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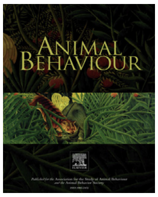

Mate availability affects the trade-off between producing one or multiple annual clutches

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Females of many iteroparous species face trade-offs between producing one or multiple broods per reproductive season, and over fertilizing broods with sperm from the same or different mates. Both trade-offs might be affected by the availability of males (i.e. absence/presence of males) and the timing and duration of male encounters. Here, we experimentally manipulated the duration of mate availability at the first brood and mate availability per se (i.e. absence/presence of mates) at the second brood, and tested their effects on female and male reproductive success, using the common lizard, *Zootoca vivipara*, as a model species. Females were either exposed to males for a long period before their first annual reproduction and they could remate before their second annual reproduction (unrestricted treatment), or they were exposed to males for a short period before their first annual reproduction and were not allowed to remate (restricted treatment). Reproductive success of first clutches was not directly affected by the duration of access to males. Remating positively affected the probability of producing a second clutch, and the proportion of viable offspring. Remating by females also affected the reproductive success of males: fewer second clutch eggs were fertilized with stored sperm in unrestricted than restricted females. Sperm presence in males was high until the end of the remating period. Our results suggest a close coevolution between male and female reproductive strategies and point to facultative skipping of second broods when fitness benefits are small. This shows that behavioural strategies are at least partially responsible for multiple annual broods. These behavioural strategies are likely to be widespread, given the multitude of taxa raising multiple broods in some but not all years, and given that in most taxa some but not all individuals produce multiple annual broods.

Females of iteroparous species face investment trade-offs between different reproductive events (Roff, 1992), i.e. between current and future reproduction (Richner & Tripet, 1999). In species in which females can (but may not) produce more than one brood per reproductive season, e.g. many mammals (Bronson, 1985), birds (Verhulst, Tinbergen, & Daan, 1997), reptiles (Tinkle, Wilbur, & Tilley, 1970), amphibians (Morrison & Hero, 2003), fish (Evans & Magurran, 2000), insects (Fritz, Stamp, & Halverson, 1982) and plants (Paige & Whitham, 1987), these investment trade-offs occur within the same season. Whether an additional seasonal brood is produced might be determined exogenously, e.g. by the optimality of the weather or the time in the season, or it might be regulated behaviourally, e.g. in response to access to conspecifics that are potential mating partners, i.e. mate availability (Adolph & Porter, 1993; Kokko & Rankin, 2006; Perrins, 1970; Roff, 1992). Mate availability should be especially important if females cannot store sperm from a previous copulation, or if stored sperm quality and/or quantity decline over time and thus replenishment of the sperm stores is required to optimally reproduce (Birkhead & Møller, 1993). Under suboptimal conditions a reproductive attempt may fail, e.g. due to a shortage of potential mates or successful copulations (Courchamp, Clutton-Brock, & Grenfell, 1999) or incorrect timing of

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encounters with potential mates (Calabrese & Fagan, 2004). In such a situation, reducing the energy allocated to a reproductive event (Roff, 1992), or completely skipping the production of an additional seasonal brood (i.e. facultative multiple breeding), may be the optimal reproductive strategy (Verhulst et al., 1997). Nevertheless, whether facultative multiple breeding has behavioural bases remains unclear.

Iteroparous females may furthermore face a trade-off between fertilizing broods with the sperm of the partner(s) that fertilized the previous brood (e.g. through stored sperm), or fertilizing them with the sperm of a new partner (e.g. through remating), and strategic sperm utilization may exist (e.g. through cryptic female choice; Eberhard, 1996). Fertilizing eggs with sperm of the same partner may be beneficial if he provides good genes (Andersson, 1994), and could allow females to avoid costs imposed by additional copulations (Bleu, Bessa-Gomes, & Laloi, 2012). On the other hand, remating may be necessary for a female to acquire sufficient sperm (Anderson, 1974), and fertilizing eggs with sperm of new males may increase the genetic diversity of the offspring, or guard against infertility or genetic defects of mates (Jennions & Petrie, 2000; Wolff & Macdonald, 2004). However, patterns of sperm usage are not necessarily under female control. For example, fresh sperm may physically displace stored sperm (Price, Dyer, & Coyne, 1999), or may have an advantage during sperm competition (Birkhead & Møller, 1993, 1998). In these cases, sperm usage may principally depend on whether females acquire fresh sperm, and thus on whether they encounter mates.

