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Decreased prolactin levels reduce parental commitment, egg temperatures, and breeding success of incubating male Adélie penguins

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ABSTRACT

Hormones regulate many aspects of an individual's phenotype, including various physiological and behavioral traits. Two hormones have been described as important players in the regulation of parental investment in birds: the glucocorticoid hormone corticosterone and prolactin, a pituitary hormone, widely involved in mediating parental behavior. In comparison with corticosterone, the role of prolactin on parental investment remains poorly documented, and most studies so far have been correlative. In this study, the effects of an experimental decrease of prolactin levels on the incubation behavior of a long-lived seabird species were assessed. Male Adélie penguins were treated with self-degradable bromocriptine pellets, inhibiting prolactin secretion. Filming and subsequent video analysis allowed the determination of a behavioral time budget for birds and their position on the nest, while dummy eggs recorded incubation parameters. Incubation duration and breeding success at hatching were also monitored. As expected, bromocriptine-treatment significantly decreased plasma prolactin levels, but did not affect corticosterone levels. The behavioral time budget of penguins was not affected by the treatment. However, treated birds spent significantly more time in an upright position on the nest. These birds also incubated their eggs at lower temperatures and turned their eggs more frequently than controls, resulting in a lengthened incubation period. Despite this, the treatment was insufficient to trigger nest desertion and eggs of treated birds still hatched, indicating that several endocrine signals are required for the induction of nest abandonment. We suggest that the decreased prolactin levels in treated birds offset their timeline of breeding, so that birds displayed behavior typical of early incubation.

Introduction

Animals face trade-offs in terms of how energy and resources are allocated to different biological functions, including self-maintenance and reproduction (Stearns, 1992). Although previously disputed, it is now clear that the incubation period of avian reproduction is considerably energy demanding (Nord and Nilsson, 2012; Nord et al., 2010), despite the fact that parents remain relatively inactive during this period. The energetic stress that parents experience then is a consequence of the restricted foraging opportunities during incubation (Reid et al., 2002). Incubation costs are especially high for species breeding in inhospitable environments, such as polar regions, where eggs may lose heat rapidly (Piersma et al., 2003; Tinbergen and Williams, 2002). An emergency life history stage (ELHS) is expressed in breeding vertebrates when the immediate survival is threatened by poor energetic conditions (Wingfield et al., 1998). This ELHS shifts energy investment away from reproduction and redirects it towards immediate survival, which is especially true for long-lived species, which should behave as 'prudent parents' (Drent and Daan, 1980). Accordingly, incubation may be prematurely terminated, when energy reserves reach a critical point of exhaustion (Ainley et al., 1983; Aldrich and Raveling, 1983; Groscolas et al., 2000, 2008; Spée et al., 2010) or when environmental conditions are severe (Astheimer et al., 1995; Conway and Martin, 2000; Davis and McCaffrey, 1986).

Activation of the ELHS is known to be mediated by several endocrine mechanisms (Wingfield, 2003; Wingfield and Kitaysky, 2002; Wingfield et al., 1998). The reduction or suppression of parental effort is associated with the activation of the hypothalamo–pituitary–adrenal axis and the resulting secretion of glucocorticoids (reviewed by Landys et al., 2006). In birds, the ELHS is activated by a release of the stress hormone corticosterone. For instance, elevated corticosterone levels have been reported to reduce brood provisioning and even trigger nest desertion in birds (Love et al., 2004; Silverin, 1986; Spée et al., 2011; Wingfield and Kitaysky, 2002). Exogenous corticosterone triggered nest desertion in 60% of treated incubating Adélie penguins *Pygoscelis*

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adeliae (Spée et al., 2011; Thierry et al., 2013), indicating that this hormone plays a major role in regulating the outcome of the trade-off between reproduction and survival, but is not necessarily sufficient on its own. Glucocorticoids not only play a major role in mediating behavioral and physiological responses to stress, but are also vital in the maintenance of a daily homeostatic balance (Sapolsky, 2000). Indeed, elevated baseline corticosterone levels have been linked with increased fitness (Bonier et al., 2009; Wingfield and Sapolsky, 2003). For example, female macaroni penguins *Eudyptes chrysolophus* with experimentally elevated corticosterone levels increased their foraging activity with positive consequences in terms of reproductive output (Crossin et al., 2012). How elevated corticosterone levels affect the expression of parental care and may trigger nest abandonment remains to be clarified (Angelier et al., 2009; Wingfield and Kitaysky, 2002).

One popular hypothesis is that corticosterone affects other endocrine mechanisms that mediate the expression of parental care. In particular, exogenous corticosterone has been shown to decrease circulating prolactin levels (Angelier et al., 2009; Buyse et al., 1987; Criscuolo et al., 2005). Several studies suggest that the suppressive effects of corticosterone on parental investment may be mediated through a negative effect on prolactin levels (Angelier and Chastel, 2009; Angelier et al., 2009). However, studies on the effects of a decrease in prolactin levels alone, i.e. without any concomitant changes in corticosterone concentrations, are rare, precluding conclusions about the respective role of these two hormones in the regulation of parental care. The pituitary hormone prolactin has been widely described as being involved in the initiation and the maintenance of avian parental (reviewed in Buntin et al., 1996; Sockman et al., 2006) and alloparental behavior (Angelier et al., 2006). Prolactin has been indicated to play an important role in incubation behavior (egg defense and thermoregulation) in birds (Buntin et al., 1996; El Halawani et al., 1986; Lormée et al., 1999). Yet, the role of prolactin on the onset and maintenance of incubation behavior seems to mostly rely on the broad temporal relationship between changes in prolactin levels and the reproductive cycle (reviewed in Williams, 2012), with little experimental evidence of a causal link between hormone levels and the onset of incubation (Sockman et al., 2006).

Most of the experimental work investigating cause and effect relationships between prolactin and parental care in breeding birds has been conducted with domesticated species. Motivated by economic interests, these correlative and experimental studies have focused on the understanding of how egg production can be increased and broodiness inhibited (Riddle et al., 1935; Sharp, 1997; Sharp et al., 1988). However, the role of prolactin in these domesticated species might differ from that in many other bird species. In fact, galliform birds show little parental care, and domesticated species have been artificially selected for different parameters, in particular reduced broodiness (Sossinka, 1982). Studies in free-living birds have mostly been correlative, describing patterns of prolactin secretion and their putative relationship with parental behavior (e.g., Garcia et al., 1996; Lormée et al., 2000; Setiawan et al., 2006). From these studies it is clear that prolactin secretion increases steadily during egg laying and incubation. Its highest level is usually reached during incubation, before it declines again, either immediately following hatching in precocial species, or at the end of the brooding period in altricial species (Vleck, 1998). In penguins, prolactin secretion is endogenously programmed and poorly influenced by external stimuli (Garcia et al., 1996; Lormée et al., 1999; Vleck et al., 2000). In these birds, prolactin levels increase during courtship, peak in mid-incubation, and remain elevated until the end of chick brooding (Vleck et al., 1999). A decrease in prolactin levels, accompanied by elevated corticosterone levels, has been reported in king penguins *Aptenodytes patagonicus* and in Adélie penguins that deserted their nest when energy stores were extremely depleted (Cherel et al., 1994; Groscolas et al., 2008; Spée et al., 2010). Such a drop in prolactin levels might decrease the incubation drive and favor nest abandonment. Besides, prolactin levels were not correlated with corticosterone levels, foraging activity, and parental effort in macaroni penguins (Crossin et al., 2012).

