Beak colouration of starling (*Sturnus unicolor*) males depends on the length of their throat feathers

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Ethical Note

We performed the study following the relevant Spanish national (Decreto 142/2013, 1 de octubre) and regional guidelines. The ethics committee of the Spanish National Research Council (CSIC) approved the protocol, and the Consejería de Medio Ambiente de la Junta de Andalucía, Spain, provided all the necessary permits for nest and birds manipulations (Ref: SGMN/GyB/JMIF). The protocols adhered to the ASAB/ABS Guidelines for the Use of Animals in Research. The authorization for carrying out the experimental work on animals in the field was granted by the Dirección General de Gestión del Medio Natural of the Consejería de Medio Ambiente of Junta de Andalucía.

Author contributions

Conceived and designed the experiments: JJS, MRR, GT and MMV. Fieldwork: MAG, JJS, MRR, GT, CRC and SDL. Analysed the data: MAG and JJS. Contributed reagents/materials/analysis tools: substantial contribution from all authors. MAG wrote the first version with supervision of JJS and MRR. All authors substantially contributed to final version.

Data accessibility

Data used in this paper can be found in CSIC Institutional Repository, with the accession numbers <xxxxxxxx>.

Lay summary

The use of signals to indicate the individual quality is widespread in nature. However, although most species show more than one signal, the relationships between different signals have almost never been experimentally studied. Here, we demonstrated that the experimental reduction of throat feathers length conditioned the beak colouration of spotless starling males at the time of reproduction. Our results are the first experimental evidence of two sexually dimorphic traits being related in natural conditions.

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Abstract

Within the context of complex sexual signalling, most research has focussed on exploring the associations between several signals and/or their relationships with different proxies of individual quality. However, very few studies have focused on checking whether the expression of one signal is conditioned by the expression of the others. Here, by experimentally shortening the throat feathers of male spotless starlings (*Sturnus unicolor*), we evaluated the influence of this trait on the colour expression of the beak base. In addition, we tested the relationship between these two sexually dimorphic characters with traits indicating individual quality such as body condition and colour reflectance at the wavelength related to carotenes in the tip of the beak. Our results show that the colouration of the beak base in males, but not in females, is positively related to body condition and to the length of ornamental throat feathers. Moreover, the experimental shortening of throat feathers in males had a negative effect on the blue chroma intensity of their beak base one year after manipulation. These results support for the first time a causal link between the expression of two sexually dimorphic characters, which is essential to understand their functionality in a multiple signalling framework.

Keywords: Beak colour, Body condition, Interacting signals, Multiple signals, Ornamental feathers length, Sexually dimorphic characters.

Introduction

Animals use a wide array of signals to inform about their phenotypic or genetic quality to conspecifics in social interactions, in contexts such as mate choice or competition for resources (Kokko 2003, Andersson and Simmons 2006, Kraaijeveld et al. 2007, Lyon and Montgomerie 2012, Edward 2015). In contexts of sexual selection, males typically possess multiple traits that may convey independent information to receivers (Møller and Pomiankowski 1993). Although most research on the evolution and function of signals has focused on single traits, the importance of studying these characters within the theoretical and more realistic framework of multiple signals has been highlighted (Candolin 2003, Hebets and Papaj 2005). Different characters might, for instance, imply multiple or redundant messages (i.e., information), or might be more efficient in particular environments or in stimulating particular sensory channels (Møller and Pomiankowski 1993, Candolin 2003, Hebets and Papaj 2005). Hebets and Papaj (2005) developed a framework of testable hypotheses for explaining the evolution and functioning of multiple signals. They highlighted (i) the importance of considering complex signals and the unit of character selection; (ii) that complex signals include several characters that function together, either facilitating the transmission (e.g., using different sensory channels) or reinforcing transmitted information to receivers (i.e., redundant information); and (iii) that individual signals or components of complex signals do not necessarily function independently, but may interact in a functional way.

Most research on complex signalling has focussed on exploring the association between several signals (Perrier et al. 2002, Bro-Jorgensen and Dabelsteen 2008, Mason et al. 2014, Chaine and Lyon 2015, Girard et al. 2015), or between signals and different proxies of fitness including phenotypic quality (Balmford et al. 1992, Martin and Lopez 2009), mating success (Møller and Pomiankowski 1993) and efficacy of signal transmission in different environments (Endler and Houde 1995). Even though the study of the interactions (i.e., associations) between different signalling characters is essential to know individual or complex signals functioning, it is one of the least explored areas within the field of signal evolution. The study of signal interactions has the potential to shed light on signal functioning because, for instance, detecting a positive association would suggest that transmitted information is redundant or complementary. Moreover, a negative association would indicate that a trade-off between signalling characters exists, while the absence of association between different signals would suggest that they convey different information to receivers (Candolin 2003, Hebets and Papaj 2005). In most instances, inter-signal interaction occurs when the presence of one signal or a signal component alters the response of the receiver to a second signal or component by amplifying or conditioning the information provided by each other.

Interactions between signals may also occur when the production of one signal influences the cost of production of another signal (Johnstone 1996, Candolin 2003). In this case, independently of the transmitted information, the phenotypic expression of one signal impinges on the resulting phenotype of the other signal. Signals are typically costly to produce (Hasson 1994, Salvador et al. 1996), to maintain (Ruiz-Rodríguez et al. 2015), or to show (i.e. social cost; Tibbetts and Dale 2004), and the expression of signals or signal components may be traded-off against each other. On the one hand, there could be a trade-off between two signals (e.g. by using the same resources as carotenes), so that a lesser expression of one increases the expression of the other (Andersson et al. 2002). On the other hand, it is also possible that the expression of one signal reduces the average costs due to social interactions (Morales and Velando 2018) and, thus, facilitates or enhances the expression of other signals. For instance, ornaments that develop before reproduction and function in social contexts others than sexual (e.g. intra-sexual), and could serve to stablish social hierarchy, may reduce agonistic social interactions and mitigate subsequent energetic costs. Saved energy could thus enhance the production of other sexual ornaments during courtship or reproduction and, therefore, the expression of ornaments developed before and during reproduction could be positively related. This might be the case of certain plumage characteristics of birds that reduce social costs before reproduction (Senar et al. 2000), and thus, could boost the expression of other sexually selected traits, such as song or other similar flexible dynamics traits, that are exclusively expressed during reproduction (Badyaev et al. 2002, Mason et al. 2014).

