



Prolactin concentrations predict parental investment and nest survival in a free-living shorebird

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ABSTRACT

The hormonal milieu that exists during reproduction is one of the key factors influencing the trade-off between reproductive investment and self-maintenance. Much previous work in birds has focused on prolactin as a physiological mediator since prolactin is involved in the onset and maintenance of parental care. However, how prolactin relates to reproductive success in terms of altering parental behavior in wild bird populations is not fully understood. Here, we report prolactin concentrations in breeding Kentish plovers (*Charadrius alexandrinus*), a small shorebird with variable mating systems and parental care, as an ecological model of mating system evolution. Throughout the breeding season, we estimated the circulating prolactin concentrations in male and female plovers during incubation. In addition, we monitored parental behavior and determined the fate of nests. We found that prolactin concentrations decreased during incubation but increased with clutch completion date. In addition, males and females with high prolactin concentrations spent more time on incubation than those with low prolactin concentrations. Importantly, higher prolactin concentrations in either males or females predict higher nest survival. Our results suggest that prolactin is an indicator of parental behavior in a wild shorebird population, although additional studies including experimental manipulation of prolactin concentrations are necessary to verify this relationship.

1. Introduction

One of the fundamental principles of life-history theory is that parents need to balance the trade-off between their reproductive investment and self-maintenance (Clutton-Brock, 1991; Székely et al., 2010; Royle et al., 2012). Investigating endogenous hormone concentrations provides insight into the mechanisms mediating resource allocation decisions since hormone concentrations change in response to both environmental and internal stimuli, affecting behavior and physiological parameters that may underlie this trade-off. (Jónsson et al., 2006; Williams, 2012; Vincze et al., 2013). Therefore, measuring the individual hormonal milieu is considered a practical approach to assess reproductive effort and reproductive success in many species (Angelier and Chastel, 2009; Norris and Lopez, 2011). It is important to further explore hormone-behavior relationships to examine the role of hormonal contributions to individual fitness and to derive hypotheses for future experimental studies.

Prolactin is an adenohypophysial hormone that exerts major pleiotropic functions in vertebrate physiology, including reproductive effort (Norris and Lopez, 2011). Circulating prolactin concentrations contribute to the regulation of reproductive behaviors, such as territory defense, incubation, and brood-rearing (Deeming, 2002; Adkins-Regan, 2005; Angelier and Chastel, 2009). In birds, prolactin levels varied

among individuals in a context-dependent way and displays annual fluctuation (Norris and Lopez, 2011; Angelier et al., 2016). For instance, low prolactin levels are thought to be associated with poor environmental conditions, poor body condition, breeding failure, and nest abandonment (Angelier and Chastel, 2009; Angelier et al., 2013; Angelier et al., 2015). In contrast, elevated prolactin levels, from early breeding season to hatching of the young, are related to the initiation and maintenance of incubation, as well as to parental care behavior (Boos et al., 2007; Crossin et al., 2012; Smiley and Adkins-Regan, 2018).

Specifically, the role of prolactin has been extensively studied in terms of sex-specific patterns of incubation attentiveness (Norris and Lopez, 2011). In general, in species that have female-only incubation, such as European starlings (*Sturnus vulgaris*) and song sparrows (*Melospiza melodia*), females have higher prolactin levels than males (Reviewed by Angelier et al., 2016). The opposite is also true in sex-role reversed species such as the Wilson's phalarope (*Phalaropus tricolor*), where males have higher prolactin levels during incubation (Oring et al., 1988). However, in species where both members of the pair incubate, prolactin profiles vary. For example, in Adélie penguins (*Pygoscelis adeliae*), females have higher prolactin levels than males, although differences between male and female Adélie penguins may depend on environmental conditions (Beaulieu et al., 2017). In

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contrast, in semipalmated sandpipers (*Calidris pusilla*) and zebra finches (*Taeniopygia guttata*), who also share equally in incubation, prolactin levels do not differ between the sexes (Gratto-Trevor et al., 1990; Christensen and Vleck, 2008).

