

Sex steroid correlates of female-specific colouration, behaviour and reproductive state in Lake Eyre dragon lizards, *Ctenophorus maculosus*

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Abstract In some species, females develop bright colouration to signal reproductive status and exhibit behavioural repertoires to incite male courtship and/or reduce male harassment and forced copulation. Sex steroids, including progesterone and testosterone, potentially mediate female reproductive colouration and reproductive behaviour. We measured associations among plasma profiles of testosterone and progesterone with variation in colour expression and reproductive behaviour, including unique courtship rejection behaviours, in female Lake Eyre dragon lizards, (*Ctenophorus maculosus*). At onset of breeding, progesterone and testosterone increased with vitellogenesis, coincident with colour intensification and sexual receptivity, indicated by acceptance of copulations. As steroid levels peaked around the inferred ovulation time, maximal colour development occurred and sexual receptivity declined. When females were gravid and exhibited maximal mate rejection behaviours, progesterone levels remained consistently high, while testosterone exhibited a discrete second peak. At oviposition, significant declines in plasma steroid levels, fading of colouration and a dramatic decrease in male rejection behaviours co-occurred. Our results indicate a generally concordant association among steroid levels, colouration, behaviour and reproductive events. However, the prolonged elevation in progesterone and a second peak of testosterone was unrelated to reproductive state or further colour change, possibly suggesting selection on

females to retain high steroid levels for inducing rejection behaviours.

Keywords Female reproduction · Sex steroids · Female colouration · Mate rejection · Lizard

Abbreviations

JND	Just noticeable difference
SE	Standard error
SD	Standard deviation
LWS	Long wavelength sensitive
GLMM	General linear mixed model
LSD	Least squared difference

Introduction

Conspicuous colours and ornaments are much less common in females than males and consequently, studies of the adaptive function and proximate basis of female ornamentation are comparatively rare (reviewed in Amundsen 2000; Amundsen and Parn 2006). Although female ornamentation is typically a reduced version of that in males, many species possess colour patterns and ornaments that are specific to females (e.g. Amundsen and Forsgren 2001; Baird 2004; Cooper and Greenberg 1992; Cuadrado 2000; Heinsohn et al. 2005; Kraaijeveld et al. 2004; Montgomerie and Thornhill 1989; Nunn 1999; Roulin et al. 2001; Rowland et al. 1991; Watkins 1997; Weiss 2006). Female-specific ornamentation may have several adaptive functions including signalling reproductive maturity, status and genetic quality to both mates and potential female competitors (Amundsen and Parn 2006; Cooper and Greenberg 1992; Nunn 1999). With respect to signalling reproductive status, females may signal the most effective time for fertilisation to stimulate male

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courtship or incite male–male competition (reviewed in Cooper and Greenberg 1992; Nunn 1999) and/or signal non-receptivity in order to decrease the direct costs of male harassment and mating (Cooper and Greenberg 1992).

Complementing variation in colour expression are often distinctive shifts in behavioural repertoires that reflect a female's transition between receptive and non-receptive breeding states (Cooper and Greenberg 1992; Nunn 1999). Changes in receptivity are typically associated with differences in a female's response towards male courtship and copulation attempts. When receptive, females may readily court and mate with males, whereas non-receptive females often actively avoid male interest by using defensive and, in some instances, highly aggressive behaviour constituting courtship rejection (Berger 1983 [horses]; Cooper and Greenberg 1992 [lizards]; Farr 1980 [fish]; 1984 [birds]; McKinney et al. 1983; Nunn 1999 [primates]). For the latter, courtship rejection behaviours may function to prevent forced male copulation and reflect potentially costly female resistance strategies that have evolved under sexual conflict (reviewed in Arnqvist and Rowe 2005; Chapman et al. 2003; Clutton-Brock and Parker 1995).

Although the evolution of female resistance strategies has received recent attention (reviewed in Arnqvist and Rowe 2005; Chapman et al. 2003; Clutton-Brock and Parker 1995), our understanding of the proximate physiological mechanisms (e.g. steroid hormones) potentially mediating courtship rejection behaviours in females is still poorly defined (Cooper and Crews 1988). This is in contrast to males where there is strong theoretical framework that links the frequency and intensity of aggression within a reproductive context and its mediation by sex steroid hormones, particularly testosterone (Wingfield et al. 1990). Specifically, in male vertebrates seasonal plasma profiles of testosterone may be distinctly different between monogamous and promiscuous males (Wingfield et al. 1990). This difference forms the basis of the challenge hypothesis (*sensu* Wingfield et al. 1990), which predicts that in promiscuous males exhibiting little paternal care, plasma levels of testosterone remain elevated to facilitate ongoing aggression, whereas monogamous males have initially high testosterone levels followed by a noticeable decrease coinciding with the onset of paternal behaviours (Wingfield et al. 2001). However in monogamous males, androgen levels may increase dramatically during certain aggressive contexts (e.g. territorial intruders, nest predators) but decrease quickly as the challenge passes and as the need to tend offspring remains. Several studies support an analogous behavioural modification of seasonal androgen profiles in females and again suggest that depending on amount of aggression exhibited during the female reproductive period, distinct profiles in plasma testosterone characterised

