Reproductive effort and oxidative stress: effects of offspring sex and number on the physiological state of a long-lived bird

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Summary

1. Individuals must trade-off between energetically costly activities to maximize their fitness. However, the underlying physiological mechanism remains elusive. Oxidative stress, the imbalance between reactive oxygen species production and antioxidant and/or repair activities, has been suggested to underlie life-history trade-offs: greater investment in reproduction supposedly generating higher oxidative damage, thus reducing life span.

2. While most studies used natural or experimental variation in offspring number to examine how reproduction affects oxidative stress, none studied the impact of offspring sex, although it could influence physiological costs and fitness, if the sexes differ in terms of energetic cost.

3. Here, we aim at further understanding how reproduction (in terms of offspring sex, experimentally manipulated and number, not manipulated) influences oxidative stress in a wild seabird, where sons are energetically costlier than daughters. We did so by conducting a chick fostering experiment (to disentangle foster and produced sex ratio) and using four oxidative stress markers plus baseline corticosterone.

4. First, the results suggest that individual physiological state before laying modulates upcoming reproductive effort. Individuals with higher pre-laying baseline corticosterone and lower antioxidant activity, estimated by their superoxide dismutase activity, subsequently invested more in reproduction, estimated by the cumulative number of days spent rearing chicks. Hence, it seems that only individuals that could afford to invest heavily in reproduction did so.

5. Then, we examined the effects of reproductive effort on individual physiological state at the end of the breeding season. Higher reproductive effort seemed to imply higher physiological costs. Oxidative stress, estimated by the ratio of oxidized over reduced glutathione, increased with more male-biased foster sex ratio among mothers but not among fathers, whereas baseline corticosterone did so in both sexes. Similarly, lipid oxidative damage to red blood cells increased with increasing cumulative number of days spent rearing chicks.

6. Our study provides the first evidence that brood sex ratio variation can affect oxidative balance, potentially in a sex-specific way, although more studies are needed to understand whether the observed physiological costs could lead to fitness costs. It also highlights the need to consider sex ratio in future studies investigating the role of oxidative stress in life-history trade-offs.

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Introduction

Energetically costly activities, such as growth, self-mainte-nance and reproduction, depend upon limited resources; individuals thus must trade them off to maximize their fit-ness. For instance, a trade-off between survival and repro-duction is common in sexually reproducing species, with a greater investment in current reproduction only being pos-sible at the expense of future reproduction or self-mainte-nance (Stearns 1992). However, the nature of the physiological mechanism(s) underlying life-history trade-offs is still a big question in evolutionary biology. One potential candidate that has recently attracted attention is oxidative stress (e.g. Costantini 2008, 2014; Monaghan, Metcalfe & Torres 2009; Isaksson, Sheldon & Uller 2011; Selman *et al.* 2012). It can be defined as an imbalance between reactive oxygen species (ROS) causing oxidative damage to biomolecules, such as DNA, lipids and proteins (Balaban, Nemoto & Finkel 2005), and their neutralization by antioxidant defences and/or repair mechanisms, such that the latter are not efficient enough to prevent excessive oxidative damage (Dickinson & Chang 2011; Murphy *et al.* 2011). Accumulation of oxidative damage is thought to contribute to ageing (Dickinson & Chang 2011). As reproduction is energetically demanding (Bryant 1997; Speakman 2008), greater investment in reproduction might lead to increased oxidative damage resulting in faster somatic deterioration and thus reduced life span, because resources allocated to reproduction (e.g. nutrients, energy, time) are no longer available for defence against ROS (e.g. Monaghan, Metcalfe & Torres 2009; Metcalfe & Alonso-Alvarez 2010; Selman *et al.* 2012; Metcalfe & Monaghan 2013).

Even though the hypothesis that oxidative stress medi-ates life-history trade-offs has generated great interest in recent years, it is still unclear whether and how oxidative stress is involved in such trade-offs (Speakman *et al.* 2015; Blount *et al.* 2016). The first set of studies investi-gating this hypothesis were mostly correlational, lab-based, and using one marker of oxidative damage or antioxidant defences and in only one tissue (reviewed in Selman *et al.* 2012; Metcalfe & Monaghan 2013). How-ever, the absence of effect of reproductive effort on one marker of oxidative damage in one tissue does not mean that reproduction does not increase oxidative stress. The lack of effect could be due to an upregulation of antioxi-dant defences and/or reproduction may have a stronger effect on another marker or in another tissue. Insights could thus be gained by considering multiple markers and tissues in free-living animals (Costantini 2008; Selman *et al.* 2012; Speakman & Garratt 2014; Speakman *et al.*

