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Oxidative status and fitness components in the Seychelles warbler

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Short title: Oxidative status and fitness in a wild bird

SUMMARY

1. Oxidative damage, caused by reactive oxygen species during aerobic respiration, is thought to be an important mediator of life-history trade-offs. To mitigate oxidative damage, antioxidant defence mechanisms are deployed, often at the cost of resource allocation to other body functions. Both reduced resource allocation to body functions and direct oxidative damage may decrease individual fitness, through reducing survival and/or reproductive output.
2. The oxidative costs of reproduction have gained much attention recently, but few studies have investigated the long-term consequences of oxidative damage on survival and (future) reproductive output under natural conditions.
3. Using a wild population of the cooperatively breeding Seychelles warbler (*Acrocephalus sechellensis*), we tested the prediction that high levels of reactive oxygen species, or high antioxidant investments to avoid oxidative damage, have fitness consequences because they reduce survival and/or reproductive output.
4. We found that individuals with higher circulating non-enzymatic antioxidant capacity had a lower probability of surviving until the next year. However, neither current reproductive output, nor future reproductive output in the surviving individuals, was associated with circulating non-enzymatic antioxidant capacity or oxidative damage.
5. The negative relationship between antioxidant capacity and survival that we observed concurs with the findings of an extensive comparative study on birds, however the mechanisms underlying this association remain to be resolved.

KEY-WORDS

Annual survival, antioxidants, cooperative breeding, current and future reproductive output, fitness, life-history, oxidative status, trade-offs.

INTRODUCTION

Variation in life-history trajectories is often shaped by constraints that result from trade-offs between traits or body functions (e.g., self-maintenance, cellular homeostasis, reproduction) (Reznick 1992; Stearns 1992). The allocation of limited resources between conflicting traits or functions is hypothesised to be the basis for such trade-offs (Reznick, Nunney & Tessier 2000; Zera & Harshman 2001). As such, resource allocation to reproductive tasks may reduce the availability of resources for self-maintenance (Kirkwood & Holliday 1979; Wiersma *et al*. 2004), and can affect survival and future reproduction (Reznick 1992; Stearns 1992). Trade-offs are thought to be driven by physiological processes (e.g., nutrient allocation) (Zera & Harshman 2001; Speakman 2008). Therefore, it is important to understand how these mechanisms shape life-history trajectories (Zera & Harshman 2001).

Recently, awareness has grown that trade-offs may be based on resources other than energy (e.g., hormones; Dowling & Simmons 2009; Isaksson, Sheldon & Uller 2011). In this context, the mechanism of oxidative damage management has gained increased attention (Costantini 2008, 2014; Monaghan, Metcalfe & Torres 2009) and has become embedded in life-history theory (Speakman & Garratt 2014). The production of reactive oxygen species (pro-oxidants) is a cost of aerobic respiration (Finkel & Holbrook 2000; but see Brand 2000; Speakman & Garratt 2014). If not nullified, these pro-oxidants can damage biomolecules ('oxidative damage'; Beckman & Ames 1998; Halliwell & Gutteridge 2011), resulting in somatic damage, accelerated ageing and early death (Finkel & Holbrook 2000). Resistance to pro-oxidants is mediated by the ‘antioxidant barrier’; a complex system containing a range of endogenous (e.g. enzymes) and exogenous (e.g. carotenoids and vitamins) antioxidant compounds that neutralize pro-oxidants (Felton & Summers 1995; Halliwell & Gutteridge 2011). The balance between pro- and antioxidants determines the rate of oxidative damage; the latter occurs when pro-oxidant production exceeds the antioxidant defences (Finkel & Holbrook 2000; Costantini & Verhulst 2009; Monaghan *et al.* 2009).