Despite these studies, it is still relatively unclear whether variation in reproductive success between individuals results from differential prolactin levels altering parental care behavior (Adkins-Regan, 2005). For instance, in common terns (*Sterna hirundo*), there is a significant and positive correlation between prolactin levels and reproductive success, but how behavior mediates this relationship was out of the scope of that particular study (Riechert et al., 2014). Miller et al. (2009) showed that, in mourning doves (*Zenaidura macroura*), prolactin levels are positively correlated with nestling weight in early nestling age, indicating that increased prolactin levels may increase reproductive success by promoting parental care (i.e., feeding). This result was further strengthened by Smiley and Adkins-Regan (2016) who demonstrated that, in another altricial bird, zebra finches, prolactin is positively correlated with chick brooding, feeding, and importantly, chick survival.

Here, we examined a small precocial shorebird, the Kentish plover (*Charadrius alexandrinus*) with a body mass of 32–56 g (Wiersma et al., 2018), how circulating prolactin concentrations vary and their relationship to variations in incubation behavior and consequently, to reproductive success. Kentish plovers are widespread and common shorebirds that breed in Eurasia and North Africa (Wiersma et al., 2018). Due to their variable mating system (monogamy, polygyny and polyandry), and parental care (biparental care and uniparental care by the male or the female), they are often used in behavioral studies of mating system evolution (Thomas et al., 2007; Székely, 2014). We investigated Kentish plovers in Bohai Bay, China, where both parents share incubation, while females desert the brood approximately one week after the young hatch, and males continue brooding the hatchlings for approximately four weeks (Que unpublished data).

Based on previous studies (Crisuolo et al., 2002; Boos et al., 2007), we expected that 1) there would be a sex difference in prolactin patterns during incubation given that males play a larger role in brood care than females, and 2) there would be a rise in prolactin levels in both males and females throughout the incubation period and variable levels throughout the breeding season. In addition, we expected that 3) prolactin would correlate with incubation efforts and 4) predict reproductive success given that incubating behavior makes heavily influences hatching success in nearly all birds (Deeming, 2002).

2. Methods

2.1. Study area and species

This study was performed on Daqinghe Saltworks (39°08' N, 118°46' E to 39°14' N, 118°52' E) in Bohai Bay, Eastern China. Fieldwork was conducted from May to July 2017. Breeding populations of Kentish plovers in Bohai are sequential polygamous individuals (Que unpublished data). Both males and females participate in incubation during an approximately 26 days incubation period (Que et al., 2014). This population exhibits a concentrated seasonal breeding pattern, with most plovers laying eggs between mid-April to late-June and the clutch size of 3 (Que et al., 2014).

2.2. Trapping and sample collection

All birds were captured between 6:00 am to 6:00 pm by placing a funnel trap directly on top of the nest for no longer than 30 min. We captured 60 incubating females and 30 incubating males for this study. Males were more alert and more difficult to trap within 30 min. On the day we found a complete nest, we estimate nest age by floating-egg method, which is based on the egg floating position and tilting angle in the water (Liu and Chen, 2002). We then calculated nest completion

date by subtracting nest age from the date we made the estimation.

For blood sampling, we punctured the brachial vein with a 26 gauge needle and collected 80 μ L blood into heparinized capillary tubes within 3 min of capture (Smiley and Adkins-Regan, 2016). Collected whole blood was immediately centrifuged for 1 min at 4000 rpm for blood plasma purification. Plasma was extracted and stored at -20°C for 3 to 50 days (average stored period = 24 days) until assayed for prolactin concentrations.