2015), while ideally experimentally manipulating repro-ductive effort (Metcalfe & Monaghan 2013). Oxidative stress would thus be expected to increase with increasing reproductive effort. Such experimental approaches have been conducted recently (e.g. Costantini *et al.* 2015; Ołdakowski *et al.* 2015; Romero-Haro, Sorci & Alonso-Alvarez 2016), but with contradictory outcomes. More-over, in a recent meta-analysis, Blount *et al.* (2016) tested this prediction and found rather unexpected results: reproductive effort and oxidative damage were positively associated (especially among blood markers) but the effect was driven by correlational and not experimental data. While this study confirmed the need for multiple markers and tissues, the low number of studies available and the ambiguous results beg for more studies investigating how reproductive state or effort and oxidative balance relate to each other.

Natural or manipulated variation in litter or brood size is commonly used to examine fitness costs of repro-duction (e.g. Partridge 1989) and studies examining phys-iological costs of reproduction are no exception (e.g. Bergeron *et al.* 2011; Stier *et al.* 2012; Costantini *et al.* 2014; Romero-Haro, Sorci & Alonso-Alvarez 2016). Off-spring sex has also been shown to induce fitness and/or physiological costs (e.g. Bérubé, Festa-Bianchet & Jor-genson 1996; Magrath *et al.* 2007) and can therefore influence the trade-off between current and future repro-duction as well (e.g. Rickard, Russell & Lummaa 2007; Rutkowska *et al.* 2011), possibly mediated by oxidative stress. Parents typically expend more energy when rearing the larger sex (e.g. Krijgsveld *et al.* 1998; Magrath *et al.* 2007; Merkling *et al.* 2015), which might provoke higher oxidative damage and/or higher investment in antioxi-dant defences or repair mechanisms. If this is the case, we might expect parents to adjust offspring sex ratio in relation to their oxidative status before laying: parents with high levels of oxidative damage should overproduce the cheapest sex in line with the 'cost of reproduction hypothesis' (Myers 1978; Cockburn, Legge & Double 2002). However, to our knowledge, no study has yet investigated how oxidative balance and sex allocation are associated.

Here, we present data from a wild population of a long-lived monogamous seabird, the black-legged kittiwake (*Rissa tridactyla*). We previously showed, in the same pop-ulation, that males (the larger sex: Helfenstein, Danchin & Wagner 2004; Merkling *et al.* 2012) are energetically cost-lier than females (Merkling *et al.* 2015); male-biased broods triggered higher parental daily energy expenditure and baseline corticosterone levels (known to facilitate higher parental workload: Ouyang *et al.* 2013; Love *et al.*

2014). To further our understanding of the energetic costs of reproductive effort (offspring sex ratio and cumulative number of days spent rearing chicks), we carried out a chick fostering experiment (i.e. parents did not necessarily rear the sex ratio they produced but they initially had the same number of chicks), and measured multiple blood markers of oxidative damage and antioxidant defence as well as baseline corticosterone (CORT), twice, before laying and at the end of the chick rearing period. Specifically, we chose to describe individual oxidative balance using (i) a specific marker of oxidative damage to the lipids, malondialdehydes, measured in plasma (MDA plasma) and red blood cells (MDA RBC), (ii) a marker of cellular oxidative stress, i.e. the ratio of oxidized over reduced glutathione (GSSG/GSH ratio), as well as glutathione disulfide (GSSG) and GSH by themselves to better understand the causes of variation in the ratio, and (iii) the activity of the antioxidant enzyme superoxide dismutase (SOD), i.e. an endogenous antioxidant. Although we acknowledge that measuring oxidative stress markers in blood only (as commonly done in long-term monitoring: Speakman *et al.* 2015) is a limitation, this is probably the first tissue to investigate as it is the only one in which an effect of reproductive effort on oxidative damage was found (Blount *et al.* 2016). Besides oxidative balance variables, we decided to include CORT as a response variable as well. Indeed, we previously showed that CORT covaried with natural brood sex ratio, but we did not manipulate sex ratio in this earlier study (Merkling *et al.* 2015). Hence, considering CORT in the present study allowed us to use it as a 'positive control' that individuals responded to the sex ratio manipulation, while validating our previous results experimentally.

