The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2016, **118**, 610–617. With 2 figures.

The timing and interval of mate encounter affects investment during mating

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Received 30 July 2015; revised 1 December 2015; accepted for publication 1 December 2015

The benefits obtained from mating are usually condition-dependent, favouring the evolution of flexible investment during copulation; for example, in terms of invested time, energy or sperm. Flexible investment strategies are predicted to depend on the likelihood of acquiring alternative mates and therefore they should depend on the timing of mate encounter. However, scarce experimental evidence for this hypothesis exists. In the present study, we manipulated the time delay until first mating and the interval between first and second mating in the polygynandrous common lizard *Zootoca vivipara*. We determined treatment effects on fertilization success and copulation duration, with the latter being a proxy for investment in mating and for the quantity of transferred sperm. The duration of the second copulation decreased with increasing inter-mating interval and depended on the fertilization success of first mates. The former provides evidence for time-dependent investment strategies, most likely resulting from the progression of the female's reproductive cycle. The fertilization success of first mates increased with increasing inter-mating interval and was higher when females were closer to ovulation, showing that flexible investment strategies significantly affected male reproductive success. This indicates fertilization assurance, which may mitigate the negative effects of low population density on reproductive success (e.g. Allee effects). © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 610–617.

KEYWORDS: copulation duration – fertilization success – *Zootoca vivipara*.

INTRODUCTION

The benefits of mate choice often depend on an organism's extrinsic and intrinsic conditions, including its physiological state and physical and social environment. The latter includes the likelihood of encountering mates, which is a function of population density and operational sex ratio (Shuster & Wade, 2003; Kokko, Klug & Jennions, 2012). For example, strong mate choice may be beneficial in dense populations but could lead to no reproduction in low-density populations or during habitat colonization because preferred mates may not be encountered (Courchamp, Clutton-Brock & Grenfell, 1999). Under such conditions, weaker mate choice may guarantee reproduction (Kokko & Rankin, 2006; Bleu, Bessa-Gomes & Laloi, 2012), potentially favouring the evolution of flexible strategies (McNamara & Houston, 1996; Jennions & Petrie, 1997; Roff, 2002).

Similarly, strategic resource investment during mating might be beneficial (Sheldon, 2000; Bonduriansky, 2001). For example, under sequential mate encounter, males may invest different amounts of sperm depending on the mating order (e.g. first/second to mate) and female mating status (e.g. virgin/previously mated) because these determine the risk of sperm competition (Parker & Pizzari, 2010; Ramm &

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Stockley, 2014). Males should invest more sperm when increased sperm competition risk exists (Parker *et al.*, 1997). However, if fertilization becomes less likely with the time passed subsequent to a female's first copulation (Huck, Quinn & Lisk, 1985; Schwagmeyer & Foltz, 1990), investment may decline. Strategic resource investment may exist not only in males (i.e. through adjustment of sperm quantity; Parker & Pizzari, 2010), but also in females, such as by controlling the quantity of sperm received or retained (i.e. cryptic female choice; Andrés & Cordero Rivera, 2000; Engqvist & Sauer, 2003; Pilastro *et al.*, 2007).

Studies indeed show that population density affects mating strategies (Palokangas, Alatalo & Korpimäki, 1992; Cornwallis & Birkhead, 2006; Mugabo *et al.*, 2013). Similarly, mating strategies may depend on the temporal pattern of mate encounter, as suggested by mathematical models (Crowley *et al.*, 1991; Gowaty & Hubbell, 2005), and the time interval between sequential mates may affect the benefits obtained through additional mating (Parker, 1990; Real, 1990; Jennions & Petrie, 1997). Therefore, flexible mate preferences and resource investment with respect to mating intervals may have evolved. However, to our knowledge, besides a few experiments in insects (Simmons, 1995; Reinhold & von Helversen, 1997; Lehmann & Lehmann, 2000), experimental studies manipulating time intervals between successive matings to investigate effects on resource investment during mating are lacking. Thus, the importance of mating intervals for the evolution of flexible mating strategies is elusive. This is surprising given that sequential mate encounter is common in natural populations and processes such as climate change and habitat fragmentation can affect the timing of mate encounter (Møller, 2004; Lane, Forrest & Willis, 2011; Morbey, Coppack & Pulido, 2012), potentially reducing fitness and population viability (e.g. through mating failure and Allee effects; Calabrese & Fagan, 2004; Saino *et al.*, 2011; Larsen *et al.*, 2013).

