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Circulating profile of the appetite-regulating hormone ghrelin during moult-fast and chick provisioning in southern rockhopper penguins (*Eudyptes chrysocome chrysocome*)

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Highlights

- Southern rockhopper penguins show elevated circulating ghrelin levels during the late stage of their annual moult fast
- Chicks show lower ghrelin levels compared to non-moulting adults
- Circulating ghrelin levels do not differ between fed and unfed chicks

Abstract

A multitude of animal species undergo prolonged fasting events at regularly occurring life history stages. During such periods of food deprivation, individuals need to suppress their

appetite. The satiety signalling gut hormone ghrelin has received much attention in this context in studies looking at mammalian systems. In wild birds, however, knowledge on the ghrelin system and its role during extended fasts is still scarce. In this study, we collected plasma samples for measurements of circulating ghrelin concentrations from adult southern rockhopper penguins (*Eudyptes chrysocome chrysocome*) during the three to four week-long moult-fast that they repeat annually to replace their feathers. We further sampled chicks before and after feeding bouts and non-moulting adults. Circulating ghrelin levels did not differ significantly between fed and unfed chicks but chicks had significantly lower plasma ghrelin levels compared to adults. Furthermore, penguins in late moult (i.e. individuals at the end of the prolonged fasting bout) had higher ghrelin levels compared to non-moulting adults. Our results show elevated levels of circulating ghrelin during moult and generally lower levels of ghrelin in chicks than in adults regardless of feeding state. Given the scarcity or absence of knowledge on the function of ghrelin in seabirds and in fasting birds in general, our results add greatly to our understanding of the avian ghrelin system.

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Keywords

Extended fasting; Appetite regulation; Moult; Ghrelin; *Eudyptes chrysocome chrysocome*; Southern rockhopper penguin; Chick development; Gut hormone

1. Introduction

Fasting, i.e. a temporary suppression of food intake, occurs in a multitude of species in the animal kingdom. For many of these species, predictable long-term fasting periods lasting several weeks to months are part of the life history and accompany environmental and/or internal cycles. Predictable and prolonged fasts are connected to important life-history stages like aestivation, development, hibernation, migration, moult, or reproduction, and individuals typically prepare for periods of food deprivation by accumulating extensive internal energy stores, mainly consisting of subcutaneous fat deposits (Secor and Carey, 2011). For example, in some phocid seal species, pups often go from continuous nursing lasting for days to sudden fasting on ice when their mothers go back to sea to hunt (Lydersen et al., 1997) and male phocid and otariid seals undergo up to three months of fasting during the breeding season, often without access to water (

Crocker and Champagne, 2018; Le Boeuf and Laws, 1994). Some anuran and lungfish species are known to fast for ten months each year during aestivation (Pinder et al., 1992; Richards, 2010; Secor and Lignot, 2010) and some waterfowl species engage in a capital breeding strategy where females do not feed during the entire egg incubation period (e.g. Ancel et al., 1998; Criscuolo et al., 2000; Parker and Holm, 1990; Spaans et al., 1999).

During extended periods of fasting, animals have to suppress their appetite and the urge to feed. In vertebrates, appetite regulation is a complex process involving external and internal factors such as environment, diet, digestive physiology, endocrine system, gut microbiota, and metabolism of nutrients (de Godoy, 2018; Jensen, 2001). In the past decade, it has been shown that the endocrine system and especially gastrointestinal hormones play a major role in the regulation of appetite, food intake, and body mass (Chaudhri et al., 2006). To date, over 20 hormones produced by enteroendocrine cells have been described, and together these cells form the largest endocrine organ in mammals (de Godoy, 2018; Rehfeld, 2004). These hormones have a variety of functions and are connected with the brain by the gut-brain-axis (Chaudhri et al., 2006). Among well-known hormones that are part of the regulatory feedback for food intake and body mass maintenance are leptin, ghrelin, adiponectin, and obestatin (Fang and Judd, 2011; Kojima and Kangawa, 2008; Zhang et al., 2005; Zhang and Chua Jr., 2011). Alteration of these endocrine systems result in a metabolic imbalance that can lead to obesity and metabolic syndromes. Another group of hormones that have been demonstrated to influence food intake are glucocorticoids. Depending on the magnitude and type of the stressor and the resulting degree of activation of the hypothalamic-pituitary-adrenal-axis, glucocorticoids can trigger either an orexigenic or anorexigenic effect, i.e. stimulating or supressing feeding behaviour. These effects are mediated by interactions between glucocorticoids and endocrine regulators of feeding such as insulin, leptin, melanocortin, and neuropeptide Y (reviewed in Maniam and Morris, 2012).

