CHARLES UNIVERSITY

FACULTY OF SCIENCE

Study programme: Zoology

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CONDITION DEPENDENCE OF SEXUALLY SELECTED ORNAMENTS IN BIRDS

KONDIČNÍ ZÁVISLOST POHLAVNĚ SELEKTOVANÝCH ORNAMENTŮ U PTÁKŮ

Doctoral thesis

Supervisor: doc. Mgr. Tomáš Albrecht, Ph.D.

Praha 2018

Title

Condition-dependence of sexually selected ornaments in birds

Title in Czech

Kondiční závislost pohlavně selektovaných ornamentů u ptáků

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Year of the submission of the study proposal: 2009
Year of the thesis publication: 2018

Keywords: sexual selection, condition-dependence, ornamental traits, signalling, condition, individual quality, ecophysiology, oxidative stres, antioxidants, redox homeostasis, carotenoids, ageing, senescence, zebra finch, Taeniopygia guttata, barn swallow, Hirundo rustica

Keywords in Czech: pohlavní výběr, kondiční závislost, ornamentální znaky, signály, kondice, kvalita jedince, ekofyziologie, oxidační stres, antioxidanty, redoxní homeostáza, karotenoidy, stárnutí, zebřička pestrá, Taeniopygia guttata, vlaštovka obecná, Hirundo rustica

ACKNOWLEDGEMENTS

I am grateful to my supervisor Tomáš Albrecht for his extensive support and, above all, for giving me the opportunity to change the direction of my life and do evolutionary research. Doors of Tom's office were always open and his mind was always open to new ideas. His extensive knowledge, experience, advises, ideas and necessary criticism helped me to develop the initial ideas further and his great material support enabled turning them into tangible results. I also owe him a great deal for introducing me to Studenec, where I have found a new home and met my wife Ája.

I would like to express my great appreciation to all my collaborators with whom I have had the pleasure to work and without whom this work would not have been possible. I wish to thank to Adéla Petrželková, Romana Michálková, Marie Adámková, Martina Němcová, Jana Albrechtová, Tereza Králová, Ondřej Kauzál, Jaroslav Cepák, Jakub Kreisinger, Pavel Munclinger and Lukáš Bobek for their work on the barn swallow project and Jana Albrechtová, Michal Vinkler, Jana Svobodová, Barbora Gabrielová, Pavlína Opatová, Martina Němcová, Jitka Vinklerová, František Vejmělka and Helena Hejlová for their help with the zebra finch experiments. I would like to extend my special thanks to Rebecca Safran, Maren Vitousek, Joey Hubbard and Amanda Hund for the possibility to collaborate on the joint projects. I am also grateful to Peter Páp and his team for providing data from their barn swallow population, Petr Maršík for carotenoid analysis and Petr Kačer and Kamila Syslová for oxidative stress analysis. I would like to extend my thanks to all the diploma and bachelor students who helped a lot with the fieldwork and data collection. I also greatly appreciate the willingness of the farm owners to let us do our research on their properties. My special thanks should be given to Dušan Havelka for his help with cage design and making and to Ondřej Kauzál, who did a great job designing the thesis cover.

Most importantly, I wish to express my grateful thanks to my loving and supportive wife Ája. Without her support and tolerance this work would not have been possible. My heartfelt thanks go also to my sons Vojta and Štěpán, who brighten up my days. Zvláštní dík patří též celé mé rodině a především mým rodičům za jejich celoživotní bezmeznou podporu.

The research included in this thesis was supported by the Grant Agency of Charles University (283811 and 146213), the Czech Science Foundation (P506/12/2472, GA15-11782S and 17 24782S) and the Ministry of Education, Youth and Sports of the Czech Republic (LH14045).

PROHLÁŠENÍ AUTORA PRÁCE

V Praze dne 12. 4. 2018

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MVDr. Oldřich Tomášek, Ph.D.

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Michálková R., Tomášek O., Adámková M., Kreisinger J. & Albrecht T. Extra-pair paternity patterns in European barn swallows *Hirundo rustica rustica* are best explained by male and female age and not male ornamentation. (*in revision for Behavioral Ecology and Sociobiology*)

Podíl O. Tomáška: sběr dat v terénu, analýza ornamentů, podíl na úpravě rukopisu

doc. Mgr. Tomáš Albrecht, Ph.D.

Tomasek O., Pap P.L, Adamkova M., Cepak J, Fulop A., Michalkova R., Stermin A.N., Vagasi C.I., Vincze O. & Albrecht T. Age-dependence and viability signalling function of tail streamer length in the European barn swallow (*in preparation for Journal of Animal Ecology*)

Podíl O. Tomáška: návrh studie, sběr dat, statistická analýza, interpretace výsledků, příprava rukopisu

doc. Mgr. Tomáš Albrecht, Ph.D.

Vitousek, M.N., Tomášek, O., Albrecht, T., Wilkins, M.R. & Safran, R.J. (2016). Signal traits and oxidative stress: a comparative study across populations with divergent signals. *Front. Ecol. Evol.*, 4, 56.

Podíl O. Tomáška: sběr dat, odběry krve, podíl na interpretaci výsledků, podíl na úpravě rukopisu

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Podíl O. Tomáška: návrh studie, provedení experimentu, sběr dat, odběry krve, analýza antioxidační kapacity, spektrofotometrická analýza ornamentu, statistická analýza, interpretace dat, příprava rukopisu

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Podíl O. Tomáška: návrh studie, provedení experimentu, sběr dat, spektrofotometrická analýza ornamentu, statistická analýza, interpretace výsledků, příprava rukopisu

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ABSTRACT

Sexual ornaments important for mating success in many species are often assumed to evolve as condition-dependent signals of individual quality. Ornament expression can be associated with age and survival, thereby signalling individual viability. Here, we have tested viability signalling function of tail streamers and their importance for within-pair and extra-pair fertilisation success in the European barn swallow (*Hirundo rustica rustica*). In contrast to previous studies on this subspecies, our data suggest that tail length is not associated with fertilisation success in our population. Instead, the most important predictors of within-pair and extra-pair fertilisation success were female and male age, respectively. Our data supported viability signalling function of male tail streamers, as documented by age-related within-individual increase in their length. There was no evidence for senescence in this trait. Contrary to some previous studies, the viability signalling function of tail streamers was further supported by observed selective disappearance of males with shorter tails.

Several physiological mechanisms have been proposed as maintaining signalling honesty. Among them, oxidative stress from highly reactive species (RS), including free radicals, attracted a considerable attention. Given that oxidative stress may be a major constraint in life history, it has been put forward as a universal mechanism maintaining signalling honesty. We analysed association between oxidative stress and ornament expression in both European and North American (*H. r. erythrogaster*) barn swallows with divergent female preference for tail length and dark pheomelanin-based ventral colouration, respectively. The results suggest that the association with oxidative state may relate to divergence in preference in some traits (tail length) and to ornament type in others (ventral colouration). We further tested the role of oxidative stress in carotenoid-based sexual signalling. We demonstrated an adverse effect of oxidative challenge on carotenoid-based beak pigmentation in male zebra finches (*Taeniopygia guttata*), providing support for redox-based hypotheses of carotenoid-based signalling honesty maintenance. Using a novel measure of lipophilic antioxidant capacity, we found support for *in vivo* antioxidant function of carotenoids (that has recently been called into question), thereby challenging several proposed mechanisms of carotenoid-based signalling honesty maintenance. Lastly, we revealed a trade-off between expression of carotenoid-based ornamentation and sperm resistance to oxidative damage. Considerable challenges remain to be addressed in future studies, such as population- and ornament-type-related differences in signalling content and associated trade-offs, as suggested by contrasting results of our and many former studies.

ABSTRAKT

Pohlavní ornamenty hrající u mnoha druhů důležitou roli při výběru partnera se mohly vyvinout jako kondičně závislé signály kvality jedince. Exprese ornamentů může asociována s věkem a přežíváním, a tím fungovat jako indikátor životaschopnosti jedince. V předkládané práci jsme testovali význam délky krajních ocasních per u vlaštovky obecné evropské (*Hirundo rustica rustica*) pro vnitropárový a mimopárový fertilizační úspěch a zda tento sekundární pohlavní znak signalizuje životaschopnost jedince. Naše výsledky na rozdíl o předchozích studií naznačují, že délka ocasních per nemá na fertilizační úspěch samce žádný vliv. Nejvýznamějším prediktorem schopnosti samce opatřit si mimopárovou partnerku byl jeho věk a přítomnost mimopárových mláďat v hnízdě nejlépe predikoval věk jeho sociální partnerky. Délka ocasních per se u samců i samic prodlužovala s věkem, což podporuje jejich funkci jako signálu životaschopnosti jedince. Nezaznamenali jsem žádný vliv stárnutí na tento znak. Signalizační funkci dále naznačuje lepší přežívání samců s delšími ocasy.

Bylo navrženo několik mechanizmů, jak by mohla být zajištěna čestnost takové signalizace. Jedním z nejvíce studovaných mechanizmů je oxidační stres vyvolaný volnými radikály a dalšími reaktivními částicemi. Vzhledem k tomu, že nutnost bránit se oxidačnímu stresu může být jedním z hlavních omezení ovlivňujících životní strategie, byl oxidační stres navržen jako možný univerzální mechanizmus zajišťující čestnost pohlavních signálů. V této práci jsme studovali vztahy mezi redoxním stavem a expresí ornamentů u evropského a severoamerického (*H. r. erythrogaster*) poddruhu vlaštovky obecné, u kterých je známa odlišná preference pro délku ocasu a tmavé pheomelaninové zbarvení ventrální části těla. Výsledky naznačují, že vztah mezi ornamentací a oxidačním stresem může mít u některých znaků souvislost s odlišnými preferencemi (ocasní pera), zatímco u jiných může souviset spíše s typem ornamentu (zbarvení břicha). Dále jsme studovali roli oxidačního stresu, jako mechanizmu zajištujícím čestnost karotenoidních signálů. Ukázali jsme, že vystavení samců zebřičky pestré (*Taeniopygia guttata*) oxidační zátěži má negativní vliv na karotenoidní zbarvení zobáku. To podporuje hypotézy, které navrhují, že nutnost udržovat redoxní homeostázu je mechanizmus zajišťující čestnost karotneoidních signálů. Použití nové metody na měření lipofilní antioxidační kapacity poskytlo nepřímý důkaz pro antioxidační funkci karotenoidů *in vivo*, což umožňuje vyloučit některé z navržených mechanizmů zajišťujících čestnost signalizace. Naše data dále naznačují trade-off mezi expresí karotenoidních ornamentů a odolnosti spermií k oxidační zátěži. Tato práce naznačuje existenci rozdílů v signalizační funkci ornamentů a s ní spojených trade-off jak mezi populacemi, tak mezi různými ornamenty, jejichž objasnění je výzvou pro budoucí studie.

CONTENTS

AIMS OF THE STUDY

The general aim of the thesis was to study patterns and mechanisms of condition-dependent expression of sexual signals. The specific aims were as follows:

- to test the importance of a classical textbook example of sexually selected trait, tail streamer length in the European barn swallow (*Hirundo rustica rustica*), for male within-pair and extra-pair fertilisation success

- to evaluate viability signalling function of tail streamer length in two populations of this subspecies by testing association of tail streamer length with individual survival and analysing age-related within-individual changes in tail streamer length

- to analyse senescence in tail streamer length in the European barn swallow

- to explore oxidative-stress related signalling content of two ornamental traits, tail streamer length and pheomelanin-based ventral colouration, by analysing their associations with oxidative stress in both the European and North American barn swallow subspecies with divergent preferences for these traits

- to assess plausibility of hypotheses proposing oxidative stress to be a mechanism maintaining honesty of carotenoid-based sexual ornamentation by experimentally testing their most fundamental assumption: an adverse effect of oxidative challenge on carotenoid-based ornament expression; using the zebra finch (*Taeniopygia guttata*) as a model species

- to test several proposed mechanisms of oxidative-stress mediated honesty of carotenoid-based signalling by evaluating *in vivo* antioxidant function of carotenoids using a novel lipophilic measure of *in vivo* antioxidant capacity

- to evaluate redox-based phenotype-linked fertility hypothesis predicting positive correlation between carotenoid-based ornamentation and sperm resistance to oxidative damage

- to test trade-off between carotenoid-based ornamentation and sperm resistance to oxidative damage

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

This thesis consists of the following five papers. The papers are referred to in the text by numbers given below:

1. Michálková R., **Tomášek O.**, Adámková M., Kreisinger J. & Albrecht T. Extra-pair paternity patterns in European barn swallows *Hirundo rustica rustica* are best explained by male and female age and not male ornamentation. (*in revision for Behavioral Ecology and Sociobiology*)
2. **Tomasek O.**, Pap P.L, Adamkova M., Cepak J, Fulop A., Michalkova R., Stermin A.N., Vagasi C.I., Vincze O. & Albrecht T. Age-dependence and viability signalling function of tail streamer length in the European barn swallow (*in preparation for Journal of Animal Ecology*)
3. Vitousek, M.N., **Tomášek, O.**, Albrecht, T., Wilkins, M.R. & Safran, R.J. (2016). Signal traits and oxidative stress: a comparative study across populations with divergent signals. *Front. Ecol. Evol.*, 4, 56.
4. **Tomášek, O.**, Gabrielová, B., Kačer, P., Maršík, P., Svobodová, J., Syslová, K., Vinkler M. & Albrecht T. (2016). Opposing effects of oxidative challenge and carotenoids on antioxidant status and condition-dependent sexual signalling. *Sci. Rep.*, 6, 23546.
5. **Tomášek, O.**, Albrechtová, J., Němcová, M., Opatová, P. & Albrecht, T. (2017). Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge. *Proc. R Soc. B*, 284, 20162444.

INTRODUCTION AND SYNTHESIS

Sexual selection and evolution of condition-dependent sexual signalling

Conspicuous ornamental traits have both fascinated and puzzled generations of researchers at least since Darwin's times and how and why such traits evolve has been intensively debated since the publication of Darwin's seminal work (e.g. Darwin 1871; Fisher 1930; Zahavi 1975; Lande 1981; Grafen 1990; Kokko *et al.* 2002; Getty 2006; Hill 2011; Cuthill *et al.* 2017). Secondary sexual characters do not increase and often even interfere with survival and as such cannot be explained by the means of natural selection (Darwin 1871; Fisher 1930; Zahavi 1975). Darwin put forward an idea that male ornamental traits evolve in response to female mate choice imposing sexual selection on males (Darwin 1871). An immense body of literature has been amassed since then supporting Darwin's hypothesis and sexual selection became widely accepted as a powerful evolutionary mechanism driving evolution of many spectacular traits and sexual dimorphism (Andersson 1994; Johnstone 1995; Kokko *et al.* 2003; Andersson & Simmons 2006; Jones & Ratterman 2009; Kuijper *et al.* 2012; Cuthill *et al.* 2017). The main driving force behind sexual selection is a competition between individuals for mates and fertilizations resulting in their differential reproductive success (Andersson 1994). Individuals with more elaborate ornamental traits are expected to enjoy reproductive advantages in terms of higher number of mates in polygamous species or higher number of extra-pair fertilisations in monogamous species, a shorter time required to obtain a mate or higher chance to obtain a high-quality mate (Johnstone 1995). As a result of such reproductive advantages, yielding higher reproductive success, genes producing elaborate ornamental traits are predicted to spread in the population (Andersson 1994).

Over time, the original Darwin's idea has been further developed. Ronald Fisher proposed that trait exaggeration can arise due to linkage disequilibrium between genes for elaborate trait expression and genes for preference of such a trait (Fisher 1930). His argumentation was based on an assumption that offspring of males with elaborate traits and females with preference for such traits will inherit genes for both trait elaboration and preference. Resultant positive genetic correlation between both gene types will then drive trait exaggeration beyond its naturally selected optimum, until survival costs of trait exaggeration counterbalance a reproductive advantage of being attractive. In its pure form, this so called Fisherian runaway process does not incorporate condition-dependent expression of ornamental traits and predicts a negative correlation between attractiveness and survival (Lande 1981; Kirkpatrick 1982; Brooks 2000). This pure Fisherian process is also known as sexy-sons hypothesis as the only reproductive advantage assumed to be obtained by females is an indirect benefit in the form of giving birth to attractive sons (Andersson & Simmons 2006).

In contrast, indicator mechanism models of sexual selection, also known as good-genes models, assume that secondary sexual characters are expressed in a condition-dependent manner and as such can honestly signal individual quality (Zahavi 1975; Grafen 1990; Getty 1998; Hill 2011). In general, positive correlation between expression of ornamental traits and survival is predicted by these models (Jennions *et al.* 2001), though it can be confounded by a trade-off between these two fitness components (Van Noordwijk & de Jong 1986) as ornamental traits are usually assumed to be costly (Zahavi 1975). In such cases, the resultant direction of correlation is influenced by resource acquisition, with correlation between attractiveness and survival being predicted to be positive under high resource abundance and negative under low resource abundance (Van Noordwijk & de Jong 1986).

More recently, a unifying model has been put forward suggesting that pure Fisherian process and indicator mechanism are just opposite ends of one sexual selection continuum (Kokko *et al.* 2002). According to this model, the direction of correlation between ornamentation and survival may depend on the costliness of female choice, and thus on the intensity of sexual selection that females impose on males. When costliness of female choice is low, the model predicts negative correlation between male ornamentation and survival due to intense sexual selection imposed on males. Viability signalling function of ornamental traits is lost under such circumstances, because reproductive advantage outweigh survival costs. In contrast, when female choice costs increase, for example due to energetic or time constraints (Milinski & Bakker 1992), higher exposure to predators (Jennions & Petrie 2007) or high prevalence of sexually transmitted diseases (Boots & Knell 2002), sexual selection on males relaxes and positive correlation between ornamentation and survival is restored. Under such conditions, secondary sexual characters can serve as viability indicators (Kokko *et al.* 2002).

Sexual signalling in the barn swallow

The barn swallow (*Hirundo rustica*) is a small migratory insectivorous songbird species, comprising eight subspecies, breeding in much of Eurasia and North America and wintering across the Southern Hemisphere (del Hoyo *et al.* 2018; Klvaňa *et al.* 2018). During the last three decades, the barn swallow has become one of the most prominent model species in the research of sexual selection; the European subspecies (*H. r. rustica*) and the North American subspecies (*H. r. erythrogaster*) being the two most studied subspecies (Møller 1994b; Safran *et* *al.* 2005; Romano *et al.* 2017). The breeding system of the barn swallow is social monogamy but sexual promiscuity and extra-pair paternity are common (Laskemoen *et al.* 2013). The adults show small to moderate degree of sexual dimorphism in several traits, with different traits reported as being under current sexual selection in different populations (Scordato & Safran 2014; Romano *et al.* 2017). Males with longer outermost tail feathers (also referred to as tail streamers) have been reported as preferred in both social and extra-pair mating in the European subspecies (Møller 1988, 1994b), whereas males with darker pheomelanin-based rusty brown ventral colouration and shorter tail streamers have been shown to be preferred as extra-pair mates by females in the North American subspecies (Safran & McGraw 2004; Safran *et al.* 2005; Eikenaar *et al.* 2011; Safran *et al.* 2016; but see Kleven *et al.* 2006). Both correlational studies and experimental manipulations of tail streamer length and ventral colouration have been carried out to discover the importance of these ornaments in mate choice (Møller 1994b; Møller *et al.* 1998; Safran & McGraw 2004; Safran *et al.* 2005; Scordato & Safran 2014). In the European subspecies, males with longer tail streamers pair and breed earlier (Møller 1988; Wilkins *et al.* 2016), produce more offspring both in the first clutches and per season (Møller 1988; Scordato & Safran 2014) and have greater both within-pair (Møller *et al.* 2003) and extra-pair fertilisation success (Møller & Tegelström 1997; Saino *et al.* 1997b). Therefore, tail streamers in European barn swallows males has become a textbook example of sexually selected trait (Møller 1994b; Møller *et al.* 1998; Scordato & Safran 2014; Romano *et al.* 2017). In the North American subspecies, early breeding, higher annual reproductive success and higher within-pair fertilisation success is associated with darker ventral colouration (Safran & McGraw 2004; Safran *et al.* 2005, 2016; Eikenaar *et al.* 2011) and shorter tail streamers (Safran *et al.* 2016). Nevertheless, differences between populations have been reported in this subspecies as longer tail streamers, but not darker ventral colouration, has been shown to be associated with higher extra-pair fertilisation success in another population (Kleven *et al.* 2006; Lifjeld *et al.* 2011).

In contrast, we found no support for either tail streamer length or ventral colouration being important predictors of within-pair (probability of paternity loss) or extra-pair (ability to obtain an extra-pair partner) fertilisation success in our population (**Paper 1**). Based on pairwise comparison, there was also no difference between cuckolded and cuckolding males with respect to tail length. No association between ventral colouration and extra-pair fertilisation success is not surprising and is in agreement with previous studies in the European barn swallow (Romano *et al.* 2017). Interestingly, our results suggest that, in contrast to previous studies on the European subspecies, tail length also does not play an important role in extra-pair mating in our population. Is it still possible, however, that tail streamer length is a sexual trait important in social mating (Møller 1988, 1994b), which is supported by the fact that long-tailed males start breeding earlier in the season in our population (Wilkins *et al.* 2016). Nevertheless, given that important role of tail streamer length in extra-pair mating has previously been reported from other populations, our data suggest between-population differences in sexual selection or signalling content (Van Noordwijk & de Jong 1986; Kokko *et al.* 2002). Indeed, such between-population differences in traits associated with mating success have been reported in the North American barn swallow (Safran & McGraw 2004; Safran *et al.* 2005; Kleven *et al.* 2006; Eikenaar *et al.* 2011) and several other songbird species (Dale *et al.* 1999; Galván & Moreno 2009; Whittingham & Dunn 2014). Whittingham & Dunn (2014) even pointed out that, between populations, differences in traits associated with extra-pair fertilisation success seem to be observed more frequently than similarities.

The only significant predictor of extra-pair fertilisation success was male's age, indicating that older birds sire more extra-pair offspring. Similar results were reported from the North American subspecies, in which age accounted for much of the variability in both within-pair and extra-pair paternity (Lifjeld *et al.* 2011). Our data are also in agreement with recent meta-analysis based on multiple species and secondary sexual traits, indicating that age is more important predictor of extra-pair fertilisation success than sexual traits in general (Hsu *et al.* 2015). The possible explanation for the link between male age and extra-pair fertilisation success is dependent on whether extra-pair mating is adaptive for females or whether its evolution is driven solely by benefits to males. According to the male manipulation hypothesis, which does not require extra-pair mating to be adaptive for females, older males may be more experienced at coercing females into extra-pair copulations, thus being more successful in gaining extra-pair fertilisations (Weatherhead & Boag 1995; Westneat & Stewart 2003; Hsu *et al.* 2015). Adaptive hypothesis assumes that females participate in extra-pair mating actively and prefer older males for their higher quality demonstrated as their ability to survive (Manning 1985; Kokko 1997, 1998). The active participation of females in extra-pair mating or higher success of older males in obtaining extra-pair copulations are difficult to assess, however, as both observing female solicitation behaviour and measuring frequency of extra-pair copulations are extremely difficult, especially in the wild. An indirect support for active female choice could be drawn from reported positive correlation between male age and sexual signal expression (Delhey & Kempenaers 2006; Bitton & Dawson 2008; Nussey *et al.* 2009; Val *et al.* 2010; Balbontín *et al.* 2011; Evans *et al.* 2011; Kervinen *et al.* 2015), suggesting that males can signal their age (Manning 1985). The plausibility of age signalling has further been supported by game-theoretic models based on life-history theory, predicting increasing investment in sexual signalling with age-related decrease in residual reproductive value (Kokko 1997, 1998).

Age-dependence and viability signalling function of tail length in the European barn swallow

In **Paper 2**, we analysed age-related changes in tail streamer length in the European barn swallow, thereby assessing the possibility that tail streamers can signal individual age. We also evaluated whether tail streamer length can signal potential viability by testing its association with survival. We used longitudinal data from two populations from the Czech Republic and Romania that has been followed since 2010 and 2011, respectively. It is important to note that phenotypic trait values in different age categories are determined by both within-individual changes in trait expression with age and between-individual variability in survival, which may by non-random with relation to the phenotypic trait measured (van de Pol & Verhulst 2006; Nussey *et al.* 2008). Therefore, we used a statistical approach enabling separation of both within-individual age-related change and selective disappearance in one linear mixed-effect model, fitted with individual age and lifespan as predictors of tail streamer length (van de Pol & Verhulst 2006). The inclusion of these two predictors in the model can also be interpreted as testing for both the proven (age) and potential (lifespan) viability signalling function of the tail streamer length.

Our data provided support for both functions. First, we found that tail streamer length increases with age in both sexes, with the change being highest at young age with subsequent slowdown and levelling-off in old age. An increase in pre-senescent ornament expression has commonly been observed in many species (Delhey & Kempenaers 2006; Bitton & Dawson 2008; Nussey *et al.* 2009; Val *et al.* 2010; Balbontín *et al.* 2011; Evans *et al.* 2011; Kervinen *et al.* 2015) and supports the hypothesis that individuals can signal their age and thus the proven ability to survive (Manning 1985; Kokko 1998). We found no evidence for senescence in tail length in our data, though previously reported in another European barn swallow populations (Balbontín *et al.* 2011). We also demonstrated that including only second-order polynomial of age as a predictor of tail length in the model can be falsely interpreted as senescence when the rate of phenotypic change is higher in the young age with subsequent plateauing and should be interpreted with caution (Bouwhuis *et al.* 2009). This could provide a possible explanation for the discrepancy between our results and the results of the previous study, which only used second-order polynomial of age (Balbontín *et al.* 2011).

Second, we found tail streamer length to be positively correlated with lifespan in males, but not females, indicating selective disappearance of short-tailed males. This further supports viability signalling function of this ornament in males. Previous studies in different European barn swallow populations provided contrasting results, reporting either positive (Møller 1991, 1994a; Saino *et al.* 1997a) or negative (Møller & Szep 2002; Balbontín *et al.* 2011; Romano *et al.* 2017) correlation. Together with our results, such a discrepancy suggest that the direction of correlation between tail streamer length and survival may differ between populations, resulting from, for example, differences in resource availability (Van Noordwijk & de Jong 1986; Catoni *et al.* 2008) or intensity of sexual selection (Kokko *et al.* 2002).

In addition, we used a second approach to test how tail length measured in the first year of life predict lifelong survival, the logistic discrete-time hazard models, which enable to test for stabilising or disruptive selection in the phenotypic trait that is included as a predictor of mortality. In the Czech population, we indeed found a non-linear relationship between first-year tail length and probability of mortality, with males having first-year tail length about 5 mm above the population median being the best survivors. This supported overall directional selection for longer tails, though combined with stabilising selection acting on males with extremely long tails.

Mechanisms maintaining signalling honesty

The indicator models of ornamental trait evolution usually assume that the expression of elaborate traits is costly and handicaps the individual. Hence, the costs of ornament elaboration have been put forward as a mechanism preventing cheating and maintaining signalling honesty (Zahavi 1975; Grafen 1990). This so called handicap principle assumes that the costs of trait elaboration are higher for the low quality males and thus only high quality individuals in a good body condition can afford elaborate ornament expression (Grafen 1990).

The existence and nature of the costs associated with ornament elaboration has been subject to intense research. Despite this effort, a broad consensus has not been reached and this topic remains to be intensely debated (Kotiaho 2001). It is highly likely that various types of ornamentation are subject to very different costs and trade-offs (Kotiaho 2001). The most straightforward cost is increased conspicuousness of more ornamented individuals increasing the risk of being predated (Selander 1965; Baker & Parker 1979; Johnstone 1995; Zuk & Kolluru 1998). There is an ample evidence for such predatory costs coming from both invertebrate (Cade 1975; Grether 1997; Fowler-Finn & Hebets 2011; Ercit & Gwynne 2015) and vertebrate species (Endler 1980, 1982, 1983; Breden & Stoner 1987; Slagsvold *et al.* 1995; Mougeot & Bretagnolle 2000; Stuart-Fox *et al.* 2003; Møller *et al.* 2006; Cabido *et al.* 2009; Hernandez-Jimenez & Rios-Cardenas 2012). In predatory species, individuals with more elaborate ornaments may also suffer costs from being more conspicuous to the prey, resulting in a reduced foraging efficiency (Grether & Grey 1996).

Apart from interspecific interactions, individuals also socially interact with their conspecifics. Such intraspecific social interactions can incur additional costs that can prevent cheating in sexual signalling. Specifically, individuals with more elaborate signals usually experience more frequent and more aggressive agonistic behaviour by their conspecifics (Møller 1987; Huhta & Alatalo 1993; Dale & Slagsvold 1996; Beani & Turillazzi 1999; van Dongen & Mulder 2007; Rick & Bakker 2008; Tibbetts & Izzo 2010; Linhart *et al.* 2012). Hence, cheating individuals run the risk of either being injured or suffering high energetic or time costs associated with more intense agonistic behaviour. Such a social punishment can result in reduced survival or reproductive success, especially in individuals of low quality, as documented by experiments with signal exaggeration (Veiga 1993, 1995; Nakagawa *et al.* 2008).

Exaggerated structural traits, such as elongated feathers, elongated fins or antlers, may also impair locomotor performance due to physical constraints (Oufiero & Garland 2007). The reduction in locomotor abilities can, for example, result from the exaggerated structures being heavy (Tullis & Straube 2017), increasing aerodynamic (Barbosa & Møller 1999) or hydrodynamic (Pettersson & Hedenström 2000) drag or reducing manoeuvrability (Buchanan & Evans 2000; Bro-Jørgensen *et al.* 2007). Consequently, elaborate structural traits may reduce individual's foraging efficiency (Møller 1989; Møller & de Lope 1994) or the ability to escape predation (Møller 1996).

Physiological mechanisms of signalling honesty maintenance

In many types of sexual signals, the signalling honesty has been proposed to be maintained, at least to some extent, through indirect physiological costs associated with signal expression or maintenance. For example, many sexual displays, such as lekking behaviour (Vehrencamp *et al.* 1989), sexual vocalisation (Ward *et al.* 2003; Hoback & Wagner 2008) or spider drumming (Kotiaho *et al.* 1998), are highly energy-demanding. Among other physiological mechanisms proposed to maintain signalling honesty, immune costs (Folstad & Karter 1992; Sheldon & Verhulst 1996; Zuk & Stoehr 2002; Roberts *et al.* 2004) and oxidative stress (von Schantz *et al.* 1999; Costantini 2008; Dowling & Simmons 2009; Garratt & Brooks 2012) gained the most attention. Physiological mechanisms and trade-offs involved in ornament expression are expected to be tightly linked to central trade-offs in life history (Zuk & Stoehr 2002; Dowling & Simmons 2009; Simons 2013). Only when this condition is met, ornamental traits can reliably signal individual genetic and phenotypic quality. Energetic, immune and oxidative costs are particularly interesting in this regard. For example, trade-off in energy allocation to different fitness components has historically been most often considered as a central constraint in life history (Stearns 1992; Zera & Harshman 2001). Importance of immune functions in life history stems from their essential role in securing survival, considering that parasitism (including parasitic bacteria and viruses) is probably the most frequent lifestyle on Earth (Price 1980). Hence, a trade-off between sexual signalling and survival may arise, if signal expression entails reduced investment in immune functions (Folstad & Karter 1992; Sheldon & Verhulst 1996; Zuk & Stoehr 2002). Oxidative stress from reactive oxygen species (ROS) is another interesting factor as ROS are inevitably formed in oxidative energy metabolism and during immune response and their formation increases in energetically demanding and stressful states and during immune activation (von Schantz *et al.* 1999; Dowling & Simmons 2009). Oxidative damage caused by reaction of ROS with vital cellular components leads to the functional decline and the loss of structural integrity (Halliwell & Gutteridge 2015) and its accumulation together with an adverse effect of ROS on telomeres are believed to be important factors involved in the process of senescence (Finkel & Holbrook 2000; von Zglinicki 2002; Hekimi *et al.* 2011; López-Otín *et al.* 2013). Therefore, the maintenance of a balanced oxidative status (redox homeostasis) has been proposed as a central constraint in life history, mediating trade-off between investment in reproduction (including sexual signalling) and survival (Dowling & Simmons 2009).

Recently, several hypotheses emerged, proposing alternative cost-free mechanisms (Hill 2011; Emlen *et al.* 2012; Warren *et al.* 2013). According to these hypotheses, ornament expression may share metabolic and signalling pathways with vital cellular processes, thereby reliably signalling individual quality and condition. Several such metabolic and signalling pathways have been put forward including insulin/insulin-like growth factor signalling (Emlen *et al.* 2012; Warren *et al.* 2013), vitamin A metabolism and signalling (Hill & Johnson 2012), pathways involved in regulation of inner mitochondrial membrane potential (Johnson & Hill 2013) or oxidative phosphorylation pathway (Hill & Johnson 2013).

Experimental test of costs associated with tail streamers in the European barn swallow

In our barn swallow population, we used experimental manipulation of male tail streamer length to test for the costs associated with this trait (**Paper 2**). In contrast to previous studies (Møller & de Lope 1994; Saino *et al.* 1997a), experimental manipulation affected neither survival nor length of tail streamers grown during subsequent moult in our study. There was also no evidence for differential effect of ornament manipulation on naturally short-tailed and long-tailed males. The discrepancy between our results and results of previous studies (Møller & de Lope 1994; Saino *et al.* 1997a) may relate to differences in methods used for tail length manipulation (see **Paper 2** for a detailed discussion). Nevertheless, our results indicate that the costs associated with tail streamers are at best mild in our population and may only be significant in males with extremely long tails, as evidenced by non-linear relationship between tail length and survival in the correlational analysis (**Paper 2**). This suggests that handicap principle may not be the major mechanism mediating condition-dependence of tail streamers and alternative cost-free mechanisms may be involved (Hill 2011; Emlen *et al.* 2012; Warren *et al.* 2013).

Redox homeostasis and oxidative stress

Below, I will describe the mechanisms proposed to maintain signalling condition dependence through redox homeostasis in greater detail, since studies into the role of oxidative stress in condition-dependent sexual signalling form a major part of my thesis (**Papers 3–5**; related also to the experimental test of tail streamer costs in **Paper 2**).

Oxidative stress arises from the action of free radicals and other oxidizing molecules reacting with cellular components and causing their oxidative damage. Free radicals are chemical species that are capable of independent existence and contain one or more unpaired electrons that are responsible for high reactivity of such species. Apart from free radicals, there are many other oxidizing molecules, such as singlet oxygen, hydrogen peroxide or hypochlorous acid that are not of free-radical nature but are also capable of causing oxidative damage (Halliwell & Gutteridge 2015). I will refer to free radicals and other oxidizing agents collectively as reactive species (RS), which covers chemical species derived from oxygen (ROS), nitrogen (reactive nitrogen species; RNS) or carbon (reactive carbonyl species; RCS) (Costantini *et al.* 2010; Pamplona & Costantini 2011).

In the body, predominant, but not exclusive, source of RS is oxidative phosphorylation in mitochondria (Valko *et al.* 2007; Halliwell & Gutteridge 2015). In this process, electrons flow through a protein complex known as electron transport chain and are transferred from NADH to oxygen in a series of controlled redox reactions. Energy released in this process is subsequently used for ATP synthesis. Some electrons can leak prematurely from the electron transport chain, however, and react with oxygen in an uncontrolled manner, leading to a formation of superoxide anion radical. Superoxide radical is a highly reactive RS with extremely short half-life that usually reacts close to the site of its formation. Nevertheless, its reactions with other molecules leads to a formation of secondary RS, some of them with much longer half-lives, which can spread the oxidative damage further to the cell (Valko *et al.* 2007; Halliwell & Gutteridge 2015). When reaching biological membranes, RS react with membrane lipids initiating lipid peroxidation resulting in generation of great variety of lipid-derived RS known as lipid hydroperoxides. Lipid peroxidation proceeds by a chain reaction, which means that one initiating RS can oxidize many molecules of lipids. This makes lipid membranes and lipid peroxidation important players in propagation of oxidative stress (Niki *et al.* 2005; Niki 2009).

To reduce generation of oxidative damage to cellular components, organisms evolved a variety of antioxidant mechanisms (Valko *et al.* 2007; Pamplona & Costantini 2011; Halliwell & Gutteridge 2015). The diversity of antioxidant mechanism is believed to be a consequence of the diversity of RS and types of molecular damage they can cause. Broadly speaking, any mechanism, structure or substance that prevents, delays, removes or protects against oxidative damage to a target molecule can be considered to be antioxidant (Pamplona & Costantini 2011). The first line of defence against oxidative stress is prevention of RS generation, which can be achieved by several mechanisms, such as reduction in the absolute content of mitochondrial electron transport chain components, particularly complexes I and III where the electron leakage is the highest (Pamplona *et al.* 2005; Pamplona & Barja 2007), or regulation of proton gradient on the inner mitochondrial membrane and stimulation of electron transfer in the electron transport chain by uncoupling proteins (Criscuolo *et al.* 2005).

The existing RS can be eliminated by various molecular antioxidant mechanisms, either of enzymatic or non-enzymatic nature. Direct enzymatic RS scavengers are superoxide dismutase (SOD), which quenches superoxide radical and catalase (CAT) and glutathione peroxidase (GPx), both responsible for elimination of hydrogen peroxide (Valko *et al.* 2007; Pamplona & Costantini 2011). Non-enzymatic antioxidants can be hydrophilic or lipophilic, which determines the compartment type where these antioxidant molecules operate. Hydrophilic antioxidants, such as glutathione and ascorbic acid (vitamin C), are active primarily in aqueous phases of cells and the circulatory system (Beyer 1994). Lipophilic antioxidants that are represented by tocopherols (vitamin E), coenzyme Q and (possibly) carotenoids protect mainly hydrophobic cellular compartments, such as lipid membranes (Beyer 1994; Young & Lowe

2001; El-Agamey *et al.* 2004). Together, hydrophilic and lipophilic antioxidants constitute a highly integrated system where individual components interact with each other. For example, both tocopherols and coenzyme Q can be regenerated by ascorbic acid, which is subsequently regenerated by glutathione. Hence, both hydrophilic antioxidants, ascorbic acid and glutathione, also participate in the antioxidant protection of lipid membranes (Beyer 1994).