Various studies have examined how multiple partner mating affects reproductive success (e.g. Jennions & Petrie, 2000; Tregenza & Wedell, 1998), and how current conditions affect trade-offs between different reproductive seasons (e.g. Cox & Calsbeek, 2009; Kaitala, 1991; McNamara & Houston, 1996; Qvarnström, Pärt, & Sheldon, 2000). Fewer studies have investigated effects on successive reproductive events within a season (Verhulst et al., 1997), and experiments that determine how mate availability affects multiple breeding within a season are restricted to a few insect species (Reinhardt, Köhler, & Schumacher, 1999; Wang & Davis, 2006; Wiklund, Kaitala, Lindfors, & Abenius, 1993). This is surprising, because a large variety of organisms can reproduce multiple times per season (see citations above), and the frequency of multiple breeding importantly affects population dynamics (Bestion, Teyssier, Richard, Clobert, & Cote, 2015; Martin, 1995). Moreover, the operational sex ratio, which is determined by the availability of individuals ready to mate, can affect reproductive behaviour, reproductive success and the intensity of sexual selection (Kvarnemo & Ahnesjö, 1996; Kvarnemo, Forsgren, & Magnhagen, 1995). This suggests that changes in the availability of mates over the course of the breeding season may affect reproductive strategies, including the production and fertilization of multiple seasonal broods.

Here, we experimentally tested whether mate availability affects the production and fertilization of successive clutches within a reproductive season, and whether the production of second broods may be facultatively skipped, using the common lizard, *Zootoca vivipara*, as a model species. Females had either restricted or unrestricted mate availability. Restricted females had short-term access to mates before producing the first clutch, and no access to mates thereafter, i.e. they were prevented from remating before producing the second clutch. Unrestricted females had continuous access to mates before and after laying the first clutch. Treatment effects on the number of copulations, maternal investment, fertilization patterns, and female and male reproductive success were quantified for first and second seasonal clutches. Additionally, we examined the seasonal pattern of sperm presence in males and performed a confirmatory experiment to unequivocally determine the relative frequencies of remating and sperm storage. Here we discuss their implications for the fertilization of second clutches.

The common lizard is ideal for investigating these questions, since it exhibits a polygynandrous mating system (Fitze, Le Galliard, Federici, Richard, & Clobert, 2005), and females produce up to three annual clutches (Horváthová et al., 2013). Within a reproductive season, females can store sperm over longer periods, and can use sperm obtained prior to the production of the first clutch to fertilize the second clutch (Heulin, 1988). Females exhibit mate choice, and the degree of choosiness depends on mating costs (resulting from male sexual harassment), population sex ratio and mating history (Fitze, Cote, & Clobert, 2010; Fitze & Le Galliard, 2008; Fitze et al., 2005). Moreover, unattractive males, i.e. males towards which females show reduced interest and increased aggression, compensate for this disadvantage by exhibiting increased interest and by performing more copulation attempts (Gonzalez-Jimena & Fitze, 2012). This points to the existence of plastic reproductive strategies in both sexes. Finally, experimental evidence demonstrates that first clutch production is independent of copulation, i.e. unmated females produce unfertilized first clutches (Bleu, Le Galliard, Meylan, Massot, & Fitze, 2011), but no evidence exists for effects of mate availability on the production of multiple broods per year, and for the existence of sperm utilization strategies in second broods.

If the duration of access to mates affects reproductive success, we predicted (1) that during the first annual reproduction, restricted females may exhibit fewer copulations, reduced maternal investment and lower reproductive success (e.g. lower viability of clutches) than unrestricted females (despite no expected differences in clutch size; Bleu et al., 2011). Moreover, we predicted (2) that males mated with restricted females may exhibit higher reproductive success, due to reduced competition for fertilizing eggs. If access to mates after first clutch production (i.e. for remating) is important for reproductive success, we predicted (3) that the probability of producing a second clutch will be lower in restricted females. Alternatively, if replenishing sperm stores, through remating, is not necessary, we predicted (4) no differences between restricted and unrestricted females in the probability of producing a second clutch. If fertilization patterns depend on the availability of males ready to mate and fresh sperm has an advantage over stored sperm, we predicted (5) that second clutches of restricted females will be fertilized with stored sperm, while second clutches of unrestricted females are more likely to be fertilized with fresh sperm. In this case, we also predicted (6) that the main sire (the male fertilizing most eggs) of a restricted female's first and second clutch will be the same male, while the main sire of an unrestricted female's first clutch will fertilize fewer eggs in her second clutch, i.e. that remating by females reduces the reproductive success of mates copulating before first clutch production. Finally, we predicted (7) that in the confirmatory experiment the main sire would fertilize fewer eggs in the female's second clutch than in the unrestricted female's second clutch, given that confirmatory females were only exposed to new, unknown males, while unrestricted females had the chance to remate with the same males.