Further studies are required to better assess the role of prolactin alone in nest desertion and incubation behavior. Indeed, the extent to which prolactin determines parental investment during incubation remains unclear and needs further investigation in free-living bird species. Experimental studies allow establishing cause and effect relationships between prolactin and parental behavior. However, studies on the effects of a modulation of prolactin levels alone on the breeding behavior of wild birds are still rare. Seabirds, and notably penguins, are ideal species to address these issues. Hormonal profiles during incubation, including prolactin levels, are well established for these species (Vleck et al., 1999). Furthermore, corticosterone-induced effects on parental behavior have been examined recently in incubating male Adélie penguins (Spée et al., 2011; Thierry et al., 2013).

In this study, we evaluated the effects of a decrease in prolactin levels alone on parental investment during incubation, using self-degradable bromocriptine pellets in male Adélie penguins. Because the effects of bromocriptine-treatment on glucocorticoid levels have not been well described (Curlewis et al., 1988; Kan et al., 2003), especially in birds, prolactin and corticosterone levels were both measured in treated birds and controls. Incubation behavior of both groups was monitored by several means: (1) dummy eggs were used to record incubation temperatures and egg rotation rates, while (2) filming and subsequent video analysis allowed assessing the detailed behavioral time budget, as well as the position of birds on the nest. Direct observation of incubation routine allowed monitoring incubation duration and hatching success. We predicted that birds with low prolactin levels would be less committed to incubate than controls. Accordingly, these birds should be less attentive to the nest, which would potentially manifest itself in decreased vigilance and a decreased frequency of nest rearrangements. Consequently, we expected that egg temperatures, egg rotations rates, and hatching success in this group would be decreased.

Materials and methods

This study was conducted at the French station Dumont d'Urville (66°40′S, 140°01′E), Terre Adélie, Antarctica, during the 2008–2009 and 2009–2010 austral summers. The study was approved by the ethical committee of the French Polar Institute Paul-Emile Victor and authorized by the French Southern and Antarctic Lands (Terres Australes et Antarctiques Françaises).

Study species

Adélie penguins are long-lived seabirds, which breed during the short austral summer in colonies around Antarctica from late October to early March. After arriving at the colony in spring, male Adélie penguins fast for five to six weeks, while they establish territories, proceed through courtship, and then incubate for about two weeks. Females usually depart to sea to refeed as soon as they complete their clutch by laying the second egg (Sladen, 1958), after a fast of two to three weeks. The incubation period for an Adélie penguin egg ranges between 30 and 39 days (Ainley, 2002), with mates alternating between foraging trips at sea and incubation bouts on land (Fig. 1).

While studies in the relationships between hormones and parental care in both sexes are of importance, previous studies on the role of corticosterone in incubation behavior and nest desertion have been mainly conducted on male Adélie penguin due to their extended fasting period (Spée et al., 2011; Thierry et al., 2013). Thus, only male Adélie penguins were considered in our study in order to get comparable data sets.

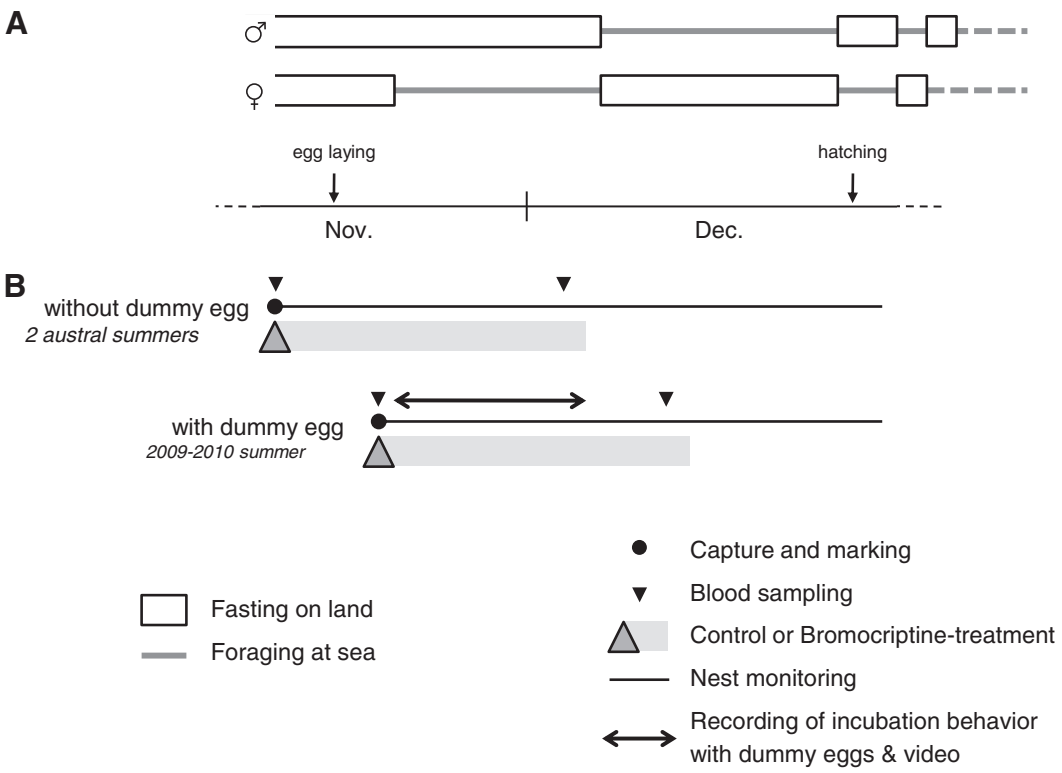

Fig. 1. Schematic view of nest attendance during the incubation period (A) and study protocol (B). Male Adélie penguins were separated into four groups depending on treatment (control or bromocriptine-treated) and whether their clutch was replaced by one dummy egg to record incubation behavior or not.

Sampling procedure and monitoring

A total of 58 Adélie penguin pairs were studied from courtship through to hatching (22 in 2008–2009 and 36 in 2009–2010). Males were first handled at the end of the courtship period, between pair formation and egg-laying (November 8 to November 23). Sex was determined by a combination of parameters, including copulatory position, cloacal inspection, and observation of the incubation routine (Sladen, 1958). To minimize handling stress, a bird's head was covered with a hood (Cockrem et al., 2008). A blood sample was collected from the alar vein (2 mL syringe, 25-gauge needle) within 3 to 5 min of capture (to obtain baseline corticosterone levels, as recommended by Romero and Reed, 2005). Handling durations not exceeding 5 min have been demonstrated to have no effect on baseline corticosterone levels in Adélie penguins (Vleck et al., 2000). In a few birds, handling time exceeded the one recommended to assess baseline corticosterone levels. Consequently, samples from these birds were not assayed for hormone levels. All blood samples were transferred into pre-treated tubes (25 μL Na-heparin or EDTA) and centrifuged at 4 °C for 10 min (5000 rpm). Aliquots of plasma were kept at −20 °C until further analyses.

Birds were then weighed with an electronic balance (Ohaus, Giessen, Germany, resolution: ±2 g), and wing length was measured to the nearest mm to calculate a scaled mass index (Peig and Green, 2009). To allow identification, penguins were individually marked using black dye (Nyanzol-D) on their chest feathers and Tesa tape that was inserted between their back feathers, before they were released on their nest.

