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Endocrine differences among colour morphs in a lizard with alternative behavioural strategies

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ABSTRACT

Alternative behavioural strategies of colour morphs are expected to associate with endocrine differences and to correspond to differences in physical performance (e.g. movement speed, bite force in lizards); yet the nature of correlated physiological and performance traits in colour polymorphic species varies widely. Colour morphs of male tawny dragon lizards *Ctenophorus decresii* have previously been found to differ in aggressive and anti-predator behaviours. We tested whether known behavioural differences correspond to differences in circulating baseline and post-capture stress levels of androgen and corticosterone, as well as bite force (an indicator of aggressive performance) and field body temperature. Immediately after capture, the aggressive orange morph had higher circulating androgen than the grey morph or the yellow morph. Furthermore, the orange morph maintained high androgen following acute stress (30 min of capture); whereas androgen increased in the grey and yellow morphs. This may reflect the previously defined behavioural differences among morphs as the aggressive response of the yellow morph is conditional on the colour of the competitor and the grey morph shows consistently low aggression. In contrast, all morphs showed an increase in corticosterone concentration after capture stress and morphs did not differ in levels of corticosterone stress magnitude (CSM). Morphs did not differ in size- and temperature-corrected bite force but did in body temperature at capture. Differences in circulating androgen and body temperature are consistent with morph-specific behavioural strategies in *C. decresii* but our results indicate a complex relationship between hormones, behaviour, temperature and bite force within and between colour morphs.

The actions and responses of steroid hormones, particularly androgens and glucocorticoids during early development and in breeding adults, appear instrumental in mediating alternative behavioural strategies and their associated life history trade-offs in many colour polymorphic species (e.g. Hayssen et al., 2002; Knapp and Moore, 1996; Pryke et al., 2007; Sinervo et al., 2000a). Organisational effects of hormones during development are thought key to "fixing" discrete phenotypic differences among colour morphs (Hews et al., 1994; Hews and Moore, 1996). However, activational roles of androgens during reproduction are known to further reinforce or potentially modulate behavioural phenotypes (Hau, 2007; Oliveira, 2004) and skeletal muscular hypertrophy (Herbst and Bhasin, 2004) with the dominant morph often showing higher levels of testosterone (Horton et al., 2014b; Küpper et al., 2016; Sinervo et al., 2000a; Swett and Breuner, 2008), larger body size (Barlow, 1976), and greater muscle volume leading to increased aggressive performance (e.g. bite force in lizards; Huyghe et al., 2009a). By contrast, in male white-throated sparrows, behavioural differences between genetic colour morphs can persist in the absence of differences in androgen (Maney et al., 2009). In this species, expression of a hormone receptor better predicted aggression than androgen levels (Horton et al., 2014a).

Initial and stress-induced levels of glucocorticoids can also differ between colour morphs with alternative strategies (Horton and Holberton, 2009; Huyghe et al., 2009b). Furthermore, glucocorticoids can often, but not always, suppress androgen levels in many vertebrates, most often through inhibition of the hypothalamic-pituitary-gonadal (HPG) axis (Greenberg and Wingfield, 1987; Rivier and Rivest, 1991). Indeed, in many colour polymorphic species, morphs may have different relationships between glucocorticoids and levels of androgen in response to stress (Knapp et al., 2003; Knapp and Moore, 1997). For example, the red and black head colour morphs of the Gouldian Finch, *Erythrura gouldiae*, differ in their hormonal response to stress in the social environment. With increased relative density of the dominant, aggressive red morph in a confined captive space, the red morph shows a simultaneous increase in the concentration of testosterone and corticosterone while the black morph shows reduced testosterone levels and unaffected corticosterone levels in response to the greater frequency of red competitors. Therefore, although red-headed males have a

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dominance advantage over black-headed males, they also show high physiological sensitivity which has potential to reduce lifespan (Pryke et al., 2007) through the costs of elevated testosterone and corticosterone (Korte et al., 2005; Wingfield et al., 2001). As this example highlights, examination of the response of corticosterone and androgens in response to stress may reveal differing life-history trade-offs (between survival and reproductive output), associated with the discrete phenotypic differences of morphs.

In this study, we investigate the endocrinology of male colour morphs of the tawny dragon lizard *Ctenophorus decresii*, to test whether previously established behavioural differences between colour morphs correspond to predicted differences in initial and stress-induced levels of plasma androgen and corticosterone concentrations, as well as bite force (an indicator of aggressive performance). *C. decresii* is a small, agamid species in which males exhibit four discrete colour morphs which can be reliably classified into orange, yellow, grey or orange-yellow (distinct orange centre surrounded by yellow) throat colouration (Teasdale et al., 2013; Fig. 1). Previous field studies have shown that colour morphs do not differ in morphology but do differ in their behavioural response to simulated conspecific intruders and predators (Teasdale et al., 2013; Yewers et al., 2016). The orange morph shows consistent high aggression to conspecifics and the grey morph shows low aggression, whereas the aggression of the yellow and orange-yellow morph is dependent on the colour of its competitor. The grey morph is also less bold towards a simulated predator than the other three morphs, which all have similar boldness. The high aggression of the orange morph implies a dominant strategy while the behaviour of the grey morph suggests a cautious strategy (Yewers et al., 2016; Fig. 1). Although the orange-yellow morph is phenotypically distinct, it remains unclear whether this morph employs a strategy similar to the pure orange or pure yellow morph or employs a unique strategy. More generally, it is important to qualify whether and how human-defined colour morph categories correspond to behavioural strategies and correlated traits, yet this is rarely done. In this study, we compared androgen, corticosterone and bite force between colour morphs and compared statistical support for models with four or three morph categories (orange-yellow grouped with either the orange or yellow morph).

We predicted that relative to other morphs, the aggressive and likely dominant orange morph should maintain higher levels of plasma androgen throughout reproduction, and potentially even during exposure to stressors. In lizards, including the closely related polymorphic species *Ctenophorus pictus*, androgen is likely to mediate aggression and therefore dominance (Olsson et al., 2007). Furthermore, in other polymorphic lizard species, males of aggressive territorial morphs appear less sensitive than less aggressive non-territorial morphs to the suppressive effects of stressors on plasma testosterone levels (Knapp and Moore, 1996; Knapp and Moore, 1997). Here, relatively modest stress-induced levels of corticosterone are thought to facilitate aggression, via direct actions on the central nervous system, or aid metabolic recovery from, or preparation for, aggressive encounters (Knapp and Moore, 1995; Moore and Jessop, 2003; van Duyse et al., 2004). We also predicted that the orange morph would have a greater aggressive performance measured as bite force. Higher levels of androgen can increase muscle volume thereby leading to increased strength, which often indicates fighting ability in lizards (Herbst and Bhasin, 2004; Lailvaux et al., 2004; Lappin and Husak, 2005; Tokarz, 1985). By contrast, we expected the cautious behavioural strategy of the grey morph to be reflected in lower initial androgen, and a greater decrease in androgen following capture stress but lower initial and higher capture stress-induced levels of circulating corticosterone due to lower metabolic demands involved with territory defence, as well as lower bite force.