It remains to be noted that oxidative stress can never be prevented completely, since RS are generated continuously as an inevitable by-product of oxidative phosphorylation (Costantini & Verhulst 2009). Moreover, RS have also important signalling and regulatory functions in the cells and certain levels of RS are necessary for the maintenance of a normal physiological cellular state (Dröge 2002; Valko *et al.* 2007). Therefore, cells are in a dynamic redox state, known as redox homeostasis, adjusting antioxidant defences or even producing RS in enzymatic reactions in order to maintain optimal levels of RS and oxidative damage (Dröge 2002; Valko *et al.* 2007). This means that elevated rate of RS generation may not result in elevated rate of oxidative damage generation, if antioxidants are upregulated accordingly. In order to simplify the description of different aspects of redox state, I will refer to the rate of RS generation as oxidative burden and to the rate of oxidative damage generation as oxidative stress. Using these terms, the aforesaid statement would read: elevated oxidative burden may not result in elevated oxidative stress, if antioxidants are upregulated accordingly. It is also important to note that the view of redox homeostasis as a single equilibrium between RS on one side and antioxidants on the other is an oversimplification of reality. Due to the existence of multiple discrete redox pathways, redox homeostasis maintenance should be rather viewed as a multi-faceted process with dysregulation in any single redox pathway having a potential to trigger tissue-specific pathologies or senescence (Jones 2006).

Redox homeostasis as a mechanism maintaining signalling honesty

Although moderate levels of RS are beneficial and necessary to maintain normal cellular redox state, dysregulation of their production and subsequent accumulation of oxidative damage is considered to be an important factor causally involved in a variety of pathologies and senescence (Valko *et al.* 2007; Hekimi *et al.* 2011; López-Otín *et al.* 2013). Therefore, the need to maintain redox homeostasis is believed to be a major constraint in life history, possibly underpinning, at least to some extent, the trade-off between reproduction and survival (Dowling & Simmons 2009). A possible central role in life history makes redox homeostasis a particularly interesting evolutionary constraint, which led to the hypothesis that redox homeostasis maintenance may also be involved in maintaining honesty of condition-dependent sexual signalling (von Schantz *et al.* 1999; Galván & Solano 2009; Garratt & Brooks 2012). Based on the handicap principle, it has been proposed that sexual signal expression entails oxidative costs, either increasing generation of RS or reducing antioxidant capacity (von Schantz *et al.* 1999; Galván & Solano 2009; Garratt & Brooks 2012). Recently, several alternative hypotheses argued against the handicap principle, suggesting instead that signalling expression depends on metabolic or signalling pathways involved in mitochondrial regulation and redox homeostasis maintenance, thereby signalling redox state and mitochondrial function without entailing any costs (Hill 2011; Hill & Johnson 2012, 2013; Johnson & Hill 2013).

Exaggerated structural traits can pose mechanical constraints either being heavy or entailing aerodynamic or hydrodynamic costs (Barbosa & Møller 1999; Buchanan & Evans 2000; Pettersson & Hedenström 2000; Bro-Jørgensen *et al.* 2007; Tullis & Straube 2017), which can result in elevated energy demands associated with elevated oxidative burden (von Schantz *et al.* 1999; Dowling & Simmons 2009; Costantini *et al.* 2010).

Another mechanism, by which ornament expression could entail oxidative costs, has been propounded by the oxidation handicap hypothesis (Alonso-Alvarez *et al.* 2007). It is based on observations that sex hormone testosterone, which promotes expression of sexual traits in many species (Folstad & Karter 1992), can also induce oxidative stress (Chainy *et al.* 1997; Alonso-Alvarez *et al.* 2007, 2008). Nevertheless, the importance of testosterone for expression of male secondary sexual characters is unclear in many species and has been questioned (Owens & Short 1995; Sheldon & Verhulst 1996).

The link between expression of sexual signals and physiological state, including redox state, can be mediated through social interactions (Rubenstein & Hauber 2008; Safran *et al.* 2008; Vitousek *et al.* 2014). Individuals with more elaborate traits may experience more aggressive agonistic behaviour from their conspecifics (Møller 1987; Huhta & Alatalo 1993; Dale & Slagsvold 1996; Beani & Turillazzi 1999; van Dongen & Mulder 2007; Rick & Bakker 2008; Tibbetts & Izzo 2010; Linhart *et al.* 2012). Frequent aggressive encounters may entail energetic costs and be stressful for the individual, which may result in elevated oxidative burden and oxidative stress (Dowling & Simmons 2009; Costantini *et al.* 2011). Conversely, higher ornament expression may lead to increased attention from females, which may result in elevated concentrations of testosterone (Rubenstein & Hauber 2008; Safran *et al.* 2008). Given that pro-oxidant effects of androgens have been proposed (Chainy *et al.* 1997; Alonso-Alvarez *et al.* 2007, 2008), this may represent another mechanism, by which ornament expression can entail oxidative costs.

Specific physiological mechanisms of signalling honesty have been hypothesised for pigment ornaments based on melanin and carotenoids. I will discuss them below.

Redox homeostasis and melanin-based ornamentation

Melanins rank among the most common natural pigments in animals (Griffith *et al.* 2006). Although the diversity of melanin pigments is much greater, two main melanin forms are recognised in animals: eumelanin, producing black, brown or grey colouration; and pheomelanin, giving rise to yellowish or reddish tones (Galván & Solano 2016). Given their colour range it is not surprising that melanins are by far the most common pigments used in animal cryptic pigmentation. Melanin colour range, the ability of animal to synthesise them and the belief that their deposition is tightly genetically controlled resulted in an opinion that melanins are not as suitable for sexual signalling as, for example, carotenoids (Gray 1996; Badyaev & Hill 2000). Nonetheless, their use in sexual selection and condition-dependent sexual signalling has been recognised recently (Roulin 1999, 2007; Roulin *et al.* 2000; Safran & McGraw 2004; Safran *et al.* 2005; Griffith *et al.* 2006; Galván & Solano 2016).

Variation in melanin-based colouration has been linked to variation in aggressiveness, sexual activity, stress response, immune response, redox state, hormone levels and survival (Ducrest *et al.* 2008; Galván & Alonso-Alvarez 2008, 2009; Galván *et al.* 2010; Galvan & Moller 2013; Saino *et al.* 2013b, a). The covariation between melanin-based colouration and physiological and behavioural traits is believed to be mediated by pleiotropic effects of proopiomelanocortin (POMC) gene, which not only controls the synthesis of melanin pigments, but is also involved in regulation of sexual and aggressive behaviour, stress response, immune response and energetic metabolism (Ducrest *et al.* 2008; Roulin & Ducrest 2011). Given that synthesis of pheomelanin consumes an important antioxidant glutathion and its key component cystein, trade-off between allocation of cystein and glutathione to ornamentation or antioxidant defence has been put forward as a mechanisms maintaining signalling honesty of pheomelanin-based ornaments (Galván & Solano 2009).

Redox state and sexual signalling in two barn swallow subspecies with divergent signals

In **Paper 3**, we analysed associations between redox state and expression of tail streamers and pheomelanin-based ventral colouration in the European barn swallow and the North American barn swallow. We tested whether ornament expression can signal redox state and whether prospective redox state signalling may be related to ornament type or to differences in ornament preference between these two subspecies. The results showed quite a complex pattern, suggesting that relationship between tail length and redox state differs between subspecies and sexes, whereas relationship between ventral colouration and redox state is stable across subspecies but differs between sexes.

In North American males, tail length was negatively correlated with antioxidant capacity and there was also negative trend with oxidative damage, indicating lower oxidative burden in long-tailed males. This may have several explanations. First, short tail streamers are costly, for example, due to having suboptimal length for manoeuvrability (Buchanan & Evans 2000). Second, long-tailed males are of higher quality and may better cope with potential tail streamer costs (Grafen 1990). Third, given that short-tailed males have been found to have higher success in within-pair fertilisations in the North American subspecies (Safran *et al.* 2016), the relationship between redox state and tail streamers can be mediated through social interactions. This may relate to higher levels of stress hormones (Costantini *et al.* 2011) or androgens (Alonso-Alvarez *et al.* 2007, 2008) due to more frequent aggressive encounters by conspecifics or higher interest from females (Vitousek *et al.* 2014).

In European males, tail length was not related to antioxidant capacity and there was a positive trend with oxidative damage, though it lost its significance when one outlier was removed. Contrary to North American males, this suggests that long-tailed males may experience slightly higher oxidative burden or there may be no relationship in European males. This is in accord with results from **Paper 2**, suggesting that costs of male tail streamers are at best mild in our population and may only be significant in males with extremely long tails. Nevertheless, oxidative costs cannot be ruled out by a correlational study, hence we are currently analysing redox markers from samples collected from experimentally manipulated males to test such costs. Female tail length showed no relationship with redox state in any of the subspecies.

Ventral colouration was correlated with oxidative damage and the direction of this correlation was the same in both populations but differed between sexes, such that higher oxidative damage has been observed in darker males and paler females. In contrast, antioxidant capacity was no related to ventral colouration. It is highly unlikely that observed relationships between pheomelanin-based ventral colouration and oxidative stress are mediated by the proposed trade-off between allocation of cytein and glutathione in pheomelanin synthesis or antioxidant defence (Galván & Solano 2009), as ventral colouration is produced during moult at the wintering grounds, long before the breeding season, limiting the potential for such an allocation trade-off. More probable explanation is based on causal effect of ornament expression on redox state through social interactions (Vitousek *et al.* 2014). For example, differently pigmented individuals may experience different rates of aggressive encounters or sexual interest from the opposite sex or can differ in their reproductive effort (Safran *et al.* 2008; Vitousek *et al.* 2013, 2014). Pleiotropic effects of POMC genes regulating melanin synthesis and deposition (Ducrest *et al.* 2008; Roulin & Ducrest 2011) also cannot be ruled out as a possible causal mechanism underpinning the observed relationships. Whatever the causal mechanism, the differing direction of correlation between the sexes indicate sex-specific differences in trade-offs and costs associated with expression of ventral colouration. Given to the correlational nature, our study merely described the pattern and further studies using experimental manipulations are needed to elucidate underlying mechanisms and trade-offs.

Redox homeostasis and carotenoid-based ornamentation

Expression of carotenoid-based ornamentation has most often been hypothesised to be linked to redox homeostasis (von Schantz *et al.* 1999; Hartley & Kennedy 2004; Vinkler & Albrecht 2010; Garratt & Brooks 2012). Carotenoids might have been the first natural pigments on Earth and, together with melanins, they rank among the most common pigments in animal kingdom (Vershinin 1999; Griffith *et al.* 2006). Carotenoids represent a group of more than 750 natural pigments of yellow, orange or red colour that can only be synthesised by photosynthetic organisms and certain bacteria and fungi (Svensson & Wong 2011), though the ability to synthesise carotenoids has recently been found in three different arthropod groups, probably gained through lateral gene transfer from fungi (Moran & Jarvik 2010; Altincicek *et al.* 2012; Cobbs *et al.* 2013). The known carotenoids can be divided into two major types: carotenes, which contain only carbon and hydrogen and oxygen-containing xanthophylls (Svensson & Wong 2011). Lipophilic nature of carotenoids determines their localisation in lipophilic cellular compartments, such as lipid membranes (Young & Lowe 2001). The position and orientation in lipid membranes differ between carotenes and xanthophylls. Hydrophobic carotenes are hidden in the hydrophobic core, whereas more polar xanthophylls are anchored in the surface of the membranes, which allows their interactions with molecules in the aqueous compartments (e.g. ascorbic acid; Young & Lowe 2001; Krinsky & Yeum 2003).

Carotenoids have traditionally been considered to be important antioxidants due to their ability to quench free radicals *in vitro* (Krinsky & Yeum 2003; El-Agamey *et al.* 2004). A trade-off in allocation of carotenoids between ornamentation and antioxidant defence has therefore been proposed as a possible mechanism maintaining signalling honesty (the carotenoid allocation trade-off hypothesis; Lozano 1994; Olson & Owens 1998; von Schantz *et al.* 1999).

The antioxidant function of carotenoids *in vivo* has recently been questioned (Hartley & Kennedy 2004; Costantini & Møller 2008; Koch *et al.* 2018), however, resulting in the emergence of alternative hypotheses. The carotenoid protection hypothesis (Hartley & Kennedy 2004) is based on the observation that RS-induced oxidation and isomerisation cause carotenoids to lose their colour (Rao & Rao 2007). Hartley & Kennedy (2004) have therefore proposed that colourful carotenoids can signal the capacity of other antioxidant mechanisms, without being important antioxidants themselves. The RS-induced oxidation can also result in carotenoid cleavage and further oxidation, giving rise to toxic metabolites, such as apocarotenals (Siems *et al.* 2000, 2002; Alija *et al.* 2005). This inspired the hypothesis that high carotenoid concentrations necessary for intense pigmentation can be harmful, thus posing a direct physiological handicap (the carotenoid maintenance handicap hypothesis; Zahavi 2007; Vinkler & Albrecht 2010).

Recently, several alternative hypotheses have been put forward, proposing that expression of carotenoid-based sexual signals need not to be costly (Hill & Johnson 2012; Johnson & Hill 2013). Instead, these hypotheses proposed that carotenoid uptake and metabolism depend on metabolic or signalling pathways involved in mitochondrial regulation and redox homeostasis maintenance. More specifically, expression of carotenoid-based ornaments have been linked to the vitamin A-mediated redox signalling (Hill & Johnson 2012) and the regulation of inner mitochondrial membrane potential (Johnson & Hill 2013).

Although oxidative stress has frequently been hypothesised to maintain carotenoid-based signalling honesty, the most fundamental assumption of adverse effect of oxidative stress on ornament expression has rarely been tested directly using controlled experimental manipulation of redox state (Isaksson & Andersson 2008; Hõrak *et al.* 2010; Alonso-Alvarez & Galván 2011; Giraudeau *et al.* 2015) and even more rarely demonstrated (Alonso-Alvarez & Galván 2011). Most commonly, experimental activation of immune system has been performed, complemented with oxidative state assessment in some, but not all, studies. Reduced ornament expression has usually been interpreted as an indirect support for the role of redox homeostasis in signalling honesty (Faivre *et al.* 2003; Alonso-Alvarez *et al.* 2004). The effect of immune challenge on ornament expression could be mediated by mechanisms different from redox homeostasis maintenance. More recently, studies using chemical substances that either induce oxidative challenge through generation of RS (e.g. diquat or paraquat generating superoxide radical through redox cycling) or reduce antioxidant capacity (e.g. buthionine sulfoximine inhibiting synthesis of important antioxidant glutathione) have been carried out to test the adverse effect of oxidative stress more directly. None of these studies found an effect of either oxidative challenge or reduced antioxidant capacity on carotenoid-based ornamentation in adult males (Isaksson & Andersson 2008; Hõrak *et al.* 2010; Giraudeau *et al.* 2015). The only study reporting significant adverse effect of oxidative challenge on carotenid-based ornament expression has been carried out on young red-legged partridges (*Alectoris rufa*) (Alonso-Alvarez & Galván 2011). There are at least two possible explanations for such discrepant result between these studies. First, the three studies reporting no effect used bird species with plumage ornaments, whereas the only study finding significant effect measured pigmented bare parts of the skin. It is therefore possible that more dynamic skin ornaments, in which carotenoids need to be continuously replenished during the breeding season, are more sensitive to oxidative stress, compared to semi-static plumage ornaments that are usually grown several weeks or months before the breeding season (Alonso-Alvarez & Galván 2011). Second possible explanation could be a difference in trade-off solutions between developing and adult birds, with the latter investing more in reproduction than in self-maintenance (Alonso-Alvarez & Galván 2011).

The role of redox homeostasis in carotenoid-based ornament expression in the zebra finch

Together with my collaborators, I therefore tested the effect of increased oxidative burden on dynamic carotenoid-based beak pigmentation in adult zebra finch males (**Paper 4**). The intense red colouration of male beaks has been reported to be a trait preferred by females in this species (Simons & Verhulst 2011; but see Forstmeier & Birkhead 2004; Wang *et al.* 2017) and has been reported to be positively correlated with immune response and antioxidant capacity (Simons *et al.* 2012b). The condition-dependence of this sexual trait has also been supported by higher survival observed in individuals with redder beaks (Simons *et al.* 2012a).

We used diquat dibromide administered in drinking water to expose males to oxidative challenge. Diquat dibromide, a redox-cycling agent known to generate superoxide radical *in vivo*, has recently been recognised as a convenient oxidative stress inducer in ecological studies (Galván & Alonso-Alvarez 2009; Hõrak & Cohen 2010; Alonso-Alvarez & Galván 2011), as well as in laboratory models of Parkinson's disease (Slaughter *et al.* 2002; Drechsel & Patel 2009; Fischer & Glass 2010). Diquat exposure resulted in a decrease in both beak redness and carotenoids circulating in blood plasma, demonstrating the adverse effect of oxidative challenge on ornament expression and carotenoid concentrations. This result provides direct experimental support for the hypotheses proposing redox homeostasis maintenance to be the mechanism ensuring honesty of carotenoid-based signalling. No loss in body mass was observed in our diquat-exposed birds, indicating that the reduced ornamentation was not a consequence of a reduced food intake or impaired total intestinal absorption, but rather an effect of increased oxidative burden.

We demonstrated that oxidative challenge affects ornament expression in adult birds, suggesting that discrepancies between previous studies (Isaksson & Andersson 2008; Hõrak *et al.* 2010; Alonso-Alvarez & Galván 2011) may not be due to different developmental stages of experimental individuals used in those studies. To date, adverse effect of oxidative stress has only been reported in bare parts (Alonso-Alvarez & Galván 2011), including our study. This suggests that the contrasting results of different studies could rather stem from differences in sensitivity to oxidative stress between semi-static plumage ornaments and more dynamic bare parts, which can change colour over a period of just a few weeks (Blount *et al.* 2003; Faivre *et al.* 2003).

In our study, we used a measure of hydrophilic antioxidant capacity traditionally used in ecological studies and also a novel measure of lipophilic antioxidant capacity derived from medical research (Yoshida *et al.* 2007, 2013). Oxidative challenge induced increase in both hydrophilic and lipophilic antioxidant capacity, resulting in only marginally non-significant increase in oxidative damage. This indicates adaptive upregulation of antioxidants in order to maintain stable redox homeostasis (Costantini & Verhulst 2009). Carotenoid supplementation reduced lipophilic, but not hydrophilic, antioxidant capacity in males experiencing both low and high oxidative burden. It is important to note that the measure of lipophilic antioxidant capacity used in our study measures *in vivo* RS-scavenging activity mediated by hydrogen donation (e.g. in tocopherols or coenzyme Q) but not radical addition, which is the most probable mechanism of carotenoid antioxidant action (Burton & Ingold 1984; El-Agamey *et al.* 2004). The reduced lipophilic antioxidant capacity following carotenoid supplementation therefore indicates down-regulation of other antioxidants in order to maintain redox homeostasis (Selman *et al.* 2006, 2008), thereby providing an indirect evidence for carotenoid antioxidant function *in vivo*. The reduction in lipophilic, but not hydrophilic, antioxidant capacity suggests that the lack of evidence for carotenoid antioxidant function *in vivo* in ecological studies could stem from the improper methods being used for assessment of antioxidant activity of lipophilic carotenoids. Our data suggest that future studies should also employ methods measuring antioxidant capacity in lipophilic compartments, when carotenoid antioxidant function is to be assessed.

Antioxidant properties of carotenoids are inconsistent with the carotenoid maintenance handicap hypothesis, which assumes carotenoids to be pro-oxidant in high concentrations. In contrast, carotenoids showed no pro-oxidant effect in our study despite supplementing birds with very high doses that were at the upper limit of that which zebra finches can absorb from the diet (Alonso-Alvarez *et al.* 2004). Moreover, antioxidant capacity is inconsistent with the pure version of carotenoid protection hypothesis, assuming no antioxidant properties of carotenoids and, instead, proposing that carotenoids needs to be protected by other antioxidants from oxidation to prevent loss of their colouration (Hartley & Kennedy 2004).

Carotenoid antioxidant function is in accord with the carotenoid allocation trade-off hypothesis, which assumes carotenoids to be traded off between their use in ornamentation and antioxidant defence. Our results are also consistent with the recent hypothesis assuming that metabolism of red carotenoids takes place in the inner mitochondrial membrane, where it shares metabolic pathways with another antioxidant coenzyme Q, thereby signalling redox state and mitochondrial functionality in a cost-free manner (Johnson & Hill 2013). We consider the latter hypothesis less likely, however, at least in zebra finches, as it assumes red carotenoids to be produced in the liver, while beak has been proposed as the site of their production in zebra finches (McGraw & Toomey 2010). Moreover, cytochrome P450 2J2 that has been identified as a catalyst of red carotenoid synthesis is probably located in the endoplasmic reticulum and not in mitochondria (Wang *et al.* 2014).

Carotenoid-based ornamentation and sperm resistance to oxidative damage

In species where female copulates with more than one male (i.e. polyandrous species or monogamous species with extra-pair copulations), sexual selection continues after copulation. In such species, sperm of two or more males compete for fertilisation of ova in a process known as sperm competition (Parker 1970, 1998). Hence, male's reproductive success is determined by an interplay between both pre- and post-copulatory phases of sexual selection. Despite many recent studies focusing on interaction between these phases, there have been no conclusive results explaining how the two processes integrate, with a positive relationship being observed in some studies (Matthews *et al.* 1997; Evans *et al.* 2003; Peters *et al.* 2004; Hosken *et al.* 2008; Polak & Simmons 2009; Helfenstein *et al.* 2010) and a negative relationship (Liljedal *et al.* 1999; Danielsson 2001; Simmons & Emlen 2006; Demary & Lewis 2007; Evans 2010; Buzatto *et al.* 2015), or no relationship, in others (Birkhead & Fletcher 1995; Devigili *et al.* 2013; Mautz *et al.* 2013).

The differing directions of correlation between studies suggest a possible trade-off between pre- and post-copulatory traits. In traits that are traded off against each other, the direction of correlation is known to depend on resource availability and can therefore differ in different contexts (Van Noordwijk & de Jong 1986; Catoni *et al.* 2008). Trade-off between pre- and post-copulatory traits is predicted by the sperm competition theory, based on an assumption that the amount of resources that can be invested in either trait type is limited (Parker 1998).

Positive correlation is predicted by the phenotype-linked fertility hypothesis (PLFH) proposing that secondary sexual characters can signal male fertility and sperm quality (Sheldon 1994). It is based on an assumption that expression of both pre- and post-copulatory traits is affected by individual quality and environmental conditions in a similar way, resulting in a positive correlation. Oxidative stress has later been put forward as a factor that can maintain honesty of such signalling in carotenoid-based ornaments (the redox-based PLFH; Blount *et al.* 2001). According to this hypothesis, more ornamented males have high-quality antioxidant system that better protects their spermatozoa to oxidative stress. By choosing such a male, female reduces the probability of mate infertility (Sheldon 1994) and obtains an indirect benefit in the form of superior DNA integrity for her offspring (Blount *et al.* 2001).

The redox-based PLFH assumes both spermatozoa and carotenoid-based ornaments to be sensitive to oxidative stress. The sensitivity of spermatozoa to oxidative stress is well known. To ensure high motility, spermatozoa are metabolically active and rich in polyunsaturated fatty acids that are more prone to oxidative damage than monounsaturated and saturated fatty acids (Tremellen 2008). Moreover, spermatozoa probably lack the capability to repair DNA (Gonzalez-Marin *et al.* 2012), and most enzymatic and non-enzymatic antioxidants are lost with the cytoplasm during sperm elongation (Henkel 2011). In contrast, the evidence for the adverse effect oxidative burden on carotenoid-based ornamentation has been inconclusive (Isaksson & Andersson 2008; Hõrak *et al.* 2010; Alonso-Alvarez & Galván 2011). Nevertheless, we have supported this assumption in our model species, the zebra finch, showing that male carotenoid-based beak pigmentation is sensitive to oxidative challenge (**Paper 4**). The redox-based PLFH also gained indirect support in a previous study on great tit (*Parus major*) males subject to experimental brood size manipulation (Helfenstein *et al.* 2010). In that study, more colourful males showed higher resistance to the adverse effects of experimental brood enlargement on sperm motility, velocity and oxidative damage, which has been interpreted as higher sperm resistance to oxidative stress from increased workload.

In **Paper 5**, we measured beak colouration and various morphological and functional sperm traits in male zebra finches in order to tested PLFH. We found no correlation between ornamentation and sperm mid-piece length (the middle part of the sperm containing mitochondria), velocity or abnormal sperm proportion. The only traits correlated to ornament expression were total sperm length and flagellum length (the sum of mid-piece length and tail length, i.e. not including head length). Total sperm length is potentially important trait that has been reported to predict fertilization success in zebra finches (Bennison *et al.* 2015). Nevertheless, the importance of sperm length was probably related to higher sperm velocity in longer spermatozoa in that study (Bennison *et al.* 2015, 2016). Sperm velocity was not correlated with either total sperm length or ornament expression, hence our results provided only little support for PLFH.

Subsequently, we used oxidative challenge induced by diquat dibromide combined with manipulation of carotenoid availability to directly test both the redox-based PLFH and a possible trade-off between expression of carotenoid-based beak pigmentation and sperm resistance to oxidative damage (**Paper 5**). Manipulation of stress level and resource availability is essential when testing trade-offs as any direction between traits that are traded off against each other depends on these factors, with a negative correlation being most likely to be revealed under stressful conditions when resources are limited (Van Noordwijk & de Jong 1986; Catoni *et al.* 2008). Our results supported a trade-off between carotenoid-based ornamentation and sperm resistance to oxidative challenge. This was evidenced by males with initially (pre-experiment) more intense beak colouration enjoying increase in sperm velocity under control conditions, but suffering a decline in sperm velocity following oxidative challenge. Interestingly, the change in sperm velocity was not related to the change in beak colouration, indicating that there is no direct resource allocation trade-off between these two traits. Instead, our results suggest a long-term trade-off between carotenoid-based ornament expression and sperm resistance to oxidative damage. This could imply a negative carry-over effect of investment in ornamentation on sperm resistance and/or life history differences among less and more ornamented males. Indeed, it has been shown that early-life stress may result in lower investment in sexual trait expression when adult (DeKogel & Prijs 1996; Ohlsson *et al.* 2002; Naguib & Nemitz 2007) and that a mismatch between early-life and adult environments may have deleterious consequences for individual fitness (Costantini *et al.* 2014; but see Briga *et al.* 2017). Accordingly, more ornamented males in our study might be adapted to low-stress environments and invest less in antioxidant mechanisms, thereby suffering a decline in sperm function under oxidative challenge.

Lower sperm resistance to oxidative challenge in more colourful males observed in our study contrasts with results of a previous study on great tits using experimental brood size manipulation (Helfenstein *et al.* 2010). It cannot be ruled out, however, that the adverse effect of brood size manipulation on sperm traits in that study was mediated by other mechanisms unrelated to oxidative stress. Alternatively, such discrepancy may suggest differences between the costs and signalling function of semi-static plumage colouration and more dynamic beak ornament. Such differences are supported by previous studies, showing an adverse effect of oxidative challenge on dynamic beak (**Paper 4**) and skin ornamentation (Alonso-Alvarez & Galván 2011), but no effect on semi-static plumage colouration (Isaksson & Andersson 2008; Hõrak *et al.* 2010).

SUMMARY OF MAIN FINDINGS

- In contrast to previous studies in the European barn swallow, tail streamer length does not seem to play an important role in within-pair and extra-pair fertilisation success in our population.
- Male age and age of his social female partner were the main predictors of extra-pair and within-pair fertilisation success, respectively.
- In two populations of European barn swallows, individual tail length increased with age in a non-linear manner, such that higher increase occurred between first and second year of adult life with subsequent slowdown and levelling-off in late age.
- We found selective disappearance of short-tailed males and extremely long-tailed males, with the males having tail streamers 5 mm longer that population median being the best survivors.
- We found no evidence for senescence in tail streamer length.
- Experimental manipulation of tail streamer length had no effect on survival or tail streamer length in the following year, suggesting low costliness of this sexual trait.
- We found a negative correlation between plasma oxidative stress and tail streamer length in North American barn swallow males and a positive trend (non-significant when one outlier was removed) in European barn swallow males.
- The direction of correlation between plasma oxidative stress and intensity of pheomelanin-based ventral colouration did not differ between subspecies, but differed between sexes, being positive in males and negative in females.
- Oxidative challenge showed an adverse effect on carotenoid-based beak pigmentation in zebra finch males, evidenced as a decrease in its redness.
- We found an indirect evidence for *in vivo* antioxidant function of carotenoids
- We demonstrated that the lack of evidence for *in vivo* antioxidant function of carotenoids probably stem from using inappropriate measures (antioxidant capacity in aqueous media) when assessing antioxidant activity of lipophilic carotenoids.

- Expression of carotenoid-based beak pigmentation of zebra finch males has only been positively correlated with total sperm length and flagellum length, but not with sperm velocity or abnormal sperm proportion. This provides only weak support for phenotype-linked fertility hypothesis.
- Males with more intense beak colouration experienced increase in sperm velocity under control treatment, decrease in this functional sperm trait following oxidative challenge. This suggests trade-off between expression of carotenoid-based ornament and sperm resistance to oxidative challenge.

CONCLUSION

In this study, we attempted to improve our understanding of signalling function and mechanisms ensuring signalling honesty of condition-dependent sexual ornamentation. During last three decades, the length of elongated tail streamer in European barn swallow males have become a textbook example of sexually selected trait (Møller 1988, 1994b; Møller *et al.* 1998). The important role of this ornamental trait in mate-choice a reproductive success have been reported by many studies (reviewed in Romano *et al.* 2017). The results from our population of the European barn swallow, however, challenge the general importance of tail streamer length for within-pair and extra-pair fertilisation success (**Paper 1**). Although we cannot rule out the importance of tail length in social mate-choice (Wilkins *et al.* 2016), the discrepancy between our results and the results of previous studies may suggest between-population differences in the role of this sexual trait at least in some aspects of mate-choice. Such between-population differences in traits associated with mating success are not rare (Dale *et al.* 1999; Safran & McGraw 2004; Safran *et al.* 2005; Kleven *et al.* 2006; Galván & Moreno 2009; Eikenaar *et al.* 2011; Whittingham & Dunn 2014) and it has been even pointed out that differences seem to be observed more frequently than similarities between populations of the same species (Whittingham & Dunn 2014). Male age was the most important predictor of the extra-pair fertilisation success in our population, which is in accordance with the results of a recent meta-analysis showing that male age is more important than ornamentation in extra-pair mating in general (Hsu *et al.* 2015).

Within-individual increase in tail streamer length and selective disappearance of short-tailed males observed in our population support viability signalling function of tail streamers (**Paper 2**). Previous studies provided mixed results, however, reporting either positive (Møller 1991, 1994a) or negative (Møller & Szep 2002; Balbontín *et al.* 2011; Romano *et al.* 2017) correlation between tail length and survival. This may suggest between-population differences in a signalling content, for example, due to differences in resource availability (Van Noordwijk & de Jong 1986) or intensity of female choice (Kokko *et al.* 2002). Age-related increase in ornament expression may be viewed as signalling of proven viability (Manning 1985) and is in agreement with life-history theory (Kokko 1998), which predicts increasing investment in sexual signalling with decreasing reproductive value. Given that physiological decline in old age indicative of senescence has previously been documented in the barn swallow (Møller & De Lope 1999; Saino *et al.* 2003), even the plateauing of the increase in tail length in the old age can be viewed as increasing investment in sexual signalling over the entire individual lifespan (Evans *et al.* 2011).

Surprisingly, we found little support for tail streamers entailing significant costs, as evidenced by no effect of tail length manipulation on survival or tail streamer length in the following year (**Paper 2**). The only indication of tail streamer costs come from the correlational analysis, suggesting lower survival probability in males with extremely long tails. This suggests that handicap principle may not be the major mechanism mediating condition-dependence of tail streamers and alternative cost-free mechanisms may be involved (Hill 2011; Emlen *et al.* 2012).

We further analysed the associations of ornament expression with redox state and tested the role of redox homeostasis in maintenance of signalling honesty. Our results from two barn swallow subspecies with divergent female preference for tail streamers and ventral colouration suggest that association with oxidative stress may be driven by trait preference in some traits and by ornament type in other traits (**Paper 3**). Observed differences between sexes indicate different oxidative-stress related costs and trade-offs in males and females.

Our experiment on zebra finch males exposed to controlled free radical attack demonstrated, for the first time in adult animals, the adverse effect of elevated oxidative burden on carotenoid-based ornament expression (**Paper 4**). Given that such an adverse effect of oxidative burden on ornament expression is a fundamental assumption of hypotheses considering oxidative stress to be the mechanism maintaining carotenoid-based signalling honesty (von Schantz *et al.* 1999; Hartley & Kennedy 2004; Alonso-Alvarez *et al.* 2007; Vinkler & Albrecht 2010; Hill & Johnson 2012), our results support plausibility of such hypotheses. Our experiment also provided support for carotenoid antioxidant function *in vivo*, which is inconsistent with hypotheses assuming carotenoids being either neutral (Hartley & Kennedy 2004) or pro-oxidant (Vinkler & Albrecht 2010) with relation to redox homeostasis. In addition, we suggest that future studies assessing carotenoid antioxidant function should use assays and markers that quantify antioxidant capacity in lipophilic cellular compartments.

We found no firm support for phenotype-linked fertility hypothesis (**Paper 5**), predicting that carotenoid-based ornament expression signals sperm function and male fertility (Sheldon 1994; Blount *et al.* 2001). Instead, our data indicate an existence of a trade-off between ornament expression and sperm resistance to oxidative challenge. The data also suggest that this trade-offs may involve a long-term negative carry-over effect of investment in ornament expression on oxidative sperm resistance. Alternatively, this trade-off may be related to divergent individual life histories (Costantini *et al.* 2014), with males investing more in ornamentation being adapted for low-stress environments and *vice versa*.

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Extra-pair paternity patterns in European barn swallows *Hirundo rustica rustica* are best explained by male and female age and not male ornamentation

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Keywords: *extra-pair fertilisations, within-pair paternity, EPP, sexual ornamentation, sexual selection, tail streamer length*

Running head: age effects on extra-pair paternity

Word count:7517; *table count*: 3; *figure count*: 2

ABSTRACT

Adaptive explanations for the evolution of extra-pair paternity (EPP) in birds often assume cuckolding males to be more attractive than cuckolded males. Several studies have confirmed that either male sexual ornamentation is associated with EPP or that phenotypes of cuckolded and cuckolding males differ. Expression of male ornamentation may increase with age, however, and a recent meta-analysis has identified age as the most important factor differentiating cuckolding and cuckolded males. The age of social female partner may also affect EPP, though this has received little attention. Here, we examine the relationship between male and female age with both within-pair and extra-pair fertilisation success in barn swallows (*Hirundo rustica rustica*). We also compared phenotypes of cuckolding and cuckolded males from 76 nests. Our results reveal that, whereas extra-pair fertilisation success increased with male age, the only predictor of the likelihood of a male being cuckolded was the age of his social partner. In contrast, several male ornaments (including tail streamer length) did not affect EPP patterns when the age of males and females were taken into account, despite cuckolding males being structurally larger than cuckolded males. Our data provide little support for the idea that extra-pair mate choice in barn swallows is ornament driven, indicating either a non-adaptive scenario for EPP evolution, with older, larger and more experienced males better able to coerce females into copulation, or extra-pair mate choice based on other than absolute mate-choice criteria associated with the expression of secondary male ornamentation.

Significance statement

We analysed patterns of extra-pair paternity in barn swallows. Derived from observation of 160 nests, our results seem to contradict some previous studies that have identified several ornamental traits as being associated with extra-pair and within-pair paternity in this iconic model taxon of sexual selection. In particular, tail streamer length was certainly not associated with a male's ability to obtain an extra-pair partner. Similarly, within-pair paternity loss was also not associated with male ornamentation. Pairwise comparisons of cuckolded and cuckolding males, involving 76 mixed paternity nests, also supported the conclusion that male ornamentation does not play a role in determining male fertilisation success. The probability of obtaining an extra-pair partner only increased with increasing age of males, while within-pair paternity was only associated with the age of their social partners. Our data provide little support for the idea that extra-pair mate choice in barn swallows is ornament driven.

INTRODUCTION

Mate choice is an important mechanism for increasing individual fitness in sexually reproducing organisms (Darwin 1871; Andersson 1994). Choice of partner may be pre-copulatory or post-copulatory, via sexual promiscuity and subsequent sperm competition or through cryptic female choice (Birkhead 2010). Over recent decades, promiscuous behaviour and multiple male mating has been identified in many taxa, including mammals (Bryja et al. 2008), reptiles (Uller and Olsson 2008) and fishes (Coleman and Jones 2011); however, the major research focus has been on extra-pair paternity (EPP) in socially monogamous birds (Griffith et al. 2002). The drivers behind evolution of promiscuous behaviour in birds remain unclear, despite intense research over the last three decades. While male interest in attending extra-pair copulations (EPC) appears to be intuitive (Forstmeier et al. 2014), female motivation to engage in EPC are much less obvious as evidence for benefits from EPP for females (as proposed by a set of "adaptive hypotheses") remains equivocal (Arnqvist and Kirkpatrick 2005; Albrecht et al. 2006; Akçay and Roughgarden 2007; Eliassen and Kokko 2008). This has led to the emergence of non-adaptive models assuming that female promiscuity may be maintained, even in the absence of any direct or indirect fitness benefit to females (reviewed in Forstmeier et al. 2014).