Nests were checked from a distance of 10 to 30 m every 2 to 3 h between 6 am and 1 am for the entire study period. Adélie penguins were monitored during courtship and incubation period, from mid-November until the end of December. Detailed incubation routine data were acquired by observing which partner was present on the nest.

42 out of 58 males were recaptured, blood sampled, and weighed before leaving the colony, either during nest relief by their partner or following nest desertion. A blood sample was collected to assess prolactin and corticosterone levels at the end of the incubation shift, and treated as reported above.

Experimental manipulation of prolactin levels

Breeding penguins

During initial handling, 30 males (11 in 2008–2009 and 19 in 2009–2010) received a bromocriptine mesylate pellet (C-231, 25 mg, 21-day release, 6.4 mm diameter) purchased from Innovative Research of America (Sarasota, FL, USA). Bromocriptine is a dopamine agonist, and has been successfully used in birds to reduce plasma prolactin levels (Angelier et al., 2006; Reddy et al., 2006). To insert the pellet subcutaneously in the nape of the neck, the skin was disinfected with 70% alcohol and a small incision was made, using a scalpel blade. After positioning the pellet subcutaneously with tweezers (disinfected with povidone-iodine), the incision was closed with sterile suture and a topical application of Alumisol® spray (CEVA, Libourne, France) was given. Handling time averaged 18 min (range: 12–24 min). During initial handling, 28 other males (11 in 2008–2009 and 17 in 2009–2010) underwent the same procedure including skin incision and positioning with tweezers, without actual implantation of a pellet (Table 1, Fig. 1).

Captive penguins

To confirm the efficacy of the bromocriptine-treatment to reduce prolactin levels, and to assess the kinetics of prolactin secretion over the course of the treatment, a further 21 birds were captured and kept in a pen (5.2 × 2.4 m) during the 2008–2009 and 2009–2010 austral summers. These captive birds were failed breeders because of predation and/or bad weather conditions, and their sex could not be determined in the field. The number of birds in the pen at any time never exceeded eight individuals. After a few days in captivity, birds were captured in

Table 1
Number of birds in each group depending on treatment (control vs. bromocriptine-treated), year (2008–2009 vs. 2009–2010) and the presence or absence of a dummy egg in the nest for the different parameters measured in the study.

		Without a dummy egg				With a dummy egg	
		Control		Treated		Control	Treated
		2008–2009	2009–2010	2008–2009	2009–2010	2009–2010	2009–2010
Breeding parameters	Body mass and scaled mass index at implantation, female departure date, no. of eggs per nest	11	11	11	11	6	8
	Laying date	11	11	8	11	0	0
	No. of chicks at hatching	11	11	11	11	0	0
	Incubation duration	11	10	8	7	0	0
Hormone levels	Pre-treatment prolactin levels	11	10	11	11	5	8
	Prolactin levels at departure	5	10	7	9	5	6
	Pre-treatment corticosterone levels	5	9	6	8	5	6
	Corticosterone levels at departure	5	9	6	8	5	6

the pen, blood sampled, and implanted as mentioned above with a bromocriptine pellet (treated birds, n = 15) or no pellet (control birds, n = 6). All captive birds were weighed every two days. Blood samples were collected at different times during a bird's captive period: on the day of implantation (day 0; before implantation), several days after implantation, and on the last day of captivity (days 7–17, depending on the initial body mass and daily body mass loss of individuals). All captive penguins were released on the edge of the breeding colony (and, thus, close to sea, which is free of sea ice at this period of the year), with a body mass average of 3.5 kg. For comparison, body mass in free-living breeding Adélie penguins can be as low as 3.2–3.3 kg in males and 2.8 kg in females (Spée et al., 2010).

Incubation

Nest success and incubation duration (Birds without a dummy egg)

During nest monitoring, the number of eggs and/or chicks was observed from the periphery of the colony at least once daily to determine laying and hatching dates, and nest success at hatching. Nest success was defined as the percentage of successful nests at hatching. Incubation duration was calculated as the difference between the hatching date of the first chick and the laying date of the first egg.

Incubation behavior (birds with a dummy egg)

During the 2009–2010 austral summer, the clutches of 14 males were replaced by dummy eggs containing several temperature probes and an accelerometer, to register key parameters of incubation behavior, i.e. incubation temperature and egg rotation rate. Eight of these males also received a bromocriptine pellet (see above for details). Dummy eggs, produced at the IPHC-DEPE (Strasbourg, France), were of the same size and shape as genuine penguin eggs, and were easily accepted and incubated by penguins (Beaulieu et al., 2010; Thierry et al., 2013). Clutch replacement occurred at the same incubation stage for all birds, between zero and two days following female departure. Egg temperature and egg position were recorded every 10 s by seven temperature probes (six external probes, located equidistantly around the mid-shell, and one internal probe) and a bi-axial accelerometer, respectively. Further technical details regarding the dummy eggs are provided in Beaulieu et al. (2010) and Thierry et al. (2013). The two real eggs of each clutch were replaced by one dummy egg between November 20 and 24 (mean: November 22). The real penguin eggs were marked and placed in surrounding nests for incubation. They were returned to the original nest at the end of the first male incubation shift, i.e. between November 30 and December 11 (mean: December 3). Two dummy eggs were predated by South Polar skuas (*Catharacta maccormicki*) on November 21 and 22, soon after the beginning of the recording. Fortunately, the eggs were found close to the colony and data were successfully downloaded.

To obtain detailed behavioral data during incubation, five control and six treated penguins with a dummy egg were filmed in the afternoons (between 1:00 pm and 8:00 pm) from November 22 to December 1 for a total recording of ~48 h. Camera's field of view allowed filming two to four birds at once. Video sessions of 30 min each were chosen randomly and analyzed using an ethogram recognizing the following behaviors: rest, vigilance, comfort, social positive interactions, agonistic interactions, and nest rearrangement and egg turning. Behaviors were defined as follows: during *rest*, birds were sleeping or otherwise inactive for at least 5 s, with birds either lying down or standing up, head still. *Vigilance* consisted of alert movements, with bird watching other birds or the surroundings, head moving for at least 5 s. *Comfort* corresponded to stretching, preening, head-shaking, ruffle-shake, swallowing, and yawning (McKinney, 1965). *Social positive interactions* included ecstatic display, bill-to-axilla display, bowing, loud and quiet mutual display (Marchant et al., 1990). *Agonistic interactions* included threat displays (direct, fixed one-sided and alternate stare, crouch), as well as fighting, charge and pecks. *Nest rearrangement and egg turning* encompassed nest-scraping, stone rearrangement, and clutch movements, with beak, body and/or feet.

Hormone assays

Hormone concentrations were measured as previously reported by Spée et al. (2010). All samples were analyzed in duplicates, except for prolactin levels of captive birds. Plasma prolactin levels were determined by a heterologous radioimmunoassay (RIA) at the Centre d'Etudes Biologiques de Chizé (CEBC, France). This prolactin assay has previously been validated for this species by the CEBC, as previously reported (see Spée et al., 2010, 2011). Intra and inter-assay variations were 5% and 13%, respectively. The minimal detectable level of prolactin was 0.43 ng/mL. Plasma corticosterone levels were determined by a quantitative competitive sandwich enzyme immunoassay technique (AssayMax Corticosterone ELISA Kit, EC3001-1, AssayPro, St. Charles, MO, USA). The corticosterone assay was run in two assays at the IPHC-DEPE according to guidelines provided by the manufacturer. Before assaying, plasma samples were diluted 1:4 into EIA diluent. Intra- and inter-assay variations for corticosterone were 9% and 15%, respectively. The minimal detectable level of corticosterone was 0.06 ng/mL.