Detecting evidence supporting the hypothesis that the expression of one signal is conditioned by the expression of other signals can be challenging. A main reason is that sexual signals are typically condition-dependent (e.g. Saino et al. 1997, Velando et al. 2006, Soler et al. 2008). Thus, detecting positive or negative associations between the expression of different signals is not enough to infer causation. Rather, this hypothesis should be tested in experimental frameworks where the modification of one signal causes or explains the phenotypic expression of other signals. As far as we know, this hypothesis has been tested experimentally only once by Henderson et al. (2018), who manipulated plumage colouration of house finch (*Haemorhous mexicanus*) males before reproduction and detected an effect on male investment in song under captivity conditions. However, the effect was dependent on experimentally modified social context (feather colouration of neighbours) and, thus, it is not completely clear that the detected effects were exclusively caused by costs associated to plumage colouration. Here, we go a step further and look for experimental evidence supporting the hypothesis in the wild in spotless starlings (hereafter starlings, *Sturnus unicolor*).

Starlings are semi colonial and sexually dimorphic birds, with males showing elongated throat feathers (Hiraldo and Herrera 1974, Lezana et al. 2000) and conspicuous yellow beak with blue coloured basal part (Navarro et al. 2010). These two sexually dimorphic~~secondary sexual~~ traits could harbour different kinds of information or, at least, information at different time scales. The apical part of these feathers is quite flexible, and males exhibit them very conspicuously during the entire year in social interactions, including courtship (Aparicio et al. 2001, Ruiz-Rodríguez et al. 2015). In addition, these feathers honestly reflect the phenotypic quality of individuals (Lezana et al. 2000, López-Rull et al. 2007, Gil and Culver 2011, Ruiz-Rodríguez et al. 2015). On the other hand, during mating and reproduction (from February to July in our study area), the otherwise black coloured beak of starlings turn to yellow colouration in both sexes, while its basal part turn to blue in males and to pink in females (Cramp 1998) (Fig.1). Beak colour in starlings is a sexually dimorphic and dynamic trait that likely reflects antioxidant capacity (Navarro et al. 2010) and, accordingly, previous studies found that the yellow colour of the beak is related to the level of carotenoids and vitamin A in the plasma in both sexes (Navarro et al. 2010). The moult of throat feathers occurs in September-October (Veiga and Polo 2016), thus far before the reproductive period. Therefore, it is likely that these feathers serve to stablish social hierarchies within the population during the whole year, allowing to reduce agonistic interactions and to mitigate its associated costs (Andersson 1994). If that was the case, the length of the throat feathers could play an important role during the non-breeding season by affecting the acquisition and allocation of resources, which could be reflected in the intensity of beak colouration in starling males. Length of throat feathers can be easily manipulated (see Material and Methods), so the hypothesis that the expression of one signal (length of throat feathers) determines the expression of the other (beak colouration) can be experimentally tested.

We manipulated the length of the throat feathers of males by cutting-off approximately the half-distal portion of the feathers before reproduction, and explored its effects on the colour (chroma and brightness) of the base of the beak during reproduction. If this manipulation increases social agonistic interactions before reproduction, energy and resources available for developing sexual colouration during reproduction will be lower for experimental than for control males. This scenario therefore predicts a negative effect of experimentally shortened throat feathers on beak colour. An alternative scenario is that the experiment did not result in differential social costs for experimental and control males before reproduction. In this case, it is possible that experimental males compensate the loss of one ~~sexual~~ signal by increasing the expression of the other. This alternative scenario therefore predicts a positive effect of the manipulation on the expression of beak colouration.

We also tested correlative predictions of the hypothesis that considered these traits as sexual signals of males but not of females. Particularly, we expected that length of throat feathers and colour (chroma and/or brightness) of the base of the beak were positively related in starling males, but not in females. Moreover, we also expected that both traits are correlated with body condition, an indicator of the phenotypic quality of individuals.

Material and Methods

Study area and study species

The study was conducted during the years 2015, 2016 and 2017 in a south-eastern region of Spain (Hoya de Guadix, 37º15'N, 3º01'W), where nest-boxes attached to tree trunks or walls at 3–4 m above-ground are available for starlings to breed in (for further information on the study area see Soler et al. (2017)).

In the studied starling population, the reproductive season starts in early April and most individuals lay a second clutch during May-June. The most common clutch size is 4-5 eggs. ~~Incubation is mostly carried out by females with sporadic help from males, and~~

~~extends for around 14 days, while the nesting period lasts 18-25 days (Cramp 1998). Both male and female parents contribute to feeding the offspring and remove nestling faecal sacs (Cramp 1998). During the whole nesting process, adults bring feathers and aromatic plants to the nest, which have been shown to have antimicrobial-beneficial functions (Ruiz-Castellano et al. 2016, Soler et al. 2017, Ruiz-Castellano et al. 2019). Reproductive success in the study area varies among years and breeding attempts, with second clutches usually showing lower reproductive success than first clutches (unpublished data).~~ Here, we will focus on the colouration of the base of the beak, a trait with a more marked sexual differentiation as we can see in its reflectance at different wavelengths (Fig. 1).

