

Article

Hormonal control of seasonal color change in female spiny-footed lizards: an observational and experimental approach

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Abstract

Breeding coloration of females often signals aspects of their reproductive status, suggesting a link between color and sex steroid hormones. In this study, we examined the relationships between 2 sex steroid hormones (progesterone and β -estradiol) and reproductive coloration in female spiny-footed lizards *Acanthodactylus erythrurus*. We first explored natural variation in female plasma hormone levels and coloration during their reproductive cycle. β -estradiol was negatively related to brightness and positively related to red saturation, whereas progesterone was not significantly related to coloration. After identifying key relationships, plasma hormone concentrations were manipulated by creating 3 experimental female groups (β -estradiol-treated, progesterone-treated, and control), and the effects on coloration were monitored. β -estradiol-treated females, in which there was a rise in both β -estradiol and progesterone levels, lost their red coloration earlier than females in the other 2 experimental groups, whereas progesterone treatment had no significant effect on female coloration. Our results suggest that high levels of either β -estradiol alone or β -estradiol together with progesterone trigger the loss of red coloration in female spiny-footed lizards, and that progesterone alone does not affect coloration. We hypothesize that changes in female breeding color might be regulated by β -estradiol in species in which conspicuous coloration is displayed before ovulation, and by progesterone in species in which this color is displayed during gravidity.

Key words: color signaling, enzyme immunoassay, hormone-induced color change, sex-specific coloration, spectrophotometry, transdermal hormone application

In species showing sexual dichromatism, the sex with lower parental investment (commonly the male) is usually more conspicuously colored, and sexual selection has been invoked to explain this phenomenon (Andersson 1994). Conspicuous female coloration, which is relatively common in some taxa (e.g., reptiles; Cooper and Greenberg 1992), has been given little attention, partly because it has traditionally been understood as the consequence of a genetic correlation with the male trait (e.g., Muma and Weatherhead 1989).

However, conspicuous female coloration may evolve independently of male color by direct selection on females (review in Amundsen 2000). Indeed, conspicuous coloration is present in females, but not in males, of some species with conventional sex roles, for example, mammals (Nunn 1999), fish (Amundsen and Forsgren 2001), birds (Heinsohn et al. 2005), or reptiles (Chan et al. 2009).

Conspicuous female coloration is frequently expressed seasonally and is therefore generally considered to signal some aspects of

female reproductive status (Nunn 1999; Amundsen and Forsgren 2001; Heinsohn et al. 2005; Chan et al. 2009). For example, depending on the species, conspicuous female coloration may advertise the most effective time for fertilization or the opposite, that is, non-receptivity (Cooper and Greenberg 1992). The relationship between female coloration and reproductive cycle has led to studies of the association between coloration and sex steroid hormones (hereafter sex hormones) in mammals (Dixon 1983), fish (Sköld et al. 2008), birds (Grindstaff et al. 2012) and reptiles (Jessop et al. 2009). However, studies of hormonal regulation of female color change are still scarce, in contrast to the relatively well studied effect of testosterone on coloration during the reproductive season in males (Cooper et al. 1987; Fargallo et al. 2007; Miles et al. 2007; Ardia et al. 2010). Studies which have analyzed the hormonal regulation of female breeding coloration in birds have mainly focused on testosterone (e.g., Eens et al. 2000), but the relationships between β -estradiol (E_2) or progesterone (P_4) and female breeding coloration have been studied in other groups and do not seem to be simple. In primates, for example, E_2 is closely related to female breeding coloration (Dixon 1983), whereas in reptiles, P_4 seems to be the hormone most commonly triggering female changes in coloration (Cooper and Greenberg 1992). In fish, however, no significant relationships between sex hormone levels and female coloration have been found (Sköld et al. 2008).

Lizards are good organisms for studying the endocrine basis of female-specific coloration because: 1) conspicuous female coloration is common in many taxa (Cooper and Greenberg 1992), 2) hormonal regulation of female reproductive cycle is relatively well known (Norris and Lopez 2011), and 3) several authors have suggested a link between female coloration, the different reproductive stages, and sex hormones (Cooper and Greenberg 1992; Calisi and Hews 2007; Jessop et al. 2009). However, experiments on the effect of sex hormones on female breeding coloration are scarce and strongly biased toward lizard families in the suborder Iguania (e.g., Agamidae, Crotaphytidae and Phrynosomatidae; Cooper and Greenberg 1992; Calisi and Hews 2007; Jessop et al. 2009), in which conspicuous coloration commonly appears during gravidity. Therefore, research on other species is necessary for a better understanding of the hormonal mechanisms affecting female coloration and to promote an integrative view of the possible role of sex hormones on the regulation of female breeding coloration.

Both E_2 and P_4 are considered the most important hormones regulating the reproductive cycle in female lizards (Norris and Lopez 2011). In general, plasma E_2 concentration rises during vitellogenesis (i.e., during follicular growth), is maximum in late vitellogenesis, just before ovulation, and falls shortly thereafter, whereas P_4 concentration increases during late vitellogenesis, reaches a peak in early gravidity (i.e., after ovulation), and maintains high levels until oviposition (Carnevali et al. 1991; Ciarcia et al. 1993; Díaz et al. 1994; Edwards and Jones 2001; Weiss et al. 2002; Kummrow et al. 2010; Al-Amri et al. 2012). The relationship between these sex hormones and female lizard coloration, however, appears to be complex. Whereas P_4 has been suggested to increase conspicuous coloration in females of several species (Cooper and Ferguson 1972a, 1972b; Medica et al. 1973; Cooper and Clarke 1982; Cooper and Crews 1987; Cooper and McGuire 1993; Jessop et al. 2009), the effect of E_2 is not clear. In most cases, a direct effect of E_2 on female coloration has not been found (e.g., Medica et al. 1973; Cooper and Clarke 1982; but see Calisi and Hews 2007), but E_2 can accelerate the effect of P_4 on coloration (Cooper and Ferguson 1973).

