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Possible reproductive benefits to female Carpetan rock lizards of pre-sensory bias towards chemical signals

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Female Carpetan rock lizards (*Iberolacerta cyreni*) might assess the quality of males from their chemical signals. Females select areas scent marked by males with secretions containing high proportions of provitamin D to ensure mating with high-quality males. However, an alternative explanation might be that females are not choosing a mate, but that females have a pre-sensory bias for chemical cues of vitamin D in the food and are, in fact, assessing habitat quality to obtain direct benefits of increasing the intake of vitamin D. We evaluated experimentally the possible benefits of a nutritional supplement of provitamin D or vitamin D in pregnant females for their clutches and offspring. However, we did not find large differences between treatments, except in the lower body condition of juveniles of mothers supplemented with provitamin D. We also tested the chemosensory interest of females in the scent of males to study the existence of a sensory bias; this response decreased when the amount of dietary vitamin D exceeded their needs. The results suggest that there are no reproductive benefits in the intake of additional vitamin D and that the potential sensory bias to scents of males could be related to the physiological needs of the females.

ADDITIONAL KEYWORDS: chemoreception – eggs – honest signals – mate choice – sensory bias – tongue flick – vitamin D_3.

INTRODUCTION

Many researchers have asked about the origin and evolution of sexual signalling in animal communication and about why the signals of males are attractive to females (Dawkins & Krebs, 1978; Johnstone, 1997; Endler & Basolo, 1998; Bradbury & Vehrencamp, 2011; Stevens, 2013). Against the classical adaptive approach (Dawkins & Guilford, 1996), mainly focused on honest signalling of male quality (Zahavi, 1975), there are other theories focusing on signal perception (Ryan 1990; Endler, 1992). These theories postulate that, originally, the receiver had a sensory bias towards the stimulus, even if it was not coding a signal. The bias for environmental perception in the receptor, without any sexual function, might have been used by the sender to implement the message (Endler & Basolo, 1998). Given that there is no agreement about whether the sensory bias is exploited by the sender or if the receiver falls into a sensory trap, the 'sensory bias hypothesis' has been proposed as a theoretical framework that integrates both approaches (Fuller *et al.*, 2005).

The sensory bias hypothesis was conceived to explain mating preferences without taking into account direct benefits to females (Dawkins & Guilford, 1996). However, if the sensory bias of the females was, for example, to dietary resources, and the sexual signals of the male reflected food availability, the sexual signalling could also have been originated in relationship to direct benefits. Females would achieve direct benefits by inhabiting a high-quality breeding territory (e.g. Giaquinto *et al.*, 2010; Ward *et al.*, 2011; Chouinard, 2012). Nevertheless, it is also plausible that the pre-existing bias could have been originated by another type of stimulus sensory by-product (Kirkpatrick, 1987; Kuijper *et al.*, 2012) and not necessarily as a result of the direct benefits from the resources available and signalled by males.

In a mate-choice context, chemical signals, which are used as territorial scent marks in many species, have an important role in assessment of the traits of potential mates (Johansson & Jones, 2007), such as quality (Moore, 1988), reproductive or health state

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(Kavaliers & Colwell, 1995), or genetic compatibility (Penn, 2002). Male lizards mark their territories with faeces and femoral gland secretions (López *et al.*, 1998; Martins *et al.*, 2006). Femoral secretions are composed of proteins and many lipophilic compounds, forming a waxy mixture. The inter-individual variability in the proportions of compounds is the source of chemical information used in mate choice (Alberts, 1993; Martín & López, 2014). For example, females of the Carpetan rock lizard, *Iberolacerta cyreni*, seem able to assess the quality of males from their femoral secretions (Martín & López, 2015) and prefer to use areas scent marked by high-quality males, such as those that are older (López *et al.*, 2003), more symmetrical (Martín & López, 2000; López *et al.*, 2002) or with a higher immune response (López *et al.*, 2006). The composition of scent marks is condition dependent, suggesting that male quality can be signalled by the proportions of specific compounds (Martín & López, 2015). Male *I. cyreni* lizards that have achieved their metabolic requirements for dietary vitamin D can increase the amount of cholesta-5,7-dien-3-ol (provitamin D_3) allocated to secretions (Martín & López, 2006a, 2015). Additionally, these males with high proportions of provitamin D have higher immune responses (Martín & López, 2010, 2012). Given that females are attracted to areas scent marked by these high-quality males, it is thought that females consider associating with these areas to enhance the probability of mating with them (Martín & López, 2006a, b, 2012).

Therefore, the attraction of females to the scent of males might have a purely sexual component, but the origin of the current attraction to these compounds might be attributable to a pre-existing sensory bias related to food stimuli (Martín & López, 2008). This was suggested because hungry females responded more strongly than satiated ones both to the chemical stimuli of provitamin D alone and to scent marks of males, which also contain this compound (Martín & López, 2008). Thus, males could be using this bias of females as a sensory trap to attract females to their home ranges and copulate with them. Conversely, females might use the scent marks of males as a source of public information (Valone, 1989) about the habitat quality, which has been reported in the timber rattlesnake *Crotalus horridus* (Clark, 2007).

Cholesta-5,7-dien-3-ol is the precursor of vitamin D_3 (Fraser, 1995; Holick, 1995). Vitamin D_3 is an essential compound in the metabolism of lizards and many other vertebrates, but its precursor, provitamin D_3, is inactive in metabolism (Allen *et al.*, 1999; Laing & Fraser, 1999). Given that vitamin D_3 is involved in calcium absorption in the intestine (Holick, 2007) and calcium accumulation in the yolk and eggshell (Stewart & Ecay, 2010), we could expect that availability of vitamin D_3 would be a relevant factor for reproduction in female lizards. Females could be choosing the area of a given male because of the quality of the resources, such as prey with a high content of vitamin D_3or its precursor, and not because of the quality of the male that lives there (Calsbeek & Sinervo, 2002; Martín & López, 2012). This alternative explanation to the simple sexual interest in male signals fits with the 'sensory bias hypothesis' framework.