1. Methods

1.1. Study species

The tawny dragon lizard, *C. decresii*, is a small, sexually dimorphic agamid lizard found on rocky outcrops of Kangaroo Island, Mt. Lofty Ranges and southern Flinders Ranges of South Australia (Houston, 1974). Males exhibit striking throat colour variation both within and between populations (Houston, 1998). In populations in the Flinders Ranges, males occur in four distinct throat colour morphs: orange, yellow, grey and orange-yellow, which can be objectively classified, independently of the human visual system (Teasdale et al., 2013; Fig. 1). Orange males have a variably sized orange patch on a grey/cream reticulated background, yellow males have a variably sized yellow patch on a grey/cream reticulated background, orange-yellow males have a

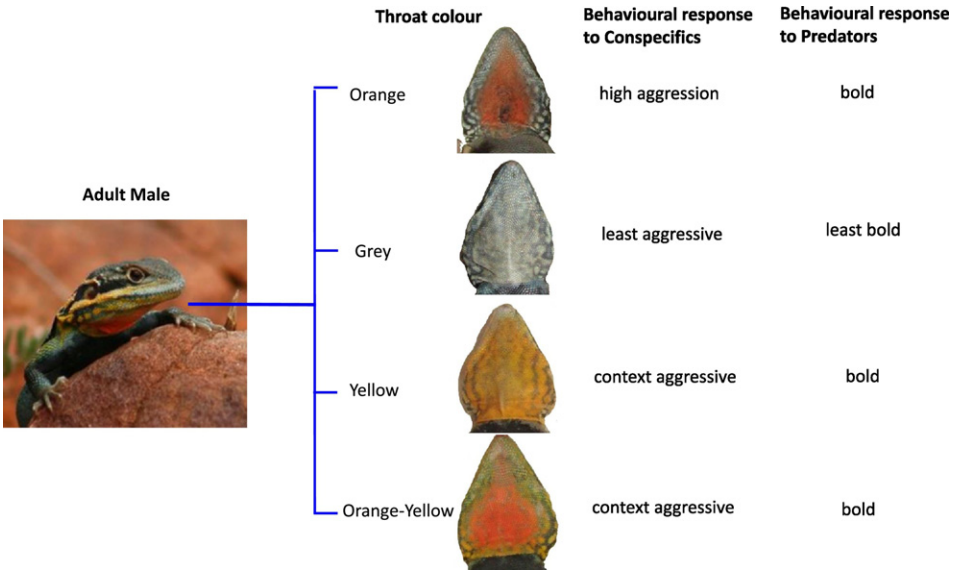

Fig. 1. Examples of *Ctenophorus decresii* throat colour morphs. From top: orange, grey, yellow and orange-yellow. Morphs have alternative behavioural strategies (Yewers et al., 2016). The orange morph is consistently aggressive to all competitors and the grey morph shows low aggression; whereas the aggression of the yellow and orange-yellow morph is dependent on the colour of its competitor, with both showing the highest aggression to like-morphs. The grey morph is also less bold towards a simulated predator than all other morphs which all have similar boldness. The high aggression of the orange morph implies a dominant strategy while the behaviour of the grey morph suggests a cautious strategy.

variably sized orange patch on a yellow background and grey males have a grey/cream reticulated throat with no yellow or orange present. These colour morphs are fixed for life from sexual maturity and heritable as confirmed by long-term field studies and captive breeding experiments (Gibbons and Lillywhite, 1981; Rankin et al., 2016). Females have uniform cream coloured throats with a variable yellow wash or yellow bib (Rankin and Stuart-Fox, 2015). There are no morphological differences (e.g. body size or relative head size) between morphs (Teasdale et al., 2013).

In colour polymorphic species, the relationship between colouration, associated behavioural or life-history strategy and proximate mechanisms is often complex. In particular, populations often contain individuals with composite colouration, which may or may not resemble 'pure' colour forms in terms of behaviour or endocrinology. For example, in the side-blotched lizard, *Uta stansburiana*, males with composite orange-yellow and orange-blue throats behave similarly to males with solid orange throats and yellow-blue throated males behave as 'pure' yellow sneaker males (Alonzo and Sinervo, 2001; Sinervo et al., 2000b; Sinervo and Zamudio, 2001). Conversely, in the tree lizard, *Urosaurus ornatus*, both morphs with either a solid orange throat or orange throat with a central blue patch coexist and show distinct behavioural strategies tied to unique hormonal profiles (Moore et al., 1998). In *C. decresii* the orange-yellow morph is both phenotypically (Teasdale et al., 2013) and genotypically (Rankin et al., 2016) distinct, but it remains unclear whether the orange-yellow morph behaviourally and/or physiologically resembles either the pure orange or pure yellow morph. In terms of aggressive behaviour, statistical models grouping the orange-yellow and pure yellow morphs together had greater support than a model with all four morphs separately; however, this was not the case for bold behaviour (Yewers et al., 2016). Because the independent status of the orange-yellow morph remains ambiguous based on behavioural data, we compared statistical support for models in which orange-yellow individuals are grouped with pure orange or yellow individuals or considered a separate morph.

1.2. Study site and population

We studied a wild population of tawny dragon lizards at the Yourambulla Caves Historic Reserve in the Flinders Ranges, South Australia, Australia (138° 37′ E 31° 95′ S) during three breeding seasons between October and December 2011, 2012 and 2013. The region is semi-arid and lizards are found in rocky areas interspersed with ground cover, casuarinas, cypress pines and low lying shrubs. We captured 25 adult male lizards in 2011, 51 male lizards in 2012 and 66 male lizards in 2013, either by hand or by noosing (using a telescopic pole and noose made of fishing line; mean capture time: 12.45 min, median: 9 min, range: 3–37 min). We permanently marked individuals using coloured elastomer implants (Northwest Marine Technology) under the skin of their hind limbs. Elastomer implants are commonly used in fish and amphibians and do not cause permanent injury compared to marking by toe clipping (Calsbeek et al., 2008; Nauwelaerts et al., 2000). For visual recognition, we wrote a temporary unique number on the back of each lizard using a nontoxic xylene-free Pilot paint pen (Pilot Corporation, Tokyo, Japan). We visually classified male lizards into discrete morphs previously defined by Teasdale et al. (2013), based on the presence or absence of orange and yellow. As detailed below, we took blood samples to assay concentrations of corticosterone and testosterone and morphological and bite force measurements of the lizards.

