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AGE, OXIDATIVE STRESS EXPOSURE AND FITNESS IN A LONG-LIVED SEABIRD

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Summary

1) The need to manage exposure to oxidative stress, which can damage macromolecules, is thought to influence the resolution of life history trade-offs. Oxidative damage is expected to increase with age as a consequence of changes in the optimal investment in defences or repair, and/or because of senescence in antioxidant defence systems, though the pattern might differ between short and long-lived species. However, data on age related changes in damage levels in wild populations are rare.
2) Using cross-sectional and longitudinal data collected over three years, we examine variation in a measure of oxidative damage exposure in known age, wild European Shags (*Phalacrocorax aristotelis*), a relatively long lived species.
3) The cross-sectional data showed a quadratic relationship between oxidative damage exposure and age: both relatively young and old adults had higher levels than those in middle age. In contrast, a measure of non-enzymatic antioxidant levels did not vary with age.
4) The cross-sectional increase in oxidative damage exposure in later life was consistent with longitudinal patterns observed within older birds (more than 10 years old).
5) However, the apparent decline in oxidative damage in early adulthood was not consistent with longitudinal patterns in younger birds, which showed individual variation but no consistent age-related change in the marker. This suggests that cross-sectional patterns reflect instead higher disappearance of individuals with high exposure to oxidative damage at this life stage
6) Our data further show that oxidative damage levels are predictive of attendance at the colony in all age classes: juveniles fledging with a high damage exposure index were less likely to be resighted in the breeding colony two years later, and adults with high levels at the end of the breeding season had reduced return rates, irrespective of age. Since this is a species that shows high colony fidelity, this is likely to reflect mortality patterns.

7) These data suggest that exposure to oxidative damage increases with age in a long lived species, but only in later life, when high investment in reproduction at the cost of defence would be predicted.

Introduction

In long lived species, individuals of different ages are expected to differ in their optimal investment into somatic maintenance (van Noordwijk & de Jong 1986). Because the forces of selection are stronger in younger age classes, genes that have positive fitness consequences early in life will be favoured even if they have negative consequences for somatic maintenance at older ages (Medawar 1946; Charlesworth 2001). Older individuals can also accumulate morphological damage (Cartar 1992) and damage to maintenance and repair mechanisms (Finkel & Holbrook 2000), lessening their ability to gather or utilise resources, and to avoid or repair damage. As individuals get older, life history theory predicts that finite resources will be channelled from somatic maintenance to reproduction as the chances of future reproduction decline (Williams 1966).

One component of somatic maintenance is investment into the prevention and repair of oxidative damage. The generation of energy by aerobic metabolism produces reactive oxygen species (ROS; Balaban, Nemoto & Finkel 2005). If not then neutralised by endogenous (e.g. enzymatic) or exogenous (e.g. from dietary sources) antioxidants, ROS react with and damage key macromolecules such as proteins, lipids and DNA (Halliwell & Gutteridge 1999). Oxidative stress is the term used to describe a state of imbalance between the production of pro-oxidants and their neutralisation by the defence systems (Finkel & Holbrook 2000). Processes such as growth and reproduction are expected to cause elevated oxidative stress exposure (Metcalfe & Alonso-Alvarez 2010, but see Metcalfe and Monaghan, 2013) or increased investment into antioxidant defences (Costantini et al., 2014).Thus, the investment into these processes may reflect a trade-off with the individual's need to minimise unrepaired damage and repair costs (Monaghan, Metcalfe & Torres 2009; Dowling & Simmons 2009). Oxidative stress can be exacerbated by molecules and compounds with other functions that also act as a catalysts for oxidation, such as glucocorticoid stress hormones (Costantini, Marasco &

Møller 2011) and proteins involved in the inflammation response (Shukla *et al.* 2006), potentially generating trade-offs between energy production and specific regulatory responses.

Organisms are expected to modulate their pattern of energy use and investment in defence and repair of oxidative damage to give the best individual fitness outcomes. Some accumulation of oxidative damage will occur with age, either because of strategic changes in investment patterns and/or accumulation of damage that is either too costly, or not possible, to repair (Harman 1956; Monaghan *et al.* 2009). It is likely that the optimal pattern of investment in somatic repair will vary with extrinsic mortality risk. Species in which such mortality risk is high are expected to invest relatively little in avoiding senescence, thus show little age-related variation among adults, while age-related changes in species with low extrinsic mortality risk might be expected to reflect the shifting balance of the trade-off (Williams 1966), with relatively little damage accumulation until the balance favours more reproductive investment, which might differ among individuals. However, it is also possible that long lived species invest heavily in repair throughout life and thus also show little age-related damage accumulation until defence systems begin to senesce (Williams 1957).