METHODS

Species Description

Zootoca vivipara is a small, ground-dwelling lacertid lizard that is widely distributed throughout Eurasia, where a large degree of variation in population density and structure exists (e.g. Heulin, Osenegg-Leconte, & Michel, 1997). The average life span of *Z. vivipara* is 2.8 years (Strijbosch & Creemers, 1988) with a maximum recorded of 11 and 7 years for females and males, respectively (Massot et al., 2011). Males emerge from hibernation between February and April, and thereafter undergo sperm maturation (Gavaud, 1991). Females emerge 2–3 weeks later, marking the start of the mating season (Fitze et al., 2010).

During copulation, a male holds on to a female with his mouth, typically producing a U-shaped scar on the female's belly (Bauwens & Verheyen, 1985; Fitze & Le Galliard, 2008), and the number of mating scars indicates a female's number of copulations (Fitze et al., 2005; Le Galliard, Fitze, Ferrière, & Clobert, 2005). Mating scars are initially light blue, subsequently becoming dark blue, black, and thereafter gradually fading, until disappearing completely (see Results).

A female's mating window initiates upon copulation, after which females remain receptive for up to 20 days (Breedveld & Fitze, 2015). Females lay one to three clutches per year, and clutch size, which is positively related to female body size, ranges from two to nine eggs (4.65 ± 0.3; mean ± SE; Horváthová et al., 2013). The mating system is polygynandrous, and eggs are fathered by one to five different males (average = 2 males; Fitze et al., 2005). No parental care exists after egg laying, i.e. maternal reproductive investment is completed upon oviposition, which occurs after 14–30 days of gestation (depending on population idiosyncrasies, e.g. in altitude; Horváthová et al., 2013). Evidence indicates that sperm cannot be stored in the female tract from one reproductive season to the next (Bleu et al., 2011).

The time of emergence from hibernation and the reproductive phenology are temperature dependent (Breedveld & Fitze, 2015; Licht, 1972) and vary between and within populations, due to interpopulation differences and annual variation in climatic conditions, respectively (Horváthová et al., 2013). This fact, together with the temperature dependence of sperm maturation (Gavaud, 1991), indicates that changing environmental conditions could produce a mismatch in the reproductive phenology of males and females, thereby potentially resulting in unsuccessful reproduction due to low density of mates (Mugabo, Perret, Legendre, & Le Galliard, 2013), or to females encountering males outside their period of receptivity (Breedveld & Fitze, 2015) or males that do not have mature sperm (Breedveld & Fitze, 2016b).

Experimental Procedures

Here, we studied oviparous individuals from the Spanish Pyrenees (Milá, Surget-Groba, Heulin, Gosá, & Fitze, 2013). All lizards originated from two natural populations belonging to the same phylogenetic lineage (Milá et al., 2013), located at Puerto de Ibañeta, (43°1′N, 1°19′W, 1105 m above sea level) and Somport (42°47′N, 0°31′W, 1640 m above sea level). In May 2009, adults were captured and transported in individual containers to the laboratory at the Instituto Pirenaico de Ecología (Jaca, Huesca, Spain). All lizards were individually marked by toe clipping for identification. Initial SVL (snout-to-vent length; mm) and body mass (mg) were measured, and lizards were housed in individual terraria (25 × 15 cm and 15 cm high) containing a shelter. Food provisioning, light regime and humidity conditions were standardized and equal for all individuals (details below). Only females without mating scars, i.e. that had not yet copulated in the current year, were used for this study.

