Original Article

Improved nutritional status may promote an “asset protection” reproductive strategy in male rock lizards

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Investing in the current reproduction requires diverting energy resources from other metabolic functions, which may compromise future reproduction and lifespan. To solve this trade-off, an individual may consider its labile state to decide how much to invest in current reproduction. We tested experimentally whether the “state quality” of male rock lizards influences their reproductive strategies. To improve the nutritional status of males before the mating season, we captured and supplemented experimental males ($N = 20$) with dietary vitamin D₃ (an essential nutrient for lizards) and had a control group of males ($N = 20$). Then, we released all these males and females ($N = 31$) in a large semi-natural outdoor enclosure where lizards could interact and mate freely during the mating period. Activity levels of males did not vary between treatments, but supplemented males started fewer intrasexual agonistic interactions and made fewer mating advances to females. When the mating season ended, we incubated eggs laid by females to obtain the offspring and estimated the paternity of males using DNA microsatellites. Supplemented males sired fewer offspring than control males. These results suggest that vitamin D₃ supplemented males used a low risk/less costly mating strategy to protect their assets (i.e., vitamin D reserves), but that still resulted in “some” current reproductive success, while likely increasing longevity and the expected future total reproductive success.

Key words: DNA microsatellites, lizards, mating behavior, paternity, reproduction-survival trade-off, reproductive success, vitamin D.

INTRODUCTION

Increasing investment in mating efforts may favor the current reproductive output, but it requires diverting energy resources from other metabolic functions, which has the cost of a reduction of somatic maintenance (Williams 1966; Gadgil and Bossert 1970; Stearns 1989). Thus, increasing reproductive investment tends to shorten life span (e.g., Barnes and Partridge 2003; Flatt and Promislow 2007; Harshman and Zera 2007). This trade-off should force individuals to consider many state variables, including life expectancy and the probability of future reproduction, to decide how much to invest in the current reproductive event.

Energy reserves, health state, hormone levels, and other temporally changing traits are all important labile state variables of an individual. These state variables may contribute to expected future reproductive success, which is one of the most important assets that may determine the current behavioral and reproductive strategies of individuals (Wolf et al. 2007; Sih et al. 2015). Within this context, the “asset protection principle” suggests that individuals

with low energy reserves or low condition need to take extra risk by intensifying their foraging time to increase their future reproductive success, while individuals with high energy reserves or high condition should risk less because of their expectations of a high future reproductive success (Clark 1994; Dall et al. 2004; Harcourt et al. 2009; Luttbegg and Sih 2010; Dosmann et al. 2014; Engqvist et al. 2015; see review in Moran et al. 2021). Conversely, the “state-dependent safety” effect suggests that taking the extra risks of high behavioral activity is beneficial for high-state individuals because their higher performance reduces risks, while they can further increase their state (Dingemanse and Wolf 2010; Luttbegg and Sih 2010; Dosmann et al. 2014; Sih et al. 2015). Finally, when likely future reproductive success is declining (e.g., by aging, decreasing condition, illness) it may also be beneficial to take every risk to maximize current reproductive efforts and forfeit unlikely future chances (“terminal investment principle”; Clutton-Brock 1984; Candolin 2000; Nielsen and Holman 2012; Engqvist et al. 2015).

For reptiles and other vertebrates, vitamin D₃ is essential in the regulation of calcium and phosphorus homeostasis for the uptake of calcium from the gastrointestinal tract, and for an appropriate

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mineralization of bones (Fraser 1995; Holick 1999). Thus, vitamin D deficiency may result in calcium deficiency, which can cause rickets, osteoporosis, and reproductive failure (Holick 1999, 2004). Moreover, the vitamin D₃ endocrine system is also a potent immune system modulator, with important functions in the establishment and maintenance of immunological self-tolerance (Griffin et al. 2003; Hayes et al. 2003). Therefore, vitamin D is very important for overall health, as its deficiency causes metabolic bone diseases and also increases the risk of cancers, autoimmune diseases, heart disease, and type I diabetes (Holick 2004). Vitamin D₃ can be synthesized in the skin from the transformation of provitamin D into vitamin after exposition to sun UV-B irradiation (Holick et al. 1995; Carman et al. 2000). However, this process depends on the availability of favorable “sunny” days and maybe costly in terms of exposition to predators (Carrascal et al. 1992), or increased levels of oxidative stress due to UV irradiation (Chang and Zheng 2003). Moreover, in many cases, sun exposition is usually not sufficient to meet physiological requirements, and many animals require additional dietary intake of vitamin D (Allen et al. 1994; Ferguson et al. 2005). Given the important functions of vitamin D₃, it may be considered an essential nutrient. Therefore, we hypothesized that availability of vitamin D₃ in the body, coming either from the diet or from solar exposition, could be an important specific state variable for many animals that may affect their risk-prone behavioral and reproductive decisions.