In the present study, we experimentally test, using the polygynandrous common lizard (*Zootoca vivipara*), whether the time interval between matings affects mating strategies and reproductive success. Previous results have demonstrated a decreasing female re-mating probability with increasing mate encounter intervals and reduced female choosiness and/or mate resistance with delayed first mate encounter (Breedveld & Fitze, 2015), pointing to flexible pre-copulatory strategies that can assure reproduction when mates are scarce (i.e. encountered late). In the present study, we determine whether flexible male or female investment strategies exist once copulation is initiated.

We manipulated the length of the interval between two successive copulations in females (re-mating delay). If the interval drives reproductive investment strategies, we predicted that re-mating delay would have an effect on the duration of second copulations and on fertilization success, and also that strategies may depend on first male fertilization success. Copulation duration is a proxy for sperm quantity invested because *Z. vivipara* exhibits non-instantaneous ejaculation (i.e. continuous sperm transfer) (Olsson *et al.*, 2004). To clarify whether investment strategies depend on the risk of sperm competition and/or intrinsic conditions (i.e. female reproductive stage), we also manipulated the delay between female emergence from hibernation and the day of first mating (mating delay). Under male strategies driven by sperm competition risk, we predicted no effects on copulation duration of first mates because these mated with virgins. However, we predicted differences in copulation duration between first and second mates because only second mates may have knowledge about potential rivals. Under strategies driven by female reproductive stage, we predicted an increased copulation duration with a longer mating delay because, in spontaneous ovulating species (Bleu *et al.*, 2011), later mating females are further advanced in their reproductive cycle (i.e. under stronger time constraints to assure fertilization).

MATERIAL AND METHODS

SPECIES DESCRIPTION

The common lizard (*Zootoca vivipara* Lichtenstein, 1823) is a small, widely distributed Lacertidae (Surget-Groba *et al.*, 2002). Males emerge from hibernation in spring (February to March) and females emerge several weeks later, initiating the mating season (Roig, Carretero & Llorente, 2000). Females mate with multiple males ($N = 1–5$) (Laloi *et al.*, 2004) and males with multiple females ($N = 1–14$) (Fitze *et al.*, 2005). Copulations last from several minutes to several hours (mean ± SE: 32.5 ± 3.8 min) (Gonzalez-Jimena & Fitze, 2012), during which sperm is transferred continuously (Olsson & Madsen, 1998). Mating is costly for females because the male's mouth-grip produces scars and mating can lead to mortality (Fitze & Le Galliard, 2008). Females exert pre-copulatory mate choice by accepting or fending off males (Fitze, Cote & Clobert, 2010), as well as exhibit time-dependent choice strategies (Breedveld & Fitze, 2015) and trade-up strategies (Fitze *et al.*, 2010; Laloi *et al.*, 2011). Males exhibit 'best-of-bad-job' strategies (Gonzalez-Jimena & Fitze, 2012).

Females cannot store sperm over winter, and thus need to acquire sperm after emergence. Ovulation (i.e. fertilization) occurs approximately 1 month after emergence (Gavaud, 1983), which is typically 3–4 weeks after mating (Bauwens & Verheyen, 1985). Although sperm storage structures (Sever & Hamlett, 2002) have not been reported, the copulation–fertilization interval suggests prolonged sperm survival in the female's reproductive tract and a possible overlap of ejaculates provided on different days (i.e. potential sperm competition) (Olsson & Madsen, 1998). Moreover, within clutches, eggs are usually fertilized by several males (Fitze *et al.*, 2005), indicating that sperm competition indeed exists (Girling, 2002). No evidence exists for cryptic choice or other fertilization-biasing mechanisms (e.g. mate guarding, copulatory plugs; Parker, 1998).