We considered the relationships between reproductive effort and physiological variables with two non-mutually exclusive questions (Fig. S1 in Appendix S1, Supporting Information). (i) Does an individual pre-laying physiological state influence the upcoming reproductive effort (sex ratio produced and cumulative number of days spent rearing chicks)? Here, predictions were that more cumulative number of days spent rearing chicks and/or more male offspring could only be afforded by individuals starting with low levels of oxidative damage and CORT (according to the 'cost of reproduction hypothesis', Cockburn, Legge & Double 2002). (ii) Does current reproductive effort (after chick fostering: change in sex ratio, foster sex ratio and cumulative number of days spent rearing chicks) influence an individual physiological state at the end of the breeding season? Predictions were that more male offspring (relatively to natural sex ratio or overall) and/or more cumulative number of days spent rearing chicks would lead to higher levels of oxidative damage and CORT at the end of the breeding season. For both questions, we also included parental sex in our analyses as previous studies revealed sex differences in the parental response to chick needs (Leclaire *et al.* 2010, 2011).

Materials and methods

STUDY SITE AND SPECIES

The black-legged kittiwake is a medium-sized, colonial genetically monogamous seabird nesting on vertical cliffs. Parents share all parental duties such as nest building, incubation and chick rearing almost equally (Hatch, Robertson & Baird 2009). Females can lay 1–3 eggs, but the vast majority (c. 85%) of clutches contain two eggs (Gill & Hatch 2002 and our unpubl. data). The study was conducted from mid-April to mid-August 2012 in a population of black-legged kittiwakes nesting on an abandoned U.S. Air Force radar tower on Middleton Island (59°26′N, 146°20′W), Gulf of Alaska. The tower is a 12-walled polygon with artificial nest sites that have been added to the upper walls, which allowed us to easily monitor and capture the breeding adults and their chicks from inside the building (for more details, see Gill & Hatch 2002).

SAMPLING AND EXPERIMENTAL DESIGN

Nests were checked twice daily (9.00 and 18.00 h) throughout the breeding season to document laying, hatching and chick mortality. Laying date was recorded and each egg was individually marked (A for the first laid egg and B for the second egg) with non-toxic waterproof ink. Eggs were put into an incubator 24 days after laying (i.e. c. 3 days before expected hatching: Hatch, Robertson & Baird 2009) to monitor hatching closely and were replaced by dummy eggs meanwhile. Details of the protocol are given in Merkling *et al.* (2014). At hatching, chicks were marked on the head with a non-toxic colour marker to identify their hatching rank and blood sampled for molecular sexing purposes (see Merkling *et al.* 2012 for a detailed protocol). Then each chick was put in a foster nest with an unfamiliar non-local sibling. We thus expected to manipulate brood sex ratio as compared to the one initially produced. However, chicks cannot be sexed morphologically at hatching (Vincenzi *et al.* 2015) and we did not have access to laboratory facilities in the field, so chick fostering had to be done blind to natural brood sex ratio.

Eighty-two adults (from 47 nests) were captured before laying (capture date ranging from 131 to 156 Julian date). We aimed at capturing them as close as possible before the female laid her first egg; we started trying to capture both parents of a nest when the nest started to be cup-shaped. Unfortunately, nest shape was not a very reliable predictor of laying date: adults were captured between 25 and 0 days before laying. Blood samples from the alar vein were taken within 3 min of capture to determine their baseline corticosterone level (Romero & Reed 2005). We used a 25G heparinized needle and a 1-mL syringe (maximum blood volume collected: 1 mL). A second capture and blood samples were attempted at the end of the rearing period (capture date ranging from 202 to 221 Julian date) on most adults who raised at least one chick close to fledging (*N* = 101 from 55 nests). Some of them had not been captured before laying. Blood samples were centrifuged at 12 000 *g* for 10 min in the field, red blood cells were separated from plasma and both fractions were frozen at −20 °C until they were brought back to the laboratory and stored at −80 °C.

MARKERS OF OXIDATIVE DAMAGE AND ANTIOXIDANT DEFENCES

We assessed the oxidative balance of males and females using several markers of oxidative damage and antioxidant defences in the blood. We measured the activity of the endogenous enzyme SOD, which catalyses the dismutation of the superoxide anion into

hydrogen peroxide (H_2O_2) and oxygen (O_2) (Halliwell & Gutteridge 2015). Oxidative damage to the lipids was assessed via levels of MDA, which are end-products of lipid peroxidation (Halliwell & Gutteridge 2015). Finally, reduced glutathione (GSH) is an endogenous tripeptide, which can be oxidized into GSSG to reduce ROS via a reaction catalysed by the enzyme glutathione peroxidase. The glutathione ratio GSSG/GSH provides accurate information on the oxidative balance of cells (Cnubben *et al.* 2001). All oxidative balance analyses were performed at the University of Neuchâtel. Further details regarding the specific protocols are given in Appendix S1.

Makers of lipid peroxidation in plasma and red blood cells

We assessed MDA (nmol mL^{-1}) by its reaction with thiobarbituric acid (TBA) to produce a pink derivate measurable by high-performance liquid chromatography (HPLC) with fluorescence detection. MDA concentration estimation was assessed using a method adapted from Losdat *et al.* (2014). More details are given in Appendix S1.