In this study, we examined the relationship of plasma P_4 and E_2 levels with color expression in female spiny-footed lizards (*Acanthodactylus erythrurus*), a species belonging to the Lacertidae family. At the beginning of the reproductive season, adult females of this species display red coloration, which gradually changes to yellowish white when they become gravid (Cuervo and Belliure 2013). We first studied temporal changes in coloration and plasma hormone concentrations in the field during the breeding season to test for possible relationships between color and hormone concentrations. Then, we experimentally manipulated plasma sex hormone concentrations in captive females to test their effects on coloration.

Materials and Methods

Study species

The spiny-footed lizard is medium-sized, with snout-vent length (SVL) and total length up to about 80 and 230 mm, respectively (Seva Román 1982; Carretero and Llorente 1993), although mean size varies among populations. Males tend to be larger than females (Castilla et al. 1992; Carretero and Llorente 1993), particularly in tail length (Barbadillo and Bauwens 1997). This species reaches sexual maturity during their second spring at approximately 57–65 mm SVL (Busack and Jaksić 1982; Bauwens and Díaz-Uriarte 1997). The phenology of the reproductive cycle varies among populations, but in central Spain, spiny-footed lizards hibernate during November–March, emerge from hibernation in April, and begin to mate in May, with females laying eggs during June–July and most eggs hatching in late August and September (Pollo and Pérez-Mellado 1990; Castilla et al. 1992). In southern Spain, reproductive events occur approximately 1 month earlier (Busack and Jaksić 1982; Seva Román 1982; Cuervo and Belliure 2013). There is no evidence of multiple clutches in populations in southern Spain (Busack and Klosterman 1987), but second clutches might be possible in central Spain (Castilla et al. 1992). Duration of gravidity (from ovulation to oviposition) is unknown, but might last up to 1 month according to the period when females with oviductal eggs are found in some populations (Busack and Klosterman 1987). Mean clutch size ranges from 3.4 to 4.4 eggs, depending on the population (Pollo and Pérez-Mellado 1990; Pérez-Quintero 1996).

Coloration in this species varies both ontogenetically and seasonally. Dorsal pattern changes from strongly-marked dark and light bands in hatchlings to a reticulated pattern in adults (Seva Román 1982). Juveniles of both sexes show red coloration on the rear part of their hind limbs and the ventrolateral part of their tails (Seva Román 1982; Carretero and Llorente 1993). Juvenile males lose their red color when they are ~1-year old, whereas juvenile females retain it through adulthood (Seva Román 1982). The red coloration of adult females increases in intensity at the beginning of the reproductive season until they are gravid, when it is gradually lost and becomes pale yellow, nearly white (Cuervo and Belliure 2013). In contrast, adult males show white coloration on the rear part of their hind limbs and the ventrolateral part of their tails during the whole reproductive season (Seva Román 1982). It remains unclear whether females regain red coloration after reproduction. The red coloration is due to drosopterin pigments (Cuervo et al. 2016), and males prefer adult females that show this color, thus suggesting that coloration provides relevant information on female reproductive status and that this information is used by males for mate selection (Belliure et al. 2018). Courtship behavior, however, has never been properly studied in this species.

Field study

A total of 18 adult females were captured during the breeding season of 2011 in central Spain (Chapinería, Region of Madrid; N40°22', W4°13'). Captures were done by noosing every 3–13 days from 5 May to 23 June, so plasma hormone levels and coloration were sampled across most of the reproductive season. Lizards were placed in individual cloth bags in the shade immediately following capture to prevent overheating and taken to the laboratory for blood sampling (see Blood sampling and hormone assays) and color measurements (see Color measurements). A single blood sample was taken from each female. Time between capture and blood sampling ranged 2.5–8.0 h (mean \pm SE = 5.0 \pm 0.3 h). Animals were also measured (SVL and total length with a ruler to the nearest 0.1 cm), weighed (with an electronic balance to the nearest 0.1 g) and toe-clipped with a unique code for future individual identification. Lizards were kept in captivity for up to 79 days for use in another study and then released in good condition (i.e., they behaved normally and were in apparent good health) at the same places where they had been captured.

Experimental study

Due to the low population density found in Madrid in 2011, the experimental study was performed in a higher-density population in southern Spain (Cabo de Gata Natural Park, Almería; N36°49', W2°18'). A total of 45 females and 45 males were captured in 2012 before the mating season (3–24 March). Lizards were taken to the laboratory and measured following the same procedures used in the 2011 field study. Pairs of animals (one female and one male) were placed in outdoor terraria (80 \times 60 cm and 43 cm high) at the Finca Experimental La Hoya, a facility of the Estación Experimental de Zonas Áridas (EEZA-CSIC) in Almería city, about 20 km from the area where lizards were captured. Weather conditions in both areas are very similar. Pairs were kept together to allow mating when females were receptive and thus egg fertilization. Shelter was provided using a brick and 2 wood panels over a thick layer of sand. Food (*Tenebrio molitor* or *Acheta domesticus*) dusted with vitamins was provided once a day and water was available *ad libitum*. All lizards were released in good condition at the same places where they had been captured after up to 64 days in captivity.

Females were randomly assigned to the following experimental groups: E₂ females were treated with E₂, P₄ females were treated with P₄, and control females were manipulated but received no hormone treatment. Each experimental group consisted of 15 females, and there were no significant morphological or colorimetric differences among groups before manipulation (one-way Analyses of Variance (ANOVAs); $F_{2,42} \leq 1.93$, $P \geq 0.16$ for body mass, SVL, total length, brightness, red saturation, and hue tests). Hormone manipulation began when all individuals had been in captivity for at least 5 days. Circulating levels of E₂ and P₄ were manipulated using the non-invasive transdermal hormone application technique (Meylan et al. 2003). Either E₂ (Sigma E8875) or P₄ (Sigma P0130) were diluted in commercial sesame oil (4.5 μ g of hormone per mL of oil) and 4.5 μ L of the respective dilution was applied daily on the dorsal side of E₂ and P₄ females for transdermal absorption. The same amount of sesame oil was applied daily to control females using the same procedure. The animals were manipulated at night, when they were inactive. Hormone dosages were determined according to previous testosterone experiments carried out in males of this species (Belliure et al. 2004), because E₂ and P₄ have never been experimentally manipulated in spiny-footed lizards. In any case, hormone doses were similar to or lower than those used in

other experiments with E₂ and P₄ in other lizard species (Cooper and Ferguson 1972b, 1973). Experimental treatments lasted until most females had started to lose their red coloration, that is, 22 days (from 29 March to 19 April).