The Carpetan rock lizard, *Iberolacerta cyreni*, is a small (70–80 mm adult snout-to-vent length, SVL) lacertid lizard that inhabits rocky habitats of some high mountains of the Central Iberian Peninsula (Martín 2015). This is a highly polygynandrous lizard species; males can reproduce until five females, with larger males fertilizing more females and siring more offspring, but many males in the population do not sire any offspring (Salvador et al. 2008; Rodríguez-Ruiz et al. 2020). Multiple paternity occurs in more than half of the clutches, with one to five different fathers per clutch (Salvador et al. 2008; Rodríguez-Ruiz et al. 2020). During the mating season, males defend territories and increase their movements to gain access to several females, which are more sedentary and have smaller home ranges (Pérez-Mellado et al. 1988; Martín and Salvador 1997a; Aragón et al. 2001). However, territories of males have an extensive overlap (each male has a mean of 13.7 overlapping neighbor males) and aggressive agonistic interactions between males are frequent (Salvador et al. 2008). When the density of males is high, dominance hierarchies often emerge, with males with larger body sizes and relatively larger head sizes being more dominant (López and Martín 2002).

The annual activity period of this lizard is limited to five or six months by the high mountain climatic conditions (Martín 2015). The very short (15–20 days) mating period occurs shortly after lizards emerge from hibernation in spring. Dominant individual males are more active and participate in more aggressive encounters, although this leads to a greater weight loss, while subordinate males are less active and avoid interactions to reduce costs (Martín and Salvador 1995; Martín and López 2000; Aragón et al. 2004). The rest of the spring and summer females develop eggs and the offspring, and all adult lizards, have to forage and acquire energy reserves to survive the long hibernation period. All lizard species within this genus have a relatively long life span (7–8 years) and can reproduce for several (at least 3–4) years (Galán and Arribas 2005; Galán 2011).

It has been proposed that labile state variables (e.g., energy reserves, health) can have a key role in the development and

maintenance of consistent between-individual behavioral variation in rock lizards (Horváth et al. 2016, 2017). A previous captivity experiment with a fully factorial design that manipulated food and dietary vitamin D₃ availability showed that male rock lizards (i) decreased activity in the high food treatment, but there was no effect of vitamin D₃, (ii) increased shelter use in the vitamin D₃ supplementation treatment, but there was no effect of food, and (iii) increased risk-taking in the low food × vitamin D₃ supplementation treatment (Horváth et al. 2017). These results suggest that short-term changes in individual state affect both behavioral consistency and behavioral type of reproductive male rock lizards, with high-state individuals becoming less active and taking less risk.

In this paper, we examined experimentally whether the current quality of the labile state or condition of male rock lizards *I. cyreni* affected their investment decisions in reproductive strategies and the consequences for the current reproductive success. Before the mating season started, we experimentally increased the “state quality” of a group of males by means of a dietary supplement of vitamin D₃, and we had a control group. Then, we released males and females in a large seminatural outdoor enclosure where lizards could interact and mate freely during the mating period. We examined their activity levels, and social and mating behavior and later measured the actual reproductive success of males (i.e., number of offspring sired) using seven DNA microsatellites.

We hypothesized that, according to the asset protection principle (e.g., Clark 1994), vitamin D supplemented males, which would have a high state (i.e., a high availability of vitamin D), and thus a high potential future reproductive success, should decrease their current mating effort (i.e., a lower involvement in agonistic interactions with other males and fewer mating attempts) to protect their asset, which would lead to lower current reproductive success. Alternatively, following the state-dependent safety effect (e.g., Dingemanse and Wolf 2010), individuals with high state might be expected to increase their mating effort and improve their current reproductive success benefits, because they may deal with the increased risk and costs of aggressive interactions and mate searching without compromising future reproduction.

However, some studies have shown that male Iberian rock lizards supplemented with vitamin D₃ produced femoral gland secretions, used to scent-mark territories, that were more “attractive” for females (Martín and López 2006a, b, 2012). Thus, independently of the potential influence of the state quality on reproductive investment decisions, supplemented males might be expected to simply change their mating strategies (i.e., by passively attracting females without needing to aggressively compete with other males and without actively looking for females), which might lead to a reproductive success equal to or even higher than that of control males.