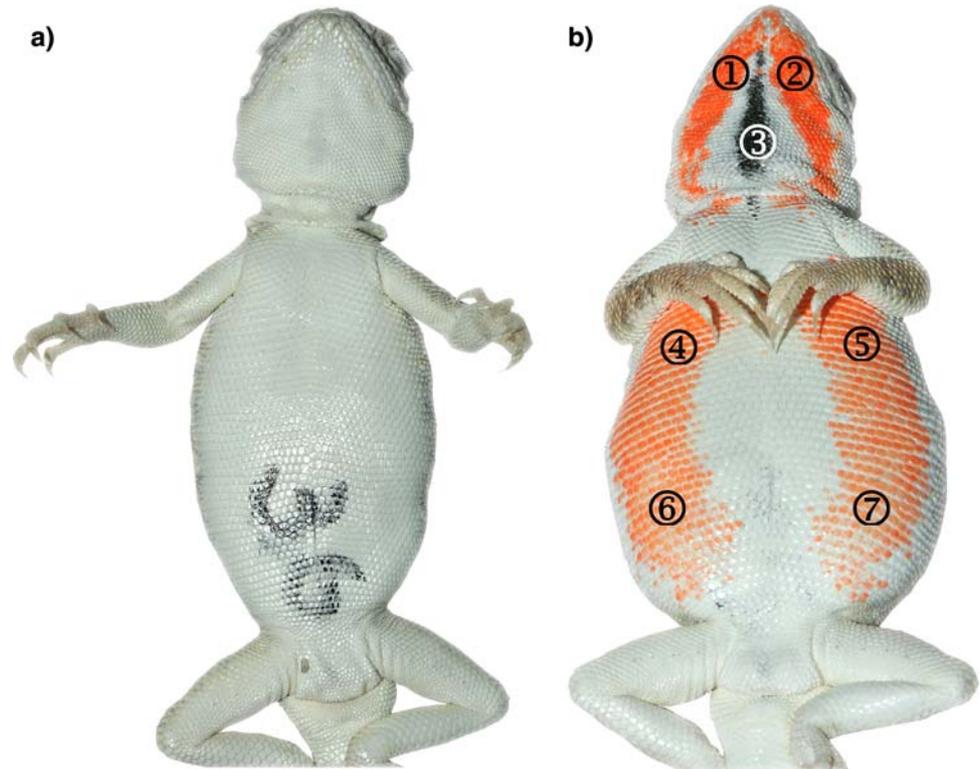
by seasonal or context dependent increases in androgens may be evident (Desjardins et al. 2006; Gill et al. 2007; Hegner and Wingfield 1986; Langmore et al. 2002; Woodley and Moore 1999a).

Lizards are good model organisms for understanding the physiological and particularly the endocrine basis of both female-specific colouration and associated behavioural transitions because in several species, the development, pattern and intensity of female colouration and corresponding behavioural repertoires may be directly regulated by sex steroids (see also Calisi and Hews 2007; reviewed in Cooper and Greenberg 1992). Previous studies have demonstrated that progesterone and testosterone, and to a lesser degree oestradiol cause development of orange pigments in adult female lizards (Cooper and Crews 1988; Cooper and Ferguson 1972a, b; Medica et al. 1973). Similarly, administration of progesterone and testosterone to ovariectomised *Holbrookia propinqua* reinstated aggressive courtship rejection behaviours (Cooper and Crews 1987). Other studies on female lizards show correlative evidence that steroid levels influence both colouration and aggressive behaviours (Calisi and Hews 2007; Chan and Callard 1974; Cooper et al. 1983; Cooper and Clarke 1982; Cooper and Crews 1988; Salvador et al. 1997; Watt et al. 2003; Weiss et al. 2002; Woodley and Moore 1999a, b).

We investigated steroid levels in relation to female colour expression and reproductive behaviour in the Lake Eyre dragon lizard, in which females exhibit both distinctive reproductive colouration and unique courtship rejection behaviours. The species is restricted to a few large salt pans in Australia's arid southern interior and shelters in loose sand beneath the salt crust (Mitchell 1973; Olsson 1995b). Females develop extensive, conspicuous, ventro-lateral orange colouration (Fig. 1b) during breeding while males remain cryptically coloured (Mitchell 1973). Males compete vigorously for access to territories and females and exhibit persistent courtship, harassment and attempted forced copulations (Olsson 1995a). Female colouration stimulates male courtship and is also emphasised during rejection displays yet there is no marked difference in colouration between receptive and unreceptive females (Chan et al. submitted). Coinciding with this colouration, female Lake Eyre dragons have evolved a unique sequence of rejection behaviours. Initially, females either flee or perform lateral displays, elevating themselves on all four legs, while laterally compressing the body to reveal their ventro-lateral orange colouration. As a last resort, however, females flip themselves onto their backs exposing the bright orange ventral colouration (a behaviour unknown in any other lizard) because this position prevents forced male intromission (Olsson 1995a).

As an initial step towards understanding the proximal regulation of the conspicuous colouration and remarkable

Fig. 1 **a** A non-reproductive female exhibiting white colouration and **b** a female exhibiting orange colouration. Reflectance measurements were taken from three body regions: the *orange* throat (1, 2), *black* gular stripe (3) and *orange* abdomen (4, 5, 6, 7)



reproductive behaviours of female Lake Eyre dragons, we examined the correlation between plasma levels of progesterone and testosterone and female colour expression and reproductive behaviour. First, we assessed if and how these hormones were correlated with development and subsequent fine-scale temporal variation in colour expression both across the breeding period and within individual females. We determined patterns of colour signal expression as perceived by the lizard, rather than human visual system by quantifying spectral reflectance of colour patterns and applying a model of colour perception based on visual pigment sensitivities for a congeneric species (Barbour et al. 2002). Next, we assessed the degree of concordance between steroid profiles and transitions in female reproductive behaviour. Specifically, we tested whether plasma steroid profiles differed in females as they progressed from receptive to non-receptive states, when females show a marked increase in mate rejection behaviours. If in Lake Eyre dragons steroid hormones regulate three interacting components of female reproduction (e.g. physiology, colouration, behaviour), we might expect to measure distinctive profiles in one or both plasma steroids (i.e. relative to female lizards where colouration or mate rejection behaviours are absent). Given persistent male harassment and forced copulation, female Lake Eyre dragons are predicted to be a likely candidate to exhibit elevated plasma steroid levels to facilitate ongoing maintenance of mate rejection behaviours until oviposition.

Methods

Animal capture and husbandry

We captured 57 lizards (25 females, 32 males) by hand from Lake Eyre, South Australia (28.95–29.05°S, 137.65–137.76°E). The animals were captured shortly after emergence from hibernation (between 22 August and 7 September 2007) and none of the females showed orange colouration until several weeks after being brought into captivity. We measured body size (snout-vent length, SVL) to the nearest mm at capture and 1 month after being brought into captivity and used the mean of these in subsequent analyses. The mean (\pm SD) size of females in this study was 56.6 ± 4 mm (range 49–64 mm) and males were 65.2 ± 3.5 mm (range 56–70 mm), which is larger than the minimum size at sexual maturity (45 mm for females and 55 mm for males; Mitchell 1973).