Data analysis

Incubation behavior data recorded with the dummy eggs were analyzed using R 2.13.2 (R. Development Core Team, 2011) and Igor Pro Software (Wavemetrics, Lake Oswego, OR, USA). Data recorded during the first 3 h of deployment were discarded to remove potential differences in initial dummy egg warming between birds. Maximum egg temperature, recorded by one of the six external probes, was

calculated by selecting the sensor that recorded the highest temperature for each measurement (every 10 s) and was averaged daily (from 0 to 24 h). In the following, 'egg temperature' refers to the mean value of the maximum temperature recorded by the six external probes. Hourly maxima of maximum temperatures (i.e. the single highest value during 1 h) were averaged daily and served as an estimate for the temperature of the brood patch, hereafter called 'maximum egg temperature'. Rotation events were counted to compare egg rotation rates between treated birds and controls (see Thierry et al. (2013) for a detailed description of the calculations regarding egg temperature and rotation rate).

Statistical analysis

All statistical analyses were performed with R 2.13.2. Results are expressed as means ± SE. Differences were considered as statistically significant when $p < 0.05$. The number of birds in each group according to the different study parameters is described in Table 1.

Birds without a dummy egg

Differences in body mass and scaled body mass at the time of implantation between birds whose clutch was not replaced with a dummy egg were assessed using a two-way ANOVA with treatment, year, and interaction between treatment and year as factors after testing for normality and homogeneity of variances using the Shapiro–Wilk and Bartlett tests, respectively. Laying date, female departure date, and the number of eggs per nest before the treatment were compared with generalized linear models (GLM) with a quasi-poisson distribution with treatment, year, and the interaction between treatment and year as factors.

ANOVAs were also used to compare prolactin and corticosterone levels between control and bromocriptine-treated birds at the time of implantation (pre-treatment) and at the end of the male incubation shift (post-treatment). Corticosterone levels were log-transformed to meet the assumptions of normality. The differences in the change in hormone levels between implantation and departure were assessed with paired *t*-tests.

The proportion of successful nests was compared with Fisher's exact tests. Differences in incubation duration and in the number of chicks at hatching between control and treated birds were assessed using a GLM with a quasi-poisson distribution, with treatment and year as factors.

Birds with a dummy egg

Due to the small sample size (6 controls and 8 treated birds), Wilcoxon tests were used to compare the different bird characteristics, including hormone levels, between groups for the subset of birds whose clutch was replaced with a dummy egg.

Generalized estimating equations (GEEs, *geeglm* function of the *geepack* package in R) were used to compare incubation behavior data between groups, taking into account the spatial and temporal correlation structure of the data (Hardin and Hilbe, 2007; Liang and Zeger, 1986). An autoregressive (AR[1]) correlation structure was assumed to estimate regression coefficient variances more appropriately, as consecutive measures of incubation temperatures for each bird lacked independence. The *geeglm* function was used to compare incubation temperatures, rotation rates, behavioral time budget, and position on the nest between treated birds and controls, with repetition on penguin identity. Models comparing incubation temperatures and rotation rates had treatment, day since implantation, and interaction between treatment and day since implantation as fixed factors. Models comparing behavioral time budget and position on the nest only included treatment as a fixed factor.

Spearman correlation coefficients between hormone levels at departure and mean egg temperature or the number of egg rotations during the 24 h prior to the blood sample were calculated to assess the relationship between hormone levels and incubation behavior.

Birds without vs. birds with a dummy egg

Initial handling dates between birds without vs. with a dummy egg were compared using a GLM with a quasi-poisson distribution.

Captive penguins

GEEs were used to assess the effect of the treatment on prolactin levels over the course of the captive period, with repetition on penguin identity, treatment, day since implantation, and interaction between treatment and day since implantation as fixed factors.

Results

Pre-treatment bird characteristics

Birds without a dummy egg

Control and treated birds, whose clutch was not replaced by a dummy egg, had a similar body mass and scaled mass index (Table 2, ANOVA, $F_{1,40} = 0.00$, $p = 0.978$ and $F_{1,40} = 0.22$, $p = 0.638$, respectively). Year and the interaction between treatment and year had no effect on body mass (year: $F_{1,40} = 0.50$, $p = 0.486$, treatment × year: $F_{1,40} = 0.29$, $p = 0.593$) and scaled mass index (year: $F_{1,40} = 1.65$, $p = 0.206$, treatment × year: $F_{1,40} = 0.78$, $p = 0.382$). There was no difference in laying date between groups (GLM with a quasi-poisson distribution, treatment: $t = 0.08$, $p = 0.937$; year: $t = 1.42$, $p = 0.164$; treatment × year: $t = -1.32$, $p = 0.194$), as well as in female departure date (treatment: $t = -0.91$, $p = 0.366$; year: $t = 0.27$, $p = 0.788$; treatment × year: $t = -0.59$, $p = 0.559$). Finally, the number of eggs per nest did also not differ significantly between control and treated birds and between years (treatment: $t = -0.56$, $p = 0.578$; year: $t = -0.56$, $p = 0.578$; treatment × year: $t = -0.02$, $p = 0.985$).

Birds with a dummy egg

For birds whose clutch was replaced by a dummy egg, there were no statistical differences in body mass, body condition, female departure date, and number of eggs per nest between groups (Table 2, Wilcoxon test, body mass: $W = 19$, $p = 0.573$; scaled mass index: $W = 19$, $p = 0.573$; female departure date: $W = 24$, $p = 1$). All females from this group laid two eggs.

Birds without vs. birds with a dummy egg

Initial handling dates were later for birds whose clutch was replaced by a dummy egg (GLM with a quasi-poisson distribution, $t = 11.40$, $p < 0.001$), regardless of the bromocriptine-treatment ($t = 0.56$, $p = 0.579$) and the interaction between the two factors ($t = -1.19$, $p = 0.851$). Consequently, body mass and scaled mass index at implantation was lower for these birds when compared with birds without a dummy egg (ANOVA, $F_{1,54} = 11.26$, $p = 0.001$; $F_{1,54} = 5.85$, $p = 0.019$, respectively), regardless of the bromocriptine-treatment ($F_{1,54} = 0.00$, $p = 0.958$; $F_{1,54} = 0.01$, $p = 0.943$) and the interaction between the two factors ($F_{1,54} = 0.16$, $p = 0.687$; $F_{1,54} = 1.10$, $p = 0.300$). However, after correcting body mass of these birds for a daily mass loss of 50 g per day (Spée et al., 2010), the estimated body mass on November 12 did not differ between both groups ($F_{1,54} = 0.04$, $p = 0.833$; scaled mass index: $F_{1,54} = 0.71$, $p = 0.402$).