Female coloration was measured weekly from 3 days before start of hormonal treatment to release (from 26 March to 7 May; 7 weeks of measurements in all). Two blood samples were taken from each female, the first sample 2 days before starting hormone manipulation (27 March), and the second 12 days later (8 April). The blood sampling technique was the same used in 2011 (see Blood sampling and hormone assays). Time between capture in the field and blood sampling ranged 9–23 days (mean \pm SE = 16.3 \pm 0.8 days). Females were also weighed 2 days before start of hormonal treatment and again the day after the manipulation was finished. One P₄ and 4 E₂ females died during the experiment, but female mortality did not differ significantly among experimental treatments (Fisher's exact test; $P = 0.11$). In addition, one pair (male and female) in the group treated with E₂ escaped during the last week of the experiment, so the effect of hormone treatment on coloration was tested in 15 control, 14 P₄ and 10 E₂ females.

Blood sampling and hormone assays

As evidence for capture stress affecting P₄ or E₂ in Squamata, the order including lizards and snakes, is weak at best (Whittier et al. 1987; Cree et al. 2000; Weiss et al. 2002; Lutterschmidt et al. 2009; Dayger et al. 2013; but see Grassman and Hess 1992) and the laboratory offered more aseptic conditions than the field for blood sampling, we decided to postpone blood sampling until lizards were taken to the laboratory. Pearson correlations between plasma hormone concentrations and time elapsed from capture to blood sampling were not statistically significant (P₄ in 2011: $r = -0.376$, $n = 18$, $P = 0.12$; E₂ in 2011: $r = -0.394$, $n = 18$, $P = 0.11$; P₄ in 2012: $r = 0.057$, $n = 45$, $P = 0.71$; E₂ in 2012: $r = -0.097$, $n = 45$, $P = 0.53$), suggesting that the effects of capture stress on P₄ and E₂ might be negligible in this study. Nevertheless, time from capture to blood sampling was taken into account in all analyses that included plasma hormone concentrations assessed during the 2011 field study and in 2012 before hormone manipulation (see Statistical analyses).

A maximum 120 μ L blood sample was taken from the *sinus orbitalis* of each animal with 20- μ L capillary tubes following Woodley and Moore (1999). This sampling technique has been used previously with no apparent effect on lizard health (Whiting et al. 2006; Jessop et al. 2009). Capillary tubes were immediately centrifuged to separate blood cells from plasma. The hematocrit was assessed as the proportion of capillary length occupied by packed red blood cells in relation to capillary length occupied by all blood components. Capillary tubes were measured with a digital caliper to the nearest 0.01 mm. Hematocrit values assessed in different capillary tubes from the same individual were averaged to obtain a single hematocrit value for each animal. Plasma was stored at -20°C until hormone analyses.

Plasma sex hormone concentrations (P₄ and E₂) in each blood sample were measured using commercial enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI, USA) and their associated protocols. Assays for P₄ and E₂ were not validated for this species, but both steroids are structurally well preserved across taxa, and the kits and protocols we used have been successfully used in numerous studies across reptiles (e.g., Jessop et al. 2009; Graham et al. 2011; Tripathy and Rai 2017). Linearity in plasma pools was tested for each hormone and found to be satisfactory. All samples were tested in duplicate in each assay. In the 2011 assays, only one

plate was run per hormone, and the within-assay coefficient of variation was 9.12% for P₄ and 15.76% for E₂. In 2012, 2 plates were run per hormone, and between- and within-assay coefficients of variation were 15.64% and 10.48% for P₄, and 12.65% and 23.62% for E₂, respectively.

Color measurements

Color measurements are described in detail elsewhere (Cuervo and Belliure 2013). Briefly, we quantified the spectral properties of female coloration by taking reflectance readings (with a USB 2000 spectrometer and a DT-MINI-2-GS tungsten halogen light source, Ocean Optics, Dunedin, USA) in the 320–700 nm range (Whiting et al. 2006). Four body regions, the rear part of both hind limbs and the ventral part of the tail at approximately 1 cm (hereafter proximal part of the tail) and 2.5 cm (hereafter distal part of the tail) from the cloaca were each measured 3 times. Coloration was always measured by the same person (B. Fresnillo) to avoid inter-observer variability. Reflectance was calculated at 1-nm intervals in the range studied using AVICOL software (Gomez 2006). From the reflectance data, 3 color parameters were calculated as follows: brightness, as the mean reflectance from 320 to 700 nm, red saturation (red chroma), as the sum of reflectance from 630 to 700 nm divided by the sum of reflectance from 320 to 700 nm, and hue, as the wavelength at which the maximum reflectance was recorded (Montgomerie 2006). Reflectance was not measured for regenerated parts of the tail or for hind limbs with wounds or scars, because all these characteristics seriously affect coloration. Thus, in some cases, body part analyses were done for different sample sizes.

Repeatability (r) of color measurements [calculated using the formula $r = S^2_A / (S^2 + S^2_A)$, where S^2_A is the among-groups variance component and S^2 is the within-group variance component (Lessells and Boag 1987)] in the 4 body parts during the 2011 field study was medium-high for brightness ($0.581 \leq r \leq 0.789$, $P < 0.001$), high for red saturation ($0.931 \leq r \leq 0.989$, $P < 0.001$), and medium for hue ($0.442 \leq r \leq 0.687$, $P \leq 0.001$). In the 2012 experimental study, repeatability was calculated using only the first week of measurements (i.e., 528 measurements on 45 females), although results were qualitatively identical using all 7 measurement weeks (i.e., 3522 measurements on 45 females; results not shown for brevity). Repeatability was medium-high for brightness ($0.381 \leq r \leq 0.728$, $P < 0.001$), high for red saturation ($0.900 \leq r \leq 0.940$, $P < 0.001$) and medium for hue ($0.307 \leq r \leq 0.529$, $P < 0.001$) in the 4 body parts. Variation in color parameters among individuals was significantly higher than measurement error in all cases in both studies. As in previous studies (Cuervo and Belliure 2013), the 3 measurements were averaged for each color parameter and body part, and then the mean of the 4 body parts was calculated to find a single brightness, red saturation and hue for each individual.