Two days after capture, each female assigned to the restricted mate availability treatment ($N = 45$) was individually and sequentially presented to six different males (i.e. six trials) over 2 days. The same male sextet was only presented to one female. Standardized mating trials were performed between 0900 and 1800 hours in escape-proof wooden boxes (2500 cm^2; Fitze et al., 2010). In each of the six trials, one male was presented to the female and after 1 h he was removed and replaced with a new male, except when copulation was in progress. In this case, the male was replaced 5 min after the end of the copulation. After the mating trials, females were released in two seminatural enclosures (details below) containing only females, where they had no access to males during the rest of the reproductive season. Females assigned to unrestricted mate availability ($N = 30$) were released in six mixed-sex seminatural enclosures (average: $N = 5$ females per enclosure) directly after measurement, and had access to nine or 10 males during the entire reproductive season, i.e. they could remate after first clutch production. Females of both treatments thus had access to 6–10 potential mates, which exceeds the maximum number of sires per clutch (Fitze et al., 2005).

All females were recaptured on a weekly basis to determine the stage of egg development via abdominal palpation. Females approaching oviposition were brought into the laboratory, and all other females were returned to their population of origin. At oviposition, clutch mass and size (i.e. number of eggs) were measured, and maternal investment was defined as relative clutch mass (i.e. the ratio of clutch mass to maternal postoviposition body mass; Shine, 1992). Clutches were incubated in the laboratory under standardized conditions, at 21 °C during the day (0900–2100 hours) and 19 °C at night (for details see San-Jose, Peñalver-Alcázar, Milá, Gonzalez-Jimena, & Fitze, 2014).

While in the laboratory, all lizards were provided with food every 3 days (*Galleria mellonella*, Pyralidae), and with water ad libitum. Light and heat were provided by a 40 W light bulb between 0900 and 1800 hours; between 1300 and 1500 hours ultraviolet (UV)-B light was also provided. Terrariums were sprayed with water twice a day for continuous humidity. Seminatural enclosures were located at the research station ‘El Boalar’ (40°33′N, 0°37′W, 700 m above sea level) of the Instituto Pirenaico de Ecología. Each enclosure (100 m^2) contained natural vegetation and prey, hides, rocks, logs and ponds, and was surrounded by escape-proof walls and covered with nets to prevent predation (San-Jose et al., 2014).

To disentangle sperm storage and remating with the same male in the unrestricted treatment, we performed a confirmatory experiment in 2011. Females received the same conditions as unrestricted females until oviposition, and thereafter were released in a new population containing only unknown males. The duration of the exposure to males thus did not differ from that of unrestricted females. If remating is the norm, males from the first mating event would fertilize few eggs (if any) in the second clutch, while a high proportion of fertilizations by males from the first mating event would indicate the importance of sperm storage.

Determination of the Number of Copulations

The number of copulations was measured by counting mating scars on the belly of all females (Fitze et al., 2005), before they laid the first clutch (2nd and 3rd week of May) and the second clutch (4th week of June). To corroborate that scars are a good proxy for copulations, the persistence of fresh mating scars and their colour change were examined in 2007. Here, 22 females were mated to different males during standardized mating trials (see above), and mating date and the number of mates were recorded. Females were thereafter examined every 1–2 weeks, and scar presence and scar colour, classified as light blue, dark blue, black, grey and light grey (corresponding to the natural order of colour change of mating scars in *Z. vivipara*; personal observation), were determined.

Determination of Sperm Presence

The seasonal pattern of sperm presence in males was analysed after the production of the first clutch, to determine the availability of fertile mates before the production of a second clutch. To this end, 31 adult males (SVL $\geq$ 48 mm) were captured in May 2009 and maintained in the laboratory. Every 2–3 weeks, males were examined to determine the presence of sperm in their epididymis (Courty & Dufaure, 1980), by gently pressing on the sides of the cloaca, to extract a droplet of seminal liquid. The extracted droplet was collected with a micropipette and directly suspended at a 1:10 ratio in Tyrode's medium (136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$ 0.4 mM NaH_2PO_4, 5.5 mM glucose). The suspension was gently mixed, aliquots were examined under a microscope, and sperm presence or absence was recorded. This noninvasive method was used to avoid killing the lizards; the method's accuracy has been previously validated (Breedveld & Fitze, 2016a).

Paternity Assignment

We collected tail tips (ca. 2 mm) from mothers, putative fathers, juveniles and dead embryos. Tissue samples were stored in 70% ethanol, and frozen at −96 °C until analyses. DNA was extracted using BioSprint 96 DNA Blood Kit (Qiagen, Hilden, Germany). Paternity was established using four to six polymorphic microsatellite DNA loci (Lv-3–19, Lv-4–72, Lv-4-alpha, Lv-2-145, Lv-4-X and Lv-4–115; Boudjemadi, Martin, Simon, & Estoup, 1999). Genetic fathers were attributed using Cervus 3.0, and attributions were controlled one by one (Fitze et al., 2005; Marshall, Slate, Kruuk, & Pemberton, 1998). Detailed methods of DNA extraction, polymerase chain reaction and allele size determination are described elsewhere (Laloi, Richard, Lecomte, Massot, & Clobert, 2004).