MATERIALS AND METHODS

Study animals and diet supplementation

In early May 2017, before the mating season started, we captured adult *I. cyreni* lizards (31 females and 40 males) by noosing at a large area around “Alto del Telégrafo” (Guadarrama Mountains, Madrid Province, Central Spain) at an elevation of 1,900 m and above. At that time and elevation, lizards had recently emerged from hibernation in late April (Martín 2015). Immediately after capture, lizards were transported inside individual cloth bags to “El Ventorrillo” field station (5 km from capture site). Here, we housed all lizards in individual plastic terraria (40 × 30 × 25 cm)

inside a climatic walk-in chamber (Ibercex V-450-D; ASL S.A.) with adequate temperatures (diurnal = 21°C; nocturnal = 7°C) and photoperiod (12 h: 12 h, light: dark) and with ultraviolet radiation from UV-lamps available during 1.5 h a day. Each terrarium was provided with a layer of coconut fiber as a substrate, a piece of roof tile as a shelter, and a hot spot (from a light bulb) for thermoregulation that allowed lizards to attain optimal body temperatures. Drinking water and food (crickets and mealworms) were provided *ad libitum*.

We measured SVL of male lizards with a metallic ruler (to the nearest mm), head height, width, and length with a digital caliper (to the nearest 0.01 mm), and body mass with a digital scale (to the nearest 0.01 g). Because social dominance in this lizard depends not only on body size but also on relative head size (López and Martín 2002), we summarized these morphological measures using a principal component analysis (PCA), which produced a PC-1 with strong negative loadings (proportion of variation explained = 61.1%; factor loadings SVL = -0.61, head height = -0.70, head width = -0.78, head length = -0.86, body mass = -0.92). For clarity, we multiplied the PC-1 scores by a negative one in order to obtain a “body size index” with higher scores indicating greater body sizes.

To avoid differences between treatments caused by body size, we ordered males by SVL and made pairs of males matched by SVL (i.e., the two males of each pair had the same SVL). One male of each pair was randomly assigned to the experimental treatment ($N = 20$) and the other male to the control treatment ($N = 20$). Thus, there were no statistically significant differences in SVL or general body size between treatments (SVL: experimental, mean $\pm$ SE = 73.6 ± 0.7 mm; control, mean $\pm$ SE = 73.2 ± 0.7 mm; one-way ANOVA: $F_{1,38} = 0.10$, $P = 0.74$; body size index: experimental, mean $\pm$ SE = 0.09 ± 0.22 ; control, mean $\pm$ SE = -0.09 ± 0.23 ; one-way ANOVA: $F_{1,38} = 0.37$, $P = 0.54$).

Every two days, during two weeks, males in the experimental group (D-males) were supplemented with $0.20 \mu\text{g}$ (= 8 IU) of vitamin D₃ (MYPROTEIN, The Hut.com Ltd., UK) dissolved in 0.25 ml of soybean oil (Sigma-Aldrich Chemicals Co., St. Louis, MO). Thus, at the end of the supplementation period, we have provided D-males with approximately 56 IU (= $1.60 \mu\text{g}$) of vitamin D₃. We selected this dose based on previous experiments that showed an appreciable positive effect of a similar amount of vitamin D₃ supplementation on several behavioral and physiological parameters (Martín and López 2006b; Horváth et al. 2017; Rodríguez-Ruiz et al. 2019). We avoided using a higher dose that might result in a possible hypervitaminosis with intoxication effects. With this supplementation, we intended to increase and maintain the body concentration of vitamin D₃ at least during the experiment, which in other lizards is known to persist for relatively long periods after being increased (Ooninx et al. 2013). Males of the control group (C-males) received in each session 0.25 ml of soybean oil alone. Soybean oil does not contain any amount of natural vitamin D₃. To ensure that all the animals received the same quantity of supplement, lizards were handled and the solution was gently delivered into their mouths with a sterile plastic syringe.

Females (SVL, mean $\pm$ SE = 74.2 ± 0.5 mm) were maintained under the same husbandry conditions as males but they did not receive any dietary vitamin supplement. We ensured that females had not mated in the field by making captures before the mating season started, which was also confirmed by the lack in

females of the characteristic abdominal mating scars (copulation marks) produced by the bites of males during copulation (Rúa and Galán 2003).

Experimental enclosure

After we finished the diet supplementation, two weeks after capture, all 40 males and the 31 females were released on 19th May in a semi-natural outdoor enclosure (28×12 m) located in a natural grass prairie surrounded by trees at “El Ventorrillo” field station facilities (Supplementary Figure S1). The enclosure walls were made of polyethylene sheet sustained by bamboo sticks every three meters and in the corners. The low part of the sheet was buried 25 cm in the substrate and the wall was 60 cm high above the ground level to prevent escapes, although lizards could not climb the plastic wall. To avoid attacks from predatory birds, the whole enclosure was covered with an anti-bird net sustained by links crossing all the enclosure at 2 m height. Since this lizard species prefers rocky microhabitats (Martín and Salvador 1997b), we placed forty similar groups of artificial rocks inside the enclosure to be used as refuges and for basking, simulating natural patches of rocks surrounded by alpine meadows where this lizard occurs. Every group of artificial rocks consisted of three concrete bricks ($39 \times 19 \times 19$ cm length $\times$ width $\times$ high) with several holes, and one mud brick semi-buried under them ($24 \times 11 \times 7.5$ cm), shaping a space with many basking platforms and shelters, which lizards used most of the time. The groups of bricks were placed in four parallel rows (240 cm of separation between rows) of ten groups each (280 cm of separation between groups within a row). There were as many artificial groups of bricks as males to ensure that every male could, theoretically, establish in the enclosure his own territory separately from those of other males. Within the enclosure, natural grass and annual plants supported enough invertebrate food for lizards.

