Comparative Biochemistry and Physiology, Part A 247 (2020) 110742

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Trade-off between aerobic performance and egg production in migratory macaroni penguins

Isabelle Jubinville[a], Tony D. Williams[b], Phil N. Trathan[c], Glenn T. Crossin[a,*]

[a] Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada
[b] Biological Sciences Department, Simon Fraser University, Burnaby, BC V5A 1S6, Canada
[c] British Antarctic Survey, Natural Environment Research Council, Cambridge CB3 0ET, United Kingdom

ARTICLE INFO

Keywords:
Migration
Hematocrit
Reticulocyte index
Vitellogenesis

ABSTRACT

When successive stages of an organism's life-history overlap, conflicts and trade-offs can emerge due to competition among physiological pathways. For example, long periods of sustained locomotion in migrating birds are supported by the androgenic up-regulation of aerobic factors, such as new red blood cell production and hematocrit. However, towards the end of migration, many female birds begin up-regulating 17ß-estradiol (E_2) to support vitellogenesis and egg production, but E_2 secretion is known to have suppressive effects on red blood cell production (anti-erythropoiesis). We explored potential trade-offs between factors related to aerobic performance (hematocrit, reticulocyte index) and the expression of factors related to E_2-mediated vitellogenesis (i.e. yolk precursor production) in female macaroni penguins (*Eudyptes chrysolophus*), a species in which the physiologies controlling egg production and migratory activity run simultaneously (e.g. females experience a migratory conflict). We collected blood samples from penguins immediately upon their return to the colony, prior to egg laying. Hematocrit was elevated when the penguins returned to the colony (50.05% ± 3.40 SD), which is similar to pre-laying values observed in other migratory bird species. Furthermore, mean reticulocyte levels were elevated (34.87% ± 2.34), which is the highest level yet recorded in birds. Similarly, both plasma vitellogenin and yolk-targeted very low density lipoprotein levels were upregulated (2.30 ± 0.06 μg Zn ml^{-1}, and 9.70 ± 0.19 mmol l^{-1}, respectively), indicating that penguins were reproductively active and producing eggs during migration and upon arrival on land. As predicted, a negative relationship between hematocrit and plasma vitellogenin was found, but we found no evidence to suggest that birds were experiencing reproductive anemia. Alternatively, we attribute the negative relationship to a hemodilution effect of yolk precursor secretion into circulation. It appears that female macaroni penguins are able to preserve hematocrit levels and new red blood cell production when migratory activity overlaps with reproductive processes.

1. Introduction

Aerobic performance, or the ability to deliver oxygen to tissues, is a critical determinant of an animal's physiological endurance during strenuous activity (Joyner, 1991; Calbet et al., 2006). In migratory birds, aerobic performance is needed to sustain work over long distances and time intervals (Morton, 1994; Piersma et al., 1996), particularly when stopover sites are few or rest periods infrequent. To bolster performance, birds can up-regulate aerobic measures prior to and during migrations to distant breeding territories (Holmgren and Hedenström, 1995). One important component of aerobic performance is hematocrit (Hct), which is the relative proportion of the total blood volume occupied by red blood cells (erythrocytes). Hct is often used as a reliable metric of aerobic performance and oxygen transport capacity, and is up-regulated in several avian species at the onset of migrations (Banerjee and Banerjee, 1977; Morton, 1994; Viscor et al., 1985; Piersma et al., 1996; Landys-Ciannelli et al., 2002; Yap et al., 2019; Minias, 2020). Functionally, Hct up-regulation results in an increase in the concentration of circulating hemoglobin, which is central to the binding and delivery of oxygen to muscles and other tissues. The physiological control of erythrocyte production and Hct up-regulation in birds has been linked to increased testosterone secretion via seasonal hypothalamic-pituitary-gonadal axis (HPG) activation (Wingfield et al., 1990; Tonra et al., 2011a).

The seasonal activation of the HPG axis is required for reproductive development in birds, but it also has some influence on aerobic factors. In response to photoperiodic cues, gonadotropin release stimulates testosterone production in both the testes and ovaries, principally for

* Corresponding author.
E-mail address: gtc@dal.ca (G.T. Crossin).

https://doi.org/10.1016/j.cbpa.2020.110742
Received 2 February 2020; Received in revised form 1 June 2020; Accepted 3 June 2020
Available online 11 June 2020

gamete production, but in male birds especially testosterone can have pleiotropic effects on physiological systems related to migratory preparedness (Wingfield et al., 1990; Gwinner, 1996; Dawson et al., 2001; Tonra et al., 2011a; Tonra et al., 2011b). For example, Tonra et al. (2011b) documented a positive correlation between testosterone and Hct in migratory male redstarts (*Setophaga ruticilla* - a neotropical migrant species), while also demonstrating a positive relationship between testosterone and breeding phenology: birds with high testosterone (and high Hct) arrived earliest at breeding grounds. The effect of testosterone secretion on erythrocyte production thus seems adaptive, as it suggests that an increase in aerobic performance is a key aspect of migratory preparedness, and thus key to migratory success and fitness. An additional way to gauge the aerobic condition of individuals is via the reticulocyte index (RI; e.g. Wagner et al., 2008), which is the relative number of new red blood cells put into circulation. Collectively, Hct and RI provide two means for assessing individual aerobic and/or migratory condition, at least in males. Indeed, most of our working knowledge about the environmental and physiological control of seasonal phenomena in birds comes from studies of males (Williams, 2012); much less is known about females.

