hormones because they interact with different receptors and have different physiological effects (Romero 2004). Nevertheless, there is still much noise in our data, as inferred from both the figures and the relatively wide effect-size estimates, which may be due at least in part to differences in downstream regulators. As knowledge on CBGs and CORT receptors becomes

available for more and more species, forthcoming comparative studies should carefully assess the impact of these modulating factors on the interspecific variation in stress response.

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Capturing and bleeding Nuttall's white-crowned sparrows at Bodega Bay Marine Station, part of the University of California Reserve Systems (photographs by John C. Wingfield).

# Reproduction and modulation of the stress response: an experimental test in the house sparrow

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The stress response is highly variable among individuals, but the causes of this variation remain largely unknown. In response to stressors, vertebrates secrete elevated levels of glucocorticoids which enhance survival, but concurrently interfere with reproduction. We tested the hypothesis that individuals flexibly modulate their stress response with respect to the reproductive value of their brood in free-living house sparrows (*Passer domesticus*). We experimentally increased or decreased clutch size during the nestling period and found that parents tending enlarged clutches responded less strongly to a stressor than those tending reduced clutches. In addition, we examined whether individuals responded less strongly to a stressor as the breeding season progressed and future reproductive opportunities declined. We found that the stress response decreased with breeding date during the birds' first breeding attempt, but it remained constant during their second breeding attempt. Within-individual variability in the stress response was related to the brood size manipulations the birds received in their two consecutive breeding attempts. These results provide the first experimental support for the hypothesis that individuals actively modulate their stress response with respect to the value of current reproduction.

**Keywords:** stress-response modulation; corticosterone; life history; parental care; brood size manipulation; *Passer domesticus*

## 1. INTRODUCTION

A key concept in life-history theory is that energy allocated to the current reproductive event can only be increased at the expense of survival and hence future reproduction (Stearns 1992). This trade-off reflects the 'cost of reproduction' (Williams 1966) which is a fundamental component determining the level of 'parental investment' (Trivers 1972). However, parental investment depends not only on its costs, but also on the benefits from the current reproduction, e.g. the value of the offspring. Although these factors have been shown to shape the animals' reproductive decisions, our knowledge is still scarce about the actual mechanisms by which these costs and benefits are assessed and integrated within the animals.

Recent theoretical models of resource allocation have shown that the life-history decisions may be influenced by the physiological state of individuals (McNamara & Houston 1996; Houston & McNamara 1999). In that respect, some hormones play a major role in the mediation of such life-history decisions (Sinervo & Svensson 1998). In vertebrates, stressors such as food limitation, predators and inclement weather induce a rapid elevation of circulating glucocorticoids, which promotes survival by the mobilization of energy resources and the occurrence of those behavioural forms that enhance the immediate survival, but may also compromise reproductive and parental behaviour in a longer term. This adrenocortical

response to stress is thought to be adaptive, because it may interrupt the current reproductive episode of an individual and promote its survival for future reproduction (Wingfield & Sapolsky 2003). The glucocorticoid levels therefore seem to have a role in mediating this fundamental life-history trade-off.

According to life-history theory, when the value of the current reproduction is high relative to the value of future reproduction and survival, the stress response should be attenuated to ensure that reproduction is not inhibited. The stress response has been shown to be suppressed during the stage of the breeding cycle when adults are providing the most care for young (also referred to as the 'parental care hypothesis'; Wingfield *et al.* 1995) in the sex that provides more care for offspring (O'Reilly & Wingfield 2001), and when breeding opportunities are limited and the probability of rebreeding is low (also referred to as the 'short season hypothesis'; Wingfield *et al.* 1995; Silverin & Wingfield 1998; Holberton & Wingfield 2003). These studies and recent empirical data (e.g. Heidinger *et al.* 2006) are consistent with the hypothesis that individuals suppress the stress response when the value of current reproduction is high. However, these studies did not use experimental manipulations, but compared different populations or individuals in different breeding stages, which may have confounded the results and therefore left the topic open for debate. Moreover, the basic yet untested underlying assumption of these predictions is that the same individuals may give different stress responses according to the value of their current

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reproduction, i.e. that individuals are able to actively modulate their hormone levels.

In this study, we simultaneously tested multiple predictions of the 'value of reproduction' hypothesis in breeding house sparrows (*Passer domesticus*). First, to test experimentally whether the value of the current brood influences the parents' stress response, we manipulated the brood size of parents by adding or subtracting two chicks and compared the response to capture/restraint stress between the experimental groups. We predicted that the rise in corticosterone levels in response to stress would be weaker in birds with increased broods than in birds with reduced broods. Second, in the study population, there is a considerable individual variation in the onset of breeding: some birds start breeding early in the season and may have up to three subsequent clutches, whereas an important proportion of the population start the reproduction later and have only one breeding attempt (Chastel *et al.* 2003). As the probability of reneating decreases over the breeding season, we used the date of breeding as a surrogate measure to test whether birds with different future breeding opportunities differ in their stress response. To control for the effects of breeding stages, in the present study, we used only parents tending large chicks, i.e. a period when the brood were close to fledging and parents soon either to reneat or finish their reproduction for the season. Specifically, we predicted that individuals breeding at the beginning of the season (high reneating probability) would have a stronger adrenocortical response to capture/restraint stress than those that bred later (low reneating probability). Finally, since several birds were sampled both in their first and second breeding attempts, we tested whether the experimental manipulations explained within-individual variance in stress response between the birds' two consecutive breeding attempts, i.e. whether individuals modulated their hormonal stress response according to the actual breeding circumstances.

## 2. MATERIAL AND METHODS

### (a) Study species and population

The study was carried out between March and July 2005 on a free-living population of house sparrows that breed in nest boxes in Chizé, France (46°09' N, 0°24' W; Chastel *et al.* 2003). A large proportion of the adults used in this study were first captured during the pre-breeding period using mist nets and marked with a unique metal ring and colour combination. The nest boxes were monitored daily to determine laying dates, clutch sizes, hatching dates and the number of hatchlings.

### (b) Brood size manipulation

To experimentally manipulate the reproductive value of the brood, the clutch sizes were either increased or decreased as follows. Two days after hatching, two nestlings were randomly chosen and transferred between two synchronous broods, resulting in enlarged and reduced clutches (Curlee & Bessinger 1995; Chastel & Kersten 2002). Owing to the moderate number of available clutches, we did not use a control group to maximize the sample sizes in the manipulated broods and to maximize the difference in brood sizes between the experimental groups. If no synchronous broods were available to exchange nestlings between nests at exactly the same age (2 days

old), or if the original brood was too small (two or three chicks) to be reduced or too large (six chicks) to be enlarged, it was not manipulated. Experimental brood sizes were within the natural variation for this population and ranged between two to four nestlings (reduced broods) and five to seven nestlings (enlarged broods). House sparrows readily accept manipulated broods and adjust their level of parental care accordingly (Seel 1969; Hegner & Wingfield 1987; Chastel & Kersten 2002; Bonneaud *et al.* 2003).

### (c) Measuring the stress response

To measure the stress response, we used a standard capture–handling–restraint protocol that has been successfully used in numerous field studies (Wingfield 1994). Breeding sparrows were captured in their nest box using a wire trap, when nestlings were 10–12 days old. Immediately after capture, a small blood sample (50–100 µl) was collected from the brachial vein and the time required to collect the sample was recorded. Mean handling time was  $2:37 \pm 0:02$  (s.e.m.) min: sec, maximum 3:09 min: sec. Corticosterone levels measured at the initial bleeding were not related to handling time ( $r=0.206$ ,  $p=0.112$ ,  $N=61$ ). Therefore, we assume that the corticosterone levels measured at capture were representative of the circulating baseline hormone levels prior to the capture. In addition, we found no significant effect of the time of capture on corticosterone.

Following the collection of initial blood samples, the birds were placed in cloth bags and subsequent samples were collected after 30 min. We chose 30 min as the time for the second sample because previous studies on this species have shown that it is a good proxy for the maximum corticosterone levels (Breuner & Orchinik 2001; Lindström *et al.* 2005). After the second blood sample was collected, we measured tarsus length ( $\pm 0.1$  mm) and body mass ( $\pm 0.1$  g) before releasing the birds. The blood samples were kept on ice and centrifuged (5000 rpm (1396 g), 6 min) as soon as possible. Plasma was separated and stored at  $-20^\circ\text{C}$  for further analyses.

### (d) Corticosterone assay

Plasma corticosterone levels were determined by radio-immunoassay at the Centre d'Etudes Biologiques de Chizé following the procedure detailed in Lormée *et al.* (2003). Corticosterone was extracted from a 20 µl plasma sample and determined in duplicates run in one assay (coefficient of intra-assay variation was 4.3%,  $N=6$  duplicates).

### (e) Data processing and analysis

To determine whether the capture–restraint protocol elevated the corticosterone levels, we used repeated measures ANOVA. The magnitude of stress response was expressed as the maximum levels of corticosterone. We also calculated the rate of increase from baseline to maximum corticosterone levels per minute ( $\text{ng ml}^{-1} \text{min}^{-1}$ ; Silverin *et al.* 1997; Lindström *et al.* 2005). The latter measure of stress response was highly correlated with the maximum levels of corticosterone ( $r=0.920$ ,  $p<0.0001$ ), and the statistical analyses of these variables gave nearly identical results, hence we report only the results for maximum corticosterone levels to facilitate the comparison with other published results. To analyse seasonal changes in the baseline and stress-induced hormone levels, we used the date of blood sampling as the independent variable in the models.

Seventy per cent of the individuals included in this study were captured only during their first or second breeding

attempt, hence the two attempts were analysed separately. No parents tending their third broods were included in this study. The inter-individual differences in the effects of manipulations were analysed only in the individuals captured in manipulated broods.

We also made two comparisons between breeding attempts. First, we compared the stress response of 'early' and 'late' breeders. Late breeders were defined as the individuals starting their first reproductive attempt when others (early breeders) were already tending their second broods (Chastel *et al.* 2003). Therefore, early and late breeders were compared in a time span where their breeding activity overlapped, i.e. from the earliest second clutch initiation until the last initiation of a first clutch (from 15 May to 13 June, figure 1). Second, for those individuals that were sampled in both their first and the second brood, we also performed within-individual comparisons to investigate whether the variation in the stress response was a function of the differences between the manipulations they received in their first and the second breeding attempts. We created three categories where: (i) the brood size manipulation induced greater brood value in the first brood compared with the second brood (i.e. the first brood was enlarged and the second was either unmanipulated or reduced), (ii) the individual either received the same type of manipulation or was left unmanipulated in both the breeding attempts, and (iii) the brood size manipulation induced lesser brood value in the first brood compared with the second brood (i.e. the first brood was reduced and the second was either unmanipulated or enlarged).

In the nests where both the male and female parent were captured, we found no relationship between the baseline corticosterone levels or the rate of increase in corticosterone levels of the two parents (all  $p > 0.1$ ), indicating that these variables reflect individual variation in the physiological response to the manipulation. Hence, we used males and females as independent data points in our analyses.

To analyse body condition, we used body mass as the dependent variable in a general linear model (GLM) controlling for the tarsus length in the model as a covariate. Dates are presented as the number of days elapsed from the initiation of the first clutch in the population (15 April). Two-tailed probabilities and mean  $\pm$  s.e.m. values are given throughout the paper. We used SPSS v. 11.0 for statistical analyses.

### 3. RESULTS

#### (a) *The study population*

During the breeding season, sparrows initiated nests in three overlapping waves (figure 1). The first wave of clutches were initiated in mid-April, whereas the second wave of clutches were initiated in mid-May at the same time as some individuals which had begun breeding in April were already initiating their second clutches. Most of the individuals in the study population were colour-ringed, hence regular observations of marked individuals and checking of nest boxes confirmed that birds initiating their first clutch had not bred elsewhere. After the first clutch, 46% of the individuals laid a second clutch. The probability of starting a second clutch decreased significantly with the date of the first clutch initiation (binary logistic regression,  $B = -0.044$ ,  $p = 0.017$ ). From the end of June, 15% of the birds that had a second clutch laid a third clutch, while most birds in the population had finished reproduction.

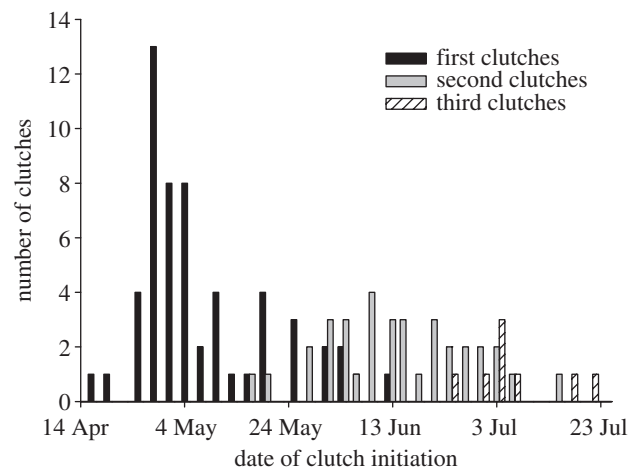


Figure 1. Number of clutches initiated by house sparrows in 2005 in 3-day periods during the breeding season.

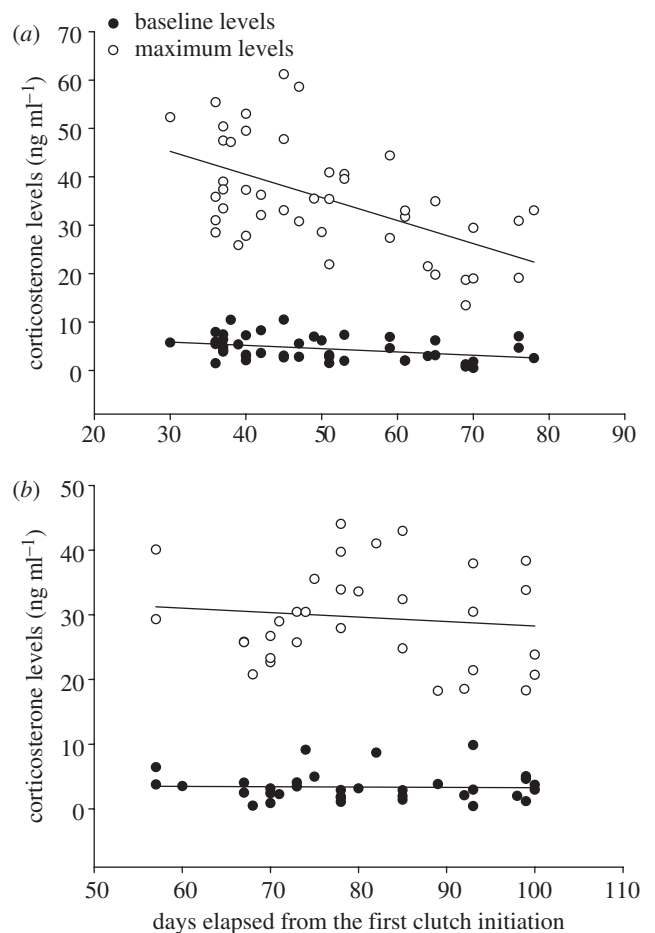


Figure 2. Seasonal change in the baseline and maximum corticosterone levels in the (a) first breeding attempt and (b) second breeding attempt.

The probability of having a third clutch depended also on the date of starting the second clutch (binary logistic regression,  $B = -0.210$ ,  $p = 0.006$ ).

#### (b) *Effects of season and manipulation on baseline corticosterone*

The baseline corticosterone levels decreased during the season in the first, but not in the second breeding attempt (first breeding attempt,  $r = -0.361$ ,  $p = 0.016$ ,  $N = 44$ ; second breeding attempt,  $r = -0.028$ ,  $p = 0.877$ ,  $N = 34$ ; figure 2). Manipulation groups did not differ in their

baseline corticosterone levels, even after controlling for the seasonal effects (first breeding attempt,  $F_{1,28}=0.500$ ,  $p=0.485$ ; second breeding attempt, manipulation:  $F_{1,21}=1.519$ ,  $p=0.231$ ). Body condition (body mass controlling for tarsus length) did not differ between the manipulation groups and the sexes either in the first or in the second breeding attempt (first breeding attempt, manipulation:  $F_{1,25}=1.181$ ,  $p=0.287$ , sex:  $F_{1,25}=0.353$ ,  $p=0.558$ , interaction:  $F_{1,25}=1.712$ ,  $p=0.203$ ; second breeding attempt, manipulation:  $F_{1,18}=2.049$ ,  $p=0.169$ , sex:  $F_{1,18}=0.354$ ,  $p=0.559$ , interaction:  $F_{1,18}=4.165$ ,  $p=0.056$ ). Body condition was positively related to baseline corticosterone levels in the first breeding attempt, whereas this relationship tended to be negative in the second breeding attempt (first breeding attempt,  $F_{1,39}=10.329$ ,  $p=0.003$ ,  $\beta=0.285$ ; second breeding attempt, manipulation:  $F_{1,39}=3.304$ ,  $p=0.079$ ,  $\beta=-0.141$ ).

### (c) The effects of season and the manipulation on the adrenocortical response to stress

Circulating plasma levels of corticosterone increased markedly following capture, handling and restraint (GLM repeated measures, first breeding attempt,  $F_{1,43}=346.475$ ,  $p<0.001$ ; second breeding attempt,  $F_{1,31}=378.408$ ,  $p<0.001$ ). The maximum corticosterone levels were not dependent on the baseline corticosterone levels (first breeding attempt,  $r=0.228$ ,  $p=0.136$ ,  $N=44$ ; second breeding attempt,  $r=0.136$ ,  $p=0.459$ ,  $N=32$ ) or the body condition (first breeding attempt,  $F_{1,39}=0.008$ ,  $p=0.930$ ; second breeding attempt,  $F_{1,29}=0.022$ ,  $p=0.883$ ). The maximum level of corticosterone was not different between the sexes (first breeding attempt,  $F_{1,42}=0.094$ ,  $p=0.761$ ; second breeding attempt,  $F_{1,30}=0.480$ ,  $p=0.494$ ).

During the first breeding attempt, the magnitude of the stress response decreased over the season and this decrease was also significant after controlling for the baseline corticosterone levels (season,  $F_{1,41}=15.392$ ,  $p<0.001$ ; baseline corticosterone,  $F_{1,41}=3.094$ ,  $p=0.086$ ; figure 2a). In the second breeding attempt, the maximum level of corticosterone was also independent from baseline corticosterone levels, but did not change over the season (season,  $F_{1,29}=0.393$ ,  $p=0.536$ ; baseline corticosterone,  $F_{1,29}=0.551$ ,  $p=0.464$ ; figure 2b).

In both the breeding attempts, individuals tending reduced broods had a higher stress response than individuals tending enlarged broods, and this difference remained significant after statistically removing the effect of the season (first breeding attempt,  $F_{1,29}=6.784$ ,  $p=0.014$ ; second breeding attempt,  $F_{1,20}=16.443$ ,  $p=0.001$ ; figure 3). We found no interaction between the brood size manipulation and the season in either breeding attempt (first breeding attempt,  $F_{1,28}=1.251$ ,  $p=0.273$ ; second breeding attempt,  $F_{1,19}=0.789$ ,  $p=0.385$ ).

### (d) Within-individual comparisons

We compared the stress response in the first and second breeding attempts for those individuals that were sampled on both occasions. We investigated whether the difference in the stress response in the first and the second breeding attempt was related to the difference in the brood size manipulation that individuals received for their first and second broods. A GLM with repeated measures showed that there was a significant change in the stress response of

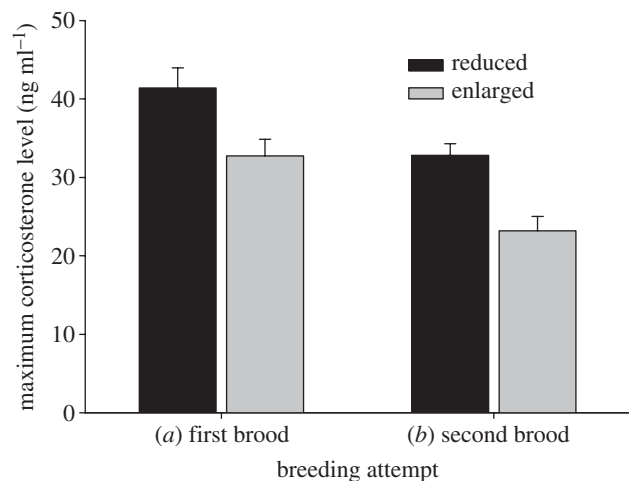


Figure 3. Maximum corticosterone levels as a function of the brood size manipulation in the (a) first and (b) second breeding attempt.

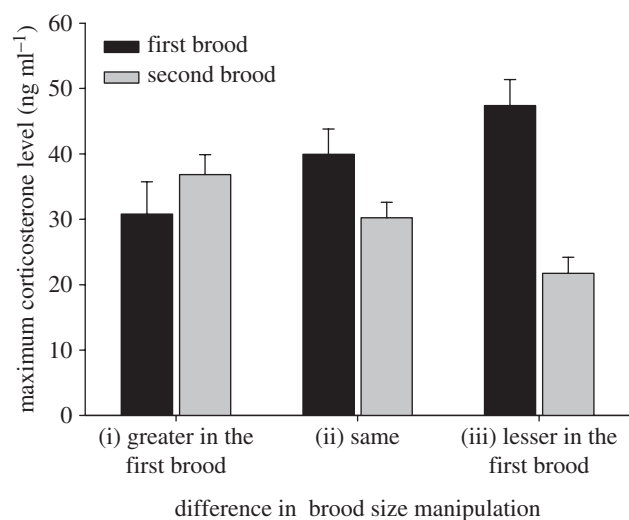


Figure 4. Within-individual comparisons of the adrenocortical response to stress as a function of the difference between the brood size manipulations received in the first and in the second breeding attempts. The brood value induced by the manipulation was either (i) 'greater in the first brood' if the first brood was enlarged and the second was either unmanipulated or reduced, (ii) 'same' if the individual either received the same type of manipulation or was left unmanipulated in both breeding attempts; and (iii) 'lesser in the first brood' if the first brood was reduced and the second was either unmanipulated or enlarged.

individuals between their first and second breeding attempts ( $F_{1,12}=34.532$ ,  $p<0.001$ ), and this change was related to the differences in manipulation ( $F_{2,12}=15.945$ ,  $p<0.001$ ; figure 4). In this model, we also controlled for the date of the first breeding ( $F_{1,12}=5.062$ ,  $p=0.044$ ) and the number of days elapsed between the two captures ( $F_{1,12}=0.955$ ,  $p=0.348$ ).

### (e) Comparison of early and late breeders

To distinguish between the seasonal effects and the individual differences in the adrenocortical response to stress, we compared the stress response of the early breeders tending their second brood and the late breeders tending their first brood, in the same time span. During this limited time span, the effects of season and

Table 1. Comparison of the stress responses of individuals during their first or second breeding attempt in 2005. (The table shows the results of a GLM model with the maximum level of corticosterone ( $\text{ng ml}^{-1}$ ) as the dependent variable. Significant effects are shown in bold.)

source of variation	$F_{1,16}$	$p$
<b>season</b>	<b>8.806</b>	<b>0.009</b>
<b>manipulation</b>	<b>7.886</b>	<b>0.013</b>
breeding attempt	0.398	0.537
breeding attempt*manipulation	0.515	0.483
<b>breeding attempt*season</b>	<b>5.635</b>	<b>0.030</b>

manipulation on the response to stress were still significant (table 1). The magnitude of the stress response did not differ between early and late breeders; however, it decreased over the season in the late breeders, while it increased slightly in early breeders resulting in a significant interaction between breeding attempt and the date of capture (table 1, figure 5).

#### 4. DISCUSSION

In this study, we found that adults with experimentally enhanced brood sizes responded less strongly to a stressor than adults with experimentally reduced brood sizes. In addition, we found that the stress response declined with increasing breeding date during the first breeding attempt. To the best of our knowledge, this study is the first experimental test of the hypothesis that individuals flexibly modulate their stress response with respect to the reproductive value of their brood. Our results lend robust support for this hypothesis and also corroborate its essential, yet untested underlying assumption, namely that the same individuals may react with a different physiological response to stress according to the actual costs and benefits of their reproductive status, and therefore are able to flexibly modulate their hormonal response to stress.

First, we found that in both breeding attempts, birds tending enlarged broods raised their circulating corticosterone levels to a smaller degree than those that tended reduced broods. Second, we found that individuals responded in different ways to a standardized stress protocol in their two consecutive breeding attempts, and this difference was explained by the different types of manipulation they received. In short, the rise in corticosterone was weaker in the breeding attempt in which their reproductive value was experimentally increased compared with the other breeding attempt.

But does an individual's flexibility to modulate its stress response work in both directions? With the lack of a control group, we cannot unambiguously determine whether the regulation of the stress response occurred in one or both experimental groups. However, in both breeding attempts, the stress response of birds tending unmanipulated broods was intermediate compared with birds tending enlarged or reduced broods (first breeding attempt, enlarged:  $32.742 \pm 2.112$ , unmanipulated:  $35.000 \pm 2.640$ , reduced:  $41.404 \pm 2.556$ ; second breeding attempt, enlarged:  $23.182 \pm 1.853$ , unmanipulated:  $30.935 \pm 2.219$ , reduced:  $32.816 \pm 1.486$ ). As the unmanipulated nests were not part of the experimental design (see §2), we did not assign these data to a control

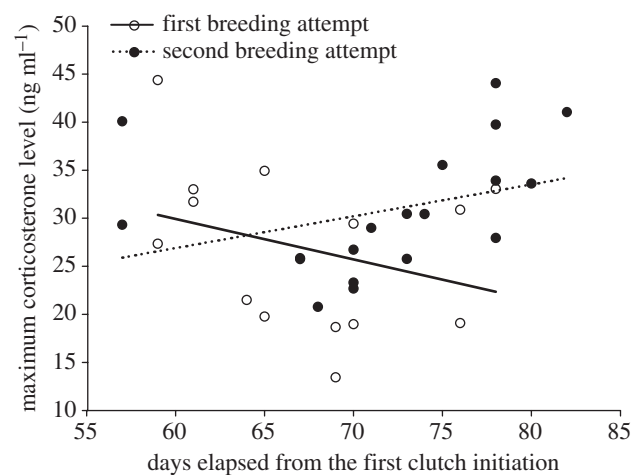


Figure 5. Comparison of the adrenocortical response to stress between individuals reproducing for the first or the second time in 2005 during the time span when they bred simultaneously. The stress response is expressed as the maximum corticosterone levels.

group *post hoc*. However, even if we had done this, the difference between the groups would be significant ( $p=0.048$  and  $p=0.007$  for the first and the second breeding attempt, respectively). To date, most of the studies have suggested that when the value of the brood is high, individuals should suppress their response to stress. However, it seems plausible that individuals experiencing a reduction in the value of the current reproduction may also upregulate their hormonal response to stress. We recommend further research to investigate this issue.

The prediction about the relationship between the parental care and the stress response is based on the idea that individuals actively modulate their response to stress to prevent the risks of nest abandonment when the value of the offspring, and therefore their level of parental care, is high, and considers this reaction as a reproductive tactic to maximize fitness (Jessop 2001). However, one may argue that these results can be interpreted without supposing an active regulation of the hormone levels. From an energetic perspective, the corticosteroid response to stress may be viewed as an energy demanding process to restore homeostasis (Nelson *et al.* 2002). Individuals should not only allocate energy between survival and reproduction, but should also balance energy between maintaining body condition and combating the stressor. Mounting an intensive response to stress may be costly because it may increase the risk of mortality from impaired body maintenance (McNamara & Buchanan 2005). Therefore, individuals whose energetic resources were mainly devoted to parental duties would show a modest response to stress. Nevertheless, in the present study, the brood size manipulations had no detectable effects on body condition or on baseline corticosterone levels, and the magnitude of the stress response was unrelated to body condition.

Although our measure of body condition is rather a rough approximation of the physiological resources available to the animal, the fact that we found no relationship between body condition and stress response does not support the view that some birds were physiologically limited to produce high corticosterone levels. On the contrary, increased workload has been evoked to explain the stronger stress response of female

pied flycatchers (*Ficedula hypoleuca*) that were not assisted by their male (Silverin & Wingfield 1998). Therefore, there is a need for more studies aiming to sort the effects of the brood value from those of parental workload to determine whether the modulation of stress response is the consequence of the animal's perception of their reproductive status or a result of a differential resource allocation between maintenance and combating the stressor.

Parents are also expected to suppress the stress response during the breeding season as the probability of renesting decreases. In accordance with this idea we found that individuals tending their first brood at the beginning of the reproductive season showed a higher response to capture and handling stress than individuals that started to breed later in the season. Were these late-breeding birds really constrained by time? We found that about half of the individuals had only one breeding attempt; moreover, the probability of starting a second clutch was significantly greater for individuals that began the reproduction early. Accordingly, it seems that the convenient circumstances for breeding are indeed limited for some individuals, and by giving up their young very easily, these individuals would be at risk of producing no offspring in a given year. Missing a year of reproduction may have serious fitness consequences for a short-lived animal such as the house sparrow. In this species, the modal lifespan is only 2 years and the most important component determining an individual's lifetime reproductive success is the number of successful broods produced per year (Jensen *et al.* 2004). However, long-lived species often maintain robust stress responses during breeding, probably to ensure the opportunity for future reproduction (Chastel *et al.* 2005).

Contrary to the first breeding attempt, birds tending their second brood showed a similar corticosterone response to stress, irrespective of the laying date. Although the probability of laying a third clutch was also related to the date of the second breeding attempt, only a small proportion (15%) of these birds started a new clutch. Hence, the individual differences in the future breeding opportunities may be smaller in the second breeding attempt compared with the first breeding. Moreover, the third clutch is generally less productive than the second in the house sparrow (e.g. McGillivray 1983). According to the latter two arguments, individuals may base their reproductive decisions on the value of the second brood rather than the possibilities of future breeding, and therefore the timing of breeding may have less influence on the response to stress in the second brood than in the first one.

Within-individual comparisons also showed that the stress response was higher in the first attempt than in the second attempt. It is possible that this resulted in habituation to capture and handling stress; however, this is unlikely because in the second breeding attempt, the stress response of the birds sampled for the first and the second time was not different ( $p=0.595$  after controlling for the effects of season and the manipulation). Moreover, most birds included in this study were captured at least once earlier in the season before the onset of breeding. Although blood samples were not collected during the early captures, the birds experienced similar capture and handling as during the stress protocol.

We also found similar seasonal patterns in the basal corticosterone levels: they decreased with the date in the first breeding attempt, whereas they were unrelated to the date in the second breeding attempt. These results might be interpreted as a part of an intrinsic seasonal change in both the baseline hormone levels and the endocrine responsiveness to stress, which would be steeper at the beginning of the reproductive season. In fact, several endocrine, metabolic or neural processes undergo changes during the annual cycle (Nelson *et al.* 2002; Romero 2002). For example, in the house sparrow, corticosterone levels, plasma concentrations of corticosteroid binding globulin (CBG) and corticosteroid receptors in the brain changed between different periods of the year, resulting together in a more pronounced corticosterone response to stress in the nesting than in the post-breeding period (Breuner & Orchinik 2001). To separate the effects of the season *per se* (i.e. some environmental variable acting as a common stimulus on the physiology of the birds, such as the day length, ambient temperature, etc.) and the differences in reproductive status of individuals (e.g. probabilities of future reproduction), we compared the stress response of the early breeders tending their second brood and the late breeders tending their first brood, in a time span when their breeding activity overlapped. The seasonal pattern of the stress response differed significantly between the two breeding attempts. This result further supports the idea that the responsiveness to the stressors depends not only on the environmental factors, but also on the individual differences, for instance, in the recent breeding history or the prospects for renesting.

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# Age and the timing of breeding in a long-lived bird: a role for stress hormones?

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## Summary

1. Young birds often reproduce later in the season than older ones, with poorer breeding success, but the proximate mechanisms involved in such a pattern remain poorly studied, especially in long-lived species. One possible mechanism is the endocrine stress response which is accompanied by the release of corticosterone. Elevated corticosterone levels can trigger physiological and behavioural responses that may delay or even suppress reproduction.

2. We tested the hypothesis that the delayed timing of breeding of young birds may be related to a greater susceptibility to stress compared with older ones during the pre-laying period of the breeding season. For this purpose, known-age (7–44 years old) pre-laying snow petrels, *Pagodroma nivea*, were monitored for baseline and acute stress-induced corticosterone levels. We examined whether baseline and stress-induced corticosterone levels were related to age, and whether they could influence the decision to breed and egg-laying date.

3. Young snow petrels were more likely to skip the breeding season and to breed later than middle-aged birds. In addition, the oldest birds bred later than middle-aged ones, suggesting a possible senescence on laying dates. Baseline corticosterone levels were independent of age but young and very old birds were more sensitive to stress than middle-aged ones. However, there was no effect of stress-induced corticosterone levels on breeding decision and egg-laying date. Elevated baseline corticosterone levels during the pre-laying period were associated with a higher probability of skipping breeding in females and a delayed timing of egg-laying in both sexes.

4. These results suggest that the greater susceptibility of young breeders to stress was not the functional mechanism explaining their delayed timing of breeding. Baseline corticosterone levels, although independent of age, appear to be a more likely mediator of breeding decision and egg-laying date. In long-lived birds, the relationship between age and timing of breeding may be rather indirect, as other age-related factors such as breeding experience or foraging skills may have a deeper impact on baseline corticosterone than age *per se*.

**Key-words:** age, glucocorticoids, laying date, snow petrel (*Pagodroma nivea*), stress response

## Introduction

Breeding at the right time is a key factor for successful reproduction in fluctuating environments (Lack 1968; Perrins 1970; Nager & van Noordwijk 1995). The adjustment of breeding schedule allows a good overlap between offspring's energy requirements and local peak in food availability (*match–mismatch hypothesis*; Visser *et al.* 1998; Stenseth & Mysterud 2002; Durant *et al.* 2007). In bird species, although early breeders usually perform better than late breeders, the decision to start breeding greatly differs between individuals. Typically, young breeders reproduce

later in the season than older ones, and have lower reproductive success (Martin 1995; DeForest & Gaston 1996; Gonzalez-Solis *et al.* 2004; Ezard, Becker & Coulson 2007; McCleery *et al.* 2008; Blas, Sergio & Hiraldo 2009). This could be explained by (i) a progressive disappearance of late-breeders over age classes (*selection hypothesis*; Mauck, Huntington & Grubb 2004), (ii) an individual adjustment of life-history strategies: as it gets older, a bird would favour current breeding rather than survival (*restraint hypothesis*; Stearns 1992), (iii) an improvement of breeding skills with age owing to the accumulation of breeding experience (*constraint hypothesis*, Curio 1983) or (iv) a progressive incorporation of early breeders over age classes (*delayed-breeder hypothesis*; Forslund & Pärt 1995; Blas, Sergio & Hiraldo

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2009). Ultimate factors explaining why young breeders reproduce later than older individuals have been discussed widely (Curio 1983; Forslund & Pärt 1995; Blas & Hiraldo 2010). However, the mechanisms underlying this age-specific timing of breeding are still poorly understood. In that respect, endocrine mechanisms deserve special attention as they mediate life-history strategies in relation to environmental conditions and to the physiological state of individuals (Wingfield *et al.* 1998; Ricklefs & Wikelski 2002).

Specifically, the endocrine stress response could play a key role in the age-specific timing of breeding. In vertebrates, stressful conditions such as harsh weather, scarce food availability or presence of predators, trigger an acute secretion of glucocorticoid hormones, that is, cortisol or corticosterone (Wingfield *et al.* 1998). In turn, this stress response allows individuals to cope with the stressors by suppressing reproductive functions (Wingfield & Sapolsky 2003; Landys, Ramenofsky & Wingfield 2006; Breuner, Patterson & Hahn 2008). Although the majority of such studies focused on the parental phase of the reproductive cycle (i.e. incubation and chick rearing in birds), very few studies have investigated relationships between glucocorticoids, known to interfere with the hypothalamic–pituitary–gonadal axis (Wingfield & Sapolsky 2003; Pérez-Rodríguez *et al.* 2006), and the timing of breeding (Salvante & Williams 2003; Schoech *et al.* 2009). These studies suggest a similar inhibiting role of corticosterone during the pre-laying period (i.e. before the onset of breeding). Elevated corticosterone levels were associated with delayed first clutch initiation during unfavourable years in Florida scrub jay (*Aphelocoma coerulescens*; Schoech *et al.* 2009). Furthermore, experimental administration of exogenous corticosterone in zebra finches (*Taeniopygia guttata*) greatly decreased the number of pairs initiating reproduction and delayed clutch initiation (Salvante & Williams 2003). Corticosterone appears therefore to be an excellent candidate adjusting the timing of breeding in response to environmental conditions (Schoech *et al.* 2009). Understanding the functional role of corticosterone implies to distinguish between (i) baseline corticosterone level, which is a marker of activities and energetic state (Kitaysky, Wingfield & Piatt 1999; Love *et al.* 2004; Landys, Ramenofsky & Wingfield 2006), and (ii) stress-induced corticosterone level, which mirrors the sensitivity to stress and less commitment to current reproduction (Lendvai, Giraudeau & Chastel 2007).

According to life-history theory, the stress response should be attenuated when the value of the current reproduction is high relative to the value of future breeding events (Wingfield & Sapolsky 2003). Recent experimental and comparative studies support this assumption (*brood value hypothesis*; Lendvai, Giraudeau & Chastel 2007; Lendvai & Chastel 2008; Bokony *et al.* 2009). Relating this to age-specific reproductive success, it has been predicted and observed that the stress response of old adults is attenuated, probably to ensure that reproduction is not inhibited (Heidinger, Nisbet & Ketterson 2006; Angelier *et al.* 2007a). During the pre-laying period, an attenuation of the stress response might therefore be the endocrine mechanism underlying early breeding in old birds.

Focusing on a long-lived species, the snow petrel (*Pagodroma nivea* Foster), we hypothesized that young birds are more sensitive to stress before the onset of reproduction than older ones. First, we tested whether young birds lay their egg later or whether they were more likely to skip the breeding season than older birds. Secondly, we tested whether baseline or stress-induced corticosterone levels varied with age. We predicted that young birds would exhibit higher baseline and/or stress-induced corticosterone levels than older birds during the pre-laying period. Thirdly, we predicted that individuals with high pre-laying baseline and/or stress-induced corticosterone levels would delay the onset of breeding or even halt their reproduction.