Where sexual promiscuity occurs in socially monogamous birds, male fitness may be determined by his ability to sire offspring in his own nest (within-pair fertilisation) and/or the ability to sire offspring at the expense of other males in the population (extra-pair fertilisation; Webster et al. 1995). Within-population studies have identified a range of phenotypic traits associated with male within-pair and extra-pair fertilisation success, including plumage colouration and/or morphological traits (e.g. Griffith et al. 2002; Bitton et al. 2007; Cleasby and Nakagawa 2012). Higher fertilisation success in more ornamented males could be interpreted as resulting from female-driven extra-pair mate choice. This kind of evidence, however, is often confounded by other variables that are often overlooked in paternity studies. As an example, values for many phenotypic traits, including male ornamentation, increase with age (Freeman-Gallant et al. 2010) and it has became apparent that some of the reported correlative relationships between male EPP success and male ornamentation could be confounded by male age effects (Kleven et al. 2006b; Freeman-Gallant et al. 2010).

Age itself may be a factor reflecting male genetic quality and, as such, may be targeted by female choice. It has been suggested that females may prefer mating with older males as age is proof of high survivorship (Kokko and Lindström 1996) or that older males may provide better signals of quality to females (Proulx et al. 2002). At the same time, however, age (and body size) could be associated with individual male experience and/or ability to coerce females into mating (Westneat and Stewart 2003). Using available data, a recent meta-analysis (Hsu et al. 2015) has shown that male age, but not male ornamentation, was the main trait differentiating between cuckolded and cuckolding males across bird populations. According to the authors, this seemed to contradict adaptive models explaining the evolution of EPC in birds as a result of indirect benefits that increase female fitness and opened space for alternative non-adaptive explanations (see also Forstmeier et al. 2014).

EPP research typically focuses on male traits, yet several studies have suggested that female characteristics may also influence variation in EPP (e.g. Grunst and Grunst 2014; Moreno et al. 2015). The relationship between female age and EPP, however, has been given only a little attention and the results of the few relevant studies (Stutchbury et al. 1997; Ramos et al. 2014; Moreno et al. 2015) are controversial. In addition, pair age compatibility could also affect the incidence of cuckoldry in avian nests (Dietrich et al. 2004; Bouwman and Komdeur 2005; Ramos et al. 2014).

Here, we use data from an extensively studied (Petrželková et al. 2015; Kreisinger et al. 2015; Vitousek et al. 2016) promiscuous population of the European barn swallow, *Hirundo* *rustica rustica*, to determine traits associated with within-pair and extra-pair mating. Barn swallows are small, socially monogamous migratory passerines commonly used in studies of sexual selection (e.g. Møller 1994; Saino et al. 1997; Safran and McGraw 2004; Vortman et al. 2011; Hasegawa et al. 2012). A range of ornamentation traits have been identified as being associated with social mate choice in barn swallow populations, including (1) *tail streamer length* (Møller 1994; Saino et al. 1997); (2) *ventral colouration* (Safran and McGraw 2004; Safran et al. 2005); (3) *white tail spot size* (Kose et al. 1999; Hasegawa et al. 2010), and (4) *throat patch colouration and size* (Hasegawa et al. 2010; Hasegawa et al. 2012). Whether similar traits play a role in extra-pair mating, however, is less well documented (but see Saino et al. 1997; Kleven et al. 2006a; Lifjeld et al. 2011). Also, surprisingly little is known about age effects on social and extra-pair mating in this species (Lifjeld et al. 2011).

As detailed data on age of individuals was available in our study population, it was possible to separate effects of male ornamentation and age in our analysis and evaluate how the age of a male's social mate affected the likelihood of cuckoldry. The main objectives of the study were as follows: (1) to evaluate the effect of male age and ornament on two potential components of male fitness in a typical promiscuous song bird population, i.e. within-pair and extra-pair fertilisation success (e.g. Webster et al. 1995; Albrecht et al. 2007; Webster et al. 2007); (2) to directly compare phenotypes of cuckolding and cuckolded males, focusing on ornament, age and body size; and (3) to test for the possibility that female age is associated with variation in within-pair paternity loss in males. The results should help clarify the mechanisms involved in extra-pair mating in barn swallows.

METHODS

Study area and general field procedure

The field study was carried out from 2010 to 2013 at two isolated farms in the Třeboňsko Protected Landscape Area (Czech Republic), Šaloun at Lomnice nad Lužnicí (49°4'7.762"N, 14°42'36.521"E) and Hamr at Lužnice (49°3'25.288"N, 14°46'10.82"E). Most of the birds breed in colonies, with just a few solitary breeding pairs not directly associated with the homestead. Adults were systematically captured with mist nets placed across window and door openings in rural buildings on several occasions during the breeding season. Each individual was marked with an aluminium ring (National Museum of Prague) and a unique combination of coloured plastic rings (AVINET) that allowed identification in the field. Social pairs were assigned by observing nest defence and repeated feeding of nestlings (or incubating eggs in the case of females). Each adult bird was measured for morphological variables (tarsus length and tail streamer length) at the time of first capture. Feather samples were collected from two body regions (throat and ventral plumage; more than ten feathers per region) for subsequent colour analysis and photographs (with a scale) were taken of the white spots on the tail. We also sampled a small amount of blood (~ 20 μL) by brachial venepuncture for parentage analysis. All nests were found during the egg-laying period and subsequently checked at three-day intervals. Clutch initiation date was estimated by observing the appearance of the first egg laid in the nest or was deduced based on the assumption that females laid one egg each day. Nestlings were ringed at the age of nine days and a blood sample taken from the brachial vein. All blood samples were stored in 96% ethanol at -20°C until DNA extraction.

Between 2010 and 2013, barn swallows initiated 160 first breeding attempts on our study plot. We used first seasonal breeding attempts only as intensity of sexual selection is highest during this period (Romano et al. 2017) and not all pairs raise two broods per year. For pairwise comparisons, we included 76 nests with mixed paternity and compared the cuckolded (social) male with the corresponding cuckolding (extra-pair) male/males directly. We knew the exact age of birds if they were ringed as nestlings in the study area (55 males and 34 females;

intense ringing of barn swallow population started in 2008). Individuals that were captured unringed and had not been captured as adults in the previous year were assumed as 1-year old birds originating from elsewhere (133 males and 72 females; see Møller 1994; Møller et al. 2003; Costanzo et al. 2017). Barn swallows show high breeding philopatry and fidelity with only exceptional cases of moving to another site within or between seasons (Møller 1994; Møller et al. 2003; Saino et al. 2013; Costanzo et al. 2017) and a high return rate (Lifjeld et al. 2011). Thus, it is reasonable to assume that new individuals in the population are first-time breeders (Lifjeld et al. 2011; Saino et al. 2013; Costanzo et al. 2017). These estimates represent minimum ages and we included these individuals into the analysis as many of them bred in multiple years, which allowed us to use these birds in assessing age-related effects (Bowers et al. 2015). The age of barn swallows ranged between 1 and 4 years. In our study area, breeding colonies were settled in isolated farms and thus it is unlikely that males were siring offspring in neighbouring colonies.

Measurement of tail spot area and colour analysis

Photographs of the white spots on the five tail feathers from the right side of the body were taken using a Nikon D40 digital camera with a millimetre scale. The area of the white tail spots was analysed from the photographs with ImageJ software, using the freehand selection function to encircle each spot.

Feather colouration was analysed using an AvaSpec 2048 reflectance spectrometer with an AvaLight-XE light source (Avantes, Netherlands). The spectrometer sensing probe was equipped with a metal adapter that shielded the measured area from ambient light and held the probe at a constant distance of 3.5 millimetres above the sample. At least ten feathers were arranged on a white paper index card in order to achieve a layer equivalent to the actual ordering of feathers on the body. Each sample was measured three times at the distal part of the feather with the probe held perpendicular. The spectrometer was calibrated against a WS-2 white standard (Avantes, Netherlands) and absolute dark after measuring eight samples. Reflectance data were analysed using the R software v. 3.1.2 (R Core Team 2014) and the pavo package (Maia et al. 2013). The three measurements from each sample were averaged and smoothed by a span of 0.25. Subsequently, the colour was analysed using avian visual model based on relative stimulation of four photoreceptor types followed by projection to the tetrahedral colour space—a method that provides the most biologically relevant quantification of how colour is perceived by a receiver (Goldsmith 1990; Endler and Mielke 2005; Stoddard and Prum 2008). The spectral sensitivity of blue tit (*Cyanistes caeruleus*) implemented in the pavo package was adopted for visual modelling (Costanzo et al. 2017) with standard daylight (D65) being used as an illuminant. Modelling in the avian tetrahedral colour space produces three colour metrics representing RGB (θ) and UV(φ) components of hue and saturation (achieved chroma; r_A). Previous analysis has indicated that all three metrics are heritable in ventral plumage of barn swallows (Hubbard et al. 2015). We only used ventral θ in our study, because all three metrics were highly intercorrelated in ventral region (θ vs. φ: $r = 0.86$; θ vs. r_A: $r = 0.96$, φ vs. r_A: $r = 0.83$). By contrast, we used both throat θ and φ, as they were independent of each other ($r = -0.11$), while both were moderately correlated to r_A (θ: $r = 0.60$; φ: $r = 0.65$). All ornament measures were performed blind with respect to the outcome of parentage assignment (see below) and/or knowledge on individual age.

Parentage assignment

DNA was extracted from blood and tissue samples using the DNeasy® Blood & Tissue kit (Qiagen). All individuals were genotyped at six highly polymorphic microsatellite autosomal loci previously developed for barn swallows (Online Resource Table S1). The combined exclusion probability for the marker set was higher than 0.9999. For a detailed description of the methods (PCR conditions, genotype scoring, binning and genotyping errors), see Petrželková et al. 2015.

Parentage assignment was undertaken using Cervus version 3.0.3 (Kalinowski et al. 2007) and Colony software version 2.0 (Wang 2004; Jones and Wang 2010). As brood parasitism and quasi-parasitism was previously detected in our study population (Petrželková et al. 2015), we had to consider this fact in parentage analysis. To undertake paternity exclusions, therefore, we defined a nestling with more than one mismatch with a social male/female as an extra-pair/parasitic young in order to avoid false exclusions caused by null alleles at single loci (Dakin and Avise 2004). For detailed methods of paternity and maternity assignment, see Petrželková et al. (2015). Using these methods, we successfully obtained genotypes of all males and females and 694 young from 160 nests. Genetic fathers were identified for 688 young (99.1%).

Statistical analysis

Statistical analysis was undertaken using the R software package v. 3.4.0 (R Core Team 2017). There was no association between male extra-pair mating success and his propensity of being cuckolded, the frequency of males able to obtain extra-pair paternity being similar in those that lost paternity and those who sired all offspring in their own nest (Chi-square test, $\chi^2 = 2.03$, df = 1, p = 0.15). Subsequently, we evaluated the effect of explanatory variables (tarsus length, tail streamer length, throat θ and φ, ventral θ, area of white tail spots, clutch initiation date, male and female age) on male extra-pair and within-pair fertilisation success separately. As a number of males (n = 33) provided more than one data point to the data set (range 2 to 4), we used the glmer function within the lme4 package for R (Bates et al. 2015) for fitting generalised linear mixed-effect models, with male identity included as a random effect. To evaluate male propensity of being cuckolded, and his ability to obtain extra-pair mate, within-pair and extra-pair success were coded as binary dependent variables (i.e. 0 = no extra-pair offspring sired outside the nest or no extra-pair offspring detected in the male's nest, respectively; 1 = at least one extra-pair offspring sired or at least one extra-pair offspring detected in the male's nest, respectively). As extra-pair offspring tend to be detected more often in larger broods, clutch size was included as a covariate when analysing within-pair paternity. We also evaluated male age x female age interaction as age incompatibility may also play a role in patterns of within-pair paternity (Dietrich et al. 2004; Bouwman and Komdeur 2005; Ramos et al. 2014). Continuous explanatory variables were scaled (z-transformed) before analysis to improve the convergence of complex initial models. The same procedure was used to identify traits that differed between cuckolded and cuckolding males, i.e. male status (cuckolded or cuckolding) was used as a binary response variable and a set of traits were used as explanatory variables. Because the aim was to perform a pairwise comparison, nest identity was incorporated into the model as a random effect along with social male identity. To obtain *p*-values, we performed likelihood ratio tests comparing models with and without specific fixed effects. Final models were identified through backward elimination of unimportant effects based on the drop1 function in R and associated changes in deviance expressed as χ^2. We estimated the variance explained by the fixed effects of our mixed-effects models as marginal R^2-values, using the r.squaredGLMM function within the MuMIn package for R (Bartoń 2016). To estimate potential multicollinearity among model predictors, variance inflation factor (VIF) was calculated for each predictor in respective model using the vif.mer function. VIF was never > 1.48, indicating low levels of collinearity among predictors (Zuur et al. 2010). We also calculated repeatability in male propensity to be cuckolded or his ability to sire at least one extra-pair offspring using the rptR package for R (number of bootstraps set to 1000; Stoffel et al. 2017).

RESULTS

Frequency of extra-pair paternity

EPP was detected in 51.2% of nests (82/160), with 19.8% (138/694) of offspring being sired by an extra-pair male (Online Resource Table S2). There was no difference between years in the proportion of EPP young in the population (Chi-squared test, $\chi^2 = 5.80$, df = 3, $n = 160$, $p = 0.12$). The number of extra-pair young per nest ranged from zero to five (mean 0.87 ± 0.8 SE), with more than one nestling sired extra-pair in 50% (41/82) of nests with mixed paternity. In 41.4% of these nests (17/41), we identified two extra-pair males, and in 2.4% of nests (1/41) we detected three extra-pair sires. The total number of offspring sired by a single male during the first breeding attempt varied from one to ten (mean 4.45 ± 0.15 SE).

Factors affecting male success in extra-pair fertilisations

As a first step, we evaluated the effect of male age on selected ornamental traits. Only tail streamer length and area of white tail spots (but not feather colouration) increased significantly with male age, while colouration remained unchanged (Online Resource Table S3). We further evaluated the effect of male characteristics on his ability to sire at least one extra-pair offspring, not accounting for male age. In the full model (Online Resource Table S4), male EPP success was negatively correlated with clutch initiation of his own brood and positively correlated with the area of white tail spots. After including male age in the model, however, these variables lost significance (Table 1). The final model included only male age (slope = 1.02 ± 0.22 [SE]; marginal pseudo$R^2 = 0.22$; $\chi^2 = 28.44$, $\Delta Df = 1$, $p < 0.001$). Males that sired at least one extra-pair offspring were older than males with no extra-pair success (Fig. 1). Repeatability in male extra-pair siring success over successive years was negligible ($r = 0.0$, $CI_{95\%} = 0 – 0.22$, n = 33 males with repeated observations).

Factors affecting male paternity loss

None of the phenotypic traits was positively correlated with male within-pair fertilisation success when male and female age were not considered (Online Resource Table S5). In the model involving male and female age, the male age x female age interaction term was not important in explaining patterns of within-pair paternity loss ($\chi^2 = 0.11$, $\Delta\mathrm{Df} = 1$, $p = 0.74$) and removed from the model. In the remaining model (Table 2) only female age was associated with the probability of a male being cuckolded. The final model for occurrence of EPP in nests only included female age (slope = 0.60 ± 0.22 [SE]; marginal pseudo$R^2 = 0.071$; $\chi^2 = 8.79$, $\Delta\mathrm{Df} = 1$, $p = 0.003$). Extra-pair offspring occurred more often in nests of older females than younger females (Fig. 2), regardless of male age. Repeatability in male propensity to be cuckolded was low ($r = 0.057$, $CI_{95\%} = 0 – 0.31$, $n = 33$ males with repeated observations).

Comparison between extra-pair sires and cuckolded males

A comparison of cuckolded and cuckolding males identified only one variable distinguishing between them (Table 3), i.e. extra-pair males were structurally larger, having longer tarsi than the males they cuckolded (effect of tarsus length: slope (cuckolded versus cuckolding) = -0.43 ± 0.17 [SE]; marginal pseudo$R^2 = 0.052$; $\chi^2 = 7.03$, $\Delta\mathrm{Df} = 1$, $p = 0.008$).

DISCUSSION

In this study, we examined patterns of within-pair and extra-pair fertilisation success in relation to a set of selected male ornaments, male age and age of his social partner in a Central European barn swallow population. Our results revealed that, whereas EPP success depends on male age, the main predictor for the likelihood of being cuckolded is the age of his social female. Our finding that repeatability in the male's ability to sire extra-pair offspring or avoid paternity loss in his nest was negligible is in agreement with this idea. Interestingly, phenotypic traits previously identified as both important determinants of male attractiveness in barn swallows and playing a role in extra-pair mating, such as tail streamer length or ventral colouration (e.g. Møller 1994; Saino et al. 1997; Safran and McGraw 2004; Vortman et al. 2011; Hasegawa et al. 2012), were not directly associated with extra-pair mating and the likelihood of being cuckolded in our barn swallow population.

Several explanations exist for the differences between our results and those of previous barn swallow studies, none of which is mutually exclusive. First, it is possible that different traits are used to choose social and extra-pair partners as the benefits to females from choosing between extra-pair and social partners differs (e.g. Griffith et al. 2002; Arnqvist and Kirkpatrick 2005; Akçay and Roughgarden 2007; Eliassen and Kokko 2008). Tail streamer length could be a trait preferred by European barn swallow females only when choosing a social mate (reviewed in Møller 1994; but see Saino et al. 1997). Second, the information content of exaggerated male traits may differ in different environments. Hence, the reliability of an ornament for signaling male quality may differ between barn swallow subspecies (Safran et al. 2016; Vitousek et al. 2016) and populations of the same subspecies may differ in traits associated with male attractiveness, as shown in North American barn swallow populations in Colorado and Ontario (Safran et al. 2005; Kleven et al. 2006a; Lifjeld et al. 2011), tree swallows *Tachycineta bicolor* (Whittingham and Dunn 2014) and collared flycatchers *Ficedula albicollis* (Dale et al. 1999; Galván and Moreno 2009).

In general, expression of male ornament typically increases with male age (Freeman-Gallant et al. 2010; Grunst et al. 2014). In our study, we identified one ornamental trait apparently correlated with extra-pair mating success. Males with larger white tail spots were more likely to obtain extra-pair offspring in other nests. Expression of white tail spots was to some extend age dependent and indeed, after including male age into the model, this trait lost its significance. In many previous studies, it remains unclear as to whether reproductive success was based solely on a male's ornamentation or his age. In this study, we were able to control for age effects statistically. Nevertheless, an experimental approach remains the best means of disentangling the effects of age and ornamentation on male fertilisation success (Safran et al. 2005; Whittingham and Dunn 2016).

The clear effect of age on a male's ability to sire extra-pair offspring could be interpreted as a combination of female preference for high quality, older males and/or male behavioural strategies that vary with age (Brooks and Kemp 2001; Westneat and Stewart 2003). Older males may be more experienced or aggressive and, consequently, better able to coerce females into mating with them (Westneat and Stewart 2003). Alternatively, males that have survived to old age should be of higher genetic quality (Richardson and Burke 1999; Brooks and Kemp 2001) as they have managed to avoid predators and parasitic diseases. Our results are in line with the idea that age is a better predictor of male EPP success than ornamental traits, and are in agreement with the conclusions of a recent meta-analysis (Hsu et al. 2015). The increased ability of older males to sire extra-pair offspring has also been documented in other bird species (Dietrich et al. 2004; Bouwman et al. 2007; Cleasby and Nakagawa 2012), though the relationship between male age and its ability to sire offspring outside his pair bond is less often measured compared with the effect of male age on within-pair fertilisation success (Cleasby and Nakagawa 2012).

In contrast to EPP, we observed a tendency for males with older social partners to loose paternity, independent of their own age. In other words, older females tended to engage more frequently in EPP than younger females. Relatively few studies have reported a correlation between female age and occurrence of EPP, and those that have provided inconsistent results, with some studies showing younger females of some species having higher levels of EPP in their nests (Stutchbury et al. 1997; Moreno et al. 2015), while in others the pattern is reversed (Kempenaers et al. 1999), and in some cases there was no correlation between female age and occurrence of extra-pair young at all (Lubjuhn et al. 2007). There is a continuing debate over the evolutionary origins and maintenance of extra-pair mating behaviour in birds (e.g. Arnqvist and Kirkpatrick 2005; Albrecht et al. 2006; Eliassen and Kokko 2008; Forstmeier et al. 2014). If EPP is driven mainly by female choice, higher occurrence of EPP in nests of older females could be interpreted as resulting from older and more experienced females being better able to evade mate guarding tactics, and hence be more likely to have multiple sired broods (Dietrich et al. 2004; Bouwman and Komdeur 2005). It is also possible that more experienced (older) females are better at obtaining high-quality genetic fathers for their offspring by engaging in EPP. This should be especially noticeable in females paired with unattractive mates, such as young males (e.g. Dietrich et al. 2004; Ramos et al. 2014). Male age was not associated with within-pair paternity loss in our study, however, and the effect of the female age x male age interaction on patterns of within-pair paternity was negligible. Older females may also be better able to manipulate the timing of extra-pair copulations, and so be more likely to obtain extra-pair fertilisations. Evidence that females actively pursue extra-pair copulations has been found in some bird species (Kempenaers et al. 1992; Double and Cockburn 2000); however, whether the ability to seek for extra-pair mates is age dependent in female birds remains unclear.

An alternative explanation for increased EPP in the nests of older females, which does not explicitly assume that EPP is beneficial to females, could be that older females are more attractive to males. Older females of most bird species tend to have higher clutch sizes and may produce higher-quality offspring than inexperienced breeders (e.g. Cichon 2003; Decker et al. 2012). In barn swallows, older females typically lay clutches earlier, have larger clutches than younger females (Turner 2006) and age of females is related to annual fecundity (Balbontín et al. 2007; own unpublished data). As extra-pair mate fecundity is a component of male fitness (Webster et al. 1995), older females could be selected preferentially as EPP partners and coerced by other males to copulate outside their social pair bond.

Pairwise comparisons between cuckolded and cuckolding males revealed that extra-pair sires had larger tarsi than males they cuckolded, supporting the findings of some previous studies (e.g. Foerster et al. 2003; Canal et al. 2011). Tarsus length is often used as a proxy for overall skeletal body size in songbirds (e.g. Kruuk et al. 2001). Møller and Ninni (1998) observed a positive effect of tarsus length and wing length on paternity in male barn swallow; hence, it is possible that larger males are better able to coerce females into copulation (Hsu et al. 2015), though tarsus length was of negligible importance in our own general model of within- and extra-pair paternity. Interestingly, comparisons between cuckolded and cuckolding males have shown no significant difference in plumage ornament or age, in contrast to previous studies on other passerines (e.g. Foerster et al. 2003; Bouwman et al. 2007; Hsu et al. 2015; Edme et al. 2016; but see Bitton et al. 2007). This is in line with our own findings that ornamentation *per se* does not affect EPP. Although the older male barn swallows in our population were better able to obtain EPP, they did it at the expense of both young and old males.

In conclusion, our results suggest that male ornamentation *per se* contributes little to overall variation in EPP, with all putative effects of male ornamentation lost when information on the age of males and females is included in the models. As in some other bird populations (Hsu et al. 2015), therefore, age may represent an important factor explaining patterns in EPP in barn swallows. It should be noted, however, that a large part of the observed variation (particularly in the case of within-pair fertilisation success) remained unexplained by our models. It would appear, therefore, that either other unmeasured factors (including genetic compatibility of social partners and self-referential, rather than absolute, criteria of mate choice; Mays and Hill 2004; Kempenaers 2007) play an important role in determining the outcome of extra-pair copulations, or that the adaptive explanation for evolution of EPP does not apply in our barn swallow population.

Acknowledgements

We would like to thank J Cepák, A. Hund, J Hubbard, P. Munclinger and A. Petrželková for help in the field. Kevin Roche provided valuable comments on the manuscript. This study would not have been possible without the collaboration of local farm owners, specifically the Kotrba family at Hamr farm, the Kraus family at Šaloun farm and the Pulec family. This research was funded through project GA15-11782S of the Czech Science Foundation.

Authors'contributions

TA conceived and designed the study; MA and OT analysed ornaments; RM, TA, OT, MA, JK performed the fieldwork; RM and JK conducted paternity analysis. TA and RM carried out statistical analyses; RM and TA wrote the manuscript. All the co-authors contributed to the final version of the manuscript and gave approval for its publication.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding This research was funded through project GA15-11782S of the Czech Science Foundation.

Ethical standards

All applicable international, national, and/or institutional guidelines for the use of animals were followed. All protocols were noninvasive and adhered to the laws and guidelines of the Czech Republic (Czech Research Permit numbers 6628/2008-10001). All protocols were approved by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011), and Charles University in Prague (4789/2008-30)

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Table 1 Male extra-pair fertilisation success in relation to male phenotype, male age and timing of breeding. Estimates are based on a generalised linear mixed model (male identity as a random grouping factor) with binary response variables (1 = at least one offspring sired extra-pair, 0 = no offspring sired extra-pair). Predictors were scaled (z-transformed) prior to analysis. Explanatory variables significantly associated with male extra-pair fertilisation success after simplification of the full model (see main text for further details) are indicated in bold.

	Estimate	Std. Error	z value	*p* value
Intercept	0.010	0.186	0.053	0.958
Tail streamer length	-0.069	0.189	-0.366	0.714
Tail spots	0.270	0.194	1.387	0.165
Ventral θ	0.326	0.195	1.670	0.095
Throat θ	0.101	0.195	0.515	0.606
Throat φ	-0.074	0.185	0.491	0.624
Tarsus length	-0.481	0.626	-0.768	0.442
Clutch initiation	-0.168	0.202	-0.830	0.407
Male age	0.970	0.243	3.993	**< 0.001**

Table 2 Male within-pair fertilisation success in relation to male phenotype, male and social partner age and timing of breeding (brood size treated as a covariate). Estimates are based on a generalised linear mixed model (male identity as a random grouping factor) with binary response variables (1 = at least one extra pair offspring occurred in the male's nest, 0 = no extra-pair offspring detected in the male's nest). Predictors were scaled (z-transformed) prior to analysis. Explanatory variables significantly associated with male within-pair fertilisation success after simplification of the full model (see main text for further details) are indicated in bold.

	Estimate	Std. Error	z value	*p* value
Intercept	0.345	0.297	1.162	0.245
Tail streamer length	0.069	0.190	0.365	0.715
Tail spots	0.205	0.190	1.082	0.279
Ventral θ	-0.258	0.194	-1.331	0.183
Throat θ	-0.056	0.197	-0.285	0.775
Throat φ	-0.126	0.189	-0.666	0.505
Tarsus length	3.308	2.625	1.260	0.207
Clutch initiation	-0.076	0.205	-0.373	0.709
Male age	-0.067	0.233	-0.287	0.774
Female age	**0.459**	**0.213**	**2.150**	**0.031**
Clutch size	0.245	0.182	1.344	0.179

Table 3 Pairwise comparison of cuckolding and culkolded males (n = 76 nests). Estimates are based on a generalised linear mixed model with social male and nest identity treated as random grouping factors and male status as a binary response variable (0 = cuckolding male, 1 = cuckolded male). Predictors were scaled (z-transformed) prior to analysis. Explanatory variables significantly associated with male status after simplifaction of the full model (see main text for further details) are indicated in bold.

	Estimate	Std. Error	z value	*p* value
(Intercept)	-0.216	0.160	-1.349	0.177
Tail streamer length	-0.052	0.171	-0.303	0.761
Tail spots	0.134	0.172	0.781	0.434
Ventral θ	0.057	0.174	0.330	0.741
Throat θ	0.099	0.168	0.594	0.552
Throat φ	-0.316	0.168	-1.881	0.060
Tarsus length	**-0.473**	**0.177**	**-2.671**	**0.008**
Male age	0.088	0.177	0.495	0.620

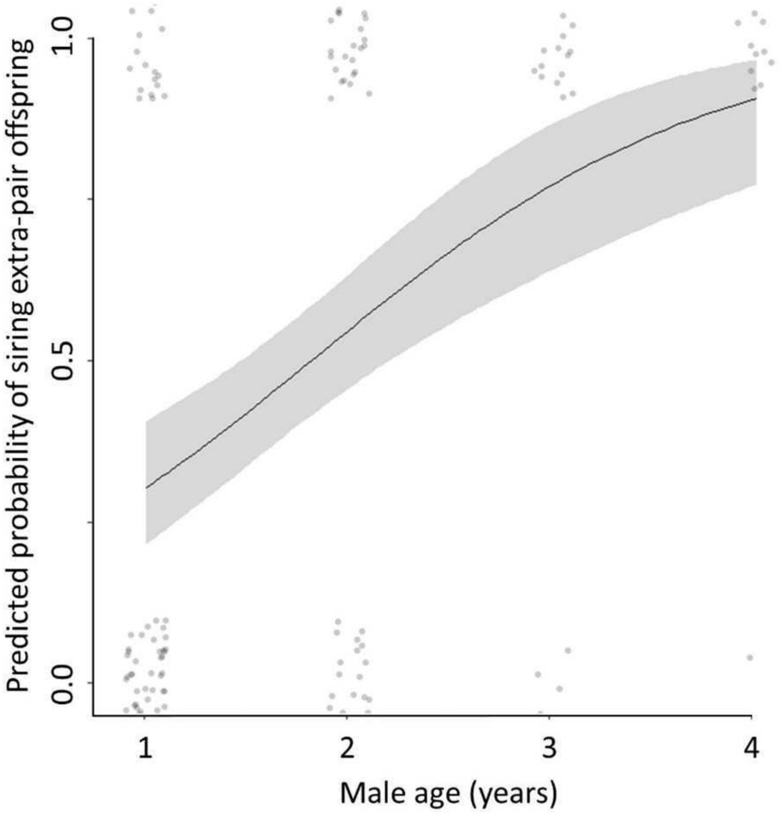

Fig. 1 Predicted probability of males siring extra-pair offspring in relation to male age. The shaded area denotes 95% confidence intervals around the predicted values, based on a generalised linear mixed effect model (binary response variable and logit link function) with male identity treated as a random grouping variable. See text for further details.

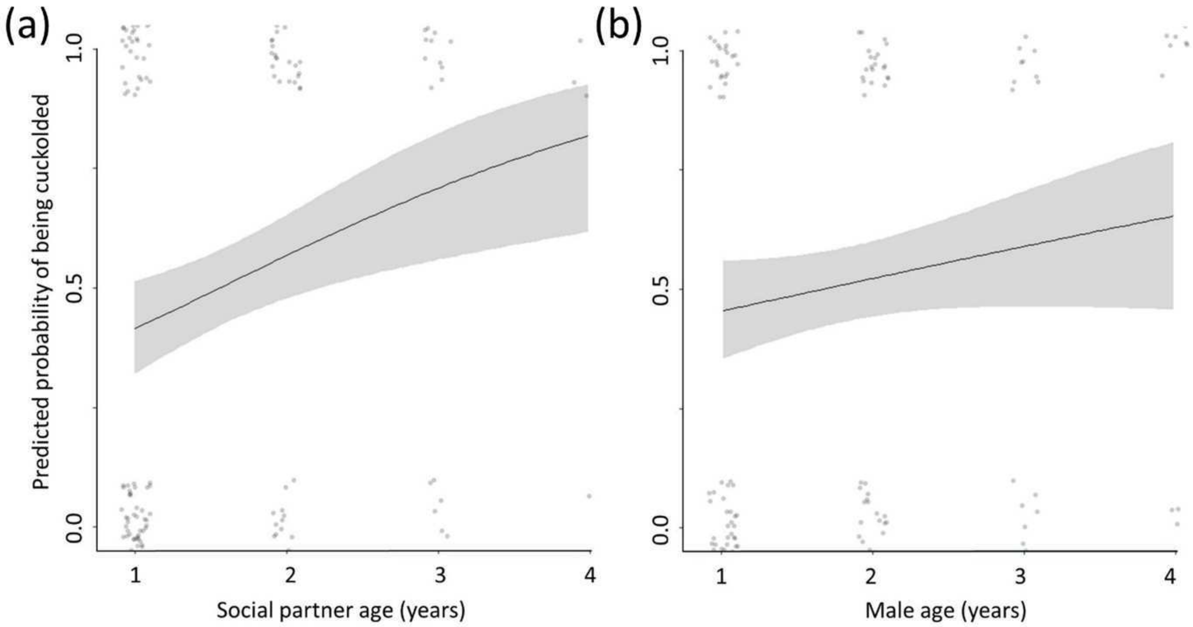

Fig. 2 Variation in the predicted probability of being cuckolded in relation to (a) female age and (b) male age. The shaded area denotes 95% confidence intervals around the predicted values based on a generalised linear mixed effect model (binary response variable and logit link function) with male identity treated as a random grouping variable. See text for further details.

Behavioral Ecology and Socobiology

Electronic Supplementary Information

for

Extra-pair paternity patterns in European barn swallows *Hirundo rustica rustica* are best explained by male and female age and not male ornamentation

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Supplementary inventory

Supplementary Table S1 Summary statistics for microsatellite loci used to determine paternity in barn swallows

Supplementary Table S2 Prevalence of extra-pair paternity and extra-pair young

Supplemetary Table S3 Results of separate analyses of the relationship between selected male phenotypes and age.

Supplementary Table S4 Results of analysis of male extrapair fertilisation success in relation to male phenotypes and the timing of breeding (male age not included).

Supplementary Table S5 Results of analysis of male within-pair fertilisation success in relation to male phenotypes and the timing of breeding (brood size treated as a covariate; male and female age not included).

Supplementary Table S1 Summary statistics for microsatellite loci used to determine paternity in barn swallows. Locus – name of locus described by Primmer et al. 1996 or Tsyusko et al. 2007; N – number of typed individual; K – number of alleles; H(obs) – observed heterozygosity; H(exp) – expected heterozygosity; PIC – polymorphic information content; F(Null) – frequency of null alleles, labelling – dye which was used for each forward primer.

Locus	K	N	H(obs)	H(exp)	PIC	F(Null)	labelling
Hir15	12	2277	0.652	0.682	0.627	0.023	FAM
Hir10	15	2277	0.788	0.835	0.815	0.027	HEX
Hir20	22	2275	0.826	0.843	0.824	0.009	HEX
Hir6	18	2276	0.857	0.842	0.822	-0.009	NED
Hir22	19	2277	0.829	0.876	0.863	0.026	NED
HrU10	49	2274	0.950	0.952	0.948	0.036	PET

Supplementary Table S2 Prevalence of extra-pair paternity (EPP) and extra-pair young (EPY) in a South Bohemian (Czech Republic) barn swallow population during the years 2010 – 2013.

Year	**Nests**	**EPP nests (%)**	**Young**	**EPY (%)**
2010	13	7 (53%)	57	16 (28%)
2011	32	13 (40%)	138	20 (14.5%)
2012	48	24 (50%)	214	51 (23.8%)
2013	67	38 (56%)	285	51 (17.8%)
Total:	160	82 (51.2%)	694	138 (19.8%)

Supplemetary Table S3 Results of separate analyses of the relationship between selected male phenotypes and age. Estimates are based on univariate GLMM models with phenotypic trait as a dependent variable, age as an explanatory variable and male identity as a random grouping factor. Phenotypic traits associated significantly with male age are indicated bold.

	Estimate	Std. Error	z value	*p* value
Tail streamer length	**0.146**	**0.058**	**2.494**	**0.012**
Tail spots	**0.129**	**0.057**	**2.247**	**0.024**
Ventral ϑ	-0.674	3.787	-0.178	0.859
Throat ϑ	-4.425	4.165	-1.063	0.288
Throat φ	0.500	2.363	0.212	0.832
Tarsus length	0.005	0.005	0.923	0.356

Supplementary Table S4 Results of analysis of male extrapair fertilisation success in relation to male phenotypes and the timing of breeding (male age not included). Estimates are based on GLMM model (male identity as a random grouping factor) with binary response variable (1 – at least one offspring sired extrapair, 0 – no offspring sired extrapair). Predictors were scaled (z-transformed) prior to analysis. Explanatory variables associated significantly with male extra-pair fertilization success after the simplification of full model (see main text for further details) are indicated bold.

	Estimate	Std. Error	z value	*p* value
(Intercept)	-0.040	0.170	-0.234	0.814
Tail streamer length	0.128	0.173	0.740	0.459
Tail spots	**0.410**	**0.180**	**2.270**	**0.023**
Ventral ϑ	0.189	0.175	1.081	0.279
Throat ϑ	-0.034	0.181	-0.189	0.849
Throat φ	-0.039	0.179	-0.222	0.824
Tarsus length	-0.334	0.492	-0.671	0.502
Clutch initiation	**-0.490**	**0.181**	**-2.692**	**0.007**

Supplementary Table S5 Results of analysis of male within-pair fertilisation success in relation to male phenotypes and the timing of breeding (brood size treated as a covariate; male and female age not included). Estimates are based on GLMM model (male identity as a random grouping factor) with binary response variable (1 – at least one extrapair offspring occurred in in male's nest, 0 – no extrapair offspring detected in male's nest). Predictors were scaled (z-transformed) prior to analysis.

	Estimate	Std. Error	z value	*p* value
(Intercept)	0.330	0.272	1.210	0.226
Tail streamer length	0.127	0.184	0.690	0.490
Tail spots	0.205	0.186	1.104	0.270
Ventral ϑ	-0.287	0.191	-1.499	0.134
Throat ϑ	-0.057	0.189	-0.301	0.763
Throat φ	-0.165	0.185	-0.894	0.371
Tarsus length	3.510	2.587	1.356	0.175
Clutch initiation	-0.205	0.185	-1.107	0.268
Clutch size	0.219	0.177	1.236	0.217

Age-dependence and viability signalling function of tail streamer length in the European barn swallow

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Keywords: sexual selection, condition-dependence, viability signalling, costs of ornament expression, senescence

Abstract

1. Sexual ornaments are often assumed to evolve as signals of individual viability. Ornaments can also signal individual age, thereby advertising proven viability. Although age-related increase in ornament expression has often been reported at the population level, only a few studies have separated the within-individual phenotypic change from selective disappearance due to differential viability of individuals with distinct phenotypes. Even less such studies analysed senescence in ornament expression.
2. In this study, we tested viability signalling function and senescence of tail streamer length in the European barn swallow (*Hirundo rustica rustica*) in two populations in the Czech Republic and Romania. In addition, we experimentally manipulated tail streamer length and analysed survival and tail length in the following year to test for tail streamer costs.
3. Using linear mixed-effect modelling (LMM) approach separating within- and between-individual age-related effects, we found non-linear within-individual increase in tail length with age in both populations and sexes, with highest increase between the first and the second year of adult life and subsequent levelling-off. There was no evidence for senescence in this ornamental trait in any of the population or sex.
4. Contrary to some previous studies on European barn swallows, the LMM approach showed a positive correlation between tail length and lifespan in males, but not females, across both populations. In addition, using logistic survival modelling, we found that tail length in the first year of life predicts lifelong survival in a non-linear way in Czech, but not Romanian, males. In the Czech population, males having tail streamers 5 mm longer than population median were the best survivors.
5. Experimental manipulations of tail streamer length had no significant effect on either survival or tail length in the following year.

