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METHODS & TECHNIQUES

Polymorphic male color morphs visualized with steroids in monomorphic females: a tool for designing analysis of sex-limited trait inheritance

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SUMMARY

In diploid animals, males and females share most of the genome (except sex-specific elements, such as sex chromosome genes), yet despite sharing the underlying genes that hard-wire traits, males and females may differ in their phenotypes when traits are controlled by proximate mechanisms, such as hormones. In color polymorphic species where coloration is only expressed in one sex, the design of studies of the inheritance of color and coevolved morph-specific traits (e.g. territorial *vs* sneaker strategies, skewed energetic investment in territorial defense *vs* sperm production, etc.) is compromised as the expression of morph-coding genes is only visualized in one sex. Here, we circumvented this problem by first characterizing oxidative stress traits in both sexes and then using testosterone implants in females to expose their otherwise 'silent' coloration. Males of our model species are highly territorial and exhibit morph-specific levels of aggression, whereas females are non-territorial and display very low levels of aggression. Interestingly, reactive oxygen species levels were found to be morph specific regardless of sex, despite considerable differences in lifestyle. Males and females did differ remarkably, however, in superoxide levels depending on whether they sported a colored throat bib or not, a trait also used in male sexual signaling. Females with throat bibs had significantly lower levels of superoxide than females without a bib, which was not the case for males.

Key words: male color, sex differences, superoxide, testosterone.

INTRODUCTION

Many organisms show variation within distinct categories, such as castes in termites and color morphs in lizards (Sinervo and Lively, 1996). To understand their evolution, researchers benefit from designing explicit breeding experiments that elucidate their patterns of inheritance so that theoretical models can be used to predict their response to selection in the wild (Lynch and Walsh, 1998). This is confounded when morph-specific traits such as color are expressed in only one sex, as it is unknown which color category one parent genetically codes for and therefore what color allele will be transferred to offspring by the colorless parent. These morphs may also encompass suites of coevolved morph characteristics from the genomic to the physiological, reproductive and ultimately evolutionary level (Sinervo and Lively, 1996). However, because the most overt part of the phenotype is only observable in one sex, one may overlook the fact that parts of the coevolved genome and trait repertoire can have profound effects on the biology of the sex in which the morph-specific characteristics are never seen (Lank et al., 1999). In order to be able to study this, the phenotypic morph characteristics (e.g. color) can be revealed experimentally.

Our model of choice for this work was the Australian painted dragon lizard (*Ctenophorus pictus*, Peters 1866). Males of this species occur in three different color morphs (red, orange and yellow headed), whilst females are cryptic in coloration and both males and females may express yellow pigmentation on their throats [a 'bib' (Healey and Olsson, 2009; Olsson et al., 2009a)]. Red males are the dominant morph in staged contests, facilitated by higher levels of testosterone, aggression and willingness to fight. They also emerge from hibernation sooner (Healey et al., 2007). Yellow males counter this aggressive tactic *via* sperm competition, siring more offspring per copulation than red males (Olsson et al., 2009b). Orange males are characteristically close to the red morphotype with respect to morph-specific traits (Healey and Olsson, 2009). We therefore asked whether the different, morph-specific strategies between males in terms of levels of aggression are also reflected at a metabolic cost level – specifically, in their levels of reactive oxygen species (ROS) and the antioxidant superoxide dismutase (SOD). This also raises the fascinating question of whether this morph specificity also applies to females, despite the striking differences in male and female lifestyles. Although females are non-territorial and non-aggressive, they do still carry the genetic underpinning of a specific morph and any co-variation between head color, ROS and SOD levels in males could also therefore be present in females.

MATERIALS AND METHODS

Model species

The Australian painted dragon, *C. pictus*, is a small (adult snout–vent length 65–95 mm, mass 8–16 g) diurnal lizard. They typically prefer sandy habitats and low vegetation in a range covering central and western New South Wales to Western Australia. Orange and yellow male lizards, and the monomorphic females, were caught by noose at Yathong Nature Reserve, New South Wales (145°35′E, 32°35′S) during the 2009 mating season (August–January), weighed, measured (only relevant measures reported) and scored for head coloration and bib presence/absence by eye (see Sinervo and Lively, 1996). Red males did not occur in high enough frequency in 2009 to be included in the experiment. The lizards were then brought

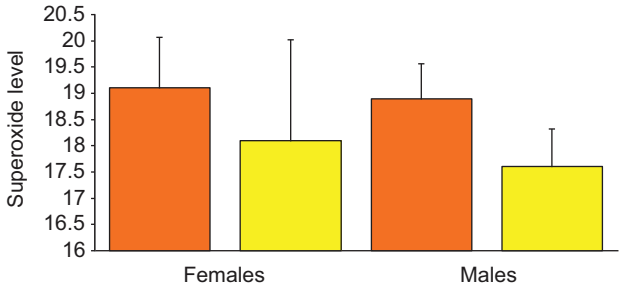

Fig. 1. Mean (±s.e.m.) relative fluorescence counts in orange (N=21) *vs* yellow (N=15) males, and orange (N=9) and yellow (N=6) females (for additional information see Materials and methods on how superoxide was quantified by flow cytometry). For test statistics see Table 1.