## Materials and methods

### STUDY SITE AND SPECIES

The study was carried out on Ile des Pétrels, Pointe Géologie Archipelago, Terre Adélie (66°40'S, 140°01'E), Antarctica. Snow petrels (250–500 g; Fig. 1) are very long-lived birds with low annual fecundity and high adult life expectancy (Chastel, Weimerskirch & Jouventin 1993; Jenouvrier, Barbraud & Weimerskirch 2005). Like all Procellariiform species, breeding pairs lay only one egg per season, with no replacement clutch in case of failure (Chastel, Weimerskirch & Jouventin 1993; Barbraud & Weimerskirch 2001). The age at first breeding is 10 years on average and birds breed annually, although a significant proportion of birds skip the breeding season (Chastel, Weimerskirch & Jouventin 1993). Nest and mate fidelity is high (Bried, Pontier & Jouventin 2003). Males and females provide roughly similar amounts of parental care (incubation of the egg and care for the chick). Birds arrive on the breeding ground in early November. The courtship and mating period typically lasts about 2 weeks. Then, individuals of both sexes forage at sea to accumulate resources and females build up reserves for the egg (pre-laying exodus). The laying period ranges from late November to mid-December. Within one breeding season, egg-laying dates differ between individuals over a period of 2 weeks (Barbraud, Lormee & LeNeve 2000).

The ongoing mark-recapture programme of the snow petrels population began in 1963 (see Chastel, Weimerskirch & Jouventin 1993; Barbraud & Weimerskirch 2001 for detailed methodology). Every



**Fig. 1.** A pair of courting snow petrels during the pre-laying period, in Terre Adélie, Antarctica. Photo by Aurélie Goutte.



chick of the monitored colonies has been banded prior to fledging every year since 1963. Thus, a part of the current population is of known age.

#### BLOOD SAMPLING, BODY CONDITION AND PHENOLOGY

Adult male and female snow petrels were handled during the 2007 pre-laying period (6–19 November, that is,  $26.93 \pm 0.76$  days before egg-laying). A total of 55 known-age adults (7–40 years old) and 11 unknown-age adults (but they were ringed as adults and they were at least 11 years old) were caught at their nest, in 65 different nests. In one nest, both partners of the pair were of known age and we randomly selected one bird of this pair. A blood sample from the alar vein was taken immediately ( $157.5 \pm 5.8$  s) after capture using a 1-mL heparinized syringe and a 25-gauge needle. For nine of them, bleeding time exceeded the time required for baseline corticosterone levels (Romero & Reed 2005), so these nine values were not used for the analysis. Birds were then placed into cloth bags, and subsequent blood samples were collected 30 min later, according to the standardized capture/restraint stress protocol (Wingfield *et al.* 1994). Skull length (head + bill) and tarsus length were measured using a calliper to the nearest 0.5 mm, wing length to the nearest 1 mm and weight to the nearest 2 g using a spring balance. As univariate metrics may be inadequate to estimate overall body size in birds, we used the first component (PC1) of a principal component analysis as an index of size. As male and female snow petrels differ greatly in size (Barbraud & Jouventin 1998), males ( $N = 38$ ) and females ( $N = 28$ ) were analysed separately. The first component accounted for 76 and 72% of the total variance in male and female measurements, respectively, and was highly positively correlated with all variables ( $r > 0.83$  for both sexes). PC1 scores increased with the size of the birds. Standardized body condition was then calculated for males and females separately from a least-squares linear regression of body mass against body size (PC1). Petrels were then marked with spots of dye on the forehead to distinguish them from their partner and were released at their nest. Then, we checked the 65 nests every 2 days to monitor the presence or absence of the marked birds, breeding decision (i.e. decision to lay an egg or to skip the breeding season) and the egg-laying date. The time between sampling and egg-laying did not differ between the sexes ( $F_{1,52} = 1.078$ ,  $P = 0.304$ ) and was not related to age ( $F_{1,44} = 0.377$ ,  $P = 0.542$ ).

In addition, we prospectively monitored other known-age birds during the incubating period to increase the sample size. A total of 109 known-age adults (6–44 years old) were indeed monitored for breeding decision, and 96 eggs were followed every day until hatching to assess hatching success and hatching date. As blood sampling and bird handling might disturb the breeding phenology, we compared breeding decision and hatching date of manipulated birds with the sample of non-manipulated birds nesting in the long-term study plots (see Chastel, Weimerskirch & Jouventin 1993), where birds were seldom handled ( $N = 221$  for the breeding decision and  $N = 142$  for the hatching date). Timing of hatching for handled birds was similar to non-handled ones ( $N = 142$ ; Wilcoxon's  $w = 2665.5$ ,  $P = 0.721$ ). The proportion of breeders tended to be slightly reduced by handling (handled birds:  $83.33 \pm 4.62\%$ ; non-handled birds:  $90.95 \pm 1.93\%$ ;  $N = 221$ ,  $\chi^2 = 3.072$ ,  $P = 0.080$ ).

#### MOLECULAR SEXING AND HORMONE ASSAY

Blood samples were centrifuged and plasma was decanted and stored at  $-20^\circ\text{C}$  until assayed. Red cells were also kept frozen for molecular

sexing at the Centre d'Etudes Biologiques de Chizé (CEBC). Sex was determined by polymerase chain reaction (PCR) amplification of a part of two highly conserved genes (CHD) present on the sex chromosomes (Fridolfsson & Ellegren 1999), as detailed in Weimerskirch, Lallemand & Martin (2005). Plasma concentrations of total baseline and stress-induced corticosterone were determined by radioimmunoassay at the CEBC, as described by Lormée *et al.* (2003). Only one assay was performed and the intra-assay coefficient of variation was 5.6% ( $N = 4$  duplicates).

#### STATISTICAL ANALYSES

All analyses were performed using R 2.8.0 (<http://r-project.org/>). We used generalized linear model (GLM) with binomial error distribution and a logit link function to explain breeding decision, and GLM with normal errors and an identity link function to explain egg-laying date and hormone levels. Dependent continuous variables were previously tested for normality with a Shapiro–Wilk test and were log-transformed when necessary. An information-theoretic approach was used to select the best models (Burnham & Anderson 2002). Model selection criteria included the corrected version of Akaike's information criterion (AICc) for small sample sizes and the difference in AICc between each candidate model and the model with the lowest AICc value ( $\Delta\text{AICc}$ ; Burnham & Anderson 2002). AICc weight can be used as a measure of the relative probability of a model for being the best model with a given data set among other potential models. Selected models were then checked for assumptions, that is, constancy of variance and residual normality. Parameters' estimates  $\pm$  SE were given for models with the highest AICc weight and by removing interactions with covariables (Quinn & Keough 2002).

#### Age, breeding decision and timing of breeding

As female body size is known to account for a great part of the variation in the egg-laying date of snow petrels (Barbraud, Lormée & LeNeve 2000), we included body size in our analysis. Therefore, we tested whether breeding decision and egg-laying date were influenced by standardized body condition, body size, sex and the interaction body condition  $\times$  sex and body size  $\times$  sex in pre-laying snow petrels ( $N = 66$ ). To test for an effect of age, we used known-age snow petrels handled during the pre-laying period and the 54 known-age birds handled after the egg-laying period. As hatching date was monitored for each nest and was highly correlated to egg-laying date (estimate =  $0.770 \pm 0.077$ ,  $F_{1,36} = 99.30$ ,  $P < 0.001$ ), we used hatching date instead of egg-laying date in this model. We tested whether breeding decision and hatching date were influenced by 'sex', 'age', 'age<sup>2</sup>' and the interaction 'sex (age + age<sup>2</sup>)'. Within breeding pairs, the age of the male was positively correlated with that of the female ( $F_{1,23} = 6.522$ ,  $P = 0.018$ ).

#### Age and stress hormones

Because standardized body condition was negatively related to sampling date (see 'Results'), we first tested whether baseline and stress-induced corticosterone levels were influenced by body condition, sex and the interaction 'sex  $\times$  body condition' by using linear regressions. Then we analysed whether baseline and stress-induced corticosterone levels were influenced by the sampling date (hereafter 'date'), 'sex', 'age', 'age<sup>2</sup>' and the interaction 'sex (age + age<sup>2</sup>)'.

Moreover, we ran the analysis again adding the 'time before egg-laying' variable (i.e. the time between sampling and egg-laying date)

to take into account a potential hormonal change with advancing time. 'Time before egg-laying' and 'sampling time' were tested separately as they were significantly negatively linked ( $F_{1,52} = 39.580$ ,  $P < 0.001$ ).

### Stress hormones, breeding decision and timing of breeding

We tested the influence of pre-laying baseline (or stress-induced) corticosterone on breeding decision and egg-laying date. As baseline and stress-induced corticosterone levels differed between sexes (see 'Results'; Table 1), we could not include the 'sex' and 'baseline (or stress-induced) corticosterone levels' variables simultaneously in a model. Thus, we standardized baseline (or stress-induced) corticosterone levels for each sex by subtracting for each sex the mean baseline (or stress-induced) corticosterone levels from each observed value, and dividing by the SD. Because sex and standardized corticosterone levels were no longer associated, we started our analyses from the general model including the 'date', 'sex', 'baseline (or stress-induced) corticosterone levels' variables and the 'sex  $\times$  baseline (or stress-induced) corticosterone levels' interaction. To test for an effect on breeding decision and on egg-laying date, we considered all monitored nests prospected during the pre-laying period and then only eggs which hatched successfully.

## Results

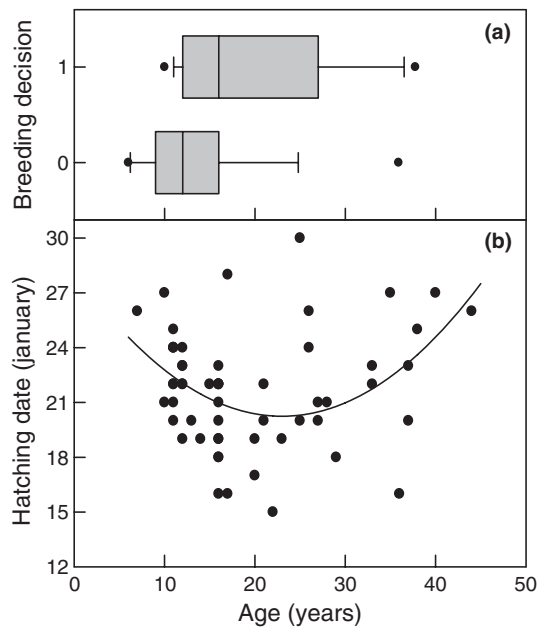
Pre-laying body condition was not related to age (estimate =  $0.009 \pm 0.014$ ,  $F_{1,53} = 0.427$ ,  $P = 0.516$ ). Pre-laying body condition decreased with the date of sampling (estimate =  $-0.111 \pm 0.029$ ,  $F_{1,64} = 14.961$ ,  $P < 0.001$ ), but was not affected by the time between sampling and egg-laying date (estimate =  $0.047 \pm 0.028$ ,  $F_{1,52} = 2.779$ ,  $P = 0.102$ ).

### AGE, BREEDING DECISION AND TIMING OF BREEDING

Breeding decision and egg-laying date were not influenced by pre-laying body condition or body size (for all tests:  $P > 0.16$ ). Breeding decision was influenced by the breeder's age ( $N = 109$ ,  $\chi^2 = 8.586$ ,  $P < 0.001$ ): birds that skipped breeding were mainly younger ( $< 13$  years old; Fig. 2a). There was a significant effect of age on hatching date, in a quadratic way: young (less than 13 years old) and very old (more than 30 years old) birds bred later than the 13–30-year-old birds (Fig. 2b;  $N = 60$ ; age: estimate =  $-0.484 \pm 0.209$ ,  $\chi^2 = 4.976$ ,  $P = 0.026$ ; age<sup>2</sup>: estimate =  $0.010 \pm 0.004$ ,

**Table 1.** Model selection using corrected version of Akaike's information criterion (AICc) to explain (a) baseline corticosterone levels ( $N = 47$ ) and (b) stress-induced corticosterone levels ( $N = 53$ ).  $K$  refers to the number of parameters. Candidate models and models without any explanatory variable (the 'intercept model') are ranked according to their AICc. The second part of the table includes the parameter estimates for the selected model: (a) 'Date; Sex' and (b) 'Date; Sex; Age; Age<sup>2</sup>'

No.	Model	AICc	$\Delta$ AICc	AICc weight	$K$
<b>(a)</b>					
1.	Date; Sex	120.58	0	37.6%	4
2.	Date	121.73	1.15	21.1%	3
3.	Date; Sex; Age	122.93	2.35	11.6%	5
4.	Date; Sex; Age; Sex $\times$ Age	123.64	3.07	8.1%	6
5.	Date; Age	124.01	3.43	6.8%	4
6.	Intercept	124.36	3.78	5.7%	2
7.	Date; Sex; Age; Age <sup>2</sup> ; Sex (Age + Age <sup>2</sup> )	125.23	4.65	3.7%	8
8.	Date; Sex; Age; Age <sup>2</sup>	125.41	4.84	3.4%	6
9.	Date; Age; Age <sup>2</sup>	126.38	5.80	2.1%	5
	Selected model	Parameters	Estimate $\pm$ SE	Chi-squared	$P$
	Date; Sex	Intercept	1.44 $\pm$ 0.48	9.11	0.003
		Date	0.08 $\pm$ 0.04	4.87	0.027
		Sex	0.44 $\pm$ 0.24	3.35	0.067
<b>(b)</b>					
1.	Date; Sex; Age; Age <sup>2</sup>	480.42	0	63.2%	6
2.	Date; Age; Age <sup>2</sup>	484.21	3.80	9.5%	5
3.	Date; Sex	484.38	3.96	8.7%	4
4.	Date; Sex; Age	485.12	4.71	6.0%	5
5.	Date; Sex; Age; Age <sup>2</sup> ; Sex (Age + Age <sup>2</sup> )	485.22	4.80	5.7%	8
6.	Date	486.23	5.81	3.5%	3
7.	Date; Sex; Age; Sex $\times$ Age	487.45	7.03	1.9%	6
8.	Date; Age	487.88	7.46	1.5%	4
9.	Intercept	502.98	22.56	0%	2
	Selected model	Parameters	Estimate $\pm$ SE	Chi-squared	$P$
	Date; Sex; Age; Age <sup>2</sup>	Intercept	75.17 $\pm$ 26.58	8.04	0.005
		Date	3.51 $\pm$ 0.94	13.87	< 0.001
		Sex	14.37 $\pm$ 5.87	5.99	0.014
		Age	-5.76 $\pm$ 2.06	7.83	0.005
		Age <sup>2</sup>	0.12 $\pm$ 0.04	6.94	0.008



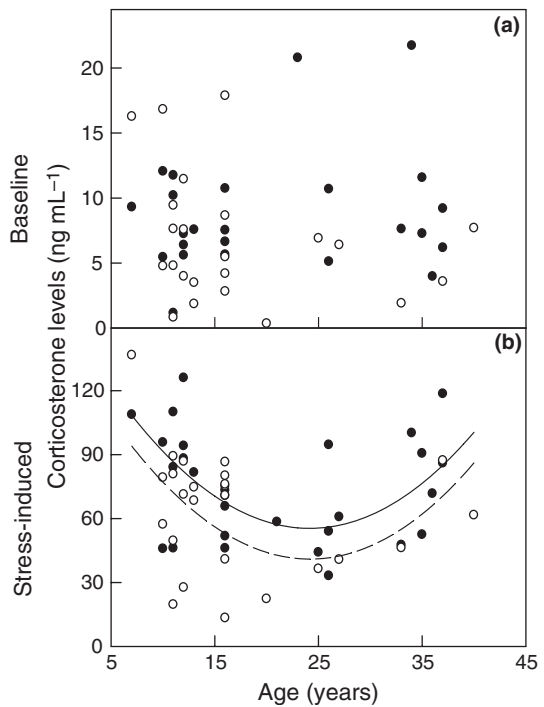
**Fig. 2.** (a) Breeding decision (0 = non-breeder; 1 = breeder) in relation to age in snow petrels (The median, 10th, 25th, 75th and 90th percentiles are plotted as horizontal boxes with error bars) and (b) hatching date in relation to age in snow petrels. The probability of breeding increased with age, whereas young and very old birds bred later than middle-aged ones.

$\chi^2 = 6.190$ ,  $P = 0.013$ ). As mentioned, the use of hatching date was more powerful (larger sample size) than the use of egg-laying date ( $N = 33$ ). However, this did not affect the detected trend (egg-laying date =  $-0.375 \times \text{age} + 0.009 \times \text{age}^2$  vs. hatching date =  $-0.484 \times \text{age} + 0.010 \times \text{age}^2$ ).

#### AGE, BODY CONDITION AND STRESS HORMONE LEVELS

Baseline corticosterone levels were not associated with pre-laying body condition (estimate =  $-0.042 \pm 0.631$ ,  $F_{1,55} = 0.006$ ,  $P = 0.940$ ) even when considering an interaction with sex (estimate =  $0.105 \pm 0.290$ ,  $F_{1,53} = 0.124$ ,  $P = 0.726$ ). However, baseline corticosterone levels were higher in males than in females ( $8.255 \pm 0.758$  and  $6.620 \pm 0.972$  ng mL<sup>-1</sup>, respectively), increased with the date of sampling (Table 1a), but were not influenced by age (Table 1a and Fig. 3a).

During the pre-laying period, males and females responded to the stress of capture by increasing their corticosterone levels significantly ( $P < 0.001$  for paired Student's *t*-tests). Stress-induced corticosterone levels were independent of baseline corticosterone levels (estimate =  $0.003 \pm 0.004$ ,  $F_{1,54} = 1.115$ ,  $P = 0.296$ ). Stress-induced corticosterone levels were negatively correlated to body condition (estimate =  $-13.690 \pm 3.139$ ,  $F_{1,62} = 19.022$ ,  $P < 0.001$ ). Moreover, stress-induced corticosterone levels were higher in males than in females ( $77.937 \pm 4.213$  and  $67.860 \pm 5.657$  ng mL<sup>-1</sup>, respectively), increased with the date of sampling and were correlated to age in a quadratic



**Fig. 3.** (a) Baseline and (b) stress-induced corticosterone levels during the pre-laying period in relation to age in males ( $N = 29$ , closed circles with solid best-fit line), and females ( $N = 25$ , open circles with dashed best-fit line) snow petrels.

way (Table 1b): young and very old birds were more sensitive to stress than middle-aged ones (Fig. 3b).

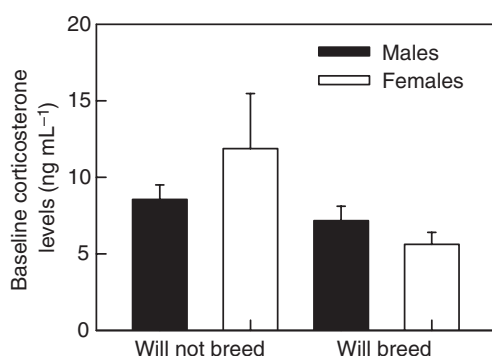
After running the analysis with the 'time before egg-laying' variable instead of the 'sampling date' variable, stress hormone levels were not associated with the time before egg-laying ( $P > 0.17$  for baseline and stress-induced corticosterone levels).

#### STRESS HORMONES, BREEDING DECISION AND TIMING OF BREEDING

Elevated baseline corticosterone levels during the pre-laying period were associated with a higher probability of skipping breeding in females (Table 2a and Fig. 4). Breeding decision was not influenced by stress-induced corticosterone levels (Table 2b). When considering all laid eggs (i.e. eggs that hatched successfully and eggs that failed to hatch), egg-laying dates were neither influenced by the standardized baseline corticosterone levels (estimate =  $0.123 \pm 0.111$ ,  $F_{1,43} = 1.236$ ,  $P = 0.272$ ), nor by the standardized stress-induced corticosterone levels (estimate =  $0.206 \pm 0.529$ ,  $F_{1,50} = 0.027$ ,  $P = 0.869$ ), even when considering an interaction effect 'sex  $\times$  standardized corticosterone levels' (baseline: estimate =  $-1.037 \pm 1.148$ ,  $F_{1,41} = 0.817$ ,  $P = 0.371$ ; stress-induced: estimate =  $0.461 \pm 1.079$ ,  $F_{1,48} = 0.183$ ,  $P = 0.671$ ). However, hatching success was negatively correlated with standardized baseline corticosterone levels (estimate =  $-0.924 \pm 0.423$ ,  $N = 40$ ,  $\chi^2 = 9.037$ ,  $P = 0.003$ ): snow petrels with elevated standardized baseline corticoste-

**Table 2.** Model selection using corrected version of Akaike's information criterion (AICc) to explain breeding decision (0 = non-breeder; 1 = breeder) in relation to (a) standardized baseline corticosterone levels (Bas. CORT,  $N = 57$ ) and standardized stress-induced corticosterone levels (Max. CORT,  $N = 64$ ) during the pre-laying period.  $K$  refers to the number of parameters. Candidate models and models without any explanatory variable (the 'intercept model') are ranked according to their AICc. The second part of the table (a) gives the estimates of parameter (absolute baseline corticosterone levels) for males ( $N = 32$ ) and females ( $N = 25$ )

No.	Model	AICc	$\Delta$ AICc	AICc weight	$K$
(a)					
1.	Bas. CORT; Sex; Bas. CORT $\times$ Sex	57.62	0	39.6%	4
2.	Intercept	57.92	0.30	34.0%	1
3.	Bas. CORT	59.14	1.52	18.5%	2
4.	Bas. CORT; Sex	60.82	3.20	8.0%	3
	Selected model	Parameters	Estimate $\pm$ SE	Chi-squared	$P$
	Bas. CORT; Sex; Bas. CORT $\times$ Sex	<i>Males</i>			
		Intercept	0.55 $\pm$ 1.02	17.301	<0.001
		Bas. CORT	0.09 $\pm$ 0.12	0.542	0.461
		<i>Females</i>			
		Intercept	3.83 $\pm$ 1.36	89.867	<0.001
		Bas. CORT	-0.26 $\pm$ 0.12	7.315	0.007
(b)					
1.	T Intercept	61.47	0	50.6%	1
2.	T Max. CORT	62.46	0.98	31.0%	2
3.	T Max. CORT; Sex	64.16	2.68	13.2%	3
4.	T Max. CORT; Sex; Max. CORT $\times$ Sex	66.04	4.56	5.2%	4



**Fig. 4.** Standardized baseline pre-laying corticosterone levels (ng mL<sup>-1</sup>, mean and SE) of snow petrels in relation to breeding decision (will skip or will breed). Black-filled bars denote males and white-filled bars denote females.

rone levels during the pre-laying period were more likely to lose their egg. When only considering eggs that hatched successfully, there was a positive and significant correlation between standardized baseline corticosterone and egg-laying date (Table 3a and Fig. 5a). Pre-laying stress-induced corticosterone levels were not associated with the egg-laying date of hatched eggs (Table 3b and Fig. 5b).

## Discussion

As found in other avian species (Forslund & Pärt 1995; Gonzalez-Solis *et al.* 2004; Ezard, Becker & Coulson 2007; McCleery *et al.* 2008), young snow petrels (less than 13 years old) bred later than middle-aged birds (from 13 to 30 years). Moreover, young snow petrels were more likely to skip a breeding attempt than old ones. In addition, the oldest birds

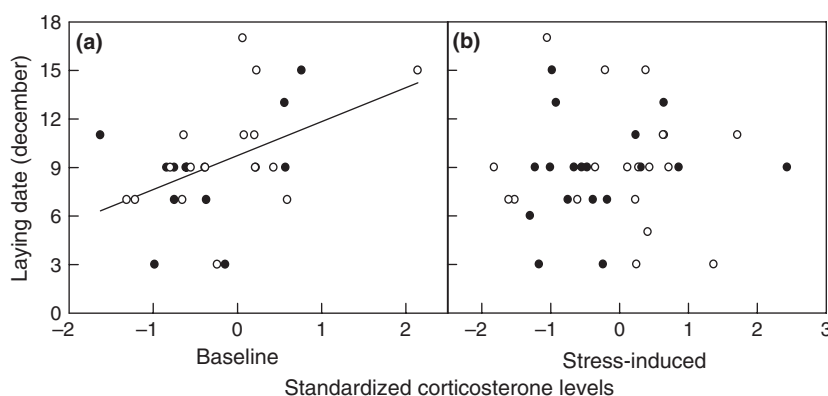
(> 30 years old) bred later than middle-aged ones, suggesting a possible effect of older age on the timing of breeding, a pattern only reported to date in the common tern (*Sterna hirundo*; Ezard, Becker & Coulson 2007) and the mute swan (*Cygnus olor*; McCleery *et al.* 2008). In parallel, young birds and very old birds were more sensitive to stress than middle-aged ones during the pre-laying period. However, there was no link between stress-induced corticosterone levels and egg-laying date. However, elevated baseline corticosterone levels were associated with a higher probability to skip breeding in females and a delayed timing of egg-laying in both sexes.

## AGE AND STRESS HORMONES

During the pre-laying period, snow petrels exhibited a 10-fold increase in corticosterone levels related to capture and handling. This magnitude was similar during incubation and was lower during chick rearing in petrel species (Adams *et al.* 2005; Angelier *et al.* 2007a, 2009). Adults in poor body condition were more sensitive to stress than those in good body condition as shown in many other species (Wingfield *et al.* 1998; Angelier *et al.* 2009). Furthermore, baseline and stress-induced corticosterone levels increased with sampling date, whereas body condition decreased with sampling date, but not with the time before egg-laying. During the pre-laying period, early-arrived petrels were indeed in better condition than late-arrived ones (Chastel, Weimerskirch & Jouventin 1995). Birds in poor condition and showing elevated corticosterone levels may then be of lower quality (e.g. low foraging skills) and may not be able to arrive early in the season. Males exhibited slightly higher baseline and stress-induced corticosterone levels than females, possibly in response to different pre-laying energetic constraints and activities (Wingfield *et al.*

**Table 3.** Model selection using corrected version of Akaike's information criterion (AICc) to explain laying date of hatched egg in relation to (a) standardized baseline corticosterone levels (Bas. CORT,  $N = 30$ ) and standardized stress-induced corticosterone levels (Max. CORT,  $N = 37$ ) during the pre-laying period.  $K$  refers to the number of parameters. Candidate models and models without any explanatory variable (the 'intercept model') are ranked according to their AICc. The second part of the table (a) includes the parameter estimates for the selected model 'Bas. CORT'

No.	Model	AICc	$\Delta$ AICc	AICc weight	$K$
(a)					
1.	Bas. CORT	156.41	0	66.8%	3
2.	Bas. CORT; Sex	158.62	2.21	22.2%	4
3.	Bas. CORT; Sex; Bas. CORT $\times$ Sex	161.26	4.85	5.9%	5
4.	Intercept	161.56	5.15	5.1%	2
	Selected model	Parameters	Estimate $\pm$ SE	Chi-squared	$P$
	Bas. CORT	Intercept	9.74 $\pm$ 0.58	277.020	< 0.001
		Bas. CORT	2.10 $\pm$ 0.75	7.618	0.006
(b)					
1.	Intercept	198.60	0	63.3%	2
2.	Max. CORT	200.59	1.99	23.4%	3
3.	Max. CORT; Sex	202.47	3.87	9.1%	4
4.	Max. CORT; Sex; Max. CORT $\times$ Sex	204.05	5.45	4.1%	5



**Fig. 5.** Relationship between the egg-laying date (December) and standardized pre-laying baseline (a) and stress-induced (b) corticosterone levels of breeders. Closed circles denote males and open circles denote females.

1998; Landys, Ramenofsky & Wingfield 2006) such as fasting and strong intrasexual competition (Barbraud & Chastel 1999).

Contrary to our prediction, pre-laying baseline corticosterone levels were not correlated with age, as reported in other long-lived birds (Angelier *et al.* 2006; Heidinger, Nisbet & Ketterson 2006; Angelier *et al.* 2007a,b). This may suggest that baseline corticosterone is more likely to be influenced daily by short-term stressful events (Landys, Ramenofsky & Wingfield 2006) and be independent of age. However, breeding experience (i.e. number of previous breeding attempts) greatly differs between snow petrels of the same age, because they frequently skip a breeding attempt and the age when first breeding is greatly variable between individuals (Chastel, Weimerskirch & Jouventin 1993). In long-lived birds, breeding experience explains better the variation of baseline corticosterone than age *per se* (Angelier *et al.* 2006, 2007b). This suggests that baseline corticosterone levels are not only influenced by short-term factors but can also be influenced by longer-term factors such as breeding experience.

According to our prediction, young pre-laying birds were more sensitive to stress than middle-aged ones. This has also been observed during incubation in another long-lived bird

(Heidinger, Nisbet & Ketterson 2006) and is commonly interpreted as a mechanism for low commitment into current reproduction (Heidinger, Nisbet & Ketterson 2006; Angelier *et al.* 2007a). Young birds should favour future reproductive opportunities, likely through high pre-laying stress-induced corticosterone levels. Stress resistance may have arisen from a habituation process to handling with age. However, known-age snow petrels have been seldom handled prior to this study and a similar study found no effect of recapture history on stress hormone levels (Heidinger, Nisbet & Ketterson 2006). The lower stress response in middle-aged snow petrels compared with younger ones may originate from a decrease in adrenal capacity as suggested by a recent study of known-age common terns (Heidinger, Ketterson & Nisbet 2008).

Interestingly, we found that the oldest birds (> 30 years) showed a stronger response to stress than the 13–30-year-old birds. To our knowledge, this is the first report of a magnified stress response at an older age in a free-living organism. The stronger stress response in the oldest birds may therefore originate from a possible physiological senescence of the hypothalamo-pituitary-adrenal (HPA) system, as observed in elderly humans and rats (Sapolsky,

Krey & McEwen 1986a,b; Wilkinson, Peskind & Raskind 1997; Sapolsky 1999; Otte *et al.* 2005).

An alternative hypothesis could be a progressive disappearance of adults excessively sensitive to stress over time. This would imply that the magnitude of the stress response is fixed early in the reproductive life of an individual. However, as shown experimentally in the house sparrow (*Passer domesticus*; Lendvai, Giraudeau & Chastel 2007), the same individual can modulate its hormonal stress response flexibly according to the value of the reproductive event. Furthermore, this 'selection hypothesis' would not explain the magnified stress response found in the oldest birds.

#### AGE, STRESS HORMONES AND TIMING OF BREEDING

As found in Florida scrub jay and zebra finches (Salvante & Williams 2003; Schoech *et al.* 2009), elevated pre-laying baseline corticosterone levels were associated with the decision to skip breeding in female snow petrels, and with a postponed egg-laying date in both sexes. Baseline corticosterone seems therefore to fine-tune the timing of breeding with regard of stress occurring during pre-laying, suggesting energetic constraints.

Male stress hormone levels were important for the timing of breeding, although it is thought that females mainly drive the egg-laying date (Caro *et al.* 2009). This suggests that baseline corticosterone levels of one partner may reflect the global state of the breeding pair (e.g. quality of the nest, male's ability to defend the nest), or an assortative mating according to stress levels. However, male corticosterone levels were not as decisive as in females for breeding decision. This highlights a potential sex difference in the response to environmental cues related to seasonal events (Ball & Ketterson 2008; Blas & Hiraldo 2010).

How could baseline corticosterone orchestrate breeding decision and timing of breeding? In domesticated animals, cortisol suppresses luteinizing hormone (LH, a pituitary hormone involved in the onset of breeding) secretion by inhibiting pituitary response to gonadotropin-releasing hormone (GnRH; Breen & Karsch 2004). Moreover, corticosterone potentially acts via the gonadotropin-inhibiting hormone (GnIH), a recently discovered peptide, that downregulates the hypothalamic–pituitary–gonadal axis (Bentley *et al.* 2006; Ubuka *et al.* 2006; Calisi, Rizzo & Bentley 2008; Greives *et al.* 2008; Kirby *et al.* 2009). Thereby, in snow petrels, stress hormones might activate the GnIH system and postpone the timing of breeding or even cancel the breeding attempt.

However, the positive correlation between baseline corticosterone levels and egg-laying date was only observed in snow petrels that incubated their egg successfully. Failed clutches belonged to parents with high baseline corticosterone levels during the pre-laying period. We did not observe any disappearance of egg owing to predation and all case of failure were because of nest desertion. In snow petrels, incubation requires petrels to fast on the nest. Birds bearing elevated baseline corticosterone levels may then be of lower quality

(e.g. low foraging skills; Angelier *et al.* 2007c) and may not be able to cope with the energetic constraints of incubation. In conclusion, higher baseline corticosterone levels in snow petrels during the pre-laying period were correlated to a higher probability (i) to skip the breeding attempt, (ii) to loose the egg or (iii) to start breeding later in the season.

Our aim was to test whether the often observed age-specific timing of breeding could be related to higher stress sensitivity in young breeders compared with older ones. Contrary to our prediction and in spite of a strong age-specific stress response during the pre-laying period, the sensitivity to stress was not the functional mechanism involved in age-specific timing of breeding pattern observed in snow petrels. Our sample size was sufficient to rule out any hidden effect. Stress-induced levels of corticosterone are not as flexible and reactive to daily environmental cues than baseline corticosterone (Kitaysky, Piatt & Wingfield 2007). Thus, stress-induced corticosterone levels might be inappropriate to rule the adjustment of timing of breeding or the decision to breed.

However, baseline corticosterone levels, which predicted breeding decision and timing of breeding, were independent of age. How can the age-specific differences in timing of breeding be explained? The link between age and timing of breeding could possibly be indirect and originate from other age-related parameters (see Blas & Hiraldo 2010). In snow petrels, timing of breeding is better explained by previous breeding experience than age *per se* (unpublished data). We suggested that baseline corticosterone levels would be higher in inexperienced birds as found in other long-lived birds (Angelier *et al.* 2006, 2007b), and in turn would explain the functional mechanism of late breeding in young birds. Regarding the very old snow petrels, their late breeding could be the consequence of reduced foraging skills (Catry *et al.* 2006) and/or the possible death of their previous mate. Newly formed pairs breed later than long-term partners (snow petrels, unpublished data) and may exhibit higher baseline corticosterone levels, as shown in black-legged kittiwakes (Angelier *et al.* 2007d). Thus age-related factors, like previous breeding experience, foraging ability or change of mates may influence the timing of breeding rather than age *per se*. In turn, these age-related factors could influence the baseline corticosterone levels of pre-laying birds.

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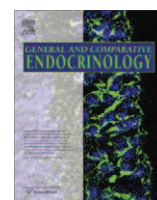
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## Stress and the timing of breeding: Glucocorticoid-luteinizing hormones relationships in an arctic seabird

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### ABSTRACT

In birds, stressful environmental conditions delay the timing of breeding but the underlying mechanisms are poorly understood. The stress hormone corticosterone appears to be a good candidate for mediating the decision to breed and when to start egg-laying, via a possible inhibition of luteinizing hormone (LH) and sex-steroids production. We used luteinizing hormone releasing hormone (LHRH) challenge in pre-laying male and female Black-legged kittiwakes (*Rissa tridactyla*) to test whether LH and testosterone secretion were depressed by elevated corticosterone levels. Females bearing high baseline corticosterone levels showed reduced baseline LH levels and a low ability to release LH, following LHRH challenge. Further, females bearing low baseline LH levels and elevated baseline corticosterone levels were more likely to skip breeding. However, non-breeding females were physiologically primed for breeding, since they mounted high LHRH-induced LH release. Egg-laying date was advanced in good body condition females but was unaffected by hormones secretion. In males, corticosterone levels had no effect on LH and/or testosterone secretion and did not affect their decision to breed. Interestingly, males with high LHRH-induced testosterone release bred early. Our study highlights clear sex-differences in the HPG sensitivity to stress hormones in pre-laying kittiwakes. Because females have to store body reserves and to build up the clutch, they would be more sensitive to stress than males. Moreover, intrasexual competition could force male kittiwakes to acquire reproductive readiness earlier in the season than females and to better resist environmental perturbations. We suggest that high testosterone releasing ability would mediate behavioural adjustments such as courtship feeding, which would stimulate early egg-laying in females.

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### 1. Introduction

Breeding at the right time is a key-component of fitness, as successful reproduction requires a good overlap between energy-demanding needs and the peak of available resources (*match-mismatch hypothesis*, Cushing, 1990; Visser et al., 1998; Stenseth and Mysterud, 2002; Durant et al., 2007; review in Visser, 2008). Hence, as adaptive responses to environmental variations, free-living organisms must exhibit considerable behavioural and physiological flexibilities in the timing of their seasonal activities (review in Wingfield, 2008). To do so, they integrated photoperiod, as a fixed cue (Wingfield and Kenagy, 1991; Dawson et al., 2001) and variable cues, such as ambient temperature, food supply, nest sites availability, and/or stimulatory social interactions (Wingfield, 1980;

Wingfield and Kenagy, 1991; Ball, 1993; Visser et al., 1998; Wingfield et al., 2003; Schoech et al., 2004; Ball and Ketterson, 2008; Both et al., 2009). At the endocrine level, the onset of breeding involves the activation of the hypothalamic-pituitary-gonadal axis (hereafter HPG axis): increasing day length activates the expression of a neuro-hormone, the Gonadotropin Releasing Hormone (GnRH) that triggers the release of two pituitary gonadotrophins: the luteinizing hormone and the follicle-stimulating hormone (LH and FSH, reviewed in Dawson et al., 2001). In turn, gonadotrophins activate the gonadal development and the release of sex steroids such as estradiol and testosterone. A wide range of sex steroid hormone-dependent behaviours is then expressed (Ball, 1993), such as nest building, courtship, and mating. However, this hormonal cascade is not only driven by photoperiod, but could also be regulated by additional non-photoperiodic cues. Specifically, pre-laying energetic constraints, such as food shortage and/or environmental cues that enable individuals to anticipate food availability (see Shultz

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et al., 2009) should be involved in the down-regulation of the HPG axis (Wingfield et al., 2003).

At the individual level, the underlying mechanisms have received increased attention (review in Schoech et al., 2009). One potential mediator is the endocrine stress response, which is known to adjust life-history strategies in relation to environmental conditions and to individual physiological state (Ricklefs and Wikelski, 2002; Wingfield and Sapolsky, 2003). Indeed, the release of glucocorticoids during stressful perturbations triggers physiological and behavioural adjustments that shift energy investment away from reproduction and redirects it towards survival (Wingfield and Sapolsky, 2003). Relating this to the timing of breeding, elevated pre-laying glucocorticoids levels might postpone or even halt the immediate breeding event, as correlatively shown in Florida scrub-jays (*Aphelocoma coerulescens*, Schoech et al., 2009), Marine iguanas (*Amblyrhynchus cristatus*, Vitousek et al., 2010), and Snow petrels (*Pagodroma nivea*, Goutte et al., 2010). Moreover, experimental administration of glucocorticoids decreased the proportion of breeding females and delayed the onset of egg-laying in captive Zebra finches (*Taeniopygia guttata*, Salvante and Williams, 2003). However, the functional action of glucocorticoids on the HPG axis remains poorly understood in free-living vertebrates (reviewed in Schoech et al., 2009).

In domesticated animals, it has been better documented how stress and stress hormones can act on the HPG system via central and peripheral sites. First, glucocorticoids can suppress LH release by inhibiting pituitary responsiveness to GnRH (e.g. Breen and Karsch, 2006). Second, glucocorticoids can act directly at the hypothalamic level by disrupting the GnRH pulse frequency (e.g. Oakley et al., 2009). Third, glucocorticoids can impair testicular development but not through the inhibition of LH release (e.g. in Common carp, *Cyprinus carpio* L., Consten et al., 2002). A novel down-regulator of the HPG axis, the gonadotropin-inhibitory hormone (GnIH), appears to inhibit the release of LH, FSH and testosterone (review in Bentley et al., 2009; Tsutsui et al., 2009; McGuire and Bentley, 2010), and is especially expressed during stressful conditions and/or glucocorticoid release (Calisi et al., 2008; Kirby et al., 2009). Thus, stress hormones seem to be candidates for the stress-related inhibition of the reproductive axis and associated physiological pathways.