6. Our data suggest that tail streamer length can signal both proven (age) and potential viability in the European barn swallows, though between-population differences in signalling content may exist. We also found that the directional selection for longer tail streamers may be combined with stabilising selection acting on males with extremely long tails. We further suggest that previous reports of senescence in tail length in this subspecies may be false positive and may have resulted from using inappropriate approaches. Interestingly, our data suggest that costs of tail streamers are at best mild, and only associated with extremely long tails.

Introduction

The indicator mechanism models of sexual selection assume that sexually selected ornamental traits are expressed in a condition-dependent manner and as such can honestly signal individual quality (Zahavi 1975; Grafen 1990; Getty 1998; Hill 2011). In such a case, sexual signals are expected to show positive relationship between their expression and survival (Jennions *et al.* 2001). Nonetheless, direction of the correlation could change due to a trade-off between ornamentation and survival as sexual signals are usually assumed to be costly (Zahavi 1975). The resultant direction of the correlation between two traits that are traded off is then influenced by resource acquisition (Van Noordwijk & de Jong 1986). In contrast, Fisherian runaway process does not incorporate condition-dependence and predict negative correlation between sexual character expression and survival (Lande 1981; Brooks 2000). It has been suggested, however, that the pure Fisherian process and the indicator mechanism are two ends of the sexual selection continuum and that the direction of correlation between ornamentation and survival may depend on the costliness of the female choice, and thus the intensity of sexual selection that females impose on males (Kokko *et al.* 2002). Hence, negative or no correlation between male attractiveness and survival indicates low costs of female choice imposing strong sexual selection on males, whereas positive correlation indicates more relaxed sexual selection due to high female choice costliness, which allows secondary sexual characters to function as viability indicators (Kokko *et al.* 2002).

Sexual traits can also advertise proven viability through signalling individual's age (Manning 1985). Game theoretic modelling has shown that such an increasing investment in attractiveness with age is in accordance with life-history theory (Kokko 1997, 1998). Although, many empirical studies have analysed age-related changes in male ornamental traits, most of them did not control for selective disappearance of particular phenotypes.

Results of such studies, even those using longitudinal data, cannot be interpreted as within-individual phenotypic change with age, because statistical separation of between-individual variation in lifespan is necessary to obtain unbiased estimates of age-related changes within individuals (van de Pol & Verhulst 2006; Nussey *et al.* 2008). The few studies that used such statistical decomposition of within- and between-individual effects usually reported increasing pre-senescent expression of secondary sexual characters with age (Delhey & Kempenaers 2006; Bitton & Dawson 2008; Nussey *et al.* 2009; Val *et al.* 2010; Balbontín *et al.* 2011; Evans *et al.* 2011; Kervinen *et al.* 2015), except for one recent study reporting no age-related change in pre-senescent carotenoid-based beak colouration in male zebra finches (Simons *et al.* 2016).

Moreover, only a proportion of these studies provided an analysis of senescence in sexual display, most probably due to the difficulty of obtaining necessary longitudinal data (Evans *et al.* 2011). The few studies available to date reported mixed results, with senescence in secondary sexual characters being detected is some studies (Balbontín *et al.* 2011; Kervinen *et al.* 2015; Simons *et al.* 2016) but not in others (Nussey *et al.* 2009; Evans *et al.* 2011). Furthermore, in some of the studies reporting senescence in male ornamental traits, such a conclusion is only based on a significant quadratic age effect (e.g. Balbontín *et al.* 2011). The information content of significant quadratic age effect as an evidence for senescence is limited, however, as significant quadratic age effect may solely result from the increase in trait expression being steeper in early life with subsequent slowdown or levelling-off (Bouwhuis *et al.* 2009). Overall, our current knowledge on how within- and between-individual effects determine the observed patterns of age-related expression of secondary sexual characters at the population level appears to be only fragmentary, and more longitudinal studies separating age effects at different levels and properly testing for senescence are needed to clarify this issue (Simons *et al.* 2016).

The evolution of condition-dependent sexual signals implies an existence of mechanisms maintaining signalling honesty. Handicap principle of signalling honesty maintenance assumes that the expression of sexual signals is costly and that the costs are higher for low quality individuals. Therefore, only individuals of high quality can express elaborate signals without reducing their overall fitness (Zahavi 1975; Grafen 1990). Recently, several alternative hypotheses have been put forward, proposing that signal expression may depend on metabolic and signalling pathways that are fundamental for organism functionality and performance, thereby signalling individual quality and condition in a cost-free manner (Hill 2011; Emlen *et al.* 2012; Warren *et al.* 2013). Testing costs associated with ornament elaboration can therefore provide a clue to the general mechanism maintaining signalling honesty.

In this study, we use longitudinal data from the two long-term studied populations of the European barn swallow (*Hirundo rustica rustica*) from the Czech Republic (CZ) and Romania (RO) to test whether expression of a sexually selected trait, the length of tail streamers, reflects individual age and survival and to estimate the effect of ageing on this trait. Tail streamers of barn swallow males are much longer than those of females and rank among the most iconic examples of sexual selection (Møller 1994b). We use linear mixed-effect models (LMM) fitted with both individual age and lifespan as predictors of tail length to decompose within- and between-individual effects of age on tail length expression in both sexes (van de Pol & Verhulst 2006). We also tested whether tail length in the first year of adult life predicts lifelong survival using logistic discrete-time hazard models with logit link-function, death set as a binary dependent variable and tail length in the first year of adult life fitted among the predictors (Singer & Willett 2003).

In addition, we performed an experimental manipulation of male tail length in the CZ population in order to test the costs of tail streamers. Specifically, we tested whether manipulation of tail streamer length affects survival to the next year or tail streamer growth during subsequent moult. The experimental manipulation is the best means to evaluate ornamentation costs, because, in observational studies, the costliness may be confounded by variability in individual genetic quality (Grafen 1990) and/or differences in resource availability (Van Noordwijk & de Jong 1986).

Methods

Study sites and data collection

We collected longitudinal data from two populations of the European barn swallow in the Czech Republic and Romania. The CZ population has been studied at four isolated farms in the Třeboňsko Protected Landscape Area: Hamr in Lužnice (49°03'24"N, 14°46'10"E); Šaloun in Lomnice nad Lužnicí (49°04'08"N, 14°42'39"E); Obora in Třeboň (48°59'07"N, 14°46'50"E); and Břilice (49°01'14"N, 14°44'17"E); during the breeding seasons 2010–2017. In this population, most of the birds breed in colonies in stables, with just a few solitary pairs breeding in separate rooms or buildings. The RO population has been studied in Cojocna village in central Transylvania (46°45’N, 23°50′E) during the breeding seasons 2011–2017. At this study site, barn swallows usually breed in stall buildings in natural nests most often solitarily, but in some cases several pairs aggregate into small loose colonies (for details see Fülöp *et al.* 2017). In total, we collected 621 observations from 398 CZ males, 469 observations from 293 CZ females, 237 observations from 160 RO males and 283 observations from 179 RO females.

Adult individuals were systematically captured with mist nets or nest traps on several occasions during the breeding season. Upon capture, each individual was marked with a unique aluminium ring and tail streamer length was measured to the nearest mm. Previous observations on barn swallows (Saino *et al.* 1999; Schaub & Von Hirschheydt 2009) and our own capture–recapture data (Pap *et al.* 2005) indicate high breeding site fidelity. Given the extremely high breeding site fidelity and because we captured and marked the vast majority of adult individuals at our study sites every year, unmarked adult birds were assumed to be one-year old birds immigrating from other colonies and birds that did not return to the study site in the next year were regarded as dead (Møller & De Lope 1999; Saino *et al.* 1999; Pap *et al.* 2005; Balbontín *et al.* 2011; Costanzo *et al.* 2017). In the CZ population, only 2.1% of adult birds that were captured at least in two years ($N = 243$) were missed in one year between captures. Sex was determined by visual examination of the presence of a brood patch, which develops only in females. In the CZ population, feather samples of at least 10 feathers were collected from the ventral region of the body for subsequent colour analysis.

Ornament manipulations

To assess costs of tail streamers and ventral colouration, we performed experimental manipulation of their expression in the CZ population during breeding seasons 2011–2015. The manipulation experiment was carried out between the first and the second nesting attempt. Tail streamers were elongated or shortened by two standard deviations (15 mm). We cut the streamers 15 mm from the base and, to the stump, we attached new streamers obtained from another male either during the same field session or during one of the previous sessions. The attachment was done by insertion of the entomological pin (total length ca. 10 mm) inside the shaft of the stump and the new streamer was slid onto the protruding half of the pin. The joint was fixed with cyanoacrylate superglue (Loctite, Henkel ČR, Czech Republic). This method preserve the natural proportions of tail streamers and prove successful in the previous experiments (Bro-Jørgensen *et al.* 2007; Vortman *et al.* 2013; Safran *et al.* 2016).

In addition, tail length manipulation was combined with experimental darkening of ventral colouration in 2014 and 2015, to evaluate its costs and importance for reproductive success. All the feathers of ventral region were darkened using a non-toxic permanent marker

(Prismacolor, light walnut) following previous experimental studies on barn swallows (Vortman *et al.* 2013; Safran *et al.* 2016).

Plumage colouration measurement

Ten feathers were composed on each other and fixed on a white paper index cards to achieve layer equivalent to real ordering of feathers in birds (Quesada & Senar 2006). Feather reflectance was measured between 300–700 using AvaSpec 2048 spectrometer with an AvaLight-XE light source (Avantes, Netherlands). Custom-made adapter was attached on the sensing spectrometer probe to eliminate ambient light and ensure constant distance 3.5 mm between the probe and the sample. Each sample was measured three times at the distal part of the feathers with the probe held perpendicular to the feather surface. Spectrometer was calibrated against a darkroom and a white standard (WS-2, Avantes, Netherlands) after measuring every eight samples. Data were analysed using R 3.4.3 (R Core Team 2017) and pavo 1.3.1 R package (Maia *et al.* 2013). Three measurements of each sample were averaged and smoothed (span set to 0.2). Tristimulus scores brightness, hue and saturation (red chroma) were calculated from the resulting curve. Brightness was calculated as average reflectance, hue as wavelength at the reflectance midpoint and red chroma as a summed reflectance of 600–700 nm divided by a summed reflectance of 300–700 nm (Butler *et al.* 2011; Simons *et al.* 2012). Given that hue and red chroma were strongly intercorrelated ($r = 0.88$) and both shown moderate correlation with brightness (hue: $r = -0.62$; red chroma: $r = -0.67$), we only used red chroma in statistical analysis. Note that the conclusions would be unchanged, if brightness or hue was used instead (data not shown).

Statistical analysis

In order to discern the effects of within-individual age-related change and selective disappearance on age-related variation in tail length at the population level, we used linear mixed-effect models (LMM) implemented in R 3.4.3. In these models, tail length was set as a dependent variable and individual identity was included as a random factor. Only random effects on intercept were fitted as only one observation was available for those individuals that did not survived past the age of one year. We fitted the models separately for each sex to avoid overly complicated model structure with higher-order interactions. We adopted two approaches to analyse data from two separate populations. First, we fitted population-specific models to test for age-related effects on tail length in each population separately. Second, we fitted joint models for both populations to test for both the average effects across populations and significant differences in effects between populations. In the joint models, populations were fitted as a continuous variable coded 0 (the CZ population) and 1 (the RO population) and centred, which allows to interpret the main effects of age and lifespan as average effect across populations. We introduced age as a cubic effect (third-order polynomial) in the models, because fitting age with only a quadratic age effect (second-order polynomial) may result in a false positive detection of senescence when phenotypic change is greater at an early age with subsequent levelling off (Bouwhuis *et al.* 2009). Non-linear effects were always fitted as orthogonal polynomials to avoid collinearity. We further introduced lifespan as one of the explanatory variables to test for the effect of selective disappearance with respect to tail length. Inclusion of both age and lifespan as the predictors in the model is important as it allows to discern within-individual changes in trait expression with age and the effect of selective disappearance (van de Pol & Verhulst 2006). In other words, this approach tests for the effect of within-individual change in analysed trait in the presence of selective disappearance and *vice versa*. The estimate of age-related changes in trait expression cannot be interpreted as sole effect of within-individual change when lifespan is not included among predictors as it also contains the between-individual effect of selective disappearance (van de Pol & Verhulst 2006). We also included the quadratic term for lifespan, as suggested by former studies and interpreted as resulting from a combination of selective disappearance and a trade-off in resource allocation between trait expression and survival (Reid *et al.* 2003; Bouwhuis *et al.* 2009).

Although the aforesaid LMM approach can test for selective disappearance, it cannot test for stabilizing or disruptive selection as the variable expressing survival (i.e. lifespan) is on the x-axis and a phenotypic trait is on the y-axis. Hence, we also used a second approach to test for differential mortality with respect to tail length, the logistic discrete-time hazard models with logit link-function, death set as a binary variable (0 = survived to next year, 1 = died during the subsequent year) and age fitted as a categorical variable (Singer & Willett 2003). We used this approach rather than Cox proportional hazards modelling, because performance of the latter is low when there are only few discrete-time periods with high number of deaths in each (Singer & Willett 2003). As tail length was highly repeatable when age and population differences were controlled for (LMM-based repeatability [95% CI]: males: 0.90 [0.88–0.92]; females: 0.88 [0.85–0.90]), we used tail length in the first year of age and tested whether it predicts individual survival in subsequent years. First-year tail length was fitted together with its quadratic effect in these models to test for non-linear relationship between ornamentation and survival, which would be indicative of either stabilizing or disruptive selection. Again, we fitted separate models for each population and a joint model including observation from both populations. Population together with two-way interactions with age, tail length and year were included among predictor terms in the joint model to test for between-population differences. Age was included as centred dummy variables. Population was centred in the same way as in LMM models, hence the main effects are interpreted as the average effects across populations. We only used age classes one to four in CZ males, CZ females and RO females and age classes one to three in RO males as probability of mortality in older age classes cannot be plausibly estimated due to the low number of individuals surviving to the late age. In a joint model fitted with male data from both populations, we only used age classes one to three to prevent rank deficiency. The models controlled for variation in mortality between years by including calendar year as a continuous variable with quadratic effect. The quadratic effect of year was supported over the linear and cubic effects, as well as over including this variable as a factor, according to an AICc-based model comparison using the joint model as a background model (Table S1 in Supplementary Information).

Our dataset included a subset of CZ males that underwent experimental manipulation of tail length and ventral colouration. We used this subset to test the costs of tail length manipulation in terms of both survival and tail streamer length grown during the subsequent moult. The effect of tail length manipulation was tested using logistic discrete-time hazard model with manipulation group (control, elongated, short) fitted as a predictor. We also included ventral colouration (control, darkened) manipulation to control for its effect. The model also controlled for age and year effects in the same way as above (i.e. included as categorical and quadratic terms, respectively). We also included both pre-manipulation tail length and ventral colouration to control for potential bias due to phenotypic differences between manipulation groups. Indeed, our approach of swapping tail streamers between males resulted in males from elongated and shortened group having pre-manipulation tail length shorter and longer, respectively, compared to control males (elongated: $\beta = -3.14 \pm 0.85$, $t = -3.69$, $P < 0.001$; shortened: $\beta = 3.19 \pm 0.74$, $t = -4.30$, $P < 0.001$). In contrast, there was no difference in pre-manipulation redness of the ventral colouration between darkened and control males ($\beta = 0.004 \pm 0.004$, $t = 0.95$, $P = 0.34$). We also tested for the previously reported differential effect of tail manipulation on short- and long-tailed males (Møller & de Lope 1994) by including interaction between tail manipulation and pre-manipulation tail length. The significance of the predictor terms was tested using likelihood ratio test (LRT).

Experimental manipulation of tail length was previously reported to affect length of the tail streamers grown during subsequent moult. We tested this using LMM with tail length in the year after experimental manipulation as a dependent variable and individual identity as a random effect. Manipulation of tail length and ventral colouration were fitted as categorical fixed effects and pre-manipulation tail length as a covariate. We also introduced a quadratic term for age to control for age-related changes in tail length. The significance of the predictor terms was tested using LRT based on maximum likelihood estimation. Ornament manipulations showed no significant effect in any of the models in this study (see Results and Table S2 and S3 in Supplementary Information), hence we only present models without these terms, except the models intended to test the manipulation effects.

Results

Tail length associations with age and lifespan

At the population level, tail length monotonically increased with age in both male and females with highest increase between the first and the second year of life and a slowdown in subsequent years (Figure 1a, b). In order to discern relative contribution of within-individual change and selective disappearance to this pattern, we fitted LMM with tail length set as dependent variable and both age and lifespan together with their third- and second-order polynomials, respectively, as predictors.

This approach revealed significant within-individual age-dependent variation in tail length in both sexes with similar dynamics to the one at the population level; i.e. highest increase in length between the first and the second year of life (steeper in the CZ population) and a slowdown in subsequent years (Table 1). Plotting the individual inter-annual changes in tail length showed no evidence of senescence in this trait in any of the population or sex (Figure 1c, d). The lack of senescence in tail length was further supported by the positive coefficient of the third-order polynomial of age in all the models, though significant only in CZ males and in the joint model for males from both populations. To investigate how removing the third-order polynomial of age from the model affects interpretation of the results, we further fitted a model with only quadratic age effect and plotted the predicted values of both models. In contrast to both the model with cubic age effect and calculated individual inter-annual changes in tail length, the results of a model including only quadratic age effect could be interpreted as indicating senescence in tail length in both sexes (Figure 1e, f). This suggests that including only second-order polynomial of age in a model could result in a false positive detection of senescence.

The LMM further tested for selective disappearance with respect to tail length. Across populations, tail length was significantly correlated with lifespan suggesting selective disappearance of short-tailed males, but not females (Table 1). Separate models for each population showed that although the estimate was also positive in RO males, the relationship between tail length and lifespan was only significant in the CZ population (Table 1; Figure 2). The non-significant interaction between lifespan and population suggests, however, that the association between tail length and lifespan does not differ between CZ and RO males.

First-year tail length and survival

We further tested whether tail length measured in the first year of age predicts individual survival in following years using logistic discrete-time hazard models. In CZ males, there was a significant second-order polynomial effect of first-year tail length suggesting that the positive relationship between tail length and survival indicated by LMM approach is reversed in males with extremely long tails (Table 2; Figure 3). This quadratic effect was only marginally non-significant when effect of ornament manipulations has been controlled for (*P* = 0.055; Table S3 in Supplementary Information), however, which suggests that the increase in mortality in long-tailed males may be mild (see also confidence intervals in Figure

3). In the RO population, this effect was not significant, though the joint model indicated no significant difference between the populations. In the CZ population, best survivors were males having first-year tail length 5 mm longer than the population median (114 mm vs. 109 mm). This provides an evidence for overall directional survival selection for longer tails (as evidenced also by LMM approach), combined with weak stabilizing selection acting on males with extremely long tails. In contrast, no such selective mortality was found in females.

Effect of tail length manipulation on male mortality

To assess costs of tail streamers in terms of survival, we tested whether experimental manipulations of tail length affected the probability of male survival to the next year in the CZ population. Tail length manipulation was combined with experimental darkening of ventral colouration in 2014 and 2015, hence we also included this effect in the model. We found no support for male tail streamers or ventral colouration being costly in terms of survival, as experimental manipulation of neither tail length nor ventral colouration showed a significant effect on male survival probability (tail length manipulation: LRT: $\Delta G^2 = 1.33$, $\Delta df = 2$, $P = 0.51$; effect of tail elongation: $\beta = 0.34 \pm 0.33$, $P = 0.31$; effect of tail shortening: $\beta = -0.05 \pm 0.32$, $P = 0.86$; manipulation of ventral colouration: LRT: $\Delta G^2 = 2.55$, $\Delta df = 1$, $P = 0.11$; effect of darkening: $\beta = -0.68 \pm 0.43$, $P = 0.11$). An addition of an interaction between pre-manipulation tail length and tail length manipulation to the model did not support previously reported differential survival of naturally short- and long-tailed males after manipulation (LRT: $\Delta G^2 = 0.75$, $\Delta df = 2$, $P = 0.69$; effect of pre-manipulation tail length in elongated males: $\beta = -0.027 \pm 0.039$, $P = 0.48$; effect of pre-manipulation tail length in shortened males: $\beta = -0.027 \pm 0.039$, $P = 0.49$).

Effect of tail length manipulation on tail streamer growth during subsequent moult

We further tested whether experimental manipulation of tail streamer length influences the length of male tail streamers grown during subsequent moult using LMM models with tail length in the next year as a dependent variable. Age and pre-manipulation tail length were controlled for in the model. Again, there was no support for the tail streamers being costly as experimental manipulation of neither tail length nor ventral colouration showed a significant effect on the length of male tail streamers in the next year (tail length manipulation: LRT: $\Delta G^2 = 4.01$, $\Delta df = 2$, $P = 0.13$; effect of tail elongation: $\beta = -0.88 \pm 0.85$, $P = 0.31$; effect of tail shortening: $\beta = -1.47 \pm 0.77$, $P = 0.072$; manipulation of ventral colouration: LRT: $\Delta G^2 = 0.29$, $\Delta df = 1$, $P = 0.59$; effect of darkening: $\beta = 0.43 \pm 0.84$, $P = 0.61$). An addition of an interaction between tail length manipulation and pre-manipulation tail length revealed no significant differential effect of ornament manipulation in birds with differing tail length (LRT: $\Delta G^2 = 0.19$, $\Delta df = 2$, $P = 0.90$; effect of pre-manipulation tail length in elongated males: $\beta = -0.067 \pm 0.121$, $t = 0.55$; effect of pre-manipulation tail length in shortened males: $\beta = -0.015 \pm 0.105$, $t = -0.14$).

Discussion

One of the main aims of our study was to test for a condition-dependence of tail streamer length in two European barn swallow populations, namely the differential mortality related to the expression of this secondary sexual trait. Using two different statistical approaches, we detected such selective mortality in males, but not females. The first approach, based on an inclusion of lifespan among predictors of tail length in the linear mixed-effect model (van de Pol & Verhulst 2006), showed a positive linear relationship between male tail length and lifespan indicating that males with longer tails live longer. Although this relationship was only significant in CZ males when each population was analysed in a separate model, the joint model supported its existence across populations. In the CZ population, such selective disappearance of short-tailed males was also supported by the second approach, the logistic discrete-time hazard modelling. This approach indicated, however, that the relationship between tail length and mortality is not linear and that males with extremely long tails also suffer higher mortality. In the CZ population, the highest survival probability was observed in males with tails 5 mm longer than the population median, providing an evidence for directional selection combined with stabilizing selection acting on males with extremely long tails. The quadratic effect of tail length on survival has become marginally non-significant when effect of ornament manipulation was controlled for in the model. Together with positive correlation between tail length and lifespan evidenced by LMM approach, this suggests that the increase in mortality in long-tailed males may only be mild. The non-linear relationship between tail length and mortality was not supported in the RO population, perhaps due to either lower number of observations or between-population differences. The differences between populations were not supported by the joint model, however, as the interaction between population and tail length was non-significant. Nevertheless, both approaches used in our study indicated directional survival selection on tail length in males but not females, either across populations (LMM approach) or in the CZ population only (discrete-time hazard modelling), which could provide one of the evolutionary mechanisms explaining maintenance of elongated tail streamers in barn swallow males.

Previous studies in different barn swallow populations provided contrasting results reporting either negative (Møller & Szep 2002; Balbontín *et al.* 2011) or positive (Møller 1991, 1994a; Saino *et al.* 1997; Romano *et al.* 2017) correlation between tail length and survival, with our results supporting the latter. Such a discrepancy suggests that the relationship between tail length and survival might not be universal across barn swallow populations. This may relate to differing conditions experienced by various populations due to, for example, between-population differences in migration routes or wintering grounds (Ambrosini *et al.* 2009; Klvaňa *et al.* 2018). Alternatively, such contrasting results may be due to resource availability being variable between populations or among years since resource acquisition can alter relationship between fitness components that are traded off against each other (Van Noordwijk & de Jong 1986). Our data suggest that at least the extremely long tail streamers are indeed traded off against survival, though our manipulation experiment suggests the survival costs of tail streamers are low. We can also speculate that the interplay between resource acquisition and allocation can possibly underpin the non-linear relationship between tail length and survival observed in our study. As resource acquisition is limited (either due to limited resource availability or due to physiological limits in resource acquisition) available resources may be sufficient in short-tailed males for both ornament expression and survival, resulting in their positive correlation, but may be limiting in males with extremely long tails. In addition, extremely long tails can even interfere with resource acquisition due to reduced manoeuvrability, an important quality for aerial foraging (Buchanan & Evans 2000).

Our results showing a link between male tail streamer length and survival support condition dependence of this sexual trait, at least in the CZ population. This is in line with a previous meta-analysis indicating that most sexually selected traits may serve as viability indicators (Jennions *et al.* 2001). The positive correlation between male tail length and viability would speak against the pure Fisherian runaway process being responsible for evolution of this sexual trait in the barn swallow (Lande 1981). Nevertheless, a unifying model has been proposed suggesting that pure Fisherian process and indicator mechanism are opposite ends of one sexual selection continuum (Kokko *et al.* 2002). According to this model, the direction of correlation between ornamentation and survival may depend on the costliness of the female choice, which determines the intensity of sexual selection that females impose on males. The model predicts that less costly female choice results in negative relationship between attractiveness and survival due to intense sexual selection, whereas more relaxed sexual selection resulting from high female choice costliness restores viability signalling function of a display (Kokko *et al.* 2002). Accordingly, positive correlation between tail length and viability observed in our study would imply high costs of female choice and, consequently, relaxed sexual selection on male tail length.

Our data confirm previously reported (Møller & De Lope 1999; Balbontín *et al.* 2011) non-linear within-individual increase in pre-senescent tail length with age with highest elongation between the first and the second year of life and a slowdown in subsequent years. Therefore, the age-related increase in tail length observed at the population level is due to combination of within-individual increase in tail length and selective disappearance of short-tailed animals in our populations. While LMM fitted with a second-order polynomial function of age indicated senescence in tail length in late life, both the individual inter-annual differences and LMM fitted with a third-order polynomial of age showed no signs of senescence. This demonstrates that fitting models with only a quadratic age effect could result in a false positive detection of senescence in tested traits in cases where the change in trait expression is steeper in early life with subsequent slowdown (Bouwhuis *et al.* 2009). This could provide a possible explanation for a discrepancy between previous studies reporting senescence in tail length in the barn swallow (Møller & De Lope 1999; Balbontín *et al.* 2011) and our study finding no senescence in this trait, despite covering the same number of age classes (from one to six years of age in both sexes). Hence, our results suggest that second-order polynomial of age effect should be in general considered with caution when analysing senescence.

The observed within-individual increase in tail length with age is a pattern reported for a variety of sexual traits in many species including the barn swallow (Delhey & Kempenaers 2006; Nussey *et al.* 2009; Val *et al.* 2010; Balbontín *et al.* 2011; Evans *et al.* 2011; Kervinen *et al.* 2015). It has been suggested that older individuals with highly expressed sexual traits can signal their proven viability and are preferred in mate choice (Manning 1985). This is in accord with our observation from the CZ population, where older males are more successful in gaining extra-pair paternity (Michálková et al. unpublished manuscript). The within-individual increase in tail length in response to age is also in accord with a game theoretic model based on life-history theory predicting increasing investment in sexual ornamentation with decreasing residual reproductive value of an individual (Kokko 1997, 1998). Considering that ageing individuals should experience decline in physiological functions (López-Otín *et al.* 2013), even maintaining a stable level of ornament expression toward the end of the life may be seen as an increasing investment in sexual signalling (Evans *et al.* 2011). In the barn swallow, such a physiological decline in old age has been documented in reproductive and immune functions (Møller & De Lope 1999; Saino *et al.* 2003). Hence, our data showing no senescence in tail length indicate that investment in sexual advertisement increases over the entire individual lifespan.

We further tested the costs of elongated male tail streamers using experimental manipulation of their length in the CZ population. In contrast to previous experiments done in different barn swallow populations (Møller & de Lope 1994; Saino *et al.* 1997), we found no significant effect of tail length manipulation on survival, despite using comparable sample size. There was also no indication of previously reported (Møller & de Lope 1994) differential survival of naturally long- and short-tailed males after manipulation as evidenced by non-significant interaction between tail manipulation and pre-manipulation tail length. We also found no effect of manipulations on the length of tail streamers grown during subsequent moult, which was also previously reported (Møller 1989). Such a discrepancy may have several possible explanations. First, the way we manipulated tail streamer length differed from previous studies. Instead of cutting out a proximal piece of tail streamer in shortened males and pasting it to the base of tail streamer in elongated males (Møller 1989; Møller & de Lope 1994), which results in both a displacement of white tail spots and unnatural proportions between wide and narrow parts of the streamer vane, we swapped whole feathers between males keeping the proportions natural (Bro-Jørgensen *et al.* 2007; Vortman *et al.* 2013; Safran *et al.* 2016). The extension of the wide part of streamer vane used in previous studies could result in feather being heavier and having different aerodynamic properties compared to streamers with natural proportions. Second, our approach needed only one joint per streamer where the feather parts were glued together instead of two, thus reducing the chance of a joint failure by 50%. This may be highly relevant as the joint must hold from the time of manipulation to a subsequent moult at the wintering grounds and its failure would cause the distal streamer part falling off possibly increasing probability of mortality due to impaired manoeuvrability. Hence, by reducing the number of joints, we reduced the chance of such an artefactual mortality affecting our results. Third, our approach based on swapping tail streamers between males resulted in males from elongated group having shorter mean pre-manipulation tail length compared to males from shortened groups, whereas there was no such difference in previous studies (Møller 1989; Møller & de Lope 1994). Nevertheless, such a difference should actually strengthen the effect of manipulation in our study, at least in the elongated group, as short-tailed males are the ones expected to suffer the highest costs of tail elongation (Møller & de Lope 1994). Fourth, we only manipulated the tail length by 1.5 cm (2 SD in our population) instead of 2 cm used in previous studies (Møller 1989; Møller & de Lope 1994), which could be another possible cause of our failure to detect any significant effect on survival. Tail shortening or elongation by 1.5 cm is still a substantial alteration compared to the distribution of tail length variability, however, suggesting that costs of bearing tail streamers are at best mild. Inferring from our correlational data, the survival costs may only be significant in males with extremely long tail streamers.

The lack of significant costs of tail streamer expression could alternatively imply that honesty of condition-dependent signalling is not maintained primarily through tail streamer costs but through expression of this sexual trait being linked to physiological processes and signalling pathways underlying individual phenotypic quality (Hill 2011). For example, insulin/insulin-like signalling has been proposed as one of the pathways possibly linking growth of exaggerated structural sexual traits with body condition without assuming ornamentation costs (Emlen *et al.* 2012; Warren *et al.* 2013). Insulin/insulin-like signalling pathway interacts with growth hormone signalling pathway, androgens and glucocorticoids and it is involved in control of metabolism, somatic growth, reproduction and ageing (Dantzer & Swanson 2012). Therefore, it represents an exciting and largely unexplored mechanism that could link exaggerated sexual traits to phenotypic quality without the need of trait costliness. Nevertheless, it should be noted that this mechanism does not exclude the possibility of exaggerated sexual traits being costly in their extreme values.

In summary, our data suggest that elongated tail streamers in barn swallow males indicates both survival and age, i.e. potential and proven viability, while only the association with age was observed in females The positive correlation between tail length and survival may attest either to relaxed sexual selection resulting from high costs of female choice (Kokko *et al.* 2002) or high resource availability in our populations (Van Noordwijk & de Jong 1986), enabling males to mitigate display costs. Alternatively, costliness may not be the main mechanism ensuring honesty in this sexual trait as our manipulation experiment suggest that costs of tail streamers are at best mild and only extremely long tails appear to be costly. Hence, the best fitting to our data may the hypothesis proposing that condition-dependence of sexual display is not mediated through its costs but through its expression being linked to metabolic and signalling pathways underlying individual phenotypic quality (Hill 2011). In addition, we found no support for senescence in tail length in either sex. We also demonstrated that including only second-order polynomial of age as a predictor in a model may result in false positive detection of senescence and should be interpreted with caution.

Acknowledgments

TA was financially supported by a grant from the Czech Science Foundation (GA15-11782S); PLP, VIC and OV by a grant from the Romanian Ministry of Research and Innovation (PN-III-P4-ID-PCE-2016-0404); PLP by a Fulbright Fellowship and by János Bolyai Research Scholarship of the Hungarian Academy of Sciences; and CIV by a postdoctoral grant of the Hungarian National Research, Development and Innovation Office (PD 121166) and János Bolyai Research Scholarship of the Hungarian Academy of Sciences. We are grateful to Adéla Petrželková, Martina Němcová, Jana Albrechtová, Jakub Kreisinger, Pavel Munclinger, Ondřej Kauzál, Lukáš Bobek, Maren Vitousek, Joey Hubbard, Amanda Hund and many undergraduate students for the help with the data collection.

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Table 1. Linear mixed-effect models estimating effects of age and selective disappearance on tail length. Population (0 = CZ, 1 = RO) was centred, hence the main age and lifespan effects represent average effects across populations.

Parameter	Estimate	SE	*t*	*P*	Estimate	SE	*t*	*P*
	Males, both populations *n = 479 individuals, 703 observations*				*Females, both populations* *n = 413 individuals, 628 observations*			
Age	**63.81**	**3.84**	**16.62**	**<0.001**	**41.77**	**2.77**	**15.08**	**<0.001**
Age^2	**−23.37**	**3.24**	**−7.20**	**<0.001**	**−13.32**	**2.48**	**−5.37**	**<0.001**
Age^3	**9.96**	**3.10**	**3.21**	**0.002**	3.78	2.44	1.55	0.12
Lifespan	**35.27**	**16.66**	**2.12**	**0.035**	−4.75	9.62	−0.49	0.62
Lifespan^2	14.57	15.32	0.95	0.34	4.20	8.98	0.47	0.64
Population	**−4.52**	**1.05**	**−4.31**	**<0.001**	**−2.47**	**0.65**	**−3.82**	**<0.001**
Population × Age	**−21.73**	**9.53**	**−2.28**	**0.024**	2.82	6.29	0.45	0.65
Population × Age^2	14.95	8.55	1.75	0.082	−6.31	6.47	−0.98	0.33
Population × Age^3	−1.91	8.46	−0.23	0.82	6.60	6.52	1.01	0.31
Population × Lifespan	−8.42	44.08	−0.19	0.85	5.40	22.96	0.24	0.81
Population × Lifespan^2	14.11	39.55	0.36	0.72	−17.03	21.71	−0.78	0.43
	Males, CZ *n = 361 individuals, 527 observations*				*Females, CZ* *n = 273 individuals, 420 observations*			
Age	**60.42**	**3.69**	**16.35**	**<0.001**	**33.82**	**2.74**	**12.34**	**<0.001**
Age^2	**−23.59**	**2.91**	**−8.11**	**<0.001**	**−9.91**	**2.23**	**−4.44**	**<0.001**
Age^3	**9.51**	**2.71**	**3.51**	**<0.001**	1.68	2.15	0.78	0.44
Lifespan	**35.08**	**16.05**	**2.19**	**0.030**	−4.37	9.34	−0.47	0.64
Lifespan^2	9.48	14.73	0.64	0.52	7.97	8.57	0.93	0.35
	Males, RO *n = 118 individuals, 176 observations*				*Females, RO* *n = 140 individuals, 208 observations*			
Age	**23.54**	**3.54**	**6.64**	**<0.001**	**24.88**	**2.50**	**9.95**	**<0.001**
Age^2	**−7.34**	**2.75**	**−2.67**	**0.010**	**−10.17**	**2.09**	**−4.85**	**<0.001**
Age^3	2.94	2.52	1.17	0.25	2.99	2.02	1.48	0.14
Lifespan	7.86	11.94	0.66	0.51	0.94	9.12	0.10	0.92
Lifespan^2	8.81	11.27	0.78	0.44	−3.36	8.53	−0.39	0.70

Table 2. Logistic discrete-time hazard models testing whether adult tail length in the first year predicts survival throughout life. Death was included as a binary dependent variable. Population (0 = CZ, 1 = RO) and year (as dummy variables) were centred, hence the main effects of tail length represent average effects across populations.