Ethical Note

The work complies with Spanish laws and was performed in line with the ABS/ASAB guidelines for ethical treatment of animals and under licences from the governments of Aragón (LC/mp/2011/577) and Navarra (permit 2009/2010). At the end of the study, all lizards were released in seminatural populations. No adverse effects of toe clipping or tail tip collection were found.

Statistics

All initial models of female reproductive success, timing and investment for both first and second clutches contained treatment (i.e. restricted versus unrestricted females) as a factor, SVL as a covariate and their interaction. The probability of producing a first or second clutch, the probability that these contained viable eggs (i.e. $\geq$1 hatched and alive juvenile), and the proportion of viable eggs within each clutch were analysed using generalized linear models (GLM) with binomial error and a logit link (quasibinomial GLM if there was overdispersion). Clutch size and oviposition date were analysed using GLMs with Poisson family and a log link (quasi-Poisson if there was overdispersion), and maternal investment using a linear model.

The number of mating scars before the first clutch and the date on which scars were grey for the first time (i.e. no longer new) were analysed using quasi-Poisson GLMs with treatment as a factor. In the latter, number of mates, date of last copulation and total copulation duration were included as covariates.

The number of sires in first and second clutches was analysed using a quasi-Poisson GLM with treatment as a factor. The proportion of eggs sired in the first and second clutch by the main sire of the first clutch was analysed using a generalized linear mixed model (GLMM) with treatment and clutch number (first or second clutch) as factors, their interaction, and female identity as a random effect. The proportion of eggs sired in the second clutch by males siring eggs in the first clutch was analysed using a linear model with treatment as a factor.

Male sperm presence was analysed using a GLMM with the proportion of males with sperm present as the dependent variable, a binomial error and a logit link. Treatment was included as a factor, day and day squared as covariates, and male as a random effect. The probability that a male fertilized at least one egg was analysed using a binomial GLM with treatment as a factor. The number of females with which a male had offspring, the number of eggs fertilized by a male and the average number of eggs fertilized per mate partner were analysed using quasi-Poisson GLMs with treatment as a factor. Post hoc tests were adjusted for repeated testing, and adjusted P values (P_{adj}) are reported.

Model selection was performed using likelihood ratio tests and nonsignificant terms were backward eliminated ($P > 0.05$). Data were analysed using R 3.0.0 (R Core Team, 2013).

RESULTS

Female Reproductive Success

Most females produced a first clutch (unrestricted: 93%; restricted: 98%). Their clutches contained 3.04 ± 0.15 (mean ± SE) and 3.66 ± 0.17 eggs, respectively. Most clutches had viable eggs (unrestricted: 75%; restricted: 86%) and 63 ± 8% and 80 ± 5% of eggs per clutch were viable, respectively. Treatment did not affect the probability of producing a first clutch, clutch size, the probability that clutches contained viable eggs and the proportion of viable eggs (no significant main and interactive effect; Table 1). SVL was significantly and positively associated with clutch size (estimate ± SE: 1.041 ± 0.007 eggs), the probability that clutches contained viable eggs (0.266 ± 0.089), and the proportion of viable eggs (0.275 ± 0.068; Table 1).

Females less frequently had second clutches (unrestricted: 30%; restricted: 16%) and clutch size was on average 3.22 ± 0.40 and 3.86 ± 0.41 eggs, respectively. For unrestricted females 89% of second clutches contained viable eggs, but for restricted females only 43%, and 79 ± 12% and 34 ± 18% of eggs per clutch were viable, respectively. The probabilities of laying a second clutch and that second clutches contained viable eggs were significantly higher in unrestricted than restricted females, and there was a trend that the proportion of viable eggs was higher in unrestricted females (Table 2). SVL was significantly and positively associated with the probability of laying a second clutch (0.186 ± 0.085; Table 2).