Despite these findings from captive or domesticated species, the functional action of glucocorticoids on gonadotropin release and timing of breeding remains unclear and controversial in free-living vertebrates (review in Schoech et al., 2009). For instance, experimental increased glucocorticoid levels did not suppress LH and testosterone levels in free-living male American Tree sparrows (*Spizella arborea*, Astheimer et al., 2000) and failed to delay breeding in food-supplemented Florida scrub jay (*Aphelocoma coerulescens*, Schoech et al., 2007). In that context, it is crucial to carefully distinguish the two levels of glucocorticoids. The baseline glucocorticoid level is a marker of activities, energetic state and food availability (Kitaysky et al., 1999; Love et al., 2004; review in Landys et al., 2006), while the stress-induced glucocorticoid level mirrors the sensitivity to stress and the commitment into current reproduction (review in Wingfield and Sapolsky, 2003; Lendvai et al., 2007). Moreover, it is difficult to draw a general picture since males and females are expected to differ in the down-regulation of the HPG axis in response to stressful events (review in Ball and Ketterson, 2008).

The aim of the present study was to address the functional action of corticosterone –the main glucocorticoid in birds– on the breeding decision and the adjustment of egg-laying date in an Arctic population of Black-legged kittiwake (*Rissa tridactyla*). At the population level, a long-term (38 years) study in Svalbard showed great inter-annual variation in the timing of breeding (Moe et al., 2009). Moreover, breeding success was higher in years when

breeding was early, and high spring sea surface temperatures were associated with early breeding (Moe et al., 2009). This population is thus well-appropriated for our study, since at the individual level, early (pre-laying) environmental and physiological conditions should be decisive for the breeding schedule. In Svalbard, kittiwakes attend the colony during two months before egg-laying (i.e. pre-laying period), thus offering the opportunity to explore corticosterone/LH relationships in pre-laying males and females, by using luteinizing hormone releasing hormone (LHRH) challenge. LHRH is an equivalent of the GnRH, and a small injection of LHRH is commonly used to assess the readiness of a bird to breed and its ability to release temporary LH and sex-steroid (Schoech et al., 1996). We predicted that elevated baseline and/or stress-induced corticosterone levels during the pre-laying period would be linked to non-breeding decision or postponed egg-laying date, through the disruption of the HPG axis. First, we tested whether baseline and/or LHRH-induced levels of LH or testosterone decreased with increasing baseline and/or stress-induced corticosterone levels. Then, we investigated whether the breeding decision was related to hormonal levels (corticosterone, LH, testosterone). Finally, we tested whether the egg-laying date was related to hormonal levels (corticosterone, LH, testosterone).

## 2. Materials and methods

### 2.1. Study area and birds

Our study was conducted on a colony of Black-legged kittiwakes at Kongsfjorden, Svalbard (78°54'N, 12°13'E), 7 km southeast of Ny-Ålesund, Norway. Black-legged kittiwakes are colonial seabirds that breed on cliffs throughout the northern parts of the Pacific and Atlantic, including the Barents Sea region up to the Svalbard Archipelago (Anker-Nilssen et al., 2000). We studied kittiwakes in one plot of around 116 pairs breeding on cliff ledges at heights of 5–10 m.

### 2.2. Blood sampling and LHRH challenge

Male and female kittiwakes were sampled from 20 May to 6 June 2008, during the pre-laying period (i.e. copulations and nest building period). In Black-legged kittiwakes, baseline LH in males and females and testosterone in males reach maximal levels during the pre-laying period (Goutte et al., unpublished data). Seventy seven birds were caught on the nests with a noose at the end of a 5 m fishing rod. In 50 of them, a first blood sample (ca. 0.3 mL) was collected immediately after capture, from the alar vein with a 1 mL heparinised syringe and a 25-gauge needle to assess baseline LH, testosterone (in males only) and corticosterone levels. Bleeding time (i.e. time elapsed from capture to the end of the first blood sample: 3 min 31 ± 5 [SE] seconds) exceeded the time recommended by Romero and Reed (2005) for some birds. Corticosterone levels were thus related to bleeding time in males (estimate of the slope: 0.067 ± 0.019,  $F_{1,25} = 14.540$ ,  $p < 0.001$ ) but not in females ( $F_{1,21} = 0.012$ ,  $p = 0.914$ ). We performed statistical tests by using the residuals of corticosterone against bleeding time, then by excluding corticosterone levels, whose bleeding time exceeded 3 min, and then by using absolute corticosterone levels. The three approaches led to similar results. Hence, we reported the results for absolute corticosterone levels to facilitate the comparison with other published results.

Immediately after this first blood sampling, kittiwakes were injected with 0.1 mL of a solution of LHRH ([Gin<sup>8</sup>], Sigma Lot 121H04314) to test the responsiveness of the pituitary gland and the gonads. The LHRH was dissolved in physiological solution to yield a final dosage of 0.6 µg/0.1 mL (1.5 µg/kg body mass in

1 mL of 0.9% saline solution). This dose of LHRH has been shown to be sufficient to elicit the maximal release of LH in other seabird species (Jouventin and Mauget, 1996). We administered 0.1 mL of LHRH solution (LHRH-injected birds,  $N = 46$ ) or saline solution (control birds,  $N = 31$ ) directly into the alar vein. Kittiwakes were then placed into cloth bags and subsequent blood samples (ca. 0.3 mL) were collected from the alar vein at 10 min and 30 min after the injection. Hence we assessed the LHRH-induced release of LH in males and females, as well as the LHRH-induced release of testosterone in males only.

Kittiwakes were individually marked with metal rings and PVC plastic bands engraved with a three-digit code and fixed to the bird's tarsus for identification from a distance. Birds were weighed to the nearest 2 g using a Pesola spring balance, and their skull length (head + bill) was measured to the nearest 0.5 mm with a sliding caliper. A scaled mass index (Peig and Green, 2009) was calculated individually for males and females separately, because of sex-difference in skull length (Moe et al., 2002). Kittiwakes were marked with spots of dye on the forehead to distinguish them from their partner during subsequent observation and were released. Using a mirror at the end of an 8 m fishing rod, we checked the whole plot (ca. 116 nests) every two days to monitor breeding decision (at least one egg is laid or no egg laid) and egg-laying dates. Breeding decision and egg-laying date were not influenced by the LHRH-injection ( $p > 0.39$  for all tests).

### 2.3. Molecular sexing and hormone assay

Blood samples were centrifuged, and plasma and red blood cells were separated and stored at  $-20^{\circ}\text{C}$  until used, respectively in hormone assays or molecular sexing, at the Centre d'Etudes Biologiques de Chizé (CEBC). Molecular sexing was performed as detailed in Weimerskirch et al. (2005). LH radioimmunoassay was conducted following the methods previously described for other seabirds (Mauget et al., 1994; Chastel et al., 2005), and validated for Black-legged kittiwake plasma. Pooled plasma samples of kittiwakes produced dose–response curves that paralleled the chicken LH standard curves ("AGM 51122F", sources: LH, Prf. Ishii and Wakabayashi, Wadesa University, Japan, Fig. 1). Parallel curves indicate that the concentration-dependent binding of LH to antibody is similar in kittiwakes and chickens, and that this heterologous RIA can be used to

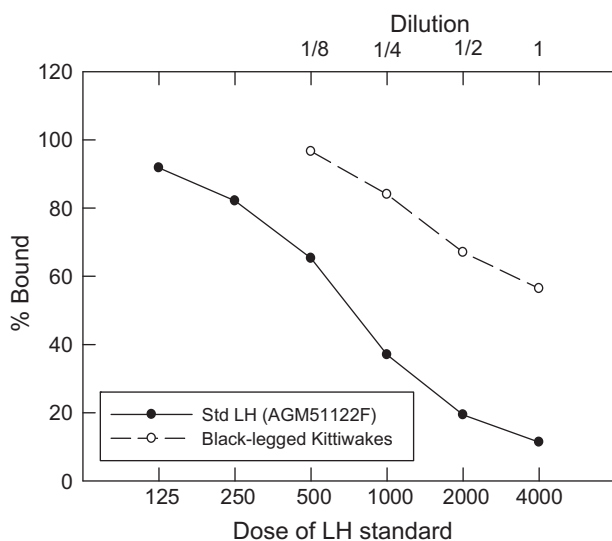


Fig. 1. Dose–response curve for LH in standard LH (AGM 51122F) and Black-legged kittiwakes. LH standard is expressed in pg per tube.

assess relative levels of plasma LH in the Black-legged kittiwakes. The lowest detectable concentration for LH was 0.06 ng/mL and the intra-assay coefficient of variation was 8.7% ( $N = 3$  duplicates). Plasma concentrations of testosterone were assayed for males only, by radioimmunoassay, at the CEBC as described by Chastel et al. (2003). The lowest detectable concentration for testosterone was 0.05 ng/mL and the intra-assay coefficient of variation was 7% ( $N = 3$  duplicates). Plasma concentrations of corticosterone were determined by radioimmunoassay at the CEBC, as described by Lormée et al. (2003). The lowest detectable concentration for corticosterone was 0.5 ng/mL. Only one assay was performed and the intra-assay coefficient of variation was 6.7% ( $N = 5$  duplicates).

### 2.4. Statistical analyses

All statistical analyses were performed using R 2.8.0 (R Development Core Team, 2008). We used generalised linear mixed-effects models (GLMM) and included bird identity as a random effect, to test the individual variation of hormone levels over 10 and 30 min after the injection of LHRH or saline solution. Then we used generalised linear models (GLM) with a normal/binomial error distribution and an identity/logit link function to test our biological assumptions (Table 1). Baseline and LHRH-induced LH and testosterone levels were tested as a function of bleeding time, sampling date and scaled mass index in males and females separately (Table 1i). Then, effects for baseline and/or stress-induced corticosterone levels on baseline and LHRH-induced LH levels were tested in pre-laying male and female kittiwakes and on baseline and LHRH-induced testosterone levels in males only (Table 1ii). Lastly, the effects for hormone levels on breeding decision and on first egg-laying date were tested in males and females (Table 1iii and 1iv). Diagnostic plots were assessed whether the data sufficiently met the assumptions of the linear model, and dependent continuous variables were log-transformed when necessary.

## 3. Results

LHRH-injection had no effect on stress-induced corticosterone levels compared to controls (10 min:  $F_{1,72} = 0.295$ ,  $p = 0.589$ , 30 min:  $F_{1,70} = 0.621$ ,  $p = 0.434$ ). Corticosterone levels significantly increased after 10 and 30 min (GLMM,  $F_{2,143} = 615.911$ ,  $p < 0.001$ ), without effect of sex (GLMM,  $F_{1,75} = 2.217$ ,  $p = 0.141$ ; interaction:  $F_{2,141} = 1.511$ ,  $p = 0.224$ ). Scaled mass index was neither correlated to sampling date (males:  $F_{1,41} = 1.127$ ,  $p = 0.295$ , females:  $F_{1,32} = 2.146$ ,  $p = 0.153$ ). Before LHRH-injection, baseline (i.e. at 0 min) hormone levels did not differ between LHRH-injected and control kittiwakes (GLM, LH:  $F_{1,41} = 0.690$ ,  $p = 0.411$ ; testosterone:  $F_{1,25} = 0.131$ ,  $p = 0.721$ , corticosterone:  $F_{1,74} = 0.501$ ,  $p = 0.481$ ). Following LHRH injection, LH levels reached maximum levels at 10 min and then returned to baseline after 30 min (GLMM, time as factor,  $F_{2,50} = 41.684$ ,  $p < 0.001$ ), without sex difference (sex:  $F_{1,42} = 2.263$ ,  $p = 0.140$ ; interaction:  $F_{2,48} = 1.640$ ,  $p = 0.205$ , Fig. 2A and B). In controls birds, LH levels significantly decreased over 10 and 30 min of handling (GLMM,  $F_{2,32} = 8.029$ ,  $p = 0.002$ , Fig. 2A and B) and were lower in males than in females at 30 min (GLM,  $F_{1,22} = 12.161$ ,  $p = 0.002$ ) but not at 10 min after the injection of saline solution ( $F_{1,21} = 1.302$ ,  $p = 0.267$ ). In LHRH-injected males, testosterone levels significantly increased over 30 min after injection (GLMM,  $F_{2,32} = 5.958$ ,  $p = 0.006$ , Fig. 2C), while in control males, testosterone significantly decreased over 30 min (GLMM,  $F_{2,28} = 5.648$ ,  $p = 0.009$ , Fig. 2C).

Concerning the factors influencing hormonal levels during the pre-laying period (Table 1i), baseline LH levels were not influenced by sampling date (males:  $F_{1,25} = 1.135$ ,  $p = 0.297$ ; females:  $F_{1,21} = 3.080$ ,  $p = 0.094$ ), by scaled mass index (males:  $F_{1,24} = 0.200$ ,

**Table 1**

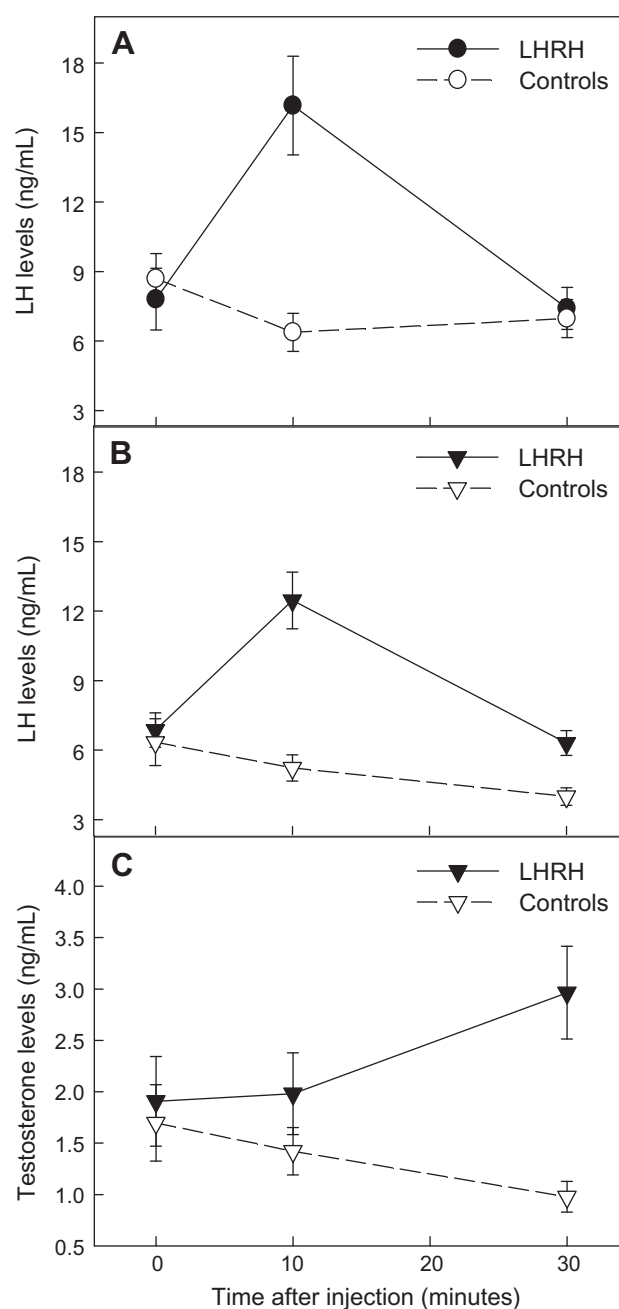
Biological assumptions tested and associated generalised linear models. Model selection was performed by a step down approach starting from the global model including all the independent variables. Sample sizes were given for males (Nm) and females (Nf). Corticosterone and luteinizing hormone levels were abbreviated as 'CORT' and 'LH'.

Dependant variable	Independent variables	Nm	Nf
<i>i-Effect of sampling date, body condition and sex on hormone levels</i>			
1-a Baseline LH	Bleeding time, sampling date, scaled mass index	27	23
1-b LHRH-induced LH	Sampling date, scaled mass index	23	17
1-c Baseline testosterone	Bleeding time, sampling date, scaled mass index	27	
1-d LHRH-induced testosterone	Sampling date, scaled mass index	22	
<i>ii-Effect of corticosterone levels on LH and testosterone levels</i>			
2-a Baseline LH	Baseline CORT	27	22
2-b Baseline LH	Stress-induced CORT	27	22
2-c LHRH-induced LH	Baseline CORT	23	16
2-d LHRH-induced LH	Stress-induced CORT	23	16
2-e Baseline Testosterone	Baseline CORT	27	
2-f Baseline Testosterone	Stress-induced CORT	26	
2-g LHRH-induced Testosterone	Baseline CORT	22	
2-h LHRH-induced Testosterone	Stress-induced CORT	22	
<i>iii-Effect of hormone levels on breeding decision (breeding or skipped breeding)</i>			
3-a Breeding decision	Body condition	43	32
3-b Breeding decision	Baseline CORT	27	21
3-c Breeding decision	Stress-induced CORT	42	29
3-d Breeding decision	Baseline LH	27	21
3-e Breeding decision	LHRH-induced LH	23	16
3-f Breeding decision	Baseline Testo	27	
3-g Breeding decision	LHRH-induced Testo	22	
<i>iv-Effect of hormone levels on first-egg-laying date</i>			
4-a First-egg-laying date	Body condition	31	20
4-b First-egg-laying date	Baseline CORT	20	15
4-c First-egg-laying date	Stress-induced CORT	30	20
4-d First-egg-laying date	Baseline LH	20	15
4-e First-egg-laying date	LHRH-induced LH	15	10
4-f First-egg-laying date	Baseline Testo	18	
4-g First-egg-laying date	LHRH-induced Testo	15	

$p = 0.659$ ; females:  $F_{1,20} = 1.425$ ,  $p = 0.247$ ), and by bleeding time (males:  $F_{1,23} < 0.001$ ,  $p = 0.997$ ; females:  $F_{1,19} = 0.014$ ,  $p = 0.908$ ). LHRH-induced LH levels (10 min) were not influenced by sampling date (males:  $F_{1,21} < 0.001$ ,  $p = 0.988$ ; females:  $F_{1,15} = 1.195$ ,  $p = 0.293$ ) and scaled mass index (males:  $F_{1,20} = 0.653$ ,  $p = 0.429$ ; females:  $F_{1,14} = 0.529$ ,  $p = 0.479$ ). In males, baseline testosterone levels were not related to bleeding time ( $F_{1,25} = 0.134$ ,  $p = 0.718$ ). Baseline and LHRH-induced (30 min) testosterone levels were neither influenced by sampling date ( $F_{1,24} = 0.717$ ,  $p = 0.406$  and  $F_{1,19} = 0.927$ ,  $p = 0.348$ ) nor by scaled mass index ( $F_{1,25} = 2.355$ ,  $p = 0.138$  and  $F_{1,20} = 3.385$ ,  $p = 0.081$ ).

### 3.1. Effect of corticosterone levels on LH and testosterone (Table 1ii)

Baseline LH levels decreased significantly with increasing baseline corticosterone levels in females (estimate:  $-0.405 \pm 0.228$ , Table 2a, Fig. 3A) but not in males (estimate:  $0.136 \pm 0.099$ , Table 2a, Fig. 3B). In addition, LHRH-induced LH levels (10 min) decreased significantly with increasing baseline corticosterone levels in females (estimate:  $-0.929 \pm 0.474$ , Table 2c, Fig. 3C) but not in males (estimate:  $-0.165 \pm 0.224$ , Table 2c, Fig. 3D). There was no effect of stress-induced corticosterone levels on baseline and LHRH-induced LH levels in both sexes (Table 2b and d). In males, baseline testosterone levels did not vary with baseline or stress-induced corticosterone levels (Table 2e and f). LHRH-induced testosterone levels did not vary with baseline and/or stress-induced corticosterone levels (Table 2g and h).



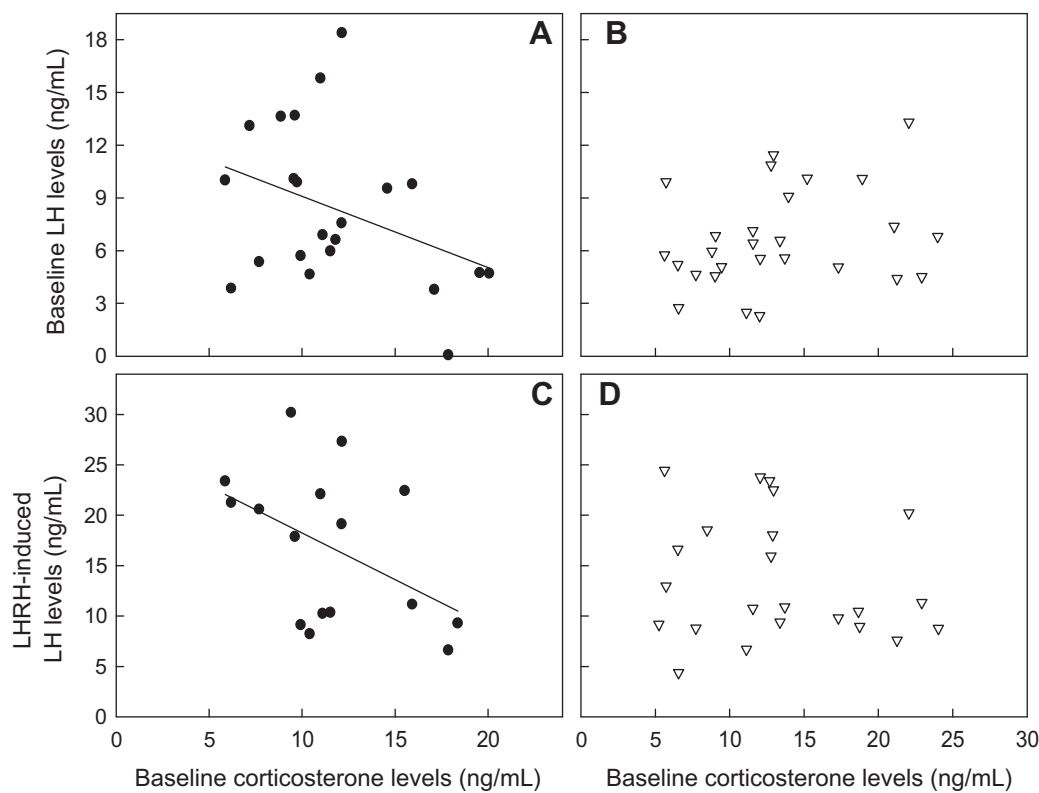
**Fig. 2.** Hormonal change after the injection of the LHRH solution (solid line, filled symbols) or of the saline solution (control, dashed lines, open symbols). LHRH-injected females (A, circle) and males (B, triangle) significantly released LH over the first 10 min then LH returned to the baseline levels over 30 min, while control levels decreased after injection. (C) Testosterone levels in males following the injection of LHRH (solid line) or saline solution (control, dashed lines). LHRH-injected males showed significantly elevated testosterone over 30 min, while control levels decreased after injection.

### 3.2. Hormonal levels and breeding decision (Table 1iii)

Breeding decision was not influenced by scaled mass index in both sexes (Table 3a). In females, breeding decision was related to pre-laying baseline LH levels and to pre-laying baseline corticosterone levels: females that will not breed show significantly lower pre-laying baseline LH levels and higher baseline corticosterone levels than females that will breed (Table 3a, Fig. 4A and B). LHRH-induced LH levels and stress-induced corticosterone levels were not linked to female breeding decision (Table 3a, Fig. 4C and D). In males, breeding decision was not related to levels of

**Table 2**  
Modelling baseline (Hyp. 2a and 2b) and LHRH-induced (Hyp. 2c and 2d) LH levels, baseline (Hyp. 2e and 2f) and LHRH-induced (Hyp. 2g and 2h) testosterone (T) levels as a function of baseline (or stress-induced) corticosterone levels (CORT), using GLMs (normal error distribution, identity link function) in pre-laying male and female kittiwakes.

Hyp	Dependant variable	Independent variables	d.f.	F	p-value
2a	Baseline LH levels	Baseline CORT (males)	1,25	1.880	0.183
		<b>Baseline CORT (females)</b>	<b>1,20</b>	<b>4.327</b>	<b>0.050</b>
2b	Baseline LH levels	Stress-induced CORT (males)	1,25	1.188	0.286
		Stress-induced CORT (females)	1,20	0.020	0.890
2c	LHRH-induced LH levels (log)	Baseline CORT (males)	1,21	0.224	0.641
		<b>Baseline CORT (females)</b>	<b>1,14</b>	<b>4.643</b>	<b>0.049</b>
2d	LHRH-induced LH levels (log)	Stress-induced CORT (males)	1,21	0.432	0.518
		Stress-induced CORT (females)	1,14	2.175	0.162
2e	Baseline T levels (log)	Baseline CORT (males)	1,25	1.370	0.253
2f	Baseline T levels (log)	Stress-induced CORT (males)	1,24	0.265	0.612
2g	LHRH-induced T levels (log)	Baseline CORT (males)	1,20	3.221	0.088
2h	LHRH-induced T levels (log)	Stress-induced CORT (males)	1,20	0.164	0.690



**Fig. 3.** In pre-laying females (filled circles), baseline (A) and LHRH-induced (C) LH levels decreased with increasing baseline corticosterone levels. In pre-laying males, baseline (B) and LHRH-induced (D) LH levels were not linked to baseline corticosterone levels.

baseline or LHRH-induced LH (Table 3a, Fig. 4A and C), baseline or stress-induced corticosterone (Table 3a, Fig. 4B and D), baseline or LHRH-induced testosterone (Table 3a).

### 3.3. Hormonal levels and egg-laying dates (Table 1iv)

In females, there was a significant effect of pre-laying scaled mass index on first egg-laying date (Table 3b): females with high scaled mass index start laying their first egg early (Fig. 5A). Males pre-laying scaled mass index had no effect on egg-laying date (Table 3b). Baseline or stress-induced corticosterone, baseline or LHRH-induced LH levels and baseline testosterone levels had no influence on first egg-laying date (Table 3b). On the other hand, males with high LHRH-induced testosterone levels bred significantly earlier than males with low LHRH-induced testosterone levels (Table 3b, Fig. 5B).

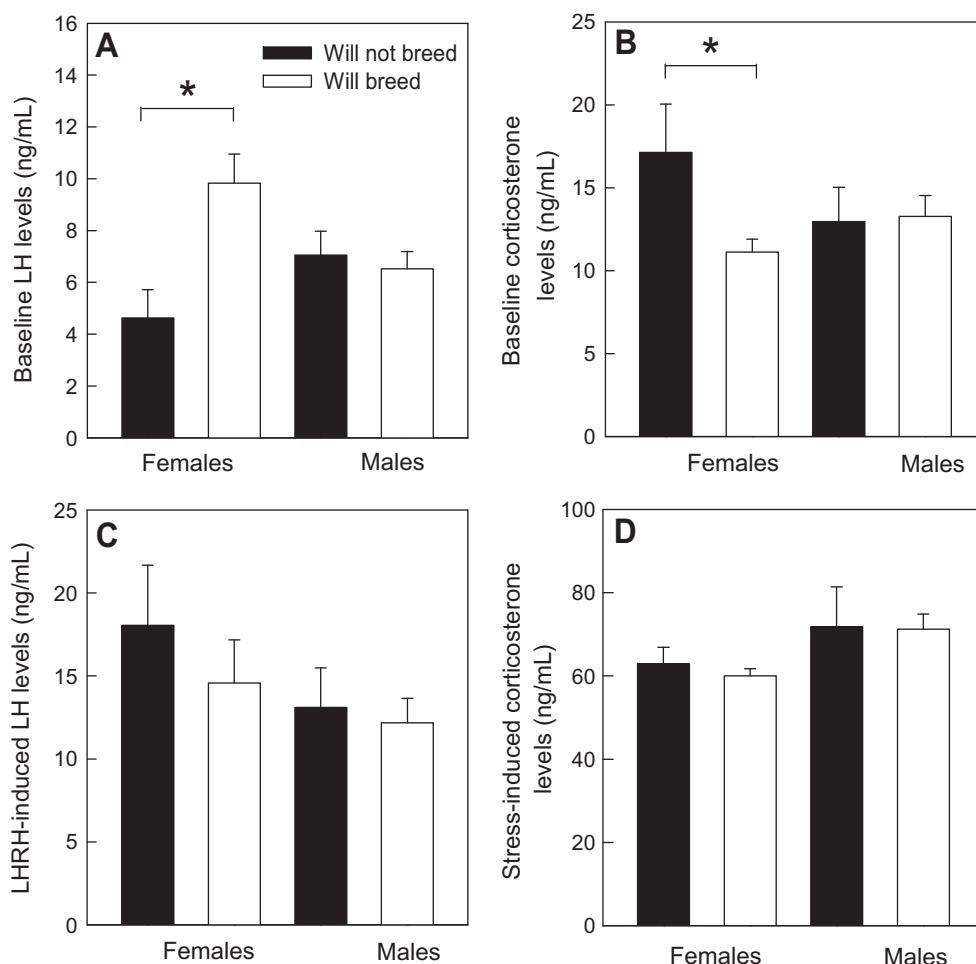
## 4. Discussion

In the present study we showed that elevated baseline, but not stress-induced, corticosterone levels have the potential to disrupt the HPG axis of female Black-legged kittiwakes. Furthermore, females with low baseline LH levels and elevated baseline corticosterone levels had the highest probability to skip breeding. On the contrary, elevated baseline and/or stress-induced corticosterone levels did not disrupt LH release in males, highlighting strong sex-difference in the HPG sensitivity to stress in Black-legged kittiwakes. Concerning the timing of breeding, females' scaled mass index, but not hormone levels, had a positive influence on egg-laying date. Males that exhibited high LHRH-induced testosterone release ability bred earlier than males with low ability to release testosterone.

**Table 3**

Modelling (a) breeding decision and (b) first-egg-laying date in 2008 as a function of scaled mass index and hormones levels using GLMs with binomial error distribution and logit link function (a) or normal error distribution and identity link function (b).

Independent variables	N	Chi <sup>2</sup>	p-value	N	Chi <sup>2</sup>	p-value
a-Breeding decision				FEMALES		
Scaled mass index	43	0.087	0.768	32	1.078	0.299
<b>Baseline CORT</b>	27	0.016	0.900	<b>21</b>	<b>7.656</b>	<b>0.006</b>
Stress-induced CORT	42	0.029	0.866	30	0.036	0.850
<b>Baseline LH</b>	27	0.173	0.678	<b>21</b>	<b>7.368</b>	<b>0.007</b>
LHRH-induced LH	23	1.599	0.206	16	0.614	0.433
Baseline Testosterone	27	0.704	0.402			
LHRH-induced Testosterone	22	0.443	0.506			
b-First egg-laying date				FEMALES		
Independent variables	d.f.	F	p-value	d.f.	F	p-value
<b>Scaled mass index</b>	1,29	0.486	0.491	<b>1,18</b>	<b>5.289</b>	<b>0.034</b>
Baseline CORT	1,18	1.622	0.219	1,13	0.004	0.953
Stress-induced CORT	1,28	0.650	0.427	1,18	0.003	0.959
Baseline LH	1,18	0.425	0.523	1,13	0.874	0.367
LHRH-induced LH	1,13	0.987	0.339	1,8	0.323	0.585
Baseline Testosterone	1,16	1.639	0.219			
<b>LHRH-induced Testosterone</b>	<b>1,13</b>	<b>7.365</b>	<b>0.018</b>			

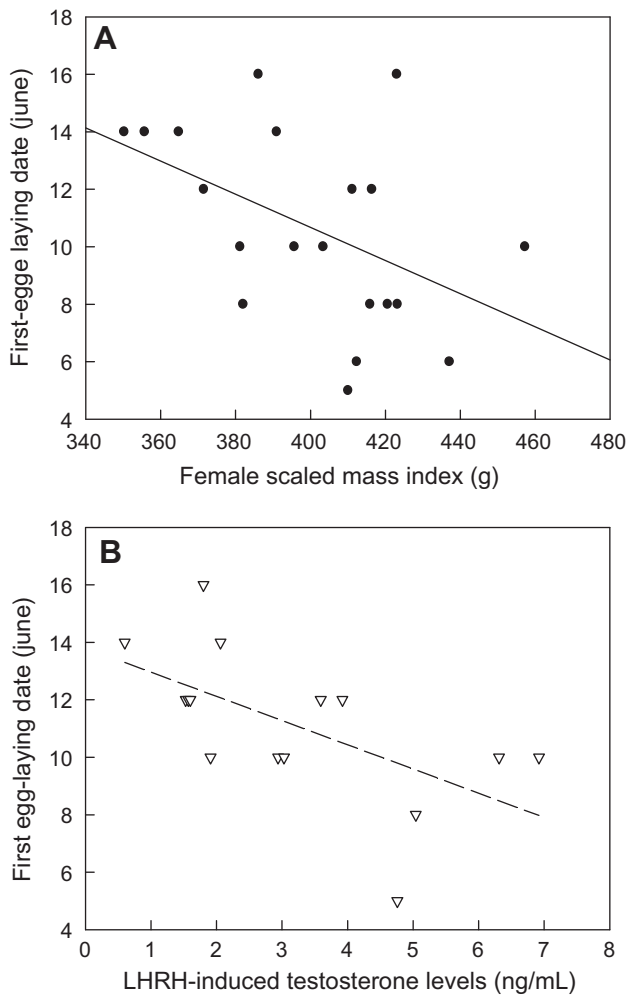


**Fig. 4.** Hormone levels in pre-laying male and female kittiwakes that will breed (white bar) and that will not breed (black bar): (A) baseline LH levels, (B) baseline corticosterone levels, (C) LHRH-induced LH levels and (D) stress-induced corticosterone levels.

#### 4.1. Effect of corticosterone levels on LH and testosterone

Our study showed, for the first time in free-living birds, that elevated baseline corticosterone was paralleled by low baseline LH levels and by low ability to release LH after a LHRH-injection. Such

down-regulation of the HPG axis by stress hormones was however strongly sex-specific in kittiwakes, since it was only observed in females. In free-living avian species, the link between LH and corticosterone levels has been seldom investigated and to our knowledge, studies were mainly biased towards males (Wilson and Follett,



**Fig. 5.** (A) Egg-laying date (June 2008) advanced with increasing females' scaled mass index; (B) Egg-laying date (June 2008) advanced with increasing males' testosterone release ability.

1975; Astheimer et al., 2000; review in Schoech et al., 2009). Our results highlight that in female kittiwakes, plasma corticosterone may act at the pituitary level, though a down-regulation of LH production and/or a low responsiveness to the GnRH input. This physiological pathway could be related to the stress-induced expression of GnRH and its associated inhibition of LH release, as recently reported in captive house sparrows and laboratory rats (Calisi et al., 2008; Kirby et al., 2009).

In males, baseline corticosterone levels had no effect on baseline LH levels and on LH releasing ability after a LHRH challenge. This supports the idea that the sensitivity of the HPG axis to stressful cues may differ between sexes, as suggested by Ball and Ketterson (2008). For instance, intrasexual competition could force male kittiwakes to acquire reproductive readiness earlier in the season than females and to better resist environmental perturbations. Moreover, females have to extract and store a sufficient amount of energy to build up the eggs, hence being more sensitive to environmental stressors, such as poor food supply, than males (Ball and Ketterson, 2008). The low susceptibility to stress of male HPG axis was further supported by the lack of relationship between baseline corticosterone and testosterone levels as experimentally observed in male American Tree sparrows (Astheimer et al., 2000).

It is also important to notice that stress-induced corticosterone levels had no effect on LH in males and females and on testosterone levels in males. In kittiwakes, corticosterone may need time

to suppress the HPG axis, therefore a short acute release of stress hormones could be inappropriate to trigger strong HPG disruption.

#### 4.2. Hormonal levels and breeding decision

As previously found (Salvante and Williams, 2003; Vitousek et al., 2010; Goutte et al., 2010), elevated pre-laying baseline corticosterone levels were associated with the decision to skip breeding in females. Females bearing elevated baseline corticosterone levels may then be of lower quality (e.g. low foraging skills, Angelier et al., 2007; Kitaysky et al., 2010) and may be unable to cope with the energetic requirements for egg-formation and future incubation effort.

Moreover, LH levels were higher in females that did breed than in females that did not breed. Since the LHRH injection did not reveal an inability to release LH, non-breeding females had a fully functional HPG axis (Schoech et al., 1996) and thus appeared to be physiologically primed for breeding. In that context, how to explain low baseline LH levels in non-breeding females? Our results suggest that non-breeding females failed to express endogenous GnRH input that is required for high baseline LH levels. Poor nest site, weak social stimulations or low mate interactions in females that will not breed could explain their low baseline LH levels (Ball, 1993; Dawson, 2008 for review). This down-regulation of LH levels might have been associated with possible reduced release of other pituitary hormones (FSH) and/or estradiol, which play a major role in the growth of reproductive organs and the expression of sexual behaviours. Estradiol is known to stimulate female begging behaviour (Hunt and Wingfield, 2004) and courtship feeding (Eda-Fujiwara et al., 2003). In pre-laying Black-legged kittiwakes, courtship feeding by the males is commonly observed and is closely linked to successful copulation (Helfenstein et al., 2003; Kempnaers et al., 2007). Hence, females with low baseline LH levels would have exhibited low estradiol levels and low mating effort, thereby skipping the breeding attempt. Therefore, in pre-laying female kittiwakes, elevated corticosterone levels appear to be strongly involved in the disruption of the HPG axis and to negatively affect breeding decision. At the ultimate level, skipped breeding could be an adaptive response to promote females' own survival, especially for long-lived prudent parents (Drent and Daan, 1980).

#### 4.3. Hormonal levels and egg-laying dates

Concerning the timing of breeding, females with low scaled mass index laid their first egg later than females with high one. However, baseline corticosterone levels were not related to the timing of breeding, contrary to recent correlative and experimental findings in another long-lived seabird, the Snow petrel (Goutte et al., 2010). Why is the hormonal regulation of the timing of breeding species-dependant? Energetic constraints associated with egg formation should differ between these two seabird species. Indeed, small Larids, like kittiwakes, build up an egg in about 6–8 days (Astheimer and Grau, 1990), while female petrels have to accumulate energy reserves at sea during an extended period of three weeks (pre-laying exodus, Warham, 1996). Therefore, the timing of breeding should be under strong energetic constraints in petrels, and thus should be greatly influenced by early environmental conditions and physiological state (baseline corticosterone levels, Goutte et al., 2010). On the contrary, kittiwakes should experience lower energetic constraints during egg formation, and in turn the timing of egg-laying would be poorly mediated by elevated baseline corticosterone levels. The functional action of corticosterone on the egg-laying date could also be environment-dependant. Indeed, favourable conditions may overcome the effect of stress hormones on the first egg-laying date, as it was shown in females Florida scrub-jays (Schoech et al., 2009). The observed inhibition of HPG axis by high baseline

corticosterone levels in females could postpone the breeding schedule, but only during poor years.