Statistical analyses

Data from the 2011 field study did not need any transformation to meet parametric assumptions (normality of residuals and homoscedasticity), but hormone concentrations, brightness and red saturation in captivity in the 2012 experiment were $\log_{10}(x + 1)$ -transformed, whereas hue was x^8 -transformed. In all cases, we chose the transformation that was more suitable for meeting parametric assumptions. After hormone treatment, hormone concentrations did not meet parametric assumptions even after transformation, so nonparametric tests were used with raw

data to check for differences in hormone concentrations among experimental groups after the treatment.

To control for possible effects of capture stress on hormone levels, natural plasma P₄ and E₂ concentrations in each year were regressed on the time elapsed between capture and blood sampling, and the residuals of these regressions (relative plasma P₄ or E₂ concentrations hereafter) were used in subsequent tests. Including both plasma hormone concentration and time elapsed between capture and blood sampling in subsequent tests instead of relative hormone concentration provided qualitatively identical results (not shown for brevity).

Possible relationships between female color parameters (brightness, red saturation and hue) and relative plasma P₄ and E₂ concentrations were tested using General Linear Models (GLM), with body mass and SVL as covariates because of a possible effect of body size or condition on timing of reproduction, and thus on coloration. In order to reduce the risk of overparameterization, we applied a backward stepwise procedure, with final models always including relative hormone concentration, and only including body mass or SVL if they were associated with $P < 0.10$. As both body mass and SVL were excluded from all final models ($P > 0.10$ in all cases), only relative plasma hormone concentration was included as predictor. In the 2012 experimental study, these relationships were analyzed before hormone manipulation, that is, before reproduction (late March). In a similar way, possible relationships between female characteristics (namely color parameters and relative plasma P₄ and E₂ concentrations) and date in 2011 were tested using GLM, with body mass and SVL as covariates, and applying a backward stepwise procedure with final models always including date. Again both body mass and SVL were excluded from all final models ($P > 0.10$ in all cases), and only date was included as predictor. These analyses were performed with STATISTICA 7.1 (StatSoft Inc. 2005).

The possible effects of the experimental manipulation of sex hormone levels (3 experimental groups: E₂, P₄ and control females) on female color parameters were tested using 2-way ANOVAs with repeated measures in one factor (measurement week). Temporal effects (7 color measurement weeks) were also tested simultaneously and the interaction between both factors was checked. When the interaction term was statistically significant, one-way ANOVAs were performed for each measurement week. Post-hoc Tukey tests were used when one-way ANOVAs showed significant differences among treatments. These analyses were performed using R software (R Development Core Team 2013) and the Linear and Nonlinear Mixed Effects Models (NLME) package (Pinheiro et al. 2013).

All statistical tests were 2-tailed and the significance level was 0.05.

Results

Natural hormone concentrations and coloration during the reproductive season

All 18 female spiny-footed lizards captured during the 2011 field study in Madrid were sexually mature according to their SVL (mean \pm SE = 72.7 \pm 0.8 mm; range: 69 - 81 mm). Mean (\pm SE) plasma P₄ and E₂ concentrations throughout the sampling period were 4.93 \pm 0.73 ng/mL (range: 0.70–12.85 ng/mL) and 0.47 \pm 0.06 ng/mL (range: 0.10–0.76 ng/mL), respectively.

We found a significant increase in relative P₄ concentration (GLM; beta \pm SE = 0.52 \pm 0.21, $F_{1,16} = 6.01$, $P = 0.026$) as P₄ levels rose until early or mid-June (Figure 1A). We also found a non-significant decrease in relative E₂ concentration (GLM; beta \pm

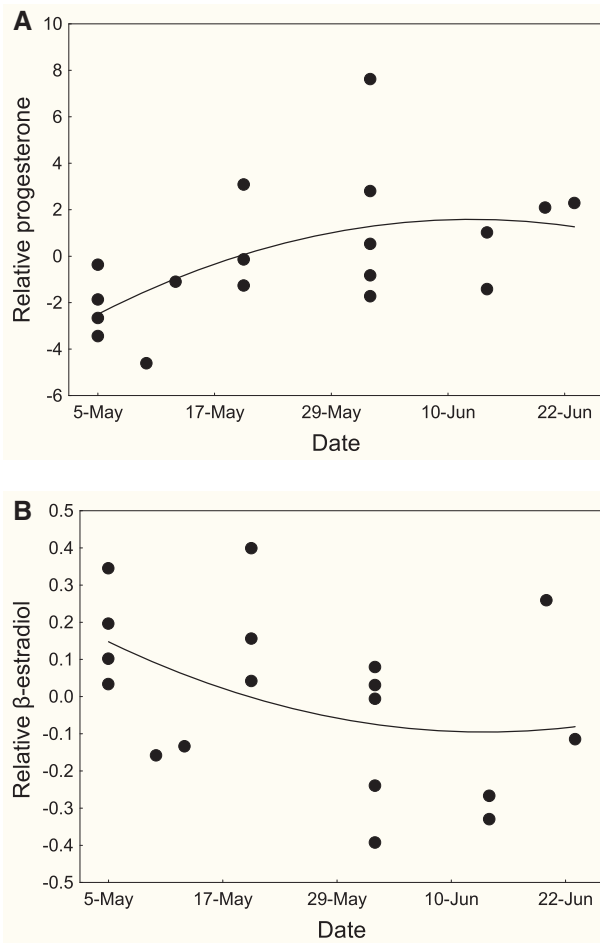


Figure 1. Relative plasma (A) progesterone and (B) β -estradiol concentrations in 18 adult female spiny-footed lizards in the Madrid population during the 2011 reproductive season. Relative hormone levels are residuals after regressing plasma hormone concentrations on the time elapsed between capture and blood sampling. Polynomial fit is represented as a continuous line for illustrative purposes. See [Supplementary Figure S1](#) for the same graphs showing absolute instead of relative hormone concentrations.