Reproductive Timing and Maternal Investment

Average oviposition date of the first clutch was 8 June ± 1.3 days (range 19 May–27 July). It was significantly affected by an interaction between treatment and SVL ($F_{1,68} = 16.098$, $P < 0.001$; Fig. 1). In unrestricted females, the day of oviposition was negatively associated with SVL, and in restricted females no significant relation existed (Fig. 1). Average oviposition date of second clutches was 8 July ± 1.9 days (range 20 June–23 July), and there were no significant differences between restricted and unrestricted females ($F_{1,14} = 0.055$, $P = 0.818$; interaction SVL$*$treatment: $F_{1,12} = 0.218$, $P = 0.649$).

Maternal investment (i.e. relative clutch mass) during the first clutch was on average 0.34 ± 0.01 (range 0.07–0.62), and increased significantly with female SVL ($F_{1,69} = 10.565$, $P = 0.002$, estimate: 0.009 ± 0.003). Treatment and its interaction with SVL were not significant (all $P > 0.1$). In the second clutch, average maternal investment was also 0.34 ± 0.03 (range 0.12–0.55), and none of the parameters was significant ($F \leq 1.3$, $P \geq 0.27$).

Table 1
Reproductive success during first clutches

Trait	Treatment	SVL	Treatment∗SVL
Pr of producing eggs	χ^2_1=0.231, P=0.631	χ^2_1=1.362, P=0.243	χ^2_1=0.777, P=0.378
Clutch size	$F_{1,69}$=0.402, P=0.528	$F_{1,70}$=34.020, ***P*<0.001**	$F_{1,68}$=3.066, P=0.084
Pr of producing viable eggs	χ^2_1=0.044, P=0.833	χ^2_1=11.488, ***P*<0.001**	χ^2_1=0.522, P=0.471
Proportion of viable eggs	$F_{1,69}$=0.045, P=0.834	$F_{1,70}$=21.286, ***P*<0.001**	$F_{1,68}$=0.156, P=0.694

Reproductive success with respect to treatment and snout-to-vent length (SVL). Probabilities (Pr), clutch size and proportion were modelled as dependent variables. Significant effects are depicted in bold.

Table 2
Reproductive success during second clutches

Trait	Treatment	SVL	Treatment∗SVL
Pr of producing eggs	χ^2_1=6.151, ***P*=0.013**	χ^2_1=5.545, ***P*=0.019**	χ^2_1=0.473, P=0.492
Clutch size	$F_{1,14}$=2.448, P=0.140	$F_{1,13}$=0.004, P=0.950	$F_{1,12}$=2.169, P=0.167
Pr of producing viable eggs	χ^2_1=4.035, ***P*=0.045**	χ^2_1=0.035, P=0.851	χ^2_1=0.499, P=0.480
Proportion of viable eggs	$F_{1,14}$=4.319, P=0.057	$F_{1,13}$=0.645, P=0.436	$F_{1,12}$=0.008, P=0.931

Reproductive success with respect to treatment and snout-to-vent length (SVL). Probabilities (Pr), clutch size and proportion were modelled as dependent variables. Significant effects are depicted in bold.