Table 1. Results of a three-factor analyses of variance with sex (males, females), head color (yellow, orange), and bib (present, absent) as factors

Traits	d.f.	SS	F	P
Sex	1	21.0	3.16	0.083
Bib (present, absent)	1	54.2	8.15	0.007
Head color (orange, yellow)	1	45.0	6.76	0.013
Sex × head color	1	3.04	0.46	0.503
Sex × bib	1	118.0	17.7	0.0001
Head color × bib	1	34.8	5.23	0.027
Onset superoxide	1	32.2	4.84	0.033
Superoxide dismutase	1	5.2	0.79	0.380

The response variable was the level of superoxide at the completion of the experiment (model $F_{8,43}$=4.19, P=0.019, R^2=0.44). Type III sum of squares (SS) are given for all factors and covariates.

back to holding facilities at Wollongong University where they were held separately in 600×600×500 mm cages with a 40 W spot light at one end to allow thermoregulation to their preferred body temperature (*ca*. 36–37°C as per cloacal temperatures of free-ranging territorial, displaying males; M.O. and E. Wapstra, unpublished). Lizards were fed crickets and mealworms dusted with calcium and multivitamins *ad libitum* every second day and sprayed with a mist of water twice daily. Lizards were allowed to acclimatize to captive conditions for 2 weeks, after which time they were blood sampled for flow cytometry and superoxide quantification.

Measuring ROS of blood cells by flow cytometry

A single sample of peripheral blood (25 µl) was drawn in the morning from the vena angularis (corner of the mouth) using a syringe to puncture the blood vessel and a capillary to collect the blood. The blood was diluted immediately with 9 volumes of phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 8 mmol l^{-1} Na_2HPO_4, pH 7.4) and stored on ice prior to analyses, which were completed within 4 h of sampling. Prior to staining, diluted blood was further diluted 50-fold with PBS and then centrifuged (300 ***g*** for 5 min) to pellet cells; each cell pellet corresponded to 10 µl of whole blood. Cells were re-suspended in 100 µl of PBS containing 5 µmol l^{-1} MitoSOX Red (MR; Molecular Probes, Invitrogen, Carlsbad, CA, USA). MR was added from a stock solution in dimethylsulfoxide (DMSO). The final concentration of DMSO was 0.2% (v/v) or less. Cells were subsequently incubated at 37°C for 30 min, then washed with PBS by centrifugation as described above and held on ice until analysed by flow cytometry; 50,000 events were acquired for all samples. Flow cytometry was performed using a Becton Dickinson LSR II (Becton Dickinson, Sydney, Australia), with excitation at 488 nm and emitted fluorescence collected using band pass filters of 515±10 nm (DHR, dihydrorhodamine 123, Molecular Probes) and 575±13 nm (MR). Data were acquired and analysed using FACSDiva v4.0.1 and FloJo software (Becton Dickinson), respectively. On the basis of forward angle laser scatter and side angle laser scatter, a number of blood cell populations were discerned. The results obtained were similar for all these populations. For each sample, the arithmetic mean fluorescence for all 50,000 cells acquired was determined using FloJo software and used to compare samples and treatments. The accuracy of flow cytometry results from two samples from the same individual has been measured in a separate experiment (Olsson et al., 2008), involving 14 males with a correlation coefficient between samples of r=0.97, P<0.0001. Thus, our flow cytometry technique can be argued to be highly consistent.

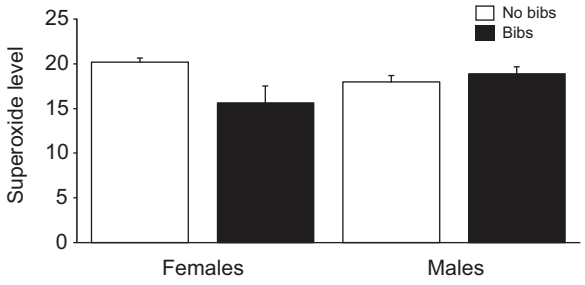

Fig. 2. Mean (±s.e.m.) relative fluorescence counts in males and females with and without a bib (for additional information see Materials and methods on how superoxide was quantified by flow cytometry). For test statistics see Table 1.