Effect of bromocriptine on hormone levels

Free-living penguins without a dummy egg

Before the treatment, prolactin levels were different between groups of birds whose clutch was not replaced by a dummy egg (ANOVA, treatment: $F_{1,39} = 7.41$, $p = 0.010$; year: $F_{1,39} = 3.51$, $p = 0.069$; treatment × year: $F_{1,39} = 8.34$, $p = 0.006$). Prolactin levels were still significantly different between groups at departure from the colony

Table 2
Study design and breeding parameters for 58 male Adélie penguins. Birds were separated into four groups, depending on treatment (control vs. bromocriptine-treated) and the presence or absence of a dummy egg in the nest.

	Without a dummy egg		With a dummy egg	
	Control (n = 22)	Treated (n = 22)	Control (n = 6)	Treated (n = 8)
Date of implantation	Nov. 12 ± 0.44	Nov. 12 ± 0.29	Nov. 21 ± 0.60	Nov. 21 ± 0.50
Male body mass at implantation (kg)	5.09 ± 0.09	5.09 ± 0.11	4.53 ± 0.23	4.66 ± 0.16
Laying date	Nov. 17 ± 0.66	Nov. 16 ± 0.52	NA	NA
Female departure date[a]	Nov. 21 ± 0.51	Nov. 20 ± 0.47	Nov. 21 ± 0.79	Nov. 22 ± 0.64
No. of eggs per nest	1.95 ± 0.21	1.86 ± 0.47	2.00 ± 0.00	2.00 ± 0.00

Results are expressed as means ± SE.
[a] Female departure date can be used as a proxy of laying date (Ainley, 2002; Sladen, 1958).

(treatment: $F_{1,27} = 17.27$, $p < 0.001$; year: $F_{1,27} = 0.99$, $p = 0.329$; treatment × year: $F_{1,39} = 0.07$, $p = 0.791$). The overall change in prolactin levels between implantation and departure was significantly different between groups ($F_{1,26} = 24.85$, $p < 0.001$; year: $F_{1,27} = 0.07$, $p = 0.797$; treatment × year: $F_{1,39} = 0.02$, $p = 0.897$). In control birds, prolactin levels significantly increased between initial handling and departure from the colony, which occurred on average 19.5 days after implantation (paired *t*-test, $t = -5.29$, $p < 0.001$). By contrast, in treated birds prolactin levels tended to decrease between these two stages ($t = 2.02$, $p = 0.061$).

Corticosterone levels were not significantly different between groups before the treatment (ANOVA, log-transformed for normality, treatment: $F_{1,24} = 3.18$, $p = 0.087$; year: $F_{1,24} = 0.05$, $p = 0.827$; treatment × year: $F_{1,24} = 0.00$, $p = 0.977$) and at departure (treatment: $F_{1,24} = 1.51$, $p = 0.231$; year: $F_{1,24} = 2.85$, $p = 0.104$; treatment × year: $F_{1,24} = 0.86$, $p = 0.363$). The change in corticosterone levels was not affected by the treatment, the year, nor the interaction between treatment and year (treatment: $F_{1,24} = 0.27$, $p = 0.610$; year: $F_{1,24} = 1.66$, $p = 0.209$; treatment × year: $F_{1,24} = 0.42$, $p = 0.521$).

Free-living penguins with a dummy egg

Prolactin and corticosterone levels before the treatment were similar in birds whose clutch was replaced with a dummy egg (prolactin: 54.8 ± 9.5 ng/mL for controls vs. 57.9 ± 5.2 for treated birds, $W = 20$, $p = 1$; corticosterone: 7.3 ± 3.7 vs. 9.8 ± 7.5, respectively, $W = 16$, $p = 0.931$). At departure, prolactin levels were decreased in treated birds compared to controls (63.7 ± 6.1 vs. 41.0 ± 7.3, respectively, $W = 27$, $p = 0.030$), while corticosterone levels were not affected by the treatment (17.4 ± 4.0 vs. 12.5 ± 2.7, respectively, $W = 20$, $p = 0.429$). The change in prolactin levels between implantation and departure was 0.51 ± 0.57 in controls vs. −1.36 ± 0.89 ng/mL in treated birds and did not differ significantly between the two groups ($W = 23$, $p = 0.178$, 5 controls and 6 treated birds). The change in corticosterone levels between the two sampling dates was similar between the two groups ($W = 17$, $p = 0.792$).

Captive penguins

Pre-treatment prolactin levels in captive penguins did not differ significantly between control and treated birds (Fig. 3; 26.7 ± 4.5 ng/mL and 32.6 ± 2.4, respectively; $W = 33$, $p = 0.381$). However, pre-treatment prolactin levels of captive birds were significantly lower than those of free-living penguins (Figs. 2–3; ANOVA, treatment: $F_{1,58} = 3.10$, $p = 0.084$; reproductive status: $F_{1,58} = 27.58$, $p < 0.001$; treatment × reproductive status $F_{1,58} = 0.47$, $p = 0.496$). The significantly decreased prolactin levels in treated birds (GEE, Wald = 26.3, $p < 0.001$) and the significant interaction between treatment and day (Wald = 3.92, $p = 0.048$) illustrate the negative effect of the bromocriptine treatment on prolactin levels, with prolactin levels at a minimum two days after implantation. Number of days since implantation had no effect on prolactin levels (Wald = 0.08, $p = 0.784$).

Incubation behavior (birds with a dummy egg)

The behavioral time budget did not differ significantly between treated birds and controls (Fig. 4A, GEE, rest: Wald = 1.64, $p = 0.20$; vigilance: Wald = 1.9, $p = 0.17$; nest rearrangement and egg turning: Wald = 0.12, $p = 0.73$; comfort: Wald = 0.02, $p = 0.89$; agonistic interactions: Wald = 1.01, $p = 0.31$; positive social interactions: Wald = 3.39, $p = 0.065$). However, treated birds spent a significantly greater proportion of time in the upright position on the nest rather than controls, while the latter spent most of their time on the nest in a prone position (Fig. 4B, GEE, Wald = 9.64, $p = 0.002$). Two treated birds (out of eight) had their dummy eggs predated by a skua, while all six control birds incubated their dummy eggs for the entire incubation bout.

Egg temperatures of treated penguins were significantly lower than those of control birds, differing on average by 7.1 °C (Fig. 5A, Table 3, $p < 0.001$). Maximum egg temperatures were also significantly lower in treated birds (by 5.2 °C), when compared with controls ($p < 0.001$). However, there was a significant increase in the egg temperature of treated birds over the course of the treatment (Fig. 5B, $p = 0.017$), while egg temperature of controls remained stable. By contrast, egg rotation rates were significantly higher in treated birds, when compared with controls (Fig. 5D, $p < 0.001$). Egg rotation rates in treated birds also changed significantly over the course of the treatment period (Fig. 5E, $p < 0.001$). However, while egg temperatures increased over time, egg rotation rates declined.