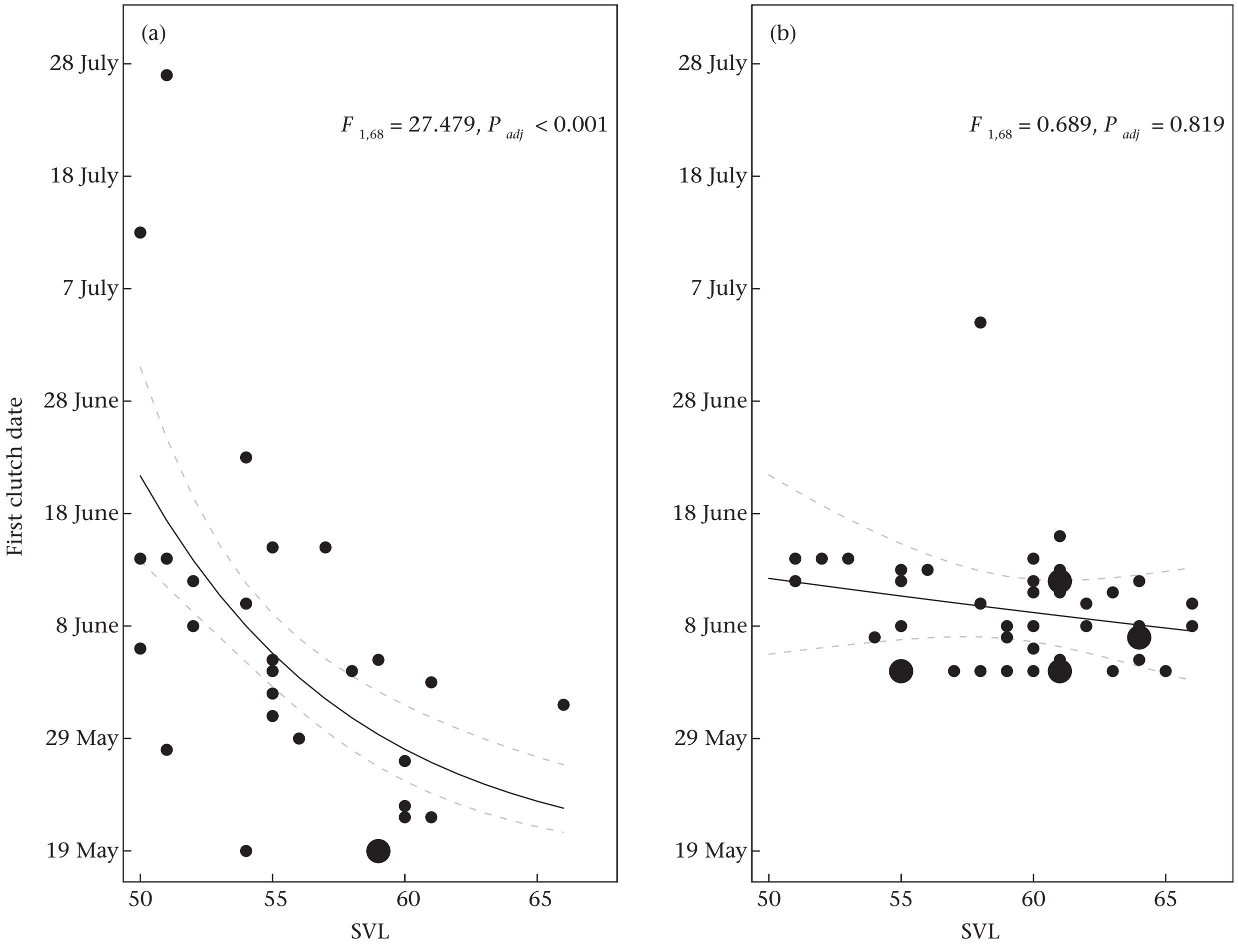

Figure 1. Date of oviposition of the first clutch in relation to female SVL in (a) unrestricted females (i.e. females with long exposure to mates) and (b) restricted females (i.e. short exposure). Raw data (small dots: $N = 1$; large dots: $N = 2$), model predictions (solid lines) and 95% confidence intervals (dashed lines) are shown.

Number of Copulations

Before laying the first clutch, 97% of the unrestricted and 96% of the restricted females exhibited mating scars (blue, black or grey). The number of scars was significantly higher in unrestricted females ($F_{1,73} = 53.855$, $P < 0.001$; mean ± SE for unrestricted and restricted females, respectively: 9.4 ± 1.2, 3.2 ± 0.2). Before laying the second annual clutch, 77% of the unrestricted and none of the restricted females exhibited black or grey mating scars.

Scar examination revealed that a few minutes up to 1 h after copulation, light blue mating scars appeared on the female's belly. These mating scars changed to dark blue on average within 3.2 ± 0.4 days (range 0–6 days), and from dark blue to black on average within 11.1 ± 0.4 days (range 8–14 days) after mating, i.e. blue scars persisted for a maximum of 2 weeks. Thereafter, black scars started to fade and became grey and light grey before they disappeared. Grey scars were observed on average 21.5 ± 1.2 days after the last mating. The first date on which scars were grey depended on the date of last copulation ($\chi^2_1 = 7.721$, $P = 0.005$, estimate: 1.075 ± 0.028 days). The number of times a female mated, the number of different males with which she mated and the copulation duration were not significant (all $P > 0.1$). All scars were light grey and most of them were very hard to spot 50.9 ± 2.2 days (range 30–60 days) after the last mating.

Fertilization Patterns

The average number of sires in the first and second clutch was 1.5 ± 0.1 and 1.4 ± 0.2, respectively. In both clutches, the number of sires did not differ significantly between restricted and unrestricted females ($P > 0.1$). In unrestricted females, 37.5% of the fertilized second clutches were at least partly sired by males that sired eggs in the first clutch, and in restricted females, 100% of the second clutches were fertilized by sires of the first clutch.