The number of studies studying the physiological mechanisms underlying oxidative status-related fitness links is growing, but especially in natural populations much about these mechanisms remains to be learnt. It has been shown that oxidative damage may shape reproductive fitness through different routes (Blount *et al.* 2004, 2015; Bizé *et al.* 2008). Oxidative damage can affect reproductive output directly, e.g. through damaging gametes/embryos or lowering embryonic environment quality (Blount *et al.* 2004; Agarwal *et al.* 2012; Garratt & Brooks 2012), or indirectly, e.g. through resource allocation to antioxidant protection rather than reproduction (von Schantz *et al.* 1999; McGraw 2005; Catoni, Peters & Schaefer 2008). Furthermore, persistent oxidative damage may cause irreversible damage to reproductive organs and thus reduce future reproductive output (Sikka 2001; Agarwal, Gupta & Sikka 2006). The oxidative 'cost' of reproduction has been broadly investigated: higher metabolism during reproductive activities (Bryant 1997; Nilsson 2002; Welcker *et al.* 2015) can elevate pro-oxidant production and exert pressure on antioxidant defences, which can be temporarily up-regulated if antioxidant availability is sufficient (e.g., Metcalfe & Alonso-Alvarez 2010; Blount *et al.* 2015). Yet, results from the small set of studies that have investigated the impact of oxidative status on reproductive output are inconclusive regarding the direction in which oxidative damage may link with reproductive performance. Higher resistance to oxidative damage has been shown to be negatively (Bertrand *et al.* 2006), positively (Bizé *et al.* 2008, Cohen *et al*. 2008), or not related to clutch size (Markó *et al.* 2011), while positive relationships with egg size (Markó *et al.* 2011), number of fledglings produced (Safran *et al.* 2010) and offspring quality (Biard, Surai & Møller 2005) have been observed. Interestingly, even within the same species, negative (Bertrand *et al.* 2006) and positive links (Kim *et al.* 2010) between antioxidant protection and fecundity have been found. A study that experimentally reduced a key antioxidant (glutathione), thus increasing oxidative stress, found delays in the start of egg laying and decreases in clutch size, but no influence on hatching/fledging success and the number of hatchlings/fledglings produced (Costantini *et al.* 2015).

Oxidative status effects on survival may also act on different levels: the gradual impairment of organs, tissues and cells caused by oxidative damage (Giugliano, Ceriello & Paolisso 1995; Beal 1995; Ozawa, Cadenas & Packer 1999) can lead to reduced survival prospects in the long term. A strong antioxidant barrier may help individuals to moderate this process, but when resources are limited, any increase in antioxidant investments is likely to come at the cost of resource allocation to other body functions (Alonso-Álvarez *et al.* 2004; Costantini *et al.* 2008). Such impairments, e.g., of the immune system, may lead to reduced survival in the short term (Bertrand *et al.* 2006; Pike *et al.* 2007; Cohen *et al.* 2008). Higher oxidative damage has been found to correlate with lower survival (Freeman-Gallant *et al.* 2011; Noguera, Kim & Velando 2011 (during development)), life span (Archer *et al.* 2013) or re-sighting probability (Blount *et al.* 2015; Costantini & Dell'Omo 2015). The evidence is less conclusive for the role of antioxidants: resistance to oxidative damage (i.e. antioxidant capacity) was either positively (Alonso-Álvarez *et al.* 2006; Bizé *et al.* 2008; Saino *et al.* 2011), negatively (Cohen *et al*. 2008;

also see Ristow & Schmeisser 2011) or not related to survival (Jang *et al.* 2009; Zhang *et al.* 2009).

To better understand the role of the oxidative balance in life-history evolution, more studies investigating such associations in free-living species are needed (Selman *et al.* 2012; Blount *et al.* 2015; Speakman *et al.* 2015). Linking oxidative parameters with both survival and reproduction within the same study is also valuable for the interpretation of inter-related fitness trade-offs (see Reznick 1992; Roff 1992; Stearns 1992), but has rarely been done so far (Costantini 2008, 2011; Monaghan *et al.* 2009; Nussey *et al.* 2009).

Here we investigate how oxidative damage and circulating non-enzymatic antioxidant capacity are linked with survival and reproductive output, using data from a long-term study of a wild population of the Seychelles warbler (*Acrocephalus sechellensis*) on Cousin Island, Seychelles. This population is well suited to investigate these links under natural conditions: nearly all birds have been individually marked (Richardson *et al.* 2001) and the annual reproductive output and age of individuals is known (Hammers *et al.* 2015). Annual survival is not confounded with dispersal, as there is virtually no inter-island migration (Komdeur *et al.* 2004). Furthermore, adult Seychelles warblers lack natural predators (van de Crommenacker *et al.* 2011c), thus extrinsic mortality contributes little to annual survival rates. These features provide the possibility to investigate long-term effects of oxidative damage on fitness components. Previous work on the Seychelles warbler showed that higher metabolism during reproduction elevates pro-oxidant production and exerts pressure on antioxidant defences (van de Crommenacker, Komdeur & Richardson 2011b).