Within gut hormones, particularly the “hunger hormone” ghrelin has gained considerable attention in past years due to its function in satiety signalling, making it of chief interest in studies on obesity and weight control. In mammals, ghrelin increases food intake and promotes gastric motility, the release of pancreatic polypeptides, and of pituitary hormones such as the growth hormone (Tschöp et al., 2000; Wren et al., 2001). Furthermore, the hormone increases during fasting and has been found to decrease post feeding (Toshinai et al., 2001). The regulation of food intake and body mass in birds is far less understood compared to mammalian species except for a few poultry species of commercial interest: like in mammals, ghrelin levels are increased in fasted chicken and quail. Yet, high levels of the hormone decrease feeding behaviour in poultry, meaning that ghrelin seems to

have an anorexigenic effect in birds as opposed to an orexigenic effect in mammals (reviewed in Kaiya et al., 2009). These findings supply evidence for a critical role of ghrelin in food intake and body mass regulation in birds. However, to date we do not know how long-fasting birds regulate or suppress the need to forage and studies on appetite regulation in wild birds are especially scarce.

Penguins are famous for engaging in long-term fasting events, which can range from a few days to two to five weeks during adult moult and from few days to four months during courtship and egg incubation, depending on the species. Moreover, penguin chicks need to cope with regular fasts lasting from 24h to five months when waiting for food provisioning by parent birds during development (reviewed in Groscolas and Robin, 2001). These characteristics make penguins attractive species to investigate hormonal correlates of predictable, extended fasts. In addition, when moulting and during development, penguins remain in the same colony and at the same terrestrial site, meaning that it is easy to track and observe them. In this study, we asked if prolonged fasting is associated with modulation of circulating ghrelin levels in southern rockhopper penguins (*Eudyptes chrysocome chrysocome*) during the annual adult moult and chick growth. Rockhopper chicks typically receive meals by both parents during the "crèche" stage, i.e. when both parents forage and chicks gather in groups in the colony, with an approximate provisioning rate of one feeding per day per parent. Parents return from their daily foraging trips in the afternoon or evening, meaning that chicks typically only receive one meal by one or both parents every 24h in this late developmental stage (Pütz et al., 2013; personal observations). Chicks are therefore subjected to a 24h fast between regularly receiving one meal every day. Moreover, like other penguin species, adult southern rockhopper penguins engage in a long fast during their annual moult, which in this species lasts three to four weeks (Pütz et al., 2013). When penguins moult, they are forced to stay ashore, as the feather renewal process greatly reduces insulation and consequently thermoregulation at sea and decreases diving abilities because of increased drag in the water (Groscolas and Cherel, 1992).

Here, we hypothesised that the levels of ghrelin would be higher at the beginning of the moult period to suppress the urge to forage and that hormonal levels would show a decrease at the end of the moult thereby signalling restored appetite. Furthermore, we predicted ghrelin levels in chicks to differ according to feeding state, with fed chicks having higher circulating ghrelin levels compared to unfed conspecifics, due to increased appetite.

2. Material and methods

2.1. Field methods

We conducted our fieldwork at the "Settlement Rookery" on New Island, Falkland Islands (Islas Malvinas, 51°42′56.6"S 61°18′43.6"W) from January 28th to February 18th, 2019. During this time of the year the colony typically hosts ca. 7000 pairs of southern rockhopper penguins, together with imperial cormorants (*Leucocarbo atriceps*), and black-browed albatrosses (*Thalassarche melanophris*).

We carried this study out in the late breeding season when chicks were already in an advanced crèche stage (ca. 60 days old), requiring both parent birds to forage in order to provide sufficient amount of food to their chicks. Our study period also comprised the major part of the three to four week-long moult of non-breeding, first year adults that started their annual moult in the beginning of the study period and finalised it by its end. We targeted rockhopper penguins in all the three aforementioned life history/ life cycle stages: chicks, breeding adults and non-breeding moulting adults. We divided chicks into two groups: individuals that had been fed by a parent (fed chicks) and chicks that were not fed (unfed chicks). In the fed chick group, we sampled chicks that had been fed 15 min before capture as the presence of food in the stomach and the initiation of the chemical breakdown of food items are a main factor in triggering the secretion/ suppression of gut hormones such as ghrelin (e.g. Murphy and Bloom, 2006; Suzuki et al., 2011). The unfed chick group included birds sampled in the morning, before they could be reached by their parents returning from a foraging trip. We caught adult breeding birds on their way to the colony upon returning from a foraging trip to ensure that they were still providing for offspring. We divided moulting individuals into two groups: early moult and late moult. Early moulting rockhopper penguins were caught at the beginning of the field season when most of their old feathers were not yet moulted (90% - 75% of old feathers left). We caught late moult birds at the end of our field season and identified them by the little proportion of unmoulted feathers left (30% - 0%). We assessed the degree of unmoulted feathers in early and late moult birds visually, before catching an individual (Fig. 1S, supplementary materials). For blood sampling, we rinsed 1 ml syringes with 0.5 M EDTA, took a maximum of 0.5 ml blood from the flipper, and then centrifuged the blood for 5 min at 2700 rcf to separate red blood cells from plasma. We then collected the plasma with a measuring pipette and added 1 M HCl at a 1:10 acid to plasma ratio to an aliquot of each sample for ghrelin measurements. We stored the remaining plasma untreated in separate tubes to be used for β-hydroxybutyrate (β–OHB) and triglyceride measurements to determine the fasting stage of individuals. Red blood cells were kept in Queen's lysis buffer for DNA extractions. We kept all plasma samples on ice until arriving at the field station where we transferred them into liquid nitrogen. We weighed all sampled penguins to the nearest g and measured flipper length (sesamoid bones to flipper tip) and foot length to the nearest mm after blood sampling. Additionally, we marked all sampled penguins with a