PRE-EXPERIMENTAL CONDITIONS

Lizards were hand-captured from populations in Roncesvalles (Spain) and individually marked (toe-clipped). Before hibernation (September 2010), they were released in 100-m^2 semi-natural enclosures, at research station 'El Boalar' (42°33′N, 0°37′W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología (San-Jose *et al.*, 2014). Males and females occupied different enclosures to prevent pre-experimental mating. Upon male emergence from hibernation (March 2011), female enclosures were searched daily (09.00 h to 05.00 h). Females were captured upon detection and males shortly before experimentation.

Lizards were measured for body mass (mg) and body size [snout–vent length (SVL) in mm]. Males and females were housed on separate shelves in individual terrariums (25 × 15 × 15 cm) under standardized conditions (Fitze *et al.*, 2010) and within-sex positions were randomized (SVL, body mass, body condition: residuals from a regression of body mass on SVL; all $P > 0.5$). Further details are provided elsewhere (Breedveld & Fitze, 2015).

MATING EXPERIMENTS

Females ($N = 26$) could mate with two randomly selected males ($N = 80$); 'first mates' were presented 2 or 9 days after female emergence from hibernation (mating delay), and 'second mates' 2, 6 or 10 days after first mates (re-mating delay; Breedveld & Fitze, 2015). During mating trials (March to April 2011; 09.00 h to 06.00 h), each female was released into a standardized box (Fitze *et al.*, 2010) and a male was introduced. Lizards were observed every 5 min to determine copulations and video-recorded for verification. If no mating occurred within 1 h, males were replaced, respecting the natural mating attempt rate (mean ± SE: 1.1 ± 0.9 h^{-1}; Heulin, 1988). If mating occurred, both individuals were removed after 1 h or, if mating continued, 5 min post-copulation. Trials ended when a female copulated with one male. After a trial, males were released in their original enclosure. Males were never presented to the same female and ≥ 2 days passed between presentations. Repeated mating by males occurred few times (in different treatments) and, excluding the repeats from the analyses, led to the same qualitative results. Twenty-three females copulated once and 20 females re-mated. Females were returned to the enclosures 20 days after capture, and thereafter recaptured weekly to determine egg presence and developmental status (abdominal palpation). Predation reduced sample size to 12 gravid females. Females near oviposition were captured and maintained in the laboratory under standardized conditions. Upon oviposition, clutch size was determined and clutches were incubated (San-Jose *et al.*, 2014).

No significant differences existed between copulating first and second mates in SVL, body condition, days between capture and copulation, and presentation history [whether they were previously presented to another female or had previously mated; analysis of variance (ANOVA), all $P \geq 0.1$]. There were no differences in emergence date, SVL or body condition between females from different (re-)mating delays, and no significant interactions existed (ANOVA, all $P \geq 0.1$).

PATERNITY ASSIGNMENT

Tissue samples (tail tips) from mothers, putative fathers, juveniles, and undeveloped embryos, were frozen in 70% ethanol. DNA was extracted using BioSprint 96 DNA Blood Kit (Qiagen). Paternity was established using 4-6 polymorphic microsatellite DNA loci (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115; Boudjemadi *et al.*, 1999). Fathers were manually attributed and verified using CERVUS, version 3.0 (Marshall *et al.*, 1998). Detailed methods of the polymerase chain reaction and allele size determination have been described previously (Laloi *et al.*, 2004).