While studies in short-lived model species such as mice and flies do indicate an increase in oxidative damage with age, even in the relatively benign and constant captive environment (Agarwal & Sohal 1994; Sohal *et al.* 1994; Yasuda *et al.* 1999; Hamilton *et al.* 2001), data on age related changes in oxidative damage levels in long lived species, and how these relate to life expectancy, are rare (Selman *et al.* 2012). The data that do exist are largely cross-sectional and find not a linear, but a quadratic relationship with age, with antioxidant capacity proving low (Devevey *et al.* 2010; Isaksson *et al.* 2011) and oxidative damage high (Bergeron *et al.* 2011) not just in older individuals, as predicted, but also in the younger age classes, relative to middle-aged individuals. This could reflect different trade-offs, for example between somatic maintenance and other energetically costly activities such as growth or age-related competition, or it could relate to selective disappearance of particular phenotypes from the population (Reid *et al.* 2003; Monaghan *et al.* 2008; Nussey *et al.* 2008).

Few studies have examined the contributions of within-individual change and selective mortality to these cross-sectional patterns (Selman *et al.* 2012). We examined the extent to which oxidative damage and total non-enzymatic antioxidant capacity varied with age in a long lived species, the European Shag (*Phalacrocorax aristotelis*). First, we examined the cross-sectional patterns in a marker of oxidative damage and a marker of antioxidant capacity. Second, using individuals sampled in more than one year, we evaluated whether cross-sectional patterns were consistent with age-related changes observed within individuals. Finally, we examined how these markers of oxidative state in birds of different ages related to survival.

Materials and methods

Study population and sample collection

The study was based on a population of shags breeding on the Isle of May National Nature Reserve, in the Firth of Forth, eastern Scotland (56811' N, 02833' W). Chicks and breeding adults in this population are individually marked with metal and coloured leg rings. Both natal and breeding fidelity are high, with around 90% of surviving young recruiting to the natal colony, and >99% of adults remaining faithful to their breeding colony (Barlow *et al.* 2013). Young birds return to the colony from age 1 (Frederiksen *et al.* 2008), and the majority of birds recruit at age 2 or 3 years. Amongst the breeders, non-breeding years occur occasionally but non-breeding individuals generally attend colonies (Aebischer 1986, Aebischer & Wanless 1992). It is therefore possible to relocate marked individuals each year when they return to the breeding site, and to use this information as a good proxy for survival. Adult return rates is a reliable proxy of survival in the study years since resighting probability was > 0.9 (S. Burthe et al. unpublished data). Survival estimates obtained from this population indicate that average annual survival is lower among juveniles than adults (0·513 ± 0·038 to age 1 year; 0·737± 0·028 to age 2 years; 0.858 ± 0.030 thereafter; Frederiksen *et al.* 2008). Occasionally, however, during protracted periods of strong onshore winds and heavy rain, all age classes may experience high mortality ('wreck' years; Frederiksen *et al.* 2008).

Blood samples were collected between April and July of each year, 2010-2012. Younger birds tend to breed late in the season (Daunt *et al.* 1999; LME hatch date ~ age in this dataset, controlling for multiple years measurement in some adults: $t = -4.03$, $p < 0.0001$,correlation: -0.89), so to separate effects of age and timing of breeding on oxidative stress exposure, hatching date was ascertained by monitoring nests for laying or hatching dates, or otherwise back-calculating hatching date from the wing length of the largest chick when the brood was visited late in rearing for chick ringing. The age of the chicks when the breeding adults were sampled for the study was estimated as sampling date - hatch date. Sex of breeding adults is determined from vocalizations, behaviour and size, with males on average 22% heavier than females (Daunt *et al.* 2001).

To examine the relationship between oxidative stress exposure and age, we collected small blood samples from 133 individually marked and known age breeding birds: 50 females (aged 3-19 years) and 83 males (aged 2-22 years). Of these, 58 were blood sampled in more than one breeding season, with 42 (13 female, 29 male) sampled in 2 years and 16 (5 female, 11 male) in all 3 years of the study. Samples were collected during chick-rearing and the brood size and age of chicks at the time of sample collection was included in the analyses since these could affect parental effort and hence oxidative status (average age of largest or only chick 22.5 ± 10 days, range 0 to 48 days). On capture, blood samples were collected within 10 minutes by syringe from either the brachial or tarsal vein. The blood was transferred from the syringe into a heparinised Eppendorf tube, and stored on ice packs for up to 2 hours in the field. On return to the field laboratory, blood samples were centrifuged at 6000 rpm to separate red blood cells from plasma. Plasma samples were then stored at -20°C for up to 1 month until they could be transferred, on dry ice, to a -80°C freezer in Glasgow.