Here, we made a first attempt to test the hypothesis that female lizards could use the scent marks of males not only as sexual signals but also as a source of environmental information related to food resources. We designed this study using two complementary approaches. First, we looked for direct benefits of increasing the food resource that might be signalled by high-quality males: provitamin D_3. To test this, we provided in the diet supplementary provitamin D_3 or vitamin D_3 to gravid female *I. cyreni*. Although the attraction of females is for provitamin D_3 and males do not secrete vitamin, we used both compounds because the isomerization from provitamin D_3 to vitamin D_3 when ultraviolet light from the sun hits the skin (Holick, 2003) would have additional costs to females in terms of energy. Second, we studied the 'chemosensory interest' of supplemented females in the sexual signals of males to check whether the sensorial perception is biased or not.

By addressing the metabolic functions of vitamin D and calcium in pregnant female lizards, and according to previous studies about the benefits of a supplementary intake of vitamin D_3 (e.g. Mattila *et al.*, 2004; Sahin *et al.*, 2009; Oonincx *et al.*, 2010; Wang *et al.*, 2017), we expected potential benefits in the following: (1) the condition and nutritional status of females; (2) the characteristics of their clutches; and (3) the condition and growth rate of their offspring. We hypothesized that having more vitamin D_3 available would be better for the health of females and their offspring. Therefore, we expected that females supplemented with vitamin D_3 or provitamin D_3 would have larger and heavier eggs and that their body condition after laying the eggs would be better. Previous studies have found a direct correlation between egg size and incubation time (Sinervo, 1990); therefore, we predicted a longer incubation time for clutches from supplemented females. We also predicted that eggshells of supplemented females would have a higher calcium content, being thicker and heavier than those of control females and, probably, that this extra availability of calcium might have an indirect effect, increasing hatching rate. Finally, juveniles born from supplemented mothers would have larger reserves of vitamin D inside their eggs and, for this reason, we expected these juveniles to be bigger and in better condition and to have faster growth rates than juveniles born from control females.

In addition, to test whether the sensory bias theory could explain female preferences, we measured the chemosensory response of supplemented females to femoral secretions of males by means of a tongue-flick (TF) bioassay (Cooper & Burghardt, 1990). We constructed our predictions based on previous studies showing that hungry females have stronger chemosensory responses than satiated ones (Martín & López, 2008). In this study, using supplementation, we tried to fulfil the vitamin requirements of females. We expected a relationship between the requirement for vitamin D_3 and the 'interest' of the females in the scents of the males, such that supplemented females would elicit lower chemosensory responses to the secretions of the males. However, if the interest of females in the secretions of males was not dependent on the availability of vitamin D_3, this would call into question a sensory bias operating at present.

MATERIAL AND METHODS

STUDY ANIMALS

During June 2016, we collected by noosing 37 live gravid female Iberian rock lizards (*I. cyreni*) in a 3 km^2 area around 'Puerto de Navacerrada' > 1800 m a.s.l. (Guadarrama Mountains, Central Spain). Lizards were transported to 'El Ventorrillo' field station, 5 km from the capture site. We housed lizards in outdoor individual plastic boxes (60 cm × 40 cm × 44 cm, length × width × height) until females laid the clutch. Given that the field station is very close to the capture site, the environmental conditions and the day–night cycle were similar to the habitat of the lizards. The terraria contained coconut fibre as a substrate, a brick for shelter and water ad libitum. We fed the lizards daily with crickets (*Achaeta domestica)* and mealworms (*Tenebrio mollitor*), dusted every 2 days with calcium powder without vitamin D_3 (Exoterra; KG Hagen Deutschland, Holm, Germany).

EXPERIMENTAL SUPPLEMENTATION OF THE DIET OF THE FEMALES

We assigned randomly each female to each of three experimental treatments: 'provitamin D' ($N = 13$), 'vitamin D' ($N = 13$) and 'control' ($N = 12$). Females received daily dietary supplementation until they laid the eggs (mean ± SE = 21 ± 1 days). A linear model, with the duration of treatment as the response variable and treatment as the explanatory factor, revealed no significant differences: ($F = 0.88$, d.f. = 2, 20, $P = 0.43$) Females in the provitamin treatment and vitamin treatment were, respectively, fed daily with 0.20 µg of provitamin D_3 (Sigma-Aldrich, St Louis, MO, USA) or 0.20 µg (= 8 IU) of vitamin D_3 (Myprotein, The Hut.com Ltd, UK), both dissolved in 0.25 mL of sunflower oil. We decided the dose of provitamin and vitamin D_3 based on previous studies conducted with the same species (Martín & López, 2006a). Females in the control treatment were fed 0.25 mL of sunflower oil alone, which does not contain vitamin D. To ensure that all lizards ingested the same amount of the supplement solution, we gently handled females and used sterile plastic syringes with a cannula to deliver the oil into their mouth.

CHARACTERISTICS OF THE CLUTCH AND THE JUVENILES

Every day, we inspected females to detect changes in their body shape that could indicate that they had laid eggs. Then, we gently looked for eggs buried in the coconut fibre substrate of the terraria. The eggs were removed from the terrarium, measured (see next paragraph) and individually placed into plastic containers (170 mL) filled with moistened perlite (1:1 proportion of water and perlite) and covered with a lid to maintain the humidity. These containers with eggs were maintained in an incubator (FRIOCELL FC-B2V-M/FC404) at 27.5 °C until hatching (Monasterio *et al.*, 2011). Females were released at their original sites of capture after laying the eggs.

