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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

The oxidative costs of parental care in cooperative and pair-breeding African starlings

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Abstract

The cost of parental care has long been thought to favor the evolution of cooperative breeding, because breeders can provide reduced parental care when aided by alloparents. Oxidative stress—the imbalance between reactive oxygen species and neutralizing antioxidants—has been proposed to mediate the cost of parental care, though results from empirical studies remain equivocal. We measured changes in oxidative status during reproduction in cooperatively breeding superb starlings (*Lamprotornis superbus*) to gain insight into the relationships among breeding status, parental care, and oxidative stress. We also compared the oxidative cost of reproduction in the cooperatively breeding superb starling to that in a sympatric non-cooperatively breeding species, the greater blue-eared glossy starling (*L. chalybaeus*), to determine whether cooperatively breeding individuals face reduced oxidative costs of parental care relative to non-cooperatively breeding individuals. Breeders and alloparents of the cooperative species did not differ in oxidative status throughout a breeding attempt. However, individuals of the non-cooperative species incurred an increase in reactive oxygen metabolites proportionally to an individual's workload during offspring care. These findings suggest that non-cooperative starlings experience an oxidative cost of parental care, whereas cooperatively breeding starlings do not. It is possible that high nest predation risk and multi-brooding in the cooperatively breeding species may have favored reduced physiological costs of parental care more strongly compared to pair-breeding starlings. Reduced physiological costs of caring for young may thus represent a direct benefit that promotes cooperative breeding.

Keywords Cost of reproduction · Cooperative breeding · Parental care · Physiological cost · Oxidative stress

Introduction

Parental care is assumed to be costly, because investing valuable resources in current reproduction can come at the expense of future survival or future reproductive success—this represents a key life-history trade-off known as the 'cost of reproduction' (Williams 1966; Nur 1988). This trade-off is thought to shape the evolution of animal mating systems (Stearns 1992), because increasing the cost of offspring care favors a transition from uni- to bi-parental care (Webb et al. 2010). Similarly, these costs may shape the evolution of animal social systems, because when the cost of reproduction becomes prohibitively high, more than two individuals may be required to successfully rear young (i.e., cooperative breeding systems with alloparental care) (Brown 1978; Emlen 1982; Crick 1992; Heinsohn and Cockburn 1994; Langen 2000; Ligon and Burt 2004). High costs of reproduction would favor the evolution of cooperative breeding behavior, because breeders can reduce their investment

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in offspring care when alloparents aid in caring for young (i.e., load lightening) (Crick 1992; Hatchwell 1999; Heinsohn 2004; Johnstone 2011).

The idea that the cost of reproduction favors cooperative breeding was first proposed nearly 40 years ago (Brown 1978; Crick 1992), though the cost of reproduction in cooperatively relative to non-cooperatively breeding species has been difficult to study. Increasingly, researchers have studied the cost of reproduction by measuring physiological costs associated with parental care (Zera and Harshman 2001; Harshman and Zera 2007; Fowler and Williams 2017), including in cooperatively breeding species. For example, researchers compared the physiological cost of offspring care across different groups of the facultative cooperative breeding white-browed sparrow weaver (*Plocepasser mahali*) to demonstrate that individuals in larger social groups have reduced physiological costs of offspring care relative to pair-breeding individuals (Cram et al. 2015b). By extending this approach to interspecific comparisons, researchers can gain additional insight into whether cooperatively breeding species have reduced costs of caring for young relative to non-cooperatively breeding species. Doing so across a large number of species remains challenging, however, as detailed field sampling is typically required to assess the physiological costs of parental care (e.g., Fowler and Williams 2017).

Although a number of physiological mechanisms have been proposed to mediate the cost of reproduction (Zera and Harshman 2001; Harshman and Zera 2007; Fowler and Williams 2017), oxidative stress has received growing interest as a potential mediator of life-history trade-offs for nearly 2 decades (Alonso-Alvarez et al. 2004; Heiss and Schoech 2012; Christe et al. 2012; Metcalfe and Monaghan 2013; Fletcher et al. 2013; Costantini and Dell'Omo 2015). Oxidative stress is broadly defined as the imbalance between reactive oxygen species and neutralizing antioxidants (Finkel and Holbrook 2000). The heightened metabolic demand associated with caring for young can result in the increased production of reactive oxygen species (Fletcher et al. 2013), harmful chemicals that may be neutralized by antioxidant defenses. If reactive oxygen species overwhelm an individual's antioxidant system, however, tissues and biomolecules can begin to accumulate harmful oxidative damage (Monaghan et al. 2009), potentially leading to reduced survival (Freeman-Gallant et al. 2011; Saino et al. 2011; Costantini and Dell'Omo 2015; van de Crommenacker et al. 2017).