We examined the relationship between the measures of oxidative stress exposure and survival using re-sightings on the Isle of May in subsequent breeding seasons (March-June). Firstly, we monitored adult survival to the next breeding season of those birds blood sampled in 2010-12, using re-sightings in 2011-13. Overall return rates of marked adults in the colony were above average in 2011 (92.6%) and 2012 (92.9%), but exceptionally low in 2013 (42.7%), following a winter of high mortality as a result of poor weather (i.e. a‘wreck’ year; Frederiksen *et al.* 2008). Due to this high

mortality between 2012 and 2013, we examined re-sightings of 2010 chicks to the age of 2 years. In 2010, we blood sampled 74 chicks from 36 nests when the chicks were approaching the end of the linear growth phase (average 28.8 ± 2.2 days, range 25 – 34 days). At blood sampling, the brood size was noted and a final mass measurement taken. Hatch rank within the brood (A, B, C or D) was followed by tagging chicks as they hatched. Growth rate was calculated as: (mass at c.30 days of age – mass at c.10 days of age)/(age at last mass measurement – age at first mass measurement). Part of the blood sample was used for molecular sexing (n= 31 females, 43 males; Griffiths, Daan & Dijkstra 1996). Re-sightings of these fledged chicks were collected throughout the 2012 season, identifying 22 fledglings that are known to have survived until 2 years (9 female, 13 male). In 2012, 7 birds (2 female, 5 male) were observed on a first breeding attempt and 11 non-breeding birds (5 female, 6 male) were identified roosting on the island. A further 2 breeding (1 male, 1 female) and 2 non-breeding birds (1 male, 1 female) that had not been observed in 2012 were recorded in 2013. It is likely that some live individuals were present on the island but missed, given the 2013 addition of 4 individuals not sighted in 2012, or may have dispersed to other colonies (Barlow et al., 2013). However, the number of chicks re-sighted is within expectations of estimates of survival rate to two years from previous years on the Isle of May (Frederiksen et al., 2008).

Permission to work with the colony was granted by Scottish National Heritage (SNH; MON/RP/116), following ethical review by SNH and the University of Glasgow. All of the experimental procedures were under licence from the UK Home Office (60/4019).

Analysis of oxidative stress exposure

Plasma oxidative damage and antioxidant capacity were measured using the d-ROMs (Reactive Oxygen Metabolites) and OXY-adsorbant test kits respectively (Diacron, Grosseto, Italy). Methods were similar to those described by Costantini and Dell'Omo (Costantini & Dell'Omo 2006). Briefly, for the d-ROMs assay, 10µl of plasma were incubated with 190µl of a chromogen solution of 0.01 mol1-1acetic acid/sodium acetate buffer and N,N-diethylphenylenediamine for 75 minutes at 37ºC,

centrifuged for 2 minutes to remove particulate matter suspended in the solution, and the absorbancy of the supernatant measured at 546nm Multiskan Spectrum, Thermo Scientific). Intra-assay variation was 10.2 % and inter-assay variation of a reference sample 4.7 %. For the OXY-adsorbants assay, plasma was diluted 1:99 in distilled water and 2µl of solution were added to a HOCl solution and incubated at 37ºC for 10 minutes before measuring absorbancy at 546nm. Intra-assay variation was 9.9 % and inter-assay variation 8.3 %.