1.3. Blood sampling protocols

We took two blood samples from each male adult lizard in the breeding season (October–December) of 2011 (25 lizards) and 2012 (42 lizards). The first blood sample was taken within 2 min after capture and the second sample was taken 30 min after capture, during which the lizard was kept in a cloth bag in the shade. For both samples, we collected 100 μL of blood from the *sinus angularis* accessed using a needle tip (26 gauge) through the corner of the mouth following the standard 'small' lizard protocol (Jessop et al., 2009; Olsson et al., 2000). Blood samples were kept on ice and centrifuged within 5 h at 10,000 rpm for 2 min and the separated plasma was collected and stored at −20 °C until assayed. When sampling, we recorded the time of day, time taken to catch the animal and the body temperature to 0.1 °C using a thermocouple (YCT) inserted into the cloaca of the animal immediately after capture as all these variables are known to affect hormone concentrations (Jessop et al., 2016; Olsson et al., 2007; Romero and Reed, 2005).

1.4. Plasma steroid analysis

We measured total (i.e. free and bound) plasma concentrations of corticosterone and testosterone using commercially available ELISA kits (Corticosterone item no: 501320; testosterone item no: 582701, Cayman Chemical, Michigan, USA). For the majority of samples we used 20 uL and 7.5 uL of plasma for corticosterone and testosterone assay, respectively. A limited number of samples, due to their small volume, required assay with decreased starting plasma volumes (corticosterone: 5–20 uL ($N = 4$); testosterone: 3.75–7.5 uL ($N = 3$)). In all instances, samples were observed to fall within the range of robust minimal assay sensitivity (i.e. 20–80% binding).

To reduce cross reaction and interference, all plasma samples were individually extracted using three repeated washes of the solvent anhydrous methylene chloride (i.e. dichloromethane; Wingfield and Farner, 1975). During extraction, each solvent phase (i.e. containing the steroids) was evaporated off using a 35 °C water bath, while being air dried by a gentle stream of compressed air. Dried steroid samples were then resuspended in EIA buffer and refrigerated overnight at 4 °C before being assayed in duplicate. We did not undertake further chromatographic separation to elute the testosterone and corticosterone plasma specific fractions contained within samples. Consequently, there is the possibility for cross reaction between the kit supplied anti-testosterone antibody and other androgens (e.g. a 27.4% cross-reactivity to 5*a*-dihydrotstosterone) also present in lizard plasma that would inflate measures of plasma testosterone. For this reason, we herein refer to plasma testosterone as plasma androgen. In contrast, because the anti-corticosterone antibody has very low cross-reactivity with other glucocorticoids (e.g. cortisol ~2.5%), we retain the use of plasma corticosterone throughout the manuscript.

We followed manufacturer's instructions to extract and measure total corticosterone and androgen concentrations in lizard plasma. We developed plates for 90 min in darkness. The enzymatic reaction was absorbed and read at 415 nm on a FLUOstar OPTIMA plate reader (BMG LABTECH, Germany).

Final steroid concentrations were calculated from hormone-specific standard curves corrected for an individual's plasma volume. We did not perform sample recovery protocols to account for plasma loss during extraction protocols (i.e. via addition of tritiated hormone); hence our final plasma hormone concentrations are expected to underestimate maximal plasma concentrations. The coefficient of variation (i.e. standard deviation / mean * 100) was calculated from duplicate measures of each hormone standard concentration and plasma sample. Intra-assay coefficient of variation for androgen and corticosterone were 1.85% and 2.72% respectively. Inter-assay coefficient of variation for androgen and corticosterone were 18.1% and 14.1%, respectively. The sensitivity was 6 pg/mL and 0.30 pg/mL for androgen and corticosterone assays respectively, which were adjusted for dilution and starting plasma values.

To validate each steroid assay protocol we tested for the presence of parallelism between kit hormone standards and endogenous corticosterone and androgen in *C. decresii* plasma. Pooled plasma samples were serially diluted with EIA buffer (1:25–1:400). We found no significant

differences ($p > 0.05$) in the slopes of the log-logit transformed curves of plasma samples and the kit standards.

1.5. Stress hormone response

To assess the stress response, we calculated the corticosterone stress magnitude (CSM) as the corticosterone concentration 30 min after capture (T30) minus the initial corticosterone concentration (T0). CSM represents the absolute amount of corticosterone synthesised in response to the stress of being captured and is arguably a functional measure of adrenal responsiveness (Payne et al., 2012). CSM was strongly correlated with T30 but not T0 and is therefore not influenced by the time taken to capture or blood sampling (Table S1).

1.6. Bite force

We measured bite force on wild-caught lizards temporarily held at the field station in 2012. We transported lizards to the field station in cloth bags and they were kept in a cool room until we were able to measure their bite force. We measured bite force using a piezoelectric isometric Kistler force transducer (type 9203, Kistler Inc., Winterthur, Switzerland) mounted on a purpose-built stand and connected to a Kistler charge amplifier (type 5995A, Kistler Inc., Switzerland; for detailed diagram and description see Herrel, 2001). We took measurements of bite force by provoking lizards to bite onto the two metal plates of the transducer. The outer side of each plate was covered with a thin foam square to standardise the point at which each lizard bit and to protect the lizards' teeth. Biting the two plates causes the upper plate to rotate around the fulcrum, exerting pull on the transducer. Before we tested the lizards bite force, we used a light source to heat lizards to between 34 and 37 °C, which is close to their preferred body temperature (36° C; Gibbons, 1977). We measured body temperature by a thermo-couple inserted into the cloaca. The average body temperature of lizards immediately before measuring their bite force was 35.27 $\pm$ 0.11 °C. We measured the bite force of each lizard five times with an interval between bite force measurements of at least 1 h. We considered maximum bite force to be the highest value out of the five measurements. We recorded maximum bite force for 51 male adult lizards: 12 grey, 13 orange, 11 orange-yellow and 15 yellow. Lizards were held for a maximum of 48 h and were released at their point of capture.

Body size and head shape can influence bite force (Herrel et al., 2007; Lailvaux et al., 2004). Therefore for each individual, we took the following morphometric measurements: mass to the nearest 0.25 g using a spring balance, snout to vent length (SVL) and head depth (deepest point of the head before the tympanum), width (widest point of the head before the tympanum) and length of the lower jaw to the nearest 0.01 mm using digital callipers. We then obtained measures of relative head size by taking the residuals of head length, width and depth regressed against SVL, and calculated condition as the residuals of weight regressed against SVL (Jakob et al., 1996).