Parameter	Estimate	SE	*z*	*P*	Estimate	SE	*z*	*P*
	Males, both populations *n = 499 individuals, 697 observations*				*Females, both populations* *n = 441 individuals, 666 observations*			
Year	4.12	2.39	1.73	0.084	**7.63**	**2.57**	**2.97**	**0.003**
Year^2	**−5.88**	**2.39**	**−2.46**	**0.014**	**−5.26**	**2.52**	**−2.09**	**0.037**
Age 2	**−0.42**	**0.20**	**−2.11**	**0.035**	−0.33	0.20	−1.62	0.11
Age 3	−0.01	0.36	−0.02	0.98	**−0.96**	**0.30**	**−3.16**	**0.002**
Age 4					0.17	0.46	0.38	0.70
Tail length	−2.46	2.58	−0.95	0.34	2.14	2.31	0.93	0.35
Tail length^2	4.52	2.53	1.79	0.073	2.71	2.31	1.17	0.24
Population	−0.06	0.24	−0.27	0.79	**−0.41**	**0.20**	**−2.11**	**0.035**
Population × Year	0.51	6.95	0.07	0.94	−7.81	5.87	−1.33	0.18
Population × Year^2	11.93	7.01	1.70	0.089	2.17	5.73	0.38	0.70
Population × Age 2	**0.99**	**0.48**	**2.06**	**0.039**	−0.10	0.42	−0.24	0.81
Population × Age 3	**2.47**	**1.14**	**2.18**	**0.029**	0.60	0.63	0.95	0.34
Population × Age 4					1.41	0.99	1.42	0.15
Population × Tail length	4.51	7.41	0.61	0.54	−6.27	4.75	−1.32	0.19
Population × Tail length^2	−4.89	6.12	−0.80	0.42	−0.49	4.83	−0.10	0.92
	Males, CZ *n = 384 individuals, 551 observations*				*Females, CZ* *n = 282 individuals, 425 observations*			
Year	**4.41**	**2.14**	**2.06**	**0.039**	**9.51**	**2.25**	**4.23**	**<0.001**
Year^2	**−7.67**	**2.14**	**−3.58**	**<0.001**	**−5.02**	**2.26**	**−2.22**	**0.026**
Age 2	**−0.65**	**0.22**	**−2.89**	**0.004**	−0.29	0.26	−1.15	0.25
Age 3	**−0.58**	**0.34**	**−1.72**	**0.086**	**−1.17**	**0.38**	**−3.08**	**0.002**
Age 4	−0.69	0.53	−1.31	0.19	−0.34	0.53	−0.63	0.53
Tail length	−1.77	2.26	−0.78	0.43	3.94	2.26	1.74	0.081
Tail length^2	**4.81**	**2.35**	**2.04**	**0.041**	2.28	2.27	1.00	0.32
	Males, RO *n = 115 individuals, 162 observations*				*Females, RO* *n = 159 individuals, 241 observations*			
Year	2.73	2.17	1.26	0.21	0.22	2.18	0.10	0.92
Year^2	1.12	2.25	0.50	0.62	−1.62	2.12	−0.77	0.44
Age 2	0.34	0.42	0.80	0.42	−0.39	0.33	−1.17	0.24
Age 3	1.89	1.08	1.74	0.081	−0.58	0.50	−1.15	0.25
Age 4					1.07	0.84	1.28	0.20
Tail length	0.14	2.15	0.07	0.95	−1.60	2.07	−0.77	0.44
Tail length^2	0.30	2.11	0.14	0.89	1.30	2.11	0.62	0.54

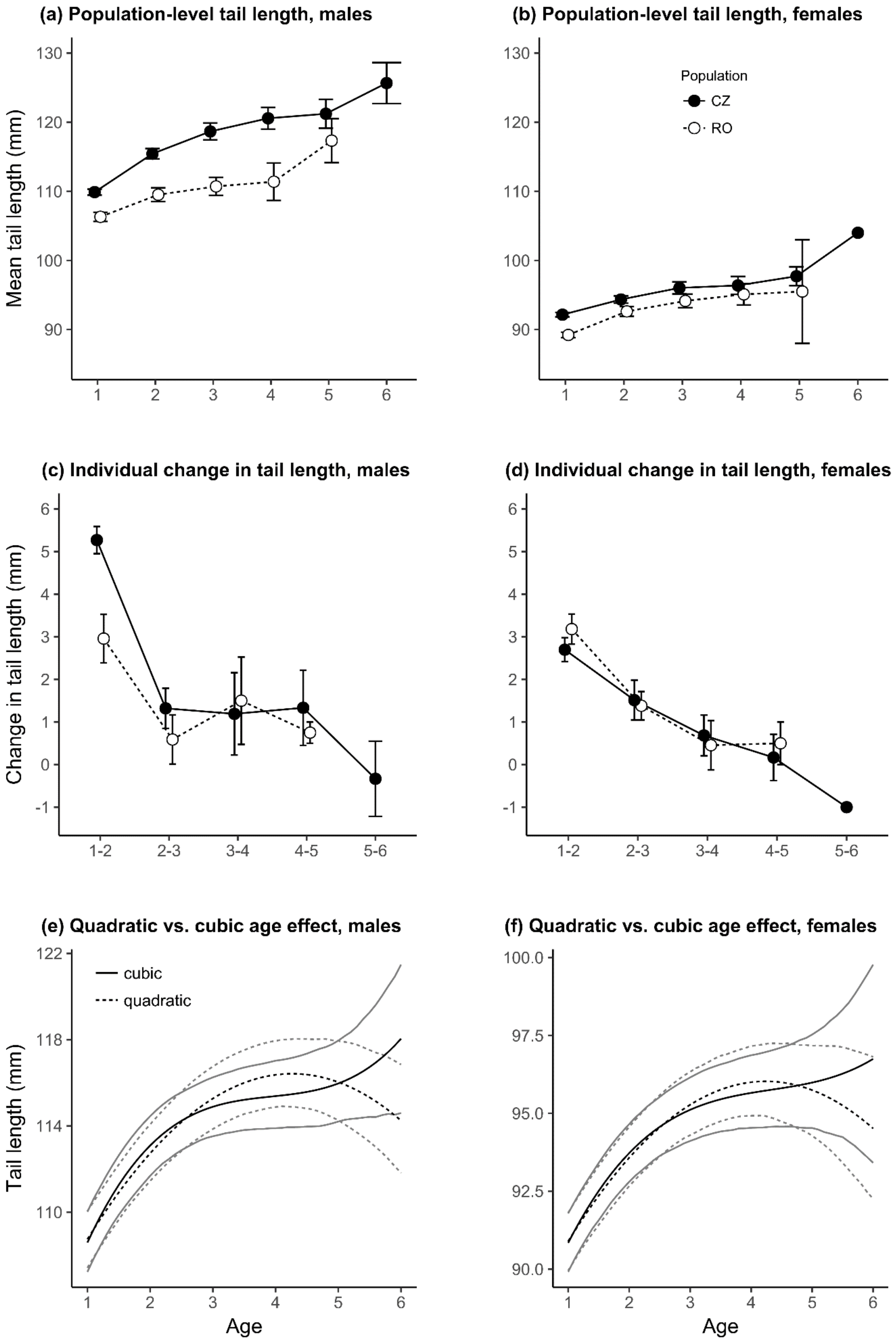

Figure 1. Age-related changes in tail length. Error bars denote standard errors of the mean (a-c) and grey lines 95% confidence intervals (e-f).

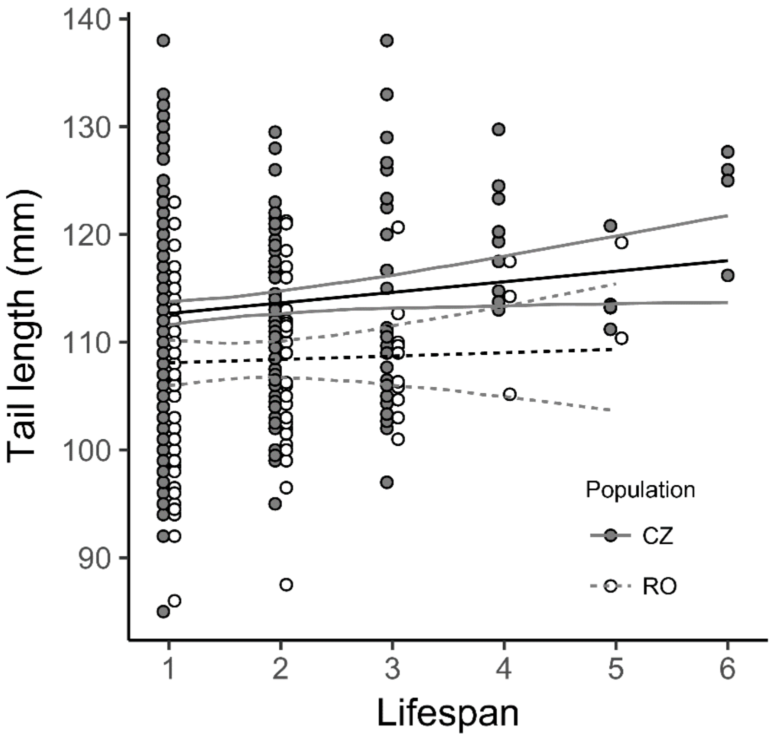

Figure 2. Relationship between tail length and lifespan. Lines are predicted values from the joint model with 95% confidence intervals.

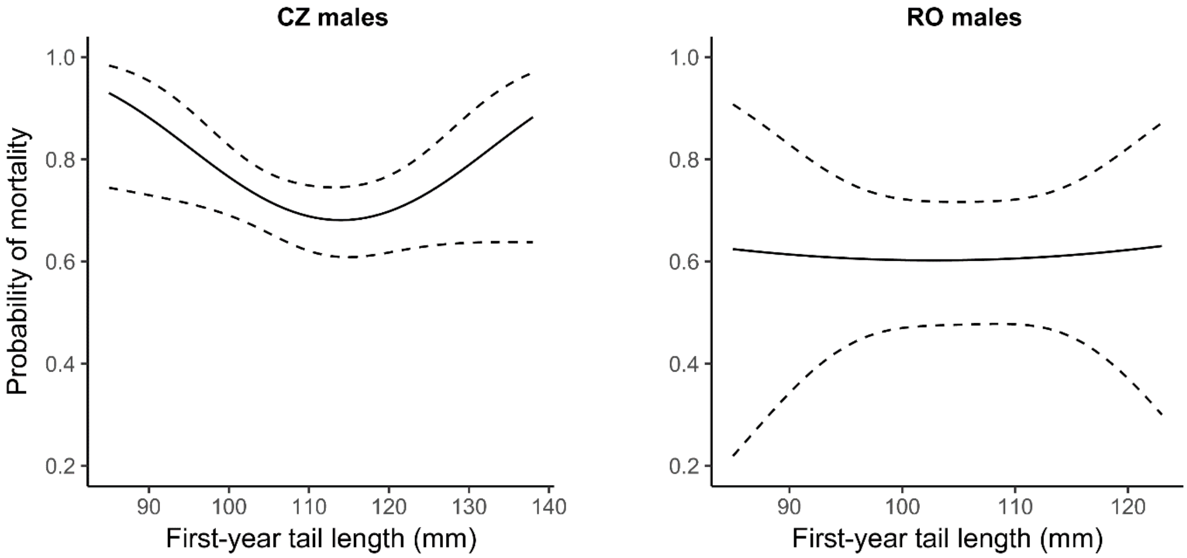

Figure 3. Probability of adult male mortality related to tail length in the first year of life. Shown are predicted values with 95% confidence intervals from the population-specific models from Table 2.

Supporting Information

for

Age-dependence and viability signalling function of tail streamer length in the European barn swallow

Oldrich Tomasek, Peter L. Pap, Marie Adamkova, Jaroslav Cepak, Attila Fulop, Romana Michalkova, Alexandru N. Stermin, Csongor I. Vagasi, Orsolya Vincze, Tomas Albrecht

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Table S1. Candidate logistic discrete-time hazard models with different parametrisation calendar year. Compared are models including calendar year as either a factor or a continuous variable with linear, quadratic or cubic effect. All the models included population, age as a factor, quadratic term for tail length in the first year of life and two-way interactions of population with all the other main effects (see also Table 2 in the main text).

Sex	Year	Parameters	logLik	AICc	ΔAICc	Weight
Males						
	quadratic	14	−438.9	906.4	0.00	0.795
	cubic	16	−438.7	910.1	3.73	0.123
	factor	19	−436.0	911.2	4.75	0.074
	linear	12	−445.7	915.8	9.35	0.007
Females						
	quadratic	16	−429.0	890.9	0.00	0.493
	linear	14	−431.8	892.2	1.31	0.255
	cubic	18	−427.8	892.7	1.75	0.206
	factor	21	−426.1	895.6	4.72	0.046

Table S2. Linear mixed-effect model estimating effects of age, selective disappearance and ornament manipulations on tail length in Czech males

Parameter	Estimate	SE	*t*	*P*
Age	**62.82**	**4.94**	**12.72**	**<0.001**
Age^2	**−26.56**	**3.31**	**−8.02**	**<0.001**
Age^3	**10.24**	**2.81**	**3.65**	**<0.001**
Lifespan	**35.85**	**16.06**	**2.23**	**0.027**
Lifespan^2	10.28	14.73	0.70	0.49
Tail manipulation				
None	0.27	0.63	0.43	0.67
Elongation	−1.03	0.85	−1.21	0.23
Shortening	−0.56	0.70	−0.80	0.42
Ventral colour darkening	1.25	0.95	1.31	0.19

Table S3. Logistic discrete-time hazard models testing whether adult tail length in the first year predicts survival throughout life, while controlling for ornament manipulations. Death was included as a binary dependent variable.

Parameter	Estimate	SE	z	P
Year	3.84	2.42	1.59	0.11
Year^2	**−9.00**	**2.24**	**−4.02**	**<0.001**
Age 2	**−0.63**	**0.23**	**−2.78**	**0.005**
Age 3	−0.55	0.34	−1.60	0.11
Age 4	−0.61	0.54	−1.14	0.25
Tail length	−0.75	2.43	−0.31	0.76
Tail length^2	4.60	2.39	1.92	0.055
Tail manipulation				
None	0.45	0.32	1.38	0.17
Elongation	0.46	0.27	1.67	0.09
Shortening	0.04	0.31	0.13	0.90
Ventral colour darkening	−0.35	0.36	−0.98	0.33

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 19 May 2016
doi: 10.3389/fevo.2016.00056

Signal Traits and Oxidative Stress: A Comparative Study across Populations with Divergent Signals

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Diverging populations often shift patterns of signal use—a process that can contribute to reproductive isolation and speciation. Yet it is not clear why most traits gain or lose signal value during divergence. One reason this could occur is because changes in the relationship between signals and relevant physiological parameters degrade the reliability of a signal, or even change its underlying information content. Here we test the hypothesis that the relationship between signal trait elaboration and a central component of organismal health—oxidative stress—differs across closely related populations that have diverged in signal use and preferences. In the recently diverged barn swallow subspecies complex (*Hirundo rustica,* Family: Hirundinidae), different populations use different traits as sexual signals. Two of these traits, ventral breast plumage color, and tail streamer length, differ markedly between North American *H. r. erythrogaster* and European *H. r. rustica.* Despite this divergence, variation in ventral plumage color was similarly associated with measures of oxidative damage across both populations. However, the directionality of these relationships differed between the sexes: darker male barn swallows had higher levels of plasma oxidative damage than their lighter counterparts, while the opposite relationship was seen in females. In contrast, relationships between tail streamer length and measures of oxidative stress were not consistent across populations. Some analyses indicated that in European *H. r. rustica,* where males bearing elongated streamers are preferred as mates, longer-streamered males were more oxidatively stressed; however, the opposite pattern was suggested in North American *H. r. erythrogaster.* Tail streamer length was not associated with measures of oxidative stress in females of either population. Differences in the physiological state of stronger signalers across populations and between the sexes may be related to costs or constraints on signal elaboration (e.g., biochemical pathways associated with melanogenesis), or reflect differences in how signal-mediated social interactions influence oxidative stress. Overall, our results suggest that while some phenotypic traits appear to be capable of conveying similar physiological information regardless of their use as signals, divergence in other phenotypic traits may be associated with shifts in their information content.

Keywords: sexual selection, social selection, speciation, physiology, antioxidants, barn swallows, *Hirundo rustica*

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 16 December 2015
Accepted: 03 May 2016
Published: 19 May 2016

Citation:
Vitousek MN, Tomášek O, Albrecht T, Wilkins MR and Safran RJ (2016) Signal Traits and Oxidative Stress: A Comparative Study across Populations with Divergent Signals. Front. Ecol. Evol. 4:56. doi: 10.3389/fevo.2016.00056

INTRODUCTION

Signal traits are aspects of behavior, morphology, or physiology, that advertise information about how their bearer is likely to perform in a competitive context (Zahavi, 1975; Schluter and Price, 1993). Because the physiological state of an individual is often a crucial aspect of performance, signals that are causally linked with physiological measures can provide honest information about performance in relevant environmental and social contexts (Lailvaux and Irschick, 2006; Vitousek et al., 2014). Signal development and display can be associated with physiological state through many mechanisms. Some signals are costly to produce or display, so their elaboration may provide information about which individuals are able to bear these costs. As such, experimental manipulations have shown causal links between many aspects of physiological state and signal elaboration, including endocrine levels and the capacity to resist oxidative threats (Evans et al., 2000; McGraw et al., 2002; Blount et al., 2003; Alonso-Alvarez et al., 2004; Safran et al., 2008). Signals can also influence the physiological state of their bearer through social mechanisms (Rubenstein and Hauber, 2008; Oliveira, 2009; Vitousek et al., 2014). In situations where signals are tested (e.g., during competition), bearing a signal that does not equate with underlying competitive ability may result in "social persecution" that induces physiological costs (Rohwer, 1977; Tibbetts and Izzo, 2010). In contrast, if signals are honestly reflective of competitive ability, then social feedback about relative signal quality could induce positive changes in physiological state by altering hormone levels or other physiological mediators (Vitousek et al., 2013, 2014).

A central question in sexual selection and speciation concerns why populations diverge in signal phenotypes and preferences. If diverging populations are exposed to new environmental and social contexts, then shifts in signal use could result from different signals being most informative in these novel contexts (Schluter and Price, 1993). While substantial research has addressed the potential for signal efficacy to change across ecological contexts (Boughman, 2002; Tobias et al., 2010; Seehausen, 2015), less is known about how the information content of signals changes during divergence (Hebets and Papaj, 2005). Signal use could change because a different physiological state is optimal in different environments—and this information is most accurately conveyed by a different signal trait—or because the same optimal phenotypic state is best conveyed by different traits in each population. Alternatively, the physiological correlates of divergent phenotypic traits could differ in ways that are unrelated to their use as signals.

While many aspects of physiological state may play a role in sexual signaling, and in organismal function more broadly, oxidative stress is believed to be a particularly important element. Oxidative stress occurs when pro-oxidants—which can be generated by a number of oxidative processes including metabolism and immune activation—overwhelm antioxidant defenses and damage important biological macromolecules including DNA, proteins, and lipids (Costantini, 2008; McGraw et al., 2010; Metcalfe and Alonso-Alvarez, 2010). Oxidative stress can also impact senescence, at least in part through its effects on telomere dynamics (Von Zglinicki, 2002; Monaghan and Haussmann, 2006; Haussmann et al., 2011). Previous analyses have found that signal traits are both influenced by and can causally affect antioxidant capacity and oxidative damage (reviewed in Costantini, 2014).

We compared the relationship between diverging morphological traits and measures of oxidative stress in two closely related but recently diverged subspecies of barn swallows (*Hirundo rustica,* Family: Hirundinidae). Recently formed sister taxa are advantageous for comparative studies because, post-speciation, the process of divergence is confounded by further evolutionary changes (Via, 2001; Coyne and Orr, 2004). Recent mtDNA phylogeographic and microsatellite analyses in the barn swallow subspecies complex suggest that this group formed rapidly and is not strongly genetically differentiated, despite marked differentiation in two sexual signals: tail streamer length and the extent of ventral color (Dor et al., 2010). In North American barn swallows (*H. r. erythrogaster*) melanin-based ventral plumage coloration is a sexual signal; males with naturally darker or experimentally darkened plumage gain a higher proportion of paternity in their nests (Safran and McGraw, 2004; Safran et al., 2005). In contrast, while elongated tail streamers do not appear to be preferred by female *H. r. erythrogaster* (Safran and McGraw 2004), this trait is strongly sexually selected in European *H. r. rustica* (Møller, 1988). Males with elongated tails pair more quickly and have higher overall paternity levels (Saino et al., 1997a). Females in both subspecies display slightly reduced versions of the same signal traits used by males (Scordato and Safran, 2014). While the specific way in which ornamental traits are used by female barn swallows is not known, some evidence suggests that these traits may hold signal value. In *H. r. erythrogaster* darker females have greater reproductive success (Safran and McGraw, 2004), and both naturally darker and experimentally darkened females experience less oxidative damage (Vitousek et al., 2013). In *H. r. rustica*, streamer length appears to be under directional selection in females via increased reproductive success (Møller, 1993).

The specific signal traits used by barn swallows could be linked to oxidative stress in many ways. Melanin-based traits like the ventral plumage of barn swallows have been causally linked with oxidative stress in many species (McGraw, 2008; Galván and Alonso-Alvarez, 2009; Costantini, 2014); however, the direction of causation varies, and the mechanisms that generate these relationships are generally not well-resolved (Costantini, 2014). Melanin production may be influenced by oxidative stress, or by an allocation tradeoff between antioxidant defense and signal development (Jawor and Breitwisch, 2003; Moreno and Møller, 2006; Galván and Alonso-Alvarez, 2008; Metcalfe and Alonso-Alvarez, 2010). Melanin-based trait expression may also be coupled with oxidative stress levels through pleiotropic links between melanogenesis and the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Xiao et al., 2003; Ducrest et al., 2008; Jenkins et al., 2013). Depending on the specific pigment type produced (pheomelanins vs. eumelanins), these links may be positive (Galván and Solano, 2009; Galván et al., 2011) or negative (Almasi et al., 2010; Roulin and Ducrest, 2011). Plumage color could also affect oxidative stress levels through other mechanisms, including by influencing thermoregulatory capability (Sirkiä et al., 2010) or predation risk (Galván et al., 2014). Tail streamer length may also be linked with oxidative stress through several pathways. The ability to produce long tail streamers could be impacted by oxidative stress when plumage is replaced annually during seasonal molt. However, it is perhaps more likely that the direct energetic cost of bearing tail streamers longer or shorter than the aerodynamic optimum (Møller et al., 1995; Rowe et al., 2001) would excessively elevate pro-oxidant levels (Dowling and Simmons, 2009).

An alternative but not mutually-exclusive mechanism to explain potential links between signal traits and oxidative stress is that bearing exaggerated signals—regardless of the specific signal type—influences social interactions in ways that alter oxidative stress levels (Vitousek et al., 2013, 2014). For example, if stronger signalers are challenged more or less by conspecifics (Rohwer, 1985; Tibbetts and Dale, 2004), or if mates alter their provisioning investment based on signal quality (Dentressangle et al., 2008; Vitousek et al., 2014), then changes in signal elaboration could indirectly influence measures of oxidative stress. Finally, observed correlations between signal traits and oxidative stress may not be underlain by direct or indirect causal links.

To test whether differences in signal-physiology relationships among subspecies are associated with divergence in mate preferences, we assessed links between signal elaboration and two components of oxidative stress: plasma antioxidant capacity and oxidative damage (reactive oxygen metabolites), in both sexes of *H. r. erythrogaster* (breeding in Colorado, USA), and *H. r. rustica* (breeding in South Bohemia, Czech Republic). If the information content of signals is directly linked with trait type, then we would predict that the physiological correlates of each trait will be consistent across populations (Hypothesis 1: same trait, same information). If a different trait is a better indicator of a preferred physiological state in each population, then we would expect to see the same physiological state predicted by dark ventral coloration in North American *H. r. erythrogaster* males and by tail streamer length in European *H. r. rustica* males (Hypothesis 2: different traits, same information). If both signal-physiology relationships and the physiological state of preferred individuals differ across populations, then we would expect to find varying signal-physiology relationships across subspecies (Hypothesis 3: same trait, different information). Alternatively, signal traits could be unrelated to the measured physiological traits (Hypothesis 4: same trait, no information).

METHODS

Capture and Sampling

Barn swallows were captured with mist nets or by hand at breeding sites in Boulder and Jefferson Counties, Colorado, USA (CO; from May–July of 2010; 76 male and 83 female *H. r. erythrogaster*), and near Luznice, South Bohemia, Czech Republic (CZ; in May and June of 2011; 35 male and 27 female *H. r. rustica*). Blood samples were taken within 3 min. of disturbance and placed on ice for several hours until centrifugation (10 min at 3500 rpm). Plasma was subsequently frozen at −70°C until analysis. Body mass was measured with a Pesola spring balance (males and females in CO; males only in CZ), and the right tail streamer measured to the nearest mm. A sample of 4–6 breast feathers was plucked from the ventral surface, mounted on an index card, and stored in the dark until spectrophotometric analysis (Safran and McGraw, 2004). All capture and handling protocols were approved by the University of Colorado's Animal Care and Use Committee (IACUC # SAF-09-07-01), and by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011), and Charles University in Prague (4789/2008-30).

Feather Color Measurements

The color of melanin-based ventral breast plumage was scored using a reflectance spectrophotometer (Ocean Optics USB4000), according to previously described methods (Safran et al., 2010). Briefly, ambient light was excluded using a metal probe holder placed against the feather sample that ensured a constant distance from the probe to the sample. A fiber-optic probe with a PX-2 pulsed xenon light source at an angle of 90° to the feather surface generated reflectance data relative to a white standard (Ocean Optics WS-1) and a dark standard (for which all light was excluded). During each sampling period, 20 spectra were averaged with an integration period of 200 ms. Each sample of breast feathers was scored three times, and average values were calculated. Previous analyses have indicated that ventral plumage brightness, a heritable trait in barn swallows (Hubbard et al., 2015), is highly correlated with hue and saturation in both populations (CO: Vitousek et al., 2013, CZ: Adámková, Albrecht, and Tomášek, unpublished data); we therefore used brightness alone in analyses.

Analyses of Oxidative Damage and Antioxidant Capacity

As a measure of oxidative damage, we used the d-ROMs kit (Diacron International, Grosseto, Italy) (Costantini et al., 2006, 2009) to assess the concentration of reactive oxygen metabolites (ROMs)—in this case primarily hydroperoxides—that derive from the oxidation of biomolecules. In this test a chromogen mixture of alkyl-substituted aromatic amine reacts with metabolites, inducing a color change proportional to the concentration of metabolites. Plasma samples were added to 200 μL of acetate buffer mixed with 2 μL of chromogen (*N*, *N*-diethyl-*p*-phenylenediamine). After 75 min. of incubation at 37°C, the samples were centrifuged and 190 μL of the supernatant was pipetted onto a microwell plate (Costantini et al., 2011b). The absorbance was read immediately at a wavelength of 505 nm (BioTek Synergy HT; VT, USA). Measured values were calibrated with a reference standard that substituted a calibrator solution of lyophilized serum for plasma, and converted to mM of H_2O_2 equivalents. Intra-assay variability was 7.9% and inter-assay variability was 6.7%.

As a measure of antioxidant capacity, we estimated the total plasma antioxidant barrier (AOC) using the OXY-adsorbent test (Diacron International, Grosseto, Italy). This test quantifies the ability of plasma antioxidants (including proteins, ascorbate, thiols, vitamin E, and carotenoids) to resist oxidation by an endogenously produced oxidant, hypochlorous acid (HOCl). Plasma samples were diluted 1:100 with distilled water, and a 200 μL aliquot of HOCl solution was incubated with 5 μL of the diluted plasma for 10 min at 37°C (Costantini et al., 2011b). Reference standards and blanks were prepared using 5 μL of calibrator solution and water, respectively, and incubated with 200 μL of HOCl. At the end of the incubation, 5 μL of chromogen solution (*N*,*N*-diethyl-*p*-phenylenediamine) was added. The absorbance was read immediately at a wavelength of 505 nm (BioTek Synergy HT), and measured values (expressed in mM of HOCl neutralized per mL of sample) were calibrated with a reference standard that neutralized 350 mM of HOCl/mL. Intra-assay variability was 5.6% and inter-assay variability was 8.9%.

Data Analyses

Data were analyzed using SAS 9.4. General linear models were used to test the predictors of antioxidant capacity and reactive oxygen metabolites. Data from both populations were combined, but separate models were run for males and females. Initial models in males contained the fixed effects: population, body mass, corrected sampling date (see below), breast brightness, streamer length, population × breast brightness, and population × streamer length. Initial models for females contained the same factors, with the exclusion of body mass, which was not measured in CZ females. Fully parameterized models are available as Supplementary Material (Table S1). Final models were identified through backwards elimination of non-significant effects ($p > 0.15$), and are presented here. Data on reactive oxygen metabolites were reciprocally transformed, and model residuals were checked to ensure they conformed to the assumption of normality. Significant interactions between population and signal traits were investigated using separate linear regressions in each population.

Because sampling dates occurred at different times relative to the initiation of the breeding season in the two populations, and it was often not possible to determine the specific reproductive stage of each captured individual, corrected sampling dates were calculated for each individual by calculating the difference between the actual sampling date and the mean sampling date of individuals in that population. Thus, while this measure provides an indication of whether an individual was sampled relatively earlier or later than other individuals in its population, it does not provide information about the breeding stage of an individual that would enable us to assess true seasonal effects.

The data sets on oxidative damage and antioxidant capacity in male barn swallows each contained a single outlier. Because of the potential for these outliers to influence model outcomes, models were run twice: once with the full data set, and once with the single outlier excluded from the full model and throughout the process of backwards elimination. Models of antioxidant capacity run with and without the outlier (a male from CO with a z-score of 3.4) did not differ qualitatively. Models of oxidative damage run with and without the outlier on this measure (a male from CZ with a *z*-score of 5.3) did differ. We present the results from both sets of models here.

RESULTS

Divergent Signaling Phenotypes

As expected, analyses of both traits indicated significant divergence between *H. r. erythrogaster* and *H. r. rustica.* North American *H. r. erythrogaster* males have significantly darker ventral breast coloration ($t = 11.8$, $df = 40.4$, $p < 0.0001$), and shorter tail streamers ($t = 11.1$, $df = 50.4$, $p < 0.0001$), than European *H. r. rustica* males (**Figure 1**). Similar relationships were seen in females: *H. r. erythrogaster* females had darker ventral breast coloration ($t = 9.4$, $df = 38.3$, $p < 0.0001$) and shorter tail streamers ($t = 13.8$, $df = 36.0$, $p < 0.0001$) than their European counterparts (**Figure 1**).

Male Signaling Phenotype and Measures of Oxidative Stress

In male barn swallows, plasma antioxidant capacity was significantly predicted by corrected sampling date (males sampled earlier in the season had higher antioxidant capacity), and by an interaction between population and streamer length (**Table 1**, Table S1; final model all males: $F_{(5,\ 100)} = 546.76$, $n = 105$, $p < 0.0001$; final model outlier removed: $F_{(5,\ 99)} = 619.28$, $n = 105$, $p < 0.0001$). Population-specific regressions of significant interactions revealed that in North American *H. r. erythrogaster,* males with shorter streamers have a higher antioxidant capacity (outlier removed: $F_1 = 4.32$, $p = 0.042$), whereas in European *H. r. rustica* streamer length does not predict antioxidant capacity ($F_1 = 0.14$, $p = 0.714$; **Figure 2**).

When all individuals are included in the analysis, oxidative damage was significantly predicted by breast brightness (**Figure 3**; higher in darker birds), and by the interaction between population and streamer length (**Table 1**, Table S1; final model: $F_{(6,\ 85)} = 833.21$, $n = 91$, $p < 0.0001$). Population-specific regressions indicated that in North American *H. r. erythrogaster*, streamer length was unrelated to oxidative damage (**Figure 2**; $F_1 = 2.32$, $p = 0.133$), whereas European *H. r. rustica* males with longer streamers have greater oxidative damage ($F_1 = 4.37$, $p = 0.045$). However, when the single outlier is excluded, oxidative damage remains significantly higher in darker birds, but the interaction between streamer length and population is no longer significant [**Table 1**, Table S1; final model: $F_{(7,\ 83)} = 791.78$, $n = 90$, $p < 0.0001$].

Female Signaling Phenotype and Measures Of Oxidative Stress

In female barn swallows, plasma antioxidant capacity was not significantly predicted by any of the morphological traits measured, but females measured earlier in the season had higher antioxidant capacity [**Table 1**; final model: $F_{(3,\ 93)} = 580.58$, $n = 96$, $p < 0.0001$]. The final model of oxidative damage includes breast brightness alone; darker females had significantly lower levels of oxidative damage in both populations [**Table 1**, **Figure 3**; final model: $F_{(2,\ 86)} = 1650.11$, $n = 88$, $p < 0.0001$].

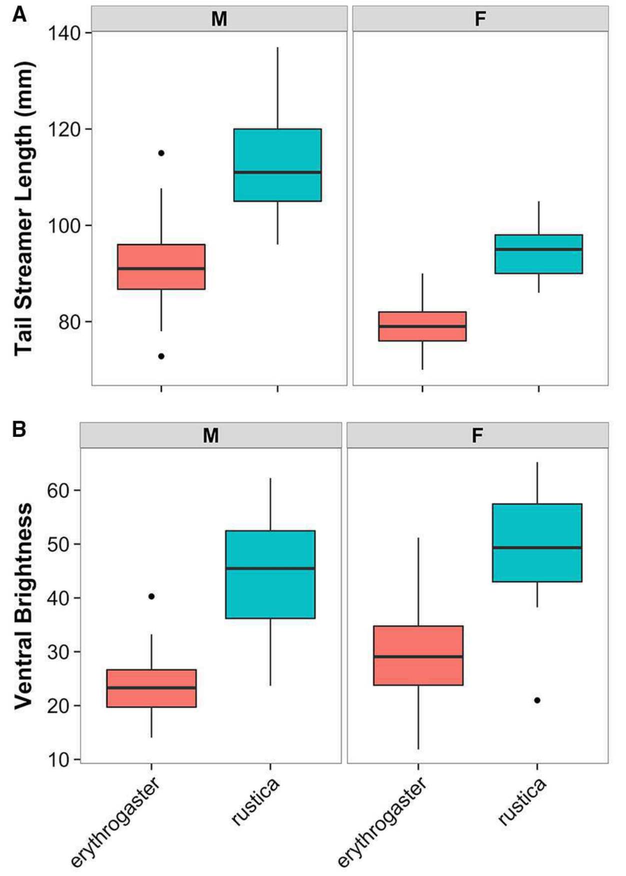

FIGURE 1 | The elaboration of sexual traits in divergent populations of barn swallows. (A) North American *H. r. erythrogaster* (red) have significantly shorter tail streamers than European *H. r. rustica* (blue) in both males and females. **(B)** *H. r. erythrogaster* of both sexes also have darker ventral breast plumage than *H. r. rustica.*

TABLE 1 | Final GLMs of antioxidant capacity and oxidative damage in male and female barn swallows.

Model	Parameter Estimate	*F*	*P*
ANTIOXIDANT CAPACITY: MALES			
Population	147.50	2.60	0.110
Corrected sampling date	**−0.790**	**6.58**	**0.012**
Streamer length	0.328	2.19	0.142
Population*streamer length	**−1.950**	**4.82**	**0.030**
ANTIOXIDANT CAPACITY—OUTLIER REMOVED: MALES			
Population	136.55	2.51	0.116
Corrected sampling date	**−0.651**	**4.96**	**0.028**
Streamer length	0.308	2.10	0.151
Population*streamer length	**−1.815**	**4.68**	**0.033**
OXIDATIVE DAMAGE: MALES			
Population	0.433	2.42	0.124
Corrected sampling date	0.002	2.60	0.111
Streamer length	0.003	0.00	0.952
Breast brightness	**−0.005**	**7.91**	**0.006**
Population*streamer length	**−0.006**	**4.48**	**0.037**
OXIDATIVE DAMAGE—OUTLIER REMOVED: MALES			
Population	0.435	2.11	0.150
Corrected sampling date	**0.002**	**4.39**	**0.039**
Streamer length	0.001	0.67	0.416
Breast brightness	**−0.003**	**9.39**	**0.003**
Population*streamer length	−0.004	2.13	0.148
Population*breast brightness	−0.006	3.18	0.078
ANTIOXIDANT CAPACITY: FEMALES			
Corrected sampling date	−1.37	17.42	**<0.001**
Streamer length	0.901	2.42	0.123
OXIDATIVE DAMAGE: FEMALES			
Breast brightness	**0.003**	**8.99**	**0.004**

Significant factors are bolded.

DISCUSSION

During divergence, shifts in signal use could occur because changes in signal-physiology relationships degrade signal reliability, or alter or eliminate its underlying information content (Schluter and Price, 1993). Yet while closely related species often differ in signal use, little is known about how the information content of signals changes during divergence. Our analyses indicate that in diverging populations of barn swallows, central components of physiological state (oxidative damage and plasma antioxidant capacity) are predicted by variation in the elaboration of two distinct plumage traits. While one of these signal traits (ventral color) is similarly associated with oxidative damage across populations, the other (tail streamer length) may not be.

The comparative approach utilized here does not enable us to determine whether signal traits are causally linked with measures of oxidative stress, or whether the observed relationships represent either a spurious relationship or result from both traits being uni-directionally influenced by a third unknown factor. Previous experiments, however, have found that both plumage color and tail length causally influence measures of oxidative stress and other physiological traits in barn swallows (Saino et al., 1997b,c; Safran et al., 2008; Vitousek et al., 2013). Whether these causal links are present across trait types, populations, and the sexes is not known, and the specific mechanisms generating these links are not well-understood. The observational data presented here do, however, provide some insight into the mechanisms that could potentially generate these links.