Although several studies have found evidence that parental care results in oxidative stress (Guindre-Parker et al. 2013; Sharick et al. 2015; Fowler and Williams 2017)—including in cooperatively breeding species (Heiss and Schoech 2012; Cram et al. 2015b)—tests of the oxidative cost of reproduction hypothesis have been equivocal (Metcalfe and Monaghan 2013). For example, a number of studies have found that oxidative stress is uncorrelated with reproductive effort (Nussey et al. 2009; Garratt et al. 2010; Wilson et al. 2012; Ołdakowski et al. 2012; King et al. 2013) or actually decreases with increasing offspring care (Garratt and Pichaud 2013; Costantini et al. 2014; Schmidt et al. 2014). In addition, non-breeding individuals often have higher oxidative stress relative to breeders, a result which seems contradictory to the hypothesis that parental care—and, therefore, breeding—is associated with increased oxidative damage (reviewed in Blount et al. 2016). To this end, cooperatively breeding species—where breeders and non-breeders provide offspring care together—make ideal study systems to test predictions of the oxidative cost of reproduction hypothesis (Costantini 2016a).

Here, we compare changes in oxidative stress over the course of parental care in a tropical cooperatively breeding passerine to gain insight into the relationship between breeding status, parental care, and oxidative stress. Specifically, we use natural variation in breeding status (breeders vs. non-breeding alloparents) and investment in offspring care behavior (offspring guarding or provisioning) to test for evidence of an oxidative cost of reproduction. We also test whether individuals that shared care among a larger group of alloparents experienced reduced oxidative costs of reproduction relative to those that divided care among a smaller group of individuals. In addition to this intraspecific analysis, we also take an interspecific approach to test the hypothesis that a cost of reproduction favors cooperative breeding behavior by comparing the oxidative cost of parental care in two co-occurring, closely related species of African starlings that differ in their social system. We selected two species that are not only syntopic with overlapping territories, but that have similar life histories and breed simultaneously: the obligate cooperatively breeding superb starling (*Lamprotornis superbus*), and the non-cooperative greater blue-eared glossy starling (*L. chalybaeus*). While greater blue-eared glossy starlings breed and care for young in pairs, superb starlings live in large social groups where both breeders and 1–14 alloparents care for young (Rubenstein 2016). We quantified oxidative stress in both species by measuring reactive oxygen metabolites and total antioxidant capacity from plasma samples. Reactive oxygen metabolites (ROM) are more stable derivatives of reactive oxygen species and represent a marker of early oxidative damage (Costantini 2016b). Although ROM are an indirect index of oxidative damage, they are correlated with reduced survival in free-living birds (Geiger et al. 2011; Costantini and Dell'Omo 2015). We quantified antioxidant defenses by measuring the general capacity of plasma antioxidants (OXY) to neutralize a strong of oxidant.

The goal of our study was twofold: to improve our understanding of how oxidative stress is related to breeding status and parental care, and to compare potential oxidative costs of parental care in cooperatively and non-cooperatively breeding individuals. We examined differences in oxidative status across breeding roles of the cooperatively breeding superb starling and made the following prediction based upon the oxidative cost of reproduction. Since we expected that mothers would experience a greater oxidative cost of offspring care, because they typically perform the greatest proportion of care relative to fathers and alloparents (Rubenstein 2016; Guindre-Parker and Rubenstein 2018), we predicted that superb starling mothers would experience a greater increase in ROM or a greater decrease in OXY from incubation to chick rearing relative to either fathers or alloparents. In addition to comparing oxidative status across breeding roles, we determined whether an oxidative cost of reproduction was correlated with social group size in the cooperative breeder. We expected that superb starling individuals caring for young as part of a larger contingent of caregivers would experience reduced costs of reproduction as measured by lower ROM or higher OXY relative to individuals from smaller contingents with fewer alloparents. Finally, we determined whether individuals of the non-cooperative and cooperative breeding species differed in their oxidative status throughout a breeding attempt. We predicted that individuals of the non-cooperative species would experience a greater increase in ROM or a greater decrease in OXY from incubation to chick rearing. We used behavioral observations to test whether an oxidative cost of reproduction was correlated with parental care behavior in both species. We predicted that individuals of either species would have higher ROM or lower OXY during chick rearing when (1) guarding the nest a greater proportion of the time, (2) feeding nestlings at a higher rate, or (3) with a greater index of workload (i.e., number of chicks in a nest multiplied by the number of days of offspring care, sensu Heiss and Schoech 2012).

Materials and methods

Study species

This study was conducted during three consecutive long-rains breeding seasons (March–June) from 2013 to 2015 at the Mpala Research Centre in central Kenya (0°17′N, 37°52′E), where we have continuously monitored populations of free-living superb and greater blued-eared glossy starlings since 2001. Non-cooperative greater blue-eared glossy starlings are socially monogamous pair-breeders, but they are seasonally gregarious and form larger flocks during the non-breeding season. In contrast, superb starlings are plural cooperative breeders that live in large social groups of up to 50 individuals with multiple breeding pairs per group (Rubenstein 2016), and between 1 and 14 alloparents at each nest (Rubenstein 2016). Individuals of both species have been marked with a unique combination of colored bands and a numbered metal band.