The d-ROMs test is an indirect quantification of hydroperoxides (ROOH) in the plasma, expressed as CARR U units, which are both oxidants and a marker of oxidative damage (Alberti et al., 2000; Iamele et al., 2002). The d-ROMs assay can be influenced to some extent by ceruloplasmin levels in humans (Erel 2005; Ganini *et al.* 2012; Kilk *et al.* 2014), a positive acute phase protein that is elevated in response to inflammation (Shukla *et al.* 2006). Interference by ceruloplasmin in avian plasma, however, is lower than in mammals (Kilk et al., 2014), with one study reporting no interference (Costantini, Casasole & Eens 2014). Following Kilk at al. (2014), we conducted two assays to estimate the contribution of ceruloplasmin to the d-ROM signal in our study species. First, in samples from 21 individuals, we added sodium azide to the reaction mixture at 1mM concentration, sufficient to inhibit ceruloplasmin activity without also inhibiting the Fenton reaction (Costantini et al., 2014; following Alberti et al., 2000). This caused a small but significant, 5.95% reduction in absorbance (range 0-10.1%; paired T test: $t = 5.37$, $p < 0.0001$), corresponding to on average 2.5 CARR U (range 0-4.84 CARR U). This signal reduction was independent of age (range 2-22, $t = 01.31$, $p = 0.21$), sex (n= 11 male, 10 female, $t = 1.10$, $p = 0.23$) or CARR U value (range 6.83 – 23.86, $t = 0.25$, $p = 0.81$). Second, we compared normal to sodium azide-treated samples when the reaction temperature was reduced from 37°C to 4°C, at which ceruloplasmin enzymatic activity is greatly reduced (e.g. mammalian activity reduced to 14% of 37°C activity at 4°C, Kilk et al., 2014). At 4°C, there was no longer a difference between normal and sodium-azide treated samples, suggesting that temperature reduction also successfully inhibited ceruloplasmin activity. Moreover, an average 62.6% reduction in signal (4 samples, range 59.2-66.3) was close to the expected reduction in Fenton reaction efficiency with temperature (59% of 37°C activity at 4°C, Kilk et al., 2014),

supporting the Fenton reaction as the main mechanism underlying the signal. Whilst, as acknowledged by Kilk et al. (2014), accurate determination of the component of the signal that is attributable to ceruloplasmin is difficult, our results and others on avian species suggest a much lower interference than in mammals. Under conditions of oxidative stress, ceruloplasmin can in fact act as a prooxidant in the blood (Shukla *et al.* 2006). In any event, therefore, this assay, abbreviated here as ROMs, is best treated as providing a general indicator of exposure to oxidative damage.

The OXY-adsorbants test (abbreviated here as OXY) measures the capacity of the non-enzymatic antioxidant compounds in the plasma to neutralise hypochlorous acid (HOCl), providing a measure of the circulating levels of antioxidant vitamins and proteins (Costantini 2011). Unlike other assays measuring total non-enzymatic antioxidant capacity, OXY values do not need to be corrected for circulating uric acid levels (Costantini 2011).

A general criticism of non-enzymatic plasma oxidative status measures, including OXY and d-ROMs, is the possible effect of short-term changes in plasma composition at the time of measurement, for example dehydration or food intake (e.g. Perez-Rodriguez et al., 2015; for review Sies, 2007). Thirty-two adults were blood sampled twice in the same season (2011), first during late incubation (average 5 ± 2 days prior to hatching, range 1 to 11 days) and second during chick-rearing (average 26 ± 8.5 days after hatching). Across these breeding stages, there was a significant increase in OXY (ANOVA: $F = 5.803$, $p = 0.022$) and marginal increase in ROMs ($F =$ (LRT $X^2 = 7.82$, $p = 0.005$) and OXY (LRT $X^2 = 3.014$, $p = 0.087$). Despite this within-season variation, both OXY ($r = 0.44$, $F = 2.59$, $p = 0.005$) and ROMs ($r = 0.52$, $F = 3.154$, $p = 0.001$) were significantly repeatable within individuals, and the differences in oxidative status among individuals were consistent across the breeding season.

Statistical methods

First, we used a mixed model to describe the cross-sectional age patterns in ROMs and OXY measured in 208 samples from 133 adults. Individual identity was specified as a random effect to control for repeated measurements from different years on 58 of those adults. We fitted linear and

quadratic $(age)^2$ to account for potential non-linear age effects. Sex, Julian hatch date, number of chicks in the brood, chick age (sampling date – hatch date of oldest chick) and the year of sampling were included as covariates.

Second, we partitioned age into within- and between-individual components and substituted these two new fixed effects for age in the original model. This distinguished cross-sectional from longitudinal patterns in ROMs and OXY (van de Pol & Wright 2009). Analyses were limited to the 132 samples from 58 birds that were sampled in multiple years. The between-individual component was the average age at which each bird was sampled. The within-individual component was (age – average age). We included identity and the slope of the within-individual component of age correlated with identity as random effects.