$SE = -0.40 \pm 0.23$, $F_{1,16} = 2.96$, $P = 0.10$) in the same period ([Figure 1B](#)). Although exact timing of the E_2 peak was unknown, it probably happened shortly before or around our first captures, that is, during late April or early May. Since maximum E_2 concentration occurs shortly before ovulation in lacertid lizards ([Carnevali et al. 1991](#); [Ciarciá et al. 1993](#); [Díaz et al. 1994](#)), and assuming that this is also the case for the spiny-footed lizard, ovulation might have occurred around early or mid-May. Maximum P_4 levels were found around early or mid-June ([Figure 1A](#)), presumably during gravidity. Although relative P_4 concentration increased and relative E_2 concentration tended to decrease along the sampling period, relative P_4 and E_2 concentrations were not significantly related (Pearson correlation; $r = -0.231$, $n = 18$, $P = 0.36$). For temporal variation of absolute instead of relative plasma hormone concentrations, see [Supplementary Figure S1](#).

We found a significant increase in brightness (GLM; $\beta \pm SE = 0.56 \pm 0.21$, $F_{1,16} = 7.40$, $P = 0.015$) and decrease in red saturation (GLM; $\beta \pm SE = -0.70 \pm 0.18$, $F_{1,16} = 15.26$, $P = 0.001$) during the sampling period, whereas hue did not change significantly (GLM; $\beta \pm SE = 0.12 \pm 0.25$, $F_{1,16} = 0.25$, $P = 0.62$) ([Figure 2](#)).

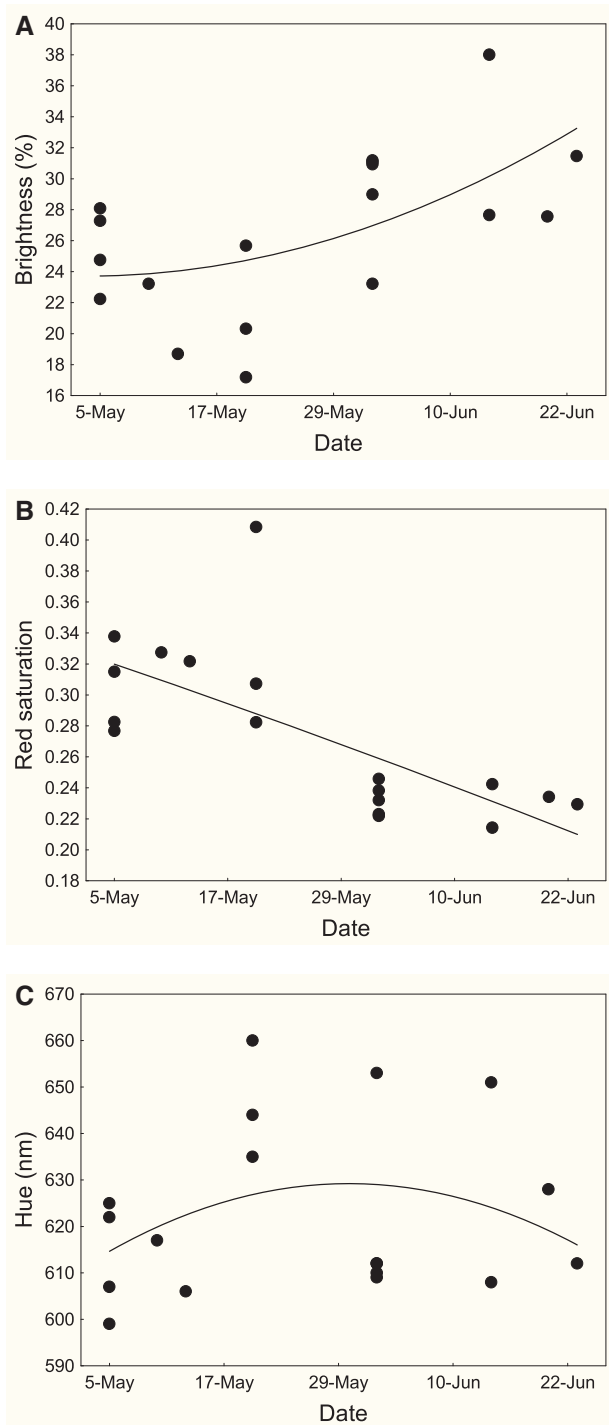


Figure 2. Mean (A) brightness, (B) red saturation, and (C) hue in 18 adult female spiny-footed lizards in the Madrid population during the 2011 reproductive season. Polynomial fit is represented as a continuous line for illustrative purposes.

Red saturation was high and brightness was low at the beginning of the period (early May), possibly coinciding with late vitellogenesis or ovulation, whereas hue tended to rise until late May and then decreased slightly ([Figure 2](#)). Females with high red saturation and low brightness were the reddest to the human eye. During June, coinciding with high P_4 levels, we found the lowest red saturation, the

Table 1. Results of GLM testing the relationship between color parameters and relative plasma levels of sex steroid hormones in female spiny-footed lizards

		β-estradiol			Progesterone		
		Beta ± SE	F	P	Beta ± SE	F	P
Madrid 2011	Brightness	-0.56 ± 0.21	7.24	0.016	0.12 ± 0.25	0.23	0.64
	Red saturation	0.52 ± 0.21	6.06	0.026	-0.45 ± 0.22	4.00	0.063
	Hue	0.02 ± 0.25	0.00	0.94	0.07 ± 0.25	0.07	0.79
Almería 2012	Brightness	-0.31 ± 0.15	4.43	0.041	0.07 ± 0.15	0.19	0.66
	Red saturation	0.25 ± 0.15	2.93	0.094	0.06 ± 0.15	0.15	0.70
	Hue	-0.06 ± 0.15	0.18	0.68	-0.12 ± 0.15	0.58	0.45

Relative hormone levels are residuals after regressing plasma hormone concentrations on the time elapsed between capture and blood sampling. Data from the Madrid population in 2011 were collected between 5 May and 23 June ($n = 18$ females). Data from the Almería population in 2012 were collected before the mating season (late March, $n = 45$ females).