Fieldwork and experimental procedure

In this population, courtship activity (e.g. singing, introducing fresh green plants and feathers in nest boxes) starts in February, more than one month before egg laying (pers. obs.). During this period, some birds roost in nest-boxes and we take advantage of this fact for conducting yearly bird trapping sessions in the study area (twice a year between February and mid-March). One hour before dawn, we closed the entrance of all nest boxes in the study area, and immediately after dawn, we captured by hand all individuals found roosting inside. Captured birds were kept individually in clean cotton bags hanging from a stick to keep birds quiet, and were released immediately after sampling. The maximum time that a captured starling was in the bag did never exceed three hours. We explored the possible effect of time that birds were kept in the bag on bird colouration and body condition measures of the males that we recaptured by classifying them as being kept in the bag less than 1 hour (N(males) = 10), between 1 and 2 hours (N = 5), and between 2 and 3 hours (N = 7). After controlling for the effect of date of first and last capture, time between captures, treatment and size of throat feathers in the first capture, results showed that retaining time in first captures did not significantly affect blue, red-yellow, or brightness colouration of the beak of males ($F_{1,15} < 2.66$, $P > 0.124$), nor body condition ($F_{1, 10} = 2.19$, $P = 0.170$) in subsequent captures. It neither had any apparent long-term consequences (see Ruiz-Rodríguez et al. (2015)), nor imply apparent negative effects on breeding performance of captured birds (Soler et al. 2008).

Every year, we also captured birds during breeding, 4-5 days after the hatching date, by using nest-box traps operated for a maximum time of one hour. All captured adults (males and females) were ringed with a numbered metal ring (if not already ringed) and with a unique combination of three colour rings. They were weighed with a hanging scale (Pesola 0–300 g, accuracy 2 g), their tarsus and beak length was measured with a digital calliper (accuracy 0.01 mm) and their wing length with a ruler (accuracy 1 mm). We also measured three times the length of throat feathers of males and females with a ruler. In addition, the colour of the base of the beak was measured with a spectrometer (see below). Finally, captured males were alternately assigned to control or experimental treatment. With the aid of scissors, we cut the distal half portion of throat feathers of experimental individuals, while we handled control males in the same way but without cutting throat feathers (Fig. 2). After manipulation, we again measured feather length of experimental males, which on average were shortened by 1.5 cm (GLM of throat-feather lengths by treatment in his first capture; Least squares means ± SE: experimental males: 1.8 ± 0.1 cm; control males: 3.2 ± 0.1; $F_{1,52} = 140.16$, $p < 0.0001$). Males that were recaptured during different study years were assigned each time to the same experimental treatment, repeating the same procedure on them, with the exception of three males. Treatment of these three males changed from control in the first to experimental in the second capture, one year later. The effects of treatment in these males were considered as independent information in the analyses. We managed to collect information from 102 females and 57 males (29 controls and 28 experimental), being recaptured after treatment 22 of these males (11 controls and 11 experimental) (see Annex 1).

Colour measurements

The colour of the base of the beak was measured in males and females with an Ocean Optics S2000 spectrometer in the field. It was connected to a deuterium-halogen light (D2-W, Mini). To standardize ambient light conditions, we used a black bag that wrapped the tip of the optical fibre and the beak and made the measurements inside a dim area (nearby building or in a tent). Before the measurement of each individual, we calibrated the spectrometer using a standard white and black reference. We obtained reflectance spectra at 1 nm intervals from 300-700 nm for all individuals. The colour was measured three times on the base of the beak, and average values were obtained.

We estimated the proportion of total reflectance within the blue range (colour to which the base of the males beak tends) of chroma of the spectrum (λ = 400-475 nm, hereafter blue colour intensity). We did the same with the yellow-red range of chroma of the spectrum (λ = 570-700nm, hereafter yellow-red colour intensity). This range coincides with the typical gradual increasing colour spectral shape of phaeomelanins (Navarro et al. 2010) and in which the reflectance values of males and females are more differentiated at the base of the beak (Fig. 1). In addition, it covers the range of red to which the base of the beak usually tends in females. We also calculated the average brightness over the entire range of the spectrum (300-700 nm). Finally, we also calculated the chroma reflectance values at the carotenoids' wavelengths (450-570 nm) as the proportion of total reflectance within the carotenoid range. All these colour variables were estimated by using the "shape model" option in the Avicol V.6 software (Gomez 2006). Prior to all analyses, negative values were set to zero and reflectance curves were corrected for noise using triangular smoothing (Gomez 2006).

Statistical analyses

The three measurements of the length of throat feathers in males and females were highly repeatable ($R = 0.96$, $F_{145,292} = 51.0$, $p < 0.0001$) and, consequently, we used the mean value for further analyses. Body condition was estimated as residuals of body mass after correcting for tarsus length ($R = 0.151$, $F_{1,241} = 5.66$, $p = 0.018$). However, residuals from this regression were positively correlated with other body size indicator, the wing length ($R = 0.289$, $F_{1,241} = 21.91$, $p < 0.00001$) and, therefore, we included this variable in the model (multiple $R = 0.323$, $F_{2,240} = 13.95$, $p < 0.0001$; partial regression coefficients: tarsus length = 0.127, $t_{240} = 2.08$, $p = 0.039$; wing length = 0.286, $t_{240} = 4.66$, $p < 0.0001$) to estimate body condition (Green 2001). Residuals of this model no longer correlated with other body size indicator variables such as beak length ($R = 0.059$, $F_{1,241} = 0.84$, $p = 0.359$).

The effect of the experimental treatment on males was estimated only for individuals that were caught at least twice (N =22). The time between captures lasted more than 315 days (N one year after treatment = 11, N two years after treatment = 6), with the exception of 5 individuals, whose second captures were performed 14 and 97 days after the first ones. We repeated the analyses by removing these 5 individuals and the results were qualitatively the same, so we show here the results with the larger sample sizes to increase statistical power since beak colour changes may occur in days or hours (Iverson and Karubian 2017). In all the analyses with the colour variables, we took into account only the captures made at the breeding season. The date of capture (day in which the measure of colour was taken within each year) was included in the models, since beak colours may vary throughout the reproductive season (Navarro et al. 2010). However, the date of capture was not included in the analyses related to the length of the throat feathers because no moulting or any major changes in feather length are expected to occur along the breeding season. Independently of the study year, the zero value of capture date corresponds to the 1st of April.