Both species of starlings are not only in the same clade (Lovette and Rubenstein 2007), but they co-occur over the majority of their ranges across East Africa (Feare and Craig 1998) and exhibit territorial overlap in our study population. Non-cooperative greater blue-eared glossy starlings are larger than cooperative superb starlings (mean 85 versus 65 g) (Feare and Craig 1998), but both species have similar clutch sizes (mean ± SD: greater blue-eared glossy = 3.0 ± 0.73; superb = 3.1 ± 0.87), and eggs of approximately the same size relative to adult body mass (7% of adult mass) (Feare and Craig 1998). Greater blue-eared glossy starlings build grassy nests in existing cavities (Feare and Craig 1998), and in our study area, pairs use natural cavities as well as human-made wooden nest boxes. Conversely, superb starlings build closed grassy-domed nests, primarily in acacia trees (Feare and Craig 1998; Rubenstein 2016). Both species line the interior of their nests with grasses and feathers. Mothers of both species perform the majority of incubation, which lasts 2 weeks (Feare and Craig 1998). Nests of both species can be parasitized by great spotted cuckoos (*Clamator glandarius*) in other populations (Feare and Craig 1998), though neither interspecific nor intraspecific brood parasitism has been observed in our study population.

Offspring care is similar in both species, consisting of guarding the nest by perching in nearby trees, as well as provisioning the young. Moreover, nestlings of both species remain in the nest up to 23 days before fledging (Feare and Craig 1998). Although adults are omnivorous, feeding on insects, berries, and seeds, both non-cooperative and cooperatively breeding starlings provision their young exclusively with insects (Feare and Craig 1998). The nests of both species attract similar predators (e.g., mongoose, snakes, birds of prey, etc.) (Rubenstein 2016), though superb starlings experienced slightly higher nest predation over the course of this study relative to their pair-breeding counterpart: approximately 90% superb starling nests were depredated compared to 75% of nests for the greater blue-eared glossy starling. Superb starlings re-nest within a breeding season at a higher rate than greater blue-eared glossy starlings (Rubenstein 2016), potentially as a result of differences in nest predation pressure and the availability of alloparents. Nest predation rates are not solely shaped by nest type, however, as another cavity nesting starling in this population—the Hildebrandt's starling, *L. hildebrandti*—has similarly high nest predation rates as the superb starling (Rubenstein 2016).

Trapping and sampling birds

We trapped individuals of both species using food-baited ground-traps or mist-nets around the focal nest. Traps were observed continuously, and a small blood sample was collected from the brachial vein within 3 min of capture. Both species were sampled during two breeding stages: (1) incubation, defined as post-clutch completion but before hatching to exclude potential costs of egg-laying or nest building; (2) chick rearing, when nestlings were between 4 and 16 days of age. While there was some variation in the exact timing of sampling across individuals (e.g., in the time lag between the incubation and chick rearing samples; mean ± SE = 15 ± 0.7 days), there was no significant difference in this lag between the two species (two-tailed t test; $t = 0.90$, $df = 10.7$, $P = 0.38$). The length of this time lag was also unrelated to within-individual changes in ROM or OXY (see Electronic Supplementary Materials). We re-sampled individuals across both stages whenever possible, but high nest predation rates made this difficult. Blood samples were centrifuged and plasma was stored frozen until analysis (up to 6 months) (Rubenstein et al. 2008). We followed all applicable institutional and national guidelines for animal care.

Monitoring reproductive effort and breeding roles

We observed active nests with a spotting scope for a minimum of 2 h per observation period (mean ± SE observation periods per nest = 3.6 ± 0.4 for superb starlings and 2.3 ± 0.2 for greater blue-eared glossy starlings) when nestlings were 4 or more days of age (mean ± SE = 4.6 ± 0.5 days for superb starlings and 4.5 ± 0.5 days for greater blue-eared glossy starlings). All focal nest observations were performed before the beginning of chick provisioning blood sample collection for breeders or alloparents at that nest. During focal observations, we monitored the identity of each bird that came within 20 m of the nest as well as those that delivered food to the nestlings (Rubenstein 2007a). We calculated two behaviors that have been shown to reflect investment in offspring care (Guindre-Parker and Rubenstein 2018): (1) *nest guarding*, defined as the proportion of time that an individual spent within 20 m of the nest—but not inside—relative to the length of the observation period; (2) *offspring provisioning*, defined as the number of trips per hour where an individual delivered food into the nest. However, since specific offspring care behaviors such as guarding or provisioning can fail to accurately represent all that is required to care for young [e.g., guarding and provisioning rates do not account for type of provisioning performed (foraging in flight vs. on the ground), the type of prey caught, the distance traveled to find prey, the nutritional value of the prey, the energy required to deter predators from approaching the nest, or time or energy invested in chasing intraspecific competitors from the territory, etc.], we also calculated a general index of breeding workload (sensu Heiss and Schoech 2012) as the number of chicks being cared for in a nest multiplied by the age of the chicks on the day of each adult's capture.