Table 2. Two-way ANOVAs with repeated measures in one factor (measurement week) testing for experimental treatment effects and temporal effects on color parameters in female spiny-footed lizards

	df	F	P
Brightness			
Treatment effect	2, 36	1.78	0.18
Temporal effect	6, 216	15.80	<0.001
Interaction time × treatment	12, 216	2.45	0.005
Red saturation			
Treatment effect	2, 36	2.72	0.080
Temporal effect	6, 216	83.25	<0.001
Interaction time × treatment	12, 216	3.52	<0.001
Hue			
Treatment effect	2, 36	0.02	0.86
Temporal effect	6, 216	5.57	<0.001
Interaction time × treatment	12, 216	0.91	0.54

There were 3 experimental groups (females treated with progesterone, females treated with β-estradiol and females with no hormone treatment) and 7 color measurement weeks (measurements taken weekly from 26 March to 7 May 2012). Data were collected from 39 lizards from the Almería population.

highest brightness, and a small decrease in hue (Figure 2). Females with low red saturation and high brightness were perceived by humans as pale yellow.

Relationships between natural hormone levels and coloration

In the 2011 field study, relative plasma E₂ concentration was negatively related to brightness and positively related to red saturation, but was not significantly related to hue (Table 1; Supplementary Figure S2). Relative plasma P₄ concentration was not significantly related to any color parameter (Table 1; Supplementary Figure S2). For temporal parallel representation of color parameters and relative plasma hormone concentrations during the 2011 reproductive season, see Supplementary Figure S3.

In the 2012 experimental study in Almería, relative plasma E₂ concentration was negatively related to brightness before the reproductive season, that is, before experimental hormone manipulation (Table 1; Supplementary Figure S2). However, all other relationships between color parameters and relative plasma hormone concentrations before the experiment were not statistically significant (Table 1; Supplementary Figure S2).

Experimental manipulation of hormone levels

Plasma sex hormone concentrations did not differ significantly among experimental groups before starting manipulation (mean (± SE) plasma E₂ concentration; control females = 0.05 ± 0.01 ng/mL, E₂ females = 0.06 ± 0.02 ng/mL, P₄ females = 0.07 ± 0.03 ng/mL; mean (± SE) plasma P₄ concentration; control females = 1.48 ± 0.26 ng/mL, E₂ females = 2.45 ± 0.68 ng/mL, P₄ females = 1.52 ± 0.24 ng/mL; one-way ANOVAs; $F_{2,42} \leq 0.67$, $P \geq 0.52$ in both tests; Supplementary Figure S4). As expected, after 12 days of experimental treatment, the plasma E₂ concentration was higher in E₂ females than in the other 2 groups (mean (± SE) plasma E₂ concentration; control females = 0.16 ± 0.06 ng/mL, E₂ females = 26.37 ± 1.30 ng/mL, P₄ females = 0.12 ± 0.02 ng/mL; Kruskal-Wallis test; $\chi^2 = 30.62$, $df = 2$, $P < 0.001$, $n_E = n_P = n_C = 15$; post-hoc multiple comparisons: between E₂ females and the other 2 groups, $Z \geq 4.18$, $P < 0.001$ in both tests, between control and P₄ females, $Z = 1.01$, $P = 0.93$; Supplementary Figure S4). However, the P₄ concentration was higher in both P₄ and E₂ females than in control females (mean (± SE) plasma P₄ concentration; control females = 1.99 ± 0.28 ng/mL, E₂ females = 7.96 ± 0.58 ng/mL, P₄ females = 7.60 ± 0.77 ng/mL; Kruskal-Wallis test; $\chi^2 = 27.72$, $df = 2$, $P < 0.001$, $n_E = n_P = n_C = 15$; post-hoc multiple comparisons: between control females and the other 2 groups, $Z \geq 4.33$, $P < 0.001$ in both tests, between E₂ and P₄ females, $Z = 0.31$, $P = 1.00$; Supplementary Figure S4), even though P₄ was only applied to P₄ females. Therefore, both E₂ and P₄ levels increased after E₂ treatment. It should be noted that mean plasma P₄ concentration after P₄ treatment (7.60 ng/mL) was within the natural range found in the 2011 field study (0.70–12.85 ng/mL), but plasma E₂ concentration after E₂ treatment (26.37 ng/mL) was much higher than natural values (0.10–0.76 ng/mL).

The interaction between measurement week and experimental treatment was statistically significant for both brightness and red saturation (Table 2). Later analyses of each measurement week revealed significant differences in brightness among experimental groups in the 4th and 5th measurement weeks (one-way ANOVAs; $F_{2,36} \geq 4.65$, $P \leq 0.016$ in both tests), and Tukey post-hoc tests showed that E₂ females were brighter than P₄ ones in the 4th and 5th measurement weeks ($P \leq 0.016$ in the 2 tests), and brighter than control females in the 5th measurement week ($P < 0.001$; Figure 3A). We found significant differences in red saturation among experimental groups in the 4th, 5th, 6th and 7th measurement weeks ($F_{2,36} \geq 4.30$, $P \leq 0.021$ in all 4 tests; Figure 3B). Following Tukey post-hoc tests showed that red in E₂ females was less saturated than in females in the other 2 groups in the 4th and