1.7. Statistical analysis

Overall we captured 142 adult males over 3 years. In two of those years we obtained data for 70 adult males for corticosterone and 56 for androgen concentrations. In a single season we measured 51 adult males for bite force. Data for all three aspects (androgen, corticosterone and bite force) were obtained for only 29 individuals due to the nature of field sampling and obtaining sufficient plasma at both time points; therefore to maximise sample size, we tested for differences between morphs in each aspect separately.

To maximise our ability to identify hormone differences between colour morphs, we assessed the potential influence of a range of additional factors that may affect morph-specific hormone levels; specifically, time of day, SVL, time to capture and body temperature immediately after capture. We tested for differences between colour morphs in each factor (PROC GLM; SAS v9.3; all adult males captured $N = 142$) as well as for correlations between either androgen or corticosterone concentration at T0 or CSM and each of these factors (PROC REG; SAS v9.3; subset of males with hormone samples: androgen $N = 53$, corticosterone $N = 58$, CSM, $N = 55$). Corticosterone and androgen concentrations and the time taken to capture an individual were log transformed to meet assumptions of normality. We included any factors with significant differences between morphs or correlations with hormone levels in our final models for androgen, corticosterone and CSM (see below).

We used general linear mixed models to test for differences between colour morphs in androgen, corticosterone concentration and CSM (PROC MIXED; SAS v9.3). Colour morph, blood sample time point (T0 or T30), and the interaction between colour morph and sample time point were included as fixed factors in all models. We initially included year (2011 or 2012) and the interaction between colour morph and year as fixed factors but these were not significant in any model so were removed from final models. Individual ID was included as a random factor to account for repeated measures on individuals (T0 and T30). Body temperature at capture differed between colour morphs (see results) and was therefore included as a covariate in all models assessing hormone differences between colour morphs. Time to capture was included as an additional covariate in the model explaining corticosterone concentrations as it was correlated with corticosterone at T0 (see results).

For the levels of androgen, corticosterone and CSM, we compared statistical models in which morphs were classified into four categories (yellow, grey, orange and orange-yellow) with models in which morphs were classified into three categories (yellow, grey and orange) with orange-yellow individuals classified as either orange or yellow. We compared four-morph and three-morph models using second-order Akaike's information criteria (AICc).

We tested for differences in bite force between colour morphs using a general linear model (PROC GLM; SAS v.9.3) with male colour morph, morphometric measures (SVL, condition, size-corrected head length, width and depth) and body temperature as fixed factors as bite force can be affected by body size and body-temperature in lizards (Anderson et al., 2008).

To assess the relationship between androgen, corticosterone and bite force, we tested whether androgen levels at either T0 or T30 were associated with CSM or bite force.

We reported effect sizes for general linear models as eta-squared (η^2) and Cohen's d for all pairwise comparisons (Cohen, 1977; Levine and Hullett, 2002).

The research was conducted under the following permits: South Australian Department of Environment and Natural Resources Permit to Undertake Scientific Research (E25861), South Australian Wildlife Ethics Committee approval (18/2010-M1) and the University of Melbourne Animal Ethics Committee approval (1011760).

2. Results

2.1. Factors affecting hormone concentrations

Morphs did not differ in most traits that could influence hormones concentrations. There were no differences between morphs in SVL ($F_{3,112} = 1.74$, $p = 0.162$, $\eta^2 = 0.03$), the time of day at capture ($F_{3,138} = 2.24$, $p = 0.086$, $\eta^2 = 0.06$) or the time it took to capture a lizard ($F_{3,139} = 2.52$, $p = 0.061$, $\eta^2 = 0.04$); however there was a difference between morphs in their body temperature at capture ($F_{3,154} = 4.19$, $p = 0.007$, $\eta^2 = 0.15$; Fig. 2) with the orange-yellow morph having a higher body temperature than the yellow morph (Tukey's test $p = 0.014$; Cohen's $d = 0.51$) or grey morph (Tukey's test $p = 0.015$; Cohen's $d = 0.55$) and the orange morph (Tukey's test $p = 0.024$; Cohen's $d = 0.57$). Cohen's effect size value for each pairwise comparison between morphs suggested a moderate practical significance. Statistically significant differences in body temperature between morphs

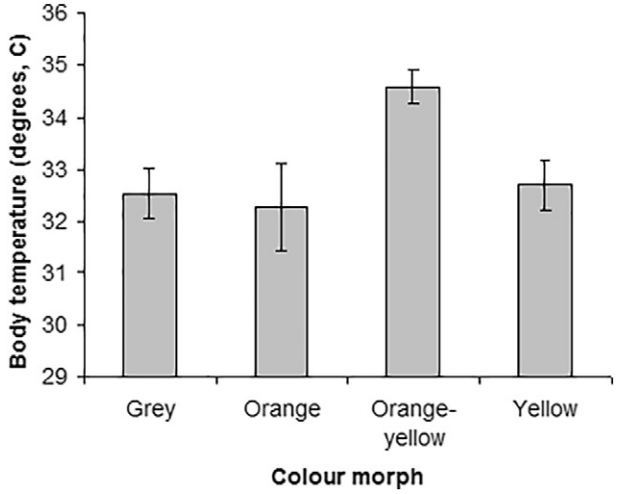

Fig. 2. Body temperature (degrees Celsius, mean ± SE) of the four colour morphs. Grey ($N = 33$), orange ($N = 19$), orange-yellow ($N = 45$) and yellow ($N = 47$). Significant comparisons are indicated by a line and *.

remained when we statistically controlled for the time of day ($F_{3,152} = 6.11$, $p < 0.001$), time to capture ($F_{3,153} = 6.41$, $p < 0.001$) or SVL ($F_{3,152} = 6.18$, $p < 0.001$). Additionally, there was a significant relationship between corticosterone at T0 (but not androgen at T0 or CSM) and time to capture ($r^2 = 0.14$, $p = 0.002$; Supplementary Fig. 2). Hormone levels were uncorrelated with other factors considered (time of day, SVL, and body temperature immediately after capture).

2.2. Androgen

When controlling for differences in body temperature at capture, for androgen levels, the three-morph model with the orange-yellow and orange morphs grouped together had the lowest AIC*i* and had the highest AIC weight (Table 1). However, the ΔAIC*i* between this model and either the three morph model with orange-yellow and yellow morphs grouped together or the four morph model was ≤2. All three models were more highly supported than the intercept only model (ΔAIC*i* > 2; Table 1).