In female birds, seasonal activation of the HPG axis also results in the conversion of testosterone to 17ß-estradiol (E_2). It is well known that E_2 and other estrogens can suppress new red blood cell production (anti-erythropoiesis; Williams et al., 2004; Wagner et al., 2008), which in breeding females can lead to reproductive anemia (Crossin et al., 2010; Williams, 2012). Specifically, estrogens inhibit the differentiation, proliferation, and survival of white and red blood cell precursors at their source of production in the bone marrow (Blobel and Orkin, 1996; Perry et al., 2000). In a study of egg-producing zebra finches (*Taeniopygia guttata*), Wagner and Williams (2007) experimentally prevented the development of anemia via treatment with the estrogen receptor antagonist tamoxifen. This provided mechanistic support to the idea that E_2-dependent suppression of erythropoiesis is responsible for the reduced Hct and reproductive anemia of breeding female birds (Wagner and Williams, 2007). However, for migratory birds, this presents a potential physiological conflict: how can migrant female birds maintain rigorous aerobic activity while simultaneously up-regulating the E_2-mediated pathway necessary for egg production?

To date, most of what we know about physiological conflicts in migrant female birds has been gleaned from studies of Eudyptid penguins, i.e. those from the genus *Eudyptes* (macaroni penguins – *Eudyptes chrysolophus* [Crossin et al., 2010; Crossin and Williams, 2016], and rockhopper penguins – *E. chyrsocome* [Crossin et al., 2012a]; but see also common eiders, *Somateria mollissima* [Hennin et al., 2016]). Eudyptid penguins are unique among birds by exhibiting a breeding system in which females lay an invariant 2-egg clutch, characterized by extreme egg size dimorphism (ESD) and obligate brood reduction (Stein and Williams, 2013; Crossin and Williams, 2016) which results in a functional clutch size of one. Such extreme ESD, in which the first laid A-egg is 56–84% smaller than the second laid B-egg (Williams, 1995; Crossin et al., 2013), occurs in no other bird species. Experimental studies with penguins have shown that the rapid yolk development phase of egg production takes ~16 days (e.g. Grau, 1982; Astheimer and Grau, 1985), but the females of some Eudyptid species like the macaroni penguin can arrive and begin laying as few as 7 days later (Williams, 1990; Crossin et al., 2010). ESD is therefore putative evidence of a conflict between migration and reproduction that results from a constraint on the E_2-mediated vitellogenic pathway underlying egg production (Crossin et al., 2010; Crossin et al., 2012b; Crossin and Williams, 2016). In the face of this conflict, how or whether the suppression of estrogenic pathways and their associated anti-erythropoietic effects influences red blood cell dynamics and aerobic state of a Eudyptid penguin is currently unknown. A reasonable prediction is that the suppression of vitellogenesis during migration facilitates or permits new red blood cell production during an aerobically demanding time.

The aim of this study is to explore the relationships between E_2-derived yolk precursors (vitellogenin [VTG], yolk-targeted very low density lipoprotein [VLDLy]) and aerobic measures (Hct, RI). We test the hypothesis that a trade-off between aerobic performance and reproductive investment exists in female macaroni penguins, which would favour aerobic performance when migratory activity and egg production occur simultaneously. We therefore predict that 1) Hct levels measured in migrant penguins arriving at a breeding colony will be at levels typical of other migrating birds (~50% packed cell volume (PCV; Piersma et al., 1996; Landys-Ciannelli et al., 2002; Hennin et al., 2016), and 2) that Hct will be negatively correlated with yolk precursor levels – VTG and VLDLy. Because migratory performance is critical to the at-sea survival and timely arrival of penguins to breeding colonies, we would expect female macaroni penguins to have some means for buffering or decoupling red blood cell production from the suppressive, anti-erythropoietic effects of E_2 secretion (Wagner et al., 2008). The known suppression of vitellogenesis underlying ESD in Eudyptid penguins could thus be the key event that preserves aerobic function at a time when migratory and reproductive processes run simultaneously. If a negative correlation between Hct and VTG or VLDLy is observed, we would then predict that 3) the RI will be elevated in these fast- and far-migrating penguins (Bost et al., 2009), as reduced E_2-mediated processes would permit new red blood cell production, resulting in elevated Hct and RI.

2. Methods

This study was conducted at a macaroni penguin colony of ~40,000 breeding pairs at Bird Island, South Georgia (54°01′S, 38°02′W). Forty-eight female macaroni penguins were sampled within 24 h of their arrival at the breeding colony, prior to laying (Crossin et al., 2010). To ensure the immediate capture of females after their arrival, ~60 lone males sitting on nest sites were marked with water-based paint. Because males arrive at the colony 1–2 weeks before females, we then monitored the nests every day until females arrived at each nest (i.e. penguins without paint marks). We then captured the females using a hand-held net, and we immediately collected blood samples and morphometrics. Sample collections occurred from 2 to 10 November 2008. All sampling protocols were approved by the British Antarctic Survey, with permits issued through the Canadian Council for Animal Care (Simon Fraser University Animal Care Permit 897B-8).