We transferred lizards to the animal facility at the University of Melbourne where they were housed individually in $61 \times 30 \times 30$ cm glass tanks containing a layer of sand and salt crust to mimic natural habitat, and separated by opaque partitions. The room was maintained at an average temperature of 28°C on a 12:12 day:night cycle, and a heat lamp was suspended above each tank to allow animals to attain their preferred body temperatures. Lizards were misted and fed live crickets (*Anchieta domesticus*) or mealworms (*Tenbrio spp.*

larvae) dusted in calcium and multivitamins three times a week.

Sampling design

Our aim was to examine changes in plasma concentrations of progesterone and testosterone in relation to female colouration and behaviour throughout the reproductive cycle, which we divided into nine stages (Table 1). To minimise stress to the animals, we did not measure colouration and behaviour or take blood samples of each individual within every stage. Instead, we aimed to obtain between five and ten sets of colour measurements and blood samples and approximately 20 behavioural trials for females representing each stage (sample sizes in Table 2). Within an ovarian cycle, we took colour measurements and blood samples of females at an average of 2.65 (± 1.2 SD, range 1–5) out of the nine stages and used females in no more than two behavioural trials per stage. We took a total of 98 sets of reflectance measurements, conducted 186 behavioural trials and took 82 blood samples (Table 2). For a subset of females, we had both blood samples and colour measurements (N = 57) for the same stage, allowing us to correlate plasma steroid concentrations with female colour intensity at the individual level. We did not take blood samples immediately before or after behavioural trials. As our aim in this study was to examine changes in steroid levels in relation to colouration and behavioural changes across the reproductive cycle, we took blood samples within the same reproductive stage, that is, within 3 days of behavioural trials. The temporal variability in blood sampling relative to behavioural trials precluded us from testing for correlations between progesterone or testosterone and behaviour at the individual level. We have described how female colour expression and reproductive behaviour vary across the

Table 2 Sample sizes for measurements of female colouration (spectrometry and area) and male, female behaviour and plasma steroid concentrations

Female stage	Spectrometry (N)	Reproductive behaviour (N)	Plasma steroid concentrations (N)
0	14 (13)	32 (16)	20 (17)
1	7 (7)	18 (9)	9 (8)
2	10 (8)	19 (10)	6 (5)
3	12 (10)	19 (10)	13 (11)
4	19 (14)	20 (10)	7 (6)
5	7 (7)	20 (10)	5 (5)
6	8 (7)	20 (10)	7 (7)
7	10 (9)	18 (9)	8 (5)
8	11 (11)	20 (10)	7 (7)
Total	98	186	82

The same individual was used in multiple stages. In each case, the number of measurements is given with the number of females represented in brackets

reproductive cycle elsewhere (Chan et al. submitted), therefore here we provide only a brief description of methods for obtaining colour and behavioural data, as well as how colour and behaviour vary across the ovarian cycle below.

Female reproductive stages

To quantify plasma steroid concentrations in relation to female colouration and behaviour we divided the reproductive cycle into nine stages (Table 1). These stages were defined in a way that would enable us to detect fine scale temporal changes in colour intensity, behaviour and plasma steroid concentrations across the reproductive cycle, since precise reproductive status (e.g. timing of ovulation) cannot be determined non-invasively in this species. Stage 0 was

Table 1 Stages defining female colour and associated reproductive state in Lake Eyre dragons

Stage	Definition	Colouration	Reproductive status
0	Prior to first developing orange	White	Non-reproductive
1	0–4 days after orange developed	Orange	Vitellogenic follicles not always detectable via palpation
2	5–8 days after orange developed	Orange	Vitellogenic follicles
3	9–12 days after orange developed	Orange	Ovulation most likely between stages 3 and 4
4	13–16 days after orange developed	Orange	Shelling oviductal eggs by the end of stage 4 at the latest
5	17–20 days after orange developed	Orange	Shelling oviductal eggs
6	21 days after orange developed to laying after orange developed	Orange	Shelled oviductal eggs
7	0–10 days post-laying	Faded orange/white	Post-oviposition
8	10+ days post-laying without orange re-intensifying	Faded orange/white	Post-reproductive

the period at the beginning of the breeding season, where female colouration was white (Fig. 1a). Some females remained white ($N = 7$) and never developed orange colouration or laid eggs (fertilised or unfertilised) over the entire breeding season (Table 1), while the remaining 18 females developed orange colouration and laid eggs. Stages 1–5 consisted of 4-day increments, from the date females developed orange colouration, and stage 6 extended to laying date (Table 1). Vitellogenic follicles could be detected via palpation during stages 2 and 3, while females had shelled oviductal eggs from stages 4 or 5 until laying. During these six stages females retained their orange colouration (Fig. 1b; Table 1). Stage 7 was defined as the 10 days following laying and stage 8 was the period greater than 10 days after laying without the female becoming reproductively active again, with colouration in these two stages being either a faded orange or white (Table 1).

We also defined three broader reproductive categories: (1) “non-reproductive” comprising females that were yet to develop orange colouration (stage 0), post-oviposition (stage 7) and post-reproductive (stage 8); (2) “receptive” comprising females from the period of developing orange colouration (stage 1) until acceptance of their last (unforced) copulation (derived from behavioural trials—see below) and (3) “unreceptive” comprising females from the date of their last (unforced) copulation until oviposition. These categories correspond to major changes in female reproductive behaviour.

Female colouration and behaviour

We provide detailed methods describing measurement of female colouration and behaviour elsewhere (Chan et al. submitted). Briefly, we quantified the spectral properties of female colouration at each reproductive stage by taking reflectance readings (USB 4000 spectrometer and PX-2 light source Ocean Optics, Dunedin, USA) in the range of 300–700 nm, the visual spectrum for most diurnal lizards (Loew et al. 2002). We measured three body regions: the throat, black gular stripe down the centre of the throat and abdomen (Fig. 1). As visual systems encode signals as contrasts between adjacent colours, we analysed colour variation as changes in the contrast between orange (throat and abdomen) or black (gular stripe) colouration against the white ventral surface, as perceived by the lizard visual system. We applied the model of Vorobyev and Osorio (1998), which has been applied to a range of vertebrates (Hemmi et al. 2006; e.g. Siddiqi et al. 2004; Stuart-Fox and Moussalli 2008 and references therein). It assumes that visual discrimination is limited by photoreceptor noise, ω_i , and can be used to estimate the discriminability of two colours in units of discrimination thresholds or just noticeable differences (JNDs). We used the same model calculations as

detailed in Siddiqi et al. (2004). We used data on the spectral sensitivities of a congeneric lizard, *Ctenophorus ornatus* (Barbour et al. 2002). We assumed that as in other vertebrates, the three single cones are used for chromatic (colour) discrimination, while the long wavelength sensitive (LWS) photoreceptors in the double cones are used for achromatic (brightness) discrimination (Osorio and Vorobyev 2005).