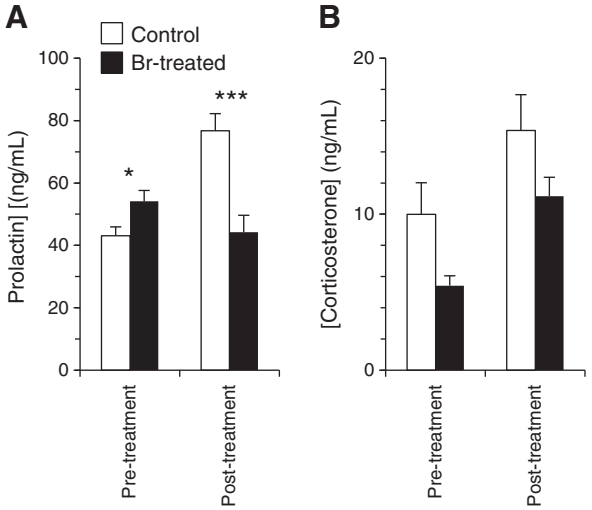

Fig. 2. Effects of bromocriptine-treatment on prolactin (A) and corticosterone (B) levels in male Adélie penguins whose clutch was not replaced with a dummy egg. Hormone levels of control and treated birds (means ± SE) are shown at the time of implantation (Pre-treatment) and at the end of the first incubation shift (Post-treatment). *$p < 0.05$, ***$p < 0.001$.

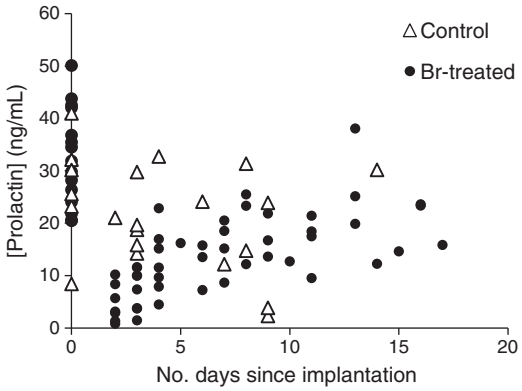

Fig. 3. Effects of bromocriptine-treatment on prolactin levels in captive control (n = 6) and treated (n = 15) Adélie penguins between the day of implantation (0) and up to 17 days post-treatment.

There was a significant correlation between egg temperatures during 24 h prior to departure and prolactin levels at departure (Fig. 5C, S = 64.58, ρ = 0.71, p = 0.015) but not between egg temperatures and corticosterone levels (S = 196.38, ρ = −0.19, p = 0.599). There was no significant correlation between egg rotation rates and hormone levels at departure (prolactin: Fig. 5F, S = 320.69, ρ = −0.46, p = 0.157; corticosterone: S = 138.84, ρ = 0.16, p = 0.662).

Nest success and incubation routine (birds without a dummy egg)

The proportion of successful nests at the end of the incubation period did not differ significantly between control (95.5%) and treated birds (68.2%) (Fisher's exact test, p = 0.505). Incubation duration was not different between years (GLM with a quasi-poisson distribution, t = −0.20, p = 0.841) and was significantly longer in treated birds (on average by 2.1 days; Fig. 6A, t = 2.52, p = 0.017). While the number of chicks at hatching did not differ between years (t = −0.34, p = 0.739), it was significantly lower in treated birds (Fig. 6B, t = −2.23, p = 0.031).

Discussion

The purpose of this study was to investigate the endocrine and behavioral consequences of experimentally decreased prolactin levels in incubating male Adélie penguins. This was achieved by monitoring the hormonal status, behavioral time budget, position on the nest, incubation parameters, and breeding success of bromocriptine-treated and control birds. The experimental decrease in prolactin levels reduced incubation commitment of male Adélie penguins and modified subsequent reproductive success, independently of any changes in corticosterone levels.

Methodological considerations

Experimental studies assessing the role of prolactin in non-domesticated bird species are rare, which is partially explained by the difficulty to pharmacologically manipulate the pituitary hormone. Bromocriptine acts as an agonist of dopamine D2 receptors which are notably localized on the hypothalamic VIP neurons (Chaiseha et al., 2003). Hence, bromocriptine reduces prolactin secretion by inhibiting the stimulation of cells secreting prolactin (Benker et al., 1990). Subcutaneous, self-degradable pellets, such as the ones used in our study, have been found to rarely deliver the hormones for the period claimed by the manufacturers when used in birds (Müller et al., 2009). In our study of free-living incubating Adélie penguins, prolactin levels were still significantly decreased in bromocriptine-treated birds 19.5 days after implantation, when compared with control birds (Fig. 2A). The significant difference in pre-treatment prolactin levels might be explained by inter-individual variability, despite the fact that birds were randomly assigned to the control and treated groups. Repeated blood sampling of captive treated and control birds (i.e. the failed breeders) allowed assessing the kinetics of prolactin secretion consecutive to pellet implantation. Prolactin levels were at their minimum two days following implantation and increased slowly thereafter (Fig. 3). It seems that there was no longer a difference in prolactin levels between control and treated birds 6–8 days post-treatment but this could not be assessed statistically. This shows a reduced efficiency of the pellets that were designed to continuously release bromocriptine during 21 days. Yet, among incubating Adélie penguins, whose prolactin levels were higher at implantation than those of captive birds, prolactin levels were still significantly reduced in treated birds compared to controls 19.5 days post-treatment. These self-degradable pellets are, thus, an effective tool for artificial manipulation of hormonal levels in field studies. However, repeated blood sampling at different times and hormone assays are required to validate the efficacy and the time-course of the treatment for each species and life-history stage.

Subcutaneous administration of bromocriptine in poultry has been shown to decrease prolactin levels, reduce broodiness (Bedrak et al., 1983), and increase egg production (Reddy et al., 2006). Other studies on mammals (Roberts et al., 2001; see also Schradin and Anzenberger, 1999 for a review) and fish (Kindler et al., 1991) have successfully used bromocriptine to decrease prolactin levels and were instrumental in establishing cause and effect relationships

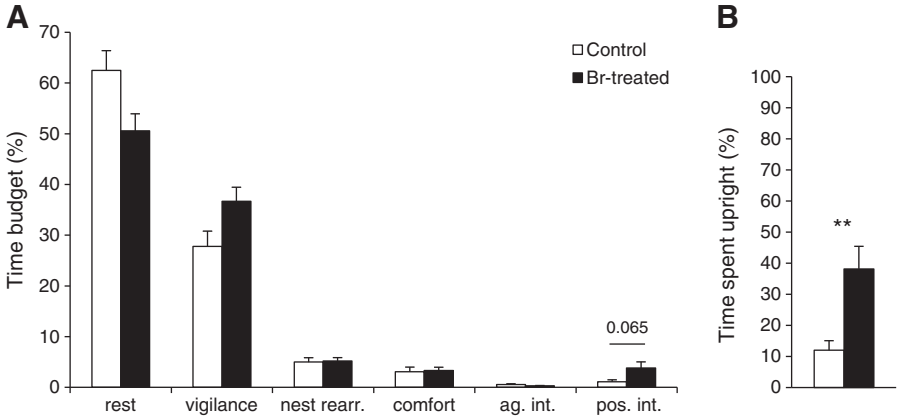

Fig. 4. Behavioral time budget (A) and percentage of time spent standing in the upright position on the nest (B) of control and bromocriptine-treated male Adélie penguins. The ethogram recognized the following behaviors: rest, vigilance, nest rearrangement and egg turning (nest rearr.), comfort, agonistic interactions (ag. int.), and social positive interactions (pos. int.). The behavioral time budget of 11 birds (5 controls and 6 treated) was analyzed, for a total of 103 video sessions of 30 min. **p < 0.01.