To explore the associations between length of throat feathers (dependent variable) and body condition (independent variable) we used General Linear Models (GLMs) that also included study year as discrete fixed independent factor. We performed this analysis for both sexes, only considering information on first captures (i.e., before manipulation). We used similar models to calculate the relationship between different beak colour variables (blue and yellow-red colour intensity and brightness; dependent variables) and body condition (independent variable) where, in addition to the year (discrete fixed independent factor), we added the date of capture within the season as an independent co-variable. Because different colour variables were significantly related to each other, associations between body condition and different colour variables were explored in separate models.

On the other hand, we explored the association between different beak colour variables (blue and yellow-red colour intensity and brightness; dependent variables) and length of throat feathers (independent variable). In this case, study year, but not date of capture was included in the models (i.e. one per colour variable). Because of the bimodal distribution for males and females of length of throat feathers and variables describing beak colouration, these models were run separately for each sex. In addition, we performed GLMs to explore the relationships among 1) blue and yellow-red chroma at the base of the beak (controlled by year and date of capture), and 2) base and tip beak colours.

Finally, the effects of the experimental manipulation (i.e., shortening the length of throat feathers) on the beak base colour and on body condition in males were tested by means of repeated-measures ANOVAs separately, with first and last capture as the within factor (i.e. dependent variable), and experimental treatment as the categorical predictor. The date of first and last captures, as well as number of days between captures were included as continuous independent variables in the statistical models. In addition, we checked whether the experiment did affect length of throat feathers after moult, by carrying out repeated-measures ANOVAs. In this model, the feather length, at first and last captures, was the dependent variables (repeated measures), the experimental treatment was the categorical predictor, and the number of days between captures was the continuous independent variable. Residuals of all statistical models were plotted and visually checked for normality. All analyses were performed with Statistica V13 (Dell-Inc. 2015).

Results

The blue chroma and the yellow-red chroma of the starlings' beak-base are negatively related in both males (Beta(SE) = -0.68(0.08), $F_{1,54}$ = 74.64, $p < 0.001$) and females (Beta(SE) = -0.92(0.03), $F_{1,100}$ = 933.58, $p < 0.001$). Moreover, the base (400-475 nm and 570-700 nm) and tip (450-570 nm) beak colours were not significantly associated in males ($blue_{400\text{-}475:}$ Beta(SE) = 0.02(0.13), $F_{1,54}$ = 0.03, p = 0.867; yellow-red $_{570\text{-}700}$: Beta(SE) = -0.08(0.11), $F_{1,54}$ = 0.49, p = 0.487), but a tendency (positive for blue and negative for yellow-red chroma) was detected in females ($blue_{400\text{-}475}$: Beta(SE) = 0.14(0.07), $F_{1,100}$ = 3.92, p = 0.050; $yellow\text{-}red_{570\text{-}700}$: Beta(SE) = -0.14(0.07), $F_{1,100}$ = 3.77, p = 0.055).

Body condition was positively and negatively related to intensity of blue and yellow-red colouration of males' beak, respectively (Table 1, Fig. 3). Neither the brightness of males' beak nor the length of their throat feathers were related to body condition (Table 1). In females, none of these variables predicted body condition (Table 1). Similarly, length of throat feathers of males, but not that of females, was positively and negatively related to blue and yellow-red colour intensity of males' beak, respectively (Table 1, Fig. 3). Beak brightness did not predict length of throat feathers of males or females (Table 1). Thus, the blue colour intensity of males' beak co-varied with the length of throat feathers, which might inform females on the phenotypic quality (body condition) of males.

Importantly, the experimental shortening of throat feathers in males provoked a reduction in the intensity of the blue, but no other, colouration of their beaks (measured one or two years after manipulation of throat feathers) (Table 2, Fig. 4). Moreover, the experimental manipulation did not affect body condition or the length of throat feathers in subsequent captures (Table 2). Neither date of first and second capture nor time between the two captures did explain additional significant proportion of variance (results not shown). These results suggest a direct link between length of throat feathers and beak colouration of males, which is independent of the association of both characters with phenotypic condition of males.

Discussion

Our main results are that (i) intensity of colouration of the beak base of spotless starling males, but not that of females, was positively related to body condition; (ii) beak colouration of males was positively related to the length of their ornamental throat feathers, and (iii) the experimental shortening of throat feathers in males had a negative effect on the blue chroma intensity of the beak of males one year after manipulation. Length of throat feathers and beak colouration are two sexually dimorphic traits that reflect phenotypic quality of males (Aparicio et al. 2001, Navarro et al. 2010) and, thus, our results demonstrate a direct connection between these two traits suggesting that they may function as a whole in a multiple signalling framework.

Starlings have several known sexually dimorphic traits and are an appropriate model system to explore functional interactions between sexual signals. Most studies on sexual signals in this species are focussed on the length of throat feathers of males, which predicts mating success (Aparicio et al. 2001), genetic heterozigosity (Aparicio et al. 2001), immune response (Gil and Culver 2011) and telomere length (Azcárate-García et al. 2020). Bill colouration of the distal yellow part has also been studied as a sexually selected trait of the species because it is related to carotenoid and vitamin A concentration in the blood of males and females, but only during the mating period (Navarro et al. 2010). Sexual differences are however more apparent at the basal part of the beak (Fig 1), and we concentrated on this trait to experimentally explore the possible association with the length of throat feathers. In agreement with the assumption that the blue colouration of the basal part of the beak has a sexual-signalling function, we found that its blue-colour intensity was positively related with both body condition and the length of the throat feathers. Thus, exploring the interaction between these two traits is justified.