In male kittiwakes, corticosterone levels were not as decisive as for females concerning the breeding decision, thereby highlighting sex differences in the response to environmental cues related to seasonal events (Ball and Ketterson, 2008; Blas and Hiraldo, 2010; Goutte et al., 2010). Furthermore, baseline and/or LHRH-induced LH and testosterone levels did not differ between breeders and non breeders, as found in Florida scrub jays (Schoech et al., 1996). Therefore, in male kittiwakes, pre-laying energetic and hormonal states appear to poorly influence the HPG system and the breeding decision.

Interestingly, the timing of breeding was closely linked to males' testosterone profile. Female kittiwakes bred earlier when their mates exhibited higher testosterone release ability after a LHRH challenge. How could males' testosterone drive the egg-laying date? High testosterone levels after a LHRH input could mediate behavioural adjustments in males, such as aggressiveness to obtain and defend a high quality nest, and/or high quality courtship display. In turn, provisioning effort was suggested to be a sexually selected trait on which female kittiwakes base decisions about timing and frequency of copulations (Kempnaers et al., 2007). Additionally, males with high testosterone release ability may enhance high body condition in pre-laying female kittiwakes, thereby triggering early breeding.

In conclusion, our study highlights clear sex-differences in the HPG sensitivity to stress hormones in pre-laying kittiwakes. This may originate from the specific constraints faced by males and females during the pre-laying period. Experimental manipulations of corticosterone levels coupled with behavioural observations (courtship feeding effort and copulation rate in corticosterone-implanted males and females) have to be conducted to confirm this hypothesis.

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# Why do some adult birds skip breeding? A hormonal investigation in a long-lived bird

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**Skipping reproduction is often observed in long-lived organisms, but proximate mechanisms remain poorly understood. Since young and/or very old snow petrels (*Pagodroma nivea*) commonly skip breeding, we tested whether they are physiologically able to breed during the pre-laying stage. To do so, we measured the ability of known-age (11–45 years old) petrels to release luteinizing hormone (LH, a crucial driver for breeding), by injecting exogenous gonadotropin-releasing hormone (GnRH). Although young petrels exhibited low baseline LH levels, they were able to elevate LH levels after a GnRH challenge. Moreover, young and very old petrels showed a stronger decrease in LH levels after the 10 min post-GnRH injection compared with middle-aged petrels. Birds that skipped breeding were as able as breeders to release LH after a GnRH challenge, indicating that they had functional pituitaries. However, the decision to skip reproduction was linked to a strong LH decrease after the 10 min post-GnRH injection. Our result suggests that the youngest and the oldest petrels fail to maintain elevated baseline LH levels, thereby do not initiate reproductive activities. Skipping reproduction in long-lived birds probably results from age-related changes in the dynamics of the hypothalamic–pituitary–gonadal (HPG) axis function.**

**Keywords:** intermittent breeding; age; GnRH challenge; luteinizing hormone; snow petrels

## 1. INTRODUCTION

Skipping reproduction (i.e. non-breeding by individuals that previously bred) is often observed in long-lived organisms [1]. The 'prudent parent hypothesis' posits that non-breeding decision should be favoured, when the value of the immediate reproduction is low relative to the value of future reproductive opportunities and survival [2], as it is expected to be in young adults. Moreover, senescent individuals may take advantage of sabbatical years to recover from previous reproductive efforts [3]. Alternatively, non-breeding events, instead of being strategically decided, might be imposed by physiological constraints in low-quality individuals [1]. Age appears to be the keystone of skipped breeding, since this behaviour is markedly observed in the youngest and in the oldest adult

birds [4,5]. Thus, some of the needed physiological requirements for successful reproduction may be deficient in young and senescent adults [6].

Although ultimate causes of skipped breeding have been widely discussed, proximate factors underlying such patterns remain poorly studied [7,8], especially in an age-specific context [5]. In birds, the onset of breeding is under strong hormonal control through the activation of the hypothalamic–pituitary–gonadal (HPG) axis [9]. In response to increased day length in spring, gonadotropin-releasing hormone (GnRH) is expressed and triggers the secretion of LH (luteinizing hormone) by the pituitary gland. LH, in concert with follicle-stimulating hormone (FSH), promotes gonadal maturation, sex steroid secretion and in turn, sexual behaviours [9]. Considering that non-breeding events are proximately regulated by the endocrine system, it is thus conceivable that birds skipping breeding do not appropriately release LH during the pre-laying period.

As very long-lived birds, snow petrels (*Pagodroma nivea*) provide an excellent model system to study non-breeding decision. Indeed, up to 60 per cent of adults can skip breeding in a given year [10], mainly the youngest and the oldest petrels [4,5]. This study was designed to test, in this monogamous species, whether age affects the ability to release LH during the pre-laying period and whether skipping petrels fail to release LH. To do so, we used a common and powerful protocol, the injection of exogenous GnRH during the photosensitive stage, and tested the ability of known-age petrels to release LH [7]. We predicted that young and very old petrels would release less LH after a GnRH injection than middle-aged ones, and that lower LH release would predict high probability to skip breeding. Moreover, LH levels are known to progressively decrease from 10 to 30 min post-GnRH injection and to return to baseline levels [7]. We therefore investigated whether this LH change (i) would be stronger in young and very old petrels than in middle-aged ones and (ii) would predict a high probability to skip breeding.

## 2. MATERIAL AND METHODS

The study was conducted on Terre Adélie (66°40' S, 140°01' E), Antarctica. Snow petrels are very long-lived birds with low fecundity (one egg per year [10]). Many birds were of known age, because chicks have been banded each year since 1964 [10]. Twenty-four males and 17 females, from 11 to 45 years old, were handled during the pre-laying period (i.e. the courtship and mating period), from 11 to 23 November 2008. Only one bird was captured at each nest and birds were observed as breeders in previous years. They were caught by hand and a first blood sample was collected from the alar vein with a syringe immediately after capture (mean  $\pm$  s.e.: 3 min and  $9 \pm 5$  s) to determine baseline LH levels. Then, birds were immediately (5 min  $51 \pm 9$  s after capture) injected with exactly 0.1 ml of a GnRH solution in the second alar vein ([Gin<sup>8</sup>], Sigma Lot 121H04314). The GnRH was dissolved in a physiological solution to yield a dosage of  $0.6 \mu\text{g} (0.1 \text{ ml})^{-1}$  (about  $1.5 \mu\text{g kg}^{-1}$  body mass in 1 ml of 0.9% saline solution, as validated for seabirds [11]). Blood samples were collected from alar veins between 10 and 30 min after the GnRH injection to measure LH levels. LH changes from 0 to 10 min post-GnRH injection  $[(\text{LH}_{t=10} - \text{LH}_{t=0})/\text{LH}_{t=0}]$  and LH changes from 10 to 30 min post-GnRH injection  $[(\text{LH}_{t=30} - \text{LH}_{t=10})/\text{LH}_{t=10}]$  were examined as individual abilities to release LH. Blood samples were centrifuged and plasma was stored at  $-20^\circ\text{C}$ . LH levels were assayed by radioimmunoassay at the CEBC (Centre d'Etudes Biologiques de Chizé) [11]. The lowest detectable concentration of LH was  $0.05 \text{ ng ml}^{-1}$  and the intra-assay coefficient of variation was 6.2 per cent (three duplicates). Following GnRH injections, LH levels

(baseline:  $8.348 \pm 0.534 \text{ ng ml}^{-1}$ ) significantly increased (10 min:  $11.544 \pm 0.842 \text{ ng ml}^{-1}$ ), then decreased (30 min:  $9.059 \pm 0.838 \text{ ng ml}^{-1}$ ; generalized linear mixed model (GLMM), time as factor:  $F_{2,76} = 21.540$ ,  $p < 0.001$ , time  $\times$  sex:  $F_{2,76} = 0.244$ ,  $p = 0.784$ ). The 41 nests were checked every 2 days, until the end of the breeding season, to monitor if birds engaged in breeding ( $n = 32$ ) or if they skipped breeding ( $n = 9$ ).

All analyses were performed using R v. 2.8.0. We used the generalized linear model (GLM) with normal errors and an identity link function to explain absolute LH levels and LH changes as functions of sampling date, sex, age (linear and/or quadratic) and interactions between sex and age (linear and/or quadratic). Similarly, we used GLM with binomial error distribution and a logit link function to test for an effect of sex, LH values and the interaction between sex and LH on the probability to skip breeding. Selected models were checked for assumptions and LH values were log-transformed when necessary.

### 3. RESULTS

Baseline LH levels were higher in males than in females ( $F_{1,38} = 7.338$ ,  $p = 0.010$ ), and significantly increased with age ( $F_{1,37} = 5.517$ ,  $p = 0.024$ ; figure 1a), with no effect of sampling date, age<sup>2</sup> and interactions ( $p > 0.16$  for all tests). Absolute LH levels at 10 min were higher in males than in females ( $F_{1,34} = 6.412$ ,  $p = 0.016$ ), with no effect of other variables ( $p > 0.07$ ). LH changes from 0 to 10 min post-GnRH injection were significantly less pronounced with increasing age ( $F_{1,36} = 6.640$ ,  $p = 0.014$ ; figure 1b), with no effect of other variables ( $p > 0.11$ ). Absolute LH levels at 30 min increased with sampling date ( $F_{1,33} = 11.031$ ,  $p = 0.002$ ), were higher in males than in females ( $F_{1,32} = 15.648$ ,  $p < 0.001$ ), and tended to be lower in very young and very old petrels than in middle-age ones (age<sup>2</sup>:  $F_{1,30} = 3.378$ ,  $p = 0.076$ ), without interaction effects ( $p > 0.11$ ). LH changes from 10 to 30 min post-GnRH injection increased with sampling date ( $F_{1,32} = 9.451$ ,  $p = 0.004$ ) and were significantly more pronounced in young and very old petrels than in middle-aged ones (age<sup>2</sup>:  $F_{1,30} = 5.439$ ,  $p = 0.027$ ; figure 1c), with no effect of sex and interactions ( $p > 0.09$ ). LH changes from 10 to 30 min post-injection predicted skipped breeding ( $n = 37$ ,  $\chi^2 = 4.066$ ,  $p = 0.044$ ). Skipped breeders showed a stronger LH decrease from 10 to 30 min post-injection ( $-35.30 \pm 8.19\%$ ) compared with breeders ( $-16.88 \pm 3.74\%$ ). Absolute levels and LH changes from 0 to 10 min post-injection did not predict skipped breeding, even when considering an effect of sex ( $p > 0.42$ ).

### 4. DISCUSSION

Although young petrels exhibited low baseline LH levels, they showed a substantial increase in LH levels from 0 to 10 min post-injection and reached similar absolute LH levels compared with older petrels at 10 min. LH changes from 0 to 10 min reflect the individual ability to release LH after a GnRH injection, relative to the baseline levels. This indicates that young petrels with previous reproductive experience had functional pituitaries. How, then, is it possible to explain their lower baseline LH levels? Although photoperiod is the primary signal controlling seasonal reproductive activities, social interactions can influence central physiology by modifying GnRH neurosecretory cells, which in turn stimulates the adjustment of

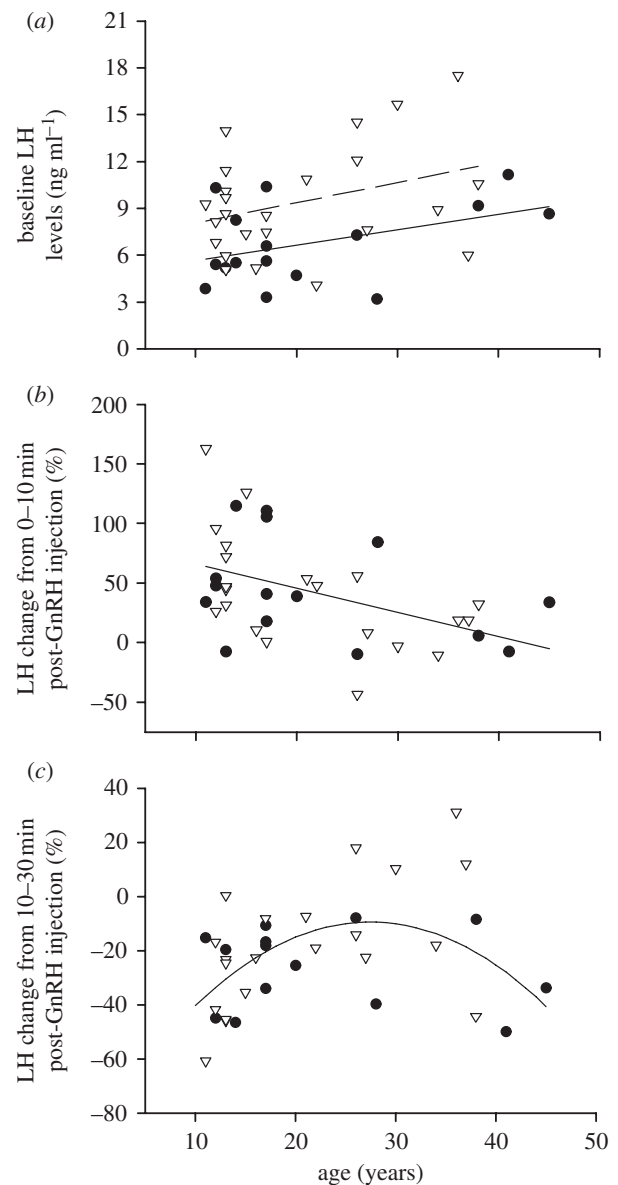


Figure 1. LH levels in relation to age in pre-laying snow petrels. (a) Baseline LH levels increased with age in males (open triangles and dashed line) and females (filled circles and solid line). (b) LH changes from 0–10 min post-GnRH injection (%) were negatively linked to age. (c) LH changes from 10–30 min post-GnRH injection (%) were quadratically linked to age.

reproductive physiology and behaviour [9]. In the monogamous snow petrel, repeated breeding attempts with the same partner could therefore provide important benefits by improving coordination of breeding activities [12]. In this context, low mate stimulations in young and newly paired petrels could explain their lower baseline LH levels. In our study, although sex steroids were not measured owing to small blood samples, we suggest that young females may reduce reproductive costs by delaying oestradiol secretion and ovarian development until establishing long-term pair bond [8]. Contrary to young petrels, old birds showed high baseline LH levels and did not strongly respond to the GnRH challenge. This may suggest that older petrels already secreted LH at their maximum levels, because of strong mate–mate interactions stimulated by long-term partnerships. Alternatively, as the

pool of available ova decreases with age, higher LH levels found in old females may have originated from a lower negative feedback at the hypothalamus level, compared with young females [13].

There was considerable inter-individual variation in relative LH changes from 10 to 30 min post-GnRH injection: LH levels rapidly decreased, did not change or continued increasing from 10 to 30 min. Compared with middle-aged petrels, the youngest and the oldest petrels failed to maintain LH release after the 10 min post-injection phase. In a previous study, we found that young and very old petrels were more sensitive to stress than middle-aged ones [5]. We suggest that this high stress response could interfere with the capacity to maintain LH production in an age-specific manner. Thus, the youngest and the oldest petrels would probably downregulate LH production during stressful conditions, such as those simulated by our manipulations. The marked decline in LH levels from 10 to 30 min post-injection in the oldest petrels may originate from a possible physiological senescence of the HPG system, as observed in ageing poultry [14]. These results are the first to show age-related differences in HPG activity of free-living birds, although more observations are needed to confirm that these patterns can be observed over several seasons and in different environmental conditions.

As found in some cooperative breeding bird species [7], skipped breeding in the monogamous snow petrel was not associated with a physiological suppression of LH secretion. However, we found that failure to maintain LH levels in the 10 min post-injection was the main predictor of the decision to skip reproduction. Specifically, the youngest and the oldest individuals, which appear to be more susceptible to stressors than middle-aged ones [5], fail to maintain elevated LH levels during the photosensitive stage and thus would be highly susceptible to skipping breeding. As previously highlighted [1], we suggest that non-breeding in long-lived free-living birds is probably the consequence of some age-related constraints acting on the dynamics of the HPG axis.

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## RESEARCH ARTICLE

# Experimentally reduced corticosterone release promotes early breeding in black-legged kittiwakes

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### SUMMARY

Breeding at the right time is important for successful reproduction. In birds, stressful environmental conditions are known to delay the timing of breeding but the underlying mechanisms are poorly understood. The stress hormone corticosterone appears to be a good candidate for mediating egg-laying date according to early environmental conditions and physiological state. By experimentally reducing the release of corticosterone in black-legged kittiwakes during the pre-laying period, we tested whether egg-laying date was mechanistically linked to corticosterone levels. Male and female kittiwakes were implanted with a low dose of exogenous corticosterone to inhibit endogenous corticosterone production. According to our predictions, the experimental reduction of corticosterone release was paralleled by a significant advancement of egg laying in females (around 4 days earlier). In addition, females with experimentally reduced corticosterone release gained mass during the pre-laying period compared with controls. Ultimately, the advancement of egg laying in females with experimentally reduced corticosterone levels was associated with an enhanced breeding success. This effect was strongly sex specific. In corticosterone-treated male kittiwakes, egg-laying date and reproductive success were not affected, but breeding probability was lower than in controls. This corticosterone treatment did not influence immediate clutch size, or return rate and breeding decision the following year. Our results support the hypothesis that corticosterone secretion during the pre-laying period mediates the timing of breeding in this long-lived seabird, possibly through the dynamics of energy reserves.

Key words: arctic seabird, fitness, *Rissa tridactyla*, stress hormones, timing of breeding.

### INTRODUCTION

Breeding at the right time is one of the most important factors for successful reproduction in a fluctuating environment (e.g. Lack, 1968; Perrins, 1970; Lyon et al., 2008). In birds, late breeding is usually associated with low breeding success. This is mainly due to a possible mismatch between the offspring's energy needs and the peak of food supply (match–mismatch hypothesis) (Visser et al., 1998; Stenseth and Myserud, 2002; Durant et al., 2007). In addition, the timing of egg laying has long-term fitness consequences because an experimental advancement and/or delay of breeding compromise future reproduction and survival (Nilsson and Svensson, 1996; Brinkhof et al., 2002). The adjustment of the timing of breeding is thus crucial and is possible through some behavioural and physiological flexibility in response to environmental and internal factors (reviewed in Wingfield, 2008; McNamara and Houston, 2008). In this context, free-living birds are thought to track pre-breeding environmental cues that enable them to anticipate the peak of food availability and then to adaptively adjust their breeding schedule (e.g. Frederiksen et al., 2004). However, this hypothesis is not always supported by correlative and experimental evidence (Hipfner et al., 2008; Shultz et al., 2009). The timing of breeding may also be imposed by pre-laying energetic constraints associated with the clutch formation (Durant et al., 2005; Visser and Both, 2005), depending on resource allocation strategies. Indeed, income-

breeding species and capital breeders are assumed to differ in their response to environmental cues regulating the onset of breeding (Drent and Daan, 1980). It is therefore essential to clarify the underlying mechanisms that link pre-laying environmental cues, physiological state and phenological decisions at the individual level.

What are the mechanisms underlying the onset of breeding? Increasing day length activates the hypothalamic–pituitary–gonadal axis (hereafter HPG axis). The expression of the gonadotropin releasing hormone (GnRH) triggers the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (reviewed in Dawson et al., 2001). In turn, gonadal development is promoted and sex steroids (estradiol and testosterone) are released, thereby mediating the adoption of sexual behaviours, such as courtship, mating and nest building (Ball, 1993). In addition to this fixed photoperiodic cue, many environmental variables, such as temperature, food supply and/or stimulatory social interactions may interact to modulate the onset of breeding (Wingfield, 1980; Wingfield and Kenagy, 1991; Ball, 1993; Wingfield et al., 2003; Dawson, 2008). The underlying mechanisms that regulate the HPG axis according to pre-laying non-photoperiodic cues have received growing attention, but it still remains poorly understood (review in Schoech et al., 2009). One potential mediator is the release of glucocorticoids in response to stressors (such as inclement weather, food shortage or predator attacks), which is known to adjust life-

history strategies according to environmental conditions and physiological state (Ricklefs and Wikelski, 2002; Wingfield and Sapolsky, 2003). In vertebrates, the acute secretion of glucocorticoids (corticosterone and/or cortisol) *via* the HPA axis promotes immediate survival through the mobilization of stored energy at the expense of current reproduction (reviewed in Breuner et al., 2008). During the pre-laying period, elevated corticosterone (hereafter CORT) levels appear to be associated with delayed breeding in females (Salvante and Williams, 2003; Schoech et al., 2009; Goutte et al., 2010a) *via* a possible perturbation of the HPG axis (reviewed in Schoech et al., 2009). In black-legged kittiwakes (*Rissa tridactyla*, Linnaeus 1758), a species showing large inter-annual variation in the timing of breeding and breeding success (Moe et al., 2009), we have shown that females with high baseline CORT levels had reduced baseline LH levels and a low ability to release LH, following GnRH challenge (Goutte et al., 2010b). In this species, reproductive success is higher in years when the timing of nesting is earlier (Byrd et al., 2008), and this also applies to our study population (Moe et al., 2009). Moreover, skipped breeding is related to high CORT levels in female kittiwakes (Lanctot et al., 2003; Kitaysky et al., 2010; Goutte et al., 2010b). Thus, CORT appears to be a potential candidate for mediating the timing of breeding according to pre-laying environmental and physiological conditions. In this respect, it is crucial to carefully distinguish the baseline and the stress-induced CORT levels. Baseline CORT levels are considered to be a marker of activity, energetic state and food availability (Kitaysky et al., 1999; Love et al., 2004) (reviewed in Landys et al., 2006) and are related to reproductive performance (reviewed in Bonier et al., 2009). Stress-induced CORT levels reflect the sensitivity to stress and the commitment to current reproduction (reviewed in Wingfield and Sapolsky, 2003; Lendvai et al., 2007; Bokony et al., 2009) and are related to the recent nutritional history of the kittiwake (Kitaysky et al., 2007; Kitaysky et al., 2010).

A low release of CORT in response to stress would be a possible mechanism to ensure early breeding through full activation of the HPG axis. In the present study, we tested this hypothesis by down-regulating the CORT release by black-legged kittiwakes during the pre-laying period. To do so, we implanted pre-laying male and female kittiwakes with a low dose of exogenous CORT to inhibit the HPA axis and endogenous CORT production. When exposed to low doses of CORT for a prolonged period, the HPA axis compensates by reducing adrenocorticotrophic hormone (ACTH) production, adrenal activity and CORT production (Akana et al., 1992; Wolkowitz, 1994). Following this CORT treatment, we addressed the following issues. (1) What are the phenological consequences of the CORT treatment? As the HPG axis of females appears to be more sensitive to stressful events than the males' in kittiwakes (Goutte et al., 2010b), we predicted that females with reduced CORT levels would breed earlier than control ones. (2) What are the fitness consequences of the CORT treatment? Hatching and breeding success the year of the treatment, as well as return rate and breeding decision the following year, were monitored. As early breeding is associated with high breeding success (Perrins, 1970), especially in kittiwakes (Byrd et al., 2008; Moe et al., 2009), and as an attenuated CORT stress response favours reproductive investment (Lendvai et al., 2007), we predict that female kittiwakes with reduced CORT levels would have a higher breeding success than controls.

## MATERIALS AND METHODS

### Study area and birds

Our study was conducted in 2009 between 19 May and 8 August in a colony of black-legged kittiwakes at Kongsfjorden, Svalbard

(78°54'N, 12°13'E), 7 km east of Ny-Ålesund, Norway. Black-legged kittiwakes are colonial seabirds that breed on cliffs throughout the northern parts of the Pacific and Atlantic, including the Barents Sea region up to the Svalbard Archipelago. We studied a plot of *ca.* 100 pairs breeding on cliffs at a height of 5–10 m. Birds were individually marked with white PVC plastic bands engraved with a three-letter code and fixed to the bird's tarsus. Thus, kittiwakes could be identified from a distance without perturbation. During the 2009 pre-laying period, foraging trip durations were longer ( $N=48$ ,  $3.75\pm 0.27$  days, mean  $\pm$  s.e.) than during the 2008 pre-laying period ( $N=81$ ,  $1.70\pm 0.13$  days,  $F_{1,126}=59.01$ ,  $P<0.001$ ), suggesting low and/or distant food availability.

### Experimental CORT treatment

The CORT implantation was conducted during the pre-laying period, from 19 to 31 May 2009. This was on average 20–30 days before the egg-laying date ( $19.2\pm 0.3$  June). A total of 21 females and 20 males were captured at their nests with a noose on the end of a 5 m fishing rod (day 0). Immediately after capture (3 min and  $2\pm 5$  s), a blood sample was collected from the alar vein with a 1 ml heparinized syringe and a 25 gauge needle, to determine baseline CORT levels. In 8 birds, handling time exceeded 3 min, the time recommended by Romero and Reed (Romero and Reed, 2005) to assess baseline CORT levels. These values were thus excluded from the analysis. Immediately after this first blood sample, we implanted the kittiwakes subcutaneously (between the shoulders) with one 25 mm Silastic tube (internal diameter 1.47 mm, external diameter 1.96 mm; Dow Corning, Midland, MI, USA) sealed at the ends with medical grade silicon (Dow Corning) and either filled with crystallized CORT (C2505, Sigma Chemical Co., St Louis, MO, USA;  $N=13$  CORT-blocked females and  $N=10$  CORT-blocked males) or empty ( $N=8$  control females and  $N=10$  control males). The implant site was disinfected with povidone iodine and then glued (Vetbond, 3M Santé, Cergy Pontoise, France). Administration of exogenous CORT is known to down-regulate the endogenous CORT production in birds over a prolonged period (Vandenborne et al., 2005; Romero et al., 2005; Busch et al., 2008; Müller et al., 2009). Two 25 mm Silastic implants significantly elevate baseline CORT levels in black-legged kittiwakes during the first 3 days after implantation (Kitaysky et al., 2001; Angelier et al., 2007; Angelier et al., 2009). We chose half of this dose (one implant) to inhibit CORT release, without strongly elevating baseline CORT levels (Akana et al., 1992; Wolkowitz, 1994).

After implantation, birds were weighed to the nearest 2 g using a Pesola spring balance. The skull length (head+bill) was measured to the nearest 0.5 mm using a sliding calliper. An index of body condition was calculated as residuals from a regression of body mass against body size (GLM,  $F_{1,38}=15.934$ ,  $P<0.001$ ). Kittiwakes were then marked with spots of dye on the forehead to facilitate subsequent observations and were released.

### Physiological effects of the CORT treatment

During the first 26 days after the CORT implantation (day 1 to day 26, still during the pre-laying period), 18 CORT-blocked kittiwakes and 19 controls were recaptured opportunistically. The number of days between the treatment and the recapture did not differ between CORT-blocked kittiwakes ( $11.72\pm 1.83$  days) and control kittiwakes ( $11.11\pm 1.54$  days, Student's *t*-test,  $t=0.258$ , d.f.=34.277,  $P=0.798$ ). Body mass was recorded, and a blood sample was collected immediately (2 min  $54\pm 5$  s) after capture to assess baseline CORT levels. The rate of body mass change was calculated as the difference between the body mass on the day of recapture ( $M_t$ ) and

the body mass on the day of implantation ( $M_0$ ), relative to the number of days elapsed since the treatment and before egg laying; that is,  $(M_T - M_0)/(t - 0)$ . A second blood sample was collected 15 min (15 min  $46 \pm 19$  s) after handling to assess the stress-induced CORT levels. Although maximum levels of CORT are reached 30 min after capture and handling in black-legged kittiwakes (Chastel et al., 2005), we decided to reduce the time of capture in order to minimize the level of disturbance. CORT levels and rate of body mass change were monitored before eggs were laid (still during the pre-laying period).

#### Timing of breeding and fitness consequences of the CORT treatment

During the egg-laying period, we checked the nests every 2 days, using a mirror on the end of an 8 m fishing rod. Breeding decision (laying egg or not), egg-laying date and clutch size (1 or 2 eggs) were thus monitored. Then, we checked the nests every 2 or 3 days to monitor the number of predated eggs per active nest, and the number of chicks that reached 12 days old per active nest (hereafter called breeding success). We assumed that CORT-blocked kittiwakes and control ones were exposed to the same level of disturbance. During the following year (from 20 May 2010 to 13 June 2010), we monitored the return rate and the breeding decision of CORT-blocked and control kittiwakes.

#### Hormone assay

The blood samples were immediately put on ice until centrifugation at the end of the day. Plasma was separated and stored at  $-20^\circ\text{C}$ . Molecular sexing was performed as detailed elsewhere (Weimerskirch et al., 2005). Plasma concentrations of CORT were determined by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé (CEBC), as previously described (Lormée et al., 2003). All samples were run in one assay. The coefficient of intra-assay variation was assessed using several reference plasmas within the assay (coefficient of variation = 5.8%,  $N=5$  duplicates). The minimum detectable CORT level was  $0.5 \text{ ng ml}^{-1}$ .

#### Statistical analyses

All statistical analyses were performed using R 2.8.0. (R Development Core Team, 2008). Regarding baseline CORT levels, the first day after treatment was distinct from the succeeding days. Thus, we first tested for an effect of CORT treatment (CORT-blocked or control), sampling period (day 0, day 1 and period between day 2 and day 26), sex and the interactions CORT treatment  $\times$  period and treatment  $\times$  sex on baseline CORT levels, by using generalized linear mixed models (GLMMs), including bird identity as a random effect. Then we focused on the period from day 2 to day 26. We tested for an effect of treatment, day of sampling (day 2 to day 26), sex and the interactions treatment  $\times$  day of sampling, and treatment  $\times$  sex on baseline CORT levels, using generalized linear models (GLMs) with normal errors and an identity link function. Second, we investigated whether stress-induced CORT levels were dependent on CORT treatment, sampling period (day 1 and the period between days 2 and 26) or day of sampling (from the 1st to the 26th day after treatment), sex and the interactions CORT treatment  $\times$  day of sampling, and treatment  $\times$  sex by using a GLM with normal errors and an identity link function. Stress-induced CORT levels were not monitored on day 0.

Baseline CORT levels have been related to breeding decision and egg-laying date (Lanctot et al., 2003; Goutte et al., 2010a; Goutte et al., 2010b; Kitaysky et al., 2010). Thus, we included the initial baseline CORT levels (prior to the CORT treatment) in our analyses. We tested for an effect of the CORT treatment, body condition,

initial baseline CORT levels and the interactions CORT treatment  $\times$  body condition and CORT treatment  $\times$  initial baseline CORT levels on breeding decision, using GLMs with binomial error distribution and a logit link function, and on laying date of the first egg, using GLMs with Poisson errors and a log link function. In addition, the CORT treatment may have affected other breeding performance in 2009 and in 2010. We thus tested for an effect of the CORT treatment, body condition and the interaction CORT treatment  $\times$  body condition on clutch size, number of lost eggs and breeding success, using GLMs with Poisson errors and a log link function, and on return rate and breeding decision the following year by using GLMs with binomial error distribution and a logit link function. Males and females were analysed separately. Finally, we tested for an effect of treatment on the rate of body mass change before egg laying, using Wilcoxon tests. Males and females were analysed separately. Only pre-laying females that did breed were analysed for body mass change, because non-breeding females are not constrained to acquire and store energy for egg building. Dependent variables were log transformed when necessary and we checked whether models met the assumptions.

## RESULTS

### Validation of the CORT treatment

On day 0, CORT-blocked kittiwakes and controls did not differ in baseline CORT levels ( $F_{1,31}=2.209$ ,  $P=0.148$ ) even when considering an interaction effect with sex (sex:  $F_{1,30}=1.813$ ,  $P=0.189$ ; treatment  $\times$  sex:  $F_{1,29}=0.019$ ,  $P=0.892$ ). Moreover, they did not differ in body condition (treatment:  $F_{1,39}=0.002$ ,  $P=0.964$ ; treatment  $\times$  sex:  $F_{1,37}=0.530$ ,  $P=0.471$ ), or in date of treatment (treatment:  $F_{1,39}=0.330$ ,  $P=0.569$ ; treatment  $\times$  sex:  $F_{1,37}=1.557$ ,  $P=0.220$ ).

CORT treatment affected baseline CORT levels, but the effect was influenced by sampling period (Table 1A, Fig. 1A): in CORT-blocked kittiwakes, baseline CORT levels increased from day 0 to day 1, but subsequently dropped below that of the control group (CORT-blocked group,  $9.47 \pm 1.28 \text{ ng ml}^{-1}$ ; control group,  $13.58 \pm 1.26 \text{ ng ml}^{-1}$ , Table 1B). In addition, males had significantly lower baseline CORT than females during this period (Table 1B). In the control group, baseline CORT levels were not affected by the sampling period (GLMM,  $F_{2,12}=0.342$ ,  $P=0.717$ ) or by the day of sampling (GLMM,  $F_{1,13}=1.1001$ ,  $P=0.313$ ). After CORT treatment, stress-induced CORT levels were significantly lower in the CORT-blocked kittiwakes ( $30.78 \pm 3.76 \text{ ng ml}^{-1}$ ) than in the controls ( $50.35 \pm 3.61 \text{ ng ml}^{-1}$ , Table 1C,D, Fig. 1B). Stress-induced CORT levels were unaffected by the sampling period or the day of sampling, sex and their interactions (Table 1C,D).

### CORT treatment and immediate phenological and fitness consequences

In females, the breeding decision did not differ between CORT-blocked kittiwakes and control ones and was not influenced by initial baseline CORT levels (Table 2A, Fig. 2A). However, the proportion of non-breeding females increased with decreasing body condition (Table 2A). In males, CORT-blocked kittiwakes showed a higher probability to skip breeding than control ones, but body condition did not affect the breeding decision (Table 2A, Fig. 2A). In addition, males that skipped the 2009 breeding season had an initially lower baseline CORT level ( $12.334 \pm 2.157 \text{ ng ml}^{-1}$ , mean  $\pm$  s.e.,  $N=8$ ) than males that did breed ( $23.062 \pm 3.476 \text{ ng ml}^{-1}$ , mean  $\pm$  s.e.,  $N=10$ , Table 2A). CORT-blocked females laid their eggs around 4 days earlier than controls (Table 2B, Fig. 2B), without any effect of body condition and/or initial baseline CORT levels (Table 2B). CORT treatment of males did not influence egg-laying date (Table 2B,

Table 1. Validation of the CORT treatment

Dependent variable	Independent variable	d.f.	F	P
A. Baseline CORT levels (ng ml <sup>-1</sup> ), N=68	Treatment	1,35	1.531	0.224
	Period	<b>2,25</b>	<b>7.436</b>	<b>0.003</b>
	Treatment × period	<b>2,25</b>	<b>6.503</b>	<b>0.005</b>
	Sex	1,35	0.303	0.586
	Treatment × sex	1,35	0.003	0.959
B. Baseline CORT levels (ng ml <sup>-1</sup> ) between days 2 and 26, N=32	Treatment	<b>1,30</b>	<b>9.378</b>	<b>0.005</b>
	Days after treatment	1,29	0.087	0.770
	Sex	<b>1,28</b>	<b>13.743</b>	<b>0.001</b>
	Treatment × day	1,27	0.002	0.962
	Treatment × sex	1,26	2.898	0.101
C. Stress-induced CORT levels (ng ml <sup>-1</sup> ), N=29	Treatment	<b>1,27</b>	<b>13.101</b>	<b>0.001</b>
	Period	1,26	2.616	0.119
	Sex	1,25	0.096	0.759
	Treatment × period	1,24	1.604	0.218
	Treatment × sex	1,23	1.604	0.218
D. Stress-induced CORT levels (ng ml <sup>-1</sup> ) between days 2 and 26, N=29	Treatment	<b>1,27</b>	<b>11.174</b>	<b>0.003</b>
	Days after treatment	1,26	1.318	0.263
	Sex	1,25	0.153	0.700
	Treatment × day	1,24	0.001	0.981
	Treatment × sex	1,23	0.180	0.675

Effect of corticosterone (CORT) treatment, period (day 0, day 1, days 2–26), sex and the interactions CORT treatment × period, CORT treatment × sex on the levels of (A) baseline CORT and (C) stress-induced CORT. Effect of CORT treatment, day (from the second to the 26th day after CORT treatment), sex and the interactions CORT treatment × day, CORT treatment × sex on the levels of (B) baseline CORT and (D) stress-induced CORT. We used GLMMs with bird identity as a random effect (A) or GLMs using normal errors and an identity link function (B,C,D).

Fig. 2B). Clutch size was not influenced by CORT treatment or body condition in male or female kittiwakes (Table 2C). In nests with a CORT-blocked female, around 25% of the eggs were predated by Glaucous gulls, *Larus hyperboreus*. This was far lower than in nests with control females, where all the eggs were predated (Table 2D, Fig. 2C). Breeding success was significantly higher in nests with CORT-blocked females than in control ones (Table 2E, Fig. 2D). In males, our experimental CORT treatment had no effect on egg predation and on breeding success (Table 2D,E, Fig. 2C,D).

During incubation, our CORT implantation treatment had a significant effect on the rate of body mass change: CORT-blocked breeding females gained mass whereas control breeding females lost mass (Wilcoxon  $W=22.5$ ,  $P=0.033$ , Fig. 3). Body mass change did not differ between CORT-blocked males and controls ( $W=11$ ,  $P=0.181$ ).

#### CORT treatment, return rate and breeding decision the following year

The return rate in 2010, the year after the CORT implantation treatment, did not differ between CORT-blocked and control females or between CORT-blocked and control males (Table 2F, Fig. 2E). For the kittiwakes observed in the colony during the pre-laying period in 2010, the decision to breed did not differ between CORT-blocked and control male and female kittiwakes (Table 2G, Fig. 2F). Body condition during the pre-laying period in 2009 did not influence the return rate and the decision to breed in 2010, even when considering an interaction effect of treatment × body condition.

#### DISCUSSION

In this study of pre-laying kittiwakes, we found that a prolonged reduction of baseline and stress-induced CORT levels was accompanied by an advancement of the egg-laying date and by an improvement of breeding success. This effect was strongly sex

specific as it was only observed in females. Moreover, breeding female kittiwakes with experimentally reduced CORT levels gained body mass during the pre-laying period, whereas control females

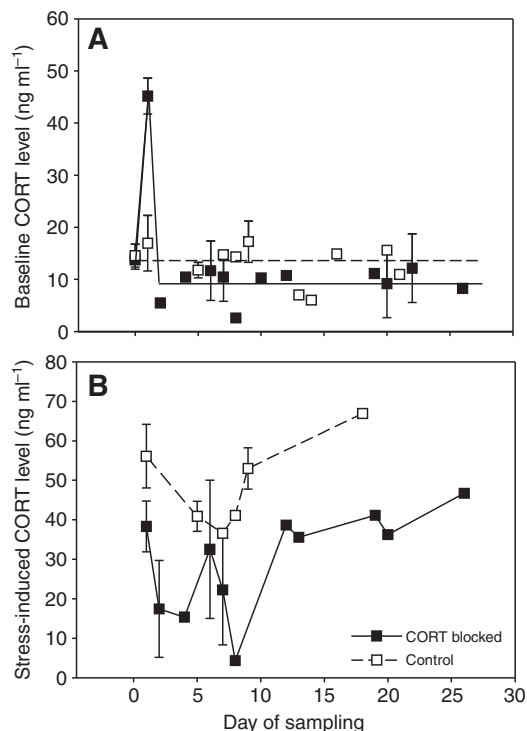


Fig. 1. Temporal change of (A) baseline corticosterone (CORT) levels and (B) stress-induced CORT levels with day of sampling after treatment (day 0 to day 26) for CORT-blocked kittiwakes and controls. Baseline CORT levels increased from day 0 to day 1 after CORT treatment, but subsequently dropped to levels below those of the control group.