From each penguin, a 2 ml blood sample was taken from a branchial vein using a heparinized syringe, fitted with a 25 gauge needle. From this 2 ml blood sample, 5 µl subsamples were collected with capillary tubes and centrifuged for 5 min at 10,000*g* to determine Hct (%). For hemoglobin analysis (Hb, g dl^{-1} whole blood), 5 µl subsamples of whole blood were diluted in 1.25 ml Drabkins reagent (D5941, Sigma-Aldrich, Oakville, Ontario) and absorbance measured at 540 nm (cyanomethoglobulin method, as per Wagner et al., 2008). Additional 5 µl whole blood subsamples were used to create blood smears on glass slides. These were stained with new methylene blue to identify reticulocytes (Reticulocyte stain R4132, Sigma-Aldrich Canada, Oakville, Ontario). The remaining blood was centrifuged for 5 min at 10,000*g*; the resultant blood plasma was pipetted into 0.6 ml cryovials and stored at − 20 °C until analysis. All blood samples were collected on average in 2.4 min (range 1.8–3.2 min). Body mass (± 10 g) was then measured using a 10 kg Pesola spring scale (penguin suspended temporarily in a bag). The entire handling procedure averaged 7.4 min for all penguins (range 5.6–8.3 min).

Plasma samples were assayed for vitellogenic zinc (Zn; zinc kit, Wako Chemicals) as an index of vitellogenin (VTG). Plasma was also assayed for total triglycerides (glycerol reagents A and B, Sigma-Aldrich Canada, Oakville, Ontario, Canada) as an index of yolk-targeted very low density lipoprotein (VLDLy), another indicator of estrogen stimulation and reproductive development in females (Crossin and Williams, 2020). All assays were measured using a Biotek 340i microplate reader. A domestic hen (*Gallus domesticus*) plasma pool was used as a control

within each VTG and VLDLy assay, to provide intraassay coefficients of variation (VTG = 5.4% to 7.1%; VLDLy = 5.5% to 6.9%).

Blood smears (i.e. slides) were preserved with glass cover slips affixed with Permount (SP15–500, Fisher Scientific Canada, Ottawa, Ontario, Canada) prior to microscopy to prevent cell displacement and damage caused by oil immersion. Slides were viewed under 1000× oil immersion microscopy, and the number of reticulocytes per 1000 erythrocytes was recorded. RI was calculated as a percentage, using an Hct correction formula (Riley et al., 2001), with an expected Hct value of 50% (Wagner et al., 2008).

$$RI = \text{reticulocyte\%} * (\text{observed Hct}/\text{expected Hct})$$

Statistical analyses were performed using R (version 3.3.1; 2016-06-21). To quickly determine whether relationships between aerobic measures and yolk precursors were present in our data, pairwise Pearson's correlations were calculated between all variables (Hct, RI, VTG, VLDLy), and residuals were examined for normality using Shapiro-Wilk tests. Generalized linear models with Gaussian response distributions were used to examine Hct levels relative to several explanatory variables: Julian date of arrival at the breeding colony, an estimate of migratory "overlap" (i.e. the time interval between colony arrival and laying), body mass, VTG, and VLDLy. AIC and ΔAIC values were used to determine the most parsimonious models. Similar models were run using RI as the response variable.

3. Results

A comparison of the blind duplicate counts of all blood smears showed that there was no significant difference between counts (Student's paired *t*-test, $T = 0.78$, df = 43, $P = .44$; correlation between pairs = 0.90), so a mean RI count was calculated for each bird. Mean RI was 34.87% ± 2.34 SEM. Additionally, mean Hct at colony arrival was 50.05% ± 0.49, while mean Hb was 24.09 ± 0.31 g dl^{-1}. Regarding yolk precursors in the blood plasma, mean VTG was 2.30 ± 0.06 μg Zn ml^{-1}, and mean VLDLy was 9.70 ± 0.19 mmol l^{-1}. Mean body mass was 4.99 ± 0.04 kg at colony arrival. Pairwise Pearson's correlation coefficients for all physiological variables, and the associated *P*-values, are presented in Table 1, which confirm the predicted negative relationship between E_2-derived yolk precursors and Hct (Table 1; Fig. 1).

Generalized linear models examining variation in Hct confirmed a negative relationship with yolk precursor levels, with VTG alone as the most predictive independent variable (Table 2; VTG parameter estimate −3.39 ± 1.19, $t = -2.83$, $P = .003$).

GLMs examining variation in RI identified VLDLy as the most parsimonious explanatory variable, although the parameter estimate was not significant (Table 2; VLDLy parameter estimate 1.54 ± 1.94, $t = 0.79$, $P = .433$).

Qualitatively, mean RI in the penguins upon their arrival at the colony, at the end of migration, was high (34.87 ± 2.34%), relative to RI values measured in other bird species to date, at various breeding and non-breeding stages (Table 3).