We fit each individual with a unique combination of colour bands and a numbered aluminum band from the National Bird Banding Center of China for individual identification purposes. We measured bill and tarsus length with Vernier calipers (to the closest 0.01 mm, Mituyoyo 500-762-10), flattened wing chord length with a wing rule (to the closest 1 mm) (Redfern and Clark, 2001), and mass using an electronic scale (to the closest 0.01 g, G&G MS501). We monitored nests every 5 days until 22 days of incubation, and daily thereafter to determine final nest fate as successfully hatched, failed or unknown. Nest success was defined as successfully hatched if at least one newly hatched chick was observed near the nest or by the images of camera traps showing chicks. A nest was considered failed when all eggs disappeared > 4 days before the predicted hatch date, eggs were flooded, eggs remained in nest after 35 days of incubation, or if camera traps revealed predation. Otherwise, when nests were unable to be defined as successful or failed, we classified them as “unknown” and did not use them in subsequent nest survival analyses (Que et al., 2014).

2.3. Monitoring of incubation behavior

After collection of both male and female samples from nests, we placed a camera trap (Loreta L710) approximately one meter away from the nest to record incubation behavior. We set camera traps to take photographs at 1-min intervals for > 24 h (Bulla et al., 2016). To avoid inducing non-natural behavior caused by banding and blood sampling, we analyzed behavioral records beginning 6 h after trapping and blood sampling. Using captured images, we quantified the duration of on-bout and off-bout for each parent.

2.4. Hormone assay

Plasma concentrations of prolactin were determined by a heterologous competitive enzyme linked immunosorbent assay (ELISA), following similar methods of Rochester et al. (2008) and Smiley and Adkins-Regan (2016). Here we used a commercial chicken prolactin ELISA kit (BIOSAMiTE CK-E60044C, Yaoyunbio company, Shanghai, China) that uses a 96-well microplate pre-coated with chicken prolactin antibodies. This kit is considered valid as pooled plasma samples from Kentish plovers resulted in a paralleled dose-response curves as chicken prolactin standard curves (Fig. S1). Assessments were performed as described in the manufacturer's instructions. All samples were analyzed singly in a single plate. The detection limit of the assay is 1.0 mIU/l (equal to 0.06 ng/mL), and the lowest measurement was 191.6 mIU/L (equal to 11.49 ng/mL). The intra- and inter- assay coefficients of variance are both 15% according to the kit manual. Ten samples from the sample pool were selected to run in triplicate for repeatability testing, showing that the prolactin assay was highly and significantly repeatable ($r = 0.91$, $F_{9,20} = 225.55$, $p < .001$, $n = 10$ individuals).

2.5. Statistical analysis

Statistical analyses were performed using R (Version 3.2.4 Core Team 2016). The lme4 package (Bates et al., 2015) was used for fitting linear mixed models (LMMs), and the MuMIn package (Bartoń 2018) was used to select the best models. We used corrected Akaike's information criterion for small sample sizes (AICc) to assess candidate models (Burnham and Anderson, 2002). Models with a change in $\text{AICc} < 2$ were considered as candidates (Burnham and Anderson,

2002). We conducted model averaging to obtain robust estimates of model parameters if there was more than one model with an $AIC_c < 6$ (Richards, 2008). We use log (10 based)-transformed prolactin concentrations for analyses to improve model fit. Data are represented as the mean \pm SEMs, and we provided two-tailed probabilities with alpha set to 0.05.

2.5.1. Prolactin, sex, and timing of breeding

We tested sex differences in prolactin concentrations by ANCOVA using nest age as a covariate. To evaluate the relationship between prolactin and temporal factors, we performed LMMs with individual ID as a random effect, and sex, clutch completion date (shown as Julian date), nest age and the interactions of these variables as fixed effects. We built a set of models with all combinations of fixed effects, including a null model.

2.5.2. Prolactin and incubation effort

Using data collected by camera traps, we identified two variables to describe incubation effort for both males and females over a 24-hour incubation period six hours after blood sampling. The following parameters were assessed: 1) the percentage of time an individual incubated; 2) the average duration of incubation bouts (Schoenle et al., 2017).