subcutaneous RFID microchip (Texas Trading, see Dehnhard et al., 2013) for individual recognition in case of recapture. Samples were kept in liquid nitrogen dry shippers and transferred to a −80 °C freezer upon arrival at our laboratory in Vienna.

All penguin capture, handling, tagging, and sampling procedures used in this study were approved by the Falkland Islands Government (Environmental Planning Office, Research Licence R15/2017).

2.2. Laboratory methods

1.) Plasma ghrelin measurements

Acylated plasma ghrelin concentration was measured at the National Cerebral and Cardiovascular Center Research Institute in Osaka, Japan by means of a radioimmunoassay (RIA) and following the methodology as previously described in Kaiya et al. (2007) and Goymann et al. (2017). In short, a primary antibody, which was raised against rabbit, and recognising the N-terminal part of octanoylated rat ghrelin (Gly1-Arg11) was used (final dilution of 1/5000000) and octanoylated chicken ghrelin (chicken ghrelin-26-C8; custom made in Daiichi Suntory Pharma Co. Ltd., Institute for Medicinal Research and Development, Gunma, Japan) was used as the standard peptide. The tracer consisted of 125I-(Tyr29)- rat ghrelin (15,000–20,000 cpm per tube), and free, and antibody-bound 125I-(Tyr29)- rat ghrelin was separated by adding an anti-rabbit IgG goat serum (Protein Purify Co., Ltd., Gunma, Japan), and the bound counts were measured by a gamma-ray counter (AccuFLEXγ8010, Nippon-RayTech Co., Ltd., Tokyo, Japan). The measurement was performed in one assay thereby inter-assay variance was not calculated, and the intra-assay variance amounted to <5%.

2.) β-hydroxybutyrate (β-OHB) measurements

Plasma β-OHB, the main ketone body in birds, is a measure of fatty acid oxidation in fasting animals and can be used to determine a shift from fatty acid to protein catabolism (Secor and Carey, 2011). It has also been proven to be a good indicator of fasting stage in penguins (Cherel et al., 1988b; Cherel et al., 1987).

Ketone body measurements were performed in duplicates with a β-OHB assay kit following the manufacturer's manual and recommendations (MAK041, Sigma-Aldrich). The assay is designed to produce a coupled enzyme reaction that results in a colorimetric product with a wavelength of 450 nm (measured with Multiskan GO microplate spectrophotometer, Thermo Scientific), which is proportional to the concentration of β-OHB in the sample.

3.) Triglyceride measurements

During fasts, triglycerides serve as an internal energy source and can be a measure of fasting status (Secor and Carey, 2011). We quantified plasma triglyceride concentration by using a triglyceride quantification kit (MAK266, Sigma-Aldrich) according to the instructions of the manufacturer.

In short, in this assay, triglycerides are converted to free fatty acids and glycerol. The glycerol is then oxidised to generate a colorimetric product measured at 570 nm. After subtracting the blank value and after usage of appropriate triglyceride standards to plot the standard curve, the amount of triglyceride present in the samples could be determined as an average of duplicates from the standard curve.

4.) Molecular sexing

As southern rockhopper penguins are not visibly sexually dimorphic we identified individual sexes by molecular sexing after Griffiths et al. (1998). We carried the amplifications for sexing-PCRs out in a total volume of 20 µl and by using extracted DNA from red blood cells, stored in Queen's lysis buffer. The PCR reaction contained: 5 µl AllTaq 4× master mix (Qiagen), 0.5 µl P2 primer (10 mM), 0.5 µl P8 primer (10 mM), 0.3 µl BSA (50 µg/ml; Invitrogen), 9.7 µl PCR-grade H_2O, 4 µl DNA (diluted to 20 ng/µl). We used a Proflex thermal cycler and PCR conditions were set as follows: an initial denaturation at 95 °C for a duration of 2 min was followed by 40 cycles of 95 °C for 2 s, 48 °C for 15 s and 72 °C for 10 s. The PCR product was cooled down to 12 °C once the PCR was finished. To separate double bands (females) from single bands (males) in the agarose gel pictures we added a digestion step after the PCR. The digestion included 5 µl of PCR product, 0.5 µl of *Hae* III (10,000 U/ml; New England Biolabs), and 0.5 µl of CutSmart Buffer 10× (New England Biolabs). This mixture was incubated at 37 °C for one hour. We separated PCR products by a 30-min gel electrophoresis at 113 V in a 2% agarose gel containing GelRed. Gel-electrophoresis was performed in TAE buffer.