Table 2. CORT treatment and fitness consequences

Dependent variable	Independent variable	Statistics (females)			Statistics (males)		
		<i>N</i>	$\chi^2$	<i>P</i>	<i>N</i>	$\chi^2$	<i>P</i>
A. Breeding decision in 2009	Treatment	21	1.318	0.251	<b>19</b>	<b>4.466</b>	<b>0.035</b>
	Body condition	<b>21</b>	<b>4.566</b>	<b>0.033</b>	19	1.715	0.190
	Treatment × body condition	21	0.566	0.452	19	0.201	0.654
	Initial CORT	19	0.143	0.705	<b>19</b>	<b>8.558</b>	<b>0.003</b>
	Treatment × initial CORT	19	0.212	0.645	19	0.076	0.783
B. Egg-laying date in 2009	Treatment	<b>13</b>	<b>4.035</b>	<b>0.045</b>	12	0.488	0.485
	Body condition	13	0.093	0.761	12	0.227	0.634
	Treatment × body condition	13	0.544	0.461	12	0.052	0.820
	Initial CORT	12	0.284	0.594	10	0.189	0.664
	Treatment × initial CORT	12	0.209	0.647	10	0.211	0.646
C. Clutch size in 2009	Treatment	13	0.036	0.849	12	0.080	0.777
	Body condition	13	<0.001	1.000	12	0.002	0.965
	Treatment × body condition	13	0.002	0.964	12	0.009	0.925
D. Lost eggs in 2009	Treatment	<b>13</b>	<b>4.010</b>	<b>0.045</b>	12	0.439	0.508
	Body condition	13	<0.001	1.000	12	0.010	0.920
	Treatment × body condition	13	0.125	0.723	12	0.205	0.650
E. Breeding success in 2009	Treatment	<b>13</b>	<b>7.355</b>	<b>0.007</b>	12	0.299	0.584
	Body condition	13	<0.001	1.000	12	0.350	0.554
	Treatment × body condition	13	0.111	0.739	12	2.943	0.086
F. Return rate in 2010	Treatment	21	1.044	0.307	20	2.848	0.092
	Body condition	21	1.737	0.188	20	0.965	0.326
	Treatment × body condition	21	2.648	0.104	20	0.185	0.667
G. Breeding decision in 2010	Treatment	16	0.760	0.383	15	0.043	0.835
	Body condition	16	2.497	0.114	15	0.161	0.689
	Treatment × body condition	16	2.048	0.152	15	0.018	0.894

Modelling (A) breeding decision, (B) egg-laying date, (C) clutch size, (D) number of predated eggs, (E) breeding success in 2009, and (F) return rate and (G) breeding decision in 2010, as functions of treatment, body condition, initial baseline corticosterone (CORT) levels for A and B and the interactions treatment × body condition, treatment × initial CORT using GLMs with binomial error distribution and logit link function (A,F,G) and GLMs with Poisson error distribution, log link function (B–E), in CORT-blocked and control kittiwakes. Females and males were analysed separately. Significant variables are indicated in bold.

lost mass. Finally, the return rate and the breeding decision the following year were not affected by CORT treatment.

#### CORT treatment and CORT secretion

The aim of the present study was to reduce the release of CORT in kittiwakes during the pre-laying period. To do so, a low dose of exogenous CORT was used to down-regulate the HPA axis and the release of endogenous CORT in response to the stress of capture (Akana et al., 1992; Wolkowitz, 1994). Thus, in male and female kittiwakes, the stress-induced CORT levels were around 60% lower after CORT treatment than after sham treatment. This down-regulation of CORT release was long lasting as it was observed at least 26 days after CORT treatment. In addition, baseline CORT levels were reduced by about 70% from the second to the 26th day. On day 1, crystallised CORT was released into the bloodstream. As a result, basal CORT plasma levels were 3.5 times higher than before the CORT treatment. This short CORT increase was within the physiological range observed for an acute stress response in kittiwakes (Kitaysky et al., 1999; Chastel et al., 2005). It was much lower than the 10 times increase of baseline CORT levels observed in previous studies after the implantation of two CORT implants (Kitaysky et al., 2001; Angelier et al., 2007; Angelier et al., 2009).

It is important to note that we only measured total CORT levels, i.e. free and bound CORT levels. The binding protein corticosteroid binding globulin (CBG) binds CORT with high affinity in the circulation (e.g. Breuner and Orchinik, 2002; Shultz and Kitaysky, 2008). Although the primary role of CBG is under debate, the free hormone hypothesis suggests that the unbound, or free, hormone in the plasma is biologically active. In European kestrel (*Falco tinnunculus*) and barn owl (*Tyto alba*) nestlings, the increase of total CORT after CORT administration was paralleled by a concomitant short-lasting increase in CBG capacity (Müller et al., 2009). However, CBG capacity was not modified by CORT treatment after the peak of circulating baseline CORT. Similar increases in plasma and serum CBG were reported in house sparrows (*Passer domesticus*) and in mouse pups after glucocorticoid administration (Zhao et al., 1997; Breuner et al., 2003), but the opposite effect was found in rats (Feldman et al., 1979). In our study, it is conceivable that CBG, and thus the free fraction of CORT, could have been affected by the low-dose CORT experiment. For instance, plasma CBG capacity would have increased just after CORT treatment. However, the lack of clear patterns among published studies makes it difficult to interpret the data. Hence, our discussion should be viewed within the context of total CORT only. Future studies are needed to understand the effect of low-dose CORT treatment on

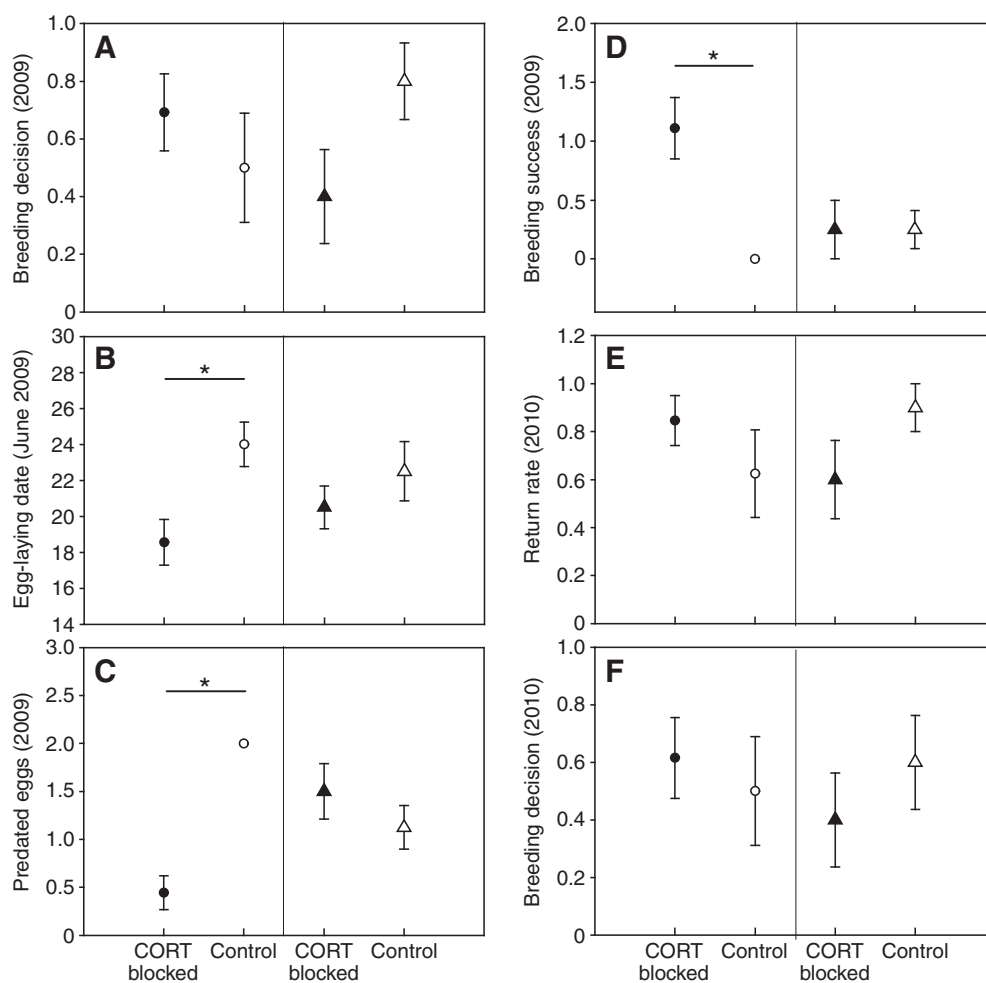


Fig. 2. (A) Breeding decision (at least one egg is laid: 1, no egg is laid: 0), (B) first egg-laying date, (C) number of predated eggs per active nest (0, 1 or 2), (D) breeding success (number of chicks >12 days old per active nest), (E) return rate the year following the treatment (not seen: 0, seen: 1), (F) breeding decision the year following the treatment for CORT-blocked and control kittiwakes. Circles denote females and triangles denote males. \*Significant difference between the CORT-blocked and control birds.

CBG capacity, and on the total and free fractions of CORT in kittiwakes.

#### CORT treatment and the timing of breeding

In agreement with our predictions, we found that the experimental reduction of CORT release during the pre-laying period was paralleled by a significant advancement of egg laying in female kittiwakes (around 4 days earlier). This experimental CORT treatment strongly supports the hypothesis that CORT levels and the timing of egg laying are mechanistically linked. This has previously been suggested by correlative observations: high baseline CORT levels were associated with late breeding in female Florida scrub-jays (*Aphelocoma coerulescens*) (Schoech et al., 2009) and in snow petrels (*Pagodroma nivea*) (Goutte et al., 2010a). Moreover, in captive female zebra finches (*Taeniopygia guttata*) (Salvante and Williams, 2003), an experimental elevation of plasma CORT levels decreased the proportion of breeding females and delayed the onset of egg laying. However, during favourable conditions, high baseline CORT levels were not associated with delayed breeding in Florida scrub-jays (Schoech et al., 2009). Furthermore, experimental CORT administration did not delay the timing of breeding, when female Florida scrub-jays were fed *ad libitum* during the pre-laying period (Schoech et al., 2007). The link between CORT and phenology appears to be environment dependent (Schoech et al., 2009), as baseline CORT level is a marker of energetic state and food availability (Kitaysky et al., 1999; Love et al., 2004) (reviewed in

Landys et al., 2006). High CORT levels may mediate late breeding, especially during high energetic constraints. Indeed, the present study showed that experimentally reduced CORT levels during unfavourable pre-laying conditions had the potential to advance the timing of breeding in free-living birds.

What is the causal link between experimentally reduced CORT levels and early breeding in female kittiwakes? In a previous study, we found that high baseline CORT levels were associated with low baseline LH levels and low LH releasing ability after GnRH injection (Goutte et al., 2010b). By experimentally reducing CORT levels, LH secretion and gonadal development may be promoted, thereby triggering early breeding. In this respect, we also found a high rate of body mass gain in breeding females with reduced CORT levels. As this high rate of body mass gain could result from rapid gonadal growth, this would support the hypothesis of an accelerated reproductive physiology, induced by the experimentally reduced CORT levels. Alternatively, body mass gain could reflect the accumulation of energetic reserves in females with experimentally reduced CORT levels, instead of gonadal development. As high body condition in pre-laying female kittiwakes predicts early breeding (Goutte et al., 2010b), females with a high body mass gain would lay their eggs earlier in the season. Consequently, female kittiwakes with experimentally reduced CORT levels may breed earlier because of a higher accumulation of energy reserves compared with controls. As the primary function of CORT is to facilitate glucose release for utilization during challenges, female kittiwakes with experimentally

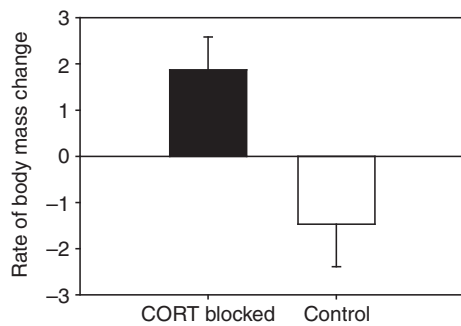


Fig. 3. Rate of body mass change between day of implantation and day of recapture, relative to the number of days elapsed since the treatment in CORT-blocked breeding females ( $N=5$ ) and control breeding females ( $N=4$ ). The number of days elapsed between the treatment and the recapture did not differ between these two groups (Wilcoxon test,  $W=12.5$ ,  $P=0.623$ ).

reduced CORT levels would have greater energy reserves than controls. Moreover, courtship feeding is a common behaviour in kittiwakes during the pre-laying period (Kempnaers et al., 2007). It is possible that females with experimentally reduced CORT levels were fed more and/or earlier than usual by their mates, hence allowing them to accumulate energy reserves and to breed earlier. Further behavioural observations must be conducted to test this hypothesis.

The effect of reduced CORT release on the timing of breeding was strongly sex specific as male kittiwakes with experimentally reduced CORT levels did not breed earlier than control birds. Indeed, the sensitivity of the HPG axis to stressful cues is thought to differ between sexes (Ball and Ketterson, 2008). Supporting this, we have recently shown that the inhibition of LH release by high baseline CORT levels was only observed in female kittiwakes and not in males (Goutte et al., 2010b). Pre-laying constraints should be strongly sex specific in free-living seabirds. Because of male–male competition, male kittiwakes must acquire reproductive readiness earlier in the season than females and must better resist environmental perturbations (Blas and Hiraldo, 2010). Moreover, females must extract and store a sufficient amount of energy to build up the eggs, and could be strongly sensitive to environmental stressors, such as a depletion of food supply early in the season (Ball and Ketterson, 2008; Blas and Hiraldo, 2010).

#### Fitness consequences of CORT treatment

First, it is important to note that the experimental reduction of CORT levels did not influence breeding decision in female kittiwakes. Only females with a low initial pre-laying body condition did not breed, which suggests that the reduction of CORT levels did not counteract energetic constraints. However, males with experimentally reduced CORT levels showed a lower breeding probability than controls, without an effect of body condition. In addition, low initial baseline CORT levels were associated with high laying failure in male kittiwakes. It is possible that male kittiwakes need to maintain relatively high baseline CORT levels to ensure sustained reproductive activity (Landys et al., 2006). During the pre-laying period, nest defence, foraging activity and courtship feeding would require moderate levels of baseline CORT in male kittiwakes; thus, a prolonged reduction of CORT levels could lead to nest and/or mate lost. This result highlights a strong sex difference in the hormonal regulation of breeding probability (Ball and Ketterson, 2008; Goutte et al., 2010b).

Second, female kittiwakes with experimentally reduced CORT levels had a higher breeding success than control birds, although clutch size did not differ between groups. Indeed, females with experimentally reduced CORT levels managed to successfully incubate their eggs ( $-0.5$  vs  $2$  lost eggs in the control group). As frequently observed in birds, early breeders succeed better than late ones (review in Verhulst and Nilsson, 2008), particularly during the incubation period in seabirds (e.g. DeForest and Gaston, 1996). In this context, we suggest that females with experimentally reduced CORT levels would have benefited more from better environmental conditions than control birds, mainly because of the advancement of egg laying. Alternatively, being heavier, CORT-blocked females may have benefited from higher body reserves during the challenging brooding period compared with control females (Moe et al., 2002). The CORT treatment might also have lasted longer than the pre-laying period and might have been effective during the incubation and chick-rearing period. As the attenuation of the CORT stress response is known to favour high parental effort (brood value hypothesis) (Lendvai et al., 2007), females with experimentally reduced stress-induced CORT levels would have attended the nest more, hence having a higher reproductive success than control females. However, this should also have been observed in CORT-blocked males, given incubating and chick-rearing roles as well as CORT levels during these periods are largely similar for male and female kittiwakes (Kitaysky et al., 1999; Chastel et al., 2005). As male kittiwakes with experimentally reduced CORT levels did not show better reproductive success than control birds, the hypothesis of a very long attenuation of CORT release is contested.

Finally, the CORT treatment had no consequences on return rate and breeding decision the following year in female kittiwakes. However, males with experimentally reduced CORT levels tended to show a lower return rate in 2010 than control males. CORT manipulation could have led to negative effects on survival, as previously found in male kittiwakes implanted with two CORT implants during the chick-rearing period (Goutte et al., 2010c). Moreover, males with experimentally lower CORT levels may have dispersed in surrounding colonies in 2010 and/or may have skipped the 2010 breeding attempt, as a possible consequence of the high laying failure in 2009 (Naves et al., 2006). Further analyses (2 years of observation after the treatment and capture–mark–recapture analyses) are needed to confirm this tendency in males. Female kittiwakes with experimentally reduced CORT levels had an identical return rate and breeding decision the following year. Brinkhof and colleagues demonstrated that female coots (*Fulica atra*) with experimentally advanced breeding had a lower survival rate than controls (Brinkhof et al., 2002). However, the advancement of hatching date in coots was higher (10 days) than in our study (*ca.* 4 days). Moreover, the production of a second brood in the experimentally advanced coot pairs could have resulted in higher reproductive costs and a higher associated mortality rate (Brinkhof et al., 2002). Finally, the higher breeding success and, hence, the higher breeding investment in females with experimentally reduced CORT levels did not compromise their breeding opportunities the following year, contrary to the hypothesis of Golet and colleagues (Golet et al., 2004).

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# The stress of being contaminated? Adrenocortical function and reproduction in relation to persistent organic pollutants in female black legged kittiwakes



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## HIGHLIGHTS

- We examined relationships between POPs, reproduction and CORT secretion in a seabird.
- POP levels were negatively related to body condition in non-breeding females only.
- Females with high levels of pesticides laid their eggs earlier.
- Females with high levels of PCBs released more CORT when subjected to a stress.
- High POP levels were not associated with poor breeding success.

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## ABSTRACT

High levels of environmental pollutants such as persistent organic pollutants (POPs) including PCB and DDT have been found in the Arctic and many of those pollutants may impair reproduction through endocrine disruption. Nevertheless, their effects on stress hormones remain poorly understood, especially in free-ranging birds. Corticosterone, the principal glucocorticoid in birds, can indirectly impair reproduction. The aim of the present study was to examine the relationships between POPs and reproduction through their potential consequences on different reproductive traits (breeding decision, egg-laying date, breeding success) and corticosterone secretion (baseline and stress-induced levels). We addressed those questions in an Arctic population of female black-legged kittiwakes during the pre-breeding stage and measured several legacy POPs (PCBs and pesticides: HCB, *p,p'*-DDE, CHL) in whole blood. POP levels were not related to breeding decision neither to breeding success, whereas females with high levels of pesticides laid their eggs earlier in the season. We found a negative relationship between POP levels and body condition index in non-breeding females. Black-legged kittiwakes with higher levels of PCB showed stronger adrenocortical response when subjected to a capture-handling stress protocol. We suggest that PCBs may disrupt corticosterone secretion whereas the positive relationship between pesticides and egg-laying date could either originate from a direct effect of pesticides or may be related to other confounding factors such as age or individual's quality. Although no direct negative reproduction output of POPs was found in this study, it is possible that the most contaminated individuals would be more sensitive to environmental stress and would be less able to maintain parental investment than less polluted individuals.

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## 1. Introduction

Environmental pollutants, such as persistent organic pollutants (POPs: pesticides, PCBs), have received an increasing attention during the last 30 years. The Arctic is considered as a sink for environmental

pollution, and for some compounds, levels may exceed that of industrialized cities (Gabrielsen and Henriksen, 2001). Because of bioaccumulation into organisms and bio-magnification along the food chain, marine apex predators such as seals, whales and seabirds are particularly vulnerable (Letcher et al., 2010; Vallack et al., 1998). Among free-living vertebrates, highly polluted individuals show decreased breeding capacities, such as abnormal breeding behaviour, reduced fertility or poor breeding success (Bustnes et al., 2003a, 2007; Colborn et al.,

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1993; Gabrielsen, 2007; Harrison et al., 1997; Taylor and Harrison, 1999; Verreault et al., 2010). Such breeding impairment could originate from the ability of POPs to act as endocrine disruptors and thus, to alter the functioning of major endocrine axes (Ottinger et al., 2013; Tyler et al., 1998). Indeed those substances are able to mimic, antagonize, alter or modify endogenous hormone functions (e.g. Amaral Mendes, 2002). In free-living vertebrates, several studies have found significant relationships between POPs and reproductive hormones such as steroids (Colborn et al., 1993; Giesy et al., 2003; Vos et al., 2000) and more recently hypothalamic and pituitary hormones (Verreault et al., 2008). Other hormones, such as those from the hypothalamic–pituitary–adrenal (HPA) axis, and especially glucocorticoids, are however known to affect reproductive behaviours and to mediate major reproductive decisions in vertebrates (reviewed in Wingfield and Sapolsky, 2003). Studies on laboratory mammals have documented a number of effects of chemicals on glucocorticoids (Odermatt and Gumy, 2008) but effects of POPs on stress hormones have been poorly studied in wildlife (Bergman et al., 2012). Hence, the concern for endocrine disruptors should also be directed towards the glucocorticoid system (Dawson, 2000; Johansson et al., 1998). Glucocorticoids (cortisol, corticosterone) are released in response to stressful events (food shortage, predation, and pathogens) to adjust life-history strategies in relation to environmental conditions and to individual physiological state (Ricklefs and Wikelski, 2002; Wingfield and Sapolsky, 2003). Indeed, the release of glucocorticoids during stressful events triggers physiological and behavioural adjustments that shift energy investment away from reproduction and redirects it towards survival (Wingfield and Sapolsky, 2003). Stress hormones have therefore a strong connection with fitness traits such as breeding success, individual quality and survival (Angelier et al., 2009, 2010; Bókonyi et al., 2009; Bonier et al., 2009; Breuner et al., 2008; Goutte et al., 2010b, 2011b; Kitaysky et al., 1999). Importantly, this means that a disruption of glucocorticoid secretion may alter the ability of an individual to adjust breeding decisions (to breed or not, when to breed) to environmental conditions. However, only a few studies have explored the impact of pollutants on both baseline and stress induced glucocorticoid levels, which depict different physiological functions: baseline corticosterone levels (CORT, the major glucocorticoid in birds) mirror the activity and metabolic rate and reflect the ratio between energy available and energy needed (Landys et al., 2006), while stress-induced CORT can be used as an index of the sensitivity to stress of an individual, this value can be modulated in order to maximize either survival, or reproduction (Bókonyi et al., 2009; Lendvai et al., 2007). Regarding contaminant/HPA axis, the pattern seems clear in fish: i.e. individuals from polluted sites (heavy metals, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls) are unable to elevate their cortisol levels (reviewed in Hontela, 2005). Studies on wild birds remain sparse and the pattern is less clear (reviewed in Verreault et al., 2010), mainly because they are difficult to compare as pollutants have been measured in different tissues (i.e. muscles, liver or feathers). And even when comparing two studies on the effects of POPs on CORT secretion, both measured in blood of Arctic seabirds, no clear pattern appeared. In black-legged kittiwakes *Rissa tridactyla* (hereafter ‘kittiwakes’) sampled early in the breeding season (April), increasing blood levels of PCBs were related to an increase of baseline CORT levels, and this relationship did not appear during the incubation period (Nordstad et al., 2012). In incubating glaucous gulls *Larus hyperboreus*, increasing blood POP levels (among which PCBs but also several pesticides) resulted in an increase of baseline CORT (Verboven et al., 2010). Moreover, male glaucous gulls subjected to a standardized stress-protocol, had decreased levels of stress-induced CORT with increasing POPs (Verboven et al., 2010). Thus the nature of POPs–CORT relationships could therefore depend on the type of pollutants, gender, types of tissue sampled and the reproductive status of the individuals. Although CORT is considered a key-stone hormone for allocation processes and reproductive effort (Wingfield and Sapolsky, 2003), there is a lack of studies investigating POPs–CORT–fitness in free-living organisms.

The aim of the present study was to consider the relationships between POPs and reproduction through their potential effects on reproductive traits and corticosterone secretion (baseline and stress-induced levels). We addressed those questions on female kittiwakes during the pre-breeding stage and measured POP levels (PCBs and pesticides: HCB, *p,p'*-DDE, CHL) from whole blood samples. In female kittiwakes CORT predicts breeding decision (Goutte et al., 2010a), egg-laying date (Goutte et al., 2011b) and breeding success (Goutte et al., 2011b). Thus, we investigated if blood POP levels would be related to 1) reproductive traits (the decision to breed or not, egg-laying date and breeding success) and 2) CORT secretion (baseline and stress-induced levels).

## 2. Materials and methods

### 2.1. Study area and birds

Our study was conducted in a colony of kittiwakes at Kongsfjorden, Svalbard (78°54'N, 12°13'E), 7 km southeast of Ny-Ålesund, Norway. Kittiwakes are colonial seabirds that breed on cliffs throughout the northern parts of the Pacific and Atlantic, including the Barents Sea region up to the Svalbard Archipelago (Anker-Nilssen et al., 2000). We studied kittiwakes in one plot of around 117 pairs breeding on cliff ledges at heights of 5–10 m. Female kittiwakes were sampled from 19 May to 7 June 2011, during the pre-laying period (i.e. copulations and nest building period), a key period for reproductive decisions during which female kittiwakes appear highly sensitive to stressors (Goutte et al., 2010a, 2011b; Tartu et al., 2013).

### 2.2. Capture and blood sampling

Forty-seven females were caught on the nests with a noose at the end of a 5 m fishing rod. A first blood sample (ca. 0.3 ml) was collected immediately after capture, from the alar vein with a 1 ml heparinised syringe and a 25-gauge needle to assess baseline CORT levels. Bleeding time (i.e. time elapsed from capture to the end of the first blood sample:  $2 \text{ min } 55 \pm 34$  (SD) seconds, on average) did not affect CORT levels (GLM,  $F_{1,45} = 1.79$ ,  $p = 0.190$ ). Kittiwakes were then placed into cloth bags and subsequent blood samples (ca. 0.3 ml) were collected from the alar vein at 30 min to assess stress-induced CORT levels.

Kittiwakes were individually marked with metal rings and PVC plastic bands engraved with a three-digit code and fixed to the bird's tarsus for identification from a distance. Birds were weighed to the nearest 2 g using a Pesola spring balance, and their skull length (head + bill) was measured to the nearest 0.5 mm with a sliding caliper. For each individual, we calculated an index of body condition by using the residuals from a linear regression of body mass against skull length (GLM,  $F_{1,46} = 7.05$ ,  $p = 0.01$ ). Kittiwakes were marked with spots of dye on the forehead to distinguish them from their partner during subsequent observation and then released. Using a mirror at the end of an 8 m fishing rod, we checked the whole plot (ca. 117 nests) every two days to monitor breeding decision (at least one egg is laid or no egg laid) and egg-laying dates. Then, with same technique, we checked the nest content every 2 or 3 days to monitor the number of chicks that reached at least 12 days of age per active nest (hereafter called ‘breeding success’).

### 2.3. Molecular sexing and hormone assay

Blood samples were centrifuged, and plasma and red blood cells were separated and stored at  $-20^\circ\text{C}$  until used respectively in hormone assays or molecular sexing, at the Centre d'Etudes Biologiques de Chizé (CEBC). Molecular sexing was performed as detailed in Weimerskirch et al. (2005). Plasma concentrations of CORT were determined by radioimmunoassay at the CEBC, as described by Lormée et al. (2003). Baseline CORT levels were not related to sampling date (GLM,  $F_{1,45} = 0.27$ ,  $p = 0.610$ ) or time of the day (GLM,  $F_{1,45} = 0.33$ ,  $p = 0.571$ ) and neither were

stress-induced levels (sampling date: GLM,  $F_{1,45} = 0.55$ ,  $p = 0.460$ ; time of the day: GLM,  $F_{1,45} = 0.72$ ,  $p = 0.399$ ). The lowest detectable concentration for CORT was 0.53 ng/ml. Only one assay was performed and the intra-assay coefficient of variation was 6.7% ( $N = 5$  duplicates).

#### 2.4. POPs analyses

POPs were analysed from 45 whole blood samples at the Norwegian Institute for Air Research (NILU) in Tromsø, for two individuals blood volumes were too low for POP measurements. The following compounds were analysed: the PCBs (CB-28, -52, -99, -101, -105, -118, -128, -138, -153, -180, -183, -187 and -194), and the pesticides (*p,p'*-DDE,  $\alpha$ -,  $\beta$ -,  $\gamma$ -HCH, HCB, oxychlordane, trans-, cis-chlordane, trans-, cis-nonachlor). The compounds chosen for further investigation were the  $\Sigma$ PCBs (CB-99, -105, -118, -128, -138, -153, -180, -183, -187 and -194), and the  $\Sigma$ Pesticides (*p,p'*-DDE, HCB, oxychlordane, trans-chlordane, trans-, cis-nonachlor). To a blood sample of 0.5 to 1.5 ml, a 100  $\mu$ l internal standard solution was added (13C-labelled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). The sample was extracted twice with 6 ml of n-hexane, after denaturation with ethanol and a saturated solution of ammonium sulphate in water. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975C MSD were performed as described by Herzke et al. (2009). The limit for detection was threefold the signal-to-noise ratio, and for the compounds investigated the limit ranged from 0.4 to 122 pg/g wet weights (ww). For validation of the results, blanks (clean and empty glass tubes treated like a sample, 3 in total) were run for every 10 samples, while standard reference material (3 in total, 1589 a human serum from NIST) was run for every 10 samples. The accuracy of the method was within the 70 and 108% range.

#### 2.5. Statistical analyses

All statistical analyses were performed using R 2.8.0 (R Development Core Team, 2008). We used generalised linear models (GLM) with a normal, binomial or Poisson error distribution and an identity, logit or log link function, respectively, to test our biological assumptions. To test the relationships between different groups of POPs (pesticides and PCBs), we categorised POP compounds based on chemical structure similarities. We summed their concentrations in four classes as follows: PCBs ( $n = 10$  congeners), CHL ( $n = 4$  compounds), *p,p'*-DDE and HCH. Because CHL, *p,p'*-DDE and HCH were all correlated (Pearson correlation coefficients,  $R = 0.70$ ,  $0.83$  and  $0.82$ ) we grouped them into a sum of pesticides (Pesticides). To describe the total blood contaminant concentration of individual kittiwakes we used the sum of POPs ( $\Sigma$ POPs) including PCBs and Pesticides as they were also highly correlated ( $R = 0.79$ ). First, we tested the relationships between body condition, organic pollutants (using POPs, PCBs and Pesticides) and breeding decision, egg-

laying date and breeding success. We also tested the relationships between organic pollutants and body condition index in non-breeders and breeders separately. Second, we checked for relationships between organic pollutants (using POPs, PCBs and Pesticides), breeding decision and interaction, and baseline and stress-induced CORT. We performed statistical tests by using absolute CORT levels to facilitate the comparison with other published results. Prior to this, we checked for possible statistical effects of bleeding time, sampling date, time elapsed between blood sampling and egg-laying, hour of the day, body condition and breeding decision on baseline and stress-induced CORT levels ( $p > 0.05$  for all tests). Diagnostic plots were used to assess whether the data sufficiently met the assumptions of the linear model, and dependent continuous variables were log-10 transformed when necessary. Values are mean  $\pm$  SD.

### 3. Results

#### 3.1. Relationships between organic pollutants, body condition and reproductive traits

The probability to breed was significantly related to the body condition as non-breeding females had a lower body-condition than breeding females (Fig. 1A; Table 1). However, the probability of breeding was not related to POPs, PCBs or Pesticides (Fig. 1B; Table 1). Additionally, in non-breeding females, we found a negative and significant relationship between body condition and POPs (GLM,  $F_{1,17} = 12.90$ ,  $p = 0.002$ ), the same relationship was found between PCBs (GLM,  $F_{1,17} = 12.46$ ,  $p = 0.003$ ) and Pesticides (GLM,  $F_{1,17} = 8.85$ ,  $p = 0.008$ ) on body condition index (Fig. 2), while no relationship was observed in breeding females ( $p > 0.51$  for all tests, Fig. 2).

In females that bred, egg-laying date was negatively related to Pesticides: females that laid early had higher levels of pesticides than females that laid later in June (Fig. 3; Table 1). This relationship did not appear when considering body condition or PCBs. No relationships were found between body condition, POPs, PCBs or Pesticides and breeding success (Table 1).

#### 3.2. Relationships between organic pollutants and CORT secretion

Baseline and stress induced CORT levels (log transformed) were not related to body condition ( $p > 0.05$  for all tests) or breeding decision (Table 2). We did not find any relationship between POPs, PCBs or Pesticides on baseline CORT levels (Fig. 4A; Table 2). However, we found a positive relationship between PCBs and stress-induced CORT levels. The most polluted individuals released more CORT when subjected to a capture-handling stress protocol (Fig. 4B; Table 2), and this relation was not found when using POPs only or Pesticides.

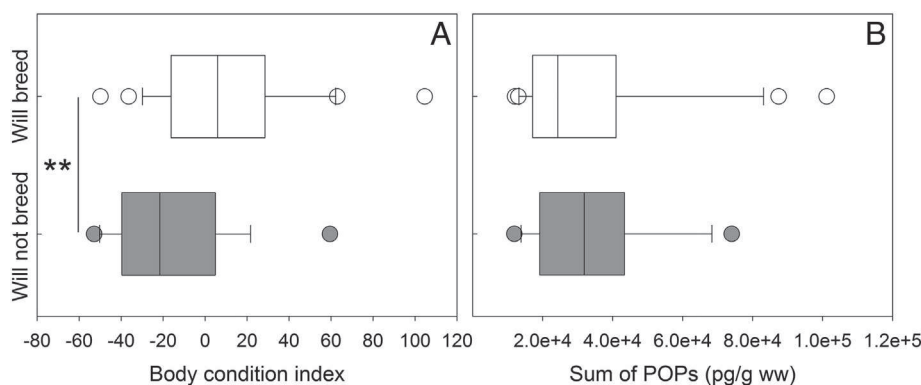


Fig. 1. Body-condition index (A) was higher in breeding (empty boxes) than in non-breeding (filled boxes, \*\*:  $p < 0.02$ ) female black-legged kittiwakes, although blood POP levels (pg/g ww, B) did not differ between breeding and non-breeding females.



**Table 1**

Relationships between body condition index (N = 47), organic pollutants (pg/g ww, N = 45) as  $\Sigma$  POPs,  $\Sigma$  PCBs and  $\Sigma$  Pesticides and reproductive traits: a) breeding decision (will breed or will not breed), b) egg-laying date (days of June) and c) breeding success in pre-laying female black-legged kittiwakes. Egg-laying dates were available for all breeding females (N = 28), while breeding success was not available for two of them. Numbers in bold indicate significant p-values ( $p < 0.05$ ).

	Independent variable	Sum of squares	Df	F or $\chi^2$	p-Value
a) Breeding decision	Body condition	–	1.45	5.60	<b>0.018</b>
	$\Sigma$ POPs	–	1.43	0.00	0.953
	$\Sigma$ PCBs	–	1.43	0.01	0.942
	$\Sigma$ Pesticides	–	1.43	0.14	0.707
b) Egg-laying date (log)	Body condition	0.20	1.26	2.36	0.136
	$\Sigma$ POPs	0.30	1.24	4.08	0.055
	$\Sigma$ PCBs	0.21	1.24	2.69	0.114
	$\Sigma$ Pesticides	0.53	1.24	8.12	<b>0.009</b>
c) Breeding success	Body condition	–	1.24	0.19	0.663
	$\Sigma$ POPs	–	1.22	0.10	0.752
	$\Sigma$ PCBs	–	1.22	0.07	0.785
	$\Sigma$ Pesticides	–	1.22	0.16	0.694

F tests were used for models with normal distribution (Egg-laying date).

$\chi^2$  tests were used for models with binomial (Breeding decision) and Poisson (Breeding success) distribution.

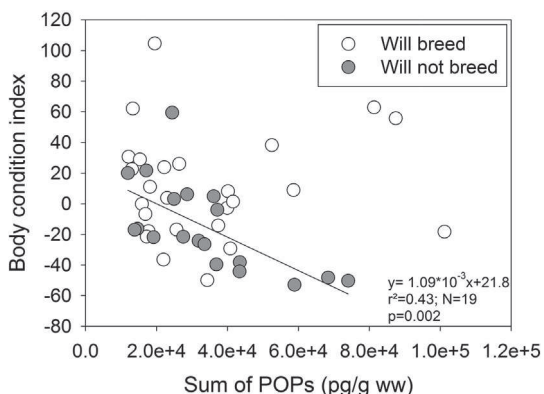
## 4. Discussion

Although kittiwakes from colonies in Svalbard are exposed to POPs (Nordstad et al., 2012; Savinova et al., 1995), the present study did not reveal any negative impact on the decision to breed or not, and breeding success. Nevertheless, females with the higher levels of pesticides laid eggs earlier. We found a negative relationship between POP levels and body condition index in non-breeding females. We also found that individuals bearing the higher levels of PCB would have a stronger adrenocortical response when subjected to a capture-handling stress protocol, and this did not appear when testing the relationship between Pesticides only and CORT secretion.

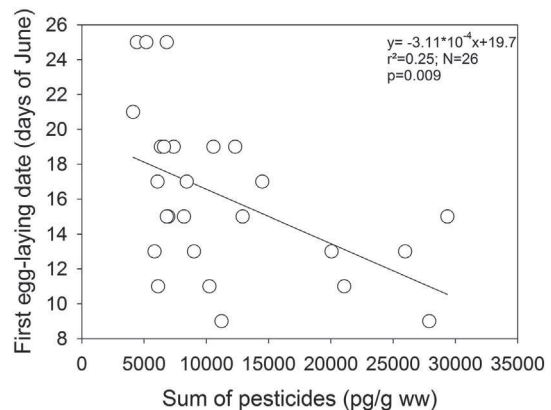
### 4.1. Relationships between organic pollutants and reproductive traits

#### 4.1.1. Breeding decision and body-condition index

In kittiwakes, and in many long-lived seabird species, a significant proportion of adult birds will not breed in a given year (Goutte et al., 2010a, 2011c). In a recent study we have shown that moderate levels of mercury could be linked to non-breeding events in kittiwakes from Svalbard (Tartu et al., 2013). In our study, however the decision to



**Fig. 2.** Relationship between blood POP concentration ( POPs pg/g ww) and body condition index in breeding female black-legged kittiwakes (empty circles) and non-breeding females (filled circles). Body condition index decreased with increasing POP levels in non-breeding females (solid line).