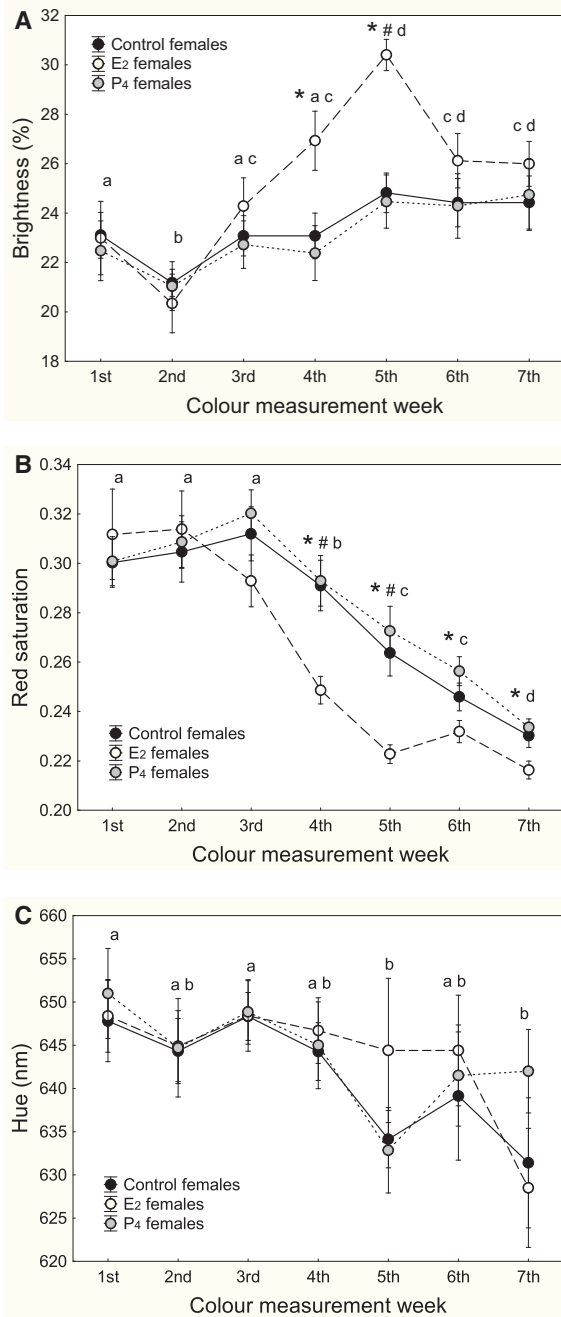


Figure 3. Mean (\pm SE) (A) brightness, (B) red saturation, and (C) hue in three experimental groups (E₂ females: treated with β -estradiol, $n = 10$; P₄ females: treated with progesterone, $n = 14$; control females: with no hormone treatment, $n = 15$) of female spiny-footed lizards from the Almería population in 7 colour measurement weeks (measurements taken weekly from 26 March to 7 May 2012). Different letters indicate significant differences in colour parameters between colour measurement weeks after Tukey post-hoc tests. Hashes indicate statistically significant differences between E₂ and control females and asterisks between E₂ and P₄ females for that measurement week after Tukey post-hoc tests.

5th measurement weeks ($P \leq 0.011$ in all 4 tests; Figure 3B) and less saturated than in P₄ females in the 5th and 7th measurement weeks ($P \leq 0.020$ in both tests; Figure 3B). The experimental treatment had no significant effect on hue in any measurement week (Table 2, Figure 3C).

Females in the 3 experimental groups lost their red coloration (higher brightness and lower red saturation) by the end of the experiment, but E₂ females lost the red color earlier than control and P₄ females (Figure 3A and 3B). Treatment with P₄ had no significant effect on female coloration as control and P₄ females did not differ in brightness, red saturation or hue in any measurement week (Figure 3). Loss of red coloration was not caused by captivity, because female lizards in the wild experience the same color change (this study; Cuervo and Belliure 2013).

We also checked whether hormonal treatments had any effect on female condition by comparing temporal changes in body mass and hematocrit among experimental groups. Both body mass and hematocrit were estimated on the day before start of hormonal treatment, and 12 (hematocrit) or 22 (body mass) days later (see Experiment in captivity in Materials and methods). We found that changes in body mass did not differ significantly among experimental groups (2-way ANOVA with repeated measures in one factor; interaction body mass \times treatment: $F_{2,38} = 0.44$, $P = 0.65$), whereas changes in hematocrit did (interaction hematocrit \times treatment: $F_{2,42} = 3.32$, $P = 0.046$). However, when changes in hematocrit were compared between pairs of treatments, the difference was statistically significant only between E₂ and P₄ females (interaction hematocrit \times treatment: $F_{1,28} = 6.23$, $P = 0.019$), but not between control and E₂ females or between control and P₄ females (interaction hematocrit \times treatment: $F_{1,28} \leq 2.55$, $P \geq 0.12$ in both tests). As changes in body mass or hematocrit did not differ significantly between control females and those treated with hormones (either E₂ or P₄), an effect of hormonal treatments on female condition was not supported.

Discussion

The significant relationships between E₂ concentration and brightness (negative) and red saturation (positive) in natural conditions (2011 breeding season) suggest that 1) females around late vitellogenesis or ovulation, characterized by high levels of E₂ (Norris and Lopez 2011), were red (i.e., high red saturation and low brightness), whereas 2) females during late gravidity, characterized by low levels of E₂ (Norris and Lopez 2011), were white (i.e., low red saturation and high brightness). These color changes agree with seasonal color changes previously described in this species (Seva Román 1982; Cuervo and Belliure 2013) and with the observation that females containing vitellogenic follicles exhibit red coloration (Castilla et al. 1992). To understand the negative relationship found between E₂ concentration and brightness before the 2012 breeding season, it should be noted that red coloration in this species increases shortly before the reproductive season (Cuervo and Belliure 2013). Consequently, females that had not begun any hormonal change for reproduction and had low E₂ levels were dark (i.e., red), but red was intensifying in females with higher E₂ levels that were already preparing for reproduction, that is, they were even darker. The increase in redness before the onset of reproduction was not observed in the 2011 breeding season, probably because it happened before our first captures.