Each egg ($N = 156$) was weighed using digital scales (to the nearest 0.001 g). Egg length and width were measured with digital callipers (to the nearest 0.01 mm). These measurements were used to calculate an ellipsoid as an estimation of egg volume [$V = (4/3)\pi \alpha b^2$, where α and b are half of the width and length of the egg, respectively; Mayhew, 1963].

To estimate whether the provitamin and vitamin treatments had any effect on the females and their reproductive parameters, we measured other potentially relevant variables of the females immediately after the clutch was found, such as body length (snout–vent length, measured with a ruler to the nearest 1 mm), body mass (measured with digital scales to the nearest 0.01 g) and body condition (the regression residuals of $\log_{10}$-transformed body mass and snout–vent length values). We also recorded clutch size, relative clutch mass (RCM; calculated as the ratio between the fresh clutch mass and the female mass after the clutch; Braña *et al.*, 1991), hatching success, incubation period of each egg (i.e. number of days between laying the clutch and hatching), and hatching duration within each litter (i.e. number of days between hatching of the first and the last egg) as an indicator of synchrony (Marco & Diaz-Paniagua, 2008). Once the juveniles hatched ($N = 113$), we measured the dry mass of the eggshell using digital scales (to the nearest 0.001 g) and the thickness of the eggshell with a spessimeter (to the nearest 0.01 mm).

At birth, we measured snout–vent length and tail length of the newborn juveniles with digital callipers (to the nearest 0.01 mm), and body mass with digital scales (to the nearest 0.001 g), and calculated body condition. We also estimated growth rate until 2 weeks after the birth by using the following formula: ln[(final mass/birth mass)/(age in days)] (Iraeta *et al.*, 2006). We maintained newborn lizards in captivity for 15 days to ensure that they were in good health before releasing them at the sites of capture of their respective mothers.

FEMALE RESPONSES TO CHEMICAL CUES OF MALES

To assess whether females supplemented with provitamin or vitamin D changed their chemosensory responses to femoral secretions of males, we used the TF rate, which is a common bioassay of chemical detection in reptiles (Cooper & Burghardt, 1990; Cooper, 1994). We compared the number of TFs elicited by female lizards in response to stimuli arising from cotton applicators bearing: (1) dichloromethane (DCM; Sigma-Aldrich Chromasol V plus for HPLC, purity > 99.9%) as a control; or (2) a mixture of femoral gland secretions of 20 *I. cyreni* males that were being used in another experiment. We collected the male secretions in a 2 mL glass vial that was closed with a Teflon-lined stopper and kept in the freezer (−20 °C) until the tests. One hour before starting the assay, the vial with the secretion was thawed at room temperature and refilled with DCM to dissolve the secretions (Khannoon *et al.*, 2010). We prepared stimuli by dipping the cotton swab attached to a long stick (50 cm) inside the corresponding glass vial. Control vials contained only DCM, whereas secretion vials contained the same volume of DCM with the dissolved femoral secretions. A new swab was used in each trial. To begin a trial, one of the experimenters slowly approached a cage containing a lizard and carefully moved the cotton swab to a position 2 cm anterior to the lizard's snout. Lizards usually allowed the approach of the swab and the test without fleeing. We recorded the latency to the first TF and, thereafter, the number of TFs directed to the swab for 60 s. Trials were conducted in outdoor conditions on 20 July, close to the end of the experimental supplementation period of females, between 11.00 and 13.00 h (GMT), when lizards were fully active. Every female was exposed to both stimuli (secretion and DCM), and the order of presentation was

Table 1. Mean (± SE) values of the variables and reproductive traits measured in female lizards, their eggs and their offspring in the different supplementation treatments

Variable	Control		Provitamin		Vitamin	
	N	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE
Maternal body mass after laying (g)	8	5.08 ± 0.38	6	6.09 ± 0.57	9	5.31 ± 0.55
Maternal body condition after laying	8	−0.02 ± 0.05	6	0.08 ± 0.10	9	−0.04 ± 0.06
Clutch size	8	5.88 ± 0.55	6	5.00 ± 0.73	9	5.11 ± 0.35
Relative clutch mass	8	33.52 ± 0.69	6	25.27 ± 2.88	9	30.62 ± 2.07
Hatching duration (days)	8	4.38 ± 0.60	6	2.83 ± 0.60	9	2.78 ± 0.43
Clutch hatching success (%)	8	90.00 ± 7.56	6	77.08 ± 10.30	9	68.57 ± 7.44
Eggs						
Weight (g)	61	0.45 ± 0.01	43	0.42 ± 0.01	52	0.46 ± 0.01
Length (mm)	61	12.70 ± 0.13	43	12.98 ± 0.13	52	13.28 ± 0.14
Width (mm)	61	8.31 ± 0.07	43	7.90 ± 0.06	52	8.16 ± 0.08
Volume (mm^3)	61	221.88 ± 4.00	43	214.89 ± 2.87	52	227.65 ± 4.07
Shell dry mass (g)	47	0.01 ± 0.00	29	0.01 ± 0.00	35	0.01 ± 0.00
Shell thickness (mm)	47	0.03 ± 0.00	29	0.02 ± 0.00	35	0.02 ± 0.00
Incubation time (days)	48	36.3 ± 0.30	27	36.8 ± 0.20	36	36.1 ± 0.20
Offspring						
Snout–vent length (mm)	48	26.63 ± 0.12	27	26.18 ± 0.18	36	26.87 ± 0.20
Tail length (mm)	48	40.89 ± 0.41	27	40.46 ± 0.52	36	41.01 ± 0.48
Body mass (g)	48	0.50 ± 0.01	27	0.50 ± 0.01	36	0.51 ± 0.01
Body condition	48	0.04 ± 0.12	27	−0.34 ± 0.14	36	0.20 ± 0.16
Growth rate (g)	47	0.00 ± 0.00	25	0.00 ± 0.00	36	−0.01 ± 0.00

alternated between them. We allowed 1.5 h between the first presentation of one stimulus and the second presentation of the other stimulus to allow females to rest and to avoid habituation.