For both starling species, the mother was identified as the bird with a brood patch, while the social father was identified as the male closely following the incubating female (Rubenstein 2007b). For superb starlings, where the large nest contingent occasionally made identifying the parents more difficult, we also confirmed parentage genetically using microsatellite markers (Rubenstein 2007c). Non-breeding birds that guarded or provisioned young at superb starling nests were classified as alloparents, whereas only breeders were observed near their own nest in greater blue-eared glossy starlings.

Physiological analyses

We measured ROM using a commercially available kit (dROM test, Diacron International, Italy) according to standard protocols (Costantini et al. 2008; Baldo et al. 2015). We diluted 10 μL of plasma with 400 μL of a 1:100 mixture of the alkyl-amine solution and acetate buffer. Similar to previous studies (Costantini et al. 2011; Guindre-Parker et al. 2013), we found that a precipitate (i.e., lipids) formed following a 75 min incubation—therefore, instead of running the assay in a 96-well plate, we ran the assay in 1.5 mL eppendorf tubes before pipetting the liquid layer into a 96-well plate. Briefly, we incubated reagents and plasma in eppendorf tubes for 75 min at 37 °C and centrifuged the tubes at 10,000 rpm for 30 s to isolate the precipitate at the bottom of the tube. We then pipetted 190 μL of the liquid into duplicate wells of a flat-bottomed 96-well plate (Costantini et al. 2011; Guindre-Parker et al. 2013). We included a standard curve of H_2O_2 (a strong oxidant; range = 0.16–5.12 mg H_2O_2/dL) on each plate. Absorbance was read at a wavelength of 490 nm and concentrations of reactive oxygen metabolites are given in mg H_2O_2/dL.

We also measured OXY using a commercial kit (OXY test, Diacron International, Italy), which measures the general capacity of plasma antioxidants to neutralize a strong oxidant in vitro (Guindre-Parker et al. 2013). We diluted plasma samples to 1:100 with deionized water and transferred 5 μL of diluted samples to the wells of a flat-bottomed 96-well plate. We then added 200 μL of the HOCl solution to each well, vortexed the plate at 450 rpm for 10 s, and incubated the plate at 37 °C for 10 min (Costantini et al. 2008, 2011; Guindre-Parker et al. 2013). On each plate, we included a standard curve of HOCl (range = 0.425–6.8 μmol HOCl/mL). We then added 2 μL of the color-changing chromogen solution to each well (*N*,*N*-diethylparaphenilendiamine) and read the plate at 490 nm after shaking for 30 s.

Concentrations of antioxidants are expressed in μmol of HOCl/mL.

Both assays were performed within 6 months of sampling, and multiple samples from a single individual were run on the same plate (for those re-trapped during both breeding stages), but positions within a plate were randomized. Each sample was run in duplicate for the dROM assay, and the mean intra-assay coefficient of variation was 5.9%, while each sample was run in triplicate for the OXY assay where the mean intra-assay coefficient of variation was 5.4%. When we used multiple plates to assay all the samples within a year, we also re-ran a subset of samples on multiple plates to calculate the inter-assay coefficient of variation, which was 10.4% for ROM and 11.9% for OXY.

Statistical analysis

ROM was square-root transformed to meet the assumptions of normality, while OXY did not require a transformation (from examining the distribution of model residuals). We used mixed-effect ANOVAs to determine whether ROM or OXY differed across breeding stage, breeding role, species, or the interactions between breeding stage and role or species. Some individuals were sampled during both breeding stages, while others were only sampled at one stage (see Table 1 for sample sizes). We also sampled multiple individuals at the same nest for both species (cooperative: 100 individuals at 27 nests; non-cooperative: 40 individuals at 21 nests), so we included individual ID nested within nest ID as a random effect. We had initially included year, rainfall, mass, and body condition as covariates, but ultimately removed these predictors from our models, because doing so did not alter the results and improved model fit ($\Delta\mathrm{AIC} > 3$). Since a subset of our data included individuals sampled during both breeding stages, we also performed ANOVAs examining within-individual changes in ROM or OXY. We found that results from these within-individual models were qualitatively similar to the more inclusive model (including non-repeatedly sampled individuals), so we present results of the inclusive analyses in the text, while within-individual analyses are found in the electronic supplementary materials.