Males and females were released at midday of 19th May all together at the central point of the enclosure to allow all of them equal opportunities to choose where to go, thus, avoiding that the distribution of males’ territories depended on differences in the initial release points. All lizards had been painted with a code of four small color dorsal points for long-distance individual identification using water-based non-toxic markers (Posca, Mitsubishi Pencil Co., Ltd., Tokyo, Japan).

In subsequent days, every day between 8:30 h and 16:30 h (GMT), during the main daily period of lizards’ activity (Martín and Salvador 1995), at least two researchers observed with binoculars the behavior of lizards from several viewpoints surrounding the enclosure. Observers were blind to the experimental treatment of each lizard. Every hour, we also made a specific 10 min systematic survey of all the enclosures (a total of 9 surveys/day) searching for and identifying all active male lizards observed in the survey. We considered lizards that were visible, either moving or static but exposed, to be active. During inactive periods, lizards remained hidden inside or under the bricks. The design of the enclosure and the location of the viewpoints surrounding the enclosure ensured that the probability that all active lizards could be observed was very high. Each male observed was identified and recorded as active in that hourly survey. The mean hourly activity level for each individual male was estimated as the percentage of days (from a total of 14 days of observation) in which that individual was seen to be active within each hourly survey. The total activity level for each individual was the mean of all hourly activity levels. We

used repeated-measures two-way ANOVAs to assess variation in activity levels (dependent variable) among hourly time periods (within-subjects fixed factor) and between vitamin D treatments (between-subjects fixed factor). The interaction between time and treatment was also included to determine whether the variation in activity level with time of day was different in control and experimental males. Statistical analyses were made with the Statistica 8.0 software (StatSoft Inc. Tulsa, OK).

We also recorded, continuously during the daily observation period, the number of intrasexual “agonistic interactions” between males. These interactions were considered to occur when one male (assigned as “starter”) rapidly approached his opponent with an aggressive behavioral display (raised, stiff forelegs with their neck arched and the snout pointing slightly down), usually causing the other individual male (assigned as “receiver”) to retreat or run away, either without physical contact between the two males or less frequently after escalated physical combat and bites. We also recorded the number of “mating advances” of males to females. These were considered to occur when a male approached a female slowly, followed her, and began to flick on the female’s tail, or the surrounding substrate. If the female did not flee from the male, the male then gripped and shook the female’s tail near the cloaca with a gentle bite, and if the female was receptive she allowed copulation during a long time period (>1 min) (assigned as “successful copulation”) (Martín and Salvador 1993; López and Martín 2002, 2011; Aragón et al. 2004). In all cases, we identified the two individuals involved in each interaction. Numbers of interactions started or received by each individual male with other males or females were included as the response variables in separated Generalized Linear Models (GLZ) with a Poisson distribution and a log link function, using the vitamin D treatment as an explanatory fixed factor and the male’s body size (PC1 scores) as a continuous covariate. Although different observers recorded these interactions, all of them had enough long-term experience in behavioral studies with this lizard species to be confident that they would coincide in their assignments of behavioral categories. Therefore, we did not consider that we needed to control statistically for an observer effect.

Lizards stayed in the enclosure for seventeen days (since 19th May until 5th June), when we considered that the usual short mating season had finished because the frequency of fights between males and matings advances to females descended drastically (i.e., frequencies changed from a mean of 13.4 agonistic interactions/day and 7.6 mating advances/day in the first week to 1.8 agonistic interactions/day and 1.0 mating advances/day in the second week, with no observations of any interactions or advances in the last three days) (Martín and Salvador 1993; Aragón et al. 2004). The mating period in the enclosure was similar in duration (about two weeks) and time of the year (second half of May to beginning of June) to the usual correspondent mating season in the very close nature population from which the lizards came (Aragón et al. 2004; Salvador et al. 2008; Rodríguez-Ruiz et al. 2020). Then, on 5th June, all the animals were recaptured (most were found inactive early in the morning inside shelters).

For paternity analyses, we used disinfected scissors to make a clean and quick cut of a little piece of the tip of the tail tissue (<0.5 cm), which was stored in absolute ethanol until DNA extraction (see below). Tail-tip clipping is a standard method used to obtain DNA in different lizard species (e.g., Halliwell et al. 2017) that has no apparent detrimental effects, giving the small amount of tail

removed that will be quickly regenerated. Thereafter, males were released at their exact capture sites after ensuring they were in a good health state. Survivorship of male lizards was 100% over the experiment.