Cooperative breeding occurs in the Cousin Island population of Seychelles warblers: a lack of suitable independent breeding vacancies drives young adult individuals into becoming subordinates within a territory (Komdeur 1994a). Some subordinates (mostly females, Komdeur 1996a) help with territory defence and the rearing of young, while others (non-helpers) do not (Richardson, Burke & Komdeur 2003a; Richardson, Komdeur & Burke 2003b). Dominant birds, constituting the breeding pair, generally remain in their breeding territory until death (Komdeur 1996a; Eikenaar *et al.* 2008). Seychelles warblers on Cousin Island typically produce one clutch per season (Komdeur 1998). Clutches generally consist of one egg (80% of all nests, Richardson *et al.* 2001). Subordinate males very rarely gain paternity, but female co-breeding frequently occurs, with ca. 44% of subordinate females (co-breeders) gaining parentage per year (Richardson *et al.* 2001; Richardson, Burke & Komdeur 2002). The period of parental investment is long for a passerine bird (*ca*. three months after fledging) (Komdeur 1996b). Throughout the breeding season, body condition of dominants is better than that of helpers and non-helpers, respectively (van de Crommenacker *et al.* 2011b).

Based on the abovementioned detrimental effects of pro-oxidants on (reproductive) organs and tissues, we predict oxidative damage levels to be negatively linked to survival and current and future reproductive output. Given the equivocal results of studies on this topic so far (see above), it is less straightforward to make a prediction for plasma antioxidant levels. Circulating antioxidant levels may indicate an individual's ability to mobilize or intake antioxidants, hence higher levels may signify better individual quality and could potentially predict higher fitness. Alternatively, as antioxidants tend to be produced in response to the production of pro-oxidants, high antioxidant levels may mean that an individual is challenged to maintain its oxidative balance, which may result in trade-offs and impair its longer term fitness.

METHODS

Study area, population and data collection

The Seychelles warbler population on Cousin Island, Seychelles (29 ha; 04°20' S, 55°40' E) has been studied intensively since 1985 (Komdeur 1994a; Richardson *et al.* 2003b; Hammers *et al.* 2016). Since 1997, nearly all individuals have been captured, individually marked (using a combination of three colour rings and a numbered British Trust for Ornithology metal ring) and blood sampled (>96% of birds, Richardson *et al.* 2001). Seychelles warblers are long-lived (average life expectancy from fledging is 5.5 years, maximum lifespan is 18 years, Komdeur 1991; Hammers *et al*. 2015). Annual survival of adults is high (average 84%) and equal for both sexes (Brouwer *et al.* 2006). As the annual re-sighting probability of breeding individuals is virtually one (0.98±0.01; Brouwer *et al.* 2010), and the probability of dispersal from the island is extremely low (0.10%; Komdeur *et al.* 2004), it can be safely assumed that all individuals not seen during the subsequent year have died (Hammers *et al.* 2013, 2016).

Data were collected during the main breeding season (June–September) in the years 2006–2010. The identity and breeding status of all birds present in each territory was recorded, and breeding activity was monitored at least once every two weeks by following the dominant female for a minimum of 15 minutes (Komdeur 1996a). Active nests were monitored every three days until their fate was determined (failed or fledged), to determine the approximate egg-laying and fledging date. The social status (i.e. dominant or subordinate) of all independent individuals (≥3 months old) was based upon field observations combined with the available long-term data. A ‘dominant’ male and female were identified in each territory, while the term ‘subordinate’ included all other independent individuals resident in the territory (Richardson *et al.* 2002; Kingma *et al.* 2016a). Subordinates are often independent young from previous breeding attempts, and are normally younger than the dominant individuals (Komdeur 1994b). To determine whether a subordinate contributed to the reproductive process of the group and could be defined as a 'helper', focal nest watches during the incubation and provisioning phase were performed (Richardson *et al.* 2003a). The number of insect prey available within a territory was used as an index of territory quality following Komdeur (1992) and Brouwer *et al*. (2009).

Sampling and analyses of oxidative parameters

Birds were caught using mist nets between 06:30h and 19:00h throughout the breeding season (see Kingma *et al*. 2016 for a description of the catching protocol). A blood sample (*ca*. 100 µl) was taken immediately after catching by brachial venipuncture. Each year we sampled between 23-28% (mean±SD = 25±2%) of the population (total = 436 samples of 312 individuals; 81 samples from 77 individuals in 2006, 108 samples from 98 individuals in 2007, 96 samples from 96 individuals in 2008, 83 samples from 82 individuals in 2009 and 68 samples from 67 individuals in 2010). From each sample, *ca*. 80 µl was centrifuged at 8,000 rpm for 8 min. within three hours of sampling. The plasma was frozen (-18ºC) for subsequent analyses of the oxidative parameters. Full details regarding the sample storage, transport and test reliability are provided in van de Crommenacker *et al*. (2011). The remaining blood (*ca.* 20 µl) was diluted in 1 ml of 100% ethanol and stored at room temperature for future DNA extraction. DNA was extracted from each sample and used to confirm sex following Griffiths *et al*. (1998).