DATA ANALYSES

To analyse morphological variables of eggs and juveniles, we used mixed models, with the experimental treatment as a fixed factor and the identity of the mother as a random factor, using the *lme4* package (Bates *et al.*, 2015) in R (R Development Core Team, 2017). We $\log_{10}$-transformed the response variables to ensure normal distributions, except for count, for which we fixed Poisson distributions. We chose the best models following the protocol of Zuur *et al.* (2009) from a beyond optimal model, which included as covariates the treatment duration, incubation time, mother mass and clutch size, and their interactions. We selected the models according to the corrected Akaike information criterion (AICc) and keeping the treatment factor as explanatory. To evaluate the significance of the chosen models, we compared them with the null models using the likelihood ratio test (lmtest R package v.0.9-36; Zeileis & Hothorn, 2002) and, only if the model was significant, the parameters for each descriptor variable were computed. Fixed effects significance was tested using ANOVA and Tukey's tests for pairwise comparisons. In those cases in which canonical assumptions could have been violated, we performed a parametric bootstrap and computed the confidence interval to ensure significance.

Variables related to mothers and reproductive biology were analysed with linear models or generalized models. The model with the lowest AIC was chosen, and its explanatory power was evaluated with R^2 and its significance with an F-test. In the event of statistical significance in the treatment, Tukey's tests were used for pairwise comparisons.

For the chemosensory tests, the experimental design was not orthogonal but of repeated measures. Thus, differences in the number of TFs elicited between treatments were tested using a repeated measures mixed model (Wang & Goonewardene, 2004), with treatment and stimuli as fixed factors and female identity as a random factor. To ensure normality, data were $\log_{10}$-transformed.

RESULTS

REPRODUCTIVE BIOLOGY AND MORPHOMETRIC MEASURES

Relative clutch mass differed significantly among experimental groups (Tables 1 and 2), with a relatively

Table 2. Results of statistical analyses comparing the response variables of reproductive biology and female state among the supplementation treatments

Response	Model *F*-test			Predictors	*F*-test			Correlation
	d.f.	*F*	*P*-value		d.f.	*F*	*P*-value	
Body mass after laying	**4, 18**	**8.39**	**0.0005**	**Clutch size**	**1**	**23.43**	**0.0001**	**0.40**
				Relative clutch mass	**1**	**14.73**	**0.001**	**–0.45**
				Treatment	2	0.24	0.79	
Body condition after laying	2, 20	0.27	0.77					
Clutch size	2, 22	0.32	0.73					
Relative clutch mass	**4, 20**	**4.04**	**0.034**	**Treatment**	**2,20**	**4.04**	**0.034**	
Hatching duration	2, 3	3.73	0.16					
Clutch hatching success	2, 20	1.89	0.18					

We chose the best models from a beyond optimal model and based on the Akaike information criterion (AIC) and, when possible, we selected the model that included the treatment factor and remained significant. We used the *F*-test for Linear Models and the *Wald* test for Generalized Linear Models (just clutch size and hatching duration variables). Significant tests and variables are shown in bold. The models with a non-significant 'omnibus test' (*F*-test) were final models that included as an explanatory factor only the experimental treatment.

lower weight for clutches from females in the provitamin group (Tukey's tests: control vs. provitamin, $P = 0.027$; control vs. vitamin, $P = 0.52$; provitamin vs. vitamin, $P = 0.17$; Fig. 1A). We did not find any significant differences among treatments in hatching duration and other mother-related variables, such as clutch size, body condition after clutch and clutch hatching success, nor in female body mass after laying the clutch, which appeared instead to be explained by the covariates clutch size ($r = 0.40$, $t = -2.00$, d.f. = 21, $P = 0.06$) and RCM ($r = -0.45$, $t = -2.30$, d.f. = 21, $P = 0.03$) (Tables 1 and 2). The graphical exploration of the relationship between body mass after laying and RCM showed some opposing trends in supplemented and control females (Fig. 1B), but an ANCOVA parallelism test did not reach significance ($F = 3.00$, d.f. = 2, 17, $P = 0.077$). Hatching success tended (Fig. 1C), although marginally insignificant, to be higher in the control group ($\chi^2 = 5.33$, d.f. = 2, $P = 0.07$; Tables 1 and 2). We also tested the relationship between RCM and average body condition per clutch as covariance (−0.15) with a Pearson correlation ($r = -0.40$, $t = 0.18$, d.f. = 21, $P = 0.90$) and with a linear model including the experimental treatment ($F = 1.02$, d.f. = 5, 17, $P = 0.44$), but both were non-significant. In the same way, we tested the correlation between RCM and hatching success ($r = 0.20$, $t = 0.90$, d.f. = 21, $P = 0.40$) and also the linear model including the treatment ($F = 0.74$, d.f. = 5,17, $P = 0.60$), with non-significant results. We can conclude that there were no relationships between RCM and average body condition or hatching success. For details see the Supporting Information (Table S1).