Plumage Brightness

Ventral plumage color, a trait that is sexually selected in North American *H. r. erythrogaster* (Safran et al., 2005) but is not predictive of pairing success (clutch initiation date) in European *H. r. rustica* (Wilkins et al., unpublished data), is similarly associated with measures of oxidative stress in males of both populations. Males with darker ventral coloration have higher levels of oxidative damage, but do not differ in antioxidant capacity. Comparative data on plumage color signals in male barn swallows are therefore consistent with our first hypothesis: that the physiological correlates of a specific trait will remain constant across diverging populations (same trait,

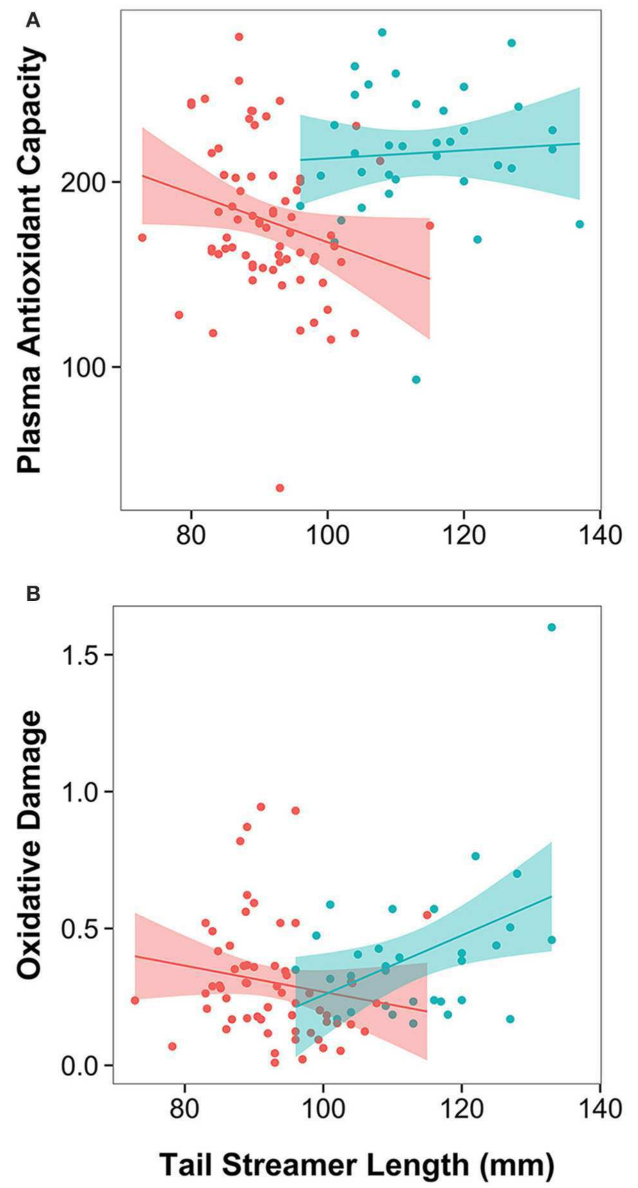

FIGURE 2 | Male tail streamer length is differently associated with measures of oxidative stress in recently diverged populations that differ in signal use. Regressions of tail streamer length on: **(A)** Plasma antioxidant capacity (mM HOCl neutralized), and **(B)** oxidative damage (mM H_2O_2 equivalents) in North American *H. r. erythrogaster* (red symbols and regression line) and European *H. r. rustica* (blue symbols and regression line). Shading represents the 95% confidence interval for the linear regression.

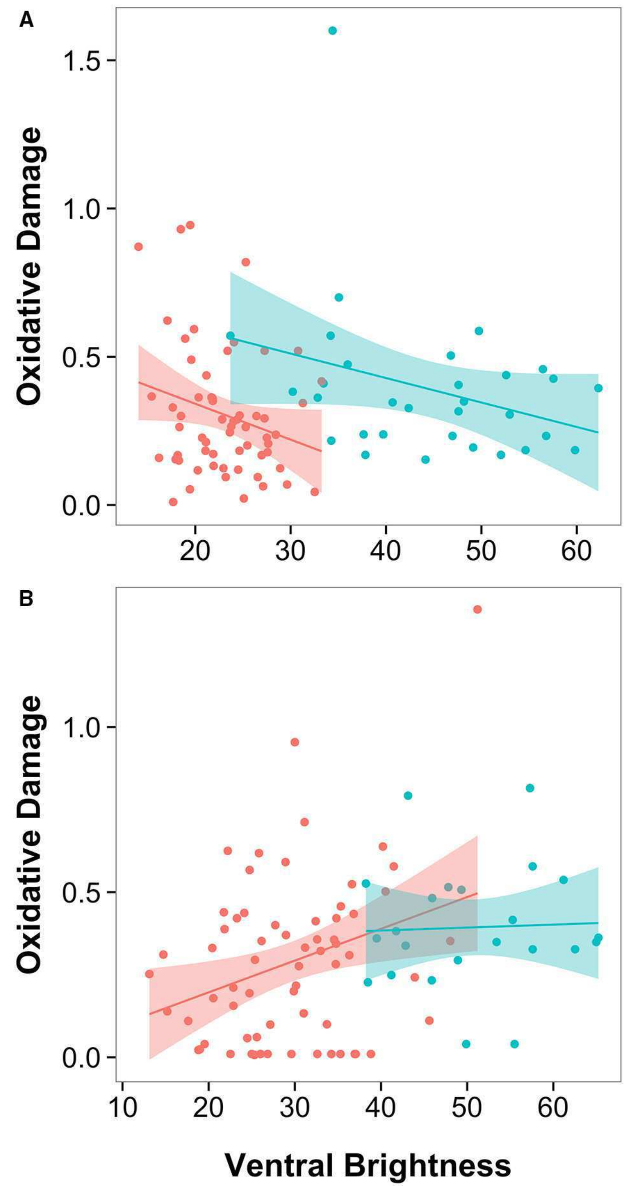

FIGURE 3 | Plumage brightness is oppositely related to plasma oxidative damage in male and female barn swallows: (A) Darker males (those with lower plumage brightness scores) have higher plasma oxidative damage (mM H_2O_2 equivalents) across both populations, (B) Darker females have lower oxidative damage. Simple regressions shown, with *H. r. erythrogaster* in red and *H. r. rustica* in blue. Shading represents the 95% confidence interval for the linear regression.

same information hypothesis). Intriguingly, while females also showed a consistent relationship between ventral plumage color and oxidative damage across populations, the direction of this relationship differed: in both North American *H. r. erythrogaster* and European *H. r. rustica,* darker females had less oxidative damage.

While we do not test the mechanisms underlying these relationships, the observed patterns provide some clues about the nature of the links between oxidative stress and plumage color. Because the plumage of these long-distance migrants is developed on the wintering grounds, long before the reproductive period, persistent links between signals and oxidative stress seem more likely to be driven by either fundamental constraints on system functionality (Hill, 2011), or by causal effects of trait display on oxidative stress (Vitousek et al., 2013). Similarly, the elevated levels of oxidative damage in darker male barn swallows is not consistent with trait elaboration providing information about the ability of an individual to resist challenges and withstand oxidative threats (von Schantz et al., 1999; Jawor and Breitwisch, 2003). The contrasting patterns found in males and females may, however, be consistent with the hypothesis that specific types of melanic pigments are differently linked with susceptibility to stress (Galván and Solano, 2015). While barn swallow plumage contains both eumelanins and pheomelanins, at least in *H. r. erythrogaster,* pheomelanin content appears to play a stronger role in male coloration than in female coloration (McGraw et al., 2005). Male ventral breast plumage contains more pheomelanins (but not eumelanins) than female plumage, and in males, but not females, the ratio of eumelanins to pheomelanins significantly predicts plumage coloration (McGraw et al., 2005). It is not known whether the sexes differ similarly in pigment composition in *H. r. rustica,* as the pigmentary basis of breast feathers has not been assessed (but see McGraw et al., 2004; Saino et al., 2013b).

Opposite relationships between these two types of melanic pigments and oxidative stress could occur through pleiotropic links between melanogenesis and the activity of the hypothalamic-pituitary-adrenal (HPA) axis—which often increases reactive oxygen metabolites (Haussmann and Marchetto, 2010; Costantini et al., 2011a). Pheomelanin production is influenced by agouti-related signaling protein, which also stimulates HPA axis activity (Xiao et al., 2003). Thus, darker pheomelanic individuals may be more sensitive to stressors (Galván and Alonso-Alvarez, 2011; Roulin and Ducrest, 2011; Saino et al., 2013a; Galván and Solano, 2015). In contrast, eumelanin pigment production is increased by melanin-stimulating hormone, which also binds to receptors in the hypothalamus that decrease HPA axis activity during the hormonal stress response (Racca et al., 2005; Ducrest et al., 2008). Thus, darker eumelanic individuals are predicted to display weaker hormonal stress responses (Almasi et al., 2010; Roulin and Ducrest, 2011)—and likely also lower levels of oxidative stress. Differences in the costs or trade-offs associated with producing these two pigment types could also be driven by other mechanisms. Pheomelanin production depends on the potent antioxidant glutathione; thus, the diversion of glutathione to pheomelanogenesis may be costly in organisms facing oxidative threats (Costantini, 2014). In contrast, eumelanogenesis can be inhibited by glutathione (Galván and Alonso-Alvarez, 2008, 2009; Hõrak et al., 2010). Thus, it is possible that, through any of several mechanisms, the consistent sex differences across populations in the directionality of the relationship between plumage brightness and oxidative stress are driven by differences in the costs or constraints of producing pheomelanic vs. eumelanic pigments.

Previous experiments in female *H. r. erythrogaster,* however, suggest that plumage coloration is causally linked with oxidative damage. Females manipulated to display darker plumage rapidly decrease plasma oxidative damage, adopting levels similar to naturally darker birds (Vitousek et al., 2013). While causal links between plumage color and oxidative damage have not been examined in males, or in *H. r. rustica, erythrogaster* males manipulated to display darker feathers rapidly decrease both testosterone and body mass (Safran et al., 2008), patterns that could be consistent with elevated levels of oxidative stress (Alonso-Alvarez et al., 2007; Costantini, 2014). Thus, at least in *H. r. erythrogaster,* plumage coloration appears to influence aspects of physiological state. The observed relationships between plumage color and oxidative damage in this study could be generated by consistent sex differences in the direct costs or benefits of displaying darker plumage—for example, if darker males suffer a thermoregulatory cost on the wing, while darker females are better able to retain heat during incubation (Sirkiä et al., 2010). Alternatively, plumage color may influence social interactions or reproductive effort in ways that alter oxidative stress (Vitousek et al., 2013, 2014). For example, if darker males invest more in costly reproductive behaviors this could increase their oxidative stress. This mechanism would be most likely to generate the observed patterns if, despite divergence of ventral coloration between the subspecies, this trait holds signal value in both populations. In *H. r. rustica,* ventral color, which is substantially lighter than in *H. r. erythrogaster* (**Figure 1**), does not predict the onset of breeding in males or females (Wilkins et al., unpublished data), as it does in *H. r. erythrogaster* (Safran and McGraw, 2004; Safran et al., 2005). However, it is not known whether in *H. r. rustica*, males with darker ventral color are preferred as extra-pair mates, or whether this trait plays a role in mediating other social interactions during or outside of the reproductive period. Experimental work in other systems has found that the value of the resource held by a signaler, or receiver motivation, can influence the likelihood that a signal will be tested (Tibbetts, 2008). If, during the breeding season, the plumage signals of male barn swallows are tested (e.g., during mate or nest site defense, or extra-pair mate assessment), while the signals of females are trusted, then we would predict that darker males would have higher levels of oxidative damage, while darker females would have lower oxidative damage—as seen here.

Previous analyses of the relationship between ventral color and susceptibility to stress have yielded mixed results. Ventral color was not related to HPA activity in *H. r. erythrogaster* males during molt or reproduction (Jenkins et al., 2013), suggesting that increased HPA activity in darker pheomelanic individuals is unlikely to drive the observed patterns with oxidative damage. Studies in a European population of *H. r. rustica* suggest that darker males—but not darker females—have lower survival rates (Saino et al., 2013a, but see Galván and Møller, 2013). It is not known, however, whether feather color is causally linked with survival in this population, and if so, whether this occurs through direct costs or constraints on pheomelanin production (Galván et al., 2011), or from physical or social costs of signal display.

Elucidating the direction and nature of causal links between ventral color and oxidative damage will require further experiments into the development and use of these traits within and across diverging populations. Additionally, as measures of oxidative stress can be highly labile, future studies that test whether the observed relationships differ across life history stages or environments, or represent context-dependent life history trade-offs (Beaulieu et al., 2015), could shed light on how and when diverging signals may convey specific, reliable information. Because we were not able to determine the reproductive sub-stage of individuals in the study, it is possible that differences between individuals, or between populations, in sample timing influenced the observed patterns. Future analyses that incorporate the specific reproductive stage of individuals—ideally as part of a repeated sampling design—could provide insight into the presence and nature of context-dependent links between signals and physiological state both within and among populations. In this analysis, we focused on a single region of ventral plumage color, the breast, as breast brightness has been shown to be strongly and causally associated with reproductive success in *H. r.* erythrogaster (Safran and McGraw, 2004; Safran et al., 2005). However, other plumage signals, including throat coloration, may hold signal value in *H. r. rustica* (Perrier et al., 2002; Wilkins et al., unpublished data). Some evidence also suggests that the relative roles of eumelanin- and pheomelanin-based pigments in determining plumage color differ between plumage patches (Saino et al., 2013b). Future analyses should be expanded to encompass other plumage traits that may hold signal value, and their pigmentary basis, and include additional key measures of physiological state and social interactions.

Tail Streamer Length

In contrast to the patterns observed with ventral color, comparative data on tail streamer length suggests that this trait is associated with different physiological information in males of these two subspecies. In European *H. r. rustica,* longer-streamered males did not differ in antioxidant capacity, but the analysis from the full data set indicated that they had greater levels of oxidative damage than shorter-streamered males. Thus, where tail streamer length is under positive sexual selection, males with longer streamers appear to experience higher levels of oxidative stress. In contrast, in North American *H. r. erythrogaster,* shorter-streamered males had a higher antioxidant capacity, but streamer length was not associated with oxidative damage. As plasma antioxidant levels are often up-regulated in response to oxidative threats, this pattern could indicate that shorter-streamered *H. r. erythrogaster* males are more oxidatively stressed (Costantini and Verhulst, 2009); however, this pattern could also result from naturally stronger antioxidant defenses in these males. Conclusions related to oxidative damage should be treated with caution due to the significant effect of the single outlier on the model outcomes. However, both models of antioxidant capacity (with and without the outlier) suggest that the physiological state of longer-streamered males differs across these diverging populations.

In male European barn swallows, *H. r. rustica,* sexual selection has resulted in the elongation of streamers past the aerodynamic optimum (Buchanan and Evans, 2000; Rowe et al., 2001). Barn swallows are aerial insectivores and so acquire all of their energetic resources during flight; thus, the apparent increase in measures of oxidative stress in longer-streamered *H. r. rustica* males could result from the increased energetic cost of foraging faced by these males. The lack of a relationship between tail streamer length and measures of oxidative stress in female *H. r. rustica,* who have shorter tail streamers than males (**Figure 1**), is consistent with females being closer to the aerodynamic optimum in this population. The elevated antioxidant levels in shorter-streamered North American *H. r. erythrogaster* males, which are consistent with elevated levels of oxidative stress, are particularly interesting. Previous analyses have indicated that short streamers could fall below the aerodynamic optimum for foraging flight (Buchanan and Evans, 2000; Rowe et al., 2001). Thus, shorter-streamered male *H. r. erythrogaster*—which appear to be preferred as mates by females (Safran et al., in revision)—could face increased foraging costs that translate into elevated oxidative stress. However, while female *H. r. erythrogaster* have even shorter tail streamers than their male counterparts (**Figure 1**), female tail length was unrelated to measures of oxidative stress. This could be because the direct costs of bearing shortened streamers differ in the sexes. For example, the increased wing-loading generated by elongated streamers might present a higher cost to shorter-winged females (Rowe et al., 2001), or differences in the foraging or reproductive behavior of female *H. r. erythrogaster* could select for shorter tail length. It is also possible that the opposing patterns in signal elaboration and physiological state in these two populations are influenced more by the social costs of trait display, or by signal-driven changes in reproductive effort, than by the direct effect of streamer length on aerial efficiency. For example, if tail length does not influence social status or social interactions in female barn swallows—but does in males—then we would expect to see social feedback-induced links between oxidative stress and tail length in males (that differ in directionality between populations) but not in females.

CONCLUSIONS

Our analyses indicate that during the process of divergence, some morphological traits remain consistent indicators of oxidative damage, despite apparent shifts in signal use. Other traits, however, differ in their relationships with physiological state, and thus their potential information content, across diverging populations. While we did not directly assess the mechanisms that link signal traits and oxidative stress in barn swallows, our findings provided some clues about the nature of these links. As populations diverge, the same signals could be associated with different physiological states because of differences in the cost of producing or displaying an ornament across different physical environments (e.g., the same ornament is more costly to display in one environment than another), or because the same signal is differently elaborated in two populations (e.g., tail streamer length above or below the aerodynamic optimum: Rowe et al., 2001). Alternatively, divergent signal-physiology patterns could be a direct result of differences in the way signals are used across populations (e.g., social feedback about signal elaboration influences physiological state in populations where the trait is used as a social signal, but not where it is not). A comprehensive understanding of the relationship between signals and physiological state during divergence will require measuring a much wider variety of traits across multiple populations and contexts, in combination with experimental tests of the diversity of mechanisms that can generate these links. Our results do, however, suggest that the relationships between specific sexual traits and oxidative stress—a central component of organismal health—may be differently altered during divergence.

AUTHOR CONTRIBUTIONS

RS, MV, and TA conceived of the study. MV, OT, and TA collected field data. MV assayed oxidative damage and antioxidant capacity, analyzed the data, and drafted the manuscript. MW aided with analysis and interpretation. All authors contributed substantially to manuscript revisions, and approved the final version of the manuscript for submission.

FUNDING

Research funding was provided by the University of Colorado, and the Ministry of Education, Youth and Sports of the Czech Republic (project LH14045).

ACKNOWLEDGMENTS

We are grateful to Kathy Chmiel, Andrew Flynn, Kate Gloeckner, Joanna Hubbard, Tessa Warner, Rachel Wildrick, Jana Albrechtová, Jaroslav Cepák, Romana Michálková, Pavel Munclinger, and Martina Němcová for assistance with field work, and to Joanna Hubbard and Monica Brandhuber for measuring feather color.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00056

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material for:

Signal traits and oxidative stress: a comparative study across populations with divergent signals.

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Table S1: Full GLMs of antioxidant capacity and oxidative damage in barn swallows.

Model	Parameter Estimate	*F*	*P*
Antioxidant Capacity: Males *(*$F_{8,95}$=334.27, *n*=103, *p*<0.0001*)*			
Population	230.15	**5.12**	**0.026**
Corrected sampling date	-0.727	**4.74**	**0.032**
Body mass	-0.731	0.04	0.833
Streamer length	0.509	1.89	0.173
Breast brightness	0.606	0.11	0.743
Population*streamer length	**-2.259**	**6.19**	**0.015**
Population*breast brightness	-1.617	1.79	0.184
Antioxidant Capacity: Male Outlier Removed *(*$F_{8,94}$=375.95, *n*=102, p<0.0001*)*			
Population	**195.05**	**4.08**	**0.046**
Corrected sampling date	**-0.658**	**4.32**	**0.040**
Body mass	-0.071	0.00	0.983
Streamer length	0.478	1.60	0.209
Breast brightness	0.610	0.06	0.812
Population*streamer length	**-2.037**	**5.61**	**0.020**
Population*breast brightness	-0.937	0.66	0.420
Oxidative Damage: Males *(*$F_{8,83}$=625.02, *n*=91, p<0.0001*)*			
Population	**-0.620**	**4.04**	**0.048**
Corrected sampling date	-0.002	3.29	0.073
Body mass	0.001	0.01	0.930
Streamer length	-0.003	0.00	0.981
Breast brightness	**0.003**	**9.87**	**0.002**
Population*streamer length	**0.006**	**5.04**	**0.027**
Population*breast brightness	0.005	1.87	0.175
Oxidative Damage: Male Outlier Removed *(*$F_{8,82}$=684.64, *n*=90, *p*<0.0001*)*			
Population	-0.436	2.10	0.152
Corrected sampling date	**-0.002**	**4.13**	**0.045**
Body mass	0.001	0.02	0.885
Streamer length	-0.001	0.62	0.432
Breast brightness	**0.002**	**9.28**	**0.003**
Population*streamer length	0.004	2.13	0.149
Population*breast brightness	0.006	2.96	0.089
Antioxidant Capacity: Females *(*$F_{7,89}$=245.79, *n*=96, *p*<0.0001*)*			
Population	-150.73	0.50	0.479
Corrected sampling date	**-1.306**	**12.06**	**0.001**
Streamer length	0.953	2.21	0.141
Breast brightness	-0.837	0.54	0.464
Population*streamer length	1.486	0.43	0.516
Population*breast brightness	0.819	0.50	0.482
Oxidative Damage: Females *(*$F_{7,78}$=453.88, *n*=85, *p*<0.0001*)*			
Population	-0.617	0.88	0.351
Corrected sampling date	-0.001	0.60	0.441
Streamer length	-0.006	0.19	0.668
Breast brightness	-0.001	1.71	0.195
Population*streamer length	0.008	1.46	0.230
Population*breast brightness	-0.002	0.30	0.587

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SCIENTIFIC REPORTS

Received: 09 October 2015
Accepted: 09 March 2016
Published: 22 March 2016

Opposing effects of oxidative challenge and carotenoids on antioxidant status and condition-dependent sexual signalling

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Several recent hypotheses consider oxidative stress to be a primary constraint ensuring honesty of condition-dependent carotenoid-based signalling. The key testable difference between these hypotheses is the assumed importance of carotenoids for redox homeostasis, with carotenoids being either antioxidant, pro-oxidant or unimportant. We tested the role of carotenoids in redox balance and sexual signalling by exposing adult male zebra finches (*Taeniopygia guttata*) to oxidative challenge (diquat dibromide) and manipulating carotenoid intake. As the current controversy over the importance of carotenoids as antioxidants could stem from the hydrophilic basis of commonly-used antioxidant assays, we used the novel measure of *in vivo* lipophilic antioxidant capacity. Oxidative challenge reduced beak pigmentation but elicited an increase in antioxidant capacity suggesting resource reallocation from signalling to redox homeostasis. Carotenoids counteracted the effect of oxidative challenge on lipophilic (but not hydrophilic) antioxidant capacity, thereby supporting carotenoid antioxidant function *in vivo*. This is inconsistent with hypotheses proposing that signalling honesty is maintained through either ROS-induced carotenoid degradation or the pro-oxidant effect of high levels of carotenoid-cleavage products acting as a physiological handicap. Our data further suggest that assessment of lipophilic antioxidant capacity is necessary to fully understand the role of redox processes in ecology and evolution.

Over the last four decades there has been a growing interest in sexually selected traits as honest indicators of individual quality and health[1,2]; however, mechanisms linking ornament expression to individual condition remain elusive[3,4]. Increasingly, redox processes (and oxidative stress in particular) have been hypothesised as providing this link and thereby maintaining honesty in many sexual signals[4–6]. Reactive oxygen and nitrogen species (ROS), the source of oxidative stress and damage, are inevitable and ever-present by-products of an oxidative metabolism. ROS can also arise through immune activation[7] or environmental pollution[8]. The need to prevent oxidative stress and maintain redox homeostasis, therefore, could provide a universal mechanism linking ornament expression to immune activation and other metabolically demanding processes (e.g. stress response, moulting or sperm production)[5,6].

Carotenoids are traditionally considered to be important antioxidants due to their ability to quench ROS *in vitro*[9]. A trade-off in allocation of carotenoids between ornamentation and antioxidant defence has therefore been proposed as a possible mechanism maintaining signalling honesty (the 'allocation trade-off hypothesis')[5,10,11]. The importance of carotenoids as antioxidants *in vivo* has recently been questioned[12–14], however, resulting in the emergence of several alternative hypotheses. The 'protection hypothesis' assumes no carotenoid

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antioxidant function, proposing instead that redox homeostasis is signalled through ROS-induced carotenoid oxidation and cleavage, which results in colour loss that can be prevented by an efficient antioxidant system[12]. An extension of this hypothesis is the 'handicap hypothesis', which proposes that high levels of carotenoids may directly aggravate oxidative stress through pro-oxidant activity of carotenoid cleavage products, thereby posing a direct physiological handicap[15,16]. More recently, the structural and functional similarities between carotenoids and ubiquinones have prompted the hypothesis that synthesis of red carotenoids could occur within the inner mitochondrial membrane (IMM) of hepatocytes, with carotenoids, redox-coupled with ubiquinones, displaying antioxidant and modulatory functions in the electron transport chain. The hypothesis proposes that the synthesis of red carotenoids could be linked to the maintenance of the IMM potential, thereby linking ornament expression not only to redox homeostasis but also to efficacy of mitochondrial respiration (the 'IMM carotenoid oxidation hypothesis')[17].

Clearly, knowledge of the actual role of carotenoids in redox homeostasis is crucial if we are to test hypotheses on the mechanisms ensuring carotenoid-based signalling honesty[18]. Studies into carotenoid antioxidant function *in vivo* have provided mixed results, however, leading to an overall low association of carotenoid levels with antioxidant capacity in a recent meta-analysis[13]. We suggest that the current controversy over carotenoid antioxidant function could stem from the use of inappropriate methods for antioxidant capacity assessment. Due to their lipophilic nature, carotenoids are located in the interior of an organism's lipid bilayers and other lipid compartments. As lipid bilayers (e.g. mitochondrial membranes) are considered a major source of highly damaging ROS[9], carotenoids have great potential to play an important role in redox homeostasis. Most commonly-used antioxidant assays (e.g. FRAP, ORAC, OXY-Adsorbent test, TEAC or TRAP), however, measure antioxidant capacity in aqueous media only, which renders them unsuitable for evaluation of lipophilic antioxidants such as carotenoids[19,20]. With this in mind, we decided to use a novel marker of lipophilic antioxidant capacity, i.e. the ratio of *ZE*- and *EE*-stereoisomeric forms of hydroxyoctadecadienoic acid (HODE). *ZE*- and *EE*-HODE are formed as oxidation products of linoleic acid, with the proportion of *ZE*-HODE to total HODE (*ZE*/tHODE) increasing with higher concentrations of hydrogen-donating lipophilic antioxidants such as vitamin E or coenzyme Q, thereby providing an integrated measure of their activity *in vivo*[21,22]. As carotenoid ROS-scavenging activity is most likely mediated through radical addition rather than hydrogen donation[9,23], carotenoid antioxidant action is probably not measured directly by *ZE*/tHODE. Despite this, the lipophilic nature of this antioxidant-capacity measure makes it far better candidate for detecting a possible effects of carotenoids on an organism's antioxidant system than traditional hydrophilic-based assays.

In this study, our aim was to assess redox-based hypotheses of carotenoid-based signalling honesty by testing the importance of carotenoids for redox homeostasis and the role of redox homeostasis in constraining carotenoid-based signal expression. Using a 2 × 2 factorial design, we analysed the effects of oxidative challenge exerted by diquat dibromide and carotenoid supplementation on beak colouration, circulating carotenoid levels and redox homeostasis in adult zebra finch (*Taeniopygia guttata*) males. Diquat dibromide, a bipyridyl compound known to generate superoxide anions *in vivo* through redox-cycling, has recently been recognised as a convenient oxidative stress inducer in ecological studies[24,25], as well as in laboratory models of Parkinson's disease[26–28]. If redox homeostasis really constrains signal expression, beak colouration should be reduced following diquat treatment. Since a change in oxidative stress intensity can be manifested as either a change in oxidative damage or in antioxidant activity (or, indeed, both together)[29], we predict that carotenoid supplementation will result in (a) a reduction in oxidative damage and/or activity of antioxidants other than carotenoids due to their reduced need[18,29–31] (lowered *ZE*/tHODE ratio) if carotenoids act as antioxidants, (b) an increase in either one or both of these parameters if carotenoids act as pro-oxidants, or (c) no effect on either parameter if carotenoids have no influence on redox homeostasis.

Results

Effect of oxidative challenge and carotenoid intake on ornament expression, plasma carotenoids and body mass.

Diquat-induced oxidative challenge of experimental males resulted in a significant decrease in beak red chroma supporting the role of redox homeostasis in maintenance of signalling honesty (Table 1; Fig. 1a). On the contrary, signal intensity was significantly enhanced following high carotenoid intake. These effects on beak pigmentation were additive with no significant interaction. Analysis of beak hue and UV chroma produced qualitatively similar results showing decreased redness and increased UV reflectance following oxidative challenge and the opposing effect of high carotenoid intake (see Supplementary Table S3).

Similar additive effects were observed in the case of total plasma carotenoids, with free oxidative challenge decreasing and high carotenoid intake increasing circulating carotenoid levels (Fig. 1b).

The treatment factors interacted in their effects on body mass, which increased slightly following either high oxidative load or high carotenoid intake, though this effect was inhibited when both treatments were combined. Change in body mass was not significant in any of the treatment groups, however, when compared to the control group (Tukey's post-hoc test on change scores: $P \geq 0.379$).

Effect of ROS exposure and carotenoid intake on blood redox state.

Interestingly, neither oxidative challenge nor carotenoid intake affected total oxidative damage measured as 8-isoprostane in red blood cells (RBC), though there was a marginally insignificant increase in 8-isoprostane following oxidative challenge. There was, however, a significant increase in RBC hydrogen-donation mediated lipophilic antioxidant capacity (*ZE*/tHODE) in response to oxidative challenge (Fig. 1c). In contrast, increased carotenoid intake provoked the opposite effect, resulting in a significant reduction in *ZE*/tHODE. There was also a significant interaction between the treatments, with substantial inhibition of the diquat-induced increase in *ZE*/tHODE by high carotenoid intake suggesting an *in vivo* antioxidant effect for carotenoids. Hydrophilic plasma antioxidant capacity, measured using

Response variables Predictors	Parameter estimates		Model statistics		Standardised effect size		
	Mean effect	SE	*t*	*P*	b^*	CI 2.5%	CI 97.5%
Beak red chroma							
initial	0.17	0.10	1.64	0.11	0.13	−0.03	0.28
ROS	−0.26	0.05	−4.81	<0.001	−0.73	−1.04	−0.43
CAR	0.50	0.05	9.38	<0.001	1.44	1.13	1.75
ROS × CAR	0.07	0.11	0.61	0.54	0.19	−0.43	0.80
Total plasma carotenoids							
initial	0.08	0.02	4.35	<0.001	0.31	0.17	0.45
ROS	−0.71	0.24	−2.99	0.005	−0.42	−0.70	−0.14
CAR	2.64	0.24	11.08	<0.001	1.56	1.28	1.84
ROS × CAR	0.29	0.48	0.62	0.54	0.17	−0.39	0.74
ZE/tHODE ratio							
initial	−0.36	0.25	−1.44	0.16	−0.06	−0.15	0.02
ROS	0.85	0.04	20.10	<0.001	1.55	1.39	1.71
CAR	−0.57	0.04	−14.88	<0.001	−1.04	−1.18	−0.90
ROS × CAR	−0.42	0.09	−4.86	<0.001	−0.77	−1.08	−0.45
OXY							
initial	0.67	0.13	5.19	<0.001	0.60	0.37	0.83
ROS	20.56	7.29	2.82	0.007	0.64	0.19	1.11
CAR	−1.15	7.05	−0.16	0.87	−0.04	−0.48	0.41
ROS × CAR	4.13	14.17	0.29	0.77	0.13	−0.77	1.03
8-isoprostane in RBC							
initial	0.41	0.09	4.48	<0.001	0.53	0.29	0.76
ROS	1.97	1.04	1.89	0.07	0.43	−0.03	0.90
CAR	1.56	1.06	1.47	0.15	0.34	−0.13	0.81
ROS × CAR	−1.35	2.12	−0.64	0.53	−0.30	−1.24	0.64
Body mass							
initial	1.01	0.11	9.62	<0.001	0.83	0.66	1.00
ROS	−0.02	0.31	−0.06	0.95	−0.01	−0.33	0.31
CAR	−0.26	0.33	−0.80	0.43	−0.14	−0.48	0.21
ROS × CAR	−1.44	0.62	−0.62	0.02	−0.75	−1.40	−0.11

Table 1. Effects of experimental manipulations on beak colouration, plasma carotenoids, body mass and blood redox state. Estimates represent coefficients from linear models with oxidative challenge (ROS) and carotenoid intake (CAR) included as factors and pre-treatment (initial) values as covariates. Low and high factor levels were coded 0 and 1, respectively and centred in order to enable the main effects to be properly interpreted without the need to remove the interaction terms from the models. Proportional variables (i.e. beak red chroma and *ZE*/tHODE ratio) and total plasma carotenoids were normalised using logit and Box-Cox ($\lambda = 0.335$) transformation, respectively. Standardised effect sizes are reported as standardised partial regression coefficients (b^*) from the same models with continuous variables *z*-standardised.

OXY, was also elevated in response to oxidative challenge (Fig. 1d) though, in contrast to lipophilic *ZE*/tHODE, it was not affected by differing carotenoid intake.

Discussion

In our study, oxidative challenge and carotenoid intake had opposing effects on beak ornamentation, plasma carotenoid levels and lipophilic antioxidant capacity in RBC. Despite the considerable adverse effect of oxidative challenge on both signal intensity and circulating carotenoid levels, it only resulted in a marginally insignificant increase in blood oxidative damage. Although blood redox state is usually used as an estimate of the body's overall redox state, we cannot completely rule out elevated oxidative damage in particular organs such as the liver or kidneys[32]. Nevertheless, oxidative challenge elicited a marked increase in both hydrophilic and lipophilic antioxidant capacity, suggesting mobilisation of antioxidants to prevent increasing oxidative damage[24,29]. Since increased antioxidant capacity accompanied with stable (or increased) oxidative damage should be interpreted as higher oxidative stress[29], this result support the pro-oxidant effect of diquat treatment. Importantly, carotenoids counteracted the effect of oxidative challenge on hydrogen-donating lipophilic antioxidants, as demonstrated by substantial inhibition of a diquat-induced increase in *ZE*/tHODE ratio by high carotenoid intake, while having no effect on oxidative damage levels. As radical addition has been proposed as the main carotenoid antioxidant mechanism[9,23], rather than hydrogen donation, which is measured by *ZE*/tHODE[21], the inhibition of the diquat-induced increase in ZE/tHODE by high carotenoid intake probably reflects down-regulation of hydrogen donating lipophilic antioxidants[29] caused by the superoxide arising from diquat redox cycling[33] being effectively

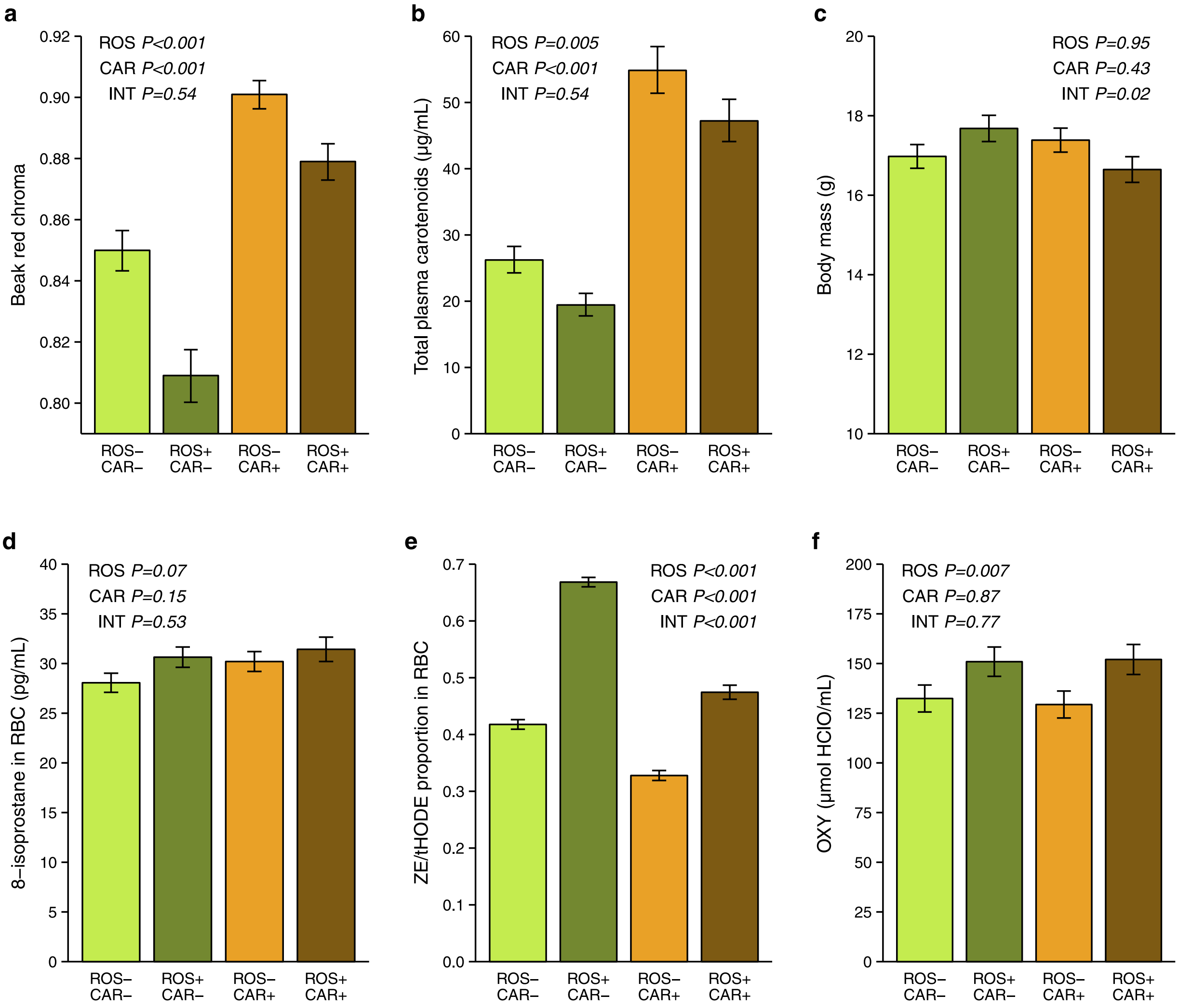

Figure 1. Effects of oxidative load and carotenoid intake on beak colouration, circulating carotenoids, body mass and blood redox state. Birds were exposed to either low (ROS−) or high oxidative load (ROS+) and to either low (CAR−) or high carotenoid intake (CAR+). Bars represent group means controlled for pre-experimental values and their standard errors obtained from the models in Table 1. Shown are model *P*-values for the main effects of oxidative challenge (ROS) and carotenoid intake (CAR), as well as their interaction (INT). (**a**,**b**) Both, beak red chroma (higher values = more saturated red colour) and total plasma carotenoids were reduced by oxidative challenge and enhanced by high carotenoid intake. (**c**) Neither treatment factor had a significant effect on body mass, though their interaction resulted in body mass in ROS + CAR + group that was lower than would be expected if both effects were additive. None of the treatment groups, however, differed from the control (Tukey's post-hoc test on change scores: $P \geq 0.379$). (**d**–**f**) Oxidative challenge elicited a marked increase in both, lipophilic (*ZE*/tHODE) and hydrophilic (OXY) antioxidant capacity, resulting in marginally insignificant increase in blood oxidative damage (8-isoprostane). Carotenoids counteracted the effect of oxidative challenge on the activity of other lipophilic antioxidants (shown as reduced *ZE*/tHODE ratio) while having no effect on oxidative damage levels. On the other hand, hydrophilic antioxidant capacity was unaffected by lipophilic carotenoids.

quenched by the carotenoids[34]. The tendency of an organism to down-regulate other antioxidant mechanisms, rather than to reduce oxidative damage levels, has previously been documented following supplementation of antioxidants such as vitamins C or E[30,31]. As reduced activity of other antioxidants, together with maintenance of stable oxidative damage is suggestive of reduced oxidative stress[29] our data support the antioxidant function of carotenoids *in vivo*.