Reactive oxygen metabolite production and antioxidant capacity of the blood plasma were measured using d-ROM and OXY-Adsorbent test kits, respectively (Diacron, Grosseto, Italy). The d-ROMs test (end-point mode) quantifies plasma concentrations of reactive oxygen metabolites (ROMs), which are generated by oxidation of a wide range of bio-molecules such as lipids, amino acids, proteins, and nucleotides. Specifically, this test is considered an accurate marker of oxidative damage (Alberti *et al.* 2000; Iamele, Fiocchi & Vernocchi 2002; Costantini 2016), as it quantifies primary oxidative damage molecules that are generated early in the oxidative cascade (mainly organic hydroperoxides, but also endoperoxides and organic chloramines, Alberti *et al*. 2000; Liang *et al*. 2012). Non-enzymatic circulating antioxidant capacity (OXY, hereafter termed 'antioxidant capacity') was analysed using the OXY-Adsorbent test. This assay measures the effectiveness of the plasma antioxidant barrier by quantifying its ability to cope with the oxidant action of hypochlorous acid (HClO). We used 20 and 10 µl of plasma for the ROMs and OXY assays respectively and measured absorbance at 505 nm (Beckman Coulter spectrophotometer, model DU-720), following the protocols provided with the kits. A detailed description of the protocols can be found in Costantini & Dell'Omo (2006). The ROMs are presented as mM of H_2O_2 equivalents, and OXY as mM HClO neutralized. In our dataset, OXY and ROMs were positively correlated (r =0.30, N=436, P<0.001).

16 Individuals were measured twice within a single breeding season, which allowed us to calculate within-individual consistency of ROMs and OXY throughout the breeding season, using the R package rptR (Nakagawa & Schielzeth 2010). Within-season consistency of measures ±SE did not significantly differ from 0 and was 0.25±0.19 (95% CI=0–0.63) for OXY and 0.34±0.21 (95% CI=0–0.70) for ROMs. Overall consistency (both within and between seasons, 436 observations of 312 individuals in five breeding seasons) ±SE was 0±0.05 (95% CI=0–0.17) for OXY and 0±0.05 (95% CI=0–0.18) for ROMs. The oxidative parameters studied here are therefore a snapshot of the oxidative status of an individual at a given point in time (as they are affected by many variables such a breeding status, food availability, etc.). Given that so

many factors will change across the breeding season, one would not expect ROMs or OXY to be a consistent characteristic of the individual.

Data selection and variables considered in the analyses

Annual survival was defined as the probability of an individual being alive one year later (Hammers *et al.* 2013). As most breeding groups without helpers produced one fledgling each year, reproductive output was treated as a binary variable (following Hammers *et al.* 2012). Producing more than one fledgling in a year would cause a large fitness gain as well as a large increase in reproductive investment, but unfortunately our dataset lacks statistical power to investigate the impact of this on oxidative balance and fitness components. To investigate reproductive output, we first focused on dominant (breeder) females that were not assisted by helpers (see Hammers et al. 2012). Limiting the analyses to unassisted females ensured that our measures were not confounded by subordinate helping (Richardson *et al.* 2003b) or co-breeding by subordinate helpers (Richardson *et al.* 2001). We focused on dominant females only as reproductive output of dominant males is difficult to estimate because it is confounded by high levels of extra-pair paternity (*ca* 40% of offspring; Richardson *et al.* 2001). In our dataset, too few offspring could be assigned to a father or to a subordinate to perform a meaningful analysis of male or subordinate reproduction. However, in the Supporting Material we provide a preliminary analysis that explores dominant male and subordinate reproduction, focusing on social (not genetic) relatedness to the offspring.

Current reproductive output was defined as the production of a fledgling during the same breeding season as in which the measures of ROMs and OXY were taken (i.e. both reproduction and sampling took place in year x). For the analysis or current reproductive output, we only considered females that were sampled before egg laying commenced (because ROMs and OXY are used to predict reproductive success) and sampled early during the breeding season (in June or July). Thirty-five out of 83 nests fulfilled these criteria. Repeating the analyses outlined below using all 83 nests (i.e. also including nests of individuals that were sampled after the start of egg laying or late in the season) gave similar results. Fledglings were successfully produced in nine nests (out of 35 nests of 29 individuals) where adult individuals were sampled for OXY and ROMs within the breeding season (year x); of these, eight nests (89%) produced one fledgling while one nest produced two. Future reproductive output was defined as the production of a fledgling during year x+1, and was tested in relation to sampling in year x. Here, we only considered dominant females that were alive and holding a dominant breeding position one year later. Of the 28 occasions (out of 69 nests of 56 individuals) where a fledgling was produced in year x + 1, one fledgling was produced in 23 (82%) cases, two fledglings in four (14%) cases and three fledglings in one (4%) case.