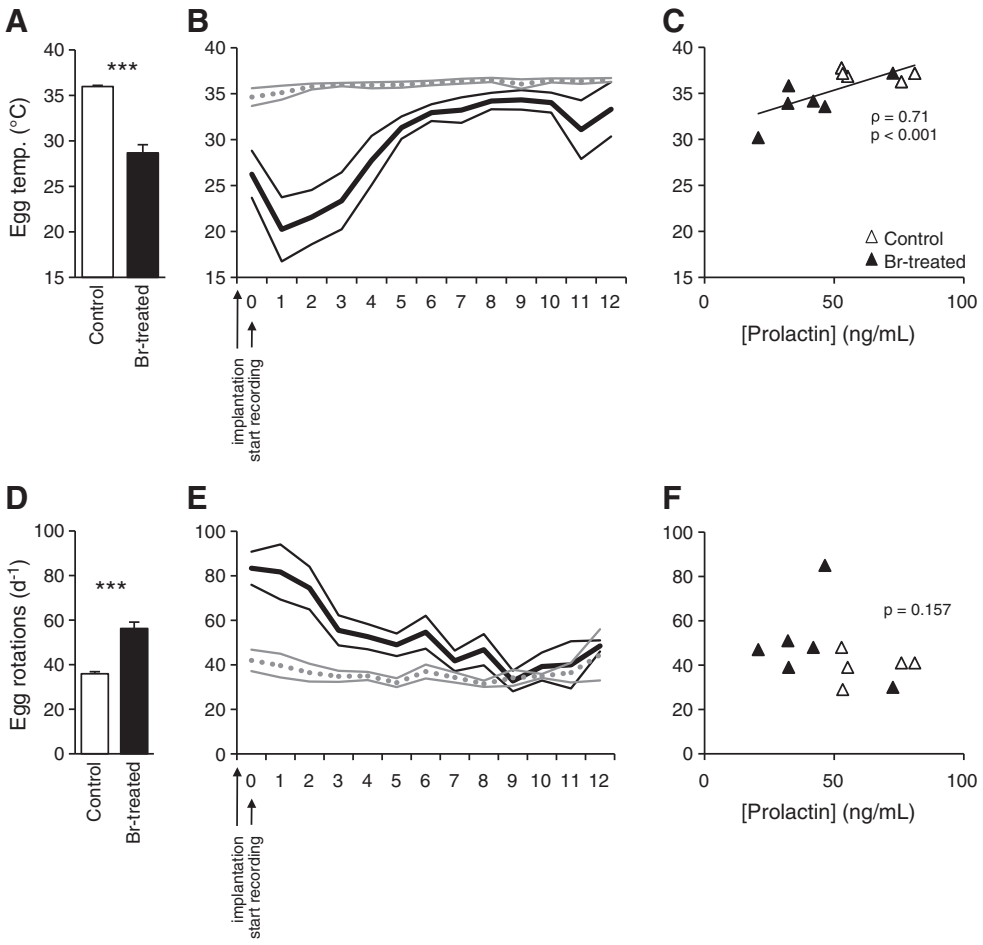

Fig. 5. Incubation behavior recorded from control and bromocriptine-treated male Adélie penguins. Parameters shown are egg temperatures (A, B, C) and egg rotations rates (D, E, F). C and F show the relationship between prolactin levels at departure and incubation parameters during the 24 h prior to departure. Values are means ± SE (n = 14; 6 control and 8 Br-treated birds; 71 vs. 65 daily observations). White bars, gray lines, and white triangles refer to control birds; while black bars, black lines, and black triangles refer to treated birds. ***p < 0.001.

between prolactin and parental care. More recently, bromocriptine-treatment has also been used in emperor penguins *Aptenodytes forsteri* to demonstrate that kidnapping behavior observed in these birds is the result of high levels of prolactin in failed breeders (Angelier et al., 2006). To our knowledge, our study is the first contribution that assessed the effects of an experimental decrease of prolactin levels, using bromocriptine implants, on the detailed incubation behavior of non-domesticated breeding birds in the wild, monitoring both prolactin and corticosterone levels.

Decreased prolactin levels and behavior

Prolactin is often described as the main hormone involved in parental care in birds. Accordingly, we expected that, low prolactin levels would reduce nest attentiveness. Surprisingly, however, the bromocriptine-treatment did not induce any changes in the behavioral time budget of incubating male Adélie penguins per se (Fig. 4A). In particular, treated birds spent the same amount of time

Table 3
Results of the GEEs, comparing egg temperatures and egg rotation rates between control and bromocriptine-treated incubating male Adélie penguins.

	Egg temperature		Maximum egg temperature		Egg rotations	
	Wald	p	Wald	p	Wald	p
Intercept	5490.3	<0.001	9513.2	<0.001	2241.2	<0.001
Treatment	43.14	<0.001	34.29	<0.001	49.31	<0.001
Day[a]	5.75	0.017	4.33	0.037		*NS*
Treatment × day	21.50	<0.001	18.91	<0.001	19.03	<0.001

[a] Day refers to the number of days since implantation.

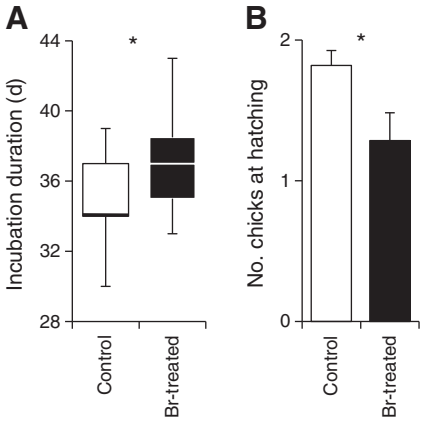

Fig. 6. Effects of bromocriptine-treatment on incubation duration (A) and breeding success at hatching (B) in Adélie penguins. Incubation duration values are plotted as median, 25th and 75th percentiles, minima and maxima. Breeding success values are means ± SE. Data (n = 44, 22 control and 22 treated birds) were averaged over two consecutive breeding seasons for birds whose clutch was not replaced by a dummy egg. *p < 0.05.

being vigilant and rearranging their nests than control birds, contrary to our expectations. Yet, experimental studies on poultry species have demonstrated that a decrease in prolactin levels can reduce and even suppress incubation behavior. For example, in one study, active immunization against prolactin led to decreased prolactin levels and prevented the expression of incubation behavior in turkey hens (Crisóstomo et al., 1998). In another study, nest deprivation in bantam hens induced a decrease in prolactin levels within two to three days, which was paralleled by the disappearance of incubation readiness (Sharp et al., 1988). Several hypotheses can be advanced to explain the absence of a decrease in nest attentiveness in our treated penguins: (1) birds could be insensitive to decreased prolactin levels at this time of the breeding cycle, (2) hormonal changes might have been insufficient to affect behavior, and/or (3) other hormones might play a role in the control of incubation behavior. Certainly, hormones such as progesterone have been shown to play a role in the regulation of nest attentiveness and parental care. For example, prolactin injection was found to be ineffective for inducing incubation behavior in the ring dove *Streptopelia risoria*, when compared with progesterone injection (Lehrman and Brody, 1961). It has also been reported that only a combination of estrogens and progesterone administration followed by prolactin administration was able to induce incubation behavior in female ovariectomized turkeys, while administration of prolactin alone was able to maintain incubation behavior after its induction (El Halawani et al., 1986). Accordingly, this justifies the sole consideration of prolactin in our study. Nevertheless, to better understand the induction of incubation behavior and its control in penguins, the role of other hormones should be examined.