To assess female behaviour over the reproductive cycle, we conducted behavioural trials during the lizards’ breeding season from September 2007 to January 2008. We placed females in male tanks, videotaped the interaction for approximately 30 min and scored female reproductive behaviours from the video footage. Behavioural interactions generally began with the male courting the female with a series of head bobs and push-ups. In response, females would perform appeasement behaviours, consisting of head bobs and arm waves, allow copulation, flee or reject the male by flipping over or using a lateral display, sometimes performed along with a slow stiffened walk. Arm waves consisted of a single extension of the forearm in a lateral forward circular motion; lateral displays consisted of females elevating their bodies by extending all four legs beneath them, lateral compression of body and arching of the back while flips consisted of the female flipping herself onto her back exposing her bright orange ventro-lateral colouration (Fig. 1). We scored the number of head bobs, female arm waves, copulation duration, and the duration of lateral displays and flips. We converted all behaviours to measures per minute of active trial time, defined as the time from when the individuals first interacted and excluding the time that each lizard spent hiding beneath the salt crust. Behavioural data were log-transformed to meet model assumptions.

Blood sampling protocols

To standardise possible circadian variation in steroid hormone levels, blood was collected between 1200 and 1400 hours from 31 adults (25 females, 6 males; Table 2) using 100 μ l heparinised capillary tubes sampling from the *sinus angularis*, which was accessed using a needle tip through the corner of the mouth, following the standard procedure for ‘small’ lizards (Olsson et al. 2000; Woodley and Moore 1999a). Blood samples were kept on ice, centrifuged within the hour at 10,000 rpm for 2 min and the plasma was separated and stored in a freezer at -20°C until assayed. Individual blood sampling occurred no more than once a week, was dependant on individual health status, and collected with a focus on obtaining samples from 5 to 10 representatives for each stage, rather than a sample per female per stage (Table 2—see [Sampling design](#)). These blood sampling procedures have been shown to not

permanently affect small lizards, with individuals performing normal behaviours (e.g. eating) within minutes after collection (Chan, pers. obs.; Whiting et al. 2006).

Plasma steroid analysis

We measured total plasma concentrations of progesterone and testosterone using commercially available ELISA kits (Caymen Chemical, Michigan, USA) and their associated protocols. Samples were analysed across three assays per hormone, with assays also containing a small number of male samples to serve as a negative and positive control for progesterone and testosterone, respectively. Preliminary assays determined that the sufficient volume of sample plasma to be used for assay was 10 μ l for progesterone and 20 μ l for testosterone (a minimum of 5 μ l used in samples where minimal plasma was available and volume corrections were performed in calculations). To reduce cross reaction and interference all samples were individually extracted under the steroid specific solvent ratios of Ethyl Acetate:Hexane (5:95) for progesterone (following Renfree et al. 1994) and Hexane:Toluene (2:1) for testosterone (following Williamson et al. 1990). To measure the efficiency of extraction 20 μ l (\approx 2,000 cpm) of tritiated steroid was added to each sample prior to extraction. To estimate steroid extraction efficiency, 50 μ l of each extracted sample was placed into a scintillation vial containing 2 ml of scintillation fluid (Ultima Gold). Sample radioactivity was estimated using a Beckman 2100R Liquid Scintillation Counter. The extraction efficiency for each sample was calculated as the quotient of radioactivity (CPM) remaining in the sample relative to the total amount of radioactivity added to each sample pre-extraction (determined from extraction controls).

Final steroid concentrations (measured in duplicate) were calculated from standard curves and corrected for individual sample recovery, individual plasma volume and the addition of tritiated steroid. For progesterone assays, average (\pm SE) female recovery was $65.7\% \pm 0.028$ with an intra-assay coefficient of variation of 22.6% and an inter-assay coefficient of variation of 13.04%. For testosterone assays, average female recovery was $67.2\% \pm 0.018$ with an intra-assay coefficient of variation of 13.6% and an inter-assay coefficient of variation of 14.0%. The assay detection limits were 0.15 and 0.10 ng/ml for plasma testosterone and progesterone, respectively.

Statistical analysis

We tested whether female plasma concentrations of progesterone and testosterone differed significantly between reproductive stages using a Generalised Linear Mixed Model (GLMM; PROC GENMOD SAS version 9.1). To

account for correlated data due to repeated use of individuals and use of the same individual over more than one reproductive cycle, we included female ID and female cycle (first, second or third) nested within female ID as random factors in GLMMs. We used least square differences (LSD) post hoc tests to test for differences between each pair of stages. We also tested whether progesterone or testosterone concentrations differed between females in the three broader reproductive categories (non-reproductive, receptive and unreceptive).

If steroid levels are a potential physiological basis for explaining stage and individual based differences we might expect to observe significant correlations between steroid level and our measures of colouration and behaviour. Thus, we calculated mean values of steroid levels, colour and behavioural traits and performed multiple regressions with each colour or behavioural trait as the dependent variable and both progesterone and testosterone as predictors. To assess which steroid best explained variation in colouration or behaviour, we applied stepwise model selection with a criterion for a variable remaining within the model set at $P < 0.1$, and, if both variables were retained, compared partial R^2 values. Finally, for the subset of females for which we had both blood samples and colour measurements within the same reproductive stage, we tested for a correlation at the individual level between plasma concentrations of progesterone or testosterone and female colouration, again using GLMMs.