SOD Activity in red blood cells

We assessed SOD activity (U mL^{-1}) using the Cayman's SOD assay kit (Cayman chemical company, Ann Arbor, MI, USA), which is based on the detection of superoxide radicals generated by xanthine oxidase and neutralized by SOD.

Glutathione in red blood cells

The reduced (GSH, ng mL^{-1}) and oxidized (GSSG, ng mL^{-1}) forms of glutathione were measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS), according to Bouligand *et al.* (2007) with some modifications. Further details are given in Appendix S1.

CORTICOSTERONE ASSAYS

All hormonal analyses were performed at the Centre d'Etudes Biologiques de Chizé. Plasma concentrations of CORT were determined in one assay following methods described in Lormée *et al.* (2003). The detection limit was 0·3 ng mL^{-1}, and the intra-assay coefficient of variation was 6% ($n = 5$ duplicates). The repeatability of CORT between pre-laying and post-hatching values was null [mixed-effects model approach using the RPTR package (Nakagawa & Schielzeth 2010): $R = 0$; CI = (0; 0·293); $P = 0.57$]. We chose not to assess free CORT and corticosteroid-binding globulin concentrations on the premise that total CORT concentration is the more biologically relevant estimate of CORT levels (Schoech *et al.* 2013). We corrected for sampling time in all models including CORT by adding it as a covariate.

STATISTICAL ANALYSES

Model selection was based on the information-theoretic approach where goodness-of-fit to the data of each model was quantified using AICc (Burnham & Anderson 2002; Burnham, Anderson & Huyvaert 2011). For all analyses, to standardize conditions before chick rearing, and based on the modal clutch and brood sizes, we focused on pairs who had two eggs and to whom we gave two foster chicks ($N = 76$; 39 females and 37 males from 40 nests). However, not every variable was measured for each individual so specific sample sizes differ depending on the analyses and are provided in the results. For each of our questions, we had a full model and compared it to all possible subset of explanatory variables (including a null model with the intercept only). The full model was carefully chosen and followed the main predictions we were testing, hence keeping the total number of possible models to a minimum (see Appendix S1 for more details). All models were mixed models with Nest ID as a random effect. They were run using the LME4 package (Bates, Maechler & Bolker 2011). For each model, we calculated its AICc and its AICc difference (ΔAICc) with the lowest AICc model (corresponding to the best model). ΔAICc provide a measure of information lost between the best model and a given model of a candidate set. Following Burnham, Anderson & Huyvaert (2011), we selected the models with ΔAICc < 4. We also calculated a weight (ωAICc) for each model, which represents the probability that a given model is the best approximating model among the subset of models (Symonds & Moussalli 2011). These parameters were calculated using the AICCMODAVG package (Mazerolle 2013) and based on maximum likelihood estimation. As this approach resulted in multiple models that were equally likely, we computed model-averaged parameter estimates, standard errors, and confidence intervals without shrinking the parameters (Burnham & Anderson 2002). Briefly, we averaged the parameter estimates of each variable for all the models in which they appeared. This allowed us to take model selection uncertainty into account. For all analyses, MDA plasma (pre-laying and near-fledging), MDA RBC (pre-laying and near-fledging), SOD (near-fledging only), GSSG/GSH ratio (pre-laying and near-fledging), GSH (pre-laying and near-fledging) and CORT (near-fledging only) values were log-transformed to meet model assumptions, whereas GSSG values were square-root transformed. Following recent recommendations (Gelman 2008), we also standardized binary and continuous input variables as it facilitates model convergence and comparison of effect sizes between models. All analyses were run using R 3.2.3 (R Core Team 2015).

Results

IMPACT OF FOSTERING EXPERIMENT ON BROOD SEX RATIO

Chick fostering was done blind to natural brood sex ratio. Unfortunately, our manipulation yielded a foster sex ratio quite similar to the natural one (from 0·51 to 0·58; Fisher's exact test for an association between initial and foster sex ratio: $P = 0.017$; Table S1 in Appendix S1).

DOES AN INDIVIDUAL PHYSIOLOGICAL STATE BEFORE LAYING INFLUENCE UPCOMING REPRODUCTIVE EFFORT?

None of the pre-laying physiological variables correlated with the natural sex ratio (i.e. sex ratio before manipulation). The null model (i.e. intercept only model) was the best model explaining variation in natural brood sex ratio for all the physiological variables considered.