Reproductive success

Females were also extracted from the enclosure and housed individually in outdoor plastic terraria (60 × 40 × 44 cm, length × width × high), with a 15 cm depth layer of coconut fiber as substrate and a clay brick for shelter. Water and food (mealworms and crickets) dusted with a vitamin complex and calcium were provided *ad libitum*. Near the expected laying dates (10th July to 1st August), we checked the terraria every day and if females showed signs of having laid the clutch (when females were pregnant, eggs were clearly apparent protruding inside the lateral part of the abdomen), we looked for eggs buried in the substrate. When a clutch was found, the eggs were carefully extracted from the terrarium and allocated individually in plastic flasks (170 mL) filled with moistened perlite (1:1 proportion of water and perlite), covered with a lid, and closed with a plastic cap to keep the humidity levels inside. The flasks with eggs were kept at 27.5°C in an incubator (FC-B2V-M/FC404 incubator, Friocell, Santa Rosa Jáuregui, Querétaro, México) until hatching. When juveniles hatched (incubation time, mean ± SE = 33 ± 1 days), we took a little piece of their tail (less than 3 mm) for paternity analyses. We released all females (after they laid the clutches) and all juveniles (a few days after birth) at the capture sites of their respective mothers after ensuring they were in a good health state.

We used DNeasy® Blood and Tissue Kit (QIAGEN®, Germantown, MD) to extract DNA from tail tissue samples. Every individual was genotyped at seven *I. cyreni*-specific microsatellite loci (Icy1, Icy2, Icy3, Icy4, Icy5, Icy6, Icy7) (Bloor 2006). Primer pairs were combined to allow the amplification of seven loci in two multiplex reactions (reaction 1 = Icy2, Icy4, and Icy7; reaction 2 = Icy1, Icy3, Icy5, and Icy6). Polymerase chain reactions (PCR) were performed in a 10 µl reaction volume containing 1 µl DNA solution, 5 µl of Quiagen® Type-it Kit, 3 µl of water and 1 µl of a mix including all primers in each multiplex at 2 µM. PCRs started with an initial denaturation step at 95°C for 5 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 90 s and extension at 72°C for 30 s. All reactions were finished with a final extension at 72°C for 30 min. One primer from each pair was fluorescently labeled to distinguish PCR products from one another. Sequencing was carried out at the SECUGEN company facilities (Madrid, Spain). PCR products were sized using Sanger sequencing. Chromatograms were visualized and scored manually by two independent observers using GeneMapper v.5 (Thermo Fisher Scientific Inc., Waltham, MA) to ensure that there were no mistakes in the data set.

Parentage assignment was performed with the software CERVUS v. 3.0.7 by calculating “logarithm of odds” (LOD) scores from the natural logarithm of the overall likelihood ratio (Kalinowski et al. 2007). LOD scores were determined by means of simulation parentage analysis with 10,000 cycles ran at two levels of confidence, strict (≥0.95) and relaxed (≥0.80). The parentage assignment of the father given the known mother at the strict level of confidence showed a 86% success rate, and at the relaxed level, a 100%. Paternity was assigned to the males with positive highest LOD scores and with significant simulation test at least, in the relaxed criterion. The mean number of alleles observed per locus was

12.29 and the mean proportion of loci typed was 0.99. The heterozygosity observed (HObs) was higher than the expected one (HExp) in all the markers except in Icy3 and Icy5, and the Hardy-Weinberg equilibrium test was not significant in all the loci (Supplementary Table S1).

We used two measures that provided different aspects of the reproductive success of males: 1) the “total number of offspring sired” and 2) the “number of females fertilized” by each individual male considering the number of clutches in which a male sired some offspring. Reproductive success measures were included as the response variable in generalized linear models (GLZ) with a Poisson distribution and a log link function, using the vitamin D treatment as an explanatory factor and male’s body size (PC1 scores) as a continuous covariate.

RESULTS

Activity levels

Activity levels of male lizards varied significantly with time of day, but C-males were not statistically significantly more active than D-males (repeated-measures two-way ANOVA, time of day: $F_{8,304} = 65.68$, $P < 0.0001$; treatment: $F_{1,38} = 0.08$, $P = 0.77$; time x treatment: $F_{8,304} = 0.54$, $P = 0.82$) (Figure 1). Results remained similar after removing the non-significant interaction (repeated-measures two-way ANOVA, time of day: $F_{8,312} = 66.45$, $P < 0.0001$; treatment: $F_{1,38} = 0.08$, $P = 0.77$).