Third, we tested for convergence between the within- and between-individual slopes of ROMs and OXY with age, to assess whether any cross-sectional age patterns were consistent with the underlying patterns of within-individual change (Sliwinski, Hoffman & Hofer 2010). We tested for convergence by substituting the within-individual and between-individual components in the previous model for the original age variable, which would contain both the within-individual (change in age per sample) and between-individual (age at sampling) information (van de Pol & Wright 2009). If the difference between models proved non-significant, then convergence would be supported. If significant, this would indicate a difference between within and between individual slopes, and hence that any cross-sectional patterns may be partly explained by processes other than within-individual change, such as selective mortality.

Finally, we tested whether ROMs or OXY predicted survival into and through adulthood. In chicks from 2010, we conducted a logistic regression of return rate to the colony aged 2 years. Re-sighting, a two level factor (0 not resighted or 1 resighted), was the response variable and we included the explanatory variables: chick growth rate (g/day), brood size at sampling, hatching rank within brood, first hatch date of the brood, sex and ROMs and OXY. Nest ID was a random effect to control for the inclusion of siblings. To examine adult survival, we conducted a logistic regression of re-

sighting in the breeding season following sampling, using the 133 adults from the age analyses. We used the first or only year of sampling per adult. Covariates were age, $(age)^2$, sex, year and ROMs and OXY. To compare normal adult mortality rates to the unusually high mortality of the 2013 'wreck' year, we included the interactions of ROMs x year and OXY x year.

Analyses were conducted using R version 2.15.1 and the package lme4 (R Core Development Team 2012). ROMs and OXY were log-transformed in all analyses to improve the model fit. Significance of variables in the cross-sectional and within- and between-individual models, convergence tests, and year interactions in the re-sighting models were tested by comparing nested models fitted with Maximum Likelihood with and without those variables with a Likelihood Ratio Test (LRT). Otherwise, full models are presented to reduce type I error by multiple comparisons (Whittingham *et al.* 2006).

Results

Age-related patterns in ROMs

The cross-sectional analysis of breeding adults indicated a U-shaped quadratic relationship between ROMs and age, with both relatively young and old adults having higher values than middle-aged individuals (Fig 1a). Sex and hatching date were also significant, with young and old adults, males and birds breeding later in the season having the highest ROMs (Table 1a). Chick age, brood size and year were non-significant in the model. Using an iterative procedure that compared the residual error when the age distribution was split into different two-segment piecewise regressions, we identified age 10 as the base of this U-shaped cross-sectional curve (Crawley 2007). To test whether age partitioned into its within-individual and between-individual components also followed a pattern of decline into middle age and increase into old age, we fitted separate piecewise slopes for adults aged 2-9 and 10-22 for the two age components, with the random effects also specified for age 2-9 and 10-22 separately. Within individuals, ROMs significantly increased over years in 10-22 year old adults, but not 2-9 year olds. There were no age-related patterns in either between-individual component of

age. Sex, chick age and Julian hatching date were also significant in the model, with males, adults in the later stages of chick rearing and late breeders having the highest ROMs (Table 1b). Brood size and year were non-significant in the model.

Removing the random slope for adults aged 10-22 did not significantly reduce model fit (LRT X^2 = 4.09, p = 0.13). For adults aged 2-9 years, however, random slopes differed significantly amongst individuals (LRT X^2 = 13.17, p =0.0014), indicating variation in the relationship between ROMs and age. The random intercept was also non-significant for individuals aged 10-22 (LRT X^2 = 2.21, p = 0.33), with relatively low repeatability of 0.13 (calculated following Nakagawa and Schielzeth, 2010). In contrast, repeatability for 2-9 year old adults was 0.35 and removal of the random intercept significantly reduced model fit (LRT X^2 = 8.40, p = 0.015), indicating greater inter-individual variability in ROMs amongst young than older adults.

To test for convergence, in a model for each age range separately, age was substituted for the within-individual and between-individual components of age, and models compared using a likelihood ratio test. For 10-22 year old adults, this simplification did not significantly reduce model fit (LRT X^2 = 0.33, p = 0.57), indicating convergence in the positive within- and between-individual slopes. For 2-9 year old adults, whilst neither component of age was significantly different to 0 in the initial model, this simplification significantly reduced model fit (LRT X^2 = 4.96, p = 0.026), indicating that they differed from each other, with a between-individual decrease but within-individual increase with age. This suggests that factors other than within individual change are responsible of the negative pattern observed in younger individuals the cross sectional data.