However, both SOD and CORT levels were important predictors of the cumulative number of days spent rearing chicks (Table S2 in Appendix S1). They were negatively correlated with SOD levels but positively with CORT (Table 1): parents with lower SOD levels and higher CORT levels before laying were more likely to have higher cumulative number of days spent rearing chicks by the end of the rearing period. Moreover, the interaction between parental sex and GSH levels also seem to predict the

cumulative number of days spent rearing chicks, although weakly as the null model was close (Table S2 in Appendix S1). GSH levels increased with the cumulative number of

Table 1. Model-averaged estimates of the pre-laying physiological variables [superoxide dismutase (SOD), reduced glutathione (GSH) and baseline corticosterone (CORT)] explaining the cumulative number of days spent rearing chicks

Parameter	Estimate	Standard error	Lower CI	Upper CI
(a) SOD (N = 55)				
Intercept	47·6212	2·5875	42·5499	52·6926
SOD	**−3·2961**	**1·3757**	**−5·9925**	**−0·5997**
Male parent*	1·8425	1·1153	−0·3434	4·0284
SOD:Male parent*	2·4469	3·1189	−3·666	8·5598
(b) GSH (N = 55)				
Intercept	47·6838	2·6318	42·5255	52·8421
GSH	0·6193	1·5092	−2·3386	3·5772
Male parent*	**2·2575**	**1·1437**	**0·0159**	**4·4991**
GSH: Male parent*	**6·1408**	**3·0572**	**0·1488**	**12·1327**
(c) CORT (N = 53)				
Intercept	48·5905	2·7882	43·1257	54·0553
CORT	**3·75**	**1·8853**	**0·0549**	**7·4451**
Male parent*	0·7212	1·3753	−1·9744	3·4167
CORT: Male parent*	−5·0353	3·5646	−12·0218	1·9512

Binary and continuous input variables were standardized to facilitate model comparisons. In bold are estimates which CI do not overlap zero. CI, confidence interval.
*Relative to female parent.

days spent rearing chicks among male parents but not among females (Table 1).

DOES CURRENT REPRODUCTIVE EFFORT (AFTER CHICK FOSTERING) INFLUENCE PHYSIOLOGICAL STATE AT THE END OF THE BREEDING SEASON?

Near-fledging values were more strongly influenced by foster sex ratio than by the change in sex ratio between natural and foster. Females, but not males, rearing more sons had a higher GSSG/GSH ratio by the end of the rearing period (Table 2; Fig. 1a). However, the fact that the null model was as likely as the model including their interaction suggests that this effect was not very strong (Table S3 in Appendix S1). This interaction was mostly due to lower GSH levels among mothers rearing all-male foster broods (Tables 2 and S3; Fig. 1b). Surprisingly, GSSG levels were higher in mixed foster broods, regardless of parental sex (Tables 2 and S3). Moreover, CORT values near-fledging were higher in parents that reared only sons (Tables 3 and S3; Fig. 2). As for covariates, sampling time positively influenced CORT values (Table 3). No physiological variable was influenced by the change in sex ratio.

Furthermore, we found a positive correlation between the cumulative number of days spent rearing chicks and MDA RBC near-fledging (Tables 4 and S4). Thus, parents with more cumulative number of days spent rearing chicks

Table 2. Model-averaged estimates of the variables explaining GSSG/GSH ratio near-fledging, reduced glutathione (GSH) and glutathione disulfide (GSSG), when considering foster sex ratio (N = 53)

Parameter	Estimate	Standard error	Lower CI	Upper CI
(a) GSSG/GSH ratio				
Intercept	1·0181	0·1218	0·7794	1·2567
Post-hatch days	−0·2245	0·1439	−0·5065	0·0575
Male parent*	0·5335	0·3517	−0·1557	1·2228
Mixed foster sex ratio[†]	0·1107	0·1704	−0·2233	0·4446
All-male foster sex ratio[†]	0·3206	0·1816	−0·0354	0·6765
Male parent*: mixed foster sex ratio[†]	−0·5816	0·3286	−1·2257	0·0625
Male parent*: all-male foster sex ratio[†]	**−1·2274**	**0·3452**	**−1·904**	**−0·5507**
(b) GSH				
Intercept	4·9935	0·1303	4·7381	5·2488
Post-hatch days	0·136	0·163	−0·1835	0·4554
Male parent*	−0·4776	0·2861	−1·0383	0·0831
Mixed foster sex ratio[†]	0·1166	0·1985	−0·2725	0·5057
All-male foster sex ratio[†]	−0·3428	0·2122	−0·7588	0·0732
Male parent*: mixed foster sex ratio[†]	0·309	0·3739	−0·4239	1·0418
Male parent*: all-male foster sex ratio[†]	**1·2773**	**0·3919**	**0·5091**	**2·0455**
(c) GSSG				
Intercept	19·9759	0·7794	18·4484	21·5035
Post-hatch days	−0·9095	0·7898	−2·4575	0·6384
Male parent*	1·2083	0·9994	−0·7505	3·1671
Mixed foster sex ratio[†]	**2·3047**	**0·9648**	**0·4138**	**4·1956**
All-male foster sex ratio[†]	−0·3198	1·025	−2·3288	1·6892
Male parent*: mixed foster sex ratio[†]	−2·391	1·7634	−5·8472	1·0652
Male parent*: all-male foster sex ratio[†]	0·7595	1·8424	−2·8516	4·3705