Table 1 Distribution of sample sizes across species, breeding stages, and breeding roles

Species	Breeding stage	Breeding role
Greater blue-eared glossy starling $N=40$ unique birds	Incubation $N=36$	Mother $N=22$ Father $N=14$
	Chick rearing $N=15$ (11)	Mother $N=8$ (8) Father $N=7$ (3)
Superb starling $N=100$ unique birds	Incubation $N=80$	Mother $N=35$ Father $N=9$ Alloparent $N=36$
	Chick rearing $N=37$ (17)	Mother $N=9$ (8) Father $N=6$ (2) Alloparent $N=22$ (7)

During chick rearing, the sample size in parentheses indicates the subset of individuals which were recaptured and for which we also collected incubation samples

Next, we used linear mixed-effect models to determine whether ROM or OXY during chick rearing were correlated to the number of individuals that cared for young at a nest in cooperatively breeding superb starlings; each model included the number of individuals providing care and Julian date as independent variables, and nest ID as a random effect.

Finally, we investigated whether ROM or OXY measured during chick rearing reflected investment in offspring care in both species using linear mixed-effect models that included nest guarding, provisioning rate, workload, their interactions with species, and Julian date as independent variables. Nest guarding, provisioning rate, and workload were not correlated to one another (Pearson's r range: -0.10–0.11; P value range: 0.24–0.85). Because we sampled multiple individuals at each nest, these models included nest ID as a random effect (conversely, each individual was included only once in these analyses, so we did not include a random effect of individual ID). All analyses were performed in R version 3.2.4 (R Core Team 2016), using 'nlme' and restricted maximum likelihood.

Results

We first examined whether oxidative stress over the course of a breeding attempt differed among breeding roles in cooperatively breeding superb starlings. We found that ROM was not related to breeding role or an interactive effect of role and breeding stage (role: $F_{3,20}=1.16$, $P=0.33$; interaction between breeding role and breeding stage; $F_{3,20}=2.61$, $P=0.08$; Fig. 1). Breeding roles also did not differ in OXY during incubation and chick rearing (role: $F_{1,92}=0.36$, $P=0.78$; interactions between breeding stage and breeding role: $F_{3,21}=0.52$, $P=0.67$; Fig. 1d). We also examined whether ROM or OXY during chick rearing were correlated to alloparent group size in the cooperatively breeding superb starling. We found that neither ROM ($t=-1.02$, $df=15$, $P=0.32$) nor OXY ($t=-1.19$, $df=18$, $P=0.25$) were correlated to the number of birds caring for young at a nest, which did not support our hypothesis that larger contingents of alloparents could reduce the oxidative cost of offspring care in superb starlings.

Next, we examined whether individuals of the non-cooperative greater blue-eared glossy starling differed in their oxidative status relative to cooperatively breeding superb starlings. We found no difference in ROM ($F_{1,82}=1.71$, $P=0.19$) or OXY ($F_{1,92}=0.06$, $P=0.81$) between the