2.3. Statistical analyses

We performed all statistical analyses in R Studio 2023.06.1+524 “Mountain Hydrangea” Release (Posit team, 2023) and R version 4.2.1 (R Core Team, 2022). To create linear models, we used the *lm* function from the native *stats* package. We performed post-hoc tests using *emmeans* from the *emmeans* package. We created all figures using the *ggplot2* and *cowplot* packages. We decided to use linear models as we only modelled continuous dependent variables and model residuals fit or were close to a normal distribution.

Flipper length is a common measure to scale individual body mass in penguins (e.g. Cappello and Boersma, 2021; Dobson et al., 2008; Robinson et al., 2005). However, due to the ongoing moult and the resulting feather wear, in our dataset individual flipper measurements varied significantly according to adult rockhoppers' status; i.e. breeding vs. early/ late moult ($F_{(2,43)}$=6.80, η^2=0.25, p<0.01), which disqualified the use of flipper length for scaling individual body mass in our data. We collected a second body size measurement, i.e. individual foot length, but this variable was not correlated with body mass ($F_{(1,47)}$=0.53, η^2=0.01, p=0.47), suggesting that foot length is not a reliable scaling variable. Therefore, when applicable we used non-scaled body mass for our analyses. Furthermore, we did not calculate a scaled body mass index for chicks as we did not know their age and indeces of condition allow to compare condition between individuals that are at the same stage of development, when the relationship between body size and limb size is stable. Thus, when applicable we used actual body mass in analyses regarding chicks.

We tested for differences in body mass between moulting and breeding adults using a linear model including body mass as dependent variable and moult state and individual sex as independent variables. We performed a pairwise post-hoc test including a Bonferroni correction for uncovering which moult states (i.e. early, late, none) differed from each other.

To test for differences in plasma ghrelin concentrations between breeding adults and adults in early and late moult, we created a linear model including ghrelin concentration (log-transformed to improve model fit) as dependent variable and moult state (i.e. early, late, none [= breeders]), and individual sex as independent variables. Thereafter, we used a post-hoc test with Bonferroni correction to uncover which moult states differed significantly from each other. To understand how plasma ghrelin concentration and body weight were distributed in moulting penguins over time (days of the moulting period), we created linear models using ghrelin concentration or body mass in moulting individuals as dependent variable and sampling date as independent variable.

To test for differences in β-OHB levels of adult moulting and breeding penguins, we created a linear model including circulating β-OHB as dependent variable and moult state and sex as independent variables. We applied the same independent variable model structure to test for differences in circulating triglyceride levels in adult rockhoppers and applied post-hoc tests with Bonferroni correction to uncover mean differences between dependent variables in the different moult states. We tested for an effect of β-OHB and triglycerides on ghrelin levels by the use of one-way ANOVAs.

We checked whether body mass in chicks differed according to feeding status by using a linear model including body mass as dependent variable and feeding status and sex as

independent variables. We created another linear model to test for potential differences in circulating ghrelin levels in fed and unfed chicks. Here we used ghrelin concentration as dependent variable and feeding status and individual sex as independent variables. To determine whether β-OHB levels differed in the fed and unfed chick groups we created a model using β-OHB level as dependent variable and feeding status and individual sex as independent variables. We used the same modelling structure for testing for differences in triglyceride levels (log-transformed to improve model fit) in fed and unfed chicks. To understand if ghrelin levels in chicks were influenced by β-OHB or triglycerides, we created one-way ANOVAs.

As we could not determine a statistically significant difference in ghrelin levels, β-OHB and triglyceride levels or body mass in fed and unfed chicks, we pooled chick data for comparisons against breeding adults. We used linear models to test whether ghrelin levels, β-OHB or triglycerides (log-transformed to improve model fit) differed between chicks and adults.

We checked model fit of all described models visually by the use of standard diagnostic plots looking at residual distribution.

3. Results

Adult rockhopper penguins' body mass was significantly influenced by moult state ($F_{(2,42)}$=48.13, η^2=0.70, p<0.001) with individuals in early moult having highest body mass (m=3.34 kg, $ci_{95\%}$=3.15–3.54), individuals in late moult having lowest body mass (m=2.03 kg, $ci_{95\%}$=1.85–2.22) and breeding birds having intermediate body mass (m=2.81 kg $ci_{95\%}$=2.58–3.03) (Fig. 1A.). Individual sex had no influence on body mass in adult penguins ($F_{(1,42)}$=0.43, η^2=0.003, p=0.52).