Along with both oxidative parameters (ROMs and OXY), the following variables which may explain variation in survival and/or reproductive output were considered in the analyses: age, age^2 (the squared effect of age was included to check for an initial increase and subsequent decline in survival and reproductive output), sex and whether a fledgling was produced during the field season of sampling (as a measure of reproductive investment potentially influencing survival and future reproductive success). In addition, the potentially confounding variables date of sampling, time of day of sampling and territory quality were considered. In our dataset, date of sampling was negatively correlated with OXY (r=-0.15, N=436, *P*=0.001), but not correlated with ROMs (r<0.01, *P*=0.956). Territory quality and time of day of sampling were both positively correlated with OXY (territory quality: r=0.21, *P*<0.001; time of day: r=0.19, *P*<0.001) and ROMs (territory quality: r=0.12, *P*=0.010; time of day: r=0.14, *P*=0.003).

Statistical analyses

Analyses were performed using generalized linear mixed models with a binomial error structure and a logit link function using the package lme4 (v1.1-12, Bates *et al*. 2014) in R (v. 3.2.5, R Core Team 2016). Year and individual identity were included as random effects in all analyses to account for the fact that some individuals were sampled more than once and to account for differences between years (*sensu* Russell *et al*. 2007; Graham *et al*. 2010). As continuous predictor variables are often on very different scales, these variables were standardized prior to analyses to have a mean of zero and a standard deviation of 0.5 using package arm (v.1.9-1, Gelman *et al*. 2009).

In the analyses of survival, survival to the next year (Y/N) was the dependent variable and ROMs, OXY, age, age^2, sex, dominance status (dominant or subordinate), fledgling production (Y/N), helper presence (Y/N), date of sampling, time of day of sampling and territory quality were included as predictors. Initial models included all main effects, the two-way interactions between age and oxidative status (ROMs or OXY), and the three-way interactions between oxidative status, sex and dominance status (and all nested two-way interaction terms between these variables). These three-way interactions were included to test the possibility that the relationship between oxidative status and survival differs between sexes and status groups. Interaction terms were removed from the models, in order of least significance, starting with the highest order interactions, and only reported when significant. Significance of the explanatory variables was determined using models containing all fixed effects (following Whittingham *et al*.

2006). Models containing only significant predictors (along with OXY and ROMs) and models derived using backwards stepwise deletion of non-significant terms (in order of least significance and keeping OXY and ROMs in the models) gave similar results (not shown).

Because the sample sizes in the analyses of reproductive output were much smaller than in the analyses of survival, it was not possible to include many independent variables simultaneously in the models as this would lead to overparameterisation. Therefore, initial models contained only ROMs and OXY as predictors, and a forward stepwise regression procedure was used to reach the final model. Specifically, we assessed the significance of each variable by entering it individually to the model. If a variable was significant ($P<0.05$), we included the variable in the model and repeated this procedure until no more significant variables could be added to the model. Significance of the explanatory variables was determined when they were last added to the model. In the analysis of current reproductive output (reproductive output in the same breeding season (year x) as in which oxidative status was measured), we used fledgling production later during the same breeding season as the dependent variable. In addition to ROMs and OXY, age, age^2, date of sampling, time of day of sampling and territory quality were considered as predictors. In the models of future reproductive output, we used fledgling production during the subsequent year (year x+1) as the dependent variable and, in addition to the predictors considered for the analysis of current reproductive output, we also considered whether the female produced a fledgling during the current breeding season (year x) and whether there was a helper as predictors.

RESULTS

Mean±SD ROMs was 2.25±1.36 (range: 0.40–12.91, N=436) mM H_2O_2 equivalents and mean±SD OXY was 141.60±31.18 (range: 63.63–238.84, N=436) mM HClO neutralized. Average survival to the next year was 80% (N=436 samples from 312 individuals). Survival was not explained by ROMs (Table 1, Figure 1). Dominants with higher OXY had lower survival until the next year and females had higher survival than males (Table 1, Figure 1). For 18 individuals that died in the year after sampling, we also had a sample available from an earlier year. OXY tended to be higher in the sample taken in the year before death (mean±SE: 153.30±7.14) than in the earlier sample (137.76±9.01), but this difference was not significant (paired t-test: t=1.33, P=0.20). ROMs were very similar in both occasions (2.02±0.25 versus 1.95±0.15; t=0.24, P=0.81).