Length of throat feathers and beak coloration of starling males provide information at different time scales. Black feathers are relatively static and would provide information of the phenotypic condition and quality of males at the time of moulting (Badyaev and Hill 2000, Hebets and Papaj 2005). Moreover, feather deterioration would also provide information on feather quality and on ability of males reducing feather degradation (Shawkey et al. 2007, Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2015). Thus, length of throat feathers might even include different kinds of information at a long-term scale. The beak colouration, however, should function at a short time scale. Like for the colouration of other bare parts of birds, beak colouration has the potential to change within weeks, days, hours, or even seconds (Iverson and Karubian 2017). Thus, this kind of dynamic characters should be continuously evaluated by receivers (Velando et al. 2006, Simons and Verhulst 2011, Dey et al. 2015). As far as we know, associations between these two types of sexually dimorphic traits have never been assessed.

Our results showed that male body condition at the time of mating was related to blue colouration of the beak, but not to the length of throat feathers, suggesting that both signals do not provide identical but, perhaps, complementary information. This could be due to the fact that throat feather length would explain body condition of males at the time of moulting, while beak colouration would be a more dynamic character that, similarly to the colour of the legs of blue-footed boobies (Sula nebouxii) (Torres and Velando 2007), shows individual condition at the time of capture. However, length of throat feathers was positively related to the intensity of blue colouration of the beak and, thus, it is possible that both traits convey redundant information to females. In agreement with the possibility that these two traits transfer complementary information to females, we experimentally showed a negative effect of length of throat feathers on the intensity of the blue chroma of the beak base of males several months after the manipulation. We know that colouration of the tip of the beak reflects the antioxidant capacity of starlings (Navarro et al. 2010). The association between beak coloration and carotenoids' concentration in the blood has also been detected in other species (Faivre et al. 2003). We did not measure concentration of carotenoids in the blood in this study and, thus, we cannot explore whether this association exists for the blue coloration of the beak base of males. Moreover, colour reflectance of the beak tip at the carotenoid wavelength, which resulted positively related to carotenoid level in starlings (Navarro et al. 2010), was not related to colouration of the base of the beak of males. Consequently, the colouration of the beak base is unlikely conveying information on antioxidant capacity to females. Thus, our experimental results should be interpreted as length of throat feathers functioning during the non-reproductive period and determining phenotypic condition of males during mating.

Like other signals operating in non-sexual scenarios such as parent-offspring communication (Morales and Velando 2018), or sibling negotiation (Johnstone and Roulin 2003, Soler and Avilés 2010), including those mediated by feather colourations (Senar 2006), the length of throat feathers of males might serve to stablish some kind of social hierarchy between males that reduce the probability of agonistic interactions among individuals of different status (Rohwer 1975, Senar 1999, McGraw and Hill 2000). Starlings moult throat feathers several months before reproduction, and males frequently display these feathers while singing in high visible places during non-reproductive periods (pers. obs.), which might have a functional significance in a context of social interactions. In some bird species, probability of social aggression by conspecifics is related to feather characteristics signalling bird status (Senar 1990, McGraw et al. 2007, Chaine and Lyon 2008)). Moreover, aggressions are more common among individuals showing similar status (Midamegbe et al. 2011), with individuals harbouring signals of higher quality eliciting lower level of aggressiveness (Lopez-Idiaquez et al. 2016). The experimental reduction of throat feathers lasts until the next moult period in autumn and, thus, it is possible that starling males with longer throat feathers experienced lower rates of social aggressions during the non-reproductive period. These costs can affect the expression of other traits related to phenotypic condition, including immune responses (Hawley et al. 2006), oxidative status (Galván and Alonso-Alvarez 2009) or the expression of sexual signals (Møller et al. 2000). Although we have no data on probability of aggression or social interactions in general in relation to length of throat feathers in starlings, we think that social costs associated to the experimental reduction of length of throat feathers during the non-breeding period is the most likely explanation for the detected experimental effects on beak colouration during reproduction. However, this mechanistic explanation deserves further research exploring for instance the expected association between feather length and aggression during the non-reproductive period.

Whatever the mechanistic explanations, our experimental results strongly suggest a causal link between expression of two sexually dimorphic traits in spotless starlings. As far as we know, causal links between two sexually selected traits have only been detected in another bird species, the house finch, a highly social species in which head and breast feathers of males show great variability from red to yellow colouration (Henderson et al. 2018). Henderson et al. (2018) found that red-feathered males are more attractive and sing more than yellow-feathered males but, when yellow males were housed with red males, they sang more than when housed with equally unattractive yellow males. Thus, males adapted their singing effort to the social environment (attractiveness) determined by the plumage coloration of the social groups. Therefore, the detected link was explained, not as a direct consequence of one of the traits, but indirectly by the social environment in terms of level of attractiveness of neighbours, which was also manipulated. Our experimental results therefore show a direct causal effect of length of throat feathers on the expression of the colouration of the base of the beak of spotless starling males, a trait that is only expressed during the reproductive period.

To conclude, we demonstrate for the first time a causal link between the expression of two sexually dimorphic characters, which is essential to understand their functionality in a multiple signalling framework. This type of interactions between sexually selected signals might be widespread in nature and could be more easily detected when considering signals that, like feather coloration or morphological traits, have signalling functions in non-reproductive contexts.

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Figure legends

Fig. 1: Mean spectral reflectance of the tip (A) and base (B) of the beak in male (empty black circles) and female (filled grey triangles) spotless starlings during the breeding season. Vertical bars denote 95% CI. Photographs show the typical colour of the beak of spotless starling males (C) and females (D) during the breeding season, as well as their black beak out of the breeding season (E).

Fig. 2: Estimation of the length of ornamental throat feathers of a male spotless starling (A), and the aspect of a non-manipulated individual (B), and of an experimental individual after cutting the feathers (C).