STATISTICAL ANALYSIS

Treatment effects on copulation duration and fertilization success were analyzed, using all females that mated, re-mated, and laid fertilized eggs ($N = 12$). Analyses were performed using R, version 3.0.0 (R Core Team, 2013).

To determine whether treatment affected the number of males presented to females until mating occurred, a generalized linear model (GLM) with

Poisson error and log link was run, including mating delay as a factor. To determine effects on the number of males presented until re-mating, a GLM with Poisson error and log link was run, including mating delay as a factor, re-mating delay as a covariate, and their interaction.

Copulation duration was measured with 5-min precision and square-root transformed. Copulation duration of the first copulation was analyzed using a linear model, including mating delay as a factor. Copulation duration of the second copulation was analyzed using a linear model with mating delay and whether or not the first mate fertilized at least one egg (i.e. first male success) as factors, re-mating delay as a covariate, and all two-way interactions. Whether or not a male had previously mated was included as a factor in the previous two models. To determine whether the copulation duration of the second copulation affected a first mate's probability of fertilizing eggs, a GLM with copulation duration of the second copulation as a covariate was run. To test for differences in copulation duration between first and second copulations, a generalized linear mixed model (GLMM) was run, with copulation order as a factor and female as a random effect. To test whether these differences depend on mating delay, the same model was run including the factor mating delay and its interaction with copulation order.

The number of eggs females laid was analyzed using a GLM with quasi-Poisson error (accounting for overdispersion), including mating delay as a factor, re-mating delay as a covariate, and their interaction. The proportion of fertilized eggs was analyzed using a GLM with quasi-Binomial error, including mating delay as a factor, re-mating delay as a covariate, and their interaction.

Fertilization success of either mate (i.e. number of eggs fertilized) was analyzed using a GLMM with Poisson error and log link, including mating delay and copulation order as factors, re-mating delay as a covariate, female as a random effect, and all two-way interactions.

Model selection was performed using likelihood ratio tests, and assumptions were tested and fulfilled. Post-hoc tests were performed using individual contrasts and *P* values were Bonferroni adjusted.

RESULTS

Treatments did not significantly affect the number of males presented to females until mating (mean ± SE: 2.1 ± 0.4 males; $N = 12$; mating delay: $\chi^2 = 0.408$, d.f. = 1, $P = 0.523$) and re-mating occurred (2.3 ± 0.4 males; $N = 12$; mating delay: $\chi^2 = 0.415$, d.f. = 1, $P = 0.520$; re-mating delay: $\chi^2 = 0.051$, d.f. = 1, $P = 0.821$; interaction: $\chi^2 = 0.794$, d.f. = 1, $P = 0.373$).

COPULATION DURATION

Copulations lasted 5–85 min (36.7 ± 3.3 min; $N = 24$). First copulations were significantly longer when the mating delay was 9 days (43.6 ± 7.6 min) compared to when it was 2 days (21.0 ± 4.8 min; $\chi^2 = 5.926$, d.f. = 1, $P = 0.015$). Whether or not a male previously mated was not significant ($\chi^2 = 0.262$, d.f. = 1, $P = 0.609$).

Second copulations were significantly shorter when the mating delay (with first mates) was 9 days (36.4 ± 4.3 min) compared to when it was 2 days (43.0 ± 5.6 min; $\chi^2 = 7.337$, d.f. = 1, $P = 0.007$) and the interaction between re-mating delay and first male success was significant ($\chi^2 = 4.182$, d.f. = 1, $P = 0.041$) (Fig. 1A). When first mates fertilized eggs, the copulation duration of the second copulation was more strongly decreased with an increasing re-mating delay compared to when first mates fertilized no eggs. There was no significant effect of whether a male previously mated ($\chi^2 = 1.011$, d.f. = 1, $P = 0.315$) and no other significant interaction (all $P \geq 0.2$). A first mate's success did not depend on the duration of second copulations ($\chi^2 = 0.045$, d.f. = 1, $P = 0.833$).