For the most highly supported model with orange-yellow and orange morphs combined, there was a significant interaction between colour morph and sample time (Table 2). The grey and yellow morphs had

Table 1
Candidate three-morph, four-morph and intercept only models, second-order Akaike's information criterion and Akaike weights for androgen, corticosterone and CSM. Models for androgen and CSM included body temperature as a covariate, models for corticosterone had time to capture and body temperature as covariates. OY represents the orange-yellow morph.

	Model	AICc	ΔAIC*i*	Akaike weight (*wi*)
Androgen	Intercept only	77.5	3.90	0.07
	Three morphs OY = orange	73.6	0.00	0.52
	Three morphs OY = yellow	75.3	1.70	0.22
	Four morphs	75.6	2.00	0.19
Corticosterone	Intercept only	99.9	39.80	0.00
	Three morphs OY = orange	60.1	0.00	0.48
	Three morphs OY = yellow	60.1	0.00	0.48
	Four morphs	64.6	4.50	0.05
CSM	Intercept only	421.6	56.40	0.00
	Three morphs OY = orange	371.0	5.80	0.05
	Three morphs OY = yellow	373.7	8.50	0.01
	Four morphs	365.2	0.00	0.94

The best model is indicated by the smallest AICc and the smallest ΔAIC*i* with ΔAIC*i* < 2 considered as an equally good fit (Richards, 2005). Akaike weight is the probability that the associated model is the best in the set.

Table 2
Test of fixed effects for the linear mixed model of androgen concentrations of the three morphs with orange-yellow individuals classified as the orange morph. Significance below 0.05 is indicated **in bold**.

Fixed effect	*DF*	*F*	*Pr* > *F*
Morph	2, 41	1.83	0.174
Sample time	1, 41	7.05	**0.011**
Morph * sample time	2, 41	3.77	**0.031**
Body temperature	1, 41	3.69	0.062

significantly less androgen at T0 than the orange morph and significantly increased androgen from T0 to T30, whereas the orange morph maintained high androgen at both sample times (Table 2, Fig. 3a). Results for the four morph model was qualitatively similar to the three morph model with the combined orange-yellow and orange morphs (although the interaction between morph and sample time was marginally non-significant: $p = 0.076$; Table S2; Fig. 3b). Specifically, the grey and yellow morphs had low androgen at T0 and significantly increased androgen at T30; whereas the orange-yellow and orange morphs maintained consistently high androgen at both time points (Table 3, Fig. 3a, Fig. 3b, Table S3). By contrast, the interaction between sample time and morph was not significant in the three morph model with orange-yellow grouped as yellow ($p = 0.17$; Table S4, Supplementary Fig. 3).

2.3. Corticosterone

When controlling for body temperature at capture and the time before capture, all four morphs showed a similar increase in corticosterone concentrations in response to capture stress (T0 to T30; Fig. 3c). Because the increase in corticosterone for the orange-yellow morph was similar to both the orange and yellow morphs, the three-morph model with orange-yellow individuals grouped as either the yellow or orange morph were approximately equally supported and more highly supported than the four morph model (ΔAIC*i* > 2; Table 1). For both of the three-morph models, corticosterone concentrations increased significantly from T0 to T30 ($p < 0.0001$ for sample time in both cases; Table 4); however, there were no differences between morphs or in the interaction between sample time and morph (Table 4; Supplementary Fig. 4).

2.4. CSM

When controlling for body temperature at capture, for CSM, the best model was a four morph model, which had an AIC weight of 0.94 and ΔAIC*i* < 5 compared to the three-morph models and intercept only model (Table 1). For the four morph model there was no effect of colour morph ($F_{3,45} = 1.41$, $p = 0.25$, $\eta^2_{partial} = 0.11$;Fig. 3d).

2.5. Bite force

SVL, condition, head width and body temperature were retained in the model explaining bite force performance; however, only SVL, condition and body temperature were statistically significant at $p < 0.05$ (SVL: $F_{1,43} = 29.69$, $p < 0.0001$, $\eta^2_{partial} = 0.27$; condition: $F_{1,43} = 10.77$, $p = 0.002$, $\eta^2_{partial} = 0.37$; head width: $F_{1,43} = 3.24$, $p = 0.08$, $\eta^2_{partial} = 0.05$; body temperature: $F_{1,43} = 4.81$, $p = 0.03$, $\eta^2_{partial} = 0.23$). As expected, hotter lizards that were bigger (greater SVL) and in better condition had a greater bite force (Fig. 4). When we accounted for these variables, there was no significant difference in bite force performance between the colour morphs ($F_{3,43} = 1.47$, $p = 0.24$, $\eta^2_{partial} = 0.09$; Table S5).

2.6. Relationship between androgen, corticosterone and bite force

There was no relationship between androgen at T0 or T30 and either CSM or bite force (overall multiple regression model results: T0: $F_{2,17} =$