In the first clutch, the proportion of eggs fertilized by the main sire did not differ between restricted and unrestricted females ($\chi^2_1 = 0.008$, $P_{adj} = 1$). In the second clutch, the main sire of the first clutch fertilized a smaller proportion of eggs in unrestricted than in restricted females ($\chi^2_1 = 12.047$, $P_{adj} = 0.001$), and the interaction between clutch number and female treatment (i.e. restricted versus unrestricted) was significant ($\chi^2_1 = 5.561$, $P = 0.018$; Fig. 2). Moreover, the proportion of second clutch eggs fertilized with sperm from males siring eggs of the first clutch was significantly lower in unrestricted than restricted females ($F_{1,9} = 6.491$, $P = 0.031$). This difference may stem from differences in sperm storage among restricted and unrestricted females or may be due to remating of unrestricted females. In the confirmatory experiment, where females could remate only with different males from before producing the first clutch, 96% of the 23 second clutches contained eggs fertilized by sperm obtained from remating, 87% of the second clutches were exclusively fertilized by sperm obtained from remating, and 96% of the 75 second clutch eggs were fertilized by sperm obtained from remating. Thus, in the confirmatory experiment only 4% of the second clutch eggs were fertilized with sperm obtained prior to the production of the first clutch (i.e. stored sperm), while in restricted females, sperm obtained prior to the production of the first clutch fertilized 33% of the second clutch eggs. This difference was statistically significant ($F_{1,26} = 9.165$, $P = 0.006$).

Male Sperm Presence and Reproductive Success

Over the largest part of the study period (30 May–18 July), we detected males that had sperm in the epididymis. The proportion of males with sperm was significantly affected by a quadratic relationship with day (linear term: $\chi^2_1 = 4.561$, $P = 0.033$; quadratic term: $\chi^2_1 = 18.254$, $P < 0.001$; Fig. 3). Sperm was on average present in 81% of the males until 24 June (range 77–87%) and thereafter declined. By the end of July, none of the males had sperm.

The probability that males fertilized at least one egg (during first and/or second clutches) did not differ significantly between males presented to restricted and unrestricted females ($\chi^2_1 = 1.114$, $P = 0.291$). Similarly, males presented to restricted and unrestricted females did not differ significantly in the number of mate partners, the total number of eggs fertilized and the average number of eggs fertilized per female (all $P > 0.1$). Males that fertilized eggs in both first and second clutches of unrestricted females fertilized significantly more eggs than males that only fertilized eggs in the first or second clutch of unrestricted females ($\chi^2_1 = 27.912$, $P < 0.001$; mean ± SE for the former and the latter group, respectively: 7.0 ± 1.6, 2.2 ± 0.3).

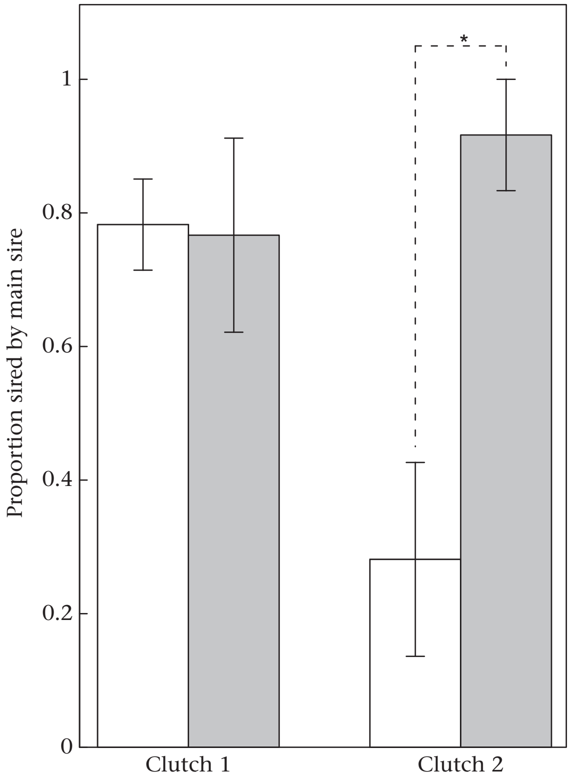

Figure 2. Proportion of eggs sired by the main sire of the first clutch, in the first and second clutch, in relation to female treatment. Grey bars represent clutches of