Social interactions and mating behavior

Larger males started statistically significant more intrasexual agonistic interactions with other males (GLZ, body size, $\chi^2 = 6.65$, $P = 0.0099$), but C-males started statistically significant more interactions than D-males (C-males: mean $\pm$ SE = 3.1 ± 1.2 interactions/male; median = 1.5; $N = 61$ interactions; D-males: 2.1 ± 0.7 interactions/male; median = 1.0; $N = 42$ interactions; treatment, $\chi^2 = 4.99$, $P = 0.025$) independently of their body size (treatment x size, $\chi^2 = 0.67$, $P = 0.41$). When this non-significant interaction was removed from the model, the results remained similar (GLZ, body size, $\chi^2 = 6.01$, $P = 0.014$; treatment, $\chi^2 = 4.39$, $P = 0.036$).

In contrast, considering the individual male that received the agonistic interaction, there were only statistically non-significant

marginal effects of body size (GLZ, body size, $\chi^2 = 3.49$, $P = 0.06$), with larger males tending to receive more interactions, and no statistically significant effects of the treatment (C-males: mean $\pm$ SE = 2.9 ± 0.6 interactions/male; median = 2.0; D-males: 2.4 ± 0.6 interactions/male; median = 2.0; treatment, $\chi^2 = 0.43$, $P = 0.51$; treatment x size, $\chi^2 = 2.22$, $P = 0.14$). After removing the interaction, the effect of body size reached significance (GLZ, body size, $\chi^2 = 4.30$, $P = 0.038$), but the treatment remained non-significant ($\chi^2 = 0.94$, $P = 0.33$).

The number of mating advances to females made by an individual male was only statistically non-significant marginally dependent on his body size (GLZ, body size, $\chi^2 = 3.52$, $P = 0.060$), with larger males tending to make more mating advances, and C-males did make statistically significant more mating advances to females than D-males (C-males: mean $\pm$ SE = 2.1 ± 0.6 advances/male; median = 1.0; $N = 42$ advances; D-males: 0.9 ± 0.3 advances/male; median = 1.0; $N = 18$ advances; treatment, $\chi^2 = 8.46$, $P = 0.0036$), independently of the male body size (treatment x size, $\chi^2 = 1.10$, $P = 0.29$). The two main effects were significant after removing the interaction (GLZ, body size, $\chi^2 = 6.91$, $P = 0.008$; treatment, $\chi^2 = 11.29$, $P = 0.0008$).

We could only observe a total of 19 apparently successful copulations made by 14 different individual males and 11 different individual females. There were no statistically significant effects of body size (GLZ, $\chi^2 = 0.49$, $P = 0.48$), or the treatment (C-males: mean $\pm$ SE = 0.5 ± 0.2 copulations/male; median = 0.0; $N = 10$ copulations made by 7 individual males; D-males: 0.4 ± 0.2 copulations/male; median = 0.0; $N = 9$ copulations made by 7 individual males; treatment, $\chi^2 = 0.01$, $P = 0.93$; treatment x size, $\chi^2 = 1.46$, $P = 0.23$). The main effects remained non-significant after removing the interaction (GLZ, body size, $\chi^2 = 0.68$, $P = 0.41$; treatment, $\chi^2 = 0.09$, $P = 0.76$).

Reproductive success

From the 31 females released in the enclosure, 24 females became pregnant and laid a total of 121 eggs (mean $\pm$ SE = 5.0 ± 0.2 eggs/clutch; $N = 24$), from which 87 offspring from 21 of the clutches were born (4.1 ± 0.3 offspring per female; $N = 21$ clutches). All unhatched eggs were unfertile eggs that did not contain embryos. These offspring were identified as sired by 25 of the 40 males that had been in the enclosure (LOD score > 0 and significant confidence test). Multiple paternity occurred in 15 from 21 (71.4 %) of the clutches that produced some offspring, the number of different fathers per clutch ranged from 1 to 4 (mean $\pm$ SE = 2.1 ± 0.2).

We assigned 58 offspring (66.6 %) to 15 of the 20 C-males and 29 offspring (33.3 %) to 10 of the 20 D-males. Thus, 5 from 20 C-males and 10 from 20 D-males did not sire any offspring (χ^2 test = 2.67, $P = 0.10$).

The GLZ model for the total number of offspring sired by males was statistically significant ($\chi^2 = 12.75$, $P = 0.005$), showing that there was a statistically significant effect of the vitamin D supplementation ($\chi^2 = 9.17$, $P = 0.002$; C-males: mean $\pm$ SE = 2.8 ± 0.6 offspring/male; range = 0–8; D-males = 1.3 ± 0.4 offspring/male; range = 0–7). Also, larger males sired statistically significant more offspring ($\chi^2 = 3.96$, $P = 0.047$), and the interaction did not reach statistical significance (treatment $\times$ size, $\chi^2 = 3.61$, $P = 0.057$), but suggested a trend to a stronger increase of offspring number with body size in D-males than in C-males (Figure 2). When this interaction was removed from the model, the effect of body size did not reach significance (GLZ, body size, $\chi^2 = 3.42$, $P = 0.064$), but the effect of treatment remained significant ($\chi^2 = 11.86$, $P = 0.0006$).