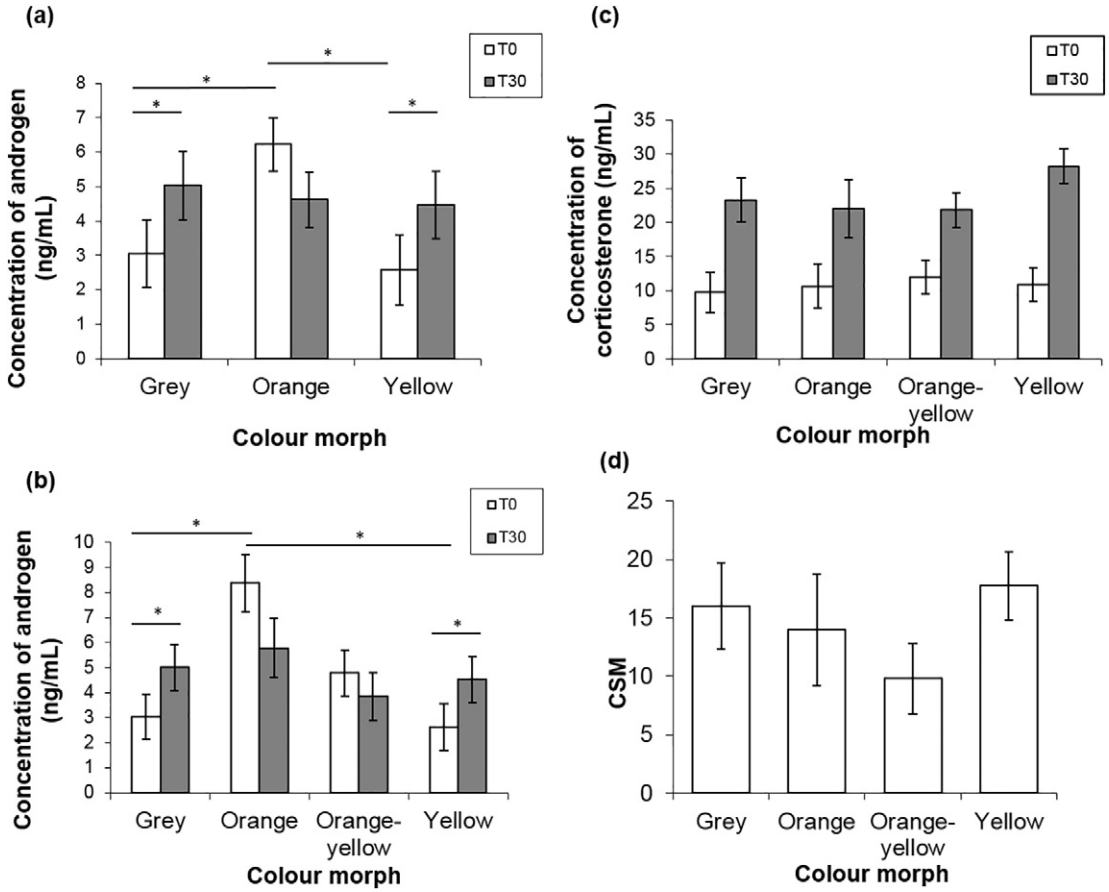

Fig. 3. (a) Concentration of androgen (mean ± SE) of three colour morphs with orange-yellow classified as orange. Grey ($N = 14$), orange ($N = 25$) and yellow ($N = 16$). (b) Concentration of androgen (mean ± SE) of the four colour morphs grey ($N = 14$), orange ($N = 10$), orange-yellow ($N = 15$) and yellow ($N = 16$). The unshaded bars are concentrations at T0 while the shaded bars are concentrations at T30. Significant pairwise comparisons are indicated by a line and *. (c) Concentration of corticosterone (mean ± SE) of four colour morphs. Grey ($N = 15$), orange ($N = 12$), orange-yellow ($N = 21$) and yellow ($N = 20$). (d) CSM (mean ± SE) of the four colour morphs. Grey ($N = 12$), orange ($N = 7$), orange-yellow ($N = 18$) and yellow ($N = 18$).

0.54, $p = 0.60$, adj. $r^2 = -0.06$; T30: $F_{2, 18} = 1.45$, $p = 0.26$, adj. $r^2 = 0.05$; for full results see Table S6).

3. Discussion

We predicted that the behavioural strategies of *C. decresii* colour morphs would be reflected by differences in androgen and corticosterone concentration as well as bite force. All morphs showed a similar increase in corticosterone concentration in response to acute capture stress and did not differ in their adrenal stress response, measured as CSM. However, morphs did differ in baseline levels of androgen, with the orange, more aggressive morph having significantly higher concentrations of androgen than the grey or yellow morphs. The grey and yellow morphs increased androgen from T0 to T30 post-capture whereas the orange (and orange-yellow) morph maintained high androgen levels between the two sampling times. There was no difference between colour morphs in size- and temperature-corrected bite force performed under standardised temperature conditions at the field station. Interestingly, however, morphs differed in body temperature immediately after capture. The orange-yellow morph had a higher body temperature than the grey, orange and yellow morphs, which may reflect anti-predator behaviour (Yewers et al., 2016). The orange-yellow morph has the smallest flight initiation distance and may therefore spend more time exposed, which could account for its higher body temperature at capture. However, the orange-yellow morph significantly differed only from the grey morph in flight initiation distance, whereas

Table 3
Pairwise *t*-tests of differences in androgen levels at T0 and T30 and for each colour morph between T0 and T30. In this model orange-yellow individuals are grouped with the orange morph. Significance below 0.05 is indicated **in bold**. Cohen's *d* values over 0.2 indicate a moderate effect size and values over 0.8 suggest a high effect size.

Group	Comparison	T (2, 41)	*p*	Cohen's *d*
T0	Grey and orange	−2.46	**0.018**	0.79
	Grey and yellow	0.22	0.824	0.08
	Orange and yellow	2.61	**0.012**	0.79
T30	Grey and orange	0.00	0.997	0.001
	Grey and yellow	0.36	0.719	0.13
	Orange and yellow	0.40	0.695	0.12
Grey	T30 vs. T0	−2.46	**0.017**	0.95
Orange	T30 vs. T0	0.52	0.523	0.18
Yellow	T30 vs. T0	−2.33	**0.025**	1.21

Table 4
Test of fixed effects for the linear mixed model of corticosterone concentration for three morph models with orange-yellow (OY) individuals classified as either the yellow or the orange morph. Significance below 0.05 is indicated **in bold**.

Model	Fixed effect	*DF*	*F*	*Pr > F*
Three morph OY = yellow	Morph	2, 47	0.59	0.560
	Sample time	1, 47	44.59	<**0.0001**
	Morph * sample time	2, 47	0.40	0.670
	Time to capture	1, 47	10.50	**0.002**
	Body temperature	1, 47	1.66	0.204
Three morph OY = orange	Morph	2, 46	0.73	0.486
	Sample time	1, 46	84.47	<**0.0001**
	Morph * sample time	2, 46	0.70	0.396
	Time to capture	1, 46	10.74	**0.003**
	Body temperature	1, 46	2.30	0.136