**Fig. 3.** Relationship between pesticide concentration in blood ( Pesticides pg/g ww) and first-egg laying date (days of June) in female black-legged kittiwakes. The females with the higher levels of pesticides ( CHL, *p,p'*-DDE and HCB) laid earlier than those with lower levels.

breed or not was unrelated to blood POP levels (neither to pesticides nor PCBs). Although Svalbard kittiwakes bear significant amounts of pesticides and PCBs in their blood (Nordstad et al., 2012, this study), their POP levels were about 10-fold lower compared to levels in glaucous gulls, the most polluted arctic seabird species (Borgå et al., 2001; Bustnes et al., 2003b; Gabrielsen et al., 1995). Thus it is possible that POP levels found in pre-laying kittiwakes were not high enough to alter reproduction. Another possibility is that pesticides and PCBs, contrary to mercury, may not interfere with hormonal pathways involved in non-breeding behaviour, such as GnRH (gonadotropin-releasing hormone) and luteinizing hormone (LH, Tartu et al., 2013).

As found in other seabird species females that did not breed had a lower body condition index (Chastel et al., 1995; Goutte et al., 2010a, 2010c). Decreasing condition in non-breeding females was associated with increasing blood POP levels. Organic pollutants are lipophilic; if body fat reserves are low, organic pollutants can be redistributed through the bloodstream and more likely to migrate to sensitive vital organs as brain, kidneys and liver (Fuglei et al., 2007; Henriksen et al., 1996). This pollutant redistribution has been confirmed in several bird species: emaciated individuals had higher levels of POPs in liver, blood and brain than individuals in better body condition (Bogan and Newton, 1977; Bustnes et al., 2010, 2012; Kenntner et al., 2003). In female kittiwakes breeding in northern Norway, average body mass decreases 20% from the beginning to the end of the breeding period, and this body mass loss was accompanied with a 4-fold increase of PCB levels in the brain (Henriksen et al., 1996). Thus birds in poor body condition are more sensitive to environmental pollution since pollutants are more available in sensitive organs. If vital organs are harmed, detoxification process would be more important, increasing the individual's metabolism. Thus, we can suppose that the most polluted non-breeders may therefore be in lower body condition because they are expending more energy in detoxification than breeders. Our study highlights that in pre-laying female kittiwakes, POP-condition relationships differ according to breeding decision. However, a question is why such relationship was found in non-breeding birds only although all females despite their breeding decision had, on average, similar blood concentrations of POPs? Maybe non-breeding individuals reached a threshold condition below which POPs/body condition relationships became apparent or non-breeding birds may also be poor quality individuals (Cam et al., 1998), which may be less able to physiologically deal with a given dose of POPs. Also, POPs may act as endocrine disruptors, and according to the physiological state of an individual, hormonal levels vary, especially if individuals chose to breed or not. Because in kittiwakes, endocrine levels differ between breeders and non-breeders (Goutte et al., 2010a; Tartu et al., 2013) this could have an effect on the potential toxicity and threat of endocrine disrupting chemicals. It has been suggested, in studies on rats, that oestrogens have a protective effect on methyl-mercury threat on the brain (Oliveira

**Table 2**

Relationships between organic pollutants (pg/g ww) as  $\Sigma$  POPs,  $\Sigma$  PCBs and  $\Sigma$  Pesticides and a) baseline and b) stress-induced CORT levels (ng/ml) in pre-laying female black-legged kittiwakes. Numbers in bold indicate significant p-values ( $p < 0.05$ ).

Dependant variable	Independent variable	Sum of squares	Df	F	p-Value
a) Baseline CORT (log)	Breeding decision	0.00	1.41	0.01	0.931
	$\Sigma$ POPs	0.63	1.41	1.95	0.170
	$\Sigma$ POPs $\times$ breeding decision	0.02	1.41	0.07	0.788
	$\Sigma$ PCBs	0.48	1.41	1.45	0.235
	$\Sigma$ PCBs $\times$ breeding decision	0.02	1.41	0.05	0.820
	$\Sigma$ Pesticides	0.95	1.41	3.03	0.089
	$\Sigma$ Pesticides $\times$ breeding decision	0.03	1.41	0.11	0.741
	Breeding decision	0.14	1.42	3.42	0.072
b) Stress-induced CORT (log)	$\Sigma$ POPs	0.14	1.42	3.43	0.071
	$\Sigma$ POPs $\times$ breeding decision	0.03	1.41	0.74	0.395
	$\Sigma$ PCBs	0.17	1.42	4.11	<b>0.049</b>
	$\Sigma$ PCBs $\times$ breeding decision	0.02	1.41	0.49	0.488
	$\Sigma$ Pesticides	0.07	1.41	1.60	0.213
	$\Sigma$ Pesticides $\times$ breeding decision	0.04	1.41	0.97	0.331

et al., 2006). Therefore, if some pollutants mimic the effects of sex steroids, and if these latter are endogenously more present in the organism, the pollutants' harm could be less effective. To our knowledge, a protective effect of reproductive hormones on POPs has never been observed. Further studies comparing POP levels in the Hypothalamo-Pituitary-Gonad axis of breeders and non-breeders, which include measurements of sex steroids, would be important to test this supposition.

#### 4.1.2. Egg-laying date and breeding success

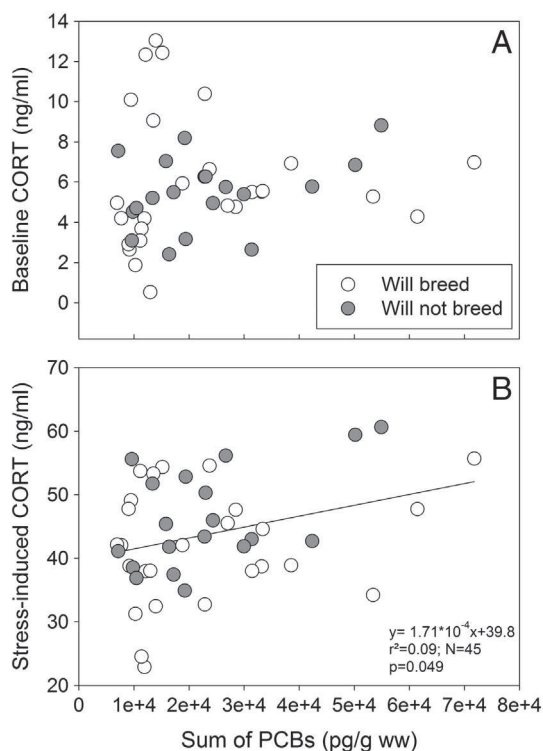
In birds, breeding at the right time is one of the most important factors for successful reproduction in a fluctuating environment (e.g. Lack, 1968) and in kittiwakes, late breeding is usually associated with low breeding success (Goutte et al., 2011b; Moe et al., 2009). Studies on free-living great black-backed gulls (*Larus marinus*) and Antarctic skuas (*Catharacta macormicki*) have reported that the most polluted individuals had a delayed egg-laying date (Bustnes et al., 2007;

Helberg et al., 2005). In our study we found the reverse pattern: females with the highest levels of pesticides laid their eggs earlier in the season, as found in some populations of glaucous gulls (Bustnes et al., 2003b) and great black-backed gulls (Bustnes et al., 2008).

This negative relationship between pesticide contamination and egg-laying date may not be causal: e.g. females laying early could be of better quality, forage at a higher trophic level and hence be more exposed to pesticide contamination. In the same line of idea, this correlation could be the result of age related processes since in birds old females often lay earlier than young ones (e.g. Goutte et al., 2010c) and those older females may possibly bear higher POP levels. In our study, no birds were of known age, so we were not able to test for a possible influence of age on POP levels. However the few studies that have explored these relationships have failed to find a relationship between age and POP concentration (Bustnes et al., 2003a; Tartu et al. unpublished data).

In mammals, some studies have described relationships between organochlorine pesticides and preterm birth (Longnecker et al., 2001; Saxena et al., 1981). DDT metabolites are able to impede the binding of some sex steroids (androgen or progesterone) to their receptor through indirect or direct paths, shortening the duration of gestation (Klotz et al., 1997; Lyon and Glenister, 1980). Some of the pesticides present in kittiwakes could also mimic or stimulate the secretion of some hormones involved in oviposition. Prostaglandin synthesis, a major hormone involved in oviposition, can be inhibited by *p,p'*-DDE in ducks (Lundholm, 1997), which is not coherent with our results, but consistent with Bustnes et al. (2007, 2008). However, many pollutants have a non-linear dose-response relationship (Calabrese, 2010; Heinz et al., 2012) and several studies have pictured an inverted U-shaped effect (e.g. Love et al., 2003), low dose of pollutant may enhance the synthesis of a hormone while important doses may inhibit it. The amount of DDE measured into the eggs to inhibit prostaglandin in Lundholm (1997) was averagely 3000-fold that of *p,p'*-DDE levels found in female kittiwakes' blood (this study) and 200-fold that of *p,p'*-DDE levels found in Svalbard kittiwakes' eggs (Barrett et al., 1996). We may speculate that in kittiwakes low dose of *p,p'*-DDE may stimulate prostaglandin synthesis, which would in this case initiate an early oviposition. Because *p,p'*-DDE and prostaglandin are tightly linked (Lundholm, 1997) and that the relationship between egg-laying date and *p,p'*-DDE was statistically the more significant among all other pesticides (i.e. HCB, chlordanes), we suppose there could exist a positive relationship between low levels of *p,p'*-DDE and prostaglandin secretion. This hypothetical disruption of prostaglandin from pesticides could however be advantageous in Polar Regions. Indeed, in regions where breeding season is short, breeding early is beneficial (Perrins, 1970).

Our study did not reveal any negative impact of persistent organic pollutants on breeding success. Average breeding success during our study was quite good,  $1.26 \pm 0.65$  chicks reached more than 12 days old per active nest, against less than 1 chick, 7 years over 11, during the period from 1997 to 2008, excluding 2001 (Moe et al., 2009),



**Fig. 4.** Relationship between PCBs concentration in blood (PCBs pg/g ww) and baseline (A) and stress-induced (B) CORT levels (ng/ml) in female black-legged kittiwakes. Baseline CORT levels were not related to PCB levels whereas stress-induced CORT levels increased with increasing PCB levels. Empty circles refer to breeding females and filled circles to non-breeding females.

suggesting favourable foraging condition at sea. It is therefore possible that birds were able to cope with POP contamination without visible reproductive penalties.

#### 4.2. Relationships between organic pollutants and CORT secretion

In the present study, stress-induced levels were higher in individuals that had the higher levels of PCBs, and this was not true for pesticides. The adrenal gland is one of the most common targets for chemically induced lesions (Rosol et al., 2001). Because of several characteristics as: its large blood supply, its lipophilicity (allowing the accumulation of lipophilic compounds), its high concentration of cytochrome P450 that can also bioactivate toxicants, and its capacity to synthesize all major classes of steroids (Falco et al., 2007; Harvey and Everett, 2003; Hinson and Raven, 2006; Rosol et al., 2001). Adrenal cells concentrate a number of toxic agents, as DDT (Lund et al., 1988) and PCB metabolites (Brandt and Bergman, 1987), those toxicants may remain inactive caught into the adrenal tissue until a period of particularly high adrenal steroid demand, as the breeding period. During the breeding period, the body mass loss may make available contaminants stored in different organs and body reserves, which at their turn would cause damage. Indeed, reduced food intake enhances biotransformation of halogenated organic contaminants and formation of metabolites which have greater toxicological impacts compared to parent POPs (Routti et al., 2013). In our study, stress-induced CORT levels increased with increasing levels of circulating PCBs. This could reflect a dysfunction coming from the adrenals (e.g. up-regulation of ACTH receptors, pollutants mimicking ACTH etc.) and/or this could mirror a dysfunction coming from the brain (e.g. pituitary loss of negative feedback from CORT on the pituitary). Relationships between PCBs and adrenocortical functions have been experimentally highlighted: PCBs can alter adrenocortical steroidogenesis, down-regulate the number of brain glucocorticoid receptors and some PCB metabolites (i.e. hydroxylor methyl PCBs) can bind competitively to glucocorticoid receptors (Aluru et al., 2004; Johansson et al., 1998; Xu et al., 2006). In addition, it seems that high concentration of PCB126 could sensitize the regulation of ACTH on adrenocortical cells by increasing ACTH receptor levels (Li and Wang, 2005), which could result in an increase of CORT secretion. Thus it is possible that some PCB congeners present in kittiwakes could act similarly by increasing the number of ACTH receptors, thus increasing CORT secretion in the most polluted individuals.

Contrary to our findings on kittiwakes, stress-induced CORT levels in the highly polluted glaucous gull decreased with increasing POP levels (Verboven et al., 2010). As mentioned previously, environmental pollutants can have a non-linear dose–response relationship (Heinz et al., 2012; Love et al., 2003), the lower stress response observed in highly polluted glaucous gulls may also be the consequence of a negative feed-back from higher baseline CORT levels (Verboven et al., 2010) due to an increased allostatic load when resources are allocated to biotransformation, detoxification and excretion of contaminants (Parkinson and Ogilvie, 2008). Finally, the lack of relationship between baseline CORT and PCBs in our study compared to that of Nordstad et al. (2012) could be explained by the difference between the two sampling periods: in the study of Nordstad et al. (2012), pre-breeding female kittiwakes were sampled in April, their blood PCB levels were average 14,900 pg/g and baseline CORT levels averaged  $7.2 \pm 1$  ng/ml. In the present study, female kittiwakes were sampled in May–June, their blood PCB levels were on average 23,030 pg/g and baseline CORT levels averaged  $6.13 \pm 3$  ng/ml. Although the compounds entering in the PCBs were not completely identical they were very close (11 PCB congeners in Nordstad et al. (2012), 10 PCB congeners in the present study, among which 8 were similar). Those findings suggest that the relationships between PCBs and CORT secretion may highly depend of the levels of PCBs present in blood, and probably also to the sampling period.

Our study adds new evidence that PCBs are linked to CORT secretion disruption. To elucidate which aspects of the HPA axis are involved in

mediating contaminant-related changes in the stress response, experimental ACTH injection would be useful to investigate if contaminant-related enhancement occurs at the level of the adrenal gland, or rather at the level of the pituitary, hypothalamus or perception of the stressor. An enhanced stress response is often the consequence of poor early-life experience across vertebrates as low body mass at birth, food restriction, and maternal deprivation (Banerjee et al., 2012; Heath and Dufty, 1998; Kitaysky et al., 2001; Müllner et al., 2004; Phillips and Jones, 2006), while in adults it often mirrors poor fitness related traits as poor parental investment (Angelier et al., 2009; Bókonyi et al., 2009; Goutte et al., 2011a; Lendvai et al., 2007) or an impacted survival (Blas et al., 2007; Goutte et al., 2010b; Romero, 2012). Still, as mentioned previously, in our study we failed to relate POP levels to breeding success. Environmental conditions were apparently favourable but in case of poor foraging conditions when CORT secretion is stimulated (Kitaysky et al., 1999; Lanctot et al., 2003), it is likely that the most contaminated individuals would be more sensitive to environmental stress and would be less able to maintain parental investment than less polluted ones.

#### 5. Conclusion

Although in this study we did not find any direct negative reproductive outputs of POPs, no information was available about the chicks or parents' survival. Also, females have been blood sampled during the pre-laying phase, and blood measures performed at the beginning of the breeding season may be less reliable to predict reproductive traits occurring at the end of the season as breeding success (Lanctot et al., 2003). This may be particularly true for POPs as levels vary significantly between the different breeding phases (Nordstad et al., 2012). Moreover a previous study on pre-laying kittiwakes has shown that the decision to defer breeding is highly related to a disruption of GnRH secretion from the hypothalamus by mercury (Tartu et al., 2013). Thus, brought together, all the effects of POPs added to the threat of other environmental pollutants could have important impacts on the long-term, depending on a birds' quality. A point to underline is that our study is correlative; we cannot confirm if there is a causal effect of POPs on reproductive traits or CORT. Feeding different concentrations of POPs to captive individuals (Love et al., 2003; Lundholm, 1997) or using silastic tubes filled with PCBs (Van den Steen et al., 2007) is a method that could enlighten on the causal effects of pollutants, yet this would be difficult to perform in free-ranging protected species. Moreover, we only measured legacy POPs, already known to be importantly related to several physiological parameters (Gabrielsen, 2007; Gabrielsen and Henriksen, 2001). In the Arctic, several emerging POPs (e.g. Perfluorinated compounds (PFC), brominated flame retardants) show increasing trends (Braune and Letcher, 2013; Braune et al., 2007; Butt et al., 2007; de Wit et al., 2006; Verreault et al., 2007). Given this perspective, in top predators those emerging POPs could be in higher levels than legacy POPs (e.g. PFC, Nøst et al., 2012) and their relationships with the parameters we measured in this study may be exacerbated, taking them into account could provide further awareness on the way environmental pollution may affect free-ranging populations.

#### Conflict of interest

The authors declare having no conflict of interest.

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## Research

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## Physiology

# To breed or not to breed: endocrine response to mercury contamination by an Arctic seabird

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Mercury, a ubiquitous toxic element, is known to alter expression of sex steroids and to impair reproduction across vertebrates but the mechanisms underlying these effects are not clearly identified. We examined whether contamination by mercury predicts the probability to skip reproduction in black-legged kittiwakes (*Rissa tridactyla*) from Svalbard. We also manipulated the endocrine system to investigate the mechanism underlying this relationship. During the pre-laying period, we injected exogenous GnRH (gonadotropin-releasing hormone) to test the ability of the pituitary to release luteinizing hormone (LH, a key hormone for the release of sex steroids and hence breeding) in relation to mercury burden. Birds that skipped reproduction had significantly higher mercury concentration in blood than breeders. Endocrine profiles of these birds also varied based on breeding status (breeders versus non-breeders), mercury contamination and sex. Specifically, in skippers (birds that did not breed), baseline LH decreased with increasing mercury concentration in males, whereas it increased in females. GnRH-induced LH levels increased with increasing mercury concentration in both sexes. These results suggest that mercury contamination may disrupt GnRH input to the pituitary. Thus, high mercury concentration could affect the ability of long-lived birds to modulate their reproductive effort (skipping or breeding) according to ongoing environmental changes in the Arctic, thereby impacting population dynamics.

## 1. Introduction

Mercury is a ubiquitous toxic element of both natural and anthropogenic sources. In its methylated form, mercury can impair reproduction and disrupt the expression of oestradiol and testosterone across vertebrates [1,2]. However, the mechanisms underlying these effects are not clearly identified [3]. In response to increased day length, gonadotropin-releasing hormone (GnRH) is secreted and triggers luteinizing hormone (LH) release from the pituitary gland. LH, in concert with follicle-stimulating hormone (FSH), promotes gonadal maturation, sex steroid secretion and in turn, the onset of reproduction. It is conceivable that mercury acts primarily on the ability of the pituitary to secrete gonadotropin hormones (LH and FSH), altering sex steroids release and ultimately impairing reproductive behaviour. Mercury may also suppress GnRH in the hypothalamus, thereby reducing LH production. Clearly, there is a need for studies investigating the mechanisms of LH suppression by mercury [3] and subsequent repercussions for breeding animals.

Recent investigations have highlighted a major role for LH on skipped breeding behaviour (non-breeding by individuals that previously bred), a common feature in long-lived birds [4,5]. In some seabirds, skipping individuals show either low levels of baseline LH (black-legged kittiwake, *Rissa tridactyla* [4]), or fail to maintain elevated LH levels after a GnRH injection (snow petrel, *Pagodroma nivea* [5]). LH secretion also appears to be sensitive to environmental stressors in kittiwakes [4]. This opens the yet unexplored possibility that some endocrine disruptors like mercury may play a role in skipping behaviour, by altering the release and secretion of LH.

Here, we test whether mercury concentrations (i) are linked to skipping behaviour, and (ii) affect patterns of LH release during the pre-breeding period in an Arctic population of black-legged kittiwakes. To evaluate LH release, we challenged the pituitary with an exogenous injection of GnRH. Kittiwakes provide an excellent model to address these questions, as they bear elevated mercury levels in Svalbard [6] and a significant proportion of adults skip breeding each year [4]. We predicted that high mercury concentration in blood would (i) be linked to a high probability to skip breeding, and (ii) impair baseline and/or GnRH-induced LH levels.

## 2. Material and methods

The study was conducted at Kongsfjorden, Svalbard (78°54' N, 12°13' E) from 20 May to 6 June 2008 (52 birds) and from 21 May to 7 June in 2011 (104 birds) during the pre-breeding period. Birds were caught on the nests and a blood sample was collected immediately after capture. In 2008, we performed a GnRH challenge immediately after the first blood sampling, kittiwakes were injected with 0.1 ml of a solution of GnRH (see the electronic supplementary material, figure S1). Blood samples were collected from the alar veins 10 and 30 min after the GnRH injection to measure baseline and GnRH-induced levels of LH (both sexes) and testosterone (males only) as detailed in the electronic supplementary material. Total mercury from all 2008 and 2011 samples was measured at LIENSs from lyophilized red blood cells, by atomic absorption spectrophotometry on 5–10 mg aliquots [7]. Mercury concentrations are expressed in  $\mu\text{g g}^{-1}$  dry weight. In both years, focal birds were measured as described in Goutte *et al.* [4] to calculate a scaled mass index and observed daily. The nest content was checked every two days, to monitor whether birds had engaged in breeding (at least one egg laid) or whether they had skipped (no egg laid). We also monitored fitness, including date of first egg-laying, clutch size and the number of chicks that reached 12 days post-hatch (hereafter breeding success). To test the effects of mercury and scaled mass index on these reproductive parameters, we pooled data from 2008 and 2011 (no birds were sampled twice). Then, for 2008, we tested the effects of mercury concentration and interaction with sex, on baseline and GnRH-induced hormone levels in skipping and breeding birds. Data are available from the Dryad Digital Repository: doi.org/10.5061/dryad.4ff07. All statistical analyses were performed using R v. 2.13.1 and we used generalized linear models (GLM) with a normal/binomial error distribution and an identity/logit link function to test our biological assumptions. Model selection was performed by a step-down approach starting from the global model including all the independent variables.

## 3. Results

Sex and year did not explain reproductive decisions (skippers versus breeders; GLM, all  $p$ -values  $> 0.10$ ). Mercury predicted

the likelihood of breeding, as skippers had significantly elevated mercury levels compared with breeders ( $\chi^2 = 14.06$ ,  $p = 0.001$ , figure 1*a,b*). This was true in both sexes (females:  $\chi^2 = 4.41$ ,  $p = 0.036$ ; males:  $\chi^2 = 4.61$ ,  $p = 0.032$ ). There was no interaction between mercury and either sex, year or scaled mass index (GLM, all  $p$ -values  $> 0.87$ ). Among birds that bred, pre-breeding mercury concentration did not predict first egg-laying date, clutch size or breeding success (all  $p$ -values  $> 0.18$ ).

In birds that bred, baseline LH levels were higher in females than in males ( $F_{1,34} = 5.9$ ,  $p = 0.02$ ), but were unrelated to mercury levels ( $F_{1,33} = 0.08$ ,  $p = 0.783$ ; mercury  $\times$  sex:  $F_{1,32} = 1.5$ ,  $p = 0.23$ , figure 1*c,d*). GnRH-induced LH levels were not linked to either sex ( $F_{1,17} = 3.23$ ,  $p = 0.09$ ) or mercury levels ( $F_{1,17} = 0.88$ ,  $p = 0.361$ ; mercury  $\times$  sex:  $F_{1,17} = 2.72$ ,  $p = 0.118$ ) in breeders.

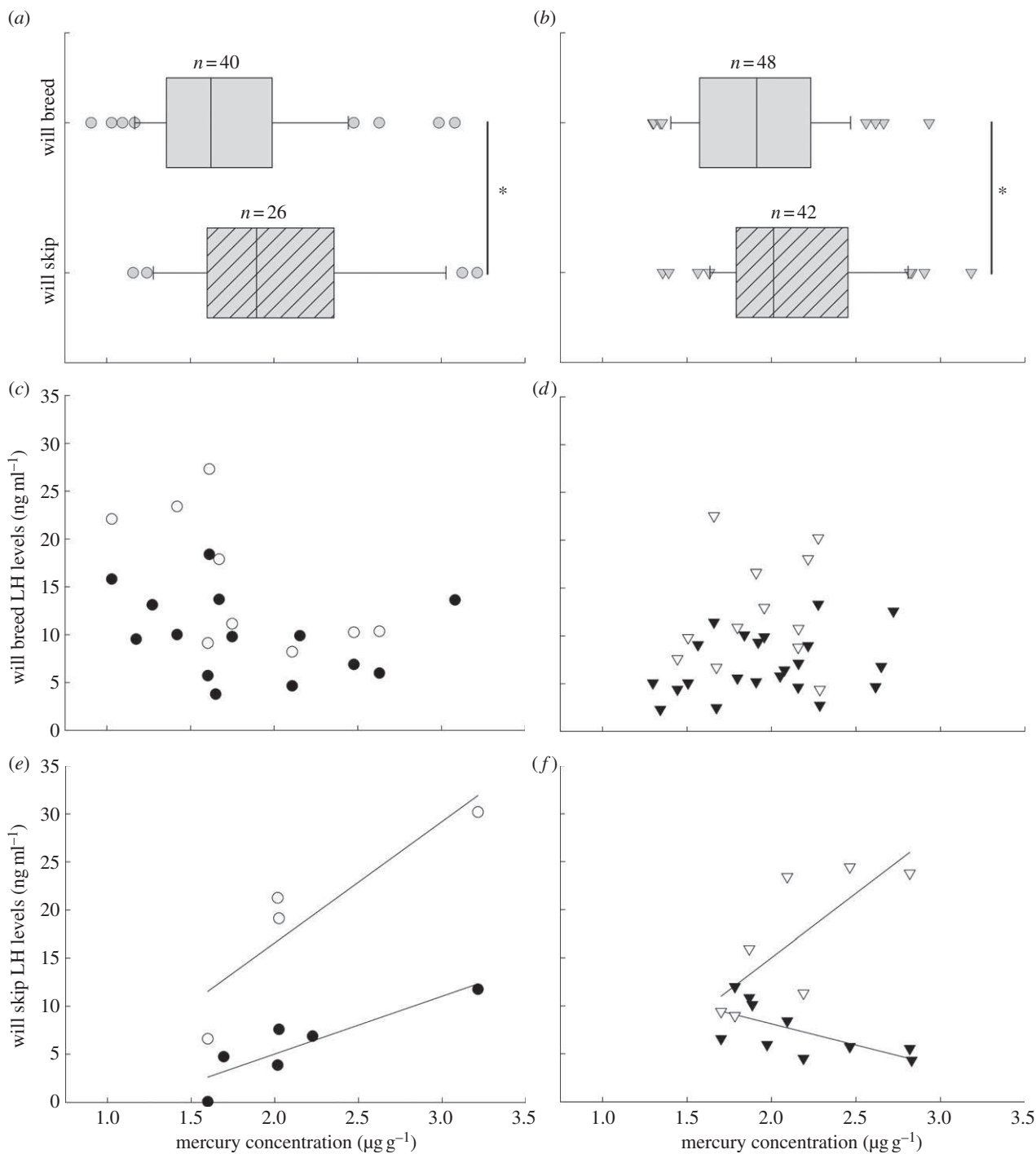
In skipping birds, baseline LH levels were not affected by sex ( $F_{1,12} = 0.43$ ,  $p = 0.523$ ) but significantly and negatively correlated to mercury levels in males, and positively in females (mercury  $\times$  sex:  $F_{1,12} = 19$ ,  $p < 0.001$ ; figure 1*e,f*). GnRH-induced LH levels significantly increased with increasing mercury concentration in skipping males and females (mercury:  $F_{1,10} = 21.6$ ,  $p < 0.001$ ; mercury  $\times$  sex:  $F_{1,8} = 0.07$ ,  $p = 0.805$ , figure 1*e,f*). Baseline testosterone levels tended to decrease in skipping males ( $F_{1,7} = 5.92$ ,  $p = 0.051$ ) but not in breeding males ( $F_{1,19} < 0.01$ ,  $p = 0.97$ ). GnRH-induced testosterone levels were not related to mercury levels neither in skipping ( $F_{1,5} = 0.1$ ,  $p = 0.761$ ) nor in breeding males ( $F_{1,9} = 0.6$ ,  $p = 0.458$ ).

## 4. Discussion

Long-lived seabirds often skip reproduction in certain years, and our previous investigations in kittiwakes demonstrate that LH levels are lower in the birds which choose to skip breeding (skippers, [4]). Could environmental toxicants influence LH and/or GnRH levels, and therefore reproductive decisions? We evaluated a possible role for mercury contamination in non-breeding black-legged kittiwakes, and investigated the endocrine mechanisms that underlie this decision.

As expected, total blood mercury measured after arrival significantly predicted the likelihood of breeding. Although our study was correlational and would require experimental manipulation of contaminants [1,8], there is support for causal effects because experimental mercury administration can alter pairing in white ibises (*Eudocimus albus*) and suppress spawning in fathead minnows (*Pimephales promelas*) [1,8]. In our study, the most contaminated males and females were less likely to breed although there was a large overlap in mercury levels between skipping and breeding birds. Some of the skipping birds may have been young or low-quality individuals [5], and therefore be intrinsically more sensitive to mercury. In birds that did breed, mercury had no effect on egg-laying dates, clutch size and breeding success. In breeders, total blood mercury averaged  $1.8 \mu\text{g g}^{-1}$  (range:  $0.91$ – $3.08 \mu\text{g g}^{-1}$ ), which could be below a threshold level beyond which breeding success is significantly impaired as shown in other bird species [9]. However, mercury–breeding success relationships appear complex in birds since administration of a low mercury dose could even enhance breeding in mallards *Anas platyrhynchos* [10].

Mercury concentration predicted LH levels in skippers, but patterns were different among males and females, and varied between baseline and GnRH-induced LH. Baseline LH and testosterone decreased with increasing blood



**Figure 1.** Pre-breeding mercury ( $\mu\text{g g}^{-1}$  dwt) and LH levels ( $\text{ng ml}^{-1}$ ) in breeding and skipping black-legged kittiwakes. Breeding birds (empty boxes) had lower mercury levels in blood than birds that would skip breeding (striped boxes), both in (a) females and (b) males. Boxes represent median, 25th and 75th percentiles and outliers. (c–d) In breeders, baseline and GnRH-induced LH levels were not affected by mercury. (e) In skipping females (circles), baseline LH increased with increasing mercury, whereas in (f) skipping males (triangles), baseline LH decreased with increasing mercury. GnRH-induced LH levels, in both skipping males and females, increased with increasing mercury levels (e–f). Filled circles and triangles denote baseline, and open circles and triangles denote GnRH-induced.

mercury concentration in males, as found in mercury-fed laboratory rats [11]. Conversely, in skipping females, LH and mercury were positively related (although levels are still significantly lower than in breeding females [4]). These data suggest that mercury–LH relationships are complex and LH disruption could also be the result of differential negative feedback from mercury given its oestrogen-like effect [2].

To test the functionality of the pituitary, we performed GnRH challenges in breeders and skippers. Based on previous work [4], we assume that the dose of GnRH we used was sufficient to elicit a significant increase of LH 10 min after injection, and interpretation of our results is based on

this assumption. If mercury is suppressing LH secretion directly at the pituitary, we would expect LH response to GnRH to be suppressed in skippers. Contrary to our predictions, skippers were clearly able to release LH, and increased LH three- to fivefold above baseline (breeders did show a slight LH increase). Although based on correlations, it suggests that mercury may act at the hypothalamic level, disrupting GnRH synthesis or secretion. Evidence from other vertebrates demonstrates that mercury can accumulate in the hypothalamus and alter GnRH content and signalling [3,12]. In response to GnRH decline, the pituitary may have upregulated GnRH receptors, explaining the large increase



in LH with GnRH injection. Thus in kittiwakes, mercury contamination could lead to a decline in GnRH release, the subsequent increase of pituitary GnRH receptors explaining why the disrupted pituitary over-releases LH when experimentally challenged by GnRH. Although we originally hypothesized that mercury was targeting the pituitary, the induced LH response also points to a problem at the hypothalamus. It is additionally possible that mercury could bind to LH [13] making LH less able to activate gonadal receptors. Further experiments evaluating LH and GnRH expression in the brain would be necessary to meter out these possibilities.

Long-lived birds often skip breeding when foraging conditions are poor, modulating their reproductive effort

according to environmental conditions [14]. The present situation in the Arctic is of concern: mercury levels in seabirds are increasing [15] and if combined with rapid climate change, we are facing a worst-case scenario [16]. Ultimately, the likelihood to skip breeding may become too high to maintain current population levels.

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## Changes in prolactin in a highly organohalogen contaminated Arctic top predator seabird, the glaucous gull

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### ABSTRACT

The factors influencing prolactin (PRL) variation in birds and in wildlife in general have rarely been investigated with respect to the physiological impacts of exposure to environmental contaminants. We investigated the associations between circulating baseline PRL levels and concentrations of eight persistent organohalogen contaminant (OHC) classes (i.e., major organochlorines and brominated flame retardants, and associated metabolic products) in blood (plasma) of free-ranging glaucous gulls (*Larus hyperboreus*), a top predator in the Norwegian Arctic, engaged in the process of incubation. We further examined whether plasma OHC concentrations were associated with the variation of PRL in glaucous gulls exposed to a standardized capture/restraint protocol. Plasma OHC concentrations in male glaucous gulls were 2- to 3-fold higher relative to females. Baseline PRL levels tended to be higher in females compared to males, although not significantly ( $p = 0.20$ ). In both males and females, the 30-min capture/restraint protocol led on average to a 26% decrease in PRL levels, which resulted in a rate of PRL decrease of 0.76 ng/mL/min. The baseline PRL levels and the rate of decrease in PRL levels tended to vary negatively with plasma OHC concentrations in males, but not in females, although several of these associations did not adhere with the criterion of significance ( $\alpha = 0.05$ ). Present results suggest that in highly OHC-exposed male glaucous gulls, the control of PRL release may be affected by the direct or indirect modulating actions of OHCs and/or their metabolically derived products. We conclude that potentially OHC-mediated impact on PRL secretion in glaucous gulls (males) may be a contributing factor to the adverse effects observed on the reproductive behavior, development and population size of glaucous gulls breeding in the Norwegian Arctic.

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### 1. Introduction

In recent years, growing research attention has been given to chemically induced changes on endocrine systems in wildlife species chronically exposed to environmental contaminants. A wide body of evidence now suggests that certain persistent contaminant classes, including various organochlorines and brominated flame retardants, and their metabolically derived products (e.g., hydroxylated (OH)-PCBs), possess structural attributes and mode of action potencies that are similar to endogenous hormones. Hence, these contaminants can perturb endocrine-related mechanisms via signaling pathways, biosynthesis and transport proteins. Alterations of natural hormone levels, beyond the thresholds of homeostatic

compensation and variation, may thus lead to detrimental and irreversible changes in the development, reproduction and cognitive and behavioral functions of exposed wildlife, including several avian species (Burger et al., 2002; Dawson, 2000; Giesy et al., 2003). However, the mechanistic linkages between contaminant exposure and endocrine system modulation in birds remain poorly understood.

Hitherto, endocrino-toxicological assessments in free-ranging birds have focused primarily on modulation of the thyroid gland and thyroid hormone levels, and to a lesser extent on reproductive steroid hormones and glucocorticosteroids (Burger et al., 2002; Dawson, 2000; Giesy et al., 2003). Nevertheless, other key hormones involved in the initiation/regulation of physiological and behavioral mechanisms and processes in birds may be affected by chemical interactions during critical phases of their life cycle. One such phase is the breeding period. Prolactin (PRL), an anterior pituitary hormone, is closely associated with reproduction and particularly in parental (Buntin, 1996; Sharp et al., 1988) and

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alloparental behaviors (Angelier et al., 2006). Circulating levels of PRL in male and female birds are known to increase at the onset of oviposition and to remain at high levels following clutch completion and throughout the incubation period. PRL levels have also been shown to decline rapidly after hatching in some avian species (Buntin, 1996), or to be maintained at elevated levels in others (Lormée et al., 1999, 2000). The current state of knowledge on the potentiation of PRL secretion in birds identifies several biological and environmental factors that may induce physiological changes in circulating PRL levels. These factors include, among others, photoperiod (i.e., diurnal and annual rhythms), reproductive and nutritional status, maturation (i.e., age and breeding experience), osmosis and stress (Angelier et al., 2006, 2007a; Chastel et al., 2005; Hall et al., 1986; Sharp et al., 1989). For example, it has been reported in long-lived seabird species that an acute stressful event can lead to a marked decrease in plasma PRL levels [e.g., black-legged kittiwakes (*Rissa tridactyla*) (Chastel et al., 2005) and snow petrels (*Pagodroma nivea*) (Angelier et al., 2007b)]. During stressful events such as the presence of a predator, severe weather conditions and sudden decrease in food supply, a decrease in plasma PRL levels can be considered adaptive as it may disrupt the current parental effort (via nest desertion) of an individual and promote its survival and future reproduction (Chastel et al., 2005). Alternatively, an attenuation of the PRL response could be considered as a hormonal tactic permitting the maintenance of parental care behaviors and maximizing current reproductive efforts during temporary stressful conditions (Chastel et al., 2005).

The factors influencing PRL homeostasis in birds and in wildlife in general have rarely been investigated with respect to the physiological impacts of exposure to anthropogenic environmental stressors such as the persistent organic contaminants. In fact, based on the primary role of PRL in avian reproduction, research on the chemically induced, endocrine-disruptive effects on PRL release may be important in understanding life-history tradeoffs in chronically exposed bird populations. The few experimental avian studies carried out *in vivo* with male and laying female mallard ducks (*Anas platyrhynchos*) have shown that the ingestion of petroleum resulted in a marked reduction in circulating PRL levels (Cavanaugh et al., 1983; Harvey et al., 1981). In contrast, in another study of mallard ducks exposed to cadmium, no effect on the plasma PRL levels was observed (Hughes et al., 2003), which may suggest a contaminant-dependent response on PRL control and release in birds. It has also been shown in breeding male American kestrels (*Falco sparverius*) that a PCB-dosed group exhibited a size reduction in brood patch (i.e., a bare area of skin that aids in transferring the body heat of the incubating bird to its eggs) (Fisher et al., 2006), which is in part under PRL control (Buntin, 1996). However, in this particular study the PRL levels were not determined, and thus no link to PRL modulation could be established.