4. Discussion

The macaroni penguins in this study arrived at their nesting sites 5–14 days before laying (Williams, 1990; Crossin et al., 2010), so egg production was clearly occurring at sea during the late stage of migration prior to landfall. Furthermore, we identified a negative correlation between VTG and Hct, consistent with prediction. It could thus be predicted that both Hct and RI would show signs of E_2-mediated suppression (Wagner et al., 2008). However, despite the known suppressive effects of estrogen secretion on new red blood cell production, neither Hct nor RI appeared to be at levels that would indicate E_2-suppression, independent of the fact that females were actively engaged in egg production during migration. Upon arrival on land, females had up-regulated levels of the yolk precursors VTG (2.30 ± 0.06 μg Zn ml^{-1}) and VLDLy (9.70 ± 0.19 mmol l^{-1}). We know from previous studies of *Eudyptes* penguins that the physiology of egg production is constrained as a result of a migratory carryover effect, which underlies their extreme egg size dimorphism (e.g. Crossin et al., 2010). Although the negative relationship between VTG and Hct is consistent with our prediction, we cannot conclude that this is due to E_2 suppression of erythrocyte production given that no females were experiencing Hct levels indicative of 'reproductive anemia' (Williams, 2012). Hct levels in these female macaroni penguins were in fact similar to those measured in other migrating penguins like Adélie penguins (*Pygoscelis adeliae*) (Vleck et al., 2000), as well as other long-distance migrating birds (e.g. bar-tailed godwits, *Limosa lapponica taymyrensis* [Landys-Ciannelli et al., 2002]; black-browed albatrosses, *Thalassarche melanophrys* [Crossin et al., 2012b]). We also found that the macaroni penguins in this study had an elevated RI relative to those measured in other non-migratory birds outside of the breeding season (Table 3). This suggests that erythropoiesis was not suppressed in the female penguins, but indeed active, which is also consistent with prediction. Unfortunately, we did not collect blood for RI analysis later in the season, so we could not compare between life history stages. We therefore assembled reticulocyte data from the literature for a variety of bird species in various life history contexts (Table 3), which shows that mean RI measured in female macaroni penguins was nearly 10-fold higher than other non-migratory, non-reproductive penguin species (Table 3). However, we note that the majority of studies that have examined relationships between E_2-pathways and red blood cell dynamics have been conducted on non-migratory birds during the egg laying period, often in captive, laboratory settings (see Table 3).

At first glance, it would appear that the negative correlation that we observed between Hct and VTG in macaroni penguins (Fig. 1) conforms to the idea of erythrocyte suppression by E_2 secretion and vitellogenic pathways. Other explanatory variables which we included in our GLMs did little to improve the fit between Hct and VTG (Table 2). E_2-mediated vitellogenic pathways underlying egg production were unequivocally operating in the penguins that we sampled – based on plasma VTG and VLDLy levels – yet Hct was high at ~50% and RI was also elevated at ~35%, which to our knowledge is the highest level yet measured in any bird species (Table 3). It therefore appears that by some unknown mechanism macaroni penguins are potentially resistant to the anti-erythropoetic effects of E_2 secretion during migration. In the absence of clear indicators of anemia, the negative correlation between

Table 1
Pearson's correlation coefficients and *P*-values for pairwise linear correlations among blood parameters measured in female macaroni penguins. Significant relationships are shown in bold.

	Hct	Hb	RI	VTG	VLDLy
Hct	–	–	–	–	–
Hb	**0.49 (*P* < .01)**	–	–	–	–
RI	0.06 (*P* = .75)	0.12 (*P* = .21)	–	–	–
VTG	**−0.43 (*P* < .01)**	0.12 (*P* = .20)	0.09 (*P* = .42)	–	–
VLDLy	0.16 (*P* = .15)	0.14 (*P* = .77)	0.15 (*P* = .69)	0.17 (*P* = .14)	–