Figure 1

Hourly activity levels (mean $\pm$ SE percentage of days) on which an individual male lizard *I. cyreni* was seen active (C-males, black points, continuous line; D-males, grey points, dashed line).

Figure 2

Relationship between number of offspring sired and body size (PC scores) in male lizards *I. cyreni* (C-males, black points, continuous line; D-males, grey points, dashed line).

However, the GLZ model for the number of females fertilized did not significantly differ from the null model in the omnibus test (GLZ, $\chi^2 = 3.82$, $P = 0.28$). Thus, neither the experimental treatment, nor body size or the interaction could significantly explain the number of females fertilized by each male (C-males: 1.4 ± 0.3 females/male; range = 0–4; D-males: 0.7 ± 0.3 females/male; range = 0–3).

The males that fertilized more females had more offspring ($r_s = 0.74$, $P < 0.0001$, $N = 25$), with a mean ($\pm$ SE) of 1.8 ± 0.2 offspring/female (range = 1–4). This relationship did not significantly differ between treatments (GLZ, $\chi^2 = 0.29$, $P = 0.59$), was independent of male body size ($\chi^2 = 0.84$, $P = 0.36$), and the interaction was not statistically significant ($\chi^2 = 0.26$, $P = 0.61$).

DISCUSSION

Our experiment showed that an increase in vitamin D levels of male rock lizards *I. cyreni* resulted in some changes in their social and mating behavior, which, in turn, had consequences for their reproductive success. Experimental D-males, which presumably attained a better nutritional status after the dietary supplementation (i.e., a higher stored amount of vitamin D), seemed to invest less in reproduction during the current mating season. This could be considered as an “asset protection” reproductive strategy (Clark 1994) that although reducing their current reproductive success also decreased many costs (see below), which may allow increasing growth and survivorship and result in a higher long-term total fitness.

Since skin irradiation with UV light from the sun is necessary to synthesize vitamin D₃ (Fraser 1995; Holick et al. 1995), we could have expected control lizards to be more active to increase time exposed to the sun or D-males to reduce activity as they would already have enough vitamin D. However, we did not find differences in general activity levels between treatments. Similarly, in a previous experiment made in small terraria with this lizard, dietary supplementation of vitamin D did not affect the consistency of activity patterns (Horváth et al. 2017). This suggests that the activity

cycles responded directly to the photoperiod and the thermal environment, as it is expected for poikilothermic animals (Adolph and Porter 1993; Huey 1982). By being active, lizards can attain and maintain an optimal body temperature, which allows maximizing foraging, running speed, growth, and reproduction (Huey 1982). Therefore, it might be expected that all individual lizards will be active in a similar way whenever weather conditions are suitable for them to achieve their preferred body temperature (Porter et al. 1973). This would be especially important in high mountain environments where thermal conditions optimal for lizards' activity are limited (Carrascal et al. 1992). Nevertheless, activity is not strictly constrained by temperature and lizards can modify their activity levels (Rose 1981; Adolph and Porter 1993; Gunderson and Leal 2015). For example, rock lizards can compensate for high predation risk, decreasing time spent active and exposed (Martín and Salvador 1995), or for food shortage, increasing activity and foraging time (Horváth et al. 2017).

Showing similar activity levels, however, does not preclude that the behavior while being active had to be the same in all individuals and many behavioral modifications are possible. Thus, males supplemented with vitamin D showed a lesser involvement in agonistic intrasexual interactions with other males, at least when referring to initiating interactions. Nevertheless, it seems that D-males could not always avoid being the target of agonistic interactions started by other males. Fighting is a very costly behavior, in terms of time and energy expenditure, increased stress, and risk of injury or predation (Huntingford and Turner 1987; Magnhagen 1991; Creel 2001), even if most of the observed interactions did not escalate in aggressive level and were resolved by fleeing from the male that started the attack. To decrease these costs, males use different fighting strategies and decision rules for resolving conflicts as quickly as possible depending on the balance between costs of fight and expected benefits (Maynard Smith and Price 1973; Enquist and Leimar 1983; López and Martín 2001, 2011). Therefore, by avoiding fights with other males, D-males may decrease these costs, increasing body growth and long-term survival, which could be linked to the asset protection principle (Clark 1994). However, avoiding fights with other males probably leads to smaller exclusive territories or lower social status, with less foraging opportunities and lower access to females (Martín and Salvador 1993), which is the main resource defended by dominant, more aggressive, territorial males during the mating season.