One seabird species in the Norwegian Arctic, the glaucous gull (*Larus hyperboreus*), has received particular research interest with regard to organohalogen contaminant (OHC) monitoring and health risk assessments. In fact, comprehensive surveys have demonstrated that the Norwegian Arctic glaucous gull, which occupies an apex position in the marine food web, accumulates some of the highest burdens of chlorinated, brominated and fluorinated contaminants among any Arctic seabirds (Verreault et al., 2005a,b,c). Current understanding of contaminant-induced biological effects in breeding glaucous gulls supports that OHCs, including their metabolically derived products, may compromise endocrine functions. Thus far, field-based studies in glaucous gulls have reported that during the breeding season highly OHC-contaminated individuals, particularly the males, exhibit altered circulating thyroid (thyroxine and triiodothyronine) (Verreault et al., 2004, 2007a) and sex steroid levels (Verreault et al., 2006a). Also documented in breeding glaucous gulls are various toxicological and reproductive effects and

endpoints that were suggested to be mediated through modulation of the thyroid and reproductive steroid hormone systems. These include: a higher rate of wing feather asymmetry (Bustnes et al., 2002), altered basal metabolism (Verreault et al., 2007a), change in reproductive behavior such as lower nest-site attendance (Bustnes et al., 2001, 2003a, 2005; Verboven et al., submitted for publication a), and reduced ability to maintain nest temperature while incubating (Verboven et al., submitted for publication b). Hence, considering the fundamental role PRL plays in avian reproduction and parental behaviors, but also in the initiation and control of molt (Dawson, 2006; Kuenzel, 2003), it could be hypothesized that the adverse effects reported in glaucous gulls on various reproductive parameters and feather growth may, in part, involve a sensitivity of baseline PRL (i.e., PRL levels measured without handling stress) secretion to chemically induced stress. Therefore, we investigated the associations between circulating baseline PRL levels and plasma concentrations of major persistent OHC classes and metabolites in free-ranging glaucous gulls from the Norwegian Arctic engaged in the process of incubation. The OHCs and metabolites investigated are known, or suspected to interfere with endocrine systems in vertebrates based on *in vitro* and *in vivo* studies (Giesy et al., 2003). Hence, we predicted that baseline PRL release should be negatively affected, and thus at lower circulating levels in highly OHC-contaminated individuals. Furthermore, we examined whether OHC concentrations were associated with the variation of PRL levels in glaucous gulls exposed to a stressful event consisting of a standardized capture/restraint protocol, which has been suggested to be a measure of reproductive investment in seabirds (Chastel et al., 2005). Because highly OHC-contaminated glaucous gulls in the Norwegian Arctic were previously reported to exhibit a lower incubation commitment (Bustnes et al., 2001, 2005; Verboven et al., submitted for publication a, submitted for publication b), we also predicted that plasma OHC concentrations and PRL levels in response to stress would be associated.

## 2. Materials and methods

### 2.1. Sample collection

Samples of blood were obtained in May and June (2006) from free-ranging adult male ( $n = 17$ ) and female ( $n = 25$ ) glaucous gulls at Bear Island (74° 22'N, 19° 05'E) in the Norwegian Arctic. The study period was characterized by continuous daylight, a mean ambient temperature of 4.4 °C (range: -0.8 to 8.8 °C), and periods of rain, strong winds and even snowfalls. In glaucous gulls, both males and females partake in the care of offspring, which includes incubation, nest defense and chick feeding (Gilchrist, 2001). Therefore, males and females were captured randomly while incubating their eggs in major colonies using a nest trap. The trap consisted of a snare placed on the edge of the nest bowl that was triggered from a distance (~100 m) with a radio transmitter. The trap was triggered approximately ten minutes after the birds had resumed incubating to ensure that these birds and the neighboring nesting pairs in the colony were unstressed.

In order to induce a stress-related response on PRL release in glaucous gulls, a standardized capture/restraint and sampling protocol developed for seabirds was applied (Chastel et al., 2005). The initial blood sample was taken from the wing vein within 3–5 min following capture. It has been shown that based on this sampling regime the initial blood sample reflects the baseline PRL levels (hereafter termed as baseline PRL). Because males and females were captured and sampled for blood at least three days following clutch completion, and throughout the first half of the incubation period (i.e., 28–30 days) at any time during the day or night, the temporal effect (i.e., day in the incubation period and time of the day) on PRL levels was examined. The influence of diurnal rhythm on PRL levels was tested by plotting the time of the initial blood sample and plasma PRL levels. No significant relationship was found for either sex ( $p \geq 0.43$ ), which suggests negligible effect of diurnal variation on PRL levels, as also documented in Adélie penguins (*Pygoscelis adeliae*) during the Antarctic summer (Vleck and van Hook, 2002). However, the effect of day of capture in the incubation period on the PRL level variation was consistently controlled for in the statistical models (see Section 2.4). Shortly after initial blood sampling, the birds were placed into individual, opaque cotton bags and left undisturbed. The final blood sample was collected once 30 min had elapsed, and was used to quantify post-handling PRL levels (hereafter termed as handling PRL) (see Section 2.3) as well as OHC concentrations and extractable plasma lipid content (see Section 2.2). Blood samples were collected, processed and stored according

to procedures optimized for hormone and OHC analyses in glaucous gulls (Verreault et al., 2004). Various morphometric measurements were recorded (i.e., head + bill, wing and tarsus length and body mass), and the birds were sexed according to methods described elsewhere (Verreault et al., 2004). All handled birds were observed in their respective nesting colonies shortly after release, and eventually resumed incubating. Capture, handling and sampling methods were approved by the Norwegian Animal Research Authority (Oslo, Norway) and the Governor of Svalbard (Longyearbyen, Norway).

## 2.2. Chemical analysis

The analytical methods (i.e., sample extraction, partitioning and clean-up, and instrumental quantification) for the determination of chlorinated benzenes (CBzs), dichlorodiphenyldichloroethanes (DDTs), chlordanes (CHLs), PCBs, polybrominated diphenyl ethers (PBDEs), methoxylated (MeO)-PBDEs, OH- and methylsulfonyl (MeSO<sub>2</sub>)-PCBs in glaucous gull plasma samples have been identified in detail by Verreault et al. (2005a,b). These methods were applied on the present plasma samples with no modification. A complete list of the congeners/compounds determined in the present study is provided in Table 1.

Chlorinated and brominated compound quantification was performed using a gas chromatograph–mass spectrometer (GC–MS) (Agilent 6890; Agilent Technologies, Palo Alto, CA, USA) operating in the electron impact (EI) (for CBzs, DDTs, CHLs and PCBs) or electron capture negative ionization (ECNI) mode (for PBDEs, MeO–PBDEs, OH–PCBs and MeSO<sub>2</sub>–PCBs). The GC–MS(EI) separation was completed using a fused silica DB-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA), whereas a fused silica DB-5 HT capillary column (15 m, 0.25 mm i.d., 0.10 μm film thickness) (J&W Scientific) was used for GC–MS(ECNI) separation. The GC–MS(ECNI) utilized methane as a buffer gas. The GC–MS(EI) and GC–MS(ECNI) analyte determinations were accomplished in the selected ion-monitoring (SIM) mode. The analytes were identified on the basis of their retention times on the DB-5 columns, and verified by matching retention times with authentic standard mixtures. An external standard method was used for CBz, DDT, CHL and PCB quantification using a spiked recovery surrogate mixture comprised of six <sup>13</sup>C-labeled PCB congeners and three tetra- through hexa-CBzs. An internal standard (IS) approach was used for quantification of PBDEs and MeO–PBDEs (IS was BDE71), OH–PCBs (IS was a 4-congener <sup>13</sup>C–OH–PCB mixture) and MeSO<sub>2</sub>–PCBs (IS was 3-MeSO<sub>2</sub>-2-CH<sub>3</sub>-2',3',4',5,5'-pentaCB).

Quality assurance and quality control procedures included a standard reference material (polar bear plasma pool #01-2004) from the National Wildlife Research Centre, method blanks, duplicate extractions and injections of authentic standards and cleaned-up glaucous gull plasma extracts for each block of six samples to monitor for quantitative reproducibility and instrument sensitivity. Mean (±1 standard error) recoveries based on the added recovery surrogates and ISs were: 86 ± 1% for <sup>13</sup>C-PCBs, 63 ± 2% for <sup>13</sup>C-CBzs, 95 ± 3% for BDE71, 70 ± 3% for <sup>13</sup>C–OH–PCBs and 103 ± 4% for 3-MeSO<sub>2</sub>-2-CH<sub>3</sub>-2',3',4',5,5'-pentaCB. The CBzs, DDTs, CHLs and PCBs were adjusted for recoveries, while the PBDEs, MeO–PBDEs, OH–PCBs and

MeSO<sub>2</sub>–PCBs, which were determined using an IS quantification approach, were inherently recovery-corrected. Blank samples showed negligible background contamination for all analyte classes, and thus no background correction was necessary. The duplicate extractions and injections demonstrated on average 10% and 5%, respectively, analytical variation of selected compound concentrations. The analyte-specific method limits of quantification (MLOQs) were set as a signal being 10 times the standard deviation of the noise.

## 2.3. Prolactin assay

Plasma levels of PRL were determined by a heterologous radioimmunoassay (RIA) based on the methods described by Cheral et al. (1994) and validated for several seabirds (e.g., Angelier et al., 2006, 2007a; Lormée et al., 2000), also including a few Charadriiforms species such as the black-legged kittiwake (Chastel et al., 2005) and common guillemot (*Uria aalge*) (Tremblay et al., 2003). Pooled plasma samples of glaucous gulls produced a dose–response curve that paralleled a chicken PRL standard curve (AFP 4444B; obtained from Dr. Parlow, NHPP, Harbor-UCLA Medical Center, Torrance, CA, USA, Fig. 1). Parallelism between the glaucous gull and chicken curve indicates that the concentration-dependent binding dynamics of the glaucous gull PRL with the antibody is similar to that of the chicken PRL (Fig. 1). Hence, this RIA was concluded to be an appropriate assay to assess relative levels of plasma PRL in incubating glaucous gulls. Only one assay was performed, the intra-assay coefficient of variation being 8.4% (n = 6 duplicates). The rate of decrease in PRL levels in glaucous gulls following the capture/restraint protocol was calculated as the decrease in PRL level (ng/mL) per handling time unit (min).

## 2.4. Data treatment

The OHCs determined in the present study were categorized and summed (Σ) in eight classes (i.e., Σ<sub>4</sub>CBz, Σ<sub>3</sub>DDT, Σ<sub>6</sub>CHL, Σ<sub>58</sub>PCB, Σ<sub>38</sub>PBDE, Σ<sub>15</sub>MeO–PBDE, Σ<sub>28</sub>OH–PCB and Σ<sub>15</sub>MeSO<sub>2</sub>–PCB) composed of closely related congeners/compounds with respect to their chemical structures if they were detected in 60% or more of the plasma samples. For these OHCs, the samples with concentrations of a given congener or compound below the method limit of quantification (MLOQ) were arbitrarily assigned a value, randomly selected from a normal distribution, between zero and the compound-specific MLOQ. In males and females, the OHC classes were positively inter-correlated (r ≥ 0.58; p < 0.0001). Hence, in order to reduce the number of predictor variables and to improve the statistical power of the analyses, principal components (PCs) were extracted based on the wet weight concentrations (log<sub>10</sub>-transformed) of these eight OHC classes. Because the first extracted PC (hereafter referred to as PC 1 OHC) accounted for 80.8% of the total variance, and because it was strongly and positively correlated with concentrations of each of the eight OHC classes (r ≥ 0.83; p < 0.0001), analyses were performed using PC 1 OHC only. The correlations between the eight OHC classes and the selected dependent and independent variables also were tested. However, because strong inter-correlations were found

**Table 1**

Mean (±1 standard error (SE)) and data range of percentage extractable plasma lipid content and organohalogen contaminant (OHC) class concentrations (ng/g wet wt.) in plasma of incubating glaucous gull males and females from the Norwegian Arctic. The levels of significance (p-level) for the differences in the eight OHC class concentrations between males and females are identified

	p-Level	Males (n = 17)		Females (n = 25)	
		Mean ± SE	Data range	Mean ± SE	Data range
Plasma lipid %	0.15	0.77 ± 0.04	0.44–1.0	0.86 ± 0.04	0.40–1.26
Σ <sub>4</sub> CBz <sup>a</sup>	0.002	24.2 ± 3.6	7.1–65.7	13.0 ± 1.6	4.3–34.5
Σ <sub>6</sub> CHL <sup>b</sup>	0.003	44.0 ± 7.0	11.9–119	22.7 ± 2.4	8.3–49.1
Σ <sub>3</sub> DDT <sup>c</sup>	0.0001	274 ± 32.9	102–625	137 ± 16.5	38.4–324
Σ <sub>58</sub> PCB <sup>d</sup>	0.001	647 ± 113	90.3–1611	263 ± 35.7	54.2–814
Σ <sub>28</sub> OH–PCB <sup>e</sup>	0.0001	19.6 ± 3.7	0.9–59.7	5.8 ± 0.9	0.8–16.3
Σ <sub>15</sub> MeSO <sub>2</sub> –PCB <sup>f</sup>	0.003	0.60 ± 0.08	0.12–1.14	0.30 ± 0.06	0.02–1.26
Σ <sub>38</sub> PBDE <sup>g</sup>	0.001	19.6 ± 3.4	4.7–54.5	8.5 ± 1.1	2.5–24.9
Σ <sub>15</sub> MeO–PBDE <sup>h</sup>	0.08	2.18 ± 0.44	0.05–5.66	0.92 ± 0.13	0.19–3.22

<sup>a</sup> Sum of 1,2,4,5-TeCBz, 1,2,3,4-TeCBz, PnCBz and HxCBz.

<sup>b</sup> Sum of heptachlor epoxide, oxychlordanes, trans-chlordane, cis-chlordane, trans-nonachlor and cis-nonachlor.

<sup>c</sup> Sum of p,p'-DDT, p,p'-DDE and p,p'-DDD.

<sup>d</sup> Sum of CB22, 28/31, 20/33, 41/64, 42, 44, 47/48, 49, 52, 56/60, 66, 70/76, 74, 85, 87, 90/101, 92, 95, 97, 99, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 167, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207 and 208.

<sup>e</sup> Sum of 4'-OH-CB79, 4-OH-CB97, 4-OH-CB107/4'-OH-CB108, 2'-OH-CB114, 4'-OH-CB120, 4'-OH-CB127, 4'-OH-CB130, 4-OH-CB134, 4-OH-CB146, 4'-OH-CB159, 4-OH-CB162, 4-OH-CB163, 4'-OH-CB177, 4-OH-CB178, 3'-OH-CB180, 3'-OH-CB182, 3'-OH-CB183, 3'-OH-CB184, 4-OH-CB187, 4-OH-CB193, 4'-OH-CB198, 4'-OH-CB199, 4'-OH-CB200, 4'-OH-CB201, 4'-OH-CB202, 4,4'-diOH-CB202, 3'-OH-CB203 and 4'-OH-CB208.

<sup>f</sup> Sum of 3-MeSO<sub>2</sub>-CB49, 4-MeSO<sub>2</sub>-CB49, 3-MeSO<sub>2</sub>-CB52, 4-MeSO<sub>2</sub>-CB52, 4-MeSO<sub>2</sub>-CB64, 3-MeSO<sub>2</sub>-CB70, 4-MeSO<sub>2</sub>-CB70, 4-MeSO<sub>2</sub>-CB87, 3-MeSO<sub>2</sub>-CB101, 4-MeSO<sub>2</sub>-CB101, 3-MeSO<sub>2</sub>-CB110, 4-MeSO<sub>2</sub>-CB110, 3-MeSO<sub>2</sub>-CB132, 4-MeSO<sub>2</sub>-CB132 and 3-MeSO<sub>2</sub>-CB149.

<sup>g</sup> Sum of BDE17, 25, 28, 47, 49, 54, 66, 75, 77, 85, 99, 100, 116, 119, 138, 139, 140, 153, 154/BB153, 155, 156, 171, 180, 181, 183, 184, 190, 191, 196, 197, 201, 202, 203, 205, 206, 207, 208 and 209.

<sup>h</sup> Sum of 6'-MeO-BDE17, 4'-MeO-BDE17, 2'-MeO-BDE28, 4-MeO-BDE42, 5-MeO-BDE47, 6-MeO-BDE47, 3-MeO-BDE47, 4'-MeO-BDE49, 6'-MeO-BDE49, 2'-MeO-BDE68, 6-MeO-BDE85, 6-MeO-BDE90, 6-MeO-BDE99, 2-MeO-BDE123 and 6-MeO-BDE137.

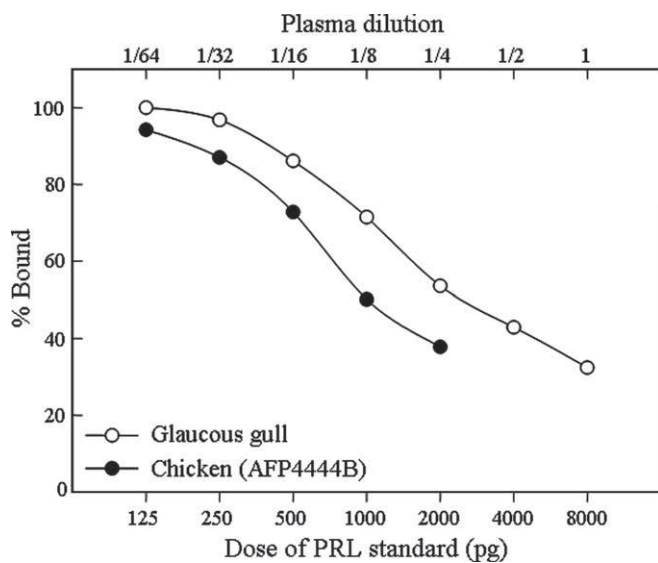


Fig. 1. Dose–response curve of prolactin in chicken (AFP 4444B) and glaucous gull from the Norwegian Arctic (Bear Island). The dose of prolactin standard is expressed in pg/tube.

between the congeners and compounds constituting those eight classes, analyses were not performed on single congeners/compounds. In order to detect potential sex-specific variation patterns in the associations between PRL levels and OHC concentrations, PC 1 OHC factor scores were tested based on a quartile categorization (i.e., groups I–IV). The quartiles generated were comprised of 4 to 5, or 6 to 7 individuals for males and females, respectively, and were significantly different from each other for both males ( $p \leq 0.03$ ) and females ( $p \leq 0.003$ ).

The structures in the relationships between baseline and handling PRL levels ( $\log_{10}$ -transformed), rate of decrease in PRL levels, PC 1 OHC as well as potentially confounding environmental and biological variables (see next paragraph) were investigated using PC analysis. The results from this multivariate exploratory technique allowed identifying the potential predictor variables to the variation of PRL levels and rate of decrease in PRL levels, which were further analyzed using general linear models (GLMs) (e.g., ANOVA). Differences between the categorical predictors (i.e., sex and PC 1 OHC quartiles) and their possible interactions, for any continuous variables, were further examined using the Fisher *post hoc* test. The statistical package utilized was Statistica® (StatSoft, Tulsa, OK, USA) and  $\alpha$  was set at 0.05.

The environmental and biological variables selected for the analyses were: day of capture in the study period (i.e., calendar dates: June 1st–June 23rd), extractable plasma lipid percentage and body condition. Body condition of an individual was defined as the body size index extracted using PC analysis from three morphological measurements (i.e., wing length, tarsus length and head + bill length), and corrected for body mass in the GLMs (Garcia-Berthou, 2001). Because PC 1 explained 76.3% and 65.1% of the variation in body size for male and females, respectively, only PC 1 was used. The body size index and body condition were generated separately for males and females as the glaucous gull is sexually dimorphic. It has previously been shown in glaucous gulls at a post-egg laying stage, for an identical suite of OHCs analyzed as in the present study (Table 1), that significant differences in plasma concentrations are found between males and females (Verreault et al., 2005a,b, 2006a, 2007a). Furthermore, in toxicological assessments on various endocrine parameters (Verreault et al., 2004, 2006a, 2007a) and reproductive and developmental endpoints (Bustnes et al., 2001, 2002, 2003a, 2005; Verboven et al., submitted for publication a, submitted for publication b) in breeding glaucous gulls, notable sex-specific disparities have been reported. As a consequence, males and females were investigated as distinct groups rather than controlling for the effect of sex in the statistical models where the variation of baseline and handling PRL levels and rate of decrease in PRL levels was examined relative to PC 1 OHC (and PC 1 OHC quartiles).

### 3. Results

#### 3.1. OHC concentrations

The sum concentrations of closely related compounds/congeners (i.e.,  $\sum_4$ CBz,  $\sum_3$ DDT,  $\sum_6$ CHL,  $\sum_{58}$ PCB,  $\sum_{38}$ PBDE,  $\sum_{15}$ MeO-PBDE,  $\sum_{28}$ OH-PCB and  $\sum_{15}$ MeSO<sub>2</sub>-PCB) determined in plasma of glaucous gull males and females can be found in Table 1. The

OHC compositional patterns and concentrations in glaucous gull plasma were comparable to those previously reported for this species during the incubation stage in which an analogous suite of OHCs was analyzed (Verreault et al., 2006a,b, 2007a). Moreover, consistent with previous glaucous gull investigations, males retained approximately 2- to 3-fold higher OHC concentrations in plasma than females, with more pronounced disparities for the  $\sum_{28}$ OH-PCB and  $\sum_{58}$ PCB (Table 1). A comprehensive description and discussion on the sex-specific factors of OHC accumulation, toxicokinetics and fate of chlorinated and brominated contaminants and metabolites in glaucous gull plasma can be found in Verreault et al. (2005a,b, 2006b, 2007b).

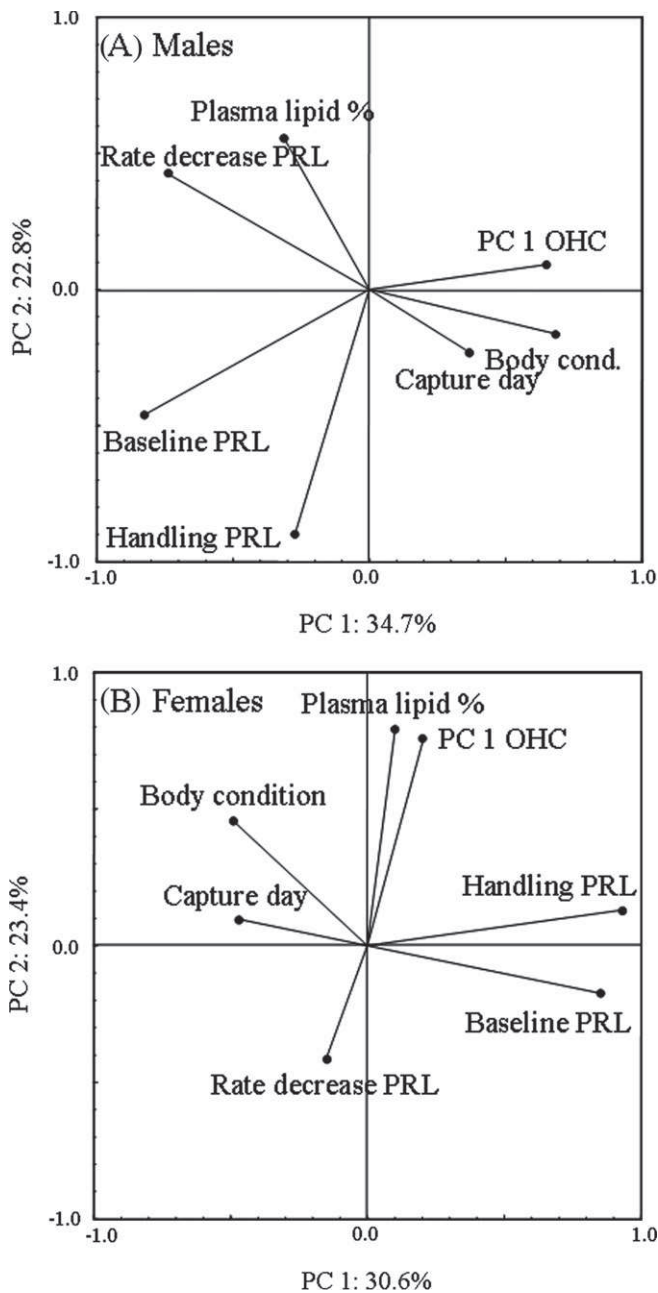
#### 3.2. PRL and stress induction

The mean baseline PRL levels in incubating glaucous gull plasma samples tended to be higher in females relative to males, although not significantly ( $p = 0.20$ ). This gender-dependent peculiarity was in accordance with several seabird species exhibiting shared parental duties for which slightly elevated baseline PRL levels were found in females during most of the breeding period (Angelier et al., 2006, 2007a; Cherel et al., 1994). The 30-min capture/restraint protocol applied on glaucous gulls led to a significant decrease in circulating PRL levels in both males and females, which was consistent with studies of several long-lived seabird species exposed to a similar treatment (Angelier et al., 2007b; Chastel et al., 2005). Following capture/restraint, the baseline PRL levels decreased on average by 26% (range: 3–45%) in males and females, which resulted in a rate of PRL decrease averaging 0.76 ng/mL/min (range: 0.08–1.61 ng/mL/min). The rate of decrease in PRL levels in glaucous gulls was identical between sexes ( $p = 0.73$ ). Baseline and handling PRL levels co-varied positively in both sexes ( $r \geq 0.64$ ;  $p \leq 0.005$ ).

#### 3.3. Associations among variables

The structures in the relationships between baseline and handling PRL levels, rate of decrease in PRL levels, PC 1 OHC as well as the potential confounding variables extractable plasma lipid percentages, body condition and capture day in the study period, were investigated separately for each sex using PC analysis (Fig. 2A and B). In males and females, the cumulated variance of PC 1 and PC 2 accounted for 57.5% and 54.0%, respectively, of the total variance. Because the contribution from PC 3 in the total variance was substantially less than PC 2 in both sexes, and because this PC did not provide noteworthy additional information on the variable association patterns, PC 3 was left out from further analyses and discussions.

In males, the spatial orientation of the variables based on PC 1 and PC 2 pointed to an inverse association between baseline PRL levels and the rate of decrease in PRL levels, and the PC 1 OHC factor scores (Fig. 2A). Using GLMs, the baseline PRL levels and rate of decrease in PRL levels in males tended to vary negatively with PC 1 OHC, although these associations did not comply with the criterion of significance (Table 2). Moreover, negative relationships were found in males between baseline PRL levels and rate of decrease in PRL levels, and the eight OHC classes tested individually, although only the  $\sum_{38}$ PBDE concentrations ( $\log_{10}$ -transformed) yielded a statistically significant correlation with the baseline PRL levels (Table 2). Baseline and handling PRL levels in males, as well as the rate of decrease in PRL levels, varied irrespective of the extractable plasma lipid percentages ( $p \geq 0.28$ ) body condition ( $p \geq 0.13$ ) and capture day in the study period ( $p \geq 0.44$ ). In contrast, in females, no significant predictive effect of PC 1 OHC was found on baseline and handling PRL level and rate of decrease in PRL level variation (Fig. 2B). In fact, PC 1 OHC scores in females



**Fig. 2.** Structures in the relationships between baseline and handling prolactin (PRL) levels ( $\log_{10}$ -transformed), rate of decrease in PRL levels, organohalogen contaminant (OHC) concentrations (PC 1 OHC), as well extractable plasma lipid percentages, body condition and capture day in the study period for incubating male (A) and female (B) glaucous gulls. The relative percentages of the total variance explained by each of the two first principal components (PCs), PC 1 and PC 2, are provided.

only varied significantly and positively as a function of the extractable plasma lipid percentages ( $r = 0.41$ ;  $p = 0.04$ ). Moreover, female baseline PRL levels were associated negatively with body condition ( $r = -0.47$ ;  $p = 0.03$ ).

The quartiles (groups I–IV) generated based on the PC 1 OHC factor score distribution were used in order to detect potential sex-specific variation (interactive or non-interactive) patterns in baseline and handling PRL levels (and rate of decrease in PRL levels) as a function of the OHC concentrations (Fig. 3A and B). In males, baseline PRL levels were significantly lower ( $p = 0.05$ ) in the top 25% quartile (IV) relative to the lowest (I). No such difference in baseline PRL levels was found for females. However, in response to the capture/re-

straint-related stress, handling PRL levels in both males and females varied following an unpredictable pattern with respect to the PC 1 OHC quartiles. No significant difference in handling PRL levels was found between any of the PC 1 OHC quartiles for both males and females. By comparison, following a similar quartile-based approach, the variation in the rate of decrease in PRL levels highly differed between the sexes (Fig. 4A and B). In males, the rate of decrease in PRL levels was significantly lower ( $p = 0.03$ ) in quartile IV relative to quartile I. In females, the rate of decrease in PRL levels fluctuated irrespective of the PC 1 OHC quartiles, although it tended to be highest in the mid-quartiles relative to the quartiles I and IV.

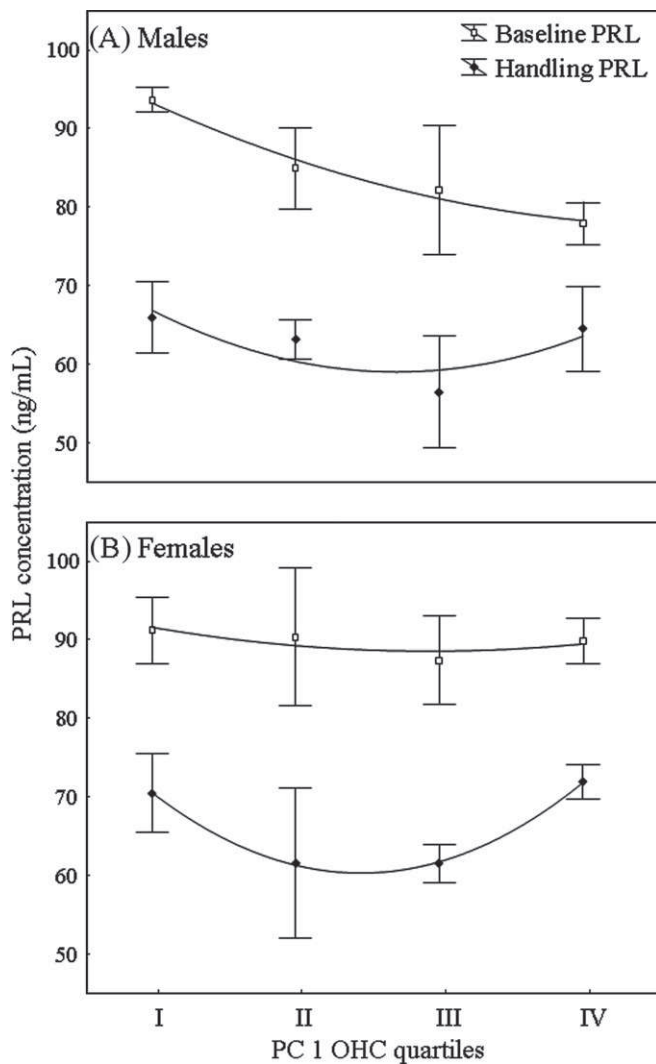
#### 4. Discussion

##### 4.1. PRL variation and OHCs

In the present field-based study of Arctic-breeding glaucous gulls exposed to a complex cocktail of OHCs in the marine ecosystem, negative associations between circulating baseline PRL levels and plasma OHC concentrations were found in males, but not in females. Furthermore, in response to a 30-min stressful event induced through a capture/restraint protocol, baseline PRL levels in males decreased at a rate that tended to be negatively associated with plasma OHC burdens. Hence, highly OHC-contaminated glaucous gull males appeared to exhibit an attenuated stress-related PRL responsiveness, which would suggest that they maintained higher PRL levels compared to lower-contaminated individuals. Interestingly, these findings were contrasting with our original predictions suggesting that the rate of decrease in PRL levels following a handling stress would be lowest in the low OHC-exposed birds. However, the reasons for this (weak) association are uncertain, and thus further studies would be required. Nonetheless, the present results suggest that in chronically OHC-exposed male glaucous gulls, the control of PRL release may be affected by the direct or indirect modulating actions, depending on the level of exposure, of OHCs and/or their metabolically derived products. In contrast, in females, the associations between PRL levels and the rate of decrease in PRL levels and plasma OHC concentrations were notably absent. This may indicate a sex-specific physiological sensitivity of PRL control mechanisms in glaucous gulls exposed to OHCs and acute stress. Alternatively, because female glaucous gulls tended to have higher baseline PRL levels than males (but not significant) and can excrete a substantial portion of their OHC burden via oviposition, they may not have reached the threshold concentrations of OHCs that are necessary to overwhelm and impair the homeostatic mechanisms of PRL release. However, based on the lack of statistical significance of most of these PRL–OHC associations in males at the established  $\alpha$ -value, which may be the consequence of a low sample size and/or a large amount of individual variation, these trends should be interpreted with great caution. In fact, inherent factors controlling PRL secretion in birds, other than body condition and time of the year (season) or day, may not have been factored out in the present field-based investigation of glaucous gulls. For instance, it has been shown in several long-lived seabirds that baseline PRL levels increase with the breeding experience and age (Angelier et al., 2006, 2007a). Moreover, PRL responsiveness to an experimental stress has been reported to be influenced by age in some seabird species where older breeders have been shown to maintain higher stress-induced PRL levels than younger individuals (Angelier et al., 2007b). While the present glaucous gull sub-population was comprised exclusively of adult and breeding individuals, and thus older than 5 years of age (Gilchrist, 2001), the exact age of the individuals was unknown. However, it has previously been established in adult breeding glaucous gulls from the Norwegian Arctic that based on recovered individuals that were ring-marked as chicks, the effect of age on the variation of OHC concentrations in blood is negligible (Bustnes et al., 2003b).

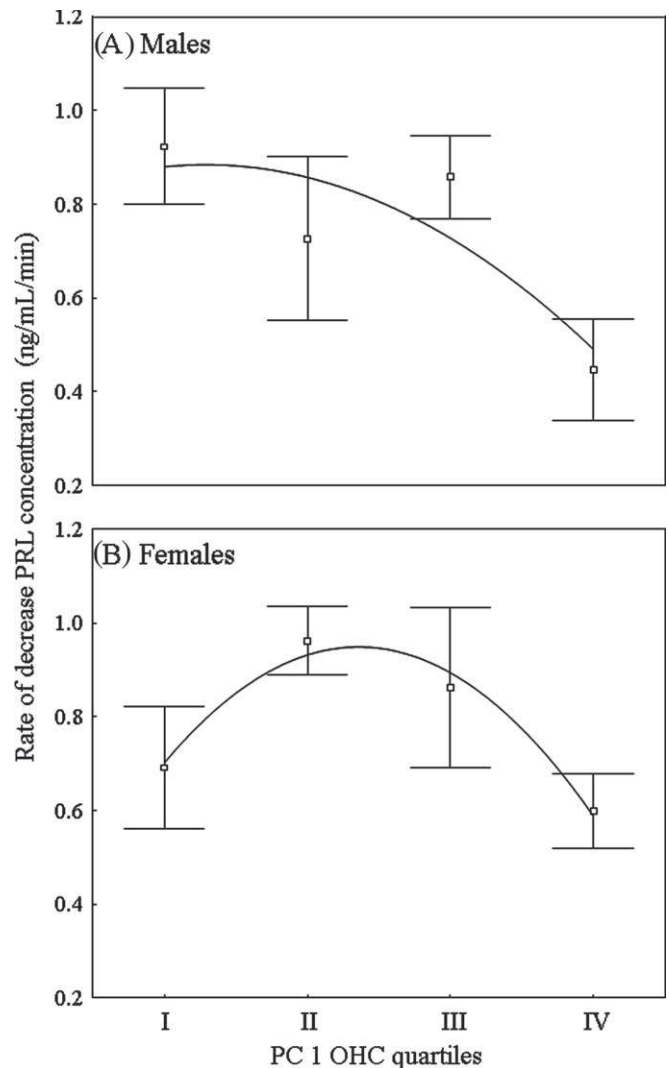
**Table 2**  
Relationships between concentrations of eight organohalogen contaminant (OHC) classes ( $\log_{10}$ -transformed ng/g wet wt.) and the first principal component extracted from OHC concentrations (PC 1 OHC) and baseline prolactin (PRL) levels ( $\log_{10}$ -transformed ng/mL) and rate of decrease in PRL levels in plasma of incubating male glaucous gulls ( $n = 17$ )

	Baseline PRL levels				Rate of decrease in PRL levels			
	$r(x,y)$	$r^2$	$t$	$p$ -Level	$r(x,y)$	$r^2$	$t$	$p$ -Level
$\sum_4$ CBz	-0.42	0.17	-1.78	0.09	-0.16	0.02	-0.61	0.54
$\sum_6$ CHL	-0.38	0.14	-1.58	0.13	-0.39	0.15	-1.62	0.12
$\sum_3$ DDT	-0.33	0.11	-1.35	0.19	-0.42	0.18	-1.79	0.09
$\sum_{58}$ PCB	-0.37	0.14	-1.56	0.13	-0.34	0.12	-1.41	0.17
$\sum_{28}$ OH-PCB	-0.33	0.11	-1.33	0.20	-0.35	0.12	-1.43	0.17
$\sum_{15}$ MeSO <sub>2</sub> -PCB	-0.35	0.12	-1.44	0.16	-0.24	0.06	-0.98	0.34
$\sum_{38}$ PBDE	-0.50	0.25	-2.24	0.04	-0.42	0.18	-1.82	0.08
$\sum_{15}$ MeO-PBDE	-0.41	0.17	-1.75	0.10	-0.31	0.09	-1.24	0.23
PC 1 OHC	-0.43	0.18	-1.84	0.08	-0.36	0.13	-1.51	0.15



**Fig. 3.** Variation of baseline and handling prolactin (PRL) levels in incubating male (A) and female (B) glaucous gulls. Mean PRL levels ( $\pm 1$  standard error) are categorized based on quartiles (i.e., groups I–IV) generated from a first principal component (PC) extracted from organohalogen contaminant (OHC) concentrations (PC 1 OHC).

There are three *in vivo* studies that have addressed the potential impacts of petroleum and cadmium ingestion on PRL levels in a captive bird; the mallard duck (Cavanaugh et al., 1983; Harvey et al., 1981; Hughes et al., 2003). These studies are to our knowledge the only studies where anthropogenic chemical stressors were investigated in relation to PRL perturbation. In line with find-



**Fig. 4.** Rate of decrease in prolactin (PRL) levels following a capture/restraint-related stress in incubating male (A) and female (B) glaucous gulls. Mean rates of decrease in PRL levels ( $\pm 1$  standard error) are categorized based on quartiles (i.e., groups I–IV) generated from a first principal component (PC) extracted from organohalogen contaminant (OHC) concentrations (PC 1 OHC).

ings in the present study, mallard ducks exposed to petroleum showed significant reductions in circulating PRL levels compared to unexposed, control specimens (Cavanaugh et al., 1983; Harvey et al., 1981). However, the laboratory conditions employed in these controlled captive studies may poorly reflect the environmental conditions experienced by free-ranging birds, which include vari-

ous stressors such as changing weather conditions and food availability, and risk of predation. In addition, avian wildlife, in contrast to most laboratory specimens, is exposed to a complex contaminant mixture that has accumulated in tissues and body compartments over time, and the multiple chemical interactions that can exist among those contaminants (e.g., additive, synergic and blocking effects). Nonetheless, investigations in captive rodent species under controlled laboratory conditions also have provided substantial evidence suggesting that *in vivo* exposure to a variety of chemicals (e.g., methyl *tert*-butyl ether, atrazine, chlordecone and PCBs) may elicit alterations in PRL secretion, including a suppressing response (de Krey et al., 1994; Rosecrans et al., 1984; Stoker et al., 1999; Williams et al., 2000).