Despite its substantial effect on lipophilic-based ZE/tHODE, carotenoid intake did not affect the hydrophilic-based OXY assay. The differing responses of these two antioxidant measures clearly demonstrate that the effect of carotenoids on an antioxidant system is missed when hydrophilic-based assays are used. As antioxidant status is usually assessed with hydrophilic-based methods, such as FRAP, ORAC, OXY, TEAC or TRAP[19,20], this provides a possible explanation for the overall weak association of carotenoid levels with antioxidant capacity that was reported in a recent meta-analysis[13].

The antioxidant effect of carotenoids observed in our study is inconsistent with the 'handicap hypothesis'[16], which assumes a pro-oxidant effect at high carotenoid concentrations, especially in the context of elevated oxidative stress. In effect, this hypothesis predicts that high carotenoid intake elicits either a significant increase in oxidative damage or a protective increase in *ZE*/tHODE in order to prevent such oxidative damage. In this study, however, we observed no adverse effect of carotenoids on blood redox state under low or high oxidative load, despite using the maximum absorbable carotenoid dose for zebra finches[35]. While (to the best of our knowledge) carotenoid intake in wild zebra finches remains unknown, work on house finches (*Haemorhous mexicanus*) suggests that wild diet in granivorous songbirds can contain as much as 90–100 μg/g of carotenoids[36]. As the 200 μg/g carotenoid dietary dose used in our study only resulted in a small increase in plasma carotenoid levels over the 100 μg/g dose in a previous study due to a plateau in carotenoid absorption at high concentrations[35], we suggest that our dose resulted in body carotenoid concentrations that were at the upper limit of that which songbirds may experience in the wild. Even if such a dose resulted in body concentrations of carotenoids that were higher than that experienced in the wild, the lack of any pro-oxidant action suggests that honesty of carotenoid-based sexual signals is not maintained through a physiological handicap mechanism based on the pro-oxidant effect of high carotenoid levels, as proposed by the 'handicap hypothesis'[16]. The observed antioxidant effect of carotenoids is also inconsistent with the 'protection hypothesis', which assumes no carotenoid effect on redox state[12].

Our results are in accordance with the 'allocation trade-off hypothesis'[5,10,11], as a key assumption of this hypothesis is the antioxidant function of carotenoids. Allocation trade-off is further supported by the fact that, following oxidative challenge, a decrease in signal intensity was accompanied by an increase in antioxidant capacity but only marginally increased oxidative damage. Rather than simply being an effect of oxidative stress, therefore, reduced ornament expression appears to result from an increased investment in self-maintenance via reallocation of resources to antioxidant defence[24,29]. Such reallocation of resources may apply not only to antioxidants (possibly including carotenoids), but also other micronutrients necessary for carotenoid metabolism (e.g. cofactors) or energy, as carotenoid absorption and conversion are believed to be energy demanding[37,38].

Our findings supporting carotenoid antioxidant function are also consistent with the recently proposed 'IMM carotenoid oxidation hypothesis'[17]. Under this hypothesis, reduced signal expression following diquat exposure is interpreted as the result of reduced red keto-carotenoid production due to mitochondrial dysfunction, rather than resource reallocation to antioxidant defence. Red keto-carotenoid synthesis, however, is believed to take place in the beak tissue in zebra finches[39] and not in the liver mitochondria, as assumed by the 'IMM carotenoid oxidation hypothesis'[17]. In addition, red keto-carotenoid synthesis has recently been proposed as being catalysed by cytochrome P450 2J2 in zebra finches[40]. Inferring from human studies, this cytochrome is probably located in the endoplasmic reticulum and not in mitochondria[41]. These two pieces of evidence suggest that the 'IMM carotenoid oxidation hypothesis' may be a less likely explanation for carotenoid-based signalling honesty, at least in this model species.

The reduced ornament expression following diquat exposure observed in our experiment is the first demonstration of an adverse effect of a redox-cycling agent on a carotenoid-based signal in adult animals. As induction of oxidative stress is a primary mechanism of diquat toxicity[28,33,42,43], this result supports the role of redox homeostasis as a major constraint ensuring honesty in carotenoid-based sexual signalling. No loss in body mass was observed in our diquat-exposed birds, indicating that the reduced ornamentation was not a consequence of a reduced food intake or impaired total intestinal absorption due to a possible diquat toxic effect on the intestinal wall. Our results therefore support the idea that energetically demanding and stressful conditions[44], immune activation[35,45] or environmental pollution (e.g. urban environment, heavy metals or pesticides)[46,47] could reduce carotenoid-based ornamentation through elevated oxidative stress[5].

Only four previous studies have directly tested the effect of oxidative status on expression of carotenoid-based ornament by either specifically increasing oxidative load through redox-cycling agents[25,48,49] or reducing antioxidant protection[50], and these produced contrasting results. In these studies, while paler beak and skin pigmentation developed following redox state disruption in juvenile red-legged partridges (*Alectoris rufa*)[25], no effect was observed on feather colouration was observed in moulting adult great tits (*Parus major*)[48], greenfinches (*Carduelis chloris*)[50] or house finches (*Haemorhous mexicanus*)[49]. One possible explanation for this discrepancy could be a difference in trade-off solutions between developing and adult birds[25], with the latter investing more in reproduction than in self-maintenance. Our demonstration of such an effect in adult sexually active zebra finch males, however, does not support this explanation. To date, the adverse effect of oxidative challenge has only been observed on bare parts only[25], perhaps suggesting a higher sensitivity to oxidative stress in the more dynamic bare parts, which can change colour over a period of just a few weeks[45,51], compared to semi-static feather ornamentation. Hence, future studies should test for possible differences in honesty mechanisms and signalling content between semi-static and dynamic carotenoid-based signals and explore possible differences in these mechanisms between different taxa and life-histories.

Conclusion

Using a novel measure of lipophilic antioxidant capacity, we demonstrate that carotenoids are able greatly inhibit the effect of oxidative challenge on redox state, thereby supporting the recently questioned antioxidant function of carotenoids *in vivo*. This result is inconsistent with both the 'protection' and the 'handicap hypotheses', but provides circumstantial support for the 'allocation trade-off hypothesis', as carotenoid antioxidant function is a key assumption of the latter. Other mechanisms, such as regulation of carotenoid absorption and/or metabolism cannot be ruled out, however, and studies using isotopic labelling of ingested carotenoids will probably be needed to ascertain the relative involvement of allocation trade-off and other mechanisms in carotenoid-based signalling honesty.

We further demonstrate that the antioxidant effect of carotenoids is missed when a common hydrophilic-based antioxidant assay is used. Our data also demonstrate an organism's impressive ability to adjust its antioxidant mechanisms and maintain stable levels of blood oxidative damage, despite large fluctuations in oxidative load or carotenoid intake. As a combination of oxidative damage markers and hydrophilic-based antioxidant assays is usually used for redox state assessment, these two observations provide a possible explanation for the generally weak support for carotenoid antioxidant function *in vivo* reported in a recent meta-analysis[13]. We suggest that the assessment of lipophilic antioxidant capacity will allow for a deeper understanding of redox processes in lipophilic compartments, such as lipid bilayers, and their ecological and evolutionary importance.

Material and Methods

Ethical approval. All experimental procedures were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union, and were approved by the animal care and ethics representatives of the Faculty of Science, Charles University in Prague, and The Czech Academy of Sciences (No. 041/2011).

Model species. The zebra finch is a traditional model species, widely used in studies exploring links between individual condition and expression of sexual signals[18,35,51,52]. Both sexes have carotenoid-based beak pigmentation, which is sexually dimorphic and is known to play an important role in mate choice[53]. Redness of the beak has been reported as predicting future survival, reproductive success[54] and immune reactivity[55]. Condition dependence in beak colouration has been demonstrated experimentally through a reduction in colouration following exercise, reduced food intake[56], immune activation[35] or cold stress[44].

Subjects and housing. Adult zebra finch males ($n = 60$) were housed individually in cages ($0.6 \times 0.4 \times 0.4$ m), at an indoor facility at the Institute for Vertebrate Biology of The Czech Academy of Sciences (Studenec, Czech Republic) from November 2010 on. All males were one to one-and-a-half years old at the time of the experiment. The birds were provided with millet seed, cuttlefish bone, grit with crushed shell and water *ad libitum*. Four weeks before the start of the experiment, millet seed was replaced by hulled millet seed, which was used for carotenoid supplementation during the experiment (see below). Photoperiod was set at 10:14 (light:dark) during winter, and gradually changed to 14:10 over April and May 2011. Eight females in separate cages were added to the experimental room at the end of May in order to stimulate breeding condition in males through visual and vocal contact. No other changes in social structure were made thereafter.

Experimental design. We manipulated the level of oxidative load (ROS) and carotenoid intake (CAR) in a fully factorial design (2 × 2), with two levels (low and high) for each factor. Adult males were randomly assigned to four treatment groups (ROS− CAR−, ROS + CAR−, ROS− CAR + or ROS + CAR +) with fifteen birds in each group. The experimental treatments lasted 10 weeks, from July to August 2011.

Diquat dibromide (Reglone 200 g/L, Syngenta, UK), a chemical compound known to generate oxidative stress *in vivo* through production of superoxide anions[24–27], was used for the controlled increase in free radical exposure (ROS + groups). A fresh diquat solution was prepared each day and added to the drinking water. Based on a preliminary experiment (four groups of six birds; 12.5, 25, 50 and 100 mg/L in drinking water for four weeks; 0%, 0%, 16.7% and 33.3% mortality, respectively; lack of mortality as a dose selection criterion), a sub-lethal concentration of 25 mg/L was chosen as it had no long-term effect on clinical condition. Free-living birds can experience much higher diquat concentrations for short periods, since concentration as high as 22.2 g/L are used in a spray form for a weed control. Nevertheless, our intention here was to imitate a mild but long-term increase in oxidative load, such that may result for example from stressful conditions or chronic infection[7,57]. Considering that zebra finches drink ca. 2–4 mL water per day[55], the concentration used in our experiment equals a daily intake of approximately 3–6 μg/g of body weight. A lethal dose for zebra finches is unknown, but our dose is much lower than LD50 reported for mallards (564 μg/g)[58]. During the main experiment, thirteen diquat-treated birds showed mild apathy and ruffled feathers at the beginning of the treatment (Yates' chi-square tests: ROS effect, $\chi^2 = 14.14$, $P < 0.001$; carotenoid effect, $\chi^2 = 0$, $p = 1$). Aside from two birds from the ROS + CAR− group that died 35- and 43-days after the beginning of the experiment, and two birds from the ROS + CAR + group that died after 2 and 41 days, most birds recovered after a few days. There was no significant difference in mortality in diquat- or carotenoid-treated birds compared to control ones (Yates' chi-square tests: ROS, $\chi^2 = 2.41$, $p = 0.12$; CAR, $\chi^2 = 0.27$, $p = 0.61$).

For the increase in carotenoid intake (CAR + groups), 1 kg of hulled millet seed was coated with 1 mL of lutein and zeaxanthin (FloraGLO Lutein 20% SAF, Kemin/DSM, France) mixed with 1 mL of safflower oil (Jules Brochenin, France). We used the highest known dose assimilated by zebra finches (200 μg of carotenoids per g of food)[35] in order to test for the hypothesised pro-oxidant effect of high carotenoid levels under elevated oxidative stress[16]. Given that FloraGLO contains 10 mg/mL α-tocopherol as an antioxidant, the control (CAR−) diet was prepared by mixing 1 kg of seed with 2 mL of safflower oil and 10 mg of α-tocopherol (T3251, Sigma-Aldrich, Czech Republic). All prepared diets were frozen at −80 °C until use.

Pre- and post-experimental values for body mass and beak reflectance were taken and blood samples (up to 120 μL) collected from the jugular vein at the beginning and end of the experiment in heparinised microhaematocrit capillaries. The blood samples were centrifuged at 9,000 × g for 5 min and the plasma and RBC separated and stored at −80 °C for biochemical analysis. There was no difference in size (tarsus length) and size-controlled body mass (tarsus length as covariate) between experimental groups prior to the experiment ($p \geq 0.12$).

Beak colour measurement. Beak reflectance was measured between 300–700 nm on an AvaSpec 2048 spectrophotometer with an AvaLight-XE light source (Avantes, Netherlands). Four points were measured on each side of the upper beak, and two on the lower beak, with the probe held perpendicular to the surface. The spectrophotometer was standardised against a darkroom and a WS-2 white standard after every five individuals. Subsequent reflectance data processing and colour analysis was undertaken using the 'pavo' package in R 3.0.2[59]. All 12 spectral curves from each individual were interpolated to 1-nm steps, merged into one average curve and smoothed with a span value of 0.15. The resulting average curves were used to calculate colorimetric measures (hue, red chroma and UV chroma) and avian visual modelling. Hue was calculated as wavelength at the reflectance midpoint, whereas red and UV chroma were calculated as a summed reflectance of 600–700 nm and 300–400 nm, respectively, divided by a summed reflectance of 300–700 nm (formulas H3, S1R and S1U in[60], red chroma modified according to[54]). Repeatability of red, UV chroma and hue measurements ($n = 25$) were 0.83, 0.50 and 0.91, respectively. While red chroma was strongly correlated with both hue (Spearman's $r_P = 0.76$) and UV chroma ($r_P = -0.88$), the correlation between hue and UV chroma was somewhat weaker ($r_P = -0.45$). There was no between-group difference in either colour metric prior to the experiment ($p \geq 0.35$). As both hue and logit transformed (see statistical analysis) UV chroma did not meet the assumption of normality (Shapiro-Wilk test: $W = 0.958$, $p = 0.039$ and $W = 0.862$, $p < 0.001$, respectively), logit transformed red chroma was used as a representative variable throughout the study and only report the effect sizes for both hue and UV chroma in the Supplementary Table S3.

Plasma carotenoids. Total carotenoid content in plasma was assessed as the absorbance of an organic extract prepared according to the slightly modified method of McGraw *et al.*[52]. Briefly, 100 µL of ethanol was added to 10 µL of the plasma and the mixture then extracted with 100 µL of hexane:*tert*-buthyl methyl ether (1:1, v/v) by vortexing. After centrifugation (2 min at 11,000 × g), the upper organic layer was transferred to a 1.5 mL vial and evaporated to dryness under a stream of nitrogen. The dry sample was dissolved in 200 µL acetonitrile and 150 µL of this solution was pipetted into a 96-well microplate. Absorbance was measured at 450 nm (local absorption maximum of the plasma extracts) on an Infinite M200 microplate-reader (Tecan, Austria). Total plasma carotenoids were expressed as an equivalent of lutein concentration, the plasma carotenoid most prevalent in the zebra finch[35]. We used this measure in our analysis in order to provide information on the amount of all the carotenoids present in the plasma. In order to control whether this measure indeed correlated with the most prevalent plasma carotenoids, we also analysed plasma lutein and zeaxanthin concentrations using HPLC (see Supplementary Information). Both, lutein and zeaxanthin, were highly correlated with our estimate of total plasma carotenoids ($r = 0.84$ and 0.79, respectively), as well as to each other ($r = 0.90$). There was no difference in either lutein, or zeaxanthin, or total plasma carotenoids between the groups prior to the experiment ($p \geq 0.86$).

Plasma antioxidant capacity. The OXY-Adsorbent test (OXY; Diacron, Italy) was used as a measure of hydrophilic antioxidant capacity in blood plasma[61]. Five microliters of plasma diluted 1:100 with double-distilled water were incubated with 200 µL of HClO-based solution for 10 min at 37 °C. After incubation, the chromogen (5 µL of *N*,*N*-diethyl-*p*-phenylenediamine solution) was added and absorbance read immediately using a Sunrise microplate-reader (Tecan, Austria) at 505 nm. The antioxidant capacity of the sample was expressed as µmol of HClO neutralised by antioxidants present in 1 mL of the sample[61]. Repeatability of the OXY measurement was 0.76. There was no difference in OXY between the groups prior to the experiment ($p = 0.12$).

Oxidative damage and lipophilic antioxidant capacity in RBC. The total RBC concentrations of 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane), a product of arachidonic acid oxidation, was used as markers of total oxidative damage[22]. In addition, the ratio of HODE stereoisomeric forms (*ZE*/*EE*-HODE) was used as a measure of *in vivo* antioxidant capacity in lipophilic cellular compartments[21,22]. We expressed the relative amount of *ZE*- and *EE*-HODE stereoisomers as a proportion of *ZE*-HODE to total HODE (*ZE*/tHODE), rather than the *ZE*/*EE*-HODE ratio[21], as proportional variables can easily be normalised through logit transformation and analysed with linear models. If necessary, the *ZE*/tHODE proportion can easily be converted to a *ZE*/*EE*-HODE ratio.

Details of the HPLC-ESI-MS/MS methods for 8-isoprostane and HODE analysis are described in Supplementary Information. The experimental groups did not differ in oxidative damage prior to the experiment ($p \geq 0.37$). Despite random assignment of the birds to the experimental groups, however, we detected a significant difference in *ZE*/tHODE with the ROS− CAR+ group having lower values (mean difference ± SE = −0.074 ± 0.029, $p = 0.012$) and the ROS+ CAR+ group higher (0.083 ± 0.029, $p = 0.005$) relative to the control group prior to the experiment. Although these differences may have influenced the effect size of the treatments, we believe that they do not change the general interpretation of our results as carotenoid supplementation produced a qualitatively similar effect in both groups, despite initial differences. Moreover, we used ANCOVA models that control for potential differences in initial values of the response variable.

Statistical analysis. The effect of experimental manipulation was tested using general linear models implemented in R 3.1.2 (The R Foundation for Statistical Computing, Austria). In each model, diquat and carotenoid treatment were included as factors, together with their interaction, and pre-experiment (initial) values of the modelled trait were included as a covariate. Factor levels were coded as 0 and 1 (low and high, respectively) and centred to enable interpretation of the main effects without the need to remove the interaction[62]. Normalising logit (R package 'car') and Box-Cox (R package 'MASS') transformations were used for proportional variables (red and UV chroma and *ZE*/tHODE) and plasma carotenoids (total carotenoids and lutein), respectively. All models in our study were also run with continuous variables *z*-transformed in order to obtain standardised partial regression coefficients (b^*) as a measure of standardised effect size[62].

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Acknowledgements

We are grateful to Bart Kempenaers and Rebecca J. Safran for valuable comments on an earlier version of the manuscript, and Kevin Roche for language editing. We would like to thank Jana Albrechtová, František Vejmělka and Jitka Vinklerová for help with data collection and Helena Hejlová for help with bird care. This study was funded through: Czech Science Foundation grant P506/12/2472 to TA and GA15-11782S to TA and MV; Charles University Grant Agency grant 283811 to OT; institutional project SVV 260313/2016 to OT and BG; through the help of the EU structural fund ("Operational Programme Prague – Competitiveness" grant No. CZ.2.16/3.1.00/22197) to PK; and through the Ministry of Education, Youth and Sports of the Czech Republic under its program "National Programme of Sustainability I" – NPU I (LO) (grant No. MSMT-34807/2013) and Contact II (MSM/LH12116) to PK.

Author Contributions

O.T., T.A. and M.V. conceived and designed the study; O.T. conducted the experiment; O.T., B.G., J.S. and M.V. collected the data; O.T. analysed the hydrophilic antioxidant capacity and spectrophotometric ornament data; B.G. and P.M. carried out carotenoid analyses; P.K. and K.S. developed and carried out LC-MS analyses of oxidative stress; O.T. carried out statistical analyses; O.T. and T.A. drafted the manuscript. All the authors contributed to the revision of the manuscript and gave final approval for publication.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Tomášek, O. *et al.* Opposing effects of oxidative challenge and carotenoids on antioxidant status and condition-dependent sexual signalling. *Sci. Rep.* **6**, 23546; doi: 10.1038/srep23546 (2016).

Supplementary Information

for

Opposing effects of oxidative challenge and carotenoids on antioxidant status and condition-dependent sexual signalling

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Supplementary inventory

Supplementary Data

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Supplementary Methods

Supplementary Table S4. Validation parameters of the LC-MS methods for determination of HODEs and 8-iso-prostaglandin $F_{2\alpha}$

Supplementary Table S1. Group mean values and SD of measured variables at the beginning of the experiment.

Treatment group	**ROS− CAR−**		**ROS− CAR+**		**ROS+ CAR−**		**ROS+ CAR+**	
Variable	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Beak red chroma	15	0.85 (0.04)	15	0.85 (0.03)	15	0.85 (0.03)	15	0.86 (0.03)
Beak hue (nm)	15	590.33 (2.87)	15	590.20 (2.21)	15	588.93 (3.76)	15	589.07 (3.37)
Beak UV chroma	15	0.018 (0.022)	15	0.015 (0.018)	15	0.011 (0.012)	15	0.007 (0.009)
Total plasma carotenoids (μg/mL)	14	27.43 (3.18)	12	27.39 (8.36)	13	26.39 (5.18)	12	26.59 (7.93)
Plasma lutein (μg/mL)	14	18.06 (3.17)	12	17.70 (4.52)	13	17.08 (3.01)	12	17.73 (5.40)
Plasma zeaxanthin (μg/mL)	14	1.32 (0.85)	10	1.57 (1.07)	13	1.21 (0.56)	10	1.36 (1.43)
8-isoprostanes in RBC (pg/mL)	15	33.49 (6.93)	14	37.06 (6.57)	15	34.90 (3.71)	14	36.20 (5.09)
ZE/tHODE ratio	15	0.44 (0.02)	14	0.42 (0.02)	15	0.44 (0.02)	14	0.46 (0.01)
OXY (μmol HClO/mL)	15	138.76 (30.16)	15	139.00 (26.52)	15	118.93 (27.28)	15	124.23 (27.02)
Body mass (g)	15	16.39 (1.65)	15	17.20 (1.62)	15	16.02 (1.40)	15	17.33 (1.34)

Supplementary Table S2. Group mean values and SD of measured variables at the end of the experiment.

Treatment group	**ROS− CAR−**		**ROS− CAR+**		**ROS+ CAR−**		**ROS+ CAR+**	
Variable	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Beak red chroma	15	0.85 (0.02)	15	0.90 (0.01)	13	0.81 (0.03)	13	0.88 (0.03)
Beak hue (nm)	15	588.33 (2.50)	15	595.73 (2.09)	13	583.69 (3.59)	13	592.38 (4.54)
Beak UV chroma	15	0.010 (0.006)	15	0.003 (0.003)	13	0.020 (0.010)	13	0.005 (0.006)
Total plasma carotenoids (µg/mL)	15	27.44 (4.91)	14	55.76 (13.40)	13	19.91 (6.99)	12	48.84 (17.11)
Plasma lutein (µg/mL)	14	17.41 (3.46)	14	40.54 (9.63)	13	16.43 (5.17)	12	45.47 (15.02)
Plasma zeaxanthin (µg/mL)	14	1.66 (0.89)	14	2.51 (1.59)	10	1.27 (0.92)	11	2.88 (1.94)
8-isoprostanes in RBC (pg/mL)	15	27.41 (4.40)	15	30.54 (4.16)	13	30.46 (4.16)	10	31.65 (4.96)
ZE/tHODE ratio	15	0.42 (0.02)	15	0.34 (0.03)	13	0.67 (0.04)	10	0.47 (0.02)
OXY (µmol HClO/mL)	15	136.52 (29.97)	15	133.66 (28.21)	13	143.45 (42.42)	12	149.62 (25.32)
Body mass (g)	15	16.71 (1.85)	15	17.93 (1.75)	13	16.85 (1.55)	13	17.15 (2.41)

Supplementary Table S3. Standardised effect sizes of oxidative load (ROS) and carotenoid intake (CAR) on beak hue and UV chroma and plasma lutein and zeaxanthin. Effect sizes are given as standardised partial regression coefficients (*b**) from linear models with oxidative load and carotenoid intake as factors, initial values as covariates, and with continuous variables *z*-standardised. Low and high factor levels were coded 0 and 1, respectively and centred in order to enable the main effects to be properly interpreted without the need to remove the interaction terms from the models. Beak UV chroma and plasma lutein were normalised using logit and Box-Cox ($\lambda = 0.116$) transformation, respectively.

Response variables	**Standardised effect size**		
Predictors	b^*	**2.5% CI**	**97.5% CI**
Beak hue			
initial	0.25	0.10	0.40
ROS	−0.61	−0.91	−0.31
CAR	1.42	1.12	1.70
ROS × CAR	0.13	−0.45	0.72
Beak UV chroma			
initial	0.16	−0.06	0.37
ROS	0.66	0.23	1.08
CAR	−1.09	−1.51	−0.67
ROS × CAR	−0.20	−1.03	0.64
Plasma lutein			
initial	0.28	0.15	0.41
ROS	0.05	−0.21	0.31
CAR	1.67	1.41	1.93
ROS × CAR	0.22	−0.30	0.74
Plasma zeaxanthin			
initial	0.53	0.31	0.76
ROS	−0.16	−0.61	0.28
CAR	0.91	0.47	1.35
ROS × CAR	0.05	−0.83	0.94

Supplementary methods

HPLC analysis of carotenoids in plasma samples. Dried organic extract of the plasma (see Material and Methods) was dissolved in a mobile phase (methanol:acetonitrile 1:1, v/v) and injected (40 µl) into the HPLC. The HPLC system (Q-grad quaternary pump, Watrex, Czech Republic; Midas autosampler, Spark, The Netherlands) was equipped with a 4.6 x 250 mm 5 µm C30 Develosil RP-Aqueous separation column with a C30 Develosil precolumn (Nomura Chemical Co., Japan). Methanol (A) and acetonitrile (B) were used as mobile phases for gradient elution (starting with 20% B, ramped to 45% at 30 min, 100% B from 33 to 43 min, followed by 10 min of equilibration with 20% B). The flow was set to 1 mL/min and the column temperature set at 35 °C (Mistral column thermostat, Spark, The Netherlands). Absorption spectra (range 300–600 nm) were measured using a DAD UV6000LP spectrometer (Thermo Finnigan, USA). Carotenoids (adonirubin, astaxanthin, cryptoxanthin, lutein, zeaxanthin) were identified by comparison of retention times and spectra with commercially available standards (Sigma-Aldrich, Czech Republic; CaroteNature, Switzerland). Quantitation of known carotenoids was performed at 450 and 470 nm using external standards.

HPLC analysis of oxidative damage and lipophilic antioxidant capacity in RBC.

Standards (13-(*Z,E*)-HODE), 9-(*Z,E*)-HODE, 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane), 9-HODE-d_4 and 13-HODE-d_4 and 8-iso-prostaglandin $F_{2\alpha}$-d_4) were obtained from Cayman Chemical Company (MI, USA). 9-(*E,E*)-HODE and 13-(*E,E*)-HODE were obtained from Larodan Fine Chemicals AB (Malmo, Sweden).

The pre-treatment step was performed according to the following procedure: internal standards 9-HODE-d_4, 13-HODE-d_4 and 8-iso-prostaglandin $F_{2\alpha}$-d_4 (each internal standard was 10 ng/50 µL of RBC), butylated hydroxytoluene (100 µM – antioxidant) and an extraction solution of methanol and acetonitrile (100 µL of methanol and 100 µL of acetonitrile/50 µL of RBC) were added to the RBC. The mixture and RBC were sonicated for 10 min at 4 °C. The sample was then centrifuged at 9,000 × g at a temperature of 4 °C and the supernatant subsequently separated. The supernatant was dried by stripping with nitrogen then dissolved in methanol and water (25 µL, methanol:water – 70:30 v/v) and immediately analysed by HPLC-ESI-MS/MS.

The LC-ESI-MS/MS system consisted of an Accela 1250 LC chromatogram (Thermo Scientific, USA), an Open Accela autosampler (Thermo Scientific, USA) and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The analytes were separated on a 100 × 2.1 mm 1.7 µm C18 Kinetex column (Phenomenex, USA) with a mobile phase (solvent A: 5 mM ammonium acetate, Solvent B: acetonitrile, solvent C: methanol) in a gradient elution with a flow rate of 200 µL/min. The HPLC elution program was as follows: 75% A, 20% B, 5% C (3 min) → 50% A, 45% B, 5% C (linear increase over 40 min, held for 5 min) → 75% A, 20% B, 5% C (linear decrease over 1 min, held for 5 min). The column temperature was maintained at 30 °C and the sample temperature at 4 °C. Injection volume was 10 µL. The analytes were eluted with the following retention times: 9-(*ZE*)-HODE = 36.6 min, 9-(*EE*)-HODE = 39.2 min, 13-(*ZE*)-HODE = 35.4 min, 13-(*EE*)-HODE = 37.2 min, and 8-iso-prostaglandin $F_{2\alpha}$ = 9.4 min. A mass spectrometer equipped with electrospray ionisation (ESI) was used for detecting HODEs, 8-iso-prostaglandin $F_{2\alpha}$ and their deuterium labelled internal standards (9-HODE-d_4, 13-HODE-d_4, 8-iso-prostaglandin $F_{2\alpha}$-d_4) in negative ionisation mode (ESI^-). The selective reaction monitoring mode was used. The scan monitoring reactions (precursor ion → fragment ion) used for the analyses and their collision induced dissociated (CID) energy were as follows: m/z = 295.4 Da (corresponding with $[M-H]^-$ ions) → m/z = 171.5 Da (corresponding with fragmentation ion) (CID = 22eV) for 9-(*ZE*)-HODE and 9-(*EE*)-HODE; m/z = 299.4 Da → m/z = 175.5 Da (CID = 22eV) for 9-HODE-d_4; m/z = 295.4 Da → m/z = 194.6 Da (CID = 21eV) for 13-(*ZE*)-HODE; and 13-(*EE*)-HODE , m/z = 299.4 Da → m/z = 198.6 Da (CID = 21eV) for 13-HODE-d_4; m/z = 299.4 Da → m/z = 198.6 Da (CID = 21eV); m/z = 353.5 Da → m/z = 193.1 Da (CID = 22eV) for 8-iso-prostaglandin $F_{2\alpha}$, and m/z = 357.5 Da → m/z = 197.1 Da (CID = 22eV) for 8-iso-prostaglandin $F_{2\alpha}$-d_4. The optimised conditions on the mass spectrometer were as follows: spray voltage = 2500 V, temperature of ion transfer tube = 300 °C, temperature of H-ESI vaporiser = 300 °C, pressure of sheath gas (nitrogen) = 35 psi, and flow of auxiliary gas (nitrogen) set at 10 arbitrary units. The data were acquired and processed using Xcalibur v. 2.1.0 software (Thermo Scientific, USA).

The validation parameters (accuracy, precision, recovery and limit of detection and quantification) were assessed by analysing five different concentration levels (LOQ, 5, 10, 50 and 100 pg/µl) of the particular substances (9-(*ZE*)-HODE, 9-(*EE*)-HODE, 13-(*ZE*)-HODE, 13-(*EE*)-HODE, and 8-iso-prostaglandin $F_{2\alpha}$. The values of the validation parameters for each particular substrate were summarised in Supplementary table S2.

Table S4. Validation parameters of the LC-MS methods for determination of HODEs and 8-iso-prostaglandin F_{2a}

Analyte	**Precision**	**Accuracy**	**Recovery**	**LOD**	**LOQ**
	RSD (%)	**RE (%)**	**(%)**	**(pg/µl)**	**(pg/µl)**
9-(*ZE*)-HODE	16.7	−15.9	84.1	0.04	0.05
9-(*EE*)-HODE	16.5	−16.2	83.8	0.05	0.06
13-(*ZE*)-HODE	18.3	−14.8	85.2	0.06	0.07
13-(*EE*)-HODE	18.1	−15.0	85.0	0.05	0.06
8-iso-$PGF_{2\alpha}$	14.6	−12.8	87.2	0.08	0.09

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Cite this article: Tomášek O, Albrechtová J, Němcová M, Opatová P, Albrecht T. 2017 Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge. *Proc. R. Soc. B* **284**: 20162444.
http://dx.doi.org/10.1098/rspb.2016.2444

Received: 7 November 2016
Accepted: 3 January 2017

Subject Category:
Evolution

Subject Areas:
behaviour, ecology, evolution

Keywords:
sexual selection, phenotype-linked fertility hypothesis, sperm competition theory, ejaculate quality, lutein, zeaxanthin

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Electronic supplementary material is available online at http://dx.doi.org/10.6084/m9.figshare.c.3666661.

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Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge

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It has been hypothesized that carotenoid-based sexual ornamentation signals male fertility and sperm competitive ability as both ornamentation and sperm traits may be co-affected by oxidative stress, resulting in positive covariation (the 'redox-based phenotype-linked fertility hypothesis'; redox-based PLFH). On the other hand, the 'sperm competition theory' (SCT) predicts a trade-off between precopulatory and postcopulatory traits. Here, we manipulate oxidative status (using diquat dibromide) and carotenoid availability in adult zebra finch (*Taeniopygia guttata*) males in order to test whether carotenoid-based beak ornamentation signals, or is traded off against, sperm resistance to oxidative challenge. Initial beak colouration, but not its change during the experiment, was associated with effect of oxidative challenge on sperm velocity, such that more intense colouration predicted an increase in sperm velocity under control conditions but a decline under oxidative challenge. This suggests a long-term trade-off between ornament expression and sperm resistance to oxidative challenge. Shortening of the sperm midpiece following oxidative challenge further suggests that redox homeostasis may constrain sperm morphometry. Carotenoid supplementation resulted in fewer sperm abnormalities but had no effect on other sperm traits. Overall, our data challenge the redox-based PLFH, partially support the SCT and highlight the importance of carotenoids for normal sperm morphology.

1. Introduction

Female preference for a particular male phenotype has long been recognized as a potent selective pressure driving the evolution of conspicuous secondary sexual characters such as carotenoid-based ornamentation [1,2]. The level of expression of such characters is known to be a major determinant of male mating opportunities, and thus reproductive success [2]. In addition, sexual selection continues after copulation in species where females copulate with multiple males, leading to competition between male spermatozoa for the fertilization of female ova [3]. In such species, male reproductive success is a result of an interplay between the precopulatory and postcopulatory phases of sexual selection [4]. Despite many recent studies focusing on interaction between these phases, there have been no conclusive results explaining how the two processes integrate, with a positive relationship being observed in some studies [5–10] and a negative relationship [11–16], or no relationship, in others [17–19].

A positive relationship is predicted by the 'phenotype-linked fertility hypothesis' (PLFH), which proposes that male fertility is signalled through secondary sexual characters [20]. The hypothesis proposes that both sexual signals and sperm traits are phenotypically plastic and co-affected by environmental effects, resulting in a positive correlation between signal expression and functional fertility [20]. As both sexual signals [21] and spermatozoa [22] are believed to be sensitive to oxidative stress, Blount *et al.* [23] hypothesized that oxidative stress could be the major factor linking such environmental effects to ornamentation and functional fertility (hereinafter 'redox-based PLFH').

Oxidative stress is characterized by an accumulation of oxidative damage to cellular components (e.g. DNA, lipids, proteins) resulting from oxidation by free radicals and other reactive oxygen and nitrogen species (ROS), and can be prevented by antioxidant mechanisms [21]. ROS are inevitable by products of oxidative metabolism and their formation generally increases under metabolically demanding and stressful conditions or during an immune response [21,24,25]. To ensure high motility, spermatozoa are metabolically active and rich in polyunsaturated fatty acids that oxidize more readily than monounsaturated and saturated fatty acids [22]. Moreover, spermatozoa may lack the capability to repair DNA as transcription and translation probably stop after spermiogenesis [26], and most enzymatic and non-enzymatic antioxidants are lost with the cytoplasm during sperm elongation [27]. Taken together, these factors render spermatozoa highly sensitive to oxidative stress, which is considered a major cause of male infertility in humans [22].

According to the redox-based PLFH [23], males that are more colourful should have a high-quality antioxidant system that better protects their sperm against oxidative stress. By choosing more ornamented males, therefore, females would obtain a direct benefit through fertility insurance, as well as an indirect benefit in the form of superior DNA integrity for their offspring.

An alternative theory, the 'sperm competition theory' (SCT), predicts a negative relationship between precopulatory and postcopulatory sexual traits based on the assumption that the amount of resources that can be invested in reproduction is limited [4]. As a result, a trade-off is expected between investment in precopulatory and postcopulatory traits. Based on this theory, we propose that expression of carotenoid-based ornamentation may be traded off against sperm resistance to oxidative challenge.

It has been proposed that the direction of any relationship between traits traded off against each other will depend on the amount of resources available to the individual [28]. Hence, a positive correlation may be observed under favourable conditions when resources are abundant, while a negative correlation may occur under more stressful conditions when resources are limited. Experimental manipulation of stress levels or resource availability are essential, therefore, to reveal trade-offs that might be masked by variation in these factors in observational studies [28–30].

In species with carotenoid-based ornamentation, carotenoids may be the key resource traded off between ornamentation and sperm traits. Particularly so given their antioxidant properties [21] and reported beneficial effects on sperm quality and male fertilization success in many taxa, including humans [7,23,31]. In birds, however, the importance of carotenoids for both antioxidant protection [32,33] and sperm competitive ability is disputed [10,31,34,35], and a pro-oxidant effect of high carotenoid concentrations has even been propounded [36]. Further studies are needed, therefore, to elucidate the importance of carotenoids as regards spermatogenesis and sperm competition in birds.