To examine the relationship between prolactin and incubation effort, we used LMMs with either the percentage of time of individual incubation or the average duration of incubation bout as the response variable, brood ID and sampling time of day as the random effect, and prolactin concentration, sex, clutch completion date, nest age and the interactions of sex and temporal variables as fixed effects. We built a set of models with all combinations of fixed effects, including a null model.

2.5.3. Prolactin and nest survival

Daily nest survival rate was defined as the probability that a nest survives a single day (Dinsmore et al., 2002). To evaluate daily nest survival, we used the day the nest was found, the last day it was observed alive and the day it was observed to have failed. If there was a gap in monitoring between the last day the nest was observed alive and the first day it was observed to have failed, we used the midpoint between the two dates as the date of failure (Weiser et al., 2017).

To evaluate the effect of prolactin on daily nest survival, we used model selection and nest survival model within Program MARK v8.2 (Dinsmore et al., 2002; Cooch and White, 2018). We constructed models of nest survival that incorporated combinations of individual covariates (prolactin, sex, clutch completion date and nest age) and compared them to the null model of constant survival rate.

3. Results

3.1. Prolactin concentrations in male and female plovers

Concentrations of prolactin in female and male Kentish plovers were normally distributed (Fig. S2), ranging from 6.5–21.2 ng/mL (mean \pm SEM: 14.1 ± 3.2 ng/mL, $n = 58$ females) and 7.3–19.7 ng/mL (13.8 ± 3.0 ng/mL, $n = 27$ males), respectively. When nest age was used as a covariate, prolactin concentrations did not differ between the sexes (Fig. 1 ANCOVA, $F = 0.01$, $\eta^2 = 0.0001$, $p = .92$). Body mass, tarsus length, bill length and wing length did not predict prolactin concentrations (Pearson correlation, Supplementary Table 1).

3.2. Prolactin, nest age and clutch completion date

Both clutch completion date and nest age predicted prolactin concentrations (Supplementary Table 2), while sex and the statistical interactions between explanatory variables were not included in the best candidate models. Thus, circulating prolactin concentrations of Kentish plovers decreased with nest age ($\beta = -0.002$, 95% CI = -0.004 ,

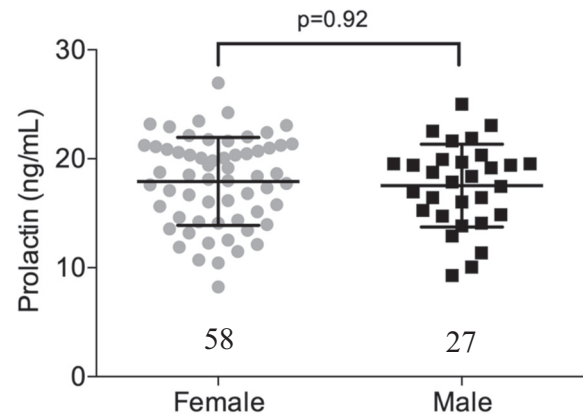


Fig. 1. Individual variation of plasma prolactin concentrations in male and female Kentish plover. Sample size for each sex are at the bottom of the panel. Each dot represents an individual. Central bars and whiskers are mean \pm SEMs.