Although natural E₂ levels were negatively related to brightness and positively related to red saturation, our experiment showed that E₂ treatment was responsible for an increase in brightness and a reduction in redness. To explain these apparently contradictory results obtained with the observational and experimental approach, it should be considered that in the natural hormone cycle of female lizards, ovulation takes place between an E₂ peak and a P₄ peak

(Norris and Lopez 2011). The loss of red coloration (lower red saturation, higher brightness) in female spiny-footed lizards starts around ovulation, when E_2 concentration is in natural decline (see Natural hormone concentrations and coloration during the reproductive season). Consequently, when the relationship between E_2 concentration and coloration was analyzed, females around late vitellogenesis or ovulation, with high E_2 levels, had darker and more saturated (i.e., redder) color, whereas females sometime after ovulation (i.e., gravid), with much lower plasma E_2 concentrations, had lighter and less saturated (i.e., less red) color. However, in the experiment, we increased plasma E_2 (and P_4) concentration in females treated with E_2 , which made them lose their red coloration earlier than females in the other experimental groups. Thus, increased E_2 levels (alone or in association with increased P_4 concentrations) trigger the loss of red coloration in females of this species. Once the mechanism for losing red is triggered, it continues even if E_2 levels decrease.

An alternative explanation for the results of our experiment is that E_2 treatment caused larger than natural E_2 levels which, in turn, resulted in deterioration of female health or condition. An association between exogenous E_2 treatment and disease or poor performance has been found in humans (e.g., Hoibraaten et al. 1999; Million Women Study Collaborators 2003) and other mammals (e.g., Holmes et al. 2002), and E_2 overdose has been suggested as the main explanatory factor (Panay 2009). According to this explanation, poorer health or condition (caused by supraphysiological E_2 levels) and not higher E_2 levels per se would have caused the earlier loss of red coloration in E_2 -treated females. A relationship between the expression of drosoprotein-based coloration and condition has been found in females of other lizard species (Weiss 2006). However, we found no evidence that E_2 -treated females experienced higher mortality (see Experimental study in Materials and methods) or poorer condition (see Experimental manipulation of hormone levels in Results) than females in the control group. Therefore, this alternative explanation is not supported by our data, although it cannot be totally ruled out because very few parameters were used to estimate condition.

It has not generally been suggested that E_2 significantly affects female breeding coloration in reptiles when this color is maintained during gravidity (Cooper and Greenberg 1992). However, the effects of E_2 on female breeding coloration have been found in different vertebrate species (e.g., primates: Czaja et al. 1977; Dixon 1983; lizards: Calisi and Hews 2007) when females develop conspicuous coloration during vitellogenesis and lose this color after ovulation, that is, the same pattern we found in female spiny-footed lizards. Therefore, we hypothesize that E_2 might affect coloration in species where conspicuous coloration is lost after ovulation, but not in species where this color is maintained during gravidity. When conspicuous female coloration is displayed before ovulation, this color may have a mating-related function, signaling for example sexual receptiveness or individual quality (Cooper and Greenberg 1992). In the spiny-footed lizard, red coloration in females intensifies during early vitellogenesis (Cuervo and Belliure 2013) and disappears gradually following ovulation (this study; Cuervo and Belliure 2013). Moreover, in this species, males prefer females showing red coloration, suggesting that the red color is a sexual signal involved in male mate selection (Belliure et al. 2018). Therefore, red coloration in females might indicate a pre-ovulatory reproductive status, that is, receptiveness. Additionally, adult females show red coloration as a retained juvenile trait, so this color might also indicate reduced risk of sperm competition (Belliure et al. 2018).

It has been previously suggested that P_4 induces conspicuous coloration in females of some lizard species (Cooper and Greenberg 1992), but our 2012 experiment suggests that P_4 does not significantly affect female coloration in spiny-footed lizards. This result agrees with those found in other animal groups (e.g., primates: Czaja et al. 1977). In the lizard species where P_4 induces conspicuous female coloration, this color begins to be expressed during vitellogenesis as P_4 levels increase, and is retained during gravidity until egg laying (Cooper and Greenberg 1992; Jessop et al. 2009). In these species, conspicuous coloration may signal that females are gravid, that is, no longer receptive, thus reducing male interest in mating. Females could then avoid male aggressive behavior associated with mating, and other potential costs of courtship such as increased predation risk or reduced foraging time (Cooper and Greenberg 1992). We hypothesize that P_4 might be responsible for color intensification in species where this color is maintained during gravidity, whereas P_4 would not affect coloration in species where the conspicuous coloration is lost after ovulation.

In our experimental study, E_2 treatment increased both E_2 and P_4 levels, and, as a consequence, we were unable to discriminate between the effects of increased levels of the 2 hormones together or E_2 alone. This increase in P_4 is difficult to interpret, but we can speculate that exogenous E_2 had a disruptive effect on key steroidogenic enzyme pathways, as has been shown in fish (Halm et al. 2002). For example, the excess of E_2 may have inhibited aromatase, an essential enzyme in the biosynthesis of E_2 (Simpson et al. 1994), and caused an accumulation of unprocessed P_4 , a precursor of testosterone and E_2 (Pieau et al. 1999). Alternatively, high P_4 levels may have simply resulted from endogenous production, although this should have led to similar P_4 levels in the control treatment, which was not the case. The differences in P_4 concentration among experimental groups suggest instead a possible interaction between endogenous sex hormones production and E_2 -induced changes in the steroidogenic pathways. Previous studies in which experimental hormone manipulation in lizards was assumed to affect only the treated hormone should be interpreted cautiously, because possible effects on other hormones have not generally been checked (Cooper and Ferguson 1972a, 1972b, 1973; Medica et al. 1973; Cooper and Crews 1987; Cooper and McGuire 1993).

In conclusion, the results of this study suggest a link between coloration and plasma E_2 concentration in female spiny-footed lizards. In a field study, female coloration was significantly associated with E_2 , but not P_4 concentration. In a hormone manipulation experiment, high P_4 levels did not have any significant effect on female coloration, but high levels of both E_2 and P_4 together caused loss of red coloration. The role of E_2 in regulating the expression of red coloration in female spiny-footed lizards agrees with other vertebrate species in which conspicuous coloration is also displayed when females are sexually receptive (before ovulation). However, it is unlike species in which conspicuous coloration is displayed when females are gravid (after ovulation), because in such cases, P_4 is the primary hormone regulating coloration. Thus, different hormones might regulate female breeding coloration depending on the reproductive phase when conspicuous color is expressed.

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Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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