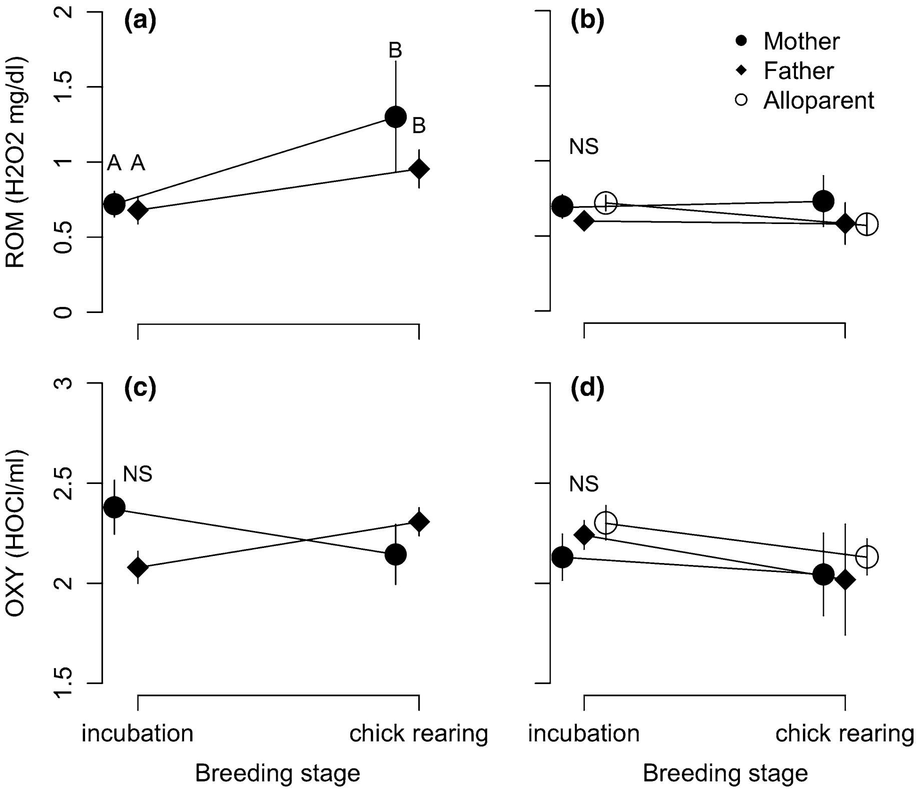

Fig. 1 **a** Reactive oxygen metabolites (ROM) increased significantly from incubation to chick rearing in non-cooperative greater blue-eared glossy starlings. In contrast, **b** ROM did not change over the course of reproduction for cooperatively breeding superb starling fathers, mothers, or alloparents. Total antioxidant capacity (OXY) did not differ significantly from incubation to chick rearing for **c** non-cooperative or **d** cooperative starlings. Symbols represent the mean ± standard error and uppercase letters represent groups that differ from each other according to Tukey's post hoc comparisons. *NS* represents cases where no significant differences were detected

species. However, we found that breeders of the non-cooperative greater blue-eared glossy starling increased significantly in ROM from incubation to chick rearing, whereas individuals of the cooperative superb starling did not (interaction of breeding stage by species: $F_{1,20} = 7.03$, $P = 0.02$; stage: $F_{1,20} = 0.68$, $P = 0.41$; Julian date: $F_{1,20} = 13.7$, $P = 0.001$; Fig. 1). In contrast, neither species exhibited a change in OXY over the course of a breeding attempt (breeding stage: $F_{1,21} = 0.57$, $P = 0.46$; Julian date: $F_{1,21} = 0.87$, $P = 0.36$; interaction between breeding stage and species: $F_{1,21} = 2.54$, $P = 0.13$; Fig. 1).

Finally, to address our hypothesis that oxidative stress may represent a cost of reproduction and would occur proportionally to investment in offspring care, we examined whether ROM or OXY measured during chick rearing reflected offspring care workload in both species of starlings. We found that chick rearing ROM was unrelated to nest guarding or provisioning rate in both species (Table 2). However, we found that chick rearing ROM increased with workload, though differently for the two species (Table 2): greater blue-eared glossy starlings increased in ROM proportionally to workload, whereas superb starlings did not (Fig. 2). This result was consistent even when we excluded the greater blue-eared glossy starling father with the highest ROM value from our analyses (i.e., ROM = 3.12 mg H_2O_2/dL), showing that this individual was not driving the observed relationship. Conversely, we found that OXY was unrelated to nest guarding, provisioning rate, and workload in both species (Table 2; Fig. 2).

Discussion

Together, our results suggest that, over the course of a breeding attempt, reactive oxygen metabolites increase proportionally to workload in non-cooperatively breeding greater blue-eared glossy starlings but not in cooperatively breeding superb starlings. Although our study examined changes in two components of oxidative stress in co-occurring species with different social breeding systems, our results are consistent with an intraspecific study in the facultative cooperatively breeding white-browed sparrow weaver, showing that the oxidative cost of reproduction was reduced in individuals breeding cooperatively (Cram et al. 2015b). Unlike white-browed sparrow weavers, however, cooperatively breeding superb starlings showed no evidence for an oxidative cost of reproduction regardless of the size of the contingent of individuals caring for young at a nest. One explanation for this difference in our findings is the reduced offspring care of individual superb starlings relative to those in white-browed sparrow weavers. For example, superb starling individuals perform up to a maximum of 5 feeds per hour (typically below 2 feeds per hour; Guindre-Parker and Rubenstein 2018), whereas white-browed sparrow weaver individuals

Table 2 Parameter estimates and test statistics for an LMM examining how (a) reactive oxygen metabolites (H_2O_2/dL) or (b) total antioxidant capacity (HOCl/mL) is related to parental and alloparental care behaviors (i.e., provisioning rate per hour, percentage of time spent chick guarding, or workload measured as chick × days of offspring care)

Predictor variable	Coefficient ± SE	*df*	*t* value	*P* value
(a) Dependent variable: ROM				
Intercept	− 0.32 ± 0.43	18	− 0.75	0.46
Provisioning rate	0.01 ± 0.03	12	0.32	0.76
Chick guarding	0.003 ± 0.005	12	0.63	0.54
Workload	**0.02 ± 0.007**	**12**	**2.76**	**0.02**
Species (superb starling)	0.41 ± 0.31	18	1.32	0.20
Julian date	**0.006 ± 0.003**	**12**	**2.53**	**0.03**
Provisioning rate × species	0.02 ± 0.10	12	0.21	0.83
Chick guarding × species	0.001 ± 0.007	12	0.17	0.87
Workload × species	**− 0.03 ± 0.01**	**12**	**− 3.03**	**0.01**
	Random effect		Variance ± SE	*N*
	Nest ID		0.13 ± 0.25	20
(b) Dependent variable: OXY				
Intercept	**1.91 ± 0.71**	**22**	**2.69**	**0.01**
Provisioning rate	0.03 ± 0.07	13	0.50	0.63
Chick guarding	0.001 ± 0.009	13	0.12	0.91
Workload	0.002 ± 0.01	13	0.17	0.87
Species (superb starling)	− 0.29 ± 0.53	22	− 0.55	0.59
Julian date	0.002 ± 0.004	13	0.41	0.69
Provisioning rate × species	0.16 ± 0.14	13	1.18	0.26
Chick guarding × species	0.001 ± 0.01	13	0.08	0.94
Workload × species	0.002 ± 0.02	13	0.14	0.89
	Random effect		Variance ± SE	*N*
	Nest ID		0.10 ± 0.51	24