Binary and continuous input variables were standardized to facilitate model comparisons. In bold are estimates which CI do not overlap zero. CI, confidence interval.
*Relative to female parent.
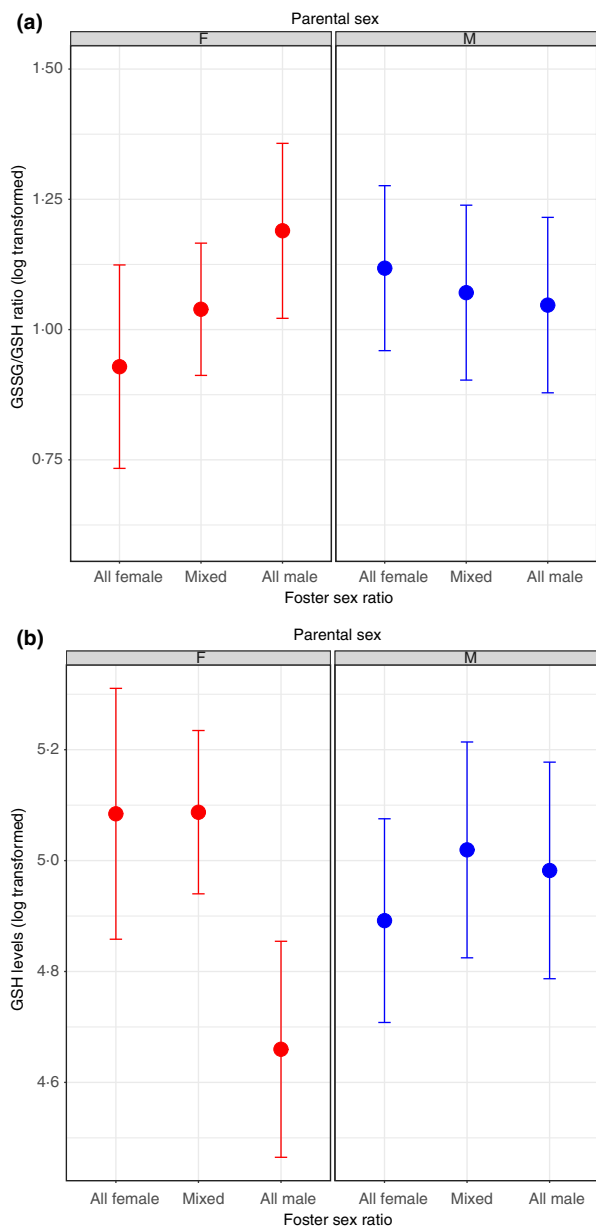[†]Relative to all-female foster sex ratio.

(a)

(b)

Fig. 1. (a) Ratio glutathione disulfide (GSSG)/reduced glutathione (GSH) (i.e. a marker of oxidative balance) at the end of the rearing period in relation to the foster sex ratio and parental sex. (b) Reduced glutathione (GSH) levels at the end of the rearing period in relation to the foster sex ratio and parental sex. Model-averaged predictions ± standard errors are represented.

(i.e. that engaged in a greater reproductive effort) had higher MDA RBC at the end of the rearing period (Fig. S2 in Appendix S1). However, no other physiological variable was related with the cumulative number of days spent rearing chicks.

Discussion

Oxidative stress has been suggested to be a potential mediator of life-history trade-offs (Metcalfe & Monaghan 2013; Speakman *et al.* 2015). Yet, solid theoretical predictions are still missing and evidence remain unclear (see

Table 3. Model-averaged estimates of the variables explaining near-fledging baseline corticosterone (CORT), when considering foster sex ratio (*N* = 54)

Parameter	Estimate	Standard error	Lower CI	Upper CI
Intercept	0·1611	0·5756	−0·967	1·2892
Post-hatch days	0·0899	0·1449	−0·1941	0·3739
Male parent*	0·1218	0·1402	−0·1531	0·3966
Sampling time	**0·4176**	**0·1746**	**0·0753**	**0·7598**
Mixed foster sex ratio	0·2137	0·1728	−0·125	0·5524
All-male foster sex ratio[†]	**0·5671**	**0·192**	**0·1907**	**0·9435**