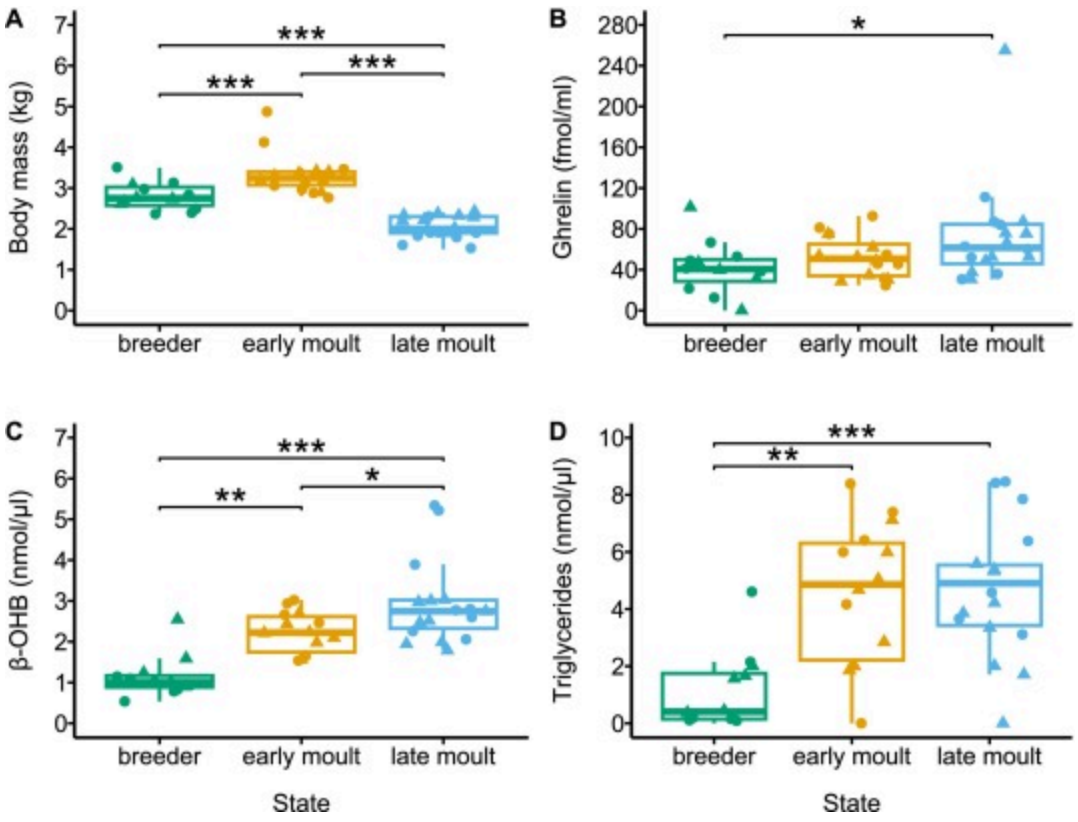

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Fig. 1. Comparison of body mass, circulating ghrelin, β-OHB, and triglycerides in moulting and breeding adults. Box and whisker plots showing median and interquartile range (IQR) of (A) body mass (kg), (B) plasma ghrelin levels (fmol/ml) (C) plasma β-hydroxybutyrate (β-OHB) levels (nmol/µl) and (D) triglycerides (nmol/µl) in adult southern rockhopper penguins. The green boxes show individuals that still provided for their chicks and were not moulting (= breeders; n=12), the yellow boxes show individuals sampled in the early moult ($n_{\text{body mass}}$=16, $n_{\text{ghrelin, β-OHB, triglycerides}}$=14) and the blue boxes show individuals in late moult state ($n_{\text{body mass, ghrelin}}$=18, $n_{\text{β-OHB}}$=17, $n_{\text{triglycerides}}$=16). The symbols represent males (closed triangles) and females (closed dots). Ends of whiskers represent highest/ lowest values within 1.5*IQR. Statistically significant relationships are marked with brackets and asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In adult rockhoppers, ghrelin levels were influenced by moult state ($F_{(2,42)}$=3.86, η^2=0.16, p=0.03; Fig. 1B) and differed significantly between individuals in late moult and breeding birds (t-ratio=−2.83, p=0.02; Fig. 1B). Plasma ghrelin levels did not differ between individuals in early and late moult (t-ratio=−0.98, p=1.00; Fig. 1B) or breeding penguins and ones in early moult (t-ratio=1.90, p=0.20; Fig. 1B). Individual sex did not influence ghrelin

levels in adult rockhoppers ($F_{(1,42)}$=0.40, η^2=0.01, p=0.53). Body mass significantly decreased over time in moulting penguins ($F_{(1,34)}$=82.11, η^2=0.71, p<0.001). On the contrary, plasma ghrelin levels increased significantly over time from early to late moult ($F_{(1,34)}$=6.46, η^2=0.16, p=0.02).

Plasma β-OHB levels differed significantly according to moult state in adult rockhopper penguins ($F_{(2,39)}$=19.07, η^2=0.50, p<0.001; Fig. 1C) but were not influenced by individual sex ($F_{(1,39)}$=0.97, η^2=0.01, p=0.33). Mean β-OHB values of breeding individuals were significantly lower than ones of birds in early moult (t-ratio=3.70, p<0.01; Fig. 1C) and late moult (t-ratio=−6.24, p<0.001; Fig. 1C). Mean β-OHB of penguins in late moult was marginally higher compared to levels of birds in early moult (t-ratio=−2.51, p=0.05; Fig. 1C). Plasma ghrelin and β-OHB levels were positively correlated in adult rockhopper penguins ($F_{(1,43)}$=5.18, η^2=0.11, p=0.03).