Fig. 3: Relationships between (A) body condition (residuals of body mass after controlling for tarsus and wing length) of starling males before applying the treatment and (B) length of their throat feathers with blue and yellow-red colour intensity of the base of their beaks. Lines are regression lines.

Fig. 4: Blue colour intensity of the base of the beak of males that were (experimental, N = 11) or were not (control, N = 11) subjected to the experimental shortening of throat feathers. Beak colouration was measured during the first capture, before the experimental manipulation, and in second captures, after the manipulation. Vertical lines denote ± 95% CI.

Table 1: Results from GLMs exploring the associations between body condition (independent variable, top) and length of the throat feathers (independent variable, down) with the different colour variables (blue (400-475 nm) and yellow-red (570-700 nm) chroma and brightness (300-700 nm)) in the first capture day (i.e. before applying the experimental treatment). The association between throat feather length and body condition of males and females is also shown. Each line shows the results of independent GLM models that also included study year and date of capture (only in the case of exploring association with body condition) as additional independent factors (statistics associated with such factors are not shown).

	Males				Females			
Variables	Beta(SE)	F	df	p	Beta(SE)	F	df	p
Body condition								
Blue	0.37(0.12)	9.72	1,52	**0.003**	0.01(0.09)	0.01	1,96	0.917
Yellow-red	-0.30(0.14)	4.72	1,52	**0.034**	-0.04(0.10)	0.16	1,96	0.686
Brightness	0.10(0.14)	0.56	1,52	0.456	-0.03(0.09)	0.14	1,96	0.71
Length of throat feathers								
Blue	0.32(0.13)	5.89	1,53	**0.019**	0.15(0.10)	2.29	1,98	0.134
Yellow-red	-0.29(0.14)	4.39	1,53	**0.041**	-0.14(0.10)	2.09	1,98	0.151
Brightness	0.16(0.13)	1.34	1,53	0.251	0.09 (0.10)	0.76	1,98	0.385
Body condition & Length of throat feathers								
Feathers	-0.08(0.14)	0.28	1,52	0.596	0.10(0.10)	0.98	1,95	0.326

Table 2: Results from Repeated Measures ANOVAs exploring the effects of experimental shortening of throat feathers (control (Ctrl.) vs experimental (Exp.)) on the body condition, feather growth (feathers) and beak colouration (blue (400-475 nm) and yellow-red (570-700 nm) chroma and brightness (300-700 nm)) among captures (repeated measures). P-values lower than 0.05 are in bold.

	First Capture		Second Capture				
	Mean $_{Ctrl.}$ (SE)	Mean $_{Exp.}$ (SE)	Mean $_{Ctrl.}$ (SE)	Mean $_{Exp.}$ (SE)	df	F	p
Blue	0.23 (0.004)	0.23 (0.004)	0.23 (0.008)	0.20 (0.008)	1,17	7.13	**0.016**
Yellow-Red	0.26 (0.008)	0.25 (0.008)	0.26 (0.016)	0.29 (0.016)	1,17	3.09	0.097
Brightness	11.80 (0.990)	11.40 (0.990)	11.42 (0.877)	10.31 (0.877)	1,17	0.10	0.753
Body condition	0.30 (0.235)	0.28 (0.221)	0.48 (0.189)	0.43 (0.177)	1,12	0.01	0.934
Feathers	3.24 (0.123)	3.38 (0.140)	3.20 (0.132)	3.09 (0.150)	1,13	1.00	0.336

Fig. 1

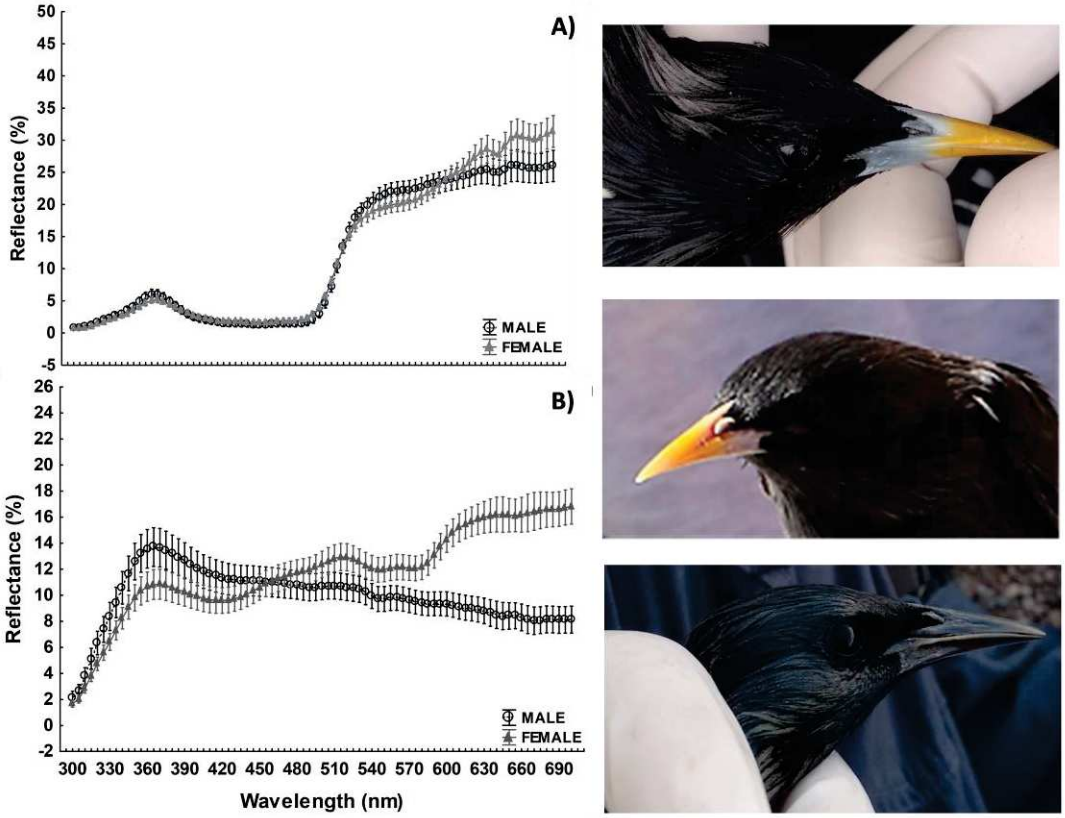

A)
B)
C)
D)
E)
Reflectance (%)
Wavelength (nm)
MALE
FEMALE
50
45
40
35
30
25
20
15
10
5
0
-5
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
300 330 360 390 420 450 480 510 540 570 600 630 660 690