Between the first and second copulations, no overall difference in copulation duration existed ($\chi^2 = 1.116$, d.f. = 1, $P = 0.291$). There was, however, a significant interaction between copulation order and mating delay ($\chi^2 = 7.036$, d.f. = 1, $P = 0.008$) (Fig. 1B). Second copulations lasted significantly longer than first copulations when the mating delay was 2 days ($Z = -3.017$, $P = 0.005$) but not when it was 9 days ($Z = 0.860$, $P = 0.780$).

FERTILIZATION SUCCESS

Mean clutch size was 3.9 ± 0.4 ($N = 12$) and the proportion of fertilized eggs was 0.7 ± 0.1. There were no significant treatment effects on the number of eggs laid or the proportion fertilized. Interactions were not significant (all $P \geq 0.2$). Of all copulating males, 42% fertilized zero eggs. Fertilizing mates sired one to six eggs per clutch (2.4 ± 0.4).

The number of eggs fertilized by either mate was significantly affected by an interaction between copulation order and mating delay ($\chi^2 = 4.405$, d.f. = 1, $P = 0.036$) (Fig. 2A); first mates fertilized significantly more eggs than second mates when the mating delay was 9 days ($Z = 2.672$, $P = 0.008$) but not when it was 2 days ($Z = 0.081$, $P = 0.935$). A significant interaction between copulation order and re-mating delay existed ($\chi^2 = 6.988$, d.f. = 1, $P = 0.008$) (Fig. 2B). The number of eggs fertilized significantly

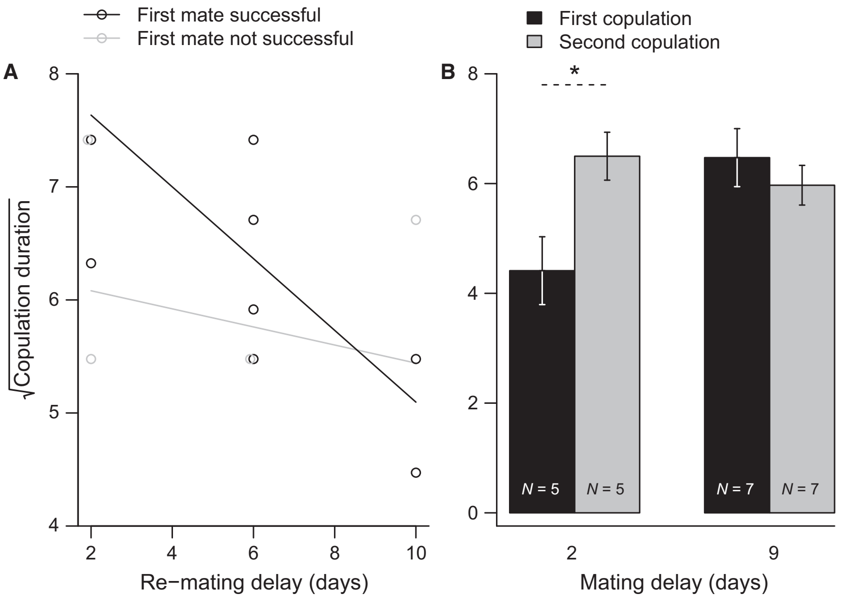

Figure 1. Treatment effects on copulation duration. A, effect of re-mating delay and first mate success on the duration of second copulations. Original data and model predictions are shown for cases where the first male fertilized no (grey) or at least one egg (black). B, significant interaction between copulation order and mating delay on copulation duration. Data are the mean ± SE. *Significant post-hoc contrasts.

increased with re-mating delay in first mates ($\chi^2 = 4.360$, d.f. = 1, $P = 0.037$, estimate = 0.153 ± 0.074) and tended to decrease with re-mating delay in second mates ($\chi^2 = 3.117$, d.f. = 1, $P = 0.077$, estimate = −0.187 ± 0.120). No other significant interactions existed (all $P \geq 0.4$).