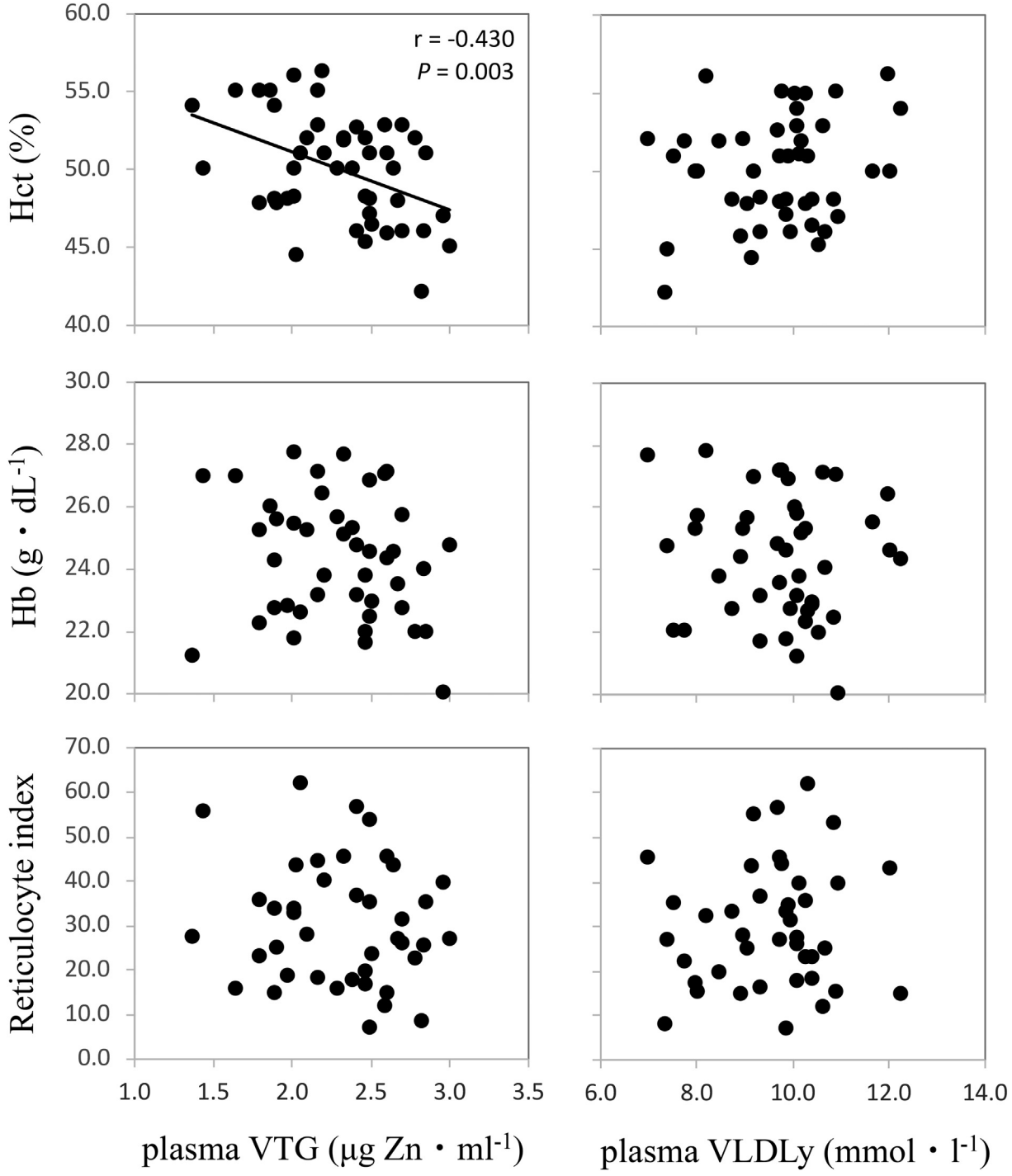

Fig. 1. Relationship of the yolk precursors levels (vitellogenin [VTG] and very-low-density lipoprotein [VLDLy] in female macaroni penguins (*Eudyptes chrysolophus*) with hematocrit (Hct), red-blood cell concentrations of hemoglobin (Hb), and reticulocyte index. The solid line represents a significant correlation ($\alpha = 0.05$). See Table 2 for statistical output from generalized linear models. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hct and VTG that we observed might therefore be best explained as an indirect effect of estrogen-dependent mobilization of yolk precursors and calcium ions needed for egg production, resulting in hemodilution (Morton, 1994; Salvante and Williams, 2002). Mobilization of these compounds in egg-producing females increases the plasma volume via the osmotic movement of water from extra-cellular spaces into the blood, all in an effort to maintain a constant plasma osmolarity and prevent the blood from becoming too viscous (Reynolds and Waldron, 1999). As a result, Hct would be reduced in those penguins with the highest yolk precursor levels, by reducing red blood cell numbers per unit plasma volume but not total cell number, and so birds would not be anemic per se. However, studies have shown that variation in Hct levels can predict the aerobic performance of birds, including macaroni penguins experiencing reproductive anemia (Hct ~ 40%) during the chick-rearing phase of the breeding season (e.g. Crossin et al., 2015).

The pre-laying Hct levels that we measured in macaroni penguins are similar to those documented in other pre-laying birds (Williams, 2012). High pre-laying Hct (~50%) may thus be a common strategy

Table 2
Generalized linear models examining variation in hematocrit (A, $N = 48$) and reticulocyte index (B, $N = 44$) relative to several explanatory parameters. The best fitting models and significant parameter estimates are indicated below in bold text.

A: Response = Hematocrit	AIC	ΔAIC
Hct ~ VTG	**239.04**	**0.00**
Hct ~ VTG * Julian date	239.15	0.11
Hct ~ VTG + Julian date	239.48	0.44
Hct ~ VTG + VLDLy + VTG*Julian date	240.38	1.34
Hct ~ VTG + Overlap	240.40	1.36
Hct ~ VTG + VLDLy + Julian date	240.54	1.50
Hct ~ VTG + VLDLy	240.81	1.77
Hct ~ VTG + VLDLy + Overlap	241.53	2.49
Hct ~ VTG * Overlap	241.62	2.58
Hct ~ VTG + VLDLy + Julian date + Body mass	242.05	3.01
Hct ~ VTG + VLDLy + VTG*Overlap	243.04	4.00
Hct ~ VTG + VLDLy + Overlap + Body mass	243.53	4.49
Hct ~ VLDLy + Julian date	245.16	6.12
Hct ~ VLDLy * Julian date	246.22	7.18
Hct ~ VLDLy + Overlap	248.86	9.82
Hct ~ VLDLy * Overlap	250.81	11.77
null (~1)	256.72	17.68

Parameter	Estimate	Standard error	t value	*P*
Intercept	58.62	2.76	21.24	< 0.001
VTG	**−3.39**	**1.19**	**−2.83**	**0.003**