Results

Variation in colouration and behaviour

Female colour expression changes significantly across the reproductive cycle, reaching its full intensity from stages 2 to 5 before fading just prior to laying or in stage 7 just after laying (Fig. 2; Chan et al. submitted). Specifically, both the achromatic and chromatic contrast of the throat and abdomen against the adjacent white ventral surface increase rapidly between stages 1 and 2, reaching a plateau until stage 5 before decreasing at stages 6 or 7.

Female behaviours vary significantly through the reproductive cycle (Chan et al. submitted; Fig. 3). Females perform arm waves and head bobs, which appear to function as appeasement behaviours, primarily when white and non-reproductive (stage 0). Females begin accepting copulation shortly after showing orange colouration (stage 1). Copulation frequency peaks during stage 2 (Fig. 3a) then falls sharply through stage 3. From stage 4 to 6, females increase the frequency of rejection behaviours—lateral displays (Fig. 3b) and flipping over (Fig. 3c).

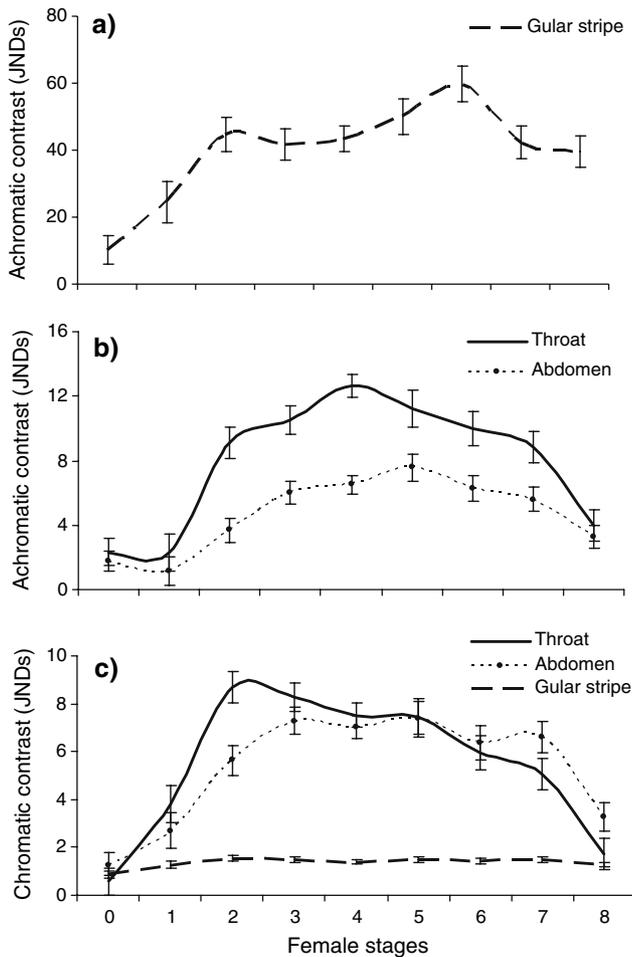


Fig. 2 Mean (\pm SE) of **a** achromatic (brightness) contrast of the gular stripe against the surrounding white throat and **b** achromatic contrast of the *orange* throat and abdomen against adjacent *white* body regions; **c** chromatic (*colour*) contrast of the throat, abdomen and gular stripe against adjacent white body regions in units of discrimination thresholds or just noticeable differences (JNDs)

Variation in steroid levels

Levels of plasma progesterone and testosterone varied significantly throughout the reproductive cycle (progesterone $F_{8,44} = 8.11$, $P < 0.0001$, testosterone $F_{8,43} = 4.65$, $P = 0.0004$). Progesterone rapidly increased and remained high in stages 3, 4 and 5 before dropping sharply prior to laying (Fig. 4). Mean values of progesterone in stages 3, 4 and 5 were significantly higher than in stages 0, 1, 7 and 8 (LSD post hoc tests: $P < 0.007$ for all pairwise comparisons). Testosterone increased steadily until stages 2–3, after which it decreased before another peak increase between stages 5 and 6, followed by a rapid decline back to initial levels at stages 7 and 8 (Fig. 4). The two testosterone peaks at stages 2–3 and 6 were significantly higher than Testosterone

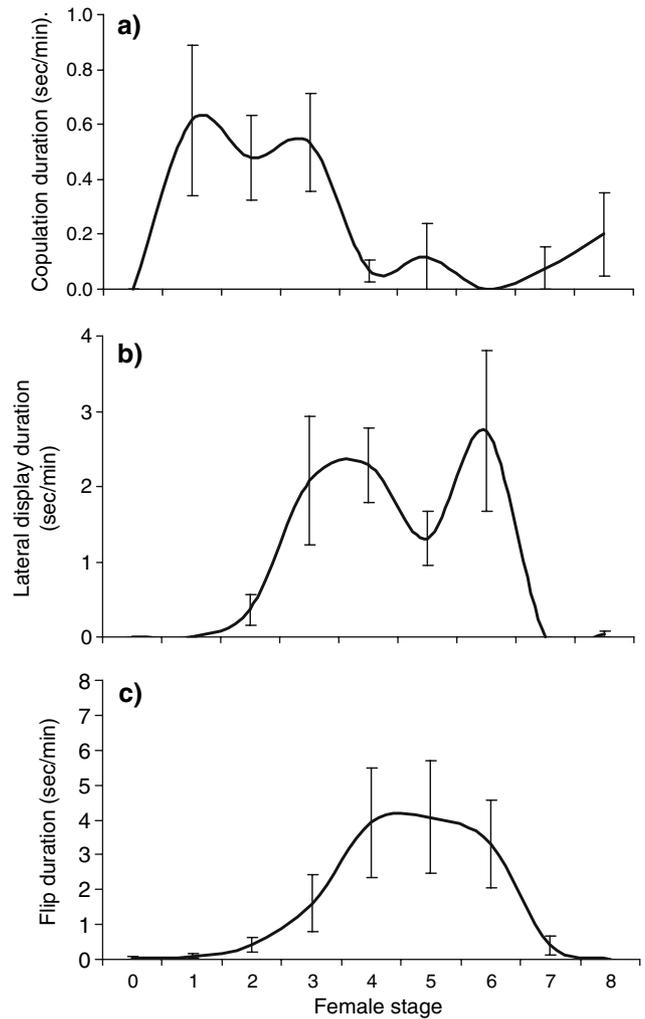


Fig. 3 Mean (\pm SE) duration (in seconds per minute of trial time) of **a** copulations and the rejection behaviours, **b** lateral display and **c** flip over performed over the eight stages of the female reproductive cycle

levels at stages 0, 7 and 8 (LSD post hoc tests: $P < 0.003$ for each pairwise comparison).