While the treatment did not affect the behavioral time budget of penguins, birds with experimentally decreased prolactin levels spent more time in the upright position on the nest (Fig. 4A). In Adélie penguins, standing in the upright position on the nest is characteristic for the early incubation phase (Derksen, 1977), when birds incubate their first egg, possibly to delay development, before the second egg is laid (Spurr, 1975). Later on, however, eggs are typically incubated between their feet and an exposed brood patch on their belly, while birds are in a prone position. The change in incubation posture we observed in treated birds probably enhanced the chances for eggs to be predated, as was the case with two dummy eggs, which were predated by skuas. One could interpret the greater proportion of time that treated birds spent in the upright position, together with their tendency to engage more often in positive social interactions, as an offset in the timing of their breeding. Accordingly, treated birds displayed behavior that is more typical for their courtship phase or early incubation, rather than their incubation period. Prolactin secretion is endogenously programmed in penguins (Vleck et al., 2000), and could therefore transmit information regarding the timing of breeding. However, the transition from egg laying to incubation in birds is not only associated with changes in the plasma level of prolactin but also with changes in steroid hormones levels (Vleck and Vleck, 2011; Wingfield and Goldsmith, 1990). Hence, in addition to decreased prolactin levels, variations in the secretion patterns of steroid hormones may also be influential and help to understand the suggested change in the timing of breeding of treated birds. While bromocriptine-treatment has no androgenic effects in fish (Kindler et al., 1991) and mammals (Taverne et al., 1982), it has been shown to increase progesterone and estradiol levels in hens (Reddy et al., 2006). Consequently, to better understand the hormonal control and the timing of incubation behavior in male Adélie penguins, further studies should examine the possible links between prolactin and steroid hormones.

Decreased prolactin levels and incubation parameters

As a consequence of the expected decrease in nest attentiveness in bromocriptine-treated penguins, we predicted a decrease in egg temperatures and egg rotation rates. However, since we found that nest attentiveness was similar in treated birds and controls, these incubation parameters should also have been similar. Contrary to that, treated penguins, with consequently decreased prolactin levels, incubated their eggs at lower temperatures (Fig. 5A) and turned their eggs more frequently (Fig. 5D) than control birds. These changes might have resulted from the higher proportion of time treated birds spent in an upright position on the nest (Fig. 4B). Standing upright during incubation might be associated with a frequent a rapid chilling of eggs, when birds move eggs with their feet, while changing position (Derksen, 1977). Lower incubation temperatures observed in treated penguins could also be the result of decreased peripheral vasodilatation in the brood patch, as suggested by the observed decrease in maximum egg temperature (Table 3). The formation of the brood patch, i.e. defeathered, vascularized, and edematous skin, is under the control of prolactin in many species (reviewed in Lea and Klandorf, 2002). Hence, decreased prolactin levels might have induced structural changes to the brood patch, therefore decreasing the amount of heat transferred to the eggs.

The differences in egg temperatures and egg rotation rates between treated birds and controls were maximal soon after implantation, and decreased over time (Fig. 5, Table 3). In our captive penguins, prolactin levels of treated birds were at a minimum two days after implantation and increased afterwards. On the other hand, prolactin levels at departure were significantly correlated with egg temperatures (Fig. 5C) but not with egg rotation rates (Fig. 5F) prior to the blood sampling. This lends support to the idea that parental investment of incubating Adélie penguins correlates negatively with prolactin levels: birds with elevated prolactin levels (80–100 ng/mL) incubate optimally, while birds with decreased prolactin levels (40–60 ng/mL) incubate their eggs sub-optimally, at lower temperatures.

Lower incubation temperatures can be harmful to embryos by reducing growth efficiency and increasing in ovo mortality (Feast et al., 1998; Nord and Nilsson, 2011; Olson et al., 2006). Incubation duration was lengthened in our treated birds (Fig. 6A), and since avian embryos are essentially ectothermic (Webb, 1987), it is very likely that the developmental rate was slowed in eggs, which were incubated by treated birds.

Decreased prolactin levels and breeding success

Incubation temperatures together with a detailed behavioral time budget can be considered to directly reflect parental investment in nestling development. The bromocriptine-treatment was expected to reduce breeding success because of reduced parental investment. Accordingly, we found that treated birds had a lower breeding success at hatching than controls (Fig. 6B). In ring doves, declining prolactin levels have been linked to the termination of incubation of infertile eggs, and to the termination of brooding of young chicks (Silver, 1984), while in penguins low prolactin levels have been associated with nest abandonment (Groscolas et al., 2008; Spée et al., 2010). However, several studies concerning free-living species, such as the semi-palmated sandpiper *Calidris pusilla* (Gratto-Trevor et al., 1990) and the pied flycatcher *Ficedula hypoleuca* (Silverin and Goldsmith, 1990), have failed to reveal a relationship between plasma prolactin levels and a decline in brooding. Furthermore, in black-legged kittiwakes *Rissa tridactyla*, a short-term increase in corticosterone levels led to a long-term decrease in prolactin levels, which was associated with lower brooding commitment and a decreased reproductive success (Angelier et al., 2009).

There is growing evidence that prolactin has to reach a threshold concentration, below which the drive to incubate is inhibited (Boos et al., 2007; Spée et al., 2010, 2011). A prolactin level below threshold, together with increased corticosterone levels, seems necessary to induce nest desertion in Adélie penguins (Spée et al., 2010, 2011). The post-treatment prolactin levels of birds in our study (40 ng/mL) were similar to post-treatment prolactin levels of corticosterone-treated

Adélie penguins (Spée et al., 2011). They were also similar to prolactin levels of non-treated Adélie penguins, which deserted their nests, i.e. about 30 ng/mL (vs. 80 ng/mL for non-treated birds, which did not desert their nest) (Spée et al., 2010). On the other hand, corticosterone levels in our study were not affected by the bromocriptine-treatment (Fig. 2B). Furthermore, nest desertion rate did not differ in our study between treated males and controls, which is unlike the situation in Adélie penguins treated with corticosterone pellets (Spée et al., 2011; Thierry et al., 2013). The current study therefore demonstrates that experimentally decreased prolactin levels alone are insufficient to trigger nest desertion in Adélie penguins, but instead decrease incubation commitment, which ultimately reduces breeding success.

Conclusion and perspectives

The experimental decrease in prolactin levels alone did not affect the birds' behavioral time budget. However, treated birds spent significantly more time in the upright position during incubation than controls. Our study supports the view that prolactin is one – but not the only – physiological agent controlling parental care in birds. Most studies investigating the hormonal control of incubation behavior have been carried out on females of domesticated bird species. To what extent their conclusions are valid for male birds in the wild remains to be examined.

We found that decreased prolactin levels alone were not sufficient to induce nest desertion in Adélie penguins, but reduced incubation commitment, especially during the early stages of the embryonic development. Our treatment led to a decrease in incubation temperature and a higher frequency of egg rotation, which was paralleled by a lengthening of the incubation period. However, the observed decrease in incubation temperature (i.e. several degrees) did not lead to a higher in ovo mortality, but instead slowed the rate of embryonic development. It would be of great interest to investigate the physiological consequences of such lengthened incubation period on chick physiology, growth, body condition at fledging, and ultimately, survival.

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