Both the redox-based PLFH and the SCT assume there is an environmental component of variation in both sexual signalling and sperm traits. In animals, sperm traits have indeed been found to be affected by environmental factors known to influence individual oxidative status, such as temperature, workload, parasites, immune activation and social environment (e.g. [37–40], reviewed in [41]). While a negative correlation between sperm oxidative damage and sperm velocity/viability has been documented in the wild [10,39], oxidative stress as a causal factor in such environmental effects cannot be inferred unless such studies use some form of controlled oxidative challenge.

In this study, we manipulate oxidative status and carotenoid availability in adult zebra finch (*Taeniopygia guttata*) males to test their effect on sperm traits and the relationship between sperm traits and carotenoid-based sexual ornamentation. The birds were subjected to controlled oxidative challenge induced by diquat dibromide (hereinafter referred to as diquat), a redox-cycling agent known to produce superoxide radicals *in vivo* that has recently been recognized as a convenient oxidative stress inducer in both ecological studies [42–45] and laboratory models of Parkinson's disease [46]. In order to assess changes in sperm quality, we analysed several sperm traits that have previously been suggested as important for male fertility and sperm competitive ability, i.e. sperm velocity [47–49], total sperm length (TSL) [50–52], relative flagellum length [50], relative midpiece length [50,53] and abnormal sperm proportion [54]. These traits could potentially be affected by oxidative stress since sperm development, as well as sperm motility, are dependent on sperm cell membrane fatty acid composition, which can be altered under oxidative stress [27,55]. Both the mitochondria-containing sperm midpiece and sperm velocity could further be affected by oxidative stress through its effect on energy and ROS production in mitochondria [56].

Experimental oxidative challenge is expected to divert resources to antioxidant defence, thereby reducing investment in sexual traits and revealing a possible trade-off [28,29]. As TSL, relative midpiece and flagellum length and sperm velocity are usually considered to be positively associated with sperm competitive ability [47,49–52], we predicted oxidative challenge-induced changes in their expression to be positively correlated with ornament expression under the redox-based PLFH, and negatively correlated under the SCT. The opposite is predicted for abnormal sperm proportion, as high occurrence of sperm abnormalities reduces male fertility [54]. In addition, we predict that carotenoids will counteract the potential effects of oxidative challenge on both sperm traits and ornamentation–sperm trait relationships if carotenoids act as antioxidants in the testes or ejaculate, or amplify such effects if they act as pro-oxidants.

2. Material and methods

(a) Study species and housing

This experiment was carried out on 60 adult zebra finch males at an indoor facility of the Institute for Vertebrate Biology (Studenec, Czech Republic) from July to August 2011. All birds were 1–1.5 years old at the time of the experiment and were housed individually in 60 × 40 × 40 cm cages with millet seed, cuttlefish bone, grit with crushed shell and water provided *ad libitum*. Four weeks before the start of the experiment, the millet seed was replaced with hulled millet seed, which was used for carotenoid supplementation during the experiment. To stimulate breeding condition in males, the original 10 : 14 (light : dark) winter photoperiod was gradually changed to 14 : 10 over April and May. Male breeding condition was further stimulated by placing eight females in separate cages in close proximity to those of the males at the end of May, thereby providing both visual and vocal contact. No further changes in social environment were made thereafter.

This same experiment was also used for testing the antioxidant function of carotenoids *in vivo* and the effect of oxidative challenge on carotenoid-based ornamentation and circulating carotenoid levels [45]. Here, we re-use the data on beak colouration for testing a different set of hypotheses.

(b) Experimental design and sample collection

Males were randomly assigned to four treatment groups with 15 individuals in each. For 70 days, we experimentally manipulated the level of oxidative burden and carotenoid intake in a 2×2 factorial design such that each treatment group received one of the four possible combinations of low (ROS−) or high (ROS+) oxidative burden and low (CAR−) or high (CAR+) carotenoid intake.

Diquat dibromide (Reglone 200 $\mathrm{g\,l^{-1}}$, Syngenta, UK), a chemical known to generate superoxide anions *in vivo* [57], was used for controlled oxidative challenge (ROS+ groups). In order to imitate a long-term mild increase in oxidative burden, diquat was administered in drinking water at a sublethal dose of 25 $\mathrm{mg\,l^{-1}}$ (approx. 3–6 $\mu\mathrm{g\,g^{-1}}$ of body weight), which has been shown to have no long-term effect on the bird's clinical condition [45]. The control groups (ROS−) received plain drinking water instead. Thirteen diquat-treated birds showed mild apathy and ruffled feathers at the beginning of the treatment (Yates' χ^2-tests: ROS effect, $\chi^2 = 14.14$, $p < 0.001$; carotenoid effect, $\chi^2 = 0$, $p = 1$). Aside from two birds from the ROS+CAR− group and two birds from the ROS+CAR+ group that died, the birds recovered after a few days. There was no significant difference in mortality between diquat- or carotenoid-treated birds and the control birds (Yates' χ^2-tests: ROS, $\chi^2 = 2.41$, $p = 0.12$; CAR, $\chi^2 = 0.27$, $p = 0.61$).

For the high carotenoid intake diet (CAR+), 200 mg of lutein and zeaxanthin (1 ml of FloraGLO Lutein 20% SAF, Kemin/DSM, France) and 1 ml of safflower oil (Jules Brochenin, France) were mixed with 1 kg of hulled millet seed. This concentration, which is close to the upper limit that zebra finches can assimilate [58], was chosen as it is most likely to reveal the hypothesized pro-oxidant effect of carotenoids. Given that assimilation of carotenoids reaches a plateau at between 100 and 200 $\mu\mathrm{g\,g^{-1}}$, and that wild songbirds are known to receive up to 100 $\mu\mathrm{g\,g^{-1}}$ carotenoids in their food [59], we argue that such a dose results in plasma concentrations that are within the range songbirds may experience in the wild (see also [45]). The control diet (CAR−) was prepared by mixing 1 kg of seed with 2 ml of safflower oil and 10 mg of α-tocopherol (T3251, Sigma-Aldrich, Czech Republic) to compensate for the safflower oil and α-tocopherol contained in the FloraGLO. Both prepared diets were frozen at −80°C until use to prevent carotenoid degradation.

Beak colour measurements and ejaculate samples were collected at both the beginning and end of the experiment. All measurements in this study were undertaken blind with respect to sample identity and experimental treatment.

(c) Beak colouration

Beak reflectance was measured between 300 and 700 nm using an AvaSpec 2048 spectrophotometer with an AvaLight-XE light source (Avantes, The Netherlands). Four points on the upper beak and two on the lower beak were measured on each side with the probe held perpendicular to the surface. The spectrophotometer was standardized against a darkroom and WS-2 white standard after measuring five individuals. Hue, red chroma and UV chroma were calculated from the reflectance data using the 'pavo' package [60] in R v. 3.0.2 (the R Foundation for Statistical Computing, Austria; for details see [45]). Because both hue and logit transformed UV chroma were strongly correlated with red chroma (Spearman's $r_s = 0.76$ and $r_s = -0.88$, respectively), but did not meet the assumption of normality (Shapiro–Wilk's test: $W = 0.958$, $p = 0.039$ and $W = 0.862$, $p < 0.001$, respectively), red chroma was used as a representative variable throughout the study.

In those cases where beak red chroma was significantly correlated with sperm traits, we calculated whether sperm trait differences could be discriminated based on beak colouration by zebra finch eyes using the colour opponency model [61,62] implemented in the 'pavo' package [60]. To this end, males were divided into three groups of 20 individuals according to the sperm trait tested (e.g. short, medium and long sperm) and the reflectance spectra in each group were averaged. Colour contrasts (ΔS) were subsequently calculated between each group pair based on neural noise [61,62], using characteristics of the zebra finch visual system [63,64]. The Weber fraction was set at 0.05 [62] and standard daylight D65 was used as the illuminant. Just noticeable differences (jnds) were used to measure ΔS, with values of $\Delta S > 1$ jnd theoretically discernible by the zebra finch visual system under the above conditions.

(d) Sperm measurement

Ejaculate samples (approx. 0.5 µl) were obtained by gentle cloacal massage [65] and immediately diluted in 60 µl of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA). Approximately 3 µl of the diluted sample was subsequently used for analysis of sperm velocity, the rest being stored in 10% formalin for analysis of sperm morphometry and abnormal sperm proportion.

Immediately after sample collection and dilution, the sample was placed on a Leja slide (Leja, The Netherlands) and sperm movement was recorded with an Olympus CX41 light microscope equipped with a heating table, phase contrast and a digital UI-1540-C camera (Imaging Development Systems, Germany). All recordings were performed at 40°C. The recordings were later analysed using the CEROS computer-assisted sperm analysis system (Hamilton Thorne, USA). As the three sperm velocity characteristics (straight, curvilinear and average path velocity) were highly inter-correlated, we used curvilinear velocity (VCL) only for all further analysis [65].

Sperm samples stored in formalin were smeared onto glass slides, left to dry and then photographed using an Olympus BX51 light microscope equipped with a camera (Visitron Systems, Germany). Sperm morphometry was subsequently measured using Olympus QuickPHOTO Industrial 2.3 imaging software. For each sample, we measured the length of the head, midpiece and tail and calculated TSL (the sum of the three components) on 30 morphologically normal spermatozoa [65]. All morphometric measurements were undertaken by the same person (M.N.).

The proportion of morphologically normal and abnormal spermatozoa in each sperm sample was assessed at 400 times magnification, with 100 sperm cells analysed per sample for each bird. Sperm not showing the typical songbird helical sperm head-shape were considered abnormal, as were the few sperm cells showing tail deformities (two-tailed spermatozoa with one head and one midpiece) [65]. All scoring was undertaken by the same person (P.O.).

(e) Statistical analysis

All data analysis was carried out using R v. 3.3.0 (the R Foundation for Statistical Computing, Austria). We first explored Pearson's correlations between individual sperm traits and ornament expression, with proportional variables (red chroma, flagellum/TSL proportion, midpiece/flagellum proportion and proportion of sperm abnormalities) normalized by logit transformation using the 'car' package. Subsequently, effects of experimental manipulation were analysed using linear models, with change scores of each sperm trait set as dependent variables. Oxidative challenge and carotenoid intake (both control and high), together with their interaction, were included as factors in order to test their effect on sperm traits. To control for the effect of initial sperm trait expression on its subsequent change, we also included pre-experimental values of the sperm trait analysed, together with its two-way interaction with both treatment factors. To test whether ornament expression signals, or is traded off against, sperm resistance to oxidative challenge, pre-experimental values for beak red chroma, along with its interaction with oxidative challenge, were inserted into the models. To test whether initial ornament expression predicts sensitivity of the sperm traits to carotenoid intake, the interaction between initial colouration and carotenoid intake was also included. Real-time trade-off in allocation of available resources between ornament expression and sperm traits was tested by including the change in beak red chroma and its two-way interaction with each of the treatment factors (see electronic supplementary material, tables S5 and S6 for a summary of all predictor terms included in each global model). Global models were simplified by removing all non-significant (i.e. $p > 0.05$) terms using a stepwise procedure. In the final models, all predictor variables were centred to obtain biologically relevant main effects in the presence of interactions [66].

3. Results

(a) Relationship between carotenoid-based ornament and sperm traits

Prior to the experiment, beak red chroma was positively correlated with TSL ($r = 0.35$, $p = 0.006$) and flagellum/TSL ratio ($r = 0.33$, $p = 0.011$), but not with sperm velocity ($r = 0.12$, $p = 0.35$), relative midpiece length ($r = -0.10$, $p = 0.44$) or abnormal sperm proportion ($r = 0.11$, $p = 0.42$). Average beak ΔS between males with low and high TSL, and low and high flagellum/TSL ratio were 12.9 and 7.0 jnds, respectively (see electronic supplementary material, table S1 for all comparisons). These results suggest that zebra finch females should be able to discriminate males differing in TSL and flagellum/TSL ratio based on beak colouration.

Neither TSL nor relative flagellum length (flagellum/TSL ratio) was correlated with sperm velocity, though there was a marginally non-significant positive correlation between sperm velocity and relative midpiece length (electronic supplementary material, table S2). Interestingly, a longer midpiece was also associated with a lower proportion of sperm abnormalities. The relationship between relative midpiece length and sperm velocity was not due to differences in abnormal sperm proportion as sperm velocity and abnormal sperm proportion were uncorrelated. Descriptive statistics for sperm trait measurements are provided in electronic supplementary material, tables S3 and S4.

(b) Effects of oxidative challenge and carotenoid intake on sperm traits and ornament–sperm trait relationships

Contrary to the predictions of the redox-based PLFH, a more intense initial beak colouration was associated with a decrease in sperm velocity under oxidative challenge, while a positive correlation was observed under control conditions (table 1, figure 1; electronic supplementary material, table S5). The observed change in sperm velocity, however, was not related to change in beak colouration or its interaction with oxidative challenge. Further, neither sperm velocity nor the effect of oxidative challenge on this trait was influenced by carotenoid intake.

Oxidative challenge also resulted in spermatozoa with a lower midpiece/flagellum ratio (table 1; electronic supplementary material, table S6). This effect was relatively weak, however, and was driven mainly by midpiece shortening, as there was no effect of oxidative challenge on TSL or flagellum/TSL ratio. Carotenoid intake had no effect on any of the morphometric traits. Interestingly, changes in both TSL and flagellum/TSL ratio were negatively correlated with initial beak colouration, irrespective of treatment.

Oxidative challenge did not affect abnormal sperm proportion in our experiment (table 1; electronic supplementary material, table S6). On the other hand, abnormal sperm proportion was reduced by elevated carotenoid intake. The significant interaction of carotenoid intake with initial abnormal sperm proportion further indicated that high carotenoid intake reduced the occurrence of sperm abnormalities most strongly in males having the highest proportion of abnormal sperm prior to the experiment (figure 2). This effect was even more significant after removing one influential observation (Cook's distance > 1; electronic supplementary material, table S7).

4. Discussion

In our study, we found little support for the PLFH [20], and especially for the redox-based variant, which proposes that high-quality carotenoid-based ornamentation signals high sperm resistance to oxidative challenge [23]. First, prior to the experiment, carotenoid-based beak colouration was positively correlated with sperm length and relative flagellum length only, but not with sperm velocity, relative midpiece length or abnormal sperm proportion. Sperm length has recently been reported as predicting fertilization success in zebra finches [52], though this was probably related to higher sperm velocity in males selected for longer spermatozoa [52,67]. In our study, however, sperm length was not correlated with sperm velocity and there was no relationship between sperm velocity and ornament expression. Second, we observed a decrease in sperm length in more colourful males (pre-experiment) during the course of the experiment, irrespective of treatment. This further challenges interpretation of a positive correlation between initial beak colouration and sperm length as supporting the PLFH. Third, initial beak colouration predicted the effect of oxidative status on sperm velocity, such that more intense beak colouration predicted an increase in sperm velocity under control conditions and a decline in sperm velocity following diquat exposure. Since the primary mechanism of diquat toxicity is induction of oxidative stress through a redox-cycling reaction generating superoxide anions [57], these results suggest that more ornamented males have sperm that are less resistant to oxidative challenge. Such a result is inconsistent with the redox-based PLFH [23]; rather, it suggests a trade-off between carotenoid-based ornamentation and sperm resistance to oxidative challenge.

No loss in body mass was observed in the diquat-exposed birds in our study, indicating that the diquat-induced effects were not a consequence of reduced food intake or impaired total intestinal nutrient absorption (see also [45]). Using this

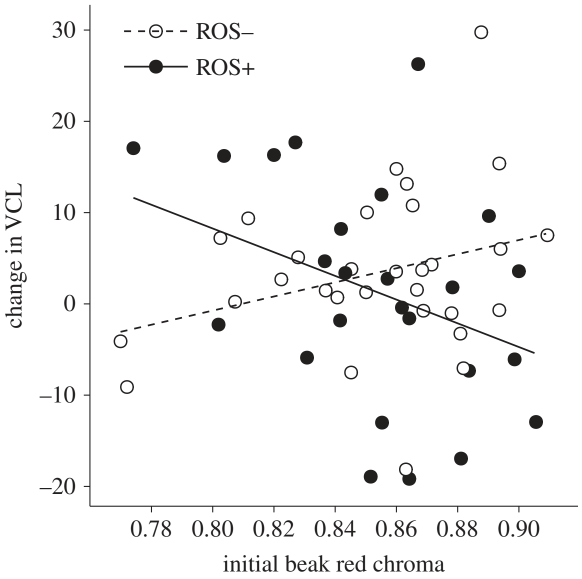

Figure 1. Change in sperm velocity (VCL) in relation to initial beak red chroma under different oxidative load levels. Lines are predicted values from the minimal adequate model.

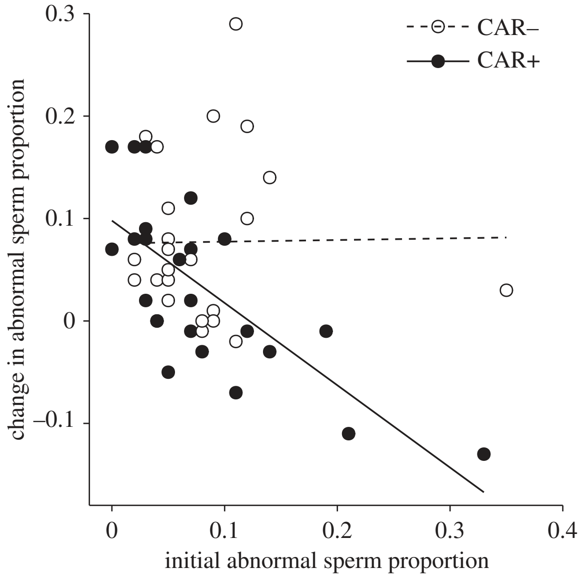

Figure 2. Effect of carotenoid availability on change in abnormal sperm proportion. Lines are predicted values from the minimal adequate model.

Table 1. Effects of oxidative challenge, carotenoid intake and ornament expression on sperm traits. Estimates are coefficients from minimal adequate models with controls and elevated treatment levels coded 0 and 1, respectively. All predictor variables were centred to enable interpretation of the main effects with interactions present. For full details of the models and the selection procedures used, see the Statistical analysis section. Full models are available in the electronic supplementary material. TSL, total sperm length.

response and predictor variables	estimate ± s.e.	*t*-value	*p*-value
change in sperm velocity ($n = 55$)			
initial sperm velocity	−0.35 ± 0.12	−2.80	0.007
initial beak red chroma	−16.86 ± 38.74	−0.44	0.67
oxidative challenge	−1.67 ± 2.55	−0.65	0.52
oxidative challenge × initial beak red chroma	−207.19 ± 77.62	−2.67	0.01
change in TSL ($n = 54$)			
initial TSL	−0.32 ± 0.08	−4.25	<0.001
initial beak red chroma	−17.64 ± 8.29	−2.13	0.04
change in flagellum/TSL ratio ($n = 54$)			
initial beak red chroma	−0.39 ± 0.11	−3.57	<0.001
change in midpiece/flagellum ratio ($n = 54$)			
initial midpiece/flagellum ratio	−0.11 ± 0.05	−2.26	0.03
initial beak red chroma	−0.38 ± 0.14	−2.69	0.01
oxidative challenge	−0.02 ± 0.01	−2.00	0.05
change in abnormal sperm proportion ($n = 47$)			
initial abnormal sperm proportion	−0.38 ± 0.15	−2.57	0.01
carotenoid intake	−0.04 ± 0.02	−2.12	0.04
carotenoid intake × initial abnormal sperm proportion	−0.82 ± 0.30	−2.74	0.009

same experiment, we previously reported that blood oxidative damage remained unchanged under diquat exposure, but that blood antioxidant capacity increased markedly [45]. This suggests that antioxidant response, rather than elevated oxidative damage, plays a major role in the diquat-induced effects observed.

A trade-off between investment in precopulatory and postcopulatory traits is predicted by the SCT, which assumes that resources available for allocation to either trait type are limited [4]. Interestingly, the change in sperm velocity noted was not related to change in beak colouration, suggesting that there is no direct trade-off in allocation of available resources between these two traits. Instead, the adverse effect of oxidative challenge on sperm velocity was related to high initial beak colouration, which suggests a long-term trade-off between ornament expression and sperm resistance to oxidative challenge. This could imply a long-term negative effect of investment in ornamentation on oxidative sperm resistance. An alternative, though not mutually exclusive, explanation may reside in differences in individual life histories. Accumulating evidence suggests that individuals are adapted for specific environments, with conditions experienced in early development shaping an individual's phenotype for the rest of its life [68]. It has been shown that early-life stress may result in lower investment in sexual trait expression when adult [69–71] and that a mismatch between early-life and adult environments may have deleterious consequences for individual fitness [72]. Accordingly, those males with redder beaks in our study may be adapted to low-stress environments and have limited antioxidant mechanisms. As a result, they may suffer a decline in sperm function under oxidative challenge. By contrast, males with paler beaks may be better adapted to higher stress environments and, therefore, perform better under oxidative challenge. The potential long-term carry-over effects of investment into sexual ornamentation on sperm traits, as well as life-history-related physiological constraints and differences in trade-off solving are not usually considered in studies investigating the relationship between precopulatory and postcopulatory sexual traits. As such, these should provide interesting areas for future research.

Our results, showing lower sperm resistance to oxidative challenge in more colourful males, contrast with those of a previous study reporting higher resistance to the adverse effects of experimental brood enlargement on sperm motility, velocity and oxidative damage in more colourful great tit (*Parus major*) males [10]. A possible explanation for such a discrepancy could lie in a difference in the signalling content of pigmented bare body parts and plumage. This is supported by their differing responses to oxidative challenge, with reduced colour intensity observed in bare-part ornamentation [44,45] but not in plumage ornamentation [73,74] following oxidative challenge. Alternatively, the contrasting results may reflect interspecific differences in ornament production and function, or differences in methodology (e.g. other effects, unrelated to oxidative stress, may come into play when using brood enlargement [10]). Whatever the explanation, our data demonstrate that carotenoid-based condition-dependent sexual traits do not generally signal sperm quality, as predicted by the PLFH [20,23].

Oxidative challenge also resulted in a shortening of sperm midpiece length in our study. Traditionally, sperm morphometry was thought to be genetically determined, at least in internal fertilizers, with low intra-individual plasticity [75,76]. Despite this, effects of season, temperature and social environment on sperm morphometry have been reported in a number of studies [38,77,78]. Our results, showing shortening of midpiece length following oxidative challenge, corroborate plasticity in morphometric sperm traits in response to environmental factors and suggest that it may be mediated by oxidative stress.

The sperm midpiece contains mitochondria that produce energy for sperm motility, and the length of the midpiece has previously been shown to be positively correlated with sperm velocity, both at the intraspecific [53] and interspecific [50] levels. In our study, however, we observed a weak and marginally non-significant correlation between midpiece length and sperm velocity, while the effects of oxidative challenge on midpiece length and sperm velocity differed, suggesting that midpiece length is probably not the major determinant of sperm velocity.

Oxidative stress is generally considered one of the most important causes of morphological defects in spermatozoa [22]. Surprisingly, we found no effect of oxidative challenge on abnormal sperm proportion in our study. We previously documented an adaptive antioxidant response in this experiment [45], however, and this is likely to have prevented any increase in oxidative damage in the testes, thereby preventing any increase in sperm abnormalities.

Carotenoid supplementation reduced the occurrence of sperm abnormalities, particularly in males with an initially high abnormal sperm proportion, suggesting that the carotenoids used in our study (i.e. lutein and zeaxanthin) are important for normal spermatogenesis. To date, evidence for the importance of carotenoids regarding normal sperm morphology has been mixed, with lycopene most often receiving support ([79–81], but see [82]). A positive correlation between normal sperm morphology and lutein or zeaxanthin concentration has only been reported in one study [83], most other studies reporting no association [81,82,84]. To the best of our knowledge, our study provides the first experimental evidence for the positive effect of lutein or zeaxanthin on normal sperm morphology. As a high occurrence of sperm abnormalities is a known cause of impaired male fertility [54], our data suggest that reduced fertility and sperm competitiveness previously reported in carotenoid deficient males [85] may be due to a high proportion of abnormal sperm in their ejaculates.

The positive effects of carotenoids on sperm traits is usually assumed to be due to their antioxidant properties [23,79–81,83]. In birds, however, the importance of carotenoids as antioxidants is disputed; indeed, carotenoids have even been proposed as pro-oxidants [31,34,36]. In our study, we observed no adverse effect of high carotenoid intake on any sperm trait, which is inconsistent with a pro-oxidant effect. By contrast, the antioxidant function of carotenoids in avian testes was supported by their reducing effect on abnormal sperm proportion. Support for their antioxidant function was mixed, however, as carotenoids did not inhibit the effects of oxidative challenge on sperm velocity or midpiece length. Overall, our results suggest that a positive carotenoid effect on sperm morphology is either not mediated by antioxidant function or that such a function is important only for specific sperm traits.

In conclusion, our data challenge the redox-based PLFH and suggest that expression of carotenoid-based sexual ornamentation is instead traded off against sperm resistance to oxidative stress, as predicted by the SCT. In their review, Dowling & Simmons [86] proposed that redox homeostasis could constitute a major constraint in life-history evolution, possibly underlying negative associations between individual sperm traits (e.g. sperm number and sperm quality) or between sperm traits and immune function. Our results suggest that redox homeostasis may also drive a trade-off between expression of sexual ornamentation and sperm competitive ability. The absence of any real-time trade-off between precopulatory and postcopulatory traits in our data implies that such a trade-off may involve long-term carry-over effects and/or physiological constraints associated with different life histories. In addition, our results suggest that carotenoids are important for normal spermatogenesis, thereby influencing male fertility and sperm competitive ability.

Ethics. All experimental procedures were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union, and were approved by the animal care and ethics representatives of the Faculty of Science of Charles University in Prague and the Czech Academy of Sciences (no. 041/2011).

Data accessibility. The dataset supporting this article is available in the Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad.f8f7g [87].

Authors' contributions. T.A. and O.T. conceived and designed the study; O.T. conducted the experiment; O.T., J.A. and T.A. collected the data; O.T. analysed the spectrophotometric ornament data; J.A., M.N. and P.O. analysed sperm data; O.T. carried out the statistical analysis and drafted the manuscript with input from T.A. All co-authors contributed to the final version of the manuscript and gave approval for its publication.

Competing interests. The authors declare no competing interests.

Funding. This study was funded through a Czech Science Foundation grant (no. P506/12/2472). O.T., J.A. and M.N. were supported through an institutional project of Charles University (no. SVV 260313/2016).

Acknowledgements. We thank Barbora Gabrielová, Jana Svobodová and František Vejmělka for help with data collection and Helena Hejlová for help with bird care.

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Supplementary Information

for

Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge

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Proceedings of the Royal Society B: Biological Sciences

Supplementary inventory

Supplementary Table S1. Average colour contrasts (ΔS) between beaks of males with different values of total sperm length (TSL) and flagellum/TSL ratio

Supplementary Table S2. Pearson's correlation coefficients between selected sperm traits prior to the experiment

Supplementary Table S3. Group mean values and standard deviations of measured variables prior to the experiment

Supplementary Table S4. Group mean values and standard deviations of measured variables at the end of the experiment

Supplementary Table S5. Selection of models analysing variation in changes in sperm velocity and abnormal sperm proportion

Supplementary Table S6. Selection of models analysing variation in changes in sperm morphometric traits

Supplementary Table S7. Effects of oxidative challenge, carotenoid intake and ornament expression on abnormal sperm proportion based on a data subset with one influential observation removed

Supplementary Table S1. Average colour contrasts (ΔS) between beaks of males with different values of total sperm length (TSL) and flagellum/TSL ratio. Males were divided into three groups of 20 individuals based on respective sperm trait (e.g. short, medium and long sperm). ΔS is measured in just noticeable differences (jnds) with values of ΔS > 1 jnd being theoretically discriminable by a modelled visual system under given conditions.

comparison	**TSL**	**flagellum/TSL**
low vs. medium	4.4	4.3
medium vs. high	8.6	2.8
low vs. high	12.9	7.0

Supplementary Table S2. Pearson's correlation coefficients between selected sperm traits prior to the experiment. Proportional variables (i.e. flagelum/TSL ratio, midpiece/flagellum ratio, and abnormal sperm proportion) were normalised using logit transformation. Abbreviations: TSL = total sperm length; VCL = sperm velocity.

pairs of sperm traits	***r***	***p***
head length : TSL	**0.40**	**0.001**
flagellum length : TSL	**0.98**	**<0.001**
flagellum length : head length	0.23	0.08
midpiece length : TSL	<0.01	0.97
midpiece length : head length	**0.33**	**0.009**
midpiece length : flagellum length	−0.06	0.64
abnormal sperm proportion : TSL	−0.05	0.71
abnormal sperm proportion : flagellum/TSL ratio	0.12	0.36
abnormal sperm proportion : midpiece/flagellum ratio	**−0.36**	**0.006**
VCL : TSL	0.04	0.78
VCL : flagellum/TSL ratio	−0.02	0.87
VCL : midpiece/flagellum ratio	0.24	0.07
VCL : abnormal sperm proportion	−0.19	0.16

Supplementary Table S3. Group mean values and standard deviations of measured variables prior to the experiment. Abbreviations: TSL = total sperm length; VCL = sperm velocity.

treatment group	**ROS− CAR−**		**ROS− CAR+**		**ROS+ CAR−**		**ROS+ CAR+**	
variable	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**
VCL	15	68.74 (10.40)	15	71.02 (8.79)	15	68.36 (10.91)	15	71.29 (11.97)
TSL	15	74.18 (3.91)	15	72.81 (3.80)	15	72.99 (4.02)	15	72.12 (2.68)
head length	15	12.60 (0.71)	15	12.32 (0.84)	15	12.19 (0.39)	15	12.31 (0.72)
flagellum length	15	61.58 (3.90)	15	60.49 (3.34)	15	60.81 (3.91)	15	59.81 (2.37)
midpiece length	15	25.55 (6.61)	15	25.90 (5.74)	15	25.11 (5.53)	15	27.65 (5.90)
flagellum/TSL ratio	15	0.830 (0.011)	15	0.831 (0.009)	15	0.833 (0.009)	15	0.829 (0.008)
midpiece/flagellum ratio	15	0.434 (0.12)	15	0.446 (0.097)	15	0.414 (0.089)	15	0.464 (0.108)
abnormal sperm proportion	14	0.071 (0.036)	15	0.093 (0.084)	14	0.103 (0.05)	15	0.058 (0.048)

Supplementary Table S4. Group mean values and standard deviations of measured variables at the end of the experiment. Abbreviations: TSL = total sperm length; VCL = sperm velocity.

treatment group	**ROS− CAR−**		**ROS− CAR+**		**ROS+ CAR−**		**ROS+ CAR+**	
variable	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**	***n***	**mean (s.d.)**
VCL	15	71.96 (11.36)	15	74.52 (11.22)	13	68.94 (11.44)	12	75.77 (12.86)
TSL	15	70.61 (3.08)	15	69.53 (2.45)	13	69.80 (3.61)	11	68.70 (3.09)
head length	15	12.00 (0.69)	15	11.77 (0.67)	13	11.55 (0.45)	11	11.69 (0.65)
flagellum length	15	58.61 (3.13)	15	57.76 (2.36)	13	58.24 (3.38)	11	57.00 (3.08)
midpiece length	15	25.03 (6.80)	15	24.93 (5.02)	13	23.24 (5.38)	11	23.81 (5.70)
flagellum/TSL ratio	15	0.830 (0.012)	15	0.831 (0.010)	13	0.834 (0.007)	11	0.830 (0.012)
midpiece/flagellum ratio	15	0.430 (0.123)	15	0.432 (0.090)	13	0.399 (0.087)	11	0.420 (0.112)
abnormal sperm proportion	14	0.160 (0.107)	13	0.123 (0.055)	12	0.150 (0.098)	10	0.102 (0.065)

Supplementary Table S5. Selection of models analysing variation in changes in sperm velocity and abnormal sperm proportion. Given are F-statistics of the predictor variables at the time of their exclusion from the model during backward stepwise model selection in cases of excluded predictors and F-statistics from the final minimal adequate models in cases of retained predictors. The predictors retained in the final models are highlighted in bold. Significances of the main effects were not tested when involved in significant interactions.

response and predictor variables	*F*	*p*
change in sperm velocity (*n* = 55)		
initial sperm velocity	**7.83**	**0.007**
initial beak red chroma	**-**	**-**
change in beak red chroma	0.09	0.77
oxidative challenge	**-**	**-**
carotenoid intake	1.05	0.31
initial sperm velocity × oxidative challenge	0.40	0.53
initial sperm velocity × carotenoid intake	0.08	0.78
initial beak red chroma × oxidative challenge	**7.12**	**0.01**
initial beak red chroma × carotenoid intake	0.44	0.51
change in beak red chroma × oxidative challenge	1.67	0.20
change in beak red chroma × carotenoid intake	1.05	0.31
oxidative challenge × carotenoid intake	0.10	0.75
change in abnormal sperm proportion (*n* = 47)		
initial abnormal sperm proportion	**-**	**-**
initial beak red chroma	0.57	0.45
change in beak red chroma	1.70	0.20
oxidative challenge	0.33	0.57
carotenoid intake	**-**	**-**
initial abnormal sperm proportion × oxidative challenge	0.47	0.50
initial abnormal sperm proportion × carotenoid intake	**7.53**	**0.009**
initial beak red chroma × oxidative challenge	2.29	0.14
initial beak red chroma × carotenoid intake	0.72	0.40
change in beak red chroma × oxidative challenge	0.88	0.35
change in beak red chroma × carotenoid intake	2.72	0.11
oxidative challenge × carotenoid intake	1.16	0.29

Supplementary Table S6. Selection of models analysing variation in changes in sperm morphometric traits. Given are F-statistics of the predictor variables at the time of their exclusion from the model during backward stepwise model selection in cases of excluded predictors and F-statistics from the final minimal adequate models in cases of retained predictors. The predictors retained in the final models are highlighted in bold. Abbreviations: TSL = total sperm length

response and predictor variables	***F***	***p***
change in TSL (*n* = 54)		
initial TSL	**18.02**	**<0.001**
initial beak red chroma	**4.53**	**0.04**
change in beak red chroma	1.69	0.20
oxidative challenge	0.71	0.40
carotenoid intake	0.01	0.93
initial TSL × oxidative challenge	0.94	0.34
initial TSL × carotenoid intake	1.01	0.32
initial beak red chroma × oxidative challenge	0.43	0.51
initial beak red chroma × carotenoid intake	0.96	0.33
change in beak red chroma × oxidative challenge	3.30	0.08
change in beak red chroma × carotenoid intake	0.03	0.86
oxidative challenge × carotenoid intake	0.00	0.99
change in flagellum/TSL ratio (*n* = 54)		
initial flagellum/TSL ratio	0.00	0.98
initial beak red chroma	**12.77**	**<0.001**
change in beak red chroma	0.79	0.38
oxidative challenge	0.00	0.99
carotenoid intake	0.04	0.84
initial flagellum/TSL ratio × oxidative challenge	0.89	0.35
initial flagellum/TSL ratio × carotenoid intake	1.34	0.25
initial beak red chroma × oxidative challenge	0.06	0.81
initial beak red chroma × carotenoid intake	0.40	0.53
change in beak red chroma × oxidative challenge	2.23	0.14
change in beak red chroma × carotenoid intake	0.01	0.91
oxidative challenge × carotenoid intake	0.05	0.83
change in midpiece/flagellum ratio (*n* = 54)		
initial midpiece/flagellum ratio	**5.09**	**0.03**
initial beak red chroma	**7.25**	**0.01**
change in beak red chroma	0.03	0.87
oxidative challenge	**4.01**	**0.05**
carotenoid intake	0.30	0.59
initial midpiece/flagellum ratio × oxidative challenge	0.11	0.74
initial midpiece/flagellum ratio × carotenoid intake	0.04	0.83
initial beak red chroma × oxidative challenge	0.03	0.87
initial beak red chroma × carotenoid intake	0.55	0.46
change in beak red chroma × oxidative challenge	0.01	0.94
change in beak red chroma × carotenoid intake	1.35	0.25
oxidative challenge × carotenoid intake	0.84	0.36

Supplementary Table S7. Effects of oxidative challenge, carotenoid intake and ornament expression on abnormal sperm proportion based on a data subset with one influential observation removed. Estimates are coefficients from a minimal adequate model with control and increased carotenoid intake coded 0 and 1, respectively. All predictor variables were centred to enable interpretation of the main effects with the interactions present.

response and predictor variables	**estimate ± s.e.**	***t***	***p***
change in abnormal sperm proportion (*n* = 46)			
initial abnormal sperm proportion	−0.12 ± 0.24	−0.49	0.63
carotenoid intake	−0.05 ± 0.02	−2.15	0.04
carotenoid intake × initial abnormal sperm proportion	−1.37 ± 0.48	−2.87	0.006

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

The following publications were published during the PhD study but are not included in the thesis.

Tomasek O., Bobek L., Kralova T., Adamkova M. & Albrecht T. Fuel for the pace of life: baseline blood glucose concentration coevolves with life history traits in songbirds. (*in revision for Functional Ecology*)

Klvaňa, P., Cepák, J., Munclinger, P., Michálková, R., **Tomášek, O.** & Albrecht, T. (2018). Around the Mediterranean: an extreme example of loop migration in a long-distance migratory passerine. *J. Avian Biol.*, 49, jav-01595.

Blažek, R., Polačik, M., Kačer, P., Cellerino, A., Řežucha, R., Methling, C., **Tomášek, O.**, Syslová, K., Terzibasi Tozzini, E., Albrecht, T., Vrtílek M. & Reichard M. (2017). Repeated intraspecific divergence in life span and aging of African annual fishes along an aridity gradient. *Evolution*, 71, 386–402.

Kreisinger, J., Kropáčková, L., Petrželková, A., Adámková, M., **Tomášek, O.**, Martin, J.-F., Michálková R. & Albrecht T. (2017). Temporal stability and the effect of transgenerational transfer on fecal microbiota structure in a long distance migratory bird. *Front. Microbiol.*, 8.

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Rymesova, D., **Tomasek, O.** & Salek, M. (2013). Differences in mortality rates, dispersal distances and breeding success of commercially reared and wild grey partridges in the Czech agricultural landscape. *Eur. J. Wildl. Res.*, 59, 147–158.