#### 4.2. Postulated mechanisms of action

It has been established that PRL secretion in birds can both be stimulated (Hall et al., 1986) and inhibited (Reddy et al., 2002) by feedback mechanisms of the gonadal steroid hormones (i.e., progesterone, 17 $\beta$ -estradiol and testosterone). For example, in pre-incubated anterior pituitary glands of broiler fowls, exposure to progesterone resulted in a marked reduction in baseline PRL release in a dose-related manner (Hall et al., 1984). Furthermore, in white leghorn chickens and girirani birds (a hybrid chicken breed) treated with the anti-PRL agent bromocriptine, levels of PRL were lower compared to the untreated groups, and were negatively correlated with those of progesterone and 17 $\beta$ -estradiol in both species (Reddy et al., 2002, 2006). The current state of knowledge on the toxicological actions of OHCs in vertebrates also suggests that certain OHCs, all identified and quantified in glaucous gull blood and tissues, may interfere with enzymes involved in steroidogenesis [e.g., hydroxysteroid dehydrogenase (HSD) and cytochrome P450 (CYP) enzymes], or less directly through modulation of the feedback mechanisms in the hypothalamus–pituitary–gonadal axis (Sanderson and van den Berg, 2003). For example, it has been suggested that OHCs may exert their effects at the anterior pituitary gland level by directly modulating PRL release (positively and negatively), as shown in *in vivo* and *in vitro* studies for various chemicals including bromocriptine in birds (Youngren et al., 1998) and estrogen-like OHCs in mammalian cell lines (Abraham and Frawley, 1997; Rousseau et al., 2002; Steinmetz et al., 1997). Other postulated mechanisms were suggested to be mediated through a chemically induced deregulation of neurotransmitters (e.g., dopamine) involved in the modulation of PRL secretion, as for instance demonstrated in rats administered orally with the pesticide methoxychlor (Lafuente et al., 2000).

In a recent study of incubating glaucous gulls also from Bear Island, plasma progesterone levels were found to be positively correlated with concentrations of PCBs, DDTs, CHLs and PBDEs in male glaucous gulls, but not in females (Verreault et al., 2006a). In this study, in which no relationship was found between OHC and testosterone and 17 $\beta$ -estradiol (not detected), it was suggested that OHCs may have the potential to interfere with certain steroidogenic enzymes, and thus impinge on circulating progesterone homeostasis in highly OHC-exposed males. Hence, it could be hypothesized that the negative relationships between baseline PRL levels and plasma OHC concentrations in the present male glaucous gulls may, in fact, be the indirect result of an OHC-mediated disturbance on gonadal progesterone synthesis and/or metabolism, leading to abnormally high progesterone levels in the circulatory system. This would in turn create a negative feedback response on PRL secretion. However, the reverse mechanism could also occur where a direct OHC-induced inhibition of PRL secretion (e.g., in the anterior pituitary gland) would influence the circulating progesterone status. Alternatively, levels of progesterone precursors (e.g., cholesterol and pregnenolone) in the steroidogenic

cascade of glaucous gull males might have been impacted through the actions of specific OHCs on HSD and/or aryl hydrocarbon receptor-responsive CYP enzymes (Sanderson and van den Berg, 2003).

It has also been suggested that in birds, levels of thyroid hormones (thyroxine and triiodothyronine) may influence the status of circulating PRL (Hall et al., 1986). However, this endocrine interactive pathway has as yet to be confirmed. Nevertheless, in glaucous gull males from the Norwegian Arctic it has been reported that plasma thyroxine levels were negatively related to blood concentrations of major organochlorines (i.e., CBzs, PCBs, DDTs and oxychlorane) (Verreault et al., 2004). In this study, adverse effects of thyroid hormone homeostasis were found in males exclusively, which again suggests possibly enhanced sensitivity of endocrine functions in breeding male glaucous gulls relative to females.

#### 4.3. Modulation of PRL and biological implications

It has been reported in Norwegian Arctic glaucous gulls, including the presently investigated colonial population from Bear Island, that behaviors related to nest-site attentiveness and incubation were negatively related to blood concentrations of major, currently determined OHCs (Bustnes et al., 2001, 2005; Verboven et al., submitted for publication a). Furthermore, in the study by Verboven et al. (submitted for publication a), it was observed that OHC-contaminated glaucous gulls were less able to maintain a constant nest temperature. Hence, as nest-site attendance and incubation are strongly influenced by hormonal fluctuations in birds, this may suggest that the concomitant effects on circulating baseline PRL (present study), sex steroid (Verreault et al., 2006a) and thyroid hormone homeostasis (Verreault et al., 2004, 2007a) are explaining, in part, the impaired reproductive behaviors observed in glaucous gulls (males). Ultimately, this may also be a possible explanation as to the poor reproductive performance documented in glaucous gulls from these same breeding colonies (Bustnes et al., 2003a, 2005; Verboven et al., submitted for publication b). However, the lower incubation commitment in male glaucous gulls apparently can not be explained based on their PRL response to a stressful event (i.e., capture/restraint-related) as the highly OHC-contaminated individuals tended to maintain higher PRL levels compared to those that had accumulated lower OHC concentrations. Moreover, feather growth is one other endocrine-dependent physiological mechanism that has been suggested to be OHC-sensitive in glaucous gulls (Bustnes et al., 2002). Because PRL, in orchestration with other hormones (e.g., thyroid and growth hormones), has been demonstrated to play a role in the control of molt (Dawson, 2006; Kuenzel, 2003), it could be speculated that the higher rate of wing feather asymmetry reported in glaucous gull males (Bustnes et al., 2002) could be partly linked to the alterations on PRL release. Taken together, these possibly OHC-associated effects on the reproductive behaviors and development of glaucous gulls from the Norwegian Arctic may contribute to adverse changes on their population size status (Bustnes et al., 2003a, 2005). In fact, it has recently been documented that since 1986 and up until the most recent census in 2006, the breeding population of glaucous gulls from these Norwegian Arctic colonies has decreased by nearly 65% (Strøm, 2006).

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## Research

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# Demographic consequences of heavy metals and persistent organic pollutants in a vulnerable long-lived bird, the wandering albatross

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Seabirds are top predators of the marine environment that accumulate contaminants over a long life-span. Chronic exposure to pollutants is thought to compromise survival rate and long-term reproductive outputs in these long-lived organisms, thus inducing population decline. However, the demographic consequences of contaminant exposure are largely theoretical because of the dearth of long-term datasets. This study aims to test whether adult survival rate, return to the colony and long-term breeding performance were related to blood mercury (Hg), cadmium (Cd) and persistent organic pollutants (POPs), by using a capture–mark–recapture dataset on the vulnerable wandering albatross *Diomedea exulans*. We did not find evidence for any effect of contaminants on adult survival probability. However, blood Hg and POPs negatively impacted long-term breeding probability, hatching and fledging probabilities. The proximate mechanisms underlying these deleterious effects are likely multifaceted, through physiological perturbations and interactions with reproductive costs. Using matrix population models, we projected a demographic decline in response to an increase in Hg or POPs concentrations. This decline in population growth rate could be exacerbated by other anthropogenic perturbations, such as climate change, disease and fishery bycatch. This study gives a new dimension to the overall picture of environmental threats to wildlife populations.

## 1. Introduction

Free-living animal populations are affected by a wide range of anthropogenic pressures. Chronic exposure to metallic and organic pollutants may compromise survival and long-term fecundity, thereby leading to population decline. For instance, mercury (Hg) is a globally distributed heavy metal of particular concern for aquatic biota, because of the harmful effects of its organic form (methyl-Hg) on embryo development, neurology, immune system, physiology and behaviour [1–3]. Another ubiquitous heavy metal, cadmium (Cd), causes irreversible renal tubular damage, leading to reduced skeletal calcium content [4]. Persistent organic pollutants (POPs), such as organochlorine pesticides (HCB, HCH, DDE, DDD and DDT), polychlorinated biphenyls (PCBs) and brominated diphenyl ethers (BDEs), can persist in the environment for decades and trigger a suite of detrimental effects in vertebrates, including endocrine disruption, immunotoxicity, embryo mortality and behavioural impairments [5–7].

Although toxic effects of heavy metals and POPs have been well described at the individual level and under controlled laboratory conditions, their population-level effects have been virtually neglected in free-living vertebrates because of the

dearth of long-term datasets. In aquatic birds, there is no evidence of an impact of heavy metals on adult mortality [8,9]. In two *Catharacta* skua species, breeding failure in the following year, but not adult survival rate, was positively related to Hg exposure [10], as highlighted by the use of long-term datasets and multi-state mark–recapture models (MSMR [11]). Concerning POPs, very high concentrations of organochlorine compounds were related to increased mortality in the glaucous gull *Larus hyperboreus* [12], but their effects on long-term fecundity were not explored in that study.

The present study aimed to relate demographic parameters to pollutants in wandering albatrosses *Diomedea exulans*. The effects of Hg and Cd in red blood cells and  $\Sigma$  POPs in plasma on apparent probabilities of adult survival, return to the breeding colony, breeding, hatching and fledgling, were investigated by using a MSMR approach. According to recent studies on long-lived seabirds [10,12], a deleterious effect of Hg was expected on long-term breeding performances and a possible lethal effect of POPs was expected in the most contaminated wandering albatrosses. Then, population-level responses to an increase in Hg and POPs levels were assessed by establishing a life cycle for the wandering albatross and by including Hg- or POP-dependent demographic traits in the matrix population models [13].

Despite high pollution burdens in albatrosses [14–16], the effects of contaminants on demographic parameters and population growth rate are unknown. Albatrosses are among the most highly endangered of the world's birds, with 18 of 22 species considered as threatened and the remaining four species considered as near threatened [17]. In this population of wandering albatrosses, population growth rate remained relatively stable during the 1960s (about 850 breeding pairs), before a first decline between the early 1970s and 1986, and a second decline since 2003 down to 380 breeding pairs [18]. Causes of decline in albatross populations have been attributed to fishery bycatch, climate change and disease [19]. This study thus gives a new dimension to the overall picture of environmental threats in albatross populations.

## 2. Material and methods

### (a) Study area and species

The study was conducted at Ile de la Possession in the Crozet Archipelago (46° S, 52° E), Southern Indian Ocean, where 300–400 pairs of wandering albatrosses nest each year. Adults return to their breeding grounds in December and females lay a single egg in late December–early January. Both parents incubate alternately until hatching in March and most young are fledged in November. Up to 6% of the birds that fledged a chick bred again in the following year, and the wandering albatross is considered to be a quasi-biennial breeding species [20]. Approximately 80% of birds that failed to breed in the previous year engage in another breeding attempt in the following year. All wandering albatrosses had been ringed and sexed as part of a long-term mark–recapture programme [21]. In December, pre-breeding adults are controlled over the whole island. From mid-January to mid-February, at least three visits are carried out every 10 days at each nest to determine the identity and breeding status (egg laid/egg hatched) of each individual. In mid-April, June and August, all nests are checked to monitor the chicks' survival.

### (b) Blood sampling

From 21 December 2007 to 04 March 2008, 147 sexually mature adults (i.e. observed as incubating or chick-rearing at least once before or during the current breeding season) were captured.

A sample of venous blood was taken from the tarsus with a 1-ml heparinized syringe and a 25-gauge needle. Only one bird was sampled per nest. The volume of the blood draws never exceeded 0.05% of the bird's body mass (8–12 kg).

### (c) Laboratory analyses

Hg and Cd were analysed in red blood cells at the Littoral Environnement et Sociétés (LIENSs), La Rochelle, France. POPs (PCBs: CB-28, -52, -101, -118, -138, -153 and -180; organochlorine pesticides: HCB (hexachlorobenzene), Gamma HCH (hexachlorocyclohexane), Heptachlore, 2,4' DDE (dichlorodiphenyldichloroethylene), Cis-chlordane, trans-nonachlor, 4,4' DDD (dichlorodiphenyldichloroethane), 2,4' DDT (dichlorodiphenyltrichloroethane), 4,4' DDT, Mirex; and BDE-47) were analysed in plasma at the EPOC-LPTC, Bordeaux, France. Further details about analyses are reported in the electronic supplementary material.

### (d) Estimating the effects of blood heavy metals and persistent organic pollutants on breeding output during the year of sampling

Generalized linear models (GLMs) with binomial error distribution and a logit link function were used to test whether breeding success in 2007–2008 was linked to blood Hg, Cd or POP levels in individuals sampled as breeders in 2007–2008. Breeding success was coded as 1 for birds that successfully fledged a chick, and as 0 for those that failed at the egg or chick stage. Analyses were performed using R [22].

### (e) Estimating the effects of blood heavy metals and persistent organic pollutants on demographic parameters

The capture–recapture data of sampled individuals from 2008 to 2012 were used to evaluate the effects of blood Hg, Cd and POPs on demographic parameters.<sup>1</sup> A MSMR model was constructed, as developed by Pardo *et al.* [23,24], and included eight states: dead, failed breeder on egg (FBE, defined as an individual that was observed with one egg that failed to hatch), failed breeder on chick (FBC, defined as an individual that was observed with one chick but that failed to fledge the chick), successful breeder (SB, defined as an individual that fledged one chick), observable non-breeder (ONB, defined as an individual that was observed at the colony but that was not observed with an egg or a chick) and three unobservable states (UNB) consisting of non-breeders that were observed at the colony during the previous breeding attempt (PONB), non-breeders whose previous breeding attempt failed (PFB) and non-breeders whose previous breeding attempt was successful (PSB). The state coded as 'dead' (†) absorbed all those individuals that had either died or permanently emigrated from the study areas. The UNBs account for temporary absence, corresponding to birds that skip breeding in one year after breeding unsuccessfully or successfully during the previous year.

Models were parametrized in terms of the probability of survival ( $s$ ), the probability of returning to the colony given survival ( $r$ ), the probability of breeding given return to the colony ( $\beta$ ), the probability of successful hatching given breeding ( $\omega$ ), the probability of successful fledgling given hatching ( $\gamma$ ) and the detection probability ( $p$ ). Transition probabilities between states were thus modelled with a five-step procedure where  $s$ ,  $r$ ,  $\beta$ ,  $\omega$  and  $\gamma$  were considered as five successive steps in transition matrices. Parameters of the model are defined in the electronic

supplementary material. We chose a MSMR approach as this allows us to take into account the probability of detecting individuals given their return to the study site, as well as the

previous breeding state of individuals in order to obtain unbiased estimates of demographic parameters [11].

This MSMR model was parametrized by the survival-transition probabilities matrix

	FBE	FBC	SB	ONB	PFB	PSB	PONB	†
FBE	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	$s(1-r)$	---	---	*
FBC	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	$s(1-r)$	---	---	*
SB	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	$s(1-r)$	---	*
ONB	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	---	$s(1-r)$	*
PFB	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	---	---	*
PSB	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	---	---	*
PONB	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	---	---	*
†	$sr\beta(1-\omega)$	$sr\beta\omega(1-\gamma)$	$sr\beta\omega\gamma$	$sr(1-\beta)$	---	---	---	*

Several constraints were made to ensure that the parameters of the model were estimable. The state 'dead' being explicitly included in the model but never being encountered, initial state probability was fixed at 0, transition probabilities from the state 'dead' to the other states were fixed at 0 and capture probability was fixed at 0 [25,26]. The probability of seeing individuals in UNBs and transitions between UNBs was constrained to 0. Since some individuals were observed breeding in the year consecutive to a successful breeding event [20],  $\beta_{SB}$  was not constrained to 0. To limit redundancy in survival parameters, models where survival probabilities all varied separately were not considered [27]. Because of the limited number of individual capture histories, the limited number of recapture occasions and the relatively large number of UNBs, we constrained (i) all parameters to be constant over time, (ii)  $r$  to be identical for ONB, PFB, PSB and PONB and (iii)  $\beta$  to be identical for PFB, PSB and PONB. With these constraints, the initial model was full-rank. Note that the model where all demographic parameters were time- and state-dependent was highly rank deficient.

Once the best model structure was identified (Model 21, electronic supplementary material), effects of blood Hg, Cd and POPs were tested on demographic parameters to investigate whether contamination levels in one breeding season may influence the long-term survival and breeding outputs of an individual over the four following years. MSMR models were built where each demographic parameter  $\theta$  was modelled as a function of an individual covariate  $C$  (standardized level of Hg, Cd or sum of POPs (log-transformed)) using a logit link function:  $\text{logit}(\theta) = a + b \times C_i$ , where  $a$  is an intercept,  $b$  is a slope and  $C_i$  is the covariate for individual  $i$ . When  $b < 0$ , or  $b > 0$ ,  $C$  has a negative or positive effect on  $\theta$ , respectively.

The effect of  $C$  was first tested on each demographic parameter separately and for different states. Because some parameters were estimated as 100% [100–100%] (electronic supplementary material), we did not test the effects of  $C$  on the return probability of males previously observed as (FBE and FBC) or as (ONB, PFB, PSB and PONB), and on the breeding probability of unobservable non-breeders. The 95% confidence interval (CI) of the slope parameters was used, as well as Akaike's information criterion corrected (AICc) for small sample size [28] for inference. We considered a contaminant's effect to be statistically supported when 0 was outside the 95% CI of the mean of the slope of the relationship [29]. A composite model was then constructed by combining all the covariates that were detected to have an effect on demographic parameters. Composite models were constructed for heavy metals and POPs separately, as sample sizes differed. In composite models, an effect was not supported if the 95% CI of the slope parameter included 0 [29].

We tested the goodness-of-fit (GOF) of the time-dependent MSMR model using U-CARE [30]. All models were run under program E-SURGE 1.8.5 allowing splitting transition probabilities

between states [26]. To avoid estimating parameters at a local minimum of the likelihood function, each model was run five times with random initial values.

### (f) Modelling population dynamics

To evaluate the population-level effects of contaminants, we constructed population models using pre-breeding matrices [13] structured by age and reproductive status classes. We built a two-sex age- and stage-classified matrix population model [13], because Hg and POPs concentrations affected the demographic parameters of males and females differently (see §3). Based on a detailed demographic study on wandering albatrosses [31], the model consists of five juvenile age classes, one pre-breeder class and seven stage-classes according to the breeding status (SB, FBE, FBC, ONB, PSB, PFB and PONB). Parameters entering the model were the recruitment probability,  $s$  of adult males, females and juveniles,  $r$  of SB and other states,  $\beta$  of SB, FB, ONB and UNB for males and females separately,  $\omega$  and  $\gamma$  of breeders and non-breeders. We assumed a 1:1 sex ratio.

We first built a deterministic matrix model with no stochasticity, which included the mean estimates of the demographic parameters from our MSMR model results and from Barbraud *et al.* [31] for juvenile survival and recruitment probability. From these matrix analyses, we estimated the deterministic population growth rate [13]. We then focused on stochastic matrix models to estimate the stochastic growth rate. Environmental stochasticity was included in two different ways. When a vital rate had no significant relationship with levels of contaminant ( $C$ ), its yearly values were sampled from a beta distribution [32], with mean and variance equal to those estimated from the MSMR model selected. When a vital rate had a significant relationship with  $C$  levels, its value was modelled as  $\theta = \text{logit}^{-1}(a + b \times \bar{C})$ , where  $\bar{C}$  is the mean value of the contaminant levels for all individuals.  $C$  values were sampled from a log-normal distribution for all individuals sampled. The values of  $a$  and  $b$  were recalculated for non-standardized  $C$  values representing the absolute values for Hg and log-transformed values for  $\Sigma$  POPs. To assess the population-level effects of  $C$ , we estimated stochastic population growth rates according to changes in mean  $C$  levels within the range of observed  $C$  values. The matrix population models were analysed by Monte Carlo simulations (10 000 iterations) using package popbio [33] implemented in R [22].

## 3. Results

### (a) Effects of blood heavy metal and persistent organic pollutants on current breeding output

Blood levels of contaminants are given in the electronic supplementary material. When considering only breeders in 2008,

**Table 1.** Modelling the effects of standardized blood heavy metals levels (*a*, Hg; *b*, Cd; and *c*, composite model) on demographic parameters ( $N = 147$  individuals). The estimated slope and 95% CIs for models with a lower AICc than the intercept model (Hg0 and Cd0) are given.

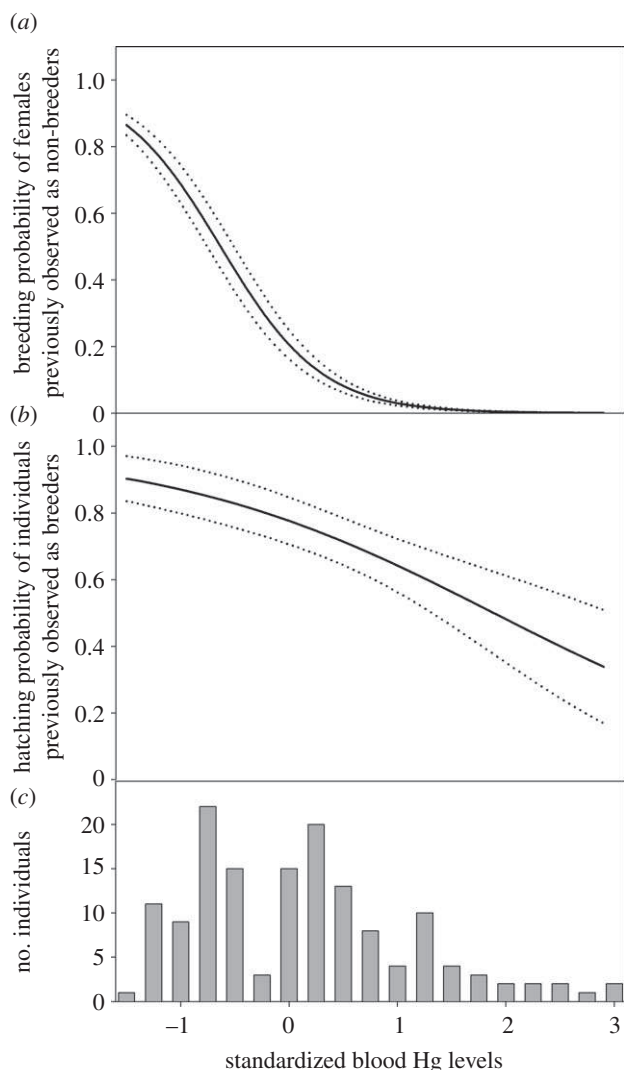
hypothesis	model	rank	deviance	$\Delta$ AICc	slope (CI-; CI+)
<i>(a) effect of blood Hg on demographic parameters</i>					
breeding probability of females previously in state ONB	Hg10	24	1442.026	0	-2.141 (-4.267; -0.014)
hatching probability of individuals previously in states FB or SB	Hg3	24	1444.431	2.405	-0.659 (-1.271; -0.047)
fledging probability of individuals previously in states FB or SB	Hg1	24	1444.560	2.533	-0.844 (-1.659; -0.029)
breeding probability of males previously in states FBE or FBC	Hg5	24	1446.133	4.107	1.569 (-0.307; 3.445)
intercept model: no effect	Hg0	23	1449.217	4.946	
breeding probability of females previously in states FBE or FBC	Hg6	24	1448.236	6.210	
breeding probability of females previously in state SB	Hg8	24	1448.881	6.854	
return probability of individuals previously in state SB	Hg12	24	1448.955	6.929	
hatching probability of individuals previously in states ONB or UNB	Hg4	24	1449.035	7.009	
breeding probability of males previously in state SB	Hg7	24	1449.159	7.133	
fledging probability of individuals previously in states ONB or UNB	Hg2	24	1449.208	7.182	
survival rate of females	Hg14	24	1449.209	7.183	
breeding probability of males previously in state ONB	Hg9	24	1449.215	7.189	
survival rate of males	Hg13	24	1449.216	7.190	
breeding probability of females previously in states PFB, PSB or PONB	Hg11	24	1449.217	7.191	
<i>(b) effect of blood Cd on demographic parameters</i>					
return probability of individuals previously in state SB	Cd12	24	1445.909	0	-0.350 (-0.758; 0.058)
hatching probability of individuals previously in states ONB or UNB	Cd4	24	1446.645	0.735	0.520 (-0.204; 1.243)
intercept model: no effect	Cd0	23	1449.217	1.063	
fledging probability of individuals previously in states ONB or UNB	Cd2	24	1447.314	1.405	
breeding probability of females previously in state ONB	Cd10	24	1447.905	1.996	
breeding probability of females previously in state SB	Cd8	24	1448.247	2.338	
breeding probability of females previously in states FBE or FBC	Cd6	24	1448.730	2.820	
hatching probability of individuals previously in states FB or SB	Cd3	24	1448.744	2.835	
survival rate of females	Cd14	24	1448.794	2.885	
survival rate of males	Cd13	24	1449.048	3.139	
probability of males previously in states FBE or FBC	Cd5	24	1449.054	3.145	
fledging probability of individuals previously in states FB or SB	Cd1	24	1449.129	3.220	
breeding probability of males previously in state SB	Cd7	24	1449.159	3.250	
breeding probability of males previously in state ONB	Cd9	24	1449.215	3.306	
breeding probability of females previously in states PFB, PSB or PONB	Cd11	24	1449.217	3.307	
<i>(c) effect of blood heavy metals on demographic parameters</i>					
composite model (Hg10, Hg3, Hg1)	Hg15	26	1433.319	0	(cf. S3(b))
intercept model: no effect	Hg0	23	1449.217	9.131	

current breeding success was not related to Hg (d.f. = 1,105,  $\chi^2 = 0.126$ ,  $p = 0.723$ ), Cd (d.f. = 1,105,  $\chi^2 = 0.008$ ,  $p = 0.929$ ) or  $\Sigma$  POPs concentrations (d.f. = 1,81,  $\chi^2 < 0.001$ ,  $p = 0.993$ ).

### (b) Demographic consequences of blood heavy metal levels

The GOF of the MSMR model was overall not significant (males:  $\chi^2 = 16.327$ , d.f. = 22,  $p = 0.799$  and females:  $\chi^2 = 7.078$ , d.f. = 16,  $p = 0.972$ ). The effects of sex and states on demographic parameters and the estimation of parameters are detailed in the electronic supplementary material.

Model selection and slope estimates suggested negative effects of Hg on the breeding probability of females previously in state ONB, and on hatching probability and fledging probability of individuals previously in states FB and SB (table 1*a*). There was no detectable effect of Cd on demographic parameters (table 1*b*). Slope estimates obtained from the composite model (table 1*c*) were -2.114 (95% CI: (-4.213; -0.015)), -0.620 (-1.234; -0.005) and -0.807 (-1.645; 0.032) for the effects of Hg on breeding probability of females previously in state ONB (figure 1*a,c*), hatching probability of individuals previously in states FB and SB (figure 1*b,c*) and fledging probability of individuals previously in states FB



**Figure 1.** Effect of standardized blood Hg levels on (a) breeding probability of females previously observable as non-breeders (ONB), and (b) hatching probability of individuals previously observed as breeders. Dotted lines represent 95% CIs estimated using the delta method [34]. Histograms represent the measured blood Hg levels (c).

and SB, respectively. The last effect was not supported, because 95% CI included 0 in the composite model.

### (c) Demographic consequences of blood $\Sigma$ POP levels

Model selection and slope estimates suggested a negative effect of  $\Sigma$  POPs on fledging probability of individuals previously in states ONB or UNB and on breeding probability of females previously in state ONB (table 2). Other models were not supported, because 95% CI of slope parameter values included 0 (table 2). Slope estimates obtained from the composite model were  $-0.976$  ( $-1.917$ ;  $-0.035$ ) and  $-0.812$  ( $-1.551$ ;  $-0.072$ ) for the effects of  $\Sigma$  POPs on breeding probability of females previously in state ONB (figure 2a,c) and on fledging probability of individuals previously in states ONB or UNB (figure 2b,c), respectively.

### (d) Modelling population dynamics

The deterministic population growth rate was 1.038, and the respective generation time was 23.8. The stochastic population growth rates were 1.008 when accounting for mean Hg effects from MSMR analyses, 1.002 when accounting for mean POPs

effects and 0.991 when accounting for both mean Hg and POPs effects (figure 3). A doubling in mean Hg concentration would decelerate the population growth rate of 0.68%. A doubling in mean  $\Sigma$  POPs concentration would decelerate the population growth rate of 0.11%. Doublings in mean Hg and  $\Sigma$  POPs concentrations would decelerate the population growth rate of 1.31% (figure 3).

## 4. Discussion

Using a unique long-term dataset and MSMR models, this study explores the demographic effects of both metallic and organic pollutants in a wild vertebrate population. Contaminant levels were associated with a lower breeding probability, a higher hatching failure and a higher fledgling failure, but not with adult survival rate in the wandering albatross. At the population level, a demographic decline was projected in response to increasing Hg and  $\Sigma$  POP levels.

### (a) Effects of contaminants on current breeding output

Contrary to previous studies [1,2,5,7], no negative effect of blood Hg, Cd and  $\Sigma$  POPs was detected on breeding success at the year of sampling. The lack of relationship is probably due to the sampling protocol, since blood sampling was mainly conducted during the incubating period. Some effect of contaminants on early nest desertion and skipped breeding [3] may have been missed.

### (b) Survival and contaminants

Estimated demographic parameters were similar to those previously estimated in the same population using all ringed individuals [20,24]. The survival rate of wandering albatrosses was not jeopardized by Hg, Cd and POPs. An effect of POPs was detected on survival rate in one of the most polluted seabirds, the glaucous gull breeding in the Norwegian Arctic, but only the most contaminated individuals had lower survival [12]. Concerning heavy metals, these findings corroborate previous studies that did not evidence an effect of Hg and Cd on adult mortality in birds [8–10]. However, our study did not exclude the possibility that contaminants could jeopardize the survival rate of immature wandering albatrosses, as they have a higher pollution burden [16] and a lower survival rate [31] than sexually mature adults.

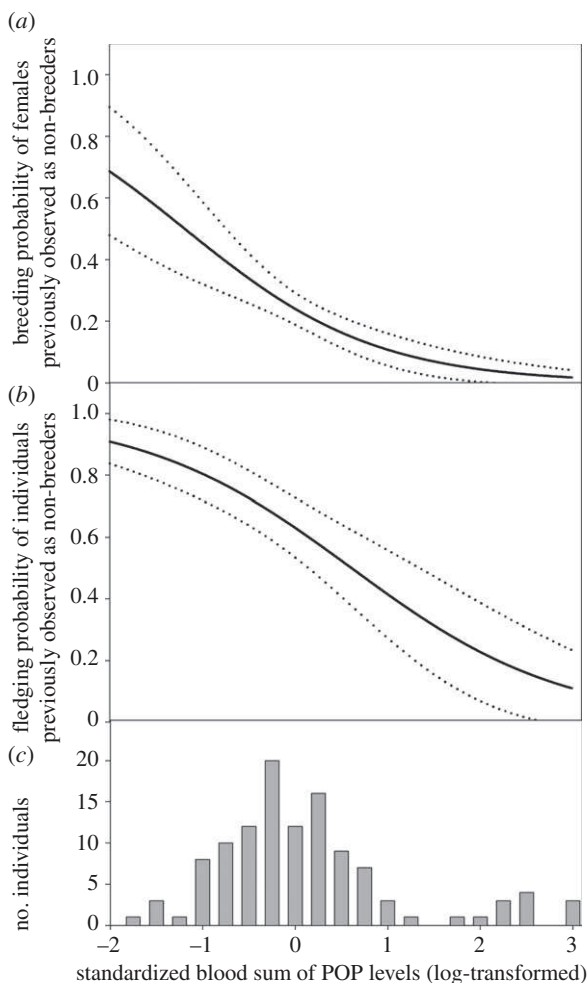
### (c) Long-term fecundity and heavy metals

A negative effect of blood Hg was detected on breeding probability of females observed as non-breeders. Concerning the proximate mechanisms, Hg, in its methylated form, is known to disrupt reproductive hormones [1] such as the luteinizing hormone, a key pituitary hormone for the onset of breeding [3].

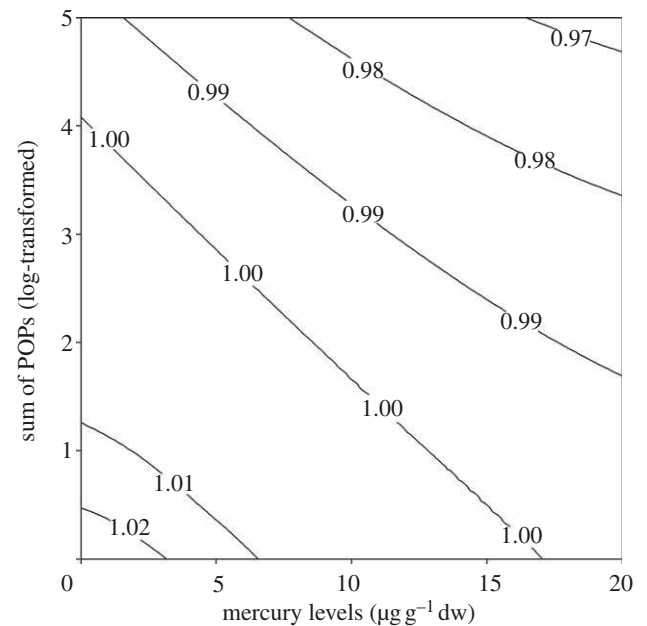
As found in two southern *Catharacta skua* species [10], Hg negatively impacted hatching probability of albatrosses, but only in individuals previously observed as breeders. Energetic and time-dependent costs of reproduction may have downstream consequences for reproductive investment during the following breeding season (carry-over effect [35]). Hence, Hg load may have exacerbated these carry-over effects in individuals that previously bred. Concerning the possible proximate mechanisms, Hg may have caused long-term endocrine disruption of the reproductive system and behavioural

**Table 2.** Modelling the effects of blood  $\Sigma$  POP levels (log-transformed and standardized) on demographic parameters ( $N = 115$  individuals). The estimated slope and 95% CIs for models with a lower AICc than the intercept model (POP0) are given.

hypothesis	model	rank	deviance	$\Delta$ AICc	slope (CI-; CI+)
breeding probability of females previously in state SB	POP8	24	1129.266	0	2.763 (-0.205; 5.731)
composite model (POP2, POP10)	POP15	25	1127.642	0.716	(cf. S3(c))
fledging probability of individuals previously in states ONB or UNB	POP2	24	1130.764	1.498	-0.878 (-1.641; -0.114)
hatching probability of individuals previously in states ONB or UNB	POP4	24	1131.111	1.845	0.841 (-0.036; 1.719)
fledging probability of individuals previously in states FB and SB	POP1	24	1131.312	2.046	1.226 (-0.075; 2.527)
hatching probability of individuals previously in states FB and SB	POP3	24	1131.434	2.168	1.006 (-0.116; 2.127)
breeding probability of females previously in state ONB	POP10	24	1131.826	2.560	-0.964 (-1.870; -0.058)
intercept model: no effect	POP0	23	1135.079	3.488	
breeding probability of females previously in states PFB, PSB or PONB	POP11	24	1133.513	4.247	
breeding probability of males previously in state SB	POP7	24	1133.847	4.581	
breeding probability of males previously in states FBE or FBC	POP5	24	1134.095	4.829	
survival rate of males	POP13	24	1134.313	5.047	
breeding probability of females previously in states FBE or FBC	POP6	24	1134.808	5.542	
return rate of individuals previously in state SB	POP12	24	1135.034	5.768	
survival rate of females	POP14	24	1135.035	5.769	
breeding probability of males previously in state ONB	POP9	24	1135.093	5.827	



**Figure 2.** Effect of standardized blood  $\Sigma$  POPs (log-transformed) on (a) breeding probability of females previously observed as non-breeders, and (b) fledging probability of individuals previously observed as non-breeders or unobserved. Dotted lines represent 95% CIs estimated using the delta method [34]. Histograms represent the measured blood  $\Sigma$  POP levels (c).



**Figure 3.** Isoclines of population growth rate in wandering albatrosses as projected with the population models, which included the responses to blood Hg levels and blood  $\Sigma$  POPs (log-transformed) within the range of observed Hg and  $\Sigma$  POP levels.

impairments [1,2]. In addition, the maternal transfer of Hg into the egg may have altered embryo development [36].

#### (d) Long-term fecundity and $\Sigma$ POPs

POP burdens reduced the long-term breeding probability of females previously observed as non-breeders and fledging probability of individuals that were previously non-breeders. Interestingly, POPs appeared to mostly affect albatrosses that skipped the preceding breeding attempt, suggesting a possible

segregation of foraging areas between breeders and non-breeders. Alternatively, non-breeding females may suffer from higher deleterious effects of POPs on long-term fecundity, because their loads of POPs were not eliminated through the egg.

During the incubating and chick-rearing periods, POPs may weaken the secretion of prolactin, a hormone closely involved in the mediation of parental care, as shown in glaucous gulls [37]. In turn, a durable Hg-induced attenuation of prolactin release may result in fledgling failure.

The link between contaminants and reduced fecundity could be a by-product of age-dependant mechanisms. However, in this population, fecundity declined in the oldest individuals (35+ years old [24,38]), while Hg levels tended to decrease with age [16] and POP levels were unrelated to age (data not shown). Moreover, age (6–48+ years old) did not affect humoral immunity, oxidative stress, antioxidant defences or hormone levels in wandering albatrosses [38]. Hence, it is unlikely that age was a confounding factor in the correlation between contaminants and physiological mechanisms underlying breeding performance.

### (e) Modelling population dynamics

Translating individual-level effects of contaminants to population-level processes is a crucial and ultimate goal of modern ecological research. Our population models suggested that the actual Hg and POPs levels could decelerate the population growth rate (0.991), whereas the population growth rate would increase (1.027) with zero concentrations of blood Hg and POPs. In addition, doublings in mean blood Hg and POPs levels would decelerate the growth rate of this wandering

albatross population by 1.31%. These predictions could undoubtedly be worsened by other anthropogenic perturbations. For instance, climate change can impact transport, distribution, bioavailability, bioaccumulation and effects of pollutants [39–41] and triggers steep population declines in albatrosses [19]. In that respect, a future avenue for ecotoxicological and conservation research could be dedicated to evaluating and predicting the coupled effects of climatic and chemical perturbations on wildlife population viability.

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### Endnote

<sup>1</sup>Dataset: demographic consequences of heavy metals and POPs in a vulnerable long-lived bird, the wandering albatross. Aurelie Goutte, Christophe Barbraud, Alizée Meillère, Alice Carravieri, Paco Bustamante, Pierre Labadie, Hélène Budzinski, Karine Delord, Yves Chérel, Henri Weimerskirch, Olivier Chastel. figshare (<http://dx.doi.org/10.6084/m9.figshare.947689>).

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