In terms of broader reproductive categories, there were significant differences among categories in concentrations of both steroids (progesterone $F_{2,27} = 18.28$, $P < 0.0001$; testosterone $F_{2,26} = 7.37$, $P = 0.003$; Fig. 5). Non-reproductive females had significantly lower levels of circulating plasma progesterone and testosterone than either receptive (progesterone $F_{27} = 3.91$, $P = 0.0006$; testosterone $F_{26} = 3.59$, $P = 0.001$) or unreceptive females (progesterone $F_{2,27} = 5.79$, $P < 0.0001$; testosterone $F_{26} = 2.75$, $P = 0.011$). There was a trend toward higher progesterone levels in unreceptive than receptive females ($F_{2,27} = 1.86$, $P = 0.07$) but no difference in testosterone levels between the two categories ($F_{2,26} = -0.76$, $P = 0.45$).

Multiple regressions of mean values at each stage showed significant relationships between both colour and

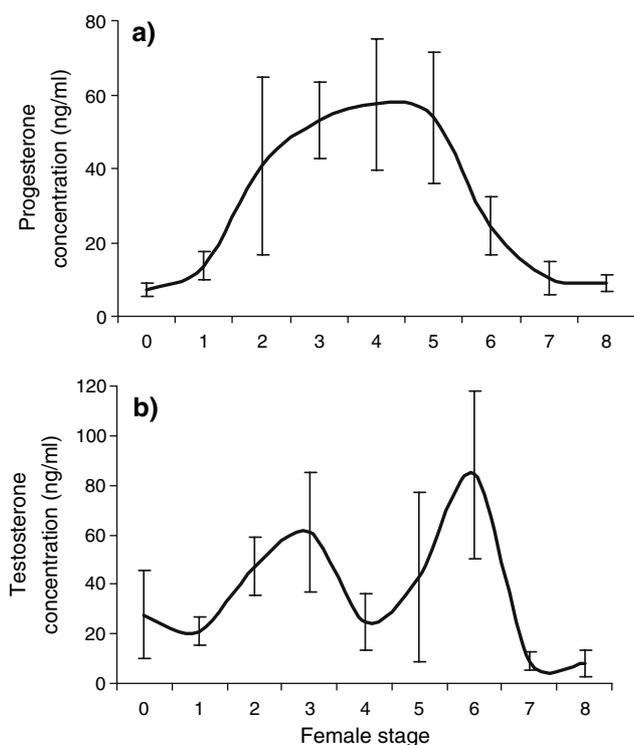


Fig. 4 Mean (\pm SE) concentration of circulating plasma: **a** progesterone and **b** testosterone for each female stage across the reproductive cycle

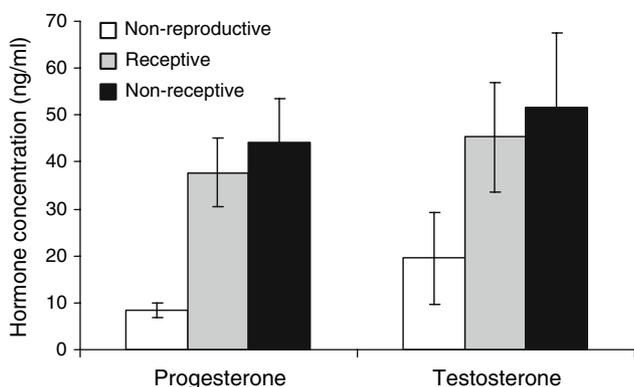


Fig. 5 Mean (\pm SE) concentration of circulating plasma progesterone and testosterone in non-reproductive, receptive and unreceptive females

behaviour and steroid levels (Table 3). Plasma progesterone level was a consistent predictor of colour expression, showing a positive relationship with both the achromatic and chromatic contrast of the throat and abdomen (Table 3). Testosterone level was a strong predictor of the frequency of arm waves and head bobs: in stages where these appeasement behaviours were frequent, testosterone values were concomitantly low (Table 3). The strongest predictor of rejection behaviour (lateral display and flip duration) was progesterone, with a positive relationship between progesterone levels and both behaviours (Table 3).

Neither steroid was associated with copulation frequency among stages.

At the individual level, there were clear correlations between plasma steroid concentrations and measures of colour intensity. Both measures of throat colouration were significantly correlated with both plasma progesterone concentrations (achromatic contrast: $F_{1,27} = 28.11$, $P < 0.0001$; chromatic contrast: $F_{1,27} = 20.81$, $P < 0.0001$) and testosterone concentrations (achromatic contrast: $F_{1,27} = 10.68$, $P < 0.003$; chromatic contrast: $F_{1,27} = 13.62$, $P = 0.001$; Fig. 6). There was also a strong relationship between the achromatic contrast of the gular stripe and progesterone concentrations ($F_{1,27} = 25.3$, $P < 0.0001$) and a near-significant trend with testosterone concentrations ($F_{1,327} = 3.97$, $P = 0.056$). Similarly, for the abdomen, there was a consistent relationship between colour intensity and progesterone concentrations (achromatic contrast: $F_{1,28} = 27.25$, $P < 0.0001$; chromatic contrast: $F_{1,28} = 28.32$, $P < 0.0001$) but not testosterone concentrations (achromatic contrast: $F_{1,28} = 1.98$, $P = 0.17$; chromatic contrast: $F_{1,28} = 2.41$, $P = 0.13$).

Discussion

During breeding female Lake Eyre dragons exhibited distinct patterns in plasma steroid profiles of both progesterone and testosterone. At the onset of the breeding cycle, plasma steroid levels rapidly increased and were directly associated with the intensification of colouration, cessation of appeasement behaviours and acceptance of male courtship. Steroid levels remained elevated throughout the remainder of the cycle, including the transition from receptive to unreceptive status, until oviposition then rapidly decreased to basal levels. During this period, colouration also remained at maximum intensity. Decreasing progesterone and testosterone levels around the time of oviposition coincided with diminished colouration and decreased male rejection behaviours. Hence our study reports a highly concordant correlation between sex steroid profiles, female colouration, mediation of specific behaviours and transition in reproductive state of female Lake Eyre dragons.