Quantifying SOD

As SOD scavenges superoxide molecules, we assayed superoxide and entered those values as a covariate in our analysis. We used Superoxide Dismutase Assay Kit II (cat. no. 574601, Calbiochem, supplied by Merck Pty Ltd, Victoria, Australia). This kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD (Cu/Zn-, Mn- and Fe-SOD).

Hormone implantation

After all animals had been characterized with respect to superoxide and SOD, and all females had laid their clutches for the season, the females (only) were implanted with testosterone to express their underlying color. Females were sterilized dorsally by wiping with 70% alcohol and injected subcutaneously with 0.2 ml lidocaine (lidocaine hydrochloride 0.4%; Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia). They were then cooled in a sterile plastic box on ice (being ectotherms, this immobilizes lizards without causing any harm) and wiped ventrally with 70% alcohol. A small incision was made between two scale rows and sterile implantation of hormone took place; subsequently, the incision was closed with surgical superglue (2-octyl cyanoacrylate; Hystoacryl). The 8 mm Silastic implant was made of a Dow Corning tube (i.d. 1.5 mm, o.d. 2.0 mm; Dow Corning, Midland, MI, USA). Testosterone crystals were packed into a 6 mm column (no. T 1500, Sigma) and one end of the implant was closed with Dow Corning 734 RTV sealant. Implanted

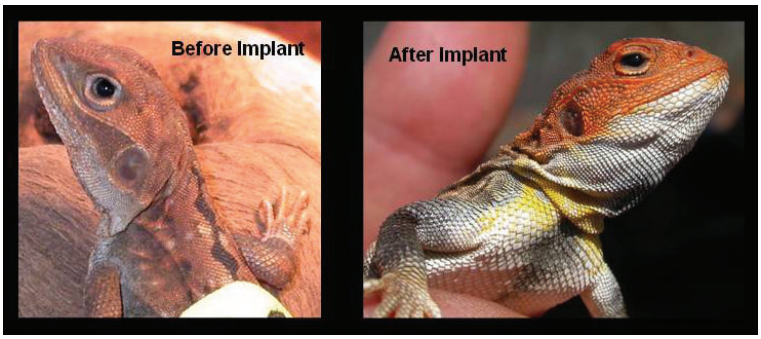

Fig. 3. Effect of testosterone treatment on female coloration.

females began to express head color within a week and after 4 weeks were brightly colored, making the assessment of the color morph by eye straightforward.

Statistical analysis

We initially used a three-factor ANOVA to assess the effect of sex, head color (orange and yellow) and bib (present, absent), while including two covariates, superoxide (controlling for among-individual variation in superoxide) and individual SOD levels. We then backward-eliminated the non-significant three-way interaction term.

RESULTS

Two of our interaction terms were significant: between head color and bib presence, and between sex and bib presence (Figs 1–3, Table 1). However, the interaction between sex and head color was non-significant, suggesting that different head colors have different superoxide levels, but with no difference between the sexes. Thus, in spite of females not showing the colors they genetically code for, they do show similar genetic morph net levels of superoxide to males in the corresponding morphs. Sex itself had a non-significant effect on superoxide (P=0.083), while both bib and head color showed significant main effects (P=0.007 and P=0.013, respectively). However, as these also interacted significantly, this casts doubt on their significant main effects.

DISCUSSION

We use testosterone treatment to reveal the otherwise ‘silent’ head color in females, allowing us to compare morph and sex-specific levels of superoxide and show that even though females do not express the phenotypic colors of males, their net levels of superoxide as a result of oxidative metabolism and antioxidation follow the same genetic morph specificities as those in males. Furthermore, additional variation in superoxide was explained by the presence or absence of a throat bib within head color morphs. Interestingly, females with bibs have less superoxide than those without bibs, and this pattern was not evident in males. What could explain these similarities and differences in a major metabolic parameter (net superoxide level) between the sexes? At least two different routes seem possible where genetic morph specificity in ROS levels could arise, regardless of sex and whether a phenotypic color is expressed or not. (i) Animals may behave the same with respect to some external ROS moderator. This could arise *via* selection on color morphs and coevolved traits in one sex (males) where only some of these traits become expressed as a result of genetic covariation in the other sex. (ii) Alternatively, there may be physiological or biochemical differences that are first under diversifying selection in both sexes, and any behavioral differences associated with them become selected for in a second stage of morph divergence or early speciation process (Sinervo and Lively, 1996). Analysis of these alternative scenarios is outside the scope of this study.

In summary, we demonstrate that exploiting hormonal manipulation to reveal hidden morphs in the monomorphic sex gives us a tool to quantify physiological and biochemical parameters involved in the evolution of polymorphism in both sexes.

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