We did not find any significant effect of treatment on the morphology of eggs and juveniles (Table 2), but the

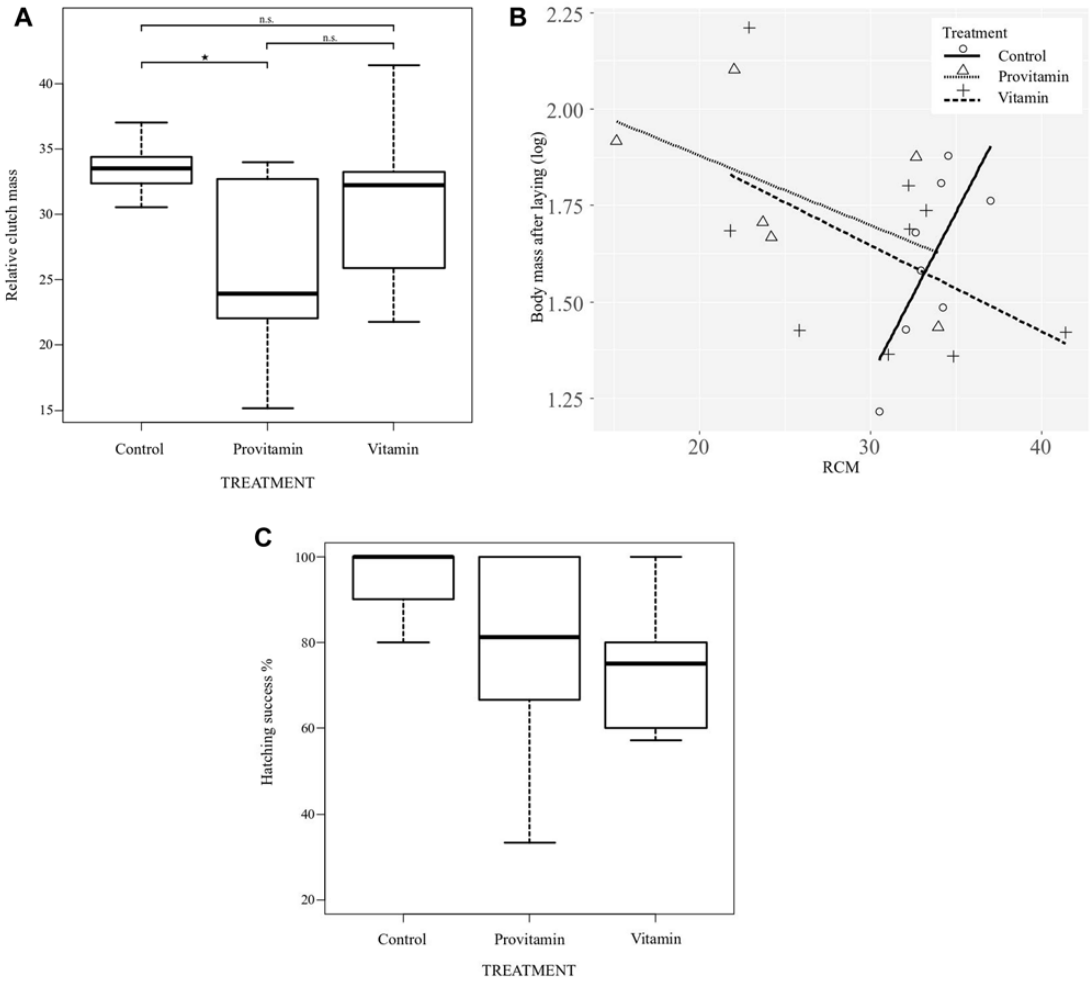

Figure 1. A, box-plots of relative clutch mass (in grams). B, body mass of females after laying the eggs (log) against relative clutch mass (RCM) for each of the supplementation treatments. Smooth lines were adjusted using linear models for each level factor: control ($F = 6.72$, d.f. = 1, 6, $P = 0.041$), provitamin ($F = 1.49$, d.f. = 1, 4, $P = 0.29$) and vitamin ($F = 2.41$, d.f. =1, 7, $P = 0.16$). C, box-plots of hatching success (as a percentage) for each of the supplementation treatments.

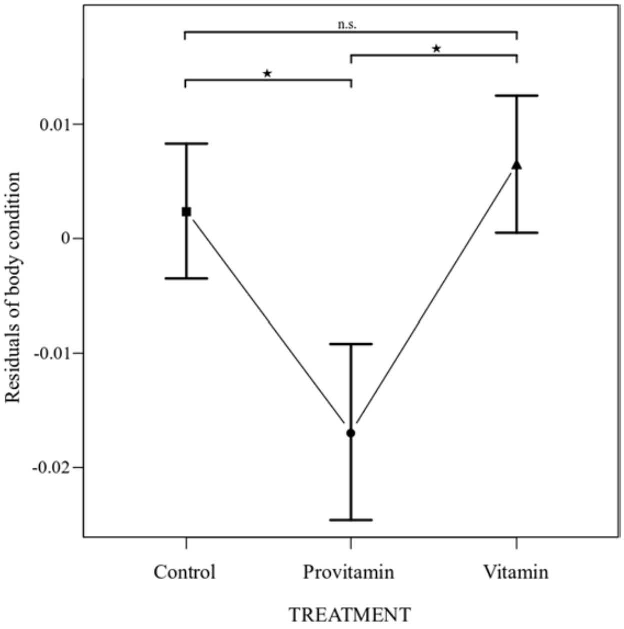

Figure 2. Mean (± SD) of residuals of body condition of juveniles for each of the supplementation treatments fitted by the mixed model.

body condition of juveniles at birth was significantly affected by the treatment, with lower condition in the juveniles born from females in the provitamin group (Tukey's tests: control vs. provitamin, $P = 0.056$; control vs. vitamin, $P = 0.97$; provitamin vs. vitamin, $P = 0.033$; Fig. 2). We included in the models other potentially relevant covariates, and some of them explained a high proportion of the variance. Covariates such as clutch size, incubation time and maternal traits, such as snout–vent length or body mass, appeared to be important, because their contributions to some models were significant (Tables 2 and 3). For details see the Supporting Information (Table S2).