Fig.2

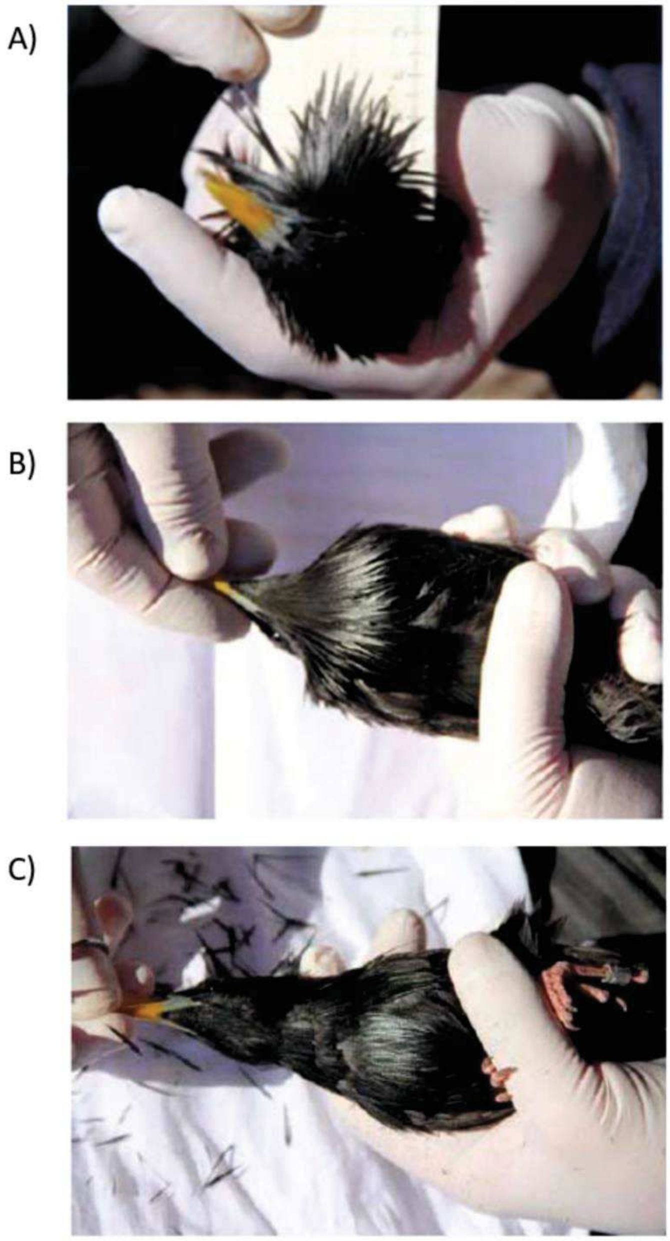
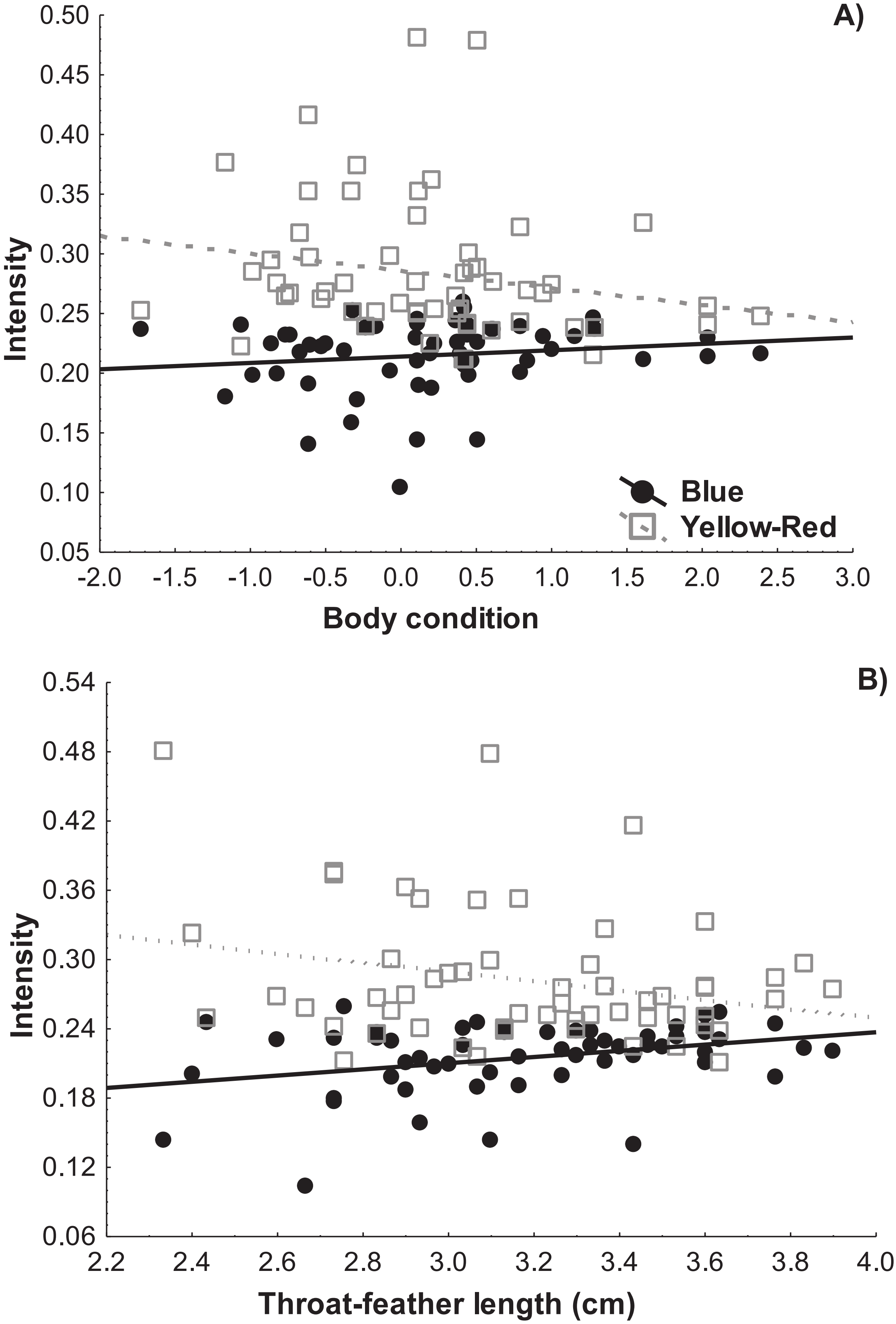
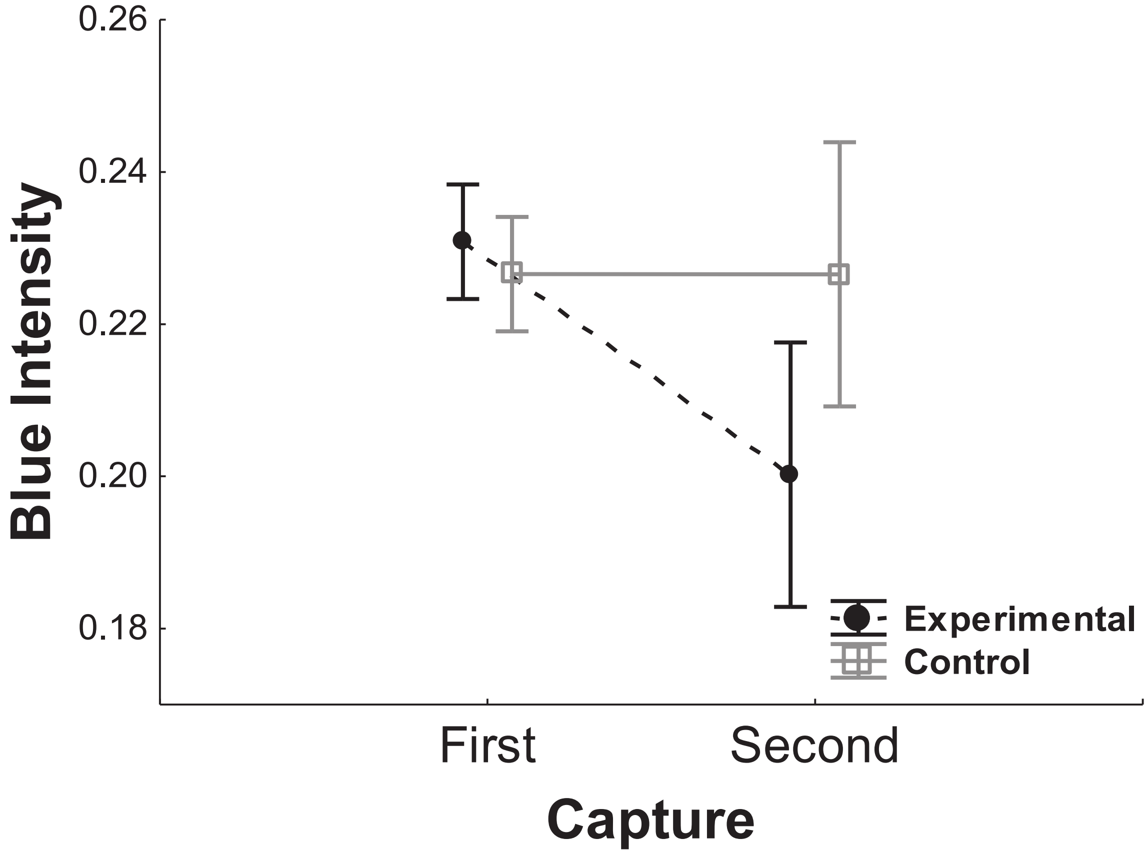