Circulating triglyceride levels were influenced by moult state in adults ($F_{(2,38)}$=9.96, η^2=0.35, p<0.001; Fig. 1D) but individual sex had no influence on triglyceride levels ($F_{(1,38)}$=2.35, η^2=0.04, p=0.13). Triglyceride levels of adult breeders were significantly lower than levels of individuals in early moult (t-ratio=−3.84, p<0.01; Fig. 1D) or late moult (t-ratio=−4.25, p<0.001; Fig. 1D). There was no significant relationship between plasma ghrelin and triglyceride levels in adult rockhopper penguins ($F_{(1,42)}$=1.26, η^2=0.03, p=0.27).

In chicks, body mass did not differ according to feeding status or sex, however there was a trend towards body mass being lower in unfed chicks compared to fed individuals (feeding status: $F_{(1,15)}$=3.65, η^2=0.20, p=0.08; sex: $F_{(1,15)}$=0.01, η^2<0.001, p=0.92). Plasma ghrelin levels were neither influenced by the feeding status (fed vs. unfed; $F_{(1,15)}$=0.31, η^2=0.02, p=0.59) nor individual sex ($F_{(1,15)}$=0.68, η^2=0.04, p=0.42) in chicks. Rockhopper chicks' ghrelin levels were further not influenced by β-OHB ($F_{(1,16)}$=0.17, η^2=0.01, p=0.69) or triglyceride levels ($F_{(1,16)}$=2.18, η^2=0.12, p=0.16). β-OHB levels did not differ significantly between fed and unfed chicks ($F_{(1,15)}$=0.44, η^2=0.03, p=0.52) and β-OHB levels were not influenced by individual sex ($F_{(1,15)}$=0.23, η^2=0.01, p=0.64). Also, chicks' triglyceride levels were not significantly influenced by feeding state ($F_{(1,15)}$=1.64, η^2=0.13, p=0.22) or individual sex ($F_{(1,15)}$=2.41, η^2=0.13, p=0.14).

When comparing ghrelin levels in chicks overall (i.e. fed and unfed states were grouped in this analysis due to not finding differences in measured hormonal or metabolite status as reported in previous paragraph) against adult breeders, we found a significant difference with adults having higher plasma ghrelin levels than chicks ($F_{(1,29)}$=15.44, η^2=0.35, p<0.001; Fig. 2). Furthermore, breeding adults had higher β-OHB levels than chicks overall

($F_{(1,29)}$=7.81, η^2=0.21, p<0.001) but there was no significant difference in triglyceride levels between adult breeders and chicks overall ($F_{(1,29)}$=0.11, η^2=0.004, p=0.74).