Responses to chemical cues

We found significant differences in the latency to the first TF (*F*-test to repeated measures linear mixed model: $F = 3.80$, d.f. = 2, 25, $P = 0.036$; Fig. 3). There was no difference between females supplemented with provitamin or vitamin, and both started tongue-flicking at longer times after presentation of the stimulus than control females (control vs. provitamin, $P = 0.04$; control vs. vitamin, $P = 0.07$; provitamin vs. vitamin, $P = 0.93$) independently of the type of stimulus (*F*-test to repeated measures linear mixed model including interaction of stimulus × treatment: $F = 0.97$, d.f. = 5, 36, $P = 0.16$).

We also found significance in the model of TFs directed to the stimuli (*F*-test to repeated measures linear mixed model including interaction of stimulus × treatment: $F = 0.97$, d.f. = 5, 36.2, $P = 0.014$). Significance was found in the treatment factor, but not in its interaction with the stimuli (treatment, $F = 6.46$, d.f. = 2, 25, $P = 0.005$; stimuli, $F = 0.01$, d.f. = 1, 25, $P = 0.91$; treatment × stimuli, $F = 2.06$, d.f. = 2, 25, $P = 0.149$). Pairwise tests revealed that the total number of TFs elicited by the control group in response to both stimuli was higher than in the provitamin and vitamin groups, which did not differ between them (control vs. provitamin, $P = 0.01$; control vs. vitamin, $P = 0.06$; provitamin vs. vitamin, $P = 0.99$; Fig. 4). Models including the duration of treatment as a covariate were not significant for latency and direct TFs; for details see the Supporting Information (Table S3).

DISCUSSION

Overall, we could not find direct benefits of the vitamin D supplement treatment for pregnant females. The lack of differences among treatments in body condition of mothers after laying the eggs suggests that vitamin D has no positive effect on energy storage by the mothers. However, we detected a slightly positive relationship between maternal body mass and clutch size, and a slightly negative relationship between maternal body mass and RCM. Despite the non-significant results, Figure 1B suggests that these relationships could be different among treatments. The trends appear to be reverses in supplemented females and control females and, according to the lower RCM in the provitamin group, we could consider an effect of vitamin supplementation on the transfer of nutrients to the eggs. However, our results are not conclusive for egg morphology, incubation time and eggshell characteristics. We do not know the cause of the lower RCM or whether it implies some kind of disadvantage, nor the relationships with other physiological aspects. It is plausible that supplementation might play a role in maternal energy storage and in the transfer of nutrients to the eggs. However, this explanation of the link between maternal investment and RCM would be too speculative, because the general relationship between RCM and reproductive effort remains unclear (e.g. Vitt & Congdon, 1978; Vitt & Price, 1982; Shine, 1992; Du *et al.*, 2005).

Offspring from the supplemented groups were expected to be bigger, with better body condition and faster growth rates than the control offspring, but there were no differences between these treatments. However, offspring from mothers in the provitamin treatment had lower body condition than juveniles born from control and vitamin-supplemented females. Although we cannot demonstrate any type

Table 3. Results of statistical analyses comparing the response variables of eggs and juvenile traits among the supplementation treatments of mothers

Model LRT test				Predictors *F*-test				
Response	d.f.	χ^2	*P*-value	Predictors	d.f.	*F*	*P*-value	Correlations
Egg mass	5, 2	0.48	0.79					
Egg length	**12, 7**	**25.12**	**0.0007**	Mother mass	1, 18.4	0.65	0.43	–0.30
				Treatment days	1, 22.8	1.18	0.29	0.26
				Clutch size	**1, 15.6**	**2.88**	**0.11**	**0.38**
				Mother mass × clutch size	**1, 22.5**	**5.31**	**0.031**	**–0.78**
				Treatment days × clutch size	**1, 7.61**	**6.33**	**0.037**	**0.25**
				Treatment	2, 22.45	0.18	0.84	
Egg width	**7, 2**	**8.82**	**0.012**	Treatment	2, 7.07	3.00	0.11	
Egg volume	8, 5	6.83	0.23					
Eggshell thickness	7, 4	5.22	0.027					
Eggshell dry mass	**7, 4**	**0.90**	**0.042**	Treatment days	1, 18	2.98	0.101	0.005
				Egg mass	**1, 25**	**7.58**	**0.011**	**–0.1**
				Treatment	2, 18.8	0.06	0.94	
Incubation time	**7, 3**	**20,21**	**0.005**	Treatment days	1, 18.7	0.004	0.85	–0.003
				Mother snout–vent length	1, 22.1	0.012	0.73	0.08
				Egg mass	1, 62.3	0.30	0.59	0.06
				Treatment days × egg mass	**1, 22.2**	**5.90**	**0.02**	**0.08**
				Mother snout–vent length × egg mass	**1, 48.3**	**9.05**	**0.004**	**0.14**
				Treatment	2, 19.9	2.90	0.08	
Snout–vent length	**7, 4**	**15.74**	**0.0034**	**Mother mass**	**1, 21.5**	**4.71**	**0.041**	**0.07**
				Incubation time	**1, 102**	**6.96**	**0.009**	**–0.03**
				Treatment	2, 23.1	2.30	0.12	
Tail length	5, 2	0.74	0.69					
Body mass	5, 2	0.30	0.86					
Body condition	**8, 3**	**15.91**	**0.001**	**Incubation time**	**1, 131**	**12.83**	**0.003**	**–0.04**
				Treatment	**2, 15.4**	**5.28**	**0.018**	
Growth rate	5, 2	0.48	0.79					