Searching for mates and mating behavior is also costly, in terms of time and energetic expenditure, and are conspicuous activities that also increases predation risk (Koga et al. 1998; Magnhagen 1991; Engqvist et al. 2015). Many animals modulate their investment in reproductive behavior according to predation risk level and the future opportunities for mating (e.g., Candolin 1997; Koga et al. 1998; Hazlett and Rittschof 2000; Martín et al. 2003). By decreasing investment in mating behavior, D-males might reduce energetic costs and predation risk and increase growth and survival, but, again, at the cost of a lower mating success.

Our data on paternity of male rock lizards show that C-males sired more offspring than D-males, suggesting that the lower investment in social and mating behavior had the cost of a lower current reproductive success (i.e., on average D-males sired less than half of offspring than C-males). Alternatively, as an opposite explanation, the differences found in reproductive success might be linked not to a lower investment in mating behavior by D-males, but to a terminal investment strategy used by C-males (Clutton-Brock 1984; Weil et al. 2006). Similar terminal

investment strategies have been observed in response to immune challenges that “simulated” a bad health state in several species (Bonneaud et al. 2003, Sköld-Chiriak et al. 2018), including rock lizards (Rodríguez-Ruiz et al. 2020). However, this explanation seems unlikely in the current study because our experimental treatment did not provoke direct negative effects on C-males, but we cannot discard the possibility that these males might be making an extra effort in obtaining matings in the current reproduction season if they assessed that their survival odds for the following years were low.

Current reproductive investment can increase current reproductive success, but it can decrease future reproductive success, as occurs in *Lacerta vivipara* lizards (Bleu et al. 2012). Moreover, numerous studies show that reproduction tends to shorten lifespan in many species (e.g., Michener and Locklear 1990; Barnes and Partridge 2003; Flatt and Promislow 2007; Harshman and Zera 2007; Tatar 2010). This is explained because reproduction, somatic maintenance, and growth are very demanding energetically, and is very difficult to maximize the allocation of resources to all of them simultaneously, such that inversion in reproduction cannot be used for somatic repair (Partridge et al. 2005). Therefore, it is likely that individuals with a better state and a potential long-life span trade-off all energetic requirements to “protect” their state and increase their total fitness.

Nevertheless, there are alternative potential explanations to our results that would be independent of an asset protection strategy. Male rock lizards might show variations in their reproductive strategies in the wild depending on the amounts of body vitamin D₃. Dietary supplementation of vitamin D to male rock lizards allows them to increase the proportions of provitamin D₃ in femoral secretions (Martín and López 2006b), an effect also found in the current experiment (unpubl. data). This compound may act as a chemical sexual signal in this lizard, correlating positively with individual quality (López et al. 2006; Martín and López 2006a) and being under positive female choice (Martín and López 2006a, b). The presence of provitamin D in territorial substrate scent marks of males may attract females to that territory (Martín and López 2006a, b, 2012), likely through a sensory bias for food mechanism in females (Martín and López 2008; Rodríguez-Ruiz et al. 2019). Therefore, it could be expected that C-males, with an average low-scent quality, take higher risks to actively find mates, while the more attractive D-males become more risk-averse, reducing costs of aggression and mating, while still successfully attracting females to their home ranges. In that case, the increased reproductive output of C-males observed in our experiment might be an artifact of the high density of males in the enclosure, which might favor a more aggressive strategy over a passive female-attracting strategy. In fact, many animals change their mating strategies depending on the social environment (i.e., density of competitor males, sex ratio, etc) (e.g., Karlsson et al. 2010).

We conclude that supplemented D-males rock lizards, in a currently better state (i.e., more vitamin D), seemed to follow a risk-averse asset protection strategy. Although decreasing mating effort reduced the current reproductive success, it probably also decreased many associated costs. Therefore, this risk-averse strategy might result in a higher growth and survivorship and increase the opportunities for future reproduction, leading to a higher total lifetime fitness. However, we cannot exclude that the presumably high attractiveness to females of scent-marks of D-males induced them to adopt a more passive strategy to attract

females, with less fights with other males and less active searching for mates. However, this strategy might not be successful when male density is high and male-male competition overcomes female mate choice. Future studies should examine the current and long-term fitness of different individuals depending on their mating behavior and their state, and on changes of state, and in different social environments (e.g., male-male competition intensity, sex ratio) to understand how animals trade-off current vs future reproduction.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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AUTHOR CONTRIBUTIONS

J.M., G.R.R., J.O., P.L., and A.S. conceived the ideas, designed the methodology, built the enclosure, captured lizards, worked in the experiment and collected the data. J.O. and G.R.R. made the genetic analyses. J.M. and G.R.R. analyzed the data and led the writing of the manuscript. All authors contributed critically to the manuscript drafts and gave final approval for submission.

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Data availability: Analyses reported in this article can be reproduced using the data provided by Rodríguez-Ruiz et al. (2021).

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