The close coincidence between initial increases in plasma steroid levels and colour development as well as peak plasma progesterone levels and peak colour expression in female Lake Eyre dragons is consistent with patterns found in other lizards. In other lizards, the development of bright female colouration also appears to be both directly regulated or correlated with elevated progesterone levels. Plasma progesterone concentration was shown to be elevated during vitellogenesis in striped plateau lizards, *Sceloporus virgatus*, with a surge related to ovulation (Weiss 2002). Brightly coloured female keeled

Table 3 Multiple regressions of mean values of each colour or behavioural trait at each stage with plasma progesterone and testosterone levels as predictor variables

Dependent Variable	Predictors retained in final model	Slope	R ²	P
Throat achromatic contrast	Progesterone	+	0.61	0.013
Throat chromatic contrast	Progesterone	+	0.73	0.004
Gular stripe achromatic contrast	None retained			
Abdomen achromatic contrast	Progesterone	+	0.43	0.056
Abdomen chromatic contrast	Progesterone	+	0.53	0.057
Arm wave frequency	Testosterone	-	0.76	0.002
Head bob frequency	Testosterone	-	0.86	0.003
Lateral display duration	Progesterone	+	0.68	0.006
Flip duration	Progesterone	+	0.60	0.01
Number of copulations per trial	None retained			

Only variables retained ($P < 0.1$) in the final model are shown

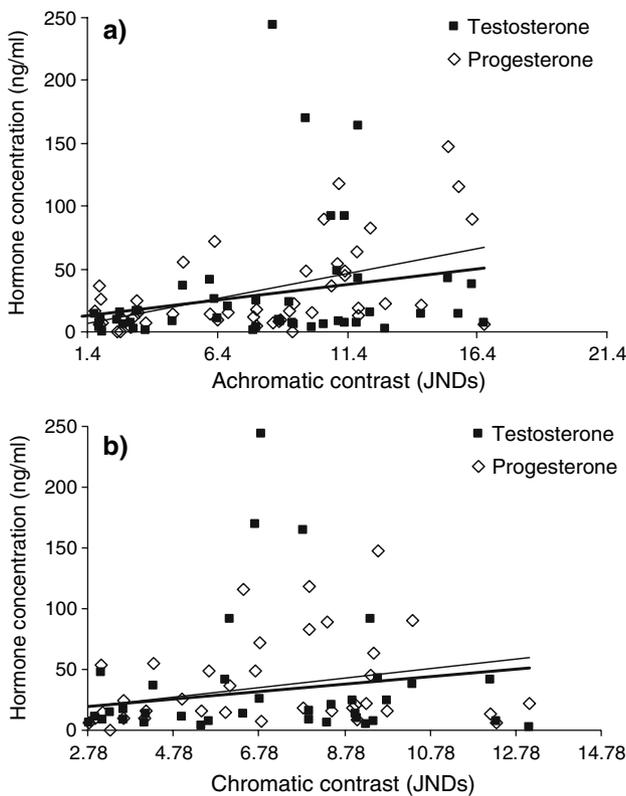


Fig. 6 Individual correlations between plasma concentrations of progesterone and testosterone and intensity of throat colouration: **a** achromatic (brightness) contrasts and **b** chromatic contrasts against adjacent white body regions in units of discrimination thresholds or just noticeable differences (JNDs). Thin *black line* is the trendline for progesterone and the *thick black line* is the trendline for testosterone

earless lizards, *Holbrookia propinqua*, also had higher levels of progesterone than non-coloured females, both in the field and the laboratory (Cooper and Crews 1988). Progesterone levels influencing colouration can be produced from both ovarian and non-ovarian sources (Cooper and Crews 1988). For example, the adrenal gland also produces progesterone during the reproductive cycle, as found in the

common lizard, *Lacerta vivipara* (Dauphinvillemant and Xavier 1985). Our results strongly suggest that circulating progesterone levels are related to colouration in Lake Eyre dragons, as orange coloured females possessed higher concentrations than white females and there was a highly significant correlation between progesterone levels and colour intensity at the individual level as well as based on mean levels among females across reproductive stages.

Across the female Lake Eyre dragon reproductive cycle, testosterone remained elevated and exhibited two peaks, one near the beginning of the reproductive cycle and the other near the end. The initial peak at the beginning of the reproductive cycle could facilitate multiple reproductive processes (e.g. oviductal hypertrophy) and associated colour development (Chan and Callard 1974). Female spiny lizards, *Sceloporus pyrocephalus*, that exhibited colouration possessed higher levels of plasma testosterone (Calisi and Hews 2007). Similarly, in both captive and wild female-keeled earless lizards, *Holbrookia propinqua*, colour expression is associated with elevated circulating testosterone (Cooper and Crews 1988). Additional evidence for the role of testosterone in female colour expression comes from experimental studies of exogenously implanted testosterone. Colouration was promoted by exogenously implanted testosterone in female eastern fence lizards, *Sceloporus undulatus*, where females normally exhibit little or greatly reduced colouration compared to males (Cox et al. 2005; Quinn and Hews 2003). Testosterone has also been implicated in female reproduction as female striped plateau lizards, *Sceloporus virgatus*, appeared to have a surge of testosterone during ovulation (Weiss 2002). Although exact occurrence of ovulation could not be determined, the initial peak of testosterone in female Lake Eyre dragons may also have been associated with ovulation as well as colour expression. The relationship between testosterone and colour expression in Lake Eyre dragons was supported by higher testosterone levels in females that were reproductively active and exhibiting orange colouration

than non-reproductive females and the significant correlation between testosterone levels and throat colour intensity at the individual level.