Nest ID was included as a random effect to account for sampling multiple individuals at the same nest. Bolding indicates statistically significant effects

can provision up to 15 times per hour (Cram et al. 2015b). Alternatively, offspring care is divided unevenly among superb starling group members (Guindre-Parker and Rubenstein 2018) and the size of the alloparent contingent varies widely among nests (Rubenstein 2016), so both workload and group size may be poor estimators of the offspring care that an individual superb starling may perform.

One key difference between the two species in our study that could explain the differences that we detected in oxidative stress throughout a breeding attempt is that superb starlings typically experience higher rates of nest failure due to nest predation (Rubenstein 2016). Although predation rates were high in both species over the course of this study, 90% of superb starling nests failed due to nest predation, whereas only 75% did for greater blue-eared glossy starlings. Nevertheless, superb starlings typically re-nest within a breeding season at a higher rate than greater blue-eared glossy starlings (Rubenstein 2016), a pattern observed more generally across the tropics, as cooperative breeders typically re-nest more frequently within a season than their non-cooperative counterparts (van den Heuvel and Ridley 2012). This raises the possibility that if a cost of reproduction accumulated with each nesting attempt, selection for reducing oxidative costs of reproduction via cooperative breeding may have been stronger in the superb starling than in the greater blue-eared glossy starling. In support of this idea, our results showed that reactive oxygen species increased in both species with Julian date, which suggests that breeding later in the season is associated with elevated oxidative damage. While this pattern potentially reflects a physiological cost to re-nesting (which becomes more likely as the breeding season progresses), the relationship between oxidative status and Julian date is potentially related to a number of other seasonal changes including habitat quality, food availability, or individual quality and the timing of breeding. It remains possible that the evolution of cooperative breeding may not just be associated with the reproductive benefits of multi-brooding (Rubenstein and Lovette 2007; Rubenstein 2011), but also the reduced physiological costs associated with re-nesting. Although we included two species in this study, the hypothesis that cooperative breeding reduces the oxidative cost of reproduction should be examined in additional species living in environments where unpredictable patterns of rainfall and resource distribution are thought to influence the incidence of cooperative breeding behavior in vertebrates (Jetz and Rubenstein 2011; Lukas and Clutton-Brock 2017).

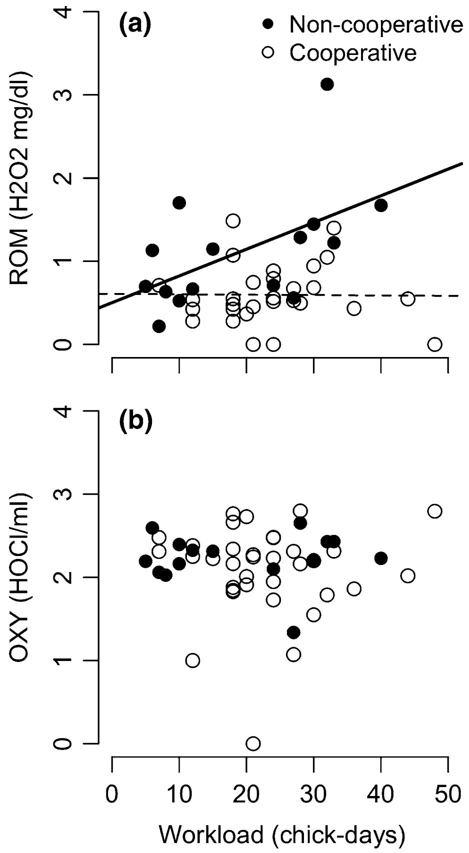

Fig. 2 Breeding workload (i.e., chick × days) was **a** positively correlated to reactive oxygen metabolites (ROM) in pair-breeding greater blue-eared glossy starlings (closed) but not cooperatively breeding superb starlings (open). Conversely, workload was unrelated to **b** total antioxidant capacity (OXY) for either species

Because sampling a greater number of species across multiple life stages can be expensive and logistically challenging, we recommend using a paired-species design (like our own study) repeated across multiple taxa that measures the cost of reproduction over a whole breeding season or multiple consecutive breeding attempts.