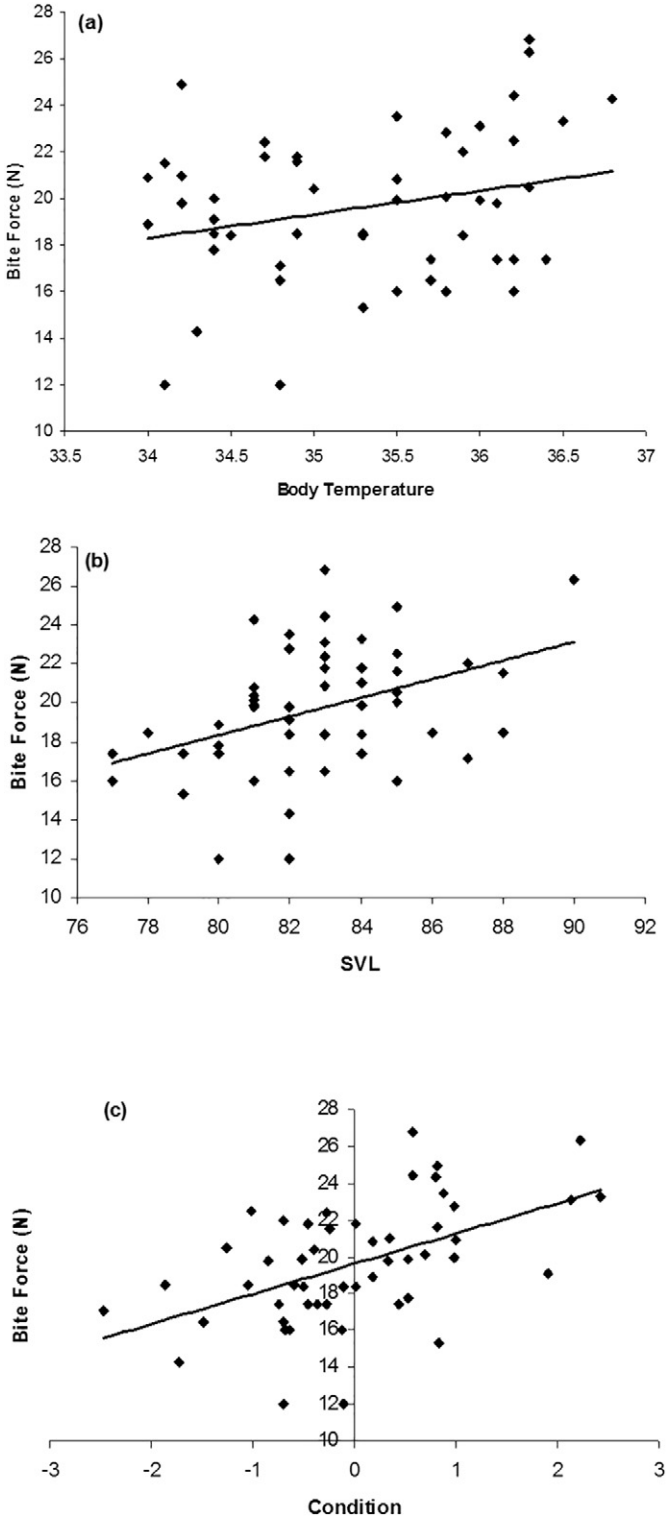

Fig. 4. Correlations between bite force (Newtons) and (a) body temperature, (b) SVL and (c) condition in *Ctenophorus decresii* colour morphs. Bite force was positively correlated with all three variables ($N = 52$).

it had a significantly higher body temperature at capture than all other morphs. Furthermore, we show here that warmer lizards have a greater bite force so body temperature could affect the aggressive ability of the orange-yellow morph in the wild.

The difference in baseline (i.e. T0) androgen between morphs is consistent with the alternative behavioural strategies in *C. decresii* colour morphs. In line with our predictions, the most aggressive orange morph had the highest levels of androgen at T0 compared to the less aggressive grey and yellow morphs. Higher levels of circulating androgen are associated with behavioural, morphological and physiological indicators of dominance (Oliveira, 2004). In many species of fish, birds, reptile and mammals including humans, dominant males exhibit the most aggression and higher androgen levels (Ehrenkranz et al., 1974; Greenberg and Crews, 1990; Knapp and Neff, 2007; Küpper et al., 2016). Furthermore, in other colour polymorphic lizard species such as *C. pictus* and *U. stansburiana*, the dominant orange morph also has higher androgen (Olsson et al., 2007; Sinervo et al., 2000a); however, this isn't always the case. In *Podarcis mellisensis* orange, blue and yellow morphs do not show significant differences in baseline testosterone (Huyghe et al., 2009b). In *Urosaurus ornatus*, differences in testosterone between morphs vary depending on environmental conditions. When conditions are favourable, both the orange and orange-blue morphs show site fidelity and high testosterone levels. However, under environmental stress, the orange morph switches from being sedentary to nomadic, which is reflected by lower testosterone (Knapp et al., 2003).

Differences in androgen levels between morphs may be a mediator of alternative behavioural strategies. For example, in *U. stansburiana* the orange, dominant morph has increased levels of circulating testosterone compared to the yellow and blue morphs. However, testosterone implants caused behavioural changes in the less dominant morphs increasing their access to females (Sinervo et al., 2000a). Conversely, morphs of the white-throated sparrow continue to display behavioural difference when levels of androgen are equal (Maney et al., 2009). Rather, expression of a hormone receptor better predicted aggression than hormone level in this species (Horton et al., 2014a). The role of other neuroendocrine and endocrine mechanisms such as the expression and abundance of neural steroid hormone receptors, the capacity of binding globulins, the activity of steroidogenic enzymes and the role of peptide hormones (e.g. gonadotropin-releasing hormone and arginine vasotocin) can also account for differences in behaviour and morphology between morphs (Arterbery et al., 2010a, b; Cain and Pryke, 2016; Foran and Bass, 1999; Genova et al., 2012; Horton et al., 2014b; Jennings et al., 2000; Zinzow-Kramer et al., 2014). There is no doubt that the endocrine control of behaviour is complex, warranting further studies of endocrine differences among colour morphs of *C. decresii*.

All four colour morphs in *C. decresii* showed increased corticosterone after acute capture stress and morphs didn't differ in the magnitude of their response. However, morphs did exhibit different androgen levels in response to capture stress with the grey and yellow morphs increasing androgen concentrations while the orange (and orange-yellow) morph maintained high androgen concentrations. The maintenance of high androgen levels could suggest that their hypothalamic-pituitary-gonadal (HPG) axis is insensitive to acute capture stress, or the effects of elevated corticosterone levels. This is contrary to many species where exposure to stressors or elevated corticosterone inhibit hormonal function of the HPG axis and suppress sex steroid production (lizards: Denardo and Licht, 1993; mice: Dong et al., 2004; humans: Fernandez-Garcia et al., 2002).

What mechanisms might permit plasma androgen to remain at normal levels in response to acute stress? There is evidence from rodents that increased activity of 11 beta-hydroxysteroid dehydrogenase alleviates corticosterone-mediated inhibition of steroidogenesis in Leydig cells (Monder et al., 1994; Tomlinson et al., 2004). Furthermore, levels of gonadotropin-inhibitory hormone (GnIH), which inhibits the release of testosterone and is often expressed during stressful conditions, may be downregulated (i.e. insensitive to stimulation from elevated corticosterone levels; Bentley et al., 2009). Similarly, dehydroepiandrosterone (DHEA) can suppress glucocorticoid receptor abundance to protect from neurotoxic levels of corticosterone (Hu et al., 2000; Kalimi et al., 1994). Irrespective of the mechanism, retention of high androgen levels may help to maximise reproductive opportunities for male lizards that have short lifespans and occupy highly variable environments. That all males have high levels of androgen with increased corticosterone is

somewhat puzzling, but may provide "physiological contingency" to buffer variation in male fitness due to environmental stress, independent of morph.