Binary and continuous input variables were standardized to facilitate model comparisons. In bold are estimates which CI do not overlap zero. CI, confidence interval.
*Relative to female parent.
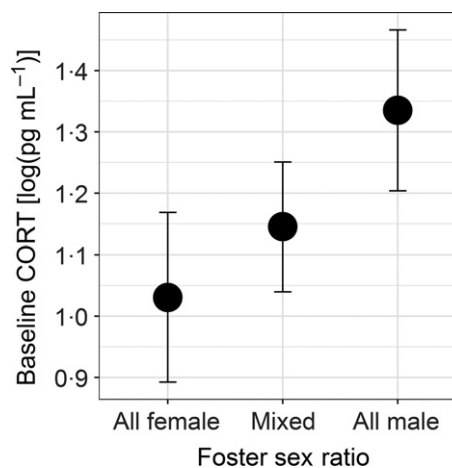[†]Relative to all-female foster sex ratio.

Fig. 2. Baseline corticosterone at the end of the rearing period (log-transformed) in relation to the foster sex ratio. Model-averaged predictions ± standard errors are represented.

Speakman & Garratt 2014; Speakman *et al.* 2015; Blount *et al.* 2016). Nonetheless, all recent reviews agree that several markers of oxidative damage and antioxidant defences need to be used to address this question (e.g. Selman *et al.* 2012; Speakman *et al.* 2015). Here, we examined how reproductive effort (offspring sex ratio, manipulated and cumulative number of days spent rearing chicks, not manipulated) relates to four oxidative stress markers and baseline CORT in a long-lived seabird. None of the tested individual pre-laying physiological variables correlated with natural brood sex ratio. However, both SOD and CORT levels predicted the upcoming cumulative number of days spent rearing chicks: parents with lower SOD levels and higher CORT levels before laying were more likely to have higher cumulative number of days spent rearing chicks by the end of the rearing period. Furthermore, some results confirmed that a higher reproductive effort may impose higher parental physiological costs at the end of the rearing period; the near-fledging GSSG/GSH ratio (a proxy of cellular oxidative balance) and CORT were higher in parents that were rearing more

Table 4. Model-averaged estimates of the variables explaining near-fledging MDA red blood cells, when considering the cumulative number of days spent rearing chicks ($N = 73$)

Parameter	Estimate	Standard error	Lower CI	Upper CI
Intercept	4·9309	0·0488	4·8353	5·0265
Cumulative number of days spent rearing chicks	**0·2265**	**0·0976**	**0·0353**	**0·4177**
Male parent*	0·1181	0·0941	−0·0664	0·3026
Chick-days: male parent*	−0·1765	0·1873	−0·5435	0·1906

Binary and continuous input variables were standardized to facilitate model comparisons. In bold are estimates which CI do not overlap zero. CI, confidence interval.
*Relative to female parent.

foster sons (among mothers only for GSSG/GSH ratio), and MDA RBC was higher in parents with higher cumulative number of days spent rearing chicks.

SEX ALLOCATION AND OXIDATIVE STRESS

The physiological costs associated with the production of one sex or the other can be various (e.g. stress-related costs: Love *et al.* 2005; energetic costs: Magrath *et al.* 2007). Sons were shown to be energetically costlier than daughters in our population (Merkling *et al.* 2015). Here, we hypothesized that producing the costlier sex may also entail oxidative costs. Therefore, in line with the 'cost of reproduction hypothesis' (Myers 1978; Cockburn, Legge & Double 2002), we expected that parents with high pre-laying oxidative damage would overproduce the cheaper sex, and that parents forced to rear more sons than they initially produced would endure more oxidative damage. However, none of the pre-laying physiological variables correlated with the natural brood sex ratio. This is not necessarily surprising for baseline CORT, as most studies finding a link between CORT and offspring sex ratio experimentally manipulated hormone levels (e.g., Pike & Petrie 2006), while those looking at baseline levels found no effect (e.g., Riechert, Chastel & Becker 2013). No study has looked at the relationship between oxidative balance variables and sex ratio bias so far. It is thus difficult to know whether the absence of results in our case is surprising or not. Looking at oxidative balance close to laying (i.e. as close as possible to the sex ratio decision) should be the best way to detect any effect (e.g., Cameron *et al.* 1999; Sheldon & West 2004), but we might have lacked statistical power to detect any relationship (sample size <120: West, Reece & Sheldon 2002).