26% (9/35) of the unassisted dominant females produced a fledgling during the same breeding season, but this was not predicted by ROMs or OXY (Table 1, Figure S1) If anything, reproductive success tended to increase with OXY (Table 1, Figure S1). Repeating this analysis including also females that were assisted by helpers (N=41 observations of 34 individuals) showed similar results (ROMs: β±SE=-0.67±1.21, z=-0.55, P=0.58; OXY: β±SE=2.10±1.25, z=1.68, P=0.09), as did a preliminary analysis including also dominant males and subordinates (see Supporting Methods and Table S1). Of the females that survived, were a dominant the next year, and were not assisted by helpers, 41% (28/69) produced a fledgling during the year after measuring oxidative status. Producing a fledgling during the next year was not associated with ROMs or OXY measured during the current breeding season (Table 1, Figure S2). Repeating this analysis including also females that were assisted by helpers (N=88 observations of 70 individuals) showed similar results (ROMs: β±SE=0.74±0.55, z=1.34, P=0.182; OXY: β±SE=-

0.19±0.60, *z*-0.32, *P*=0.753). A preliminary analysis (see Supporting Methods) that included also dominant males and subordinates showed that, overall, future reproductive output appeared not significantly related to ROMs or OXY (Table S1). Although none of the three-way interactions of interest were significant, the two-way interactions between ROMs and sex, and between OXY and dominance status were significant. Although these results should be interpreted with caution (see Methods), these interactions suggest that the negative trend between ROMs and future reproductive output is steeper for males than for females and the negative trend between OXY and future reproductive output is steeper for subordinates than for dominants (Table S1).

DISCUSSION

We found that adult Seychelles warblers with higher circulating antioxidant capacity (OXY) had a lower probability of surviving until the next year. This result supports the findings of an extensive study across North-American bird species (Cohen *et al*. 2008), but contrasts with other studies that found positive associations (e.g., Alonso-Álvarez *et al.* 2006; Bizé *et al.* 2008; Saino *et al.* 2011) or no associations (Blount *et al.* 2015). The inter-specific study of Cohen *et al*. (2008) characterized species rather than individuals, and the associations found could have been driven by species-dependent differences in mass-adjusted metabolic rates which could (but not necessarily, e.g. see Brand 2000) be translated into the levels of ROS production measured. The results of Cohen *et al*. (2008) emphasize the importance of species differences in pace of life: i.e., "live fast, die young" versus "live slow, die old". In this framework, the 'live fast' types would have higher basal antioxidant levels to cope with higher and more acute needs for protection from pro-oxidant production (due to a higher metabolism). In contrast, the 'live slow' types would have lower basal antioxidant levels, as they probably experience more constant environments and can invest in the tight regulation of their internal physiology, with elevations occurring only as a direct response to higher levels of pro-oxidants produced. The Seychelles warbler, with its high adult survival, low reproductive rates and benign environment probably falls within the second group. Based on this view, high antioxidant levels in the Seychelles warbler may occur as a result of an oxidative challenge (e.g. a disease such as malaria, see van de Crommenacker *et al*. 2011). The avoidance of oxidative imbalance may be important, e.g. for preserving the potential for future reproduction in this relatively long-lived passerine. Indeed, we found a strong positive correlation between ROMs and OXY within individuals (P<0.001), which could indicate that high ROMs are facultatively counteracted by high OXY. Contrary to our expectations, high OXY was related with lower survival, but high ROMs were not. We can only speculate about the underlying mechanism of this relationship; causal links between oxidative status and fitness parameters may be hard to resolve without an experimental approach, however manipulations are not feasible in the Seychelles warbler due to its conservation status. Therefore, when interpreting our results, it is important to keep in mind that other unmeasured variables may play a role in explaining variation in both oxidative status and fitness. For example, physiological mechanisms we are not aware of may be regulating the interplay between oxidative damage and antioxidant activity, and may explain why high antioxidant capacity do not necessarily reflect a positive over-all body condition (see Costantini & Verhulst 2009). One possibility in our study is that the detrimental effects of high ROMs are successfully mitigated by the elevated OXY, but that the elevation in OXY comes at a cost. Resource use for antioxidant protection may be traded off with reduced resource allocation into other traits, such as the regulation of immune function and other physiological processes (Monaghan *et al.* 2009), thereby increasing short-term mortality. For example, 44% of adult Seychelles warblers on

Cousin Island carry the GRW1 *Haemoproteus nucleocondensus* strain of 'malaria-like' blood parasite (Hammers *et al.* 2016). Although infection itself is not directly related with adult survival in this species (Hammers *et al.* 2016), it may – in combination with other challenging environmental factors – contribute to an increased pressure for antioxidant investments (van de Crommenacker *et al*. 2011), which might lead to lower survival in some infected individuals.