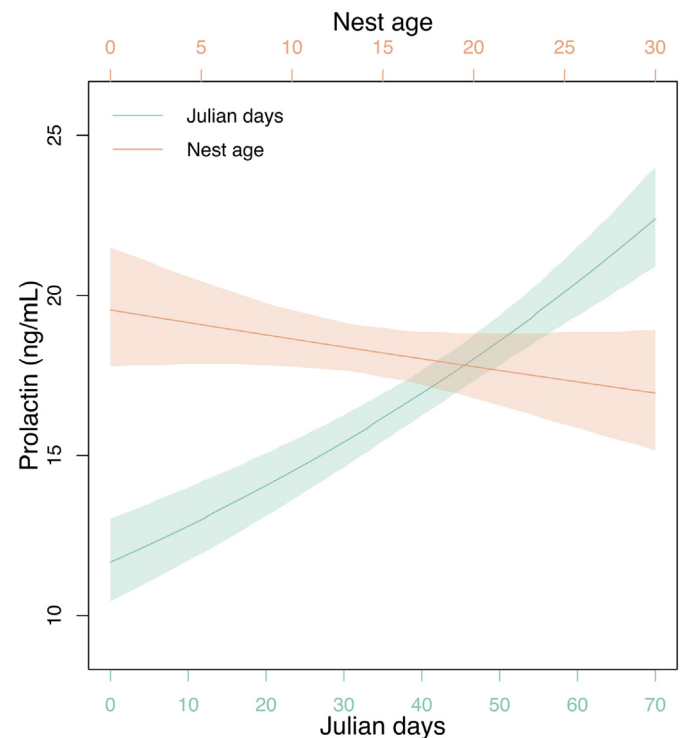


Fig. 2. Relationship between clutch completion date (shown as Julian day, green) and nest age (days, orange) and plasma prolactin concentrations in Kentish plover. Julian day: Day1 = May 17th. Regression line is derived from the averaging model predicting prolactin concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

-0.0003 , $p = .024$, $n = 85$ individuals, Supplementary Table 2, Fig. 2) and increased with clutch completion date ($\beta = 0.004$, 95% CI = 0.003 , 0.005 , $p < .001$, $n = 85$ individuals, Supplementary Table 2, Fig. 2).

3.3. Prolactin and incubation effort

The percentage of time an individual spent on incubation increased with prolactin concentration ($\beta = 0.559$, 95% CI = 0.037 , 1.081 , $p = .036$, $n = 25$ incubation time, Supplementary Table 3B, Fig. 3). Sex did not affect the relationship between percentage of time spent on incubation and prolactin ($\beta = 0.324$, 95% CI = -1.070 , 1.718 ,

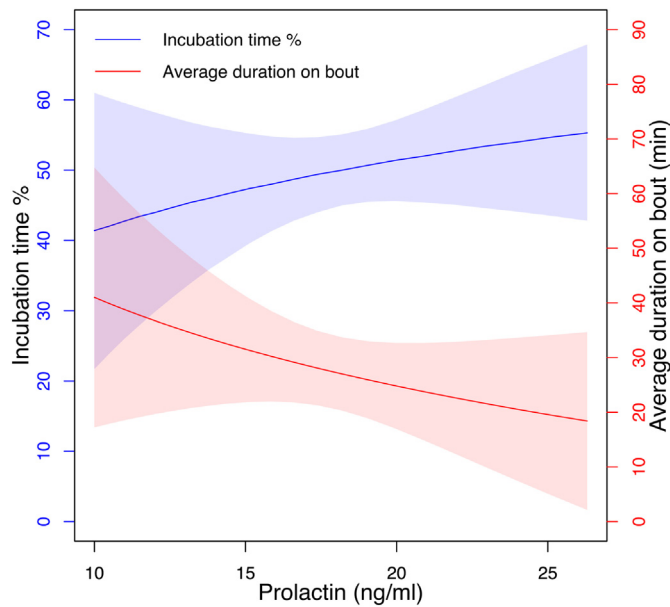


Fig. 3. Incubation effort varied with prolactin concentrations in Kentish. Incubation effort is indicated by average duration on bout (blue) and the percentage of time an individual incubated (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$p = .648$, $n = 25$ incubation time, Supplementary Table 3B). Meanwhile, the average duration on bout was negatively correlated to prolactin concentration ($\beta = -7.820$, 95% CI = $-148.576, -7.825$, $p = .029$, $n = 25$ incubation time, Supplementary Table 4B, Fig. 3). Sex was a non-significant predictor of duration on bout and prolactin concentration ($\beta = -0.753$, 95% CI = $-12.198, 10.691$, $p = .897$, $n = 25$ incubation time, Supplementary Table 4B) suggesting that prolactin concentrations predict the bout duration consistently between males and females.