All linear mixed models included mother as a random factor. The best models were chosen from a beyond optimal model and based on the Akaike information criterion (AIC) and, when was possible, we selected the model that included the treatment factor and remained significant. Final models, with a non-significant 'omnibus test' [Likelihood-ratio test (LRT)], included as an explanatory factor only the experimental treatment. Significant tests and variables are marked in bold.

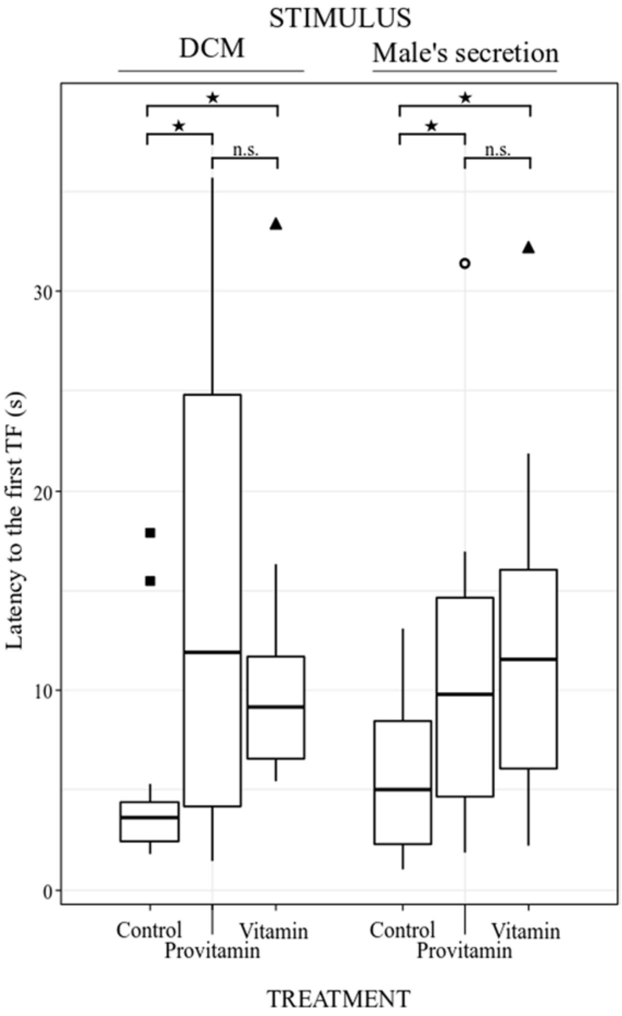

Figure 3. Box-plots of latency (in seconds) to first tongue-flick (TF) for each of the supplementation treatments in response to dichloromethane (DCM) as the control and male femoral secretion as the stimulus.

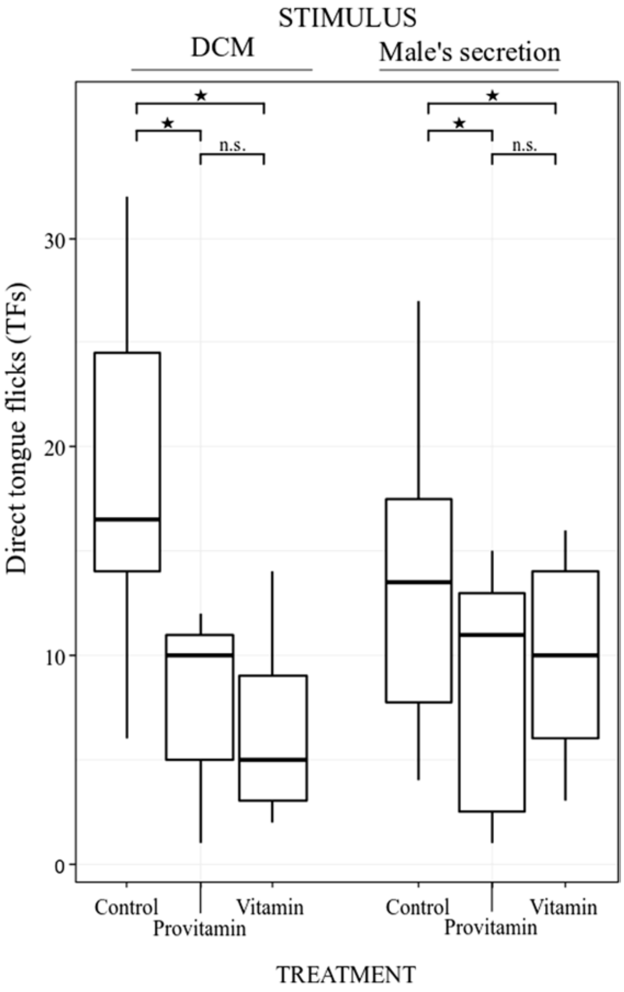

Figure 4. Box-plots of tongue-flicks (frequency) for each of the supplementation treatments in response to dichloromethane (DCM) as the control and male femoral secretion as the stimulus.

of relationship between the lower RCM and the lower body condition of juveniles, this result seems to suggest that too much provitamin D_3 could be disadvantageous. Despite their lower body condition, juveniles from the provitamin D group had similar growth rates to the control and vitamin groups. Therefore, growth does not seem to be affected by body condition or energy reserves, although it is possible that the effects of the supplement on growth could arise later during development.