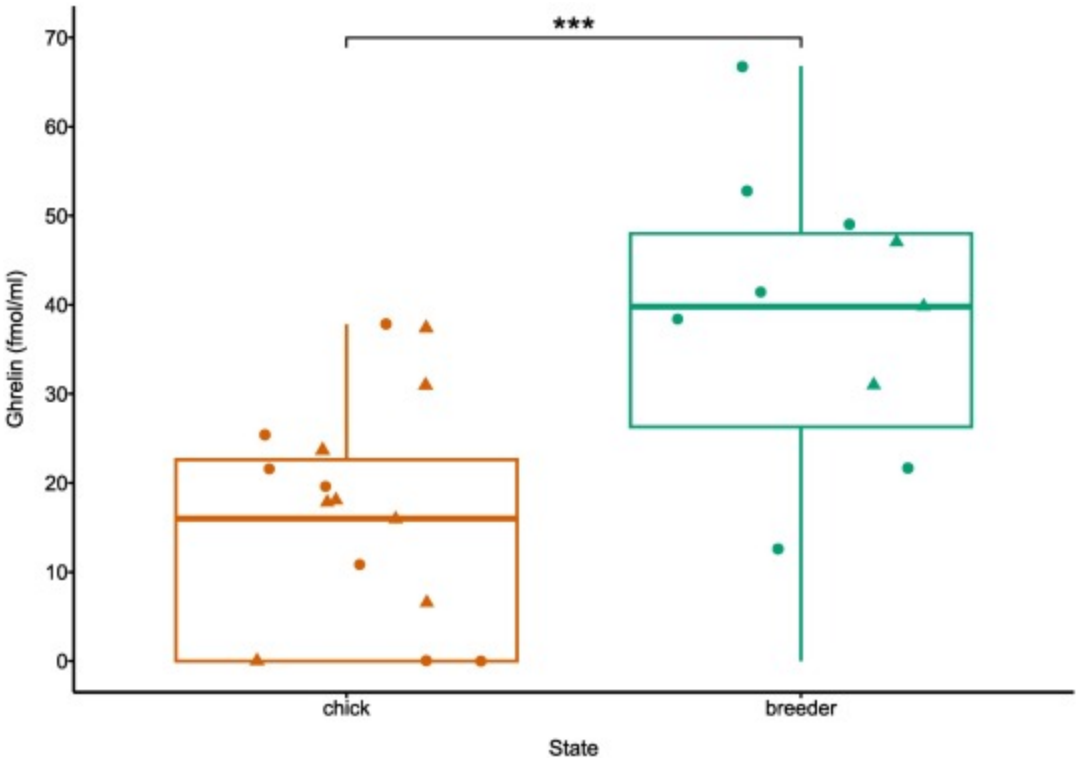

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Fig. 2. Comparison of circulating ghrelin between chicks and adults. Box and whisker plots show median and interquartile range (IQR) of plasma ghrelin levels (fmol/ml) in chicks and adult southern rockhopper penguins. The orange boxes show values for chicks (n=19) and the green boxes show data for adults that still provided for their chicks (= breeders; n=12). The symbols represent males (closed triangles) and females (closed dots). Ends of whiskers represent highest/ lowest values within 1.5*IQR. The statistically significant relationship (p≤0.001) is highlighted with a bracket and asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this paper, we show that adult rockhopper penguins maintained stable levels of circulating ghrelin throughout their naturally occurring weekslong fasting period associated with moult. Birds in an advanced moult state had higher concentrations of ghrelin compared to active adult breeders, which had a body mass intermediate between birds in early moult, that had accumulated fat to sustain the prolonged fast, and those in late moult,

which had lost body mass caused by fasting. Concentrations of key metabolites in late moult individuals indicate that these animals were still in good body condition and had not entered the critical late phase of moult and fasting that requires immediate ingestion of food to prevent starvation. Ghrelin concentrations in rockhopper chicks did not vary according to feeding status, however, they were lower than those found in adults.

Moulting first year adults in the late stage of moult had the highest plasma ghrelin levels and lowest body mass in this study, however, contrary to our expectations there was no difference in plasma ghrelin between individuals that had recently started moult and those that were at an advanced stage of moult (Fig. 1B). To our knowledge, no other study has looked at ghrelin levels during avian moult and there is a lack of studies considering digestive hormones in the context of naturally occurring extended fasting periods. Studies conducted in recent years show that low circulating ghrelin levels in birds are associated with higher food intake and lower body condition. High circulating levels of the hormone were found to be associated with decreased appetite and a higher fat score in birds (Buyse et al., 2009; Furuse et al., 2001; Geelissen et al., 2006; Goymann et al., 2017; Saito et al., 2005; Shousha et al., 2005), whereas in contrast to birds, low ghrelin levels are associated with satiety in mammals (Kojima and Kangawa, 2005). In golden-mantled ground squirrels (*Callospermophilus lateralis*) plasma ghrelin levels are low during hibernation, a state of natural fasting, which may be important in controlling the energy balance during this period of the life cycle and keeping the animals from arousing and going into a state of euthermia (Healy et al., 2010). Our results show no reduction of ghrelin levels in penguins in the late phase of moult, suggesting that maintaining high plasma ghrelin in late moult may supress the urge to feed and enter the sea water. Returning to sea with incompletely moulted feathers would prove fatal for penguins as they require a smooth and waterproof coat to maximise hydrodynamics and thermal insulation during swimming and diving (Groscolas and Cherel, 1992; Lovvorn et al., 2001; Wilson et al., 1992). Interestingly, studies on domestic fowl found that ghrelin levels are already increased after short fasting events (12–24h) (Kaiya et al., 2007; Ocłoń and Pietras, 2011; Shousha et al., 2005). These results may further hint to ghrelin playing a crucial role during fasting events. In penguins, circulating ghrelin levels might only decrease markedly when individuals are close to or reach a threshold negative energy balance where endogenous energy stores are at a minimum or depleted, as already shown for many other factors involved in metabolic regulation (e.g. Cherel et al., 1988b; Groscolas and Robin, 2001; Robin et al., 1998; Secor and Carey, 2011). Penguin moult fast consists of three distinctive phases: The first two phases are characterised by body fat mobilisation and protein sparing combined with low circulating corticosterone levels but high thyroxine (T_4) and triiodothyronine (T_3) levels. In contrast, the third phase of fasting consists of protein catabolism, as fat stores are depleted,

and high circulating corticosterone but low T_4 and T_3 levels. It is important to mention that the third fasting stage is often detrimental if individuals do not manage to restart feeding in a timely manner (Groscolas and Cherel, 1992; Groscolas and Robin, 2001). From studies on king penguins it is known that β-OHB ketone levels are highest in phase two of fasting and drastically drop throughout phase three due to protein catabolism, meaning that this metabolite is a good indicator of fasting stage (Bernard et al., 2002; Cherel and Le Maho, 1985; Cherel et al., 1988a; Cherel et al., 1988c). Furthermore, due to catabolism of lipid stores, penguin triglyceride levels decrease at the end of a fasting period (Groscolas, 1982; Groscolas and Robin, 2001). As plasma β-OHB concentrations were highest in the late moulting group in our study (Fig. 1C) and we could not detect significant differences in triglyceride levels (Fig. 1D), we deduce that penguins in our study had not yet entered phase three of fasting and still carried sufficient body fat reserves. In future studies it will be crucial to capture this last phase of fasting to understand if the complete depletion of body fat reserves brings about substantial changes in ghrelin levels and to draw a more complete picture of ghrelin patterns and ghrelin's potential interactions with other hormones throughout fasting.