B: Response = Reticulocyte Index	AIC	ΔAIC
RI ~ VLDLy	**350.94**	**0.00**
RI ~ VLDLy * Julian date	352.53	1.59
RI ~ VTG + VLDLy	352.64	1.70
RI ~ VLDLy + Overlap	352.81	1.87
RI ~ VTG + VLDLy + Body mass + Julian date	352.90	1.96
RI ~ VLDLy + Julian date	352.91	1.97
RI ~ VTG + Overlap	353.08	2.14
RI ~ VTG + Julian date	353.11	2.17
RI ~ VTG + VLDLy + Body mass + Overlap	353.49	2.55
RI ~ VLDLy * Overlap	353.82	2.88
RI ~ VTG + VLDLy + Overlap	354.62	3.68
RI ~ VTG + VLDLy + Julian date	354.64	3.70
RI ~ VTG * Overlap	354.91	3.97
RI ~ VTG * Julian date	355.07	4.13
RI ~ VTG + VLDLy + VTG*Overlap	356.53	5.59
RI ~ VTG + VLDLy + VTG*Julian date	356.60	5.66
null (~1)	364.03	13.09

Parameter	Estimate	Standard error	t value	*P*
Intercept	16.46	18.72	0.88	0.385
VLDLy	1.54	1.94	0.79	0.433

among birds to deal with the aerobic challenges of reproduction, including sustained increases in metabolic rate during the course of incubation and chick rearing, as well as the inevitable anemia that ensues at clutch completion (e.g. Williams et al., 2004; Wagner et al., 2008). For example, in breeding female birds Hct can show marked decreases by as much as 10% (e.g. Crossin et al., 2010), the recovery of which can require weeks and extend well beyond the hatching period. Eventually, reticulocytosis returns Hct to pre-breeding levels, which then prepares females for the high aerobic demands of foraging that occur when growing chicks require increasingly more frequent food deliveries.

Mechanistically, the maintenance of high pre-laying Hct and erythropoiesis in migrating *Eudyptes* penguins may depend on a key investment decision between the androgenic pathway that supports aerobic performance via reticulocytosis and high circulating Hct levels, and the estrogenic pathway favouring yolk precursor production, perhaps at the level of the aromatase enzyme (Shen et al., 1995). In the context of a migration-reproduction conflict, limitations on the conversion efficiency of aromatase would provide females with a standing pool of testosterone to support erythropoiesis and oxygen delivery to working tissues, but with the indirect effect of constraining E_2-mediated vitellogenesis underlying egg production, which we know occurs in *Eudyptes* penguins (Williams, 1990; Crossin et al., 2010; Crossin et al., 2012a). When the physiologies underlying migration and reproduction overlap in this way, the consequent low degree of estrogenic yolk production forming the first egg of the clutch leads to the extreme egg size dimorphism which characterizes *Eudyptes* penguins. Indeed, that RI was so high immediately after migration indicates the degree to which individual females optimize aerobic performance right up until the point of colony landfall. This is important because macaroni penguins are expeditious travelers during their return migrations (Bost et al., 2009), and must make landfall quickly so as to avoid predation by seals (e.g. Edwards et al., 2009). Despite being elevated, our GLMs did not identify a significant predictor for variation in RI (Table 2).

The best evidence for a migration/reproduction overlap in female birds comes from the penguins. If we consider the family as a whole (Spheniscidae), 11 of 18 species are migratory (Crossin and Williams, 2016). Pelagic migrants like the emperor penguins (*Aptenodytes forsteri*), chinstrap penguins (*Pygoscelis antarcticus*), and Adélie penguins make extraordinarily long migrations as the macaroni penguins do, but when they arrive back at breeding colonies they spend upwards of a month on land prior to egg laying (Crossin and Williams, 2016). Similarly, other non-migratory but nevertheless sea-dispersing penguins do the same; when they return to breeding colonies after a non-breeding period at sea, they spend weeks to months on land before laying. Recalling that egg production takes approximately 16–20 days in penguins (Grau, 1982; Astheimer and Grau, 1985), the temporal offset between migratory activity and egg production ensures that the regulatory systems governing each process are separated in all penguins, with the curious exception of the *Eudyptes*. It is only in *Eudyptes* where a significant overlap between these processes occurs (Williams, 1990; Crossin et al., 2010; Crossin and Williams, 2016).

Collectively, these results add a new dimension to the physiological intricacies related to extreme egg-size dimorphism in *Eudyptes* penguins. Recent phylogenetic work on the penguins suggests that ESD evolved maladaptively in response to selection for a pelagic, migratory

Table 3
Reticulocyte indices of female macaroni penguins (*Eudyptes chrysolophus*) in this study, and values assembled from the literature for other wild or captive bird species at varying life history stages.