Age-related patterns in OXY

The cross-sectional analysis of OXY showed no relationship with age or $(age)^2$ (Table 1a). The only significant variable in the model was year, with OXY significantly lower in 2012 than 2010 or 2011, and marginally higher in birds with large broods (Table 1a). When partitioned into within- and

between-individual components, the only significant correlate was year, with OXY significantly higher in 2011 than 2010, but lower in 2012 than 2010 (Table 1b).

In contrast to ROMs, there was greater variability in OXY amongst old than young birds. There was significant variation in the relationship between age and OXY in adults aged 10-22, with removal of the random slope from the model significantly reducing model fit (LRT $X^2 = 11.99$, $p = 0.003$), but not in adults aged 2-9 (LRT $X^2 = 2.32$, $p = 0.31$). And repeatability of OXY in adults aged 10-22 was 0.33 , with model fit significantly reduced by the removal of the random intercept (LRT $X^2 = 7.79$, $p = 0.020$), whilst repeatability in adults aged 2-9 was 0.20 and the random intercept not significant in the model LRT $X^2 = 2.04$, $p = 0.36$).

For 2-9 year old adults, simplification of the model to a single age component did not significantly reduce model fit (LRT $X^2 = 0.15$, $p = 0.70$), indicating convergence between within-individual and between-individual slopes, neither of which had differed significantly from 0 in the model. For 10-22 year old adults, simplification significantly reduced model fit (LRT $X^2 = 6.51$, $p = 0.012$), indicating non-convergence between the within-individual increase and between-individual decrease in OXY. This suggests that processes other than within-individual change may be masking within-individual age-related patterns in older birds, with an age slope not significantly different to 0 in the cross-sectional model.

Oxidative Stress and Re-sighting

Chick return rate to the colony aged 2 was predicted by ROMs measured towards the end of their linear growth period (Table 2a): chicks with relatively low ROMs in 2010 were most likely to be re-sighted in the colony in 2012 (Fig 2a). Chicks with fast growth rates and early hatch dates were also marginally more likely to be re-sighted. OXY, brood size, sex, hatching rank and hatching date were non-significant in the model. The non-significance of brood size, identified in a previous study as a correlate of recruitment (Harris *et al.* 1994), was not due to collinearity with growth rate, as growth

rate was independent of brood size (LME controlling for repeated measurements of Nest ID: t = -1.46, p 0.15).

For adults, whilst re-sightings were significantly lower in 2013, the wreck year, there was no interaction between ROMs and year (LRT X^2 = -2.69, p = 0.27) or OXY and year (LRT X^2 = -1.57, p = 0.46), suggesting that this did not alter patterns of survival in relation to oxidative stress levels. There was a significant negative relationship between ROMs and re-sighting probability (Fig 2b), but no main effect of OXY. Males were also more likely to survive than females, and there was a marginal quadratic effect (p = 0.083) of age on survival: young and old individuals were less likely to survive between years than middle-aged birds.

Discussion

We identified a within-individual increase with age in a measure of oxidative stress exposure in a wild population of European Shags, but this occurred only in older birds, aged 10-22 years. In these older birds, there was little variation in level of ROMs amongst individuals of the same age, nor in the rate of increase in ROMs over years amongst individuals. In contrast, younger birds, aged 2-9, showed significant and consistent inter-individual variation in ROMs over years, and no consistent pattern of age-related change. The cross sectional analysis had suggested a U-shaped quadratic relationship with age, decreasing into middle-age and increasing thereafter. In older birds, within- and between-individual slopes converged, supporting within-individual change as a mechanism for the cross-sectional increase observed in later life. Slopes did not converge, however, for younger individuals, and this discrepancy in the within- and between individual patterns is consistent with selective mortality in young, high ROM individuals. Re-sighting data from both chicks and adults support selective mortality underlying the apparent cross-sectional decrease in ROMS with age in the 2-9 age group: chicks with high pre-fledging ROMs were least likely to be re-sighted in the colony as 2 year old adults, and adults with high ROMs suffered highest mortality. In contrast, OXY showed only weak evidence of age-related patterns and no link to survival.

There are considerable logistical constraints in following longitudinal change throughout the lifespan of a long-lived wild animal. Instead, age-related patterns are often inferred from the cross-sectional patterns in an age-heterogenous sample of a given population (Nussey *et al.* 2008). However, our data highlight the dangers inherent in inferring the pattern of age related change from cross sectional data. As the lack of convergence in our within- and between-individual analyses highlights, selective mortality can disguise longitudinal processes within cross-sectional studies (Sliwinski *et al.* 2010).