It is plausible that vitamin D is not a very important resource that requires supplementary access in pregnant females in this species. On the contrary, it appears that it could even cause some damage if it is consumed in excess. Hypervitaminosis D has been reported in reptiles and other taxa, such as mammals (Roberson *et al.*, 2000; Holz, 2018) and it might be the reason for the lower weights found in our experiment. An unnecessary supplementary contribution could explain why the predicted effects did not appear in our experiment. However, according to the benefits of vitamin D supplementation in other taxa (Mattila *et al.*, 2004; Oonincx *et al.*, 2010; Wang *et al.*, 2017), we cannot discount the possibility of an unobserved beneficial effect for the mother or the offspring. There are many potential unobserved benefits, such as maternal effects, dispersal behaviour, increased growth rates, etc. It is also possible that provitamin D_3 might be signalling habitat quality to the offspring after hatching. New long-term studies should be carried out to explore the consequences of having a diet 'too rich' in provitamin D_3 and the benefit or harmful effects on energy storage in mothers and eggs, maternal investment or development of the embryos and juveniles.

The absence of increased chemosensory responses to the secretions of the males by the females suggests that their chemosensory interest in male secretions is related to the vitamin D_3 content inside the body. Based on previous studies where, at the beginning of the mating season, hungry females responded with higher intensity to scents of males than satiated females (Martín & López, 2008), we expected lower responses of supplemented females. Nevertheless, in the present experiment, similar responses to male secretion were elicited in all the females. However, a significant difference was noted in the total number of TFs elicited in response to both stimuli, being lower in supplemented females than in the control females. The lack of interest in the scent of males in supplemented females in comparison to the control females could be interpreted in the same way, as the absence of need. But it might be also explained because the assay was performed outside the mating season, when femoral secretions of males might not be functional or have no sexual interest for females after they have already finished reproduction for that year.

This experiment cannot provide evidence about the use of male sexual signals by females in the field, but the results suggest that the response to male secretions could be dependent on the need for vitamin D; taking into account that supplemented females, whose requirements for vitamin D were probably in surplus, elicited lower TF rates. In addition, we did not find reproductive benefits of having extra access to vitamin D, in comparison to control females. We assumed that control females would represent a deficit state of vitamin D in comparison to supplemented females. However, the control females could have obtained a sufficient amount of vitamin D_3, because the dietary prey that females could eat ad libitum contained a certain amount of vitamin D_3: *T. mollitor* 150 IU/kg dry matter (individual mean mass = 0.01 g; expected amount of vitamin D_3=0.011 IU) and *A. domestica* 934 IU/kg dry matter (individual mean mass = 0.16 g; expected amount of vitamin D_3 = 1.07 IU) (Oonincx *et al.*, 2010). New studies should be designed to determine the requirements and body contents of vitamin D_3 in gravid females in nature.

However, our findings do not discount a pre-existing sensory bias as the origin of the sexual signal, nor can we be sure that the signal is, currently, only sexual and discard a food bias, considering the results of previous studies. A relevant suggestion that we could extract from our results is that, if the available food is sufficient and the requirements for vitamin D are covered, the male signal might be ignored by females. For example, in favourable seasons, vitamin D would be easy to obtain, whereas in unfavourable seasons, the environment might in some way constrain vitamin D acquisition. In these unfavourable conditions, selective pressure would act on the signalling system, and it would be then when the bias could be acting more strongly.

In fact, this could be the reason for the lack of response in TF tests, because the assay was carried out in July, whereas the mating season is in May. Maybe, the requirements for vitamin D are higher during the mating season, when a few weeks of activity have passed after winter (Pérez-Mellado, 1982), and synthesis in the sunlight could be difficult (Kimlin, 2008). Furthermore, our results provide new answers about the mechanism underlying the origin of signalling. In the context of mate choice, if there was a differential response to signals dependent on the physiological utility or need for the signalling compound by the female receiver, the effectiveness of the signal might be dependent not only on the quality of the male signaller. In this sense, mate-choice preferences would be determined by both direct benefits and sensory bias.

We conclude that our results support the role of provitamin D_3 in the femoral secretions males as a communicative signal and not as a sensory trap used by males to attract females. In light of this, new experiments should be conducted to improve our knowledge about the origin of the signal and the relationship between the biases of the receivers and the production of the chemical signal by senders.

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SUPPORTING INFORMATION

Table S1. Information about the model selection comparing the response variables of reproductive biology and female state among supplementation treatment of mothers. Initial, final and null models are shown. The corrected

Akaike information criterion (AICc) and weight values which allow selection, Phi-value as overdispersion measure in count variables (Poisson models) and variance inflation factor (VIF) as collinearity measure. Significant tests and variables are shown in bold.

Table S2. Information about the model selection comparing the response variables of egg and juvenile traits among supplementation treatments of the mothers. Initial, final and null models are shown. The corrected Akaike information criterion (AICc) and weight values that allow selection and variance inflation factor (VIF) as collinearity measure. Significant tests and variables are shown in bold.

Table S3. Results of statistical analyses comparing the tongue flick responses. Variables were analysed with linear mixed models for repeated measures, including the females as a random factor; in the table, only the fixed factors are shown. Four different models were developed statistical computing. Vienna: R Foundation to test the relevance of the treatment factor, treatment duration as a covariate (treatment days), and the interaction between treatment and stimuli to explain the response variables of latency and tongue-flick (TF). Significant tests and variables are shown in bold.