Fig. 3



A)
Intensity
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Body condition
Blue
Yellow-Red
B)
Intensity
0.54
0.48
0.42
0.36
0.30
0.24
0.18
0.12
0.06
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
Throat-feather length (cm)

Fig.4


0.26
0.24
0.22
0.20
0.18
Blue Intensity
Experimental
Control
First
Second
Capture

Annex 1

Table A1: Number of captures made for each of the recaptured spotless starling males during the study and the number of times the treatment was applied to each male.

Ring	Treatment	Year 2015	Year 2016	Year 2017	Number of treatments
3256561	Control	2			1
3256564	Control	1	1		1
3256565	Control	1	1		1
3256567	Control	2	1		1
3256590	Control	2		1	1
3301955	Control	3		1	1
3368660	Control	2			1
3368681	Control	1	1	1	2
3369509	Control	1	1	1	2
3406027	Control	1	1		1
3418841	Control		1	1	1
3256556	Experimental	2			1
3285646	Experimental	2			1
3387759	Experimental	1	2	1	2
3387764	Experimental	2	1		1
3387774	Experimental	1		1	1
3387838	Experimental	1	1		1
3392095	Experimental		1	1	1
3428304	Experimental			2	1
3256564b	Experimental		1	2	1
3256565b	Experimental		1	1	1
3256567b	Experimental		1	1	1