The only age-related pattern identified in OXY was a marginally significant within-individual decrease in older birds, which differed significantly in direction from the between-individual slope, though neither slope differed from 0. However, we found significant annual variation in OXY, and greater variation amongst old (10-22 year old) than younger (2-9 year old) birds. As a measure of circulating non-enzymatic antioxidants including vitamins (Costantini 2011), rather than endogenous antioxidant defences, the lack of consistent age effects is unsurprising, as it is most likely to reflect environmentally-imposed differences in dietary antioxidant availability.

We also found that ROMs measured close to fledging were predictive of the probability of return to the colony as a prospecting or breeding bird two years later, suggesting that it related to either survival, age of recruitment or to natal fidelity. The former seems most likely because natal fidelity is high in this species/population. Moreover, re-sighting in this cohort was independent of sex, with 23% of both male and female 2 year olds resighted, despite females not usually recruiting until age 3. Plasma oxidative damage was also found to be associated negatively with chick recruitment in the Cies Island population of European shags (Galicia, Spain) (Noguera, Kim & Velando 2012). In both the Cies study and this study on the Isle of May, pre-fledging plasma antioxidant capacity did not predict chick return rate; interestingly, nor did brood size, previously identified as an important correlate of recruitment in the Isle of May population (Harris *et al.* 1994). Fast growth rate and early hatching, however, did marginally predict probability of return, which may be linked to individual or parental quality. Studies manipulating conditions in the nest suggest stress levels (Herborn et al., 2014) and parasite burden (Granroth-Wilding et al., 2014) can have lasting physiological impacts on

fledgling shags. In the first two years of life after fledging, young shags are less efficient foragers than older adults (Daunt *et al.* 2007b; a). However, several of the factors influencing recruitment are likely to co-vary, and it is not possible in a study of this kind to tease these apart. How successfully chicks navigate these post-fledging challenges appears from our data to be related to oxidative stress exposure, which is likely to be indicative of poor condition.

ROMs were also found to be higher towards the end of the breeding season, and towards the end of chick rearing in birds breeding at any time. Two processes may elevate energetic expenditure in the late-breeding birds: the availability of prey is lower, hence foraging effort increased (Daunt *et al.* 2007b), and the parasite burden is greater than in early breeding birds (Burthe *et al.* 2013). These birds are also likely to be younger, and late breeding is generally associated with poor quality individuals in seabirds (Furness & Monaghan 1987). Interestingly, adult ROMs did not increase with brood size. Indeed, OXY and brood size were marginally positively correlated. This is most likely because brood size is positively linked to parental quality, and because individuals will modulate their effort to reduce oxidative damage where possible (Metcalfe & Monaghan 2013). That ROMs appear to increase towards the end of chick rearing may be indicative of parents approaching the point at which parental investment should cease.

In summary, oxidative stress exposure, indicated by high levels of ROMs, differed significantly amongst individuals in early adulthood, but only showed consistent patterns of within-individual increase in later life in the European shag. The observed cross-sectional patterns in younger birds, however, appear to be shaped by selective mortality of high ROM individuals. It is perhaps surprising to find that, given that this is a non-experimental study, exposure to oxidative damage increases even in a long-lived species like the shag. However, a consistent age related change was not observed until the birds were 10 years of age and older. This suggests that the age-related increase in damage exposure observed in these older individuals is a consequence of age-related deterioration in defence processes. The variation in exogenous antioxidant defences may reflect individual variation in such senescence, but is perhaps more likely to be a consequence of dietary variation. More information on endogenously produced antioxidant levels would be needed to fully understand how

defences change with age. More likely is that the balance with reproductive output and somatic maintenance has shifted in the older age classes and that damage accumulation is no longer minimised.

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Data accessibility

Data have been deposited in the Dryad Digital Repository: doi:10.5061/dryad.c555g (Herborn *et al*, 2015)

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Table and figures legends

Figure 1 Age-related patterns in ROMs in adults. a) Cross-sectional patterns, illustrated using the mean and confidence intervals per age class but analysed as continuous variable, n = 133 adults, 208 samples; b) within-individual patterns for adults aged 10+ years, using (age – average age) per measurement, n = 26 adults, 58 samples. The y axis is logarithmic.

Figure 2 Relationship between ROMs and re-sighting of chicks and adults. a) Re-sightings of chicks fledged in 2010 demonstrating survival to at least 2 years, n = 74 chicks, and b) re-sightings of adults in the breeding season subsequent to year of first or only sampling, n = 133 adults. The x axis is logarithmic.