We found a positive correlation between ghrelin and β-OHB concentrations in adult rockhoppers, suggesting a link between ghrelin and β-OHB. Indeed, in humans, elevated β-OHB levels seem to be correlated with lower ghrelin concentrations (Stubbs et al., 2018; Vestergaard et al., 2021). Yet, to the best of our knowledge, the results reported here are the first ones to reveal a link between ghrelin and β-OHB in an avian species and further studies are needed to better understand this hormone-metabolite relationship.

Based on previous findings in birds we hypothsised that rockhopper chicks' plasma ghrelin concentrations would differ according to their feeding status, i.e. whether an individual was recently provided with food by a parent bird or whether it was not yet provided with food and had not fed since the last food intake. However, contrary to our expectations, we could not find a significant difference between fed and unfed chicks regarding their ghrelin, body mass, β-OHB or triglyceride concentrations. This suggests that the daily ca. 24 h fast of rockhopper chicks is not comparable to a prolonged fast in adults. However, it should be mentioned that the time interval between feeding and blood sampling in the fed chick group (15 min) may have been not sufficiently long to detect a significant feeding-induced change in ghrelin, β-OHB or triglyceride levels. Since we could not find any previous report of penguin ghrelin profiles, we do not have information on the dynamics of ghrelin in relation to food intake. From past studies in humans and sheep, we know that circulating ghrelin levels start changing immediately after having a meal, however, it takes roughly 1–2 h after food intake for the hormone to reach a peak (Cummings et al., 2001;

Takahashi et al., 2012). In future, it will be interesting to collect blood samples at different time points after chick feeding to determine the time curve of ghrelin levels after feeding events.

Chicks had overall lower ghrelin levels compared to adults (Fig. 2). This result seems to hint to relatively lower levels of ghrelin in rockhopper chicks independent of recent feeding events. In mice, gastric ghrelin mRNA levels start increasing ca. three-fold in the first week postpartum, peak at day 21 and remain elevated until ca. 120 days after birth (Liu et al., 2002). Previous work on chicken offers evidence that the ghrelin system undergoes changes across development. For example, Yamato et al. (2005) found that neonatal chickens have a lower density of ghrelin-immunopositive cells in their proventriculus compared to adult birds and further have low plasma ghrelin levels after hatching. This result was confirmed by Yu et al. (2016) who showed that ghrelin-immunopositive cell density in the proventriculus of chicken older than 72 h was increased in comparison to younger individuals, and the authors further found that plasma ghrelin levels in neonatal chickens were lower compared to those of birds of 72–120 h of age. However, both studies mention that the increase in ghrelin levels post hatching is additionally influenced by the initiation of food intake. Another study on African ostriches (*Struthio camelus*) at different developmental stages and with regular access to food, found that the number of ghrelin-immunopositive cells increased steadily between postnatal day 1 and day 45, when numbers peaked (Wang et al., 2009). Altogether, the above findings are in line with the hypothesis that baseline ghrelin levels increase during rockhopper penguin development.

5. Conclusion

Our novel study on circulating ghrelin levels in a seabird species during a prolonged fast associated with moult contributes to our still scarce knowledge of the ghrelin system in wild birds. In penguins, ghrelin appears to be differently regulated at different developmental stages and at different annual cycle stages. Our results are compatible with a role of ghrelin in controlling hunger during the later stages of moult, which requires fasting in seabirds because of reduced thermoregulatory efficiency of moulting feathers in water. Further studies should investigate the interplay between ghrelin, glucocorticoids, and thyroid hormones in regulating behavioural and physiological mechanisms associated with long fasting during moult.

The following are the supplementary data related to this article.

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Figure 1S. Photos showing moult states of southern rockhopper penguins during the sample collection period between 28th January and 18th February 2019. The top photo (A) is representative of a penguin in early moult state (all/most old feathers are still visible; new feathers are still covered). The bottom photo (B) is representative of an individual in late moult state (only few old feathers are still visible; most new feathers are already visible).

Ethics statement

All penguin capture, handling, tagging, and sampling procedures used in this study were approved by the Falkland Islands Government (Environmental Planning Office, Research Licence R15/2017).

CRediT authorship contribution statement

Julia Slezacek: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Petra Quillfeldt:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Hiroyuki Kaiya:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Alba Hykollari:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Leonida Fusani:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

Recommended articles

Data availability

Data will be made available on request.

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