We found no significant association between oxidative status (ROMs and OXY) and the probability of successfully producing a fledgling during the current or subsequent breeding season. However, although not statistically significant, we observed a positive trend between OXY and current reproductive success. A study by Markó *et al.* (2011) found that variation in oxidative status in collared flycatchers (*Ficedula albicollis*) did not result in differences in reproductive performance measured as clutch size, but found that performance measured as egg size was positively correlated with antioxidant capacity. Our results contrast with the expected negative correlation between ROMs and reproduction. A possible explanation for this may be that individuals may successfully manage to counteract oxidative challenges (at the expense of lowered survival), and keep their oxidative balance in such a way that their reproductive output is not impaired. In turn, higher antioxidant capacity could be expected to result in higher reproductive output as it may boost the antioxidant machinery and condition of the gametes/eggs/offspring (Blount, Houston & Møller 2000; Surai, Speake & Sparks 2001). That we only find a non-significant trend between current reproductive output and antioxidant capacity may be explained by the fact that there are many more factors determining the survival of the fledgling survival during its course of development. It should also be considered that in Seychelles warblers, annual reproductive investment early in the reproductive cycle may be low compared to other passerines (e.g. mainly single egg clutches), thereby obscuring any impact of oxidative status on reproduction in the short-time (however their period of parental care is long compared to other passerine species – thus overall investment in breeding may be relatively high). Furthermore, we cannot exclude that oxidative status is linked with other aspects of reproductive output that were not measured in this study, such as egg size, antioxidant and other nutrient contents in the eggs, and offspring condition.

In general, care should be taken while interpreting the results, as high circulating antioxidants (as measured in this study) are not necessarily reflective of the total antioxidant status of the individual. The OXY-test that we used here measures a range of circulating micro-molecular antioxidants, but does not account for antioxidants that occur mainly in cells (e.g., endogenously produced enzymes and other biomolecules) and which are likely to represent costs involving allocation of resources in favour of antioxidant protection. The cellular (endogenous) and circulating (exogenous) components of the total antioxidant capacity encompass a complex interplay (Selman *et al.* 2006; Monaghan *et al.* 2009), e.g., more dietary antioxidants may be ingested to compensate for a low (potential of the) endogenous cellular antioxidant defence. Without being able to investigate the full antioxidant barrier it is difficult to understand such regulatory mechanisms.

The circulating antioxidant barrier itself, which plays an important role in the total antioxidant defence system (Cohen, Klasing & Ricklefs 2007), is also a collection of different components. It comprises non-enzymatic antioxidants (vitamin E and C, uric acid, carotenoids, proteins) of which a large proportion is derived from the diet (Vertuani, Angusti & Manfredini 2004; Catoni *et al.* 2008; Cohen, McGraw & Robinson 2009). It also involves endogenous processes such as protein catabolism products and indicates mobilization of stored molecules (Cohen, Klasing & Ricklefs 2007; Cohen *et al*. 2008). It is difficult to describe and interpret the costs of dietary versus endogenously produced circulating antioxidants, as much remains unknown about how much both types contribute to the circulating antioxidant barrier, or how important they are. For example, a high concentration of circulating dietary antioxidant levels might mean that an individual is better able to obtain high quality food, or could indicate that there is little need to allocate antioxidant compounds to other functions such as up-regulation of immune function. Alternatively, it could be a sign of increased protein catabolism which may be caused by a prolonged stress response (Koehn & Bayne 1989). To understand the contribution of different types of antioxidants/compounds that we measured with our OXY test, it would also be useful to measure each individual antioxidant simultaneously, but unfortunately this is not currently feasible in this small species of conservation concern (Cohen *et al.* 2007). Analyses of dietary intake would also be valuable to help interpreting the results.

All assessments of antioxidant capacity will be influenced by the choice of assay used. We chose our assays for a number of practical reasons. First, both the d-ROMs and OXY tests are commonly used measures that have proven to give valuable information on oxidative status (e.g., Costantini & Møller 2008; Costantini 2016). Second, the tests require only small amounts of plasma, which is important when working with this small and vulnerable species. Third, both ROMs and OXY can be analysed efficiently at the same time, so that repeated freezing⁄thawing of samples can be avoided. Furthermore, the OXY-test measures the extent to which the circulating micro-molecular antioxidant mechanism can withstand an oxidative attack, rather than quantifying single antioxidants (Costantini & Møller 2009; Monaghan *et al*. 2009). The OXY test can measure the contribution of both hydrophilic and lipophilic antioxidants, and – unlike other methods – the contribution of uric acid to the serum antioxidant capacity measured is low and does not influence the antioxidant level test results (Costantini 2011). However, like all methods these tests have their shortcomings. For example, in humans the d-ROMs results can be influenced by ceruloplasmin, an inflammation-induced acute phase protein in the blood (Alberti *et al.* 2000; Colombini, Carratelli & Alberti 2016). However, in birds the contribution of ceruloplasmin to d-ROMs values has been found to be absent (Costantini, Casasole & Eens 2014) or small (*ca*. 6%, Herborn *et al*. 2015). Although accurate measurement of the contribution of ceruloplasmin is difficult, and biochemical mechanisms behind it are yet to be resolved, it is important to keep its potential role in mind when interpreting d-ROMs results.