Despite finding that oxidative stress during breeding reflects workload and differs between a cooperative and non-cooperative breeder, we did not find evidence that an oxidative cost of reproduction occurred proportionally to offspring guarding or provisioning rates. One interpretation for this result is that our measure of workload better reflects total breeding effort relative to distinct behaviors like nest guarding or nestling provisioning rate—breeding successfully requires a complex set of behaviors that may be poorly captured by a single behavioral measure. For example, nest guarding and provisioning rates do not necessarily reflect the cost of cleaning the nest, brooding the young, deterring predators, foraging, or mate guarding. If workload is a better index of the effort required to raise young, it is, not surprising that we found no relationship between oxidative status and workload in superb starlings where offspring care is shared unevenly among a large group of individuals. Instead, we might expect that alloparent contingent size would be a better indicator of the degree to which individuals experience a cost of offspring care in superb starlings (rather than total nest workload), because this measure does not account for how workload is divided among group members of a cooperatively breeding group. This was not supported by our study, however, as superb starlings did not appear to change in oxidative status across a breeding attempt, and the oxidative status of individuals was unrelated to workload or the size of the alloparent contingent at a nest.

Although the oxidative differences which we observed between superb and greater blue-eared glossy starlings are consistent with differences in their social behavior, it is difficult to rule out other factors in a comparison of two species. For example, while both species have similar life histories and behaviors, it is possible that differences in ROM over the course of a breeding attempt would be related to factors other than offspring care. Indeed, differences in body size or mass (Schull et al. 2016), territory size or defense (Guindre-Parker et al. 2013), social rank or social conflict (Cram et al. 2015a), parental or social interactions (Beaulieu et al. 2017), or even plumage coloration (Henschen et al. 2016) can also covary with oxidative status. However, we would expect differences in oxidative status caused by these alternative explanations to be expressed during incubation as well as chick provisioning, which is not the case in our study. Thus, our findings are most consistent with the conclusion that differences in social behavior and offspring care shaped differences in an oxidative cost of reproduction. Methodologically, it is also possible that the oxidative cost of reproduction may be tissue-specific, and damage may accumulate in different tissues or organs rather than circulating plasma markers of oxidative stress (Schmidt et al. 2014). Although this is a possibility in our study system, it seems unlikely that greater blue-eared glossy starlings would incur damage that can be measured in plasma, whereas a close relative living in the same environment would accumulate damage exclusively in different tissues. Similarly, multiple physiological systems likely interact to shape the cost of parental care (Christe et al. 2012; Fowler and Williams 2017) and the relative importance of these alternative mechanisms may differ across our study species. Future comparative work investigating alternative physiological mechanisms or a greater number of markers of oxidative status across a greater number of tissues would be needed before we can conclude whether different species experience the cost of reproduction differently.

We did not find evidence that oxidative status differed between breeding roles for either species. Nevertheless, it is possible that an individual's oxidative state prior to breeding may affect subsequent breeding decisions, particularly in cooperatively breeding species. For example, only individuals with low pre-breeding oxidative stress invested in reproduction in cooperatively breeding Seychelles warblers (*Acrocephalus sechellensis*) (van de Crommenacker et al. 2011) and Florida scrub-jays (*Aphelocoma coerulescens*) (Heiss and Schoech 2012). While superb starling breeders and alloparents did not differ in their ROM and OXY during the incubation stage, oxidative status in the dry pre-breeding season could influence breeding decisions—such as the adoption of different roles—which would represent an oxidative constraint on reproduction that we did not explore in this study. Since previous work in superb starlings demonstrated that pre-breeding physiology (i.e., glucocorticoid hormones) is central to shaping breeding roles (Rubenstein 2007d), it will be interesting to examine carryover effects of oxidative stress across life-history stages in this and other species. Similarly, an alternative to the oxidative cost of reproduction hypothesis is the oxidative shielding hypothesis—where mothers of oviparous species may lower their oxidative stress prior to egg-laying to shield their young from potential oxidative damage (Blount et al. 2016). Although this hypothesis is not mutually exclusive from the oxidative cost of reproduction hypothesis, future work testing the oxidative shielding hypothesis in birds should focus upon sampling oxidative status at multiple additional stages (including during the pre-breeding season, during mating, during follicular development, and during egg-laying) and use an experimental approach to disentangle predictions of both hypotheses.

Ultimately, our study suggests that oxidative status differs across species with different breeding systems. Specifically, cooperatively breeding species may have reduced or absent oxidative costs of reproduction relative to non-cooperative species, supporting the hypothesis that the cost of reproduction could be one of the many potential factors favoring the evolution of cooperative breeding. Thus, in social species where the costs of rearing young are high, reduced physiological costs of re-nesting likely represent a direct benefit of cooperative breeding.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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