Table 1 Summary of models of adult log(ROMs) and log(OXY). a) Cross-sectional model (n = 133 adults, 58 with repeated measures; n = 208 samples), and b) within- and between-individual model (n = 58 adults, n = 132 samples). All p-values obtained using likelihood ratio tests between the full model and model excluding that variable.

Table 2 Summary of models investigating re-sighting in chicks and adults. a) mixed model of chick re-sighting (n = 74 chicks, n = 36 nests) and b) GLM of adult survival and re-sighting (n = 133 adults)

Table 1

	ROMs			OXY		
Variable	**Coefficient±SE**	**t**	**p**	**Coefficient±SE**	**t**	**p**
a) Cross-sectional models						
σ Identity	0.002±0.042			0.001±0.029		
σ Residual	0.028±0.168			0.008±0.088		
Intercept	0.799±0.245	3.25		2.093±0.131	16.05	
Sex Male	0.099±0.027	3.68	**0.0003**	0.012±0.014	0.86	0.38
Hatching date	0.004±0.001	3.15	**0.002**	0.001±0.001	1.48	0.14
Chick age	0.003±0.001	2.25	**0.023**	0.001±0.001	1.57	0.11
Brood size	-0.026±0.017	-1.56	0.11	0.016±0.009	1.84	0.062
Age	-0.031±0.012	-2.60		-0.005±0.006	-0.72	0.44
$(Age)^2$	0.002±0.001	2.58	**0.009**	0.0002±0.0003	0.56	0.56
Year 2011	0.015±0.032	0.46	0.36	0.020±0.017	1.22	**<0.0001**
2012	0.042±0.030	1.38		-0.118±0.016	-7.41	
b) Within- and between-individual models						
σ Identity age 2-9	0.002±0.045			0.001±0.028		
σ Identity age 10+	0.007±0.080			0.002±0.046		
σ Within-individual age 2-9	0.012±0.109			0.001±0.038		
σ Within-individual age 10+	0.024±0.155			0.007±0.083		
σ Residual	0.013±0.115			0.004±0.066		
Intercept	0.404±0.316	1.28		1.847±0.176	10.48	
Sex Male	0.143±0.032	4.50	**< 0.0001**	0.035±0.019	1.85	0.066
Hatching date	0.006±0.002	3.29	**0.001**	0.002±0.001	1.98	0.051
Chick age	0.005±0.002	2.50	**0.019**	0.002±0.001	1.94	0.070
Brood size	0.001±0.022	0.02	0.99	0.021±0.012	1.75	0.075
Between-individual 2-9	-0.003±0.009	-0.36	0.71	0.002±0.005	0.34	0.73

	10+	-0.005±0.005	-1.02	0.29	-0.001±0.003	-0.33	0.73
Within-individual	2-9	0.109±0.065	1.69	0.09	-0.007±0.033	-0.22	0.82
	10+	0.129±0.062	2.09	**0.034**	-0.052±0.037	-1.33	0.15
Year 2011		-0.038±0.052	-0.74	0.35	0.045±0.029	1.54	**< 0.0001**
2012		-0.122±0.100	-1.23		-0.065±0.057	-1.14	

Table 2

Variable	**Coefficient±SE**	**z-value**	**p-value**
a) Chick re-sighting			
σ Nest ID	<0.001±<0.001		
Intercept	11.971±9.580	1.25	0.21
Sex Male	-0.562±0.672	-0.84	0.40
Growth rate (g/day)	0.162±0.094	1.72	0.085
Brood size	0.109±0.390	0.28	0.78
Hatching rank B	-0.624±0.723	-0.86	0.39
C/D	0.156±0.687	0.23	0.82
Hatching date	-0.079±0.047	-1.68	0.093
Log(ROMs)	-5.457±2.392	-2.28	**0.023**
Log(OXY)	-0.723±1.140	-0.63	0.53
b) Adult re-sighting			
Intercept	9.205±8.421	1.09	0.27
Age	0.302±0.230	1.31	0.19
$(Age)^2$	-0.020±0.012	-1.73	0.083
Sex	1.594±0.631	2.53	**0.012**
Log(ROMs)	-4.244±1.794	-2.37	**0.018**
Log(OXY)	-1.070±3.349	-0.32	0.75
Year 2011	0.158±0.776	0.20	0.84
2012	-2.478±0.767	-3.23	**0.0012**