Species	Wild/Captive	Reticulocyte index (%)	Sex	Migratory state	Breeding state	Source
Macaroni penguin *(E. chrysolophus)*	Wild	34.87 ± 2.34	F	End of migration	Pre-breeding	Current study
African penguin *(Spheniscus dermersus)*	Captive	3.5 ± 1.2	F,M	Not migrating	Post-breeding	Mazzaro et al. 2013
Rhinoceros auklet (Cerrorhinca monocerata)	Wild	4.6 ± 2.3	F,M	Not migrating	Breeding	Newman et al. 1999
Domestic chicken *(Gallus gallus domesticus)*	Captive	9.93	F,M	Not migrating	Breeding	Coates and March 1966
Pigeon *(Columbidae spp)*	Captive	12	F,M	Not migrating	Non-breeding	Vaughan et al. 1930
Mallard duck (Anas platyrhyncos)	Captive	15	F,M	Not migrating	Non-breeding	Roscoe et al. 1979
Zebra finch (Taeniopygia guttata)	Captive	10.3 ± 1.3 15.5 ± 1.3	F	Not migrating	Pre-breeding	Wagner et al., 2008

non-breeding biology and a slow life history (Stein and Williams, 2013; Crossin and Williams, 2016). This is corroborated by our results, which suggest that aerobic capacity is a necessary part of a pelagic lifestyle, despite the apparent cost to egg production when migratory activity and reproductive physiology run simultaneously.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

Thanks to Fabrice Le Bouard for assistance in the field, and to Chris Harvey-Clark for advice on microscopy. Financial support was provided by a Natural Sciences and Engineering Research Council of Canada Discovery Grant to GTC (#04044-2014-RGPIN).