3.4. Prolactin and nest survival

Daily nest survival rate increased with prolactin concentration in both parents ($\beta = 0.006$, 95% CI = $0.0002, 0.012$, $n = 57$ nests, Fig. 4), based on the average of four top models ($\Delta\text{AICc} < 2$, Supplementary Table 5). Consistently with the aforementioned result, the model that included the statistical interaction between parental sex and prolactin concentration was not among the best candidate models ($\beta = -0.027$, 95% CI = $-0.154, 0.099$, $n = 57$ nests, Supplementary

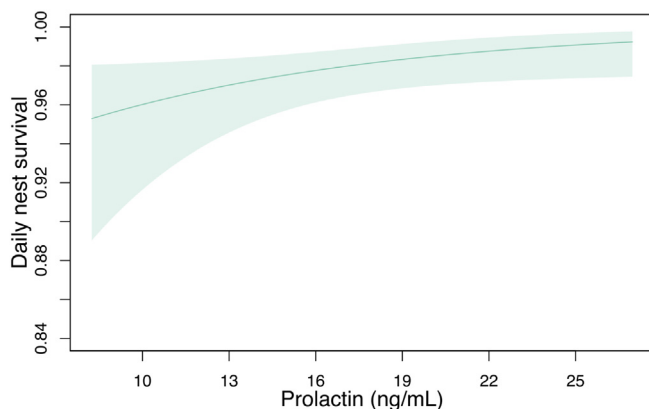


Fig. 4. Daily nest survival rate in relation to prolactin concentrations of male or female Kentish plovers.

Table 5), suggesting that male and female prolactin concentrations predict nest survival in a similar way.

4. Discussion

Our study of free-living Kentish plovers produced four main results. First, prolactin concentrations do not differ between sexes. This finding is consistent with a former plover study (Skrade et al., 2017), even though after hatching of eggs male Kentish plovers look after the young for longer than the females. Therefore, the lack of sex differences likely results from both male and female Kentish plovers developing brood patches and participating in incubation, which in turn stimulates prolactin secretion in the same manner (Norris and Lopez, 2011; Kosztolányi et al., 2012; Smiley and Adkins-Regan, 2018). Both males and females were captured on the nest when they carried out incubation: females usually incubate in the morning and mid-day, whereas males usually attend the nest in tend to incubate in the evening (Vincze et al., 2013). In addition, body traits (i.e., body mass) of Kentish plovers do not predict prolactin concentrations, unlike studies in Manx shearwater (*Puffinus puffinus*), lesser snow goose (*Anser caerulescens caerulescens*) and Gould's petrel (*Pterodroma leucoptera*). (Jónsson et al., 2006; O'dwyer et al., 2006; Riou et al., 2010). We assumed that Kentish plovers may have alternative physiological mechanisms to maintain prolactin concentrations, even at low body mass. In addition, the absence of differences between individuals, irrespective of their sex and body traits, may be related to good environmental conditions experienced by birds during the time of our study, allowing all individuals to produce optimal concentrations of prolactin.

Second, contrary to our hypothesis, our results showed that in both parents prolactin concentrations gradually decreased with incubation days. Previous studies in precocial birds demonstrated that maximal circulating prolactin concentrations are reached toward the end of incubation and drop steeply or remain stable only a little while after the young hatch (Kosztolányi and Székely, 2002; Norris and Lopez, 2011). One possible explanation for our result is that prolactin in Kentish plovers is more likely to prime the onset of incubation. In other words, prolactin may rapidly rise from base-line to a relatively high concentration before the start of incubation and, though decreasing, maintain this concentration above certain threshold during the incubation period (Crossin et al., 2012).