The second peak in testosterone (at stage 6) and the prolonged elevation in progesterone is distinctive and we suggest that both steroids could mediate and maintain behaviours used to reject males (i.e. lateral displays and flips) up until almost the end of the female's reproductive cycle. Several lines of evidence support this assertion. First, the physiological and colouration aspects of reproduction are effectively decreasing during this late stage and intuitively this should not require elevated testosterone and progesterone levels to facilitate down regulation. Second, other female lizards, including agamids, in which colouration and aggression are absent, exhibit decreases in these sex steroids shortly after ovulation (Amey and Whittier 2000; Whittier and Tokarz 1992). Third, in other female lizards, testosterone and progesterone are directly implicated in mediating female aggression, such as in the spiny lizard, *Sceloporus jarrovi* (Woodley and Moore 1999a) and female-keeled earless lizards *Holbrookia propinqua* (Cooper and Crews 1987). Similarly, female marine iguanas, *Amblyrhynchus cristatus*, also exhibit elevated testosterone levels following copulation coinciding with periods of post-mating aggression with males (Rubenstein and Wikelski 2005). The specific role for testosterone in female aggression is suspected to be indirect and via its aromatisation into oestrogen at various sites including the ovaries, spine and/or brain (Woodley and Moore 1999b). For instance, female lizards with experimentally increased or decreased oestrogen induced or reduced female aggression, respectively (Woodley and Moore 1999b). Further there is some evidence that progesterone may also influence female aggression in leopard geckos, *Eublepharis macularius* (Rhen et al. 1999), suggesting multiple hormones could contribute to mediating female aggression in lizards. In Lake Eyre dragons, elevated testosterone levels corresponded with cessation of appeasement behaviours and mean progesterone levels were significantly correlated with the duration of rejection behaviours. There was no general correlation, however, between testosterone levels and duration of rejection behaviours because during the period of receptivity (stages 1–3), testosterone levels were very high (the first peak) while rejection behaviours were absent or rare. This does not exclude the possibility that testosterone could mediate rejection behaviour, particularly given that testosterone levels remained elevated (and even exhibited a second peak) during periods when females exhibited maximal courtship rejection (stages 4–6).

More broadly in female vertebrates, the potential mediation of female mate rejection or aggressive behaviours by sex steroids, particularly testosterone, is now documented in multiple lizards, birds and fish (Desjardins et al. 2006;

Gill et al. 2007; Hegner and Wingfield 1986; Langmore et al. 2002; Woodley and Moore 1999a). However the relationships between sex steroids and female aggression are diverse and potentially reflect the multiple contexts in which selection for female aggressive behaviours (including mate rejection behaviours) can evolve. Consequently, a prolonged elevation in plasma progesterone and testosterone in Lake Eyre dragons may suggest a potential proximal response to mediate male rejection behaviours consistent with a prolonged period of ongoing male harassment post-receptivity. Superficially, Lake Eyre dragons exhibit seasonal plasma sex steroid profiles consistent with those of male vertebrates exhibiting promiscuous mating systems, high levels of aggression and limited parental care (Wingfield et al. 1990). In other female vertebrates where aggression is required but sporadic and typically coupled with periods of parental care, plasma testosterone is not chronically elevated, but may increase rapidly during specific aggressive contexts via behavioural androgen responses (Desjardins et al. 2006; Gill et al. 2007). These differences in the interactions between behaviour and testosterone (and presumably other sex steroids) in relation to the relative frequency of aggression supports the applicability of the challenge hypothesis to explaining the role of testosterone in female vertebrates (Desjardins et al. 2006; Gill et al. 2007). However, recent studies have also suggested that testosterone levels of females can reflect a correlated response to direct selection on testosterone levels in males, as opposed to direct selection on females themselves (Ketterson et al. 2005; Mank 2007). This is especially pertinent in the case of female lizards where testosterone can be consistently elevated across the breeding cycle. To demonstrate a non-correlated response and provide evidence that elevated testosterone in female Lake Eyre dragons is directly selected for mediating female mate rejection behaviours we would need to show an uncoupling between male and female seasonal androgen profiles and potentially also demonstrate that females exhibit some capacity for behavioural androgen responses. For the latter we could simply measure plasma testosterone in females before and after bouts of mate rejection behaviour.

Elevated levels of testosterone in both sexes are thought to be associated with potential fitness costs including decreased parental care, reduced fecundity and suppressed immunocompetence (e.g. Fite et al. 2005; Folstad and Karter 1992; Rutowska et al. 2005; Searcy 1988). While some of these costs are irrelevant for Lake Eyre dragons (e.g. parental care), any potential direct costs of testosterone may be negligible relative to the costs of forced male copulation, which include potential injury, death and reduced offspring fitness (Olsson 1995a). Consequently, female Lake Eyre dragons may maintain elevated plasma testosterone, despite associated costs, in order to increase

male rejection behaviours post-ovulation because these behaviours reduce the frequency of costly forced copulations (Chan et al. submitted).

Other steroids, including oestradiol, are presumably important in regulating or co-regulating physiology, colouration and behaviour in female Lake Eyre dragons. For example, the regulation of sexual receptivity in lizards appears to be a synergistic interaction between oestradiol and progesterone (McNicol and Crews 1979; Whittier and Tokarz 1992). In Lake Eyre dragons receptivity inferred by maximal copulation frequency peaked during stages 1–3 whilst progesterone levels were still increasing and both progesterone and testosterone levels remained high when females were no longer receptive. Consequently, for Lake Eyre dragons' female receptivity was poorly correlated with plasma steroid profiles suggesting that other hormones, such as oestradiol, could be mediating this behaviour in Lake Eyre dragons.

In summary, our study reports a generally concordant relationship between sex steroid profiles, female colouration, mediation of specific behaviours and transitions in reproductive state of female Lake Eyre dragons. However, further manipulative research, via exogenous steroid hormone administration (e.g. via silastic implants or oil patches), is necessary to directly assess the role of progesterone, testosterone and other steroids in mediating colour expression and female aggression. The patterns of testosterone and progesterone and associated changes in colouration and behaviour appear similar to those reported for North American Iguanid lizards with similar colour and behavioural traits, including female *Holbrookia propinqua* (Cooper and Crews 1987). This concordance suggests a potentially conserved regulatory role for these steroids in mediating these reproductive traits in female lizards. Further research is necessary to understand if the distinctive testosterone profile of Lake Eyre dragons is an example of direct selection on females to mediate behaviours preventing forced male copulations, or a consequence of correlated selection on the steroid for male reproductive traits.

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