We hope that our study provides a valuable contribution in the attempt to better understand the role of the oxidative balance in shaping life-history trade-offs. With the increased interest in oxidative status-related patterns, it is tempting to generalize trade-offs between oxidative damage and fitness parameters found in separate studies into one overall pattern. However, the interesting and somewhat surprising relationships found here show that much is still to be learnt, and emphasize the importance of keeping the biology of the species in mind. In other species, experimental manipulation of individual oxidative status could be used to test causality and provide important insights into associations between oxidative status and fitness components. Such studies are needed to build on the mainly correlative nature of studies in this area so far. In addition, more individual-based studies on free-living species – including measures of both survival and fecundity – are needed to better understand the role of oxidative balance in mediating fitness, and to place results from captive, experimental studies in a broader ecological perspective.

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AUTHOR CONTRIBUTIONS

The study was conceived by JvdC, DSR and JK. The fieldwork was conducted by JvdC, MH, JvdW, ML and PS. Lab analyses were conducted by JvdC, JvdW, ML and PS. The statistical analyses were performed by MH and JvdC. The manuscript was written by JvdC and MH (equal contributions) and all authors revised and approved the manuscript.

DATA ACCESSIBILITY

Data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.1d0m9 (van de Crommenacker et al. 2017)

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TABLES AND FIGURES

Table 1. Annual survival (A), reproductive output in the current breeding season (B), and reproductive output in the next year (C) in relation to oxidative parameters (reactive oxygen metabolites (ROMs) and non-enzymatic circulating antioxidant capacity (OXY)). Significant terms are shown in bold and are underlined. Reference categories are given in brackets.

A) Survival	**Estimate**	**SE**	***z***	***P***
Intercept	**2.53**	**0.56**	**4.50**	**<0.001**
OXY	**-0.97**	**0.35**	**-2.78**	**0.006**
ROMs	0.67	0.42	1.60	0.109
Sex (male)	**-0.70**	**0.34**	**-2.05**	**0.040**
Dominance status (subordinate)	-0.62	0.48	-1.29	0.198
Age	-0.49	1.15	-0.42	0.672
Age^2	-0.03	0.95	-0.03	0.974
Fledgling produced? (yes)	0.20	0.33	0.62	0.537
Helper present? (yes)	0.00	0.36	0.01	0.993
Day of catching	0.64	0.33	1.92	0.055
Catch hour	0.34	0.32	1.05	0.296
Territory quality	-0.55	0.38	-1.45	0.148

B) Current reproduction (year x)	**Estimate**	**SE**	***z***	***P***
Intercept	**-1.66**	**0.62**	**-2.67**	**0.008**
OXY	2.18	1.27	1.72	0.086
ROMs	-0.55	1.14	-0.49	0.627
Age	-0.01	1.23	-0.01	0.991
Age^2	-7.57	9.81	-0.77	0.441
Day of catching	**-3.87**	**1.29**	**-3.01**	**0.003**
Catch hour	0.14	0.21	0.68	0.499
Territory quality	1.85	1.51	1.23	0.220

C) Future reproduction (year x+1)	**Estimate**	**SE**	***z***	***P***
Intercept	-0.40	0.30	-1.33	0.183
OXY	-0.40	0.62	-0.64	0.521
ROMs	0.79	0.60	1.31	0.189
Age	-0.16	0.10	-1.65	0.098
Age^2	-3.73	2.79	-1.34	0.181
Fledgling produced? (yes)	-0.87	0.53	-1.63	0.103
Helper present? (yes)	-0.73	0.88	-0.83	0.405
Day of catching	0.02	0.54	0.03	0.975
Catch hour	0.92	0.54	1.71	0.088
Territory quality	1.45	0.94	1.54	0.124

Figure 1. Relationship ± 95% C.I. between annual survival and (A) non-enzymatic circulating antioxidant capacity (OXY) and (B) reactive oxygen metabolites (ROMs) in male and female Seychelles warblers on Cousin island. Lines are model predicted regression slopes from a model containing ROMs, OXY and sex (see Table 1). The models contained 436 samples from 312 individuals (198 samples from 152 females; 238 samples from 160 males). The relationship between annual survival and ROMs is depicted with a dashed line because this relationship was not significant.