Although androgen levels often decrease in response to acute stress, in some cases stress may cause levels of plasma androgen to increase (Heiblum et al., 2000; Narayan et al., 2012; van Hout et al., 2010). The neuroendocrine system responsible for testosterone regulation is complex, but mechanisms might include stimulation of the HPG axis to produce increased androgen or cognitive mechanisms differentially activating the endocrine system (Chichinadze and Chichinadze, 2008). More specifically, activation of luteinizing hormone receptors (that regulate expression of 17-β hydroxysteroid dehydrogenase) or increased synthesis of steroidogenic acute regulatory protein (StAR) is fundamental rate determining processes involved in increasing androgen synthesis within the testis (Huffman et al., 2012). Furthermore, morphs of male white-throated sparrows differ in the expression of the follicle stimulating hormone receptor gene in the gonad (Zinzow-Kramer et al., 2014) and differ in the extent of testosterone increases following injection of gonadotropin-releasing hormone (Spinney et al., 2006) This, alongside morph-specific variation in levels or activities of testosterone metabolising enzymes (e.g. 5α-reductase, aromatase, 17β-hydroxysteroid dehydrogenase), may underlie increased androgen levels during acute stress (Burns et al., 2014).

We remain cautious in interpreting the functional significance of increased androgen during acute stress in grey and yellow morphs because hormone responses to capture stress may not be the same as to behavioural stressors (e.g. Deviche et al., 2014).We are yet to conduct direct experiments to examine hormone responses to staged challenges between *C. decresii* morphs (e.g. Knapp and Moore, 1996). Nevertheless, numerous examples suggest that androgen responses to capture stress can be indicative of behavioural variation among males. Good evidence comes from social species with male-dominant hierarchies, including primates. Here, an increase in testosterone following acute, not chronic, stress is observed in dominant males (Elofsson et al., 2000; Rejeski et al., 1989). In *C. decresii* the more aggressive orange morph did not increase androgen concentrations; rather it was the less aggressive grey and yellow morphs. The orange morph is highly aggressive to all competitors regardless of colour and may maintain consistently high levels of androgen (insensitive to acute stress) to facilitate territorial patrolling during the breeding season (in which this study was conducted). By contrast, under situations of acute stress, (e.g. when faced with a competitor and the prospect of escalated conflict) the yellow and grey morphs may increase concentrations of androgen and exhibit greater aggression.

We expected previously defined differences in aggressive behaviour of *C. decresii* morphs to be reflected in greater bite force, which can give a competitive advantage in contests (Huyghe et al., 2009a; López and Martín, 2001). However, this was not the case. Similarly, in *U. ornatus*, morphs do not differ in bite force (Meyers et al., 2006), despite clear differences in dominance status during staged encounters (Thompson and Moore, 1991). In species in which bite force is an honest indicator of fighting ability, it is generally correlated with head size, particularly head depth and width (Huyghe et al., 2005; Lailvaux et al., 2004); however, we observed no such relationship in *C. decresii*. Thus, like *U. ornatus*, bite force may be a poor predictor of fighting ability or dominance in *C. decresii*, perhaps because contests rarely escalate to biting in the wild (in contrast to staged laboratory contexts; Osborne, 2004; Stuart-Fox and Johnston, 2005). In staged captive contests, larger *C. decresii* adult males were more likely to win but residency also strongly predicted contest success. Indeed, smaller residents were more likely to win contests than larger intruders (Umbers et al., 2012). Therefore, territory size could potentially be a better indicator of competitive ability and dominance than body size, head dimensions or associated bite force as larger territories often reflect social dominance through the monopolisation of resources (Fox et al., 1981; Stamps and Krishnan, 1998; Stone and Baird, 2002). Indeed, in the closely related lizard *Ctenophorus ornatus*, larger territories overlap the range of more females (Lebas, 2001).

Based on behavioural data, whether the orange-yellow morph employs a distinct strategy remains ambiguous; therefore, we compared statistical support for models in which orange-yellow individuals were grouped with either pure orange or yellow males or considered as a separate morph. For androgen, the most highly supported model grouped orange-yellow and orange individuals together because they showed a similar pattern, with high androgen concentrations maintained between T0 and T30. This contrasts with aggressive behaviour, for which orange-yellow individuals grouped with yellow rather than orange in the most highly supported model (Yewers et al., 2016). However, for androgen, the three morph model with orange-yellow grouped as yellow and the four morph model were both within $\Delta AICi < 2$ of the best model, although each of these models had less than half the AIC weight of the best model. Furthermore, statistical support for models grouping orange-yellow individuals with either yellow or orange or separately didn't differ for other traits including boldness (Yewers et al., 2016), corticosterone concentrations, stress response, or bite force. Given shared alleles between the orange-yellow and both the pure orange and pure yellow morphs (Rankin et al., 2016; and expected linked or common genetic mechanisms underlying correlated traits), the orange-yellow morph may exhibit an intermediate phenotype or strategy between the pure orange and pure yellow morphs.

4. Conclusions

Here we have shown that colour morphs of *C. decresii* differ in levels of androgen immediately after capture and following acute stress but do not differ in concentration of corticosterone, CSM or bite force. Our findings appear consistent with previously established behavioural differences of morphs (Yewers et al., 2016). The orange morph shows consistently high aggression and has a higher concentration of androgen immediately after capture than the grey or yellow morphs, and maintains high androgen following acute stress. The cautious strategy of the grey morph is in agreement with low androgen concentrations immediately after capture. The grey and yellow morphs greatly increase androgen concentrations following acute stress, which may reflect their aggressive response. Despite differences in endocrinology between morphs in *C. decresii*, the extent to which orange-yellow individuals employ a distinct strategy remains unclear. Furthermore, the role of other neuroendocrine and endocrine mechanisms such as receptor abundance, binding globulin capacity, other steroidogenic enzymes and peptide hormones can also account for differences in behaviour and morphology between morphs and should be considered (Knapp, 2003). Which of these, or indeed other, candidate processes could help explain our results requires further research. More broadly, addressing how these proximate mechanisms operate within and between the sexes is fundamental to understanding life-history tactics that shape important evolutionary phenomena. Our findings add to our growing understanding of the complex relationship between polymorphic sexual signals, behaviour, endocrinology and performance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2017.05.001.

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