References

Astheimer, L.B., Grau, C.R., 1985. The timing and energetic consequences of egg formation in the Adélie penguin. Condor 87, 256–268.
Banerjee, V., Banerjee, M., 1977. Variations of erythrocyte number and hemoglobin content of a migratory bird - *Philomachus pugnax* (Linnaeus). Zool. Anz. 199, 261–264.
Blobel, G.A., Orkin, S.H., 1996. Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. Mol. Cell. Biol. 16, 1687–1694.
Bost, C.A., Thiebot, J.B., Pinaud, D., Cherel, Y., Trathan, P.N., 2009. Where do penguins go during the inter-breeding period? Using geolocation to track the winter dispersion of the macaroni penguin. Biol. Lett. 5, 473–476.
Calbet, J.A., Lundby, C., Koskolou, M., Boushel, R., 2006. Importance of hemoglobin concentration to exercise: acute manipulations. Resp. Phys. Neurobiol. 151, 132–140.
Coates, V., March, B.E., 1966. Reticulocyte counts in the chicken. Poultry Sci. 6, 1302–1305.
Crossin, G.T., Williams, T.D., 2016. Migratory life histories explain the extreme egg-size dimorphism of *Eudyptes* penguins. Proc. R. Soc. B 283, 1413–1418.
Crossin, G.T., Williams, T.D., 2020. Using physiology to infer the reproductive status and breeding performance of cryptic or at-risk bird species. In: Madliger, C.L., Franklin, C., Love, O.P., Cooke, S.J. (Eds.), Conservation Physiology: Applications for Wildlife Conservation and Management. Oxford University Press, Oxford.
Crossin, G.T., Trathan, P.N., Phillips, R.A., Dawson, A., Le Bouard, F., Williams, T.D., 2010. A carryover effect of migration underlies individual variation in reproductive readiness and extreme egg size dimorphism in macaroni penguins. Am. Nat. 176, 357–366.
Crossin, G.T., Poisbleau, M., Demongin, L., Chastel, O., Williams, T.D., Eens, M., Quillfeldt, P., 2012a. Migratory constraints on yolk precursors limit yolk androgen deposition and underlie a brood reduction strategy in rockhopper penguins. Biol. Lett. 8, 1055–1058.
Crossin, G.T., Phillips, R.A., Trathan, P.N., Fox, D.S., Dawson, A., Wynne-Edwards, K.E., Williams, T.D., 2012b. Migratory carryover effects and endocrinological correlates of reproductive decisions and reproductive success in female albatrosses. Gen. Comp. Endocrinol. 176 (2), 151–157.
Crossin, G.T., Trathan, P.N., Crawford, R.J.M., 2013. The macaroni penguin (*Eudyptes chrysolophus*) and the royal penguin (*E. schlegeli*). In: Garcia-Borboroglu, P., Boersma, P.D. (Eds.), Penguins: Natural History and Conservation. University of Washington Press, Seattle, pp. 185–208.
Crossin, G.T., Takahashi, A., Sakamoto, K.Q., Trathan, P.N., Williams, T.D., 2015. Habitat selection by foraging macaroni penguins correlates with hematocrit, an index of aerobic condition. Mar. Ecol. Prog. Ser. 530, 163–176.
Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythm. 16, 365–380.
Edwards, E.W.J., Forcada, J., Crossin, G.T., 2009. First documentation of leopard seal predation of South Georgia pintail duck. Polar Biol. 33, 403–405.
Grau, C.R., 1982. Egg formation in fiordland crested penguins (*Eudyptes pachyrhynchus*). Condor 84, 172–177.
Gwinner, E., 1996. Circannual clocks in avian reproduction and migration. Ibis 138, 47–63.
Hennin, H.L., Bêty, J., Legagneux, P., Gilchrist, H.G., Williams, T.D., Love, O.P., 2016. Energetic physiology mediates individual optimization of breeding phenology in a migratory Arctic seabird. Am. Nat. 188, 434–445.
Holmgren, N., Hedenström, A., 1995. The scheduling of molt in migratory birds. Evol. Ecol. 9, 354–368.
Joyner, M.J., 1991. Modeling optimal marathon performance on the basis of physiological factors. J. Appl. Physiol. 70, 683–687.
Landys-Ciannelli, M.M., Jukema, J., Piersma, T., 2002. Blood parameter changes during stopover in a long-distance migratory shorebird, the bar-tailed godwit *Limosa lapponica taymyrensis*. J. Avian Biol. 33, 451–455.
Mazzaro, L.M., Meegan, J., Sarran, D., Romano, T.A., Bonato, V., Deng, S., Dunn, J.L., 2013. Molt-associated changes in hematologic and plasma biochemical values and stress hormone levels in African penguins (*Spheniscus demersus*). J. Avian Med. Surg. 27, 285–293.
Minias, P., 2020. Ecology and evolution of blood oxygen-carrying capacity in birds. Am. Nat. 195, 788–801 (In press).
Morton, M.L., 1994. Hematocrits in montane sparrows in relation to reproductive schedule. Condor 96, 119–126.
Newman, S.H., Mazet, J.K., Ziccardi, M.H., Lieske, C.L., Fauquier, D.A., Gardner, I.A., Zinkl, J.G., Christopher, M.M., 1999. Haematological changes and anaemia associated with captivity and petroleum exposure in seabirds. Comp. Haematol. Int. 9, 60–67.
Perry, M.J., Samuels, A., Bird, D., Tobias, J.H., 2000. Effects of high-dose estrogen on murine hematopoietic bone marrow precede those on osteogenesis. Am. J. Physiol. Endocrinol. Metab. 279, E1159–E1165.
Piersma, T., Everaarts, J.M., Jukema, J., 1996. Build-up of red blood cells in refuelling bar-tailed godwits in relation to individual migratory quality. Condor 98, 363–370.
Reynolds, S.J., Waldron, S., 1999. Body water dynamics at the onset of egglaying in the zebra finch *Taeniopygia guttata*. J. Avian Biol. 30, 1–6.
Riley, R.S., Ben-Ezra, J.M., Goel, R., Tidwell, A., 2001. Reticulocytes and reticulocyte enumeration. J. Clin. Lab. Anal. 15, 267–294.
Roscoe, D.E., Nielsen, S.W., Lamola, A.A., Zuckerman, D., 1979. A simple, quantitative test for erythrocytic protoporphyrin in lead-poisoned ducks. J. Wildl. Dis. 15, 127–136.
Salvante, K.G., Williams, T.D., 2002. Vitellogenin dynamics during egg-laying: daily variation, repeatability and relationship with egg size. J. Avian Biol. 33, 391–398.
Shen, P., Schlinger, B., Campagnoni, A., Arnold, A., 1995. An atlas of aromatase mRNA expression in the zebra finch brain. J. Comp. Neurol. 360, 172–184.
Stein, R.W., Williams, T.D., 2013. Extreme intraclutch egg-size dimorphism in *Eudyptes* penguins: an evolutionary response to clutch-size maladaptation. Am. Nat. 182, 260–270.
Tonra, C.M., Marra, P.P., Holberton, R.L., 2011a. Early elevation of testosterone advances migratory preparation in a songbird. J. Exp. Biol. 214, 2761–2767.
Tonra, C.M., Marra, P.P., Holberton, R.L., 2011b. Migration phenology and winter habitat quality are related to circulating androgen in a long-distance migratory bird. J. Avian Biol. 42, 397–404.
Vaughan, J.M., Muller, G.L., Zetrel, L., 1930. The response of grain-fed pigeons to substances effective in pernicious anaemia. Brit. J. Exp. Path. 11, 456–468.
Viscor, G., Marques, M., Palomeque, J., 1985. Cardiovascular and organ weight adaptations as related to flight activity in birds. Comp. Biol. Physiol. A 82, 597–599.
Vleck, C.M., Vertalino, N., Vleck, D., Bucher, T.L., 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adélie penguins. Condor 102, 392–400.
Wagner, E.C., Williams, T.D., 2007. Experimental (antiestrogen-mediated) reduction in egg size negatively affects offspring growth and survival. Physiol. Biochem. Zool. 80, 293–305.
Wagner, E.C., Stables, C.A., Williams, T.D., 2008. Hematological changes associated with egg production: direct evidence for changes in erythropoiesis but a lack of resource dependence? J. Exp. Biol. 211, 2960–2968.
Williams, T.D., 1990. Growth and survival in macaroni penguin, *Eudyptes chrysolophus*, A- and B-chicks: do females maximise investment in the large B-egg? Oikos 59, 349–354.
Williams, T.D., 1995. The Penguins: Spheniscidae. Oxford University Press, Oxford.
Williams, T.D., 2012. Physiological Adaptations for Breeding in Birds. Princeton University Press, Princeton.
Williams, T.D., Challenger, W., Christians, J., Evanson, M., Love, O., Vezina, F., 2004. What causes the decrease in haematocrit during egg production? Funct. Ecol. 18, 330–336.
Wingfield, J., Schwabl, H., Mattocks Jr., P., 1990. Endocrine mechanisms of migration. In: Gwinner, E. (Ed.), Bird Migration. Springer, Berlin Heidelberg, pp. 232–256.
Yap, K.N., Tsai, O., Williams, T.D., 2019. Haematological traits co-vary with migratory status, altitude and energy expenditure: a phylogenetic, comparative analysis. Sci. Reports 9, 6351.