DISCUSSION

Flexible mating strategies with respect to the timing of mate encounter may allow optimization of reproductive investment when mates are scarce. In the present study, we manipulated the timing of mate encounter and analyzed the effects on copulation duration and fertilization success in *Z. vivipara*. The results obtained reveal investment strategies in response to the length of the interval between copulations and first male success. Copulation duration with second mates decreased faster with increasing re-mating delay when first mates fathered eggs (Fig. 1A) and first male success was independent of copulation duration with second mates. Copulation duration decreased more slowly with re-mating delay when first mates did not fertilize any eggs, suggesting that adjustment of copulation duration with second mates did not occur with respect to whether a female had previously mated or not. By contrast, it depended on whether or not eggs were fertilized by sperm from a previous mate (i.e. dependent on sperm representation; García-González, 2004). Nonsperm representation is the result of male sterility, insemination failures or failures to fertilize the ova. The latter cannot explain the mating strategies detected in the present study because, in *Z. vivipara*, fertilization occurs several weeks after copulation (Bauwens & Verheyen, 1985). By contrast, temporal sterility is common in male *Z. vivipara* and previous observations showed that male *Z. vivipara* copulated regardless of whether or not they carried sperm (M. C. Breedveld, pers. observ.). The finding that copulation duration with second mates depended on first male success is thus congruent with bet-hedging strategies of females against sterility or genetic defects (Thornhill & Alcock, 1983; Wolff & Macdonald, 2004).

First copulations occurring 9 days after female emergence lasted longer than first copulations 2 days post-emergence, and the copulation duration with virgin and mated females (i.e. first and second copulations) did not differ. These findings are in contrast to predictions under strategic male investment in response to sperm competition risk but are in line with strategic investment with respect to female reproductive stage. Virgin females emerging 9 days before copulation are closer to ovulation (i.e. under stronger pressure to assure fertilization) than those emerging 2 days before, potentially explaining the longer copulations. Similarly, shorter copulations after longer re-mating delays by females that already obtained sperm from first mates may be the result of females reducing reproductive investment (e.g. to conserve energy/avoid unnecessary mating costs)

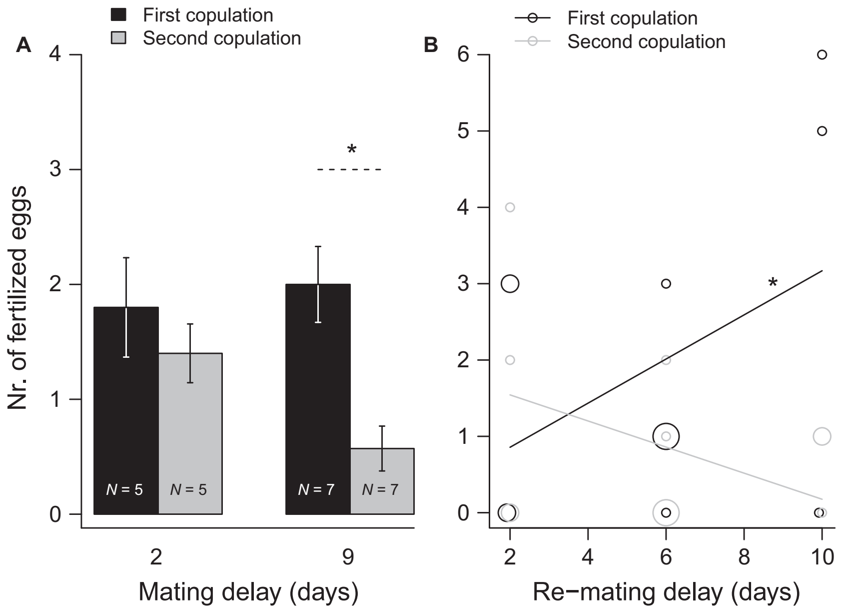

Figure 2. Effects of copulation order, mating delay, and re-mating delay on the number of fertilized eggs. A, interaction between copulation order and mating delay. Data are the mean ± SE. B, interaction between copulation order and re-mating delay. Original data and linear predictions are shown. Dot size is proportional to sample size (small dots: $N = 1$, large dots: $N = 3$). Asterisks indicate significant post-hoc contrasts, or slopes significantly different from 0.