Meanwhile, prolactin concentrations increased with clutch completion date. Since there is an overlap between reproduction and molt in the life-history of Kentish plovers (Cramp, 1983), prolactin likely participates in the trade-off between reproductive investment and self-maintenance, which is a known hormonal conflict (Williams, 2012). Early studies have established a role for prolactin in the regulation of parental behavior in birds (Norris and Lopez, 2011). At the same time, as shown in European starlings, molt is associated with decreasing prolactin concentrations and is inhibited when prolactin concentrations are high (Dawson, 2006). However, a previous study showed that male southern giant petrels (*Macronectes giganteus*) begin molting early in incubation, even when prolactin concentrations are still high (Crossin et al., 2012). Our previous study (Que unpublished data) demonstrated that by late breeding season (early July), both incubating male and female Kentish plovers began to molt, during which time they have the highest prolactin concentrations of the entire breeding season (this study). We assumed that they are similar to southern giant petrels, which may have alternative physiological pathways, such as the thyroid hormone axis (Reinert and Wilson, 1997), that promotes molting while prolactin concentrations are high.

Third, as predicted by our hypothesis, incubation effort was positively correlated with prolactin concentrations. Specifically, higher circulating prolactin concentrations contributed to a greater share of incubation time, indicating individuals with higher prolactin concentrations exhibit higher incubation effort. However, since our study was observational, this finding may actually be in reverse. That is,

because birds spent more time in contact with eggs, they have higher prolactin concentrations. Nevertheless, there was a negative relationship between prolactin concentrations and average duration on bout. Because average duration on bout may vary based on different exposure concentrations to disturbance and tend to represent the stress level of birds, our results suggest that individuals with higher prolactin concentrations may be more sensitive to these disturbances. Furthermore, corticosterone more likely contributes to the frequency of on-off bouts (Skrade et al., 2017). It is believed that prolactin alone is not sufficient to cause or maintain incubation behavior. Former studies have shown that in domestic female turkeys (*Meleagris gallopavo*), the action of prolactin on incubation behavior is facilitated by the combined action of estradiol and progesterone (el Halawani et al., 1986). Moreover, in Adélie penguins, nest abandonment was related to a combination of high corticosterone levels and low prolactin levels (Spée et al., 2010). Thus, it is possible that additional steroid hormones may contribute to incubation behavior in both male and female Kentish plovers.

Finally, high prolactin concentrations in both parents predicted high nest daily survival rate. This result may occur via parents with higher prolactin concentrations defending their nests more vigorously and/or successfully from predators than those with lower concentrations or by parents speeding up offspring development through intense incubation.

Currently, our results have shown both a positive relationship between prolactin concentration-incubation effort and prolactin concentration-nest survival. However, future studies are required to determine whether prolactin causes changes in nest survival rate in terms of incubation effort in a causative way. Experimental manipulation of prolactin levels has been performed in domestic birds, such as turkey and bantam hens (*Gallus gallus*), indicating that injection of prolactin induces incubation, while immunization against VIP inhibits prolactin secretion, causing incubating bantam hens to desert their nests (Sharp et al., 1988; el Halawani et al., 1996). Nevertheless, stress-induced decreases in prolactin did not trigger desertion in free-living Kentish plovers (Kosztolányi et al., 2012). Experimental inhibition of prolactin secretion in Adélie penguins was not sufficient to induce nest desertion either, although it did decrease incubation effort (Thierry et al., 2013). Therefore, future research should not only investigate the causative relationship between circulating prolactin concentrations and incubation behavior but also whether prolactin causes changes in nest survival rates in terms of incubation effort.

In conclusion, our study suggests that 1) there are no sex differences in prolactin concentrations among incubating individuals, nor do body traits correlate with prolactin concentrations; 2) prolactin concentrations gently decreased with increasing nest age but increased with clutch completion date; 3) individuals with higher circulating prolactin concentrations pay greater incubation effort into the current bout; 4) parents with high prolactin concentrations achieve high nest survival. Further investigations examining the independent and combined effects of multiple hormones are necessary. Overall, combined observational and experimental studies across additional avian species with different life history stages may provide fruitful insights into the evolution of hormone-mediated behavior.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2019.104633>.

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