Our fostering experiment unfortunately led to most birds rearing the same sex ratio as they initially produced and thus had low statistical power to detect an effect of the change in sex ratio. However, some results concerning foster sex ratio were in line with our predictions. Baseline CORT is known to facilitate higher parental workload (Ouyang *et al.* 2013; Love *et al.* 2014). Hence, the increased CORT levels in foster broods with more sons (Fig. 2) corroborated the prediction that sons are energetically costlier than daughters for both parents. However, this did not translate into a similar effect of brood sex ratio on oxidative balance, as only females, but not males, rearing all-male foster broods had higher GSSG/GSH ratio at the end of the breeding season than those rearing at least one daughter (Fig. 1a). As GSH is transformed into GSSG during ROS scavenging (Surai 2002) and as GSH levels were lower among mothers rearing only foster sons (Fig. 1b), the increase in the GSSG/GSH ratio with foster sex ratio suggests that females rearing more sons faced a higher oxidative threat than those rearing daughters (i.e. the cheaper sex).

It remains unclear, however, why brood sex ratio affected male CORT but not the GSSG/GSH ratio. In line with the present data, our previous study on the costs of rearing sons did not reveal any parental differences in terms of CORT (Merkling *et al.* 2015). However, other studies on the same population have revealed behavioural and physiological differences among parents. For instance, handicapped males were willing to lose mass to sustain similar feeding rates than controls (Leclaire *et al.* 2011), while females seem to favour their own survival before their chicks' (Leclaire *et al.* 2010). Moreover, a carotenoid supplementation experiment revealed sex differences in resource allocation to coloration or oxidative balance (Leclaire *et al.* 2015). Hence, it is possible that males rearing sons passed the resulting energetical burden to an unmeasured physiological component (e.g. coloration, immunity, weight loss), thereby preventing us from detecting an effect. Yet, given that the GSSG/GSH ratio model with interaction between parental sex and brood sex ratio was equally likely as the null model, and although the confidence interval did not overlap zero, care in the interpretation of our sex-specific effect is required. We therefore need more data to determine whether parents are responding differently to sex ratio in terms of antioxidant activity and whether another component of their physiology is affected too.

REPRODUCTIVE EFFORT AND OXIDATIVE STRESS

Several lines of evidence suggest that an individual's physiological state influenced his parental effort in terms of cumulative number of days spent rearing chicks. Parents with lower SOD (an antioxidant enzyme) and higher CORT levels before laying were more likely to have higher upcoming cumulative number of days spent rearing chicks. As antioxidant activity may reflect the rate of ROS production (Costantini & Verhulst 2009), low levels of SOD activity before laying may reflect a more efficient mitochondrial efficiency, for instance via uncoupling (Brand 2000), thus saving energy for a subsequent greater investment in reproductive effort. Furthermore, the fact that parents with higher CORT levels before laying invested more in reproduction suggests that these individuals were prepared for their higher upcoming workload.

However, longer chick rearing still seemed to induce physiological costs given that parents with more

cumulative number of days spent rearing chicks had higher MDA RBC levels at the end of the breeding season. Increased reproductive effort thus led to more oxidative damage to lipids in red blood cells, but apparently not in plasma. Yet, our data do not allow us to speculate whether this result is a manifestation of a cost of reproductive effort. All parents could 'choose' how many chicks to rear and for how long (i.e. how much to invest) and this result may highlight differences in individual quality, with only high quality individuals being able to deal with increased oxidative damage without detrimental effects on residual reproductive value.

Conclusion

All studies so far investigating the role of oxidative stress as a mediator of life-history trade-offs have focused on variation in brood size, but our study reveals that considering variation in brood sex ratio could be a fruitful avenue of research. As kittiwakes are long-lived and we manipulated sex ratio over one season only, we are unable to conclude whether the observed physiological costs of rearing sons could lead to fitness costs (i.e. a reduction in residual reproductive value). Experiments over multiple seasons where brood sex ratio is consistently manipulated to be all-male or all-female and where parental oxidative balance parameters as well as survival are monitored should bring interesting insight into the role of offspring sex ratio in the trade-off between reproduction and survival.

Authors' contributions

T.M., P.B., E.D. and F.H. designed the study; S.A.H. provided logistic support in the field; T.M. and P.B. collected the data; O.C., G.G., A.V.-M. and F.H. conducted the physiological analyses; T.M. sexed the chicks; T.M., P.B. and F.H. conducted the statistical analyses; T.M. wrote the manuscript and all authors contributed comments.

Acknowledgements

Data accessibility

The data are available on the Open Science Framework website at http://doi.org/10.17605/OSF.IO/45AXS (Merkling *et al.* 2016).

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Supporting Information

Details of electronic Supporting Information are provided below.

Appendix S1. Offspring sex ratio and physiological costs, supplementary methods, tables and results.