when smaller fitness returns of re-mating are predicted, in line with a declining willingness to re-mate and trade-up strategies (Fitze *et al.*, 2010; Bleu *et al.*, 2012). The fact that clutch size and fertilization success were unaffected by mating and re-mating delay indicates that females were not sperm limited and that different strategies had no negative effects on female reproduction. This suggests that the observed investment strategies allow females to maintain reproductive success under low mate availability by relying on first mates for fertilization, especially when these are encountered late.

Re-mating delay positively and significantly affected the number of eggs first mates fertilized, whereas it negatively affected the number fertilized by second mates (marginally significant). This shows that mating intervals importantly determine male fertilization success, in accordance with previous studies of time-dependent sperm competition patterns (Schwagmeyer & Foltz, 1990). The fact that mating delay also influenced fertilization may result from the interval between insemination and ovulation (Olsson & Madsen, 1998). Females with first copulations occurring longer after emergence may have been at a more advanced reproductive stage (Gavaud, 1991), potentially leading to a higher fertilization probability. However, although, at a mating delay of 9 days, first mates fertilized more eggs than second mates (Fig. 2A), the length of first and second copulations did not differ (Fig. 1B). Moreover, fertilization success did not differ when the mating delay was 2 days, although copulation with first mates was shorter. This is in contrast to other lizard species, where copulation duration is a good predictor of fertilization success (Olsson *et al.*, 2004), at least when two males copulate with the same female on different days. This again suggests that the observed investment strategies with respect to mate encounter time are likely female (and not male) driven. However, additional experiments are needed to confirm this hypothesis.

Overall, we provide evidence suggesting that the timing of successive matings can importantly determine investment strategies. Additional experiments should investigate similar strategies in other species, and studies manipulating time of mate encounter in males would refine knowledge about male roles in investment strategies. The detected strategies could mitigate the potential negative effects of mate scarcity, strong choosiness, and male sterility on female reproductive success (e.g. through adjusting choosiness and/or investment) and population dynamics, in line with investment strategies that optimize reproductive success, potentially explaining rapid post-glacial population expansions in *Z. vivipara* (Surget-Groba *et al.*, 2002). Because sequential mating is common in most species, investment strategies with respect to inter-mating intervals might be widespread. Understanding how animals deal with temporal variation in mate encounter is important for predicting how individuals, and ultimately populations, will respond to changes in population demography. If individual investment strategies are flexible, the effects of variation in mate encounter timing on population dynamics might be small (as suggested by the present study), whereas inflexible responses can negatively impact fitness and population viability (Calabrese & Fagan, 2004; Saino *et al.*, 2011).

ACKNOWLEDGEMENTS

We thank the anonymous referees for their helpful comments; Miguel Peñalver-Alcázar and Cristina Romero-Díaz for field/laboratory assistance; Sébastien Biollay for molecular work; and Luis M. San-Jose for paternity assignments. MCB was supported by a PhD grant (JAEPre_09_01372) from the Consejo Superior de Investigaciones Científicas and co-financed by the European Social Fund. The work was funded by the Spanish Ministry of Education and Science (CGL2008-01522, CGL2012-32459); and the Swiss National Science Foundation (PPOOP3_128375, PP00P3_152929/1). Capture and experimentation licences were obtained from Gobierno de Aragón/Navarra, and comply with Spanish laws and ASAB/ABS Guidelines for Treatment of Animals in Behavioural Research. The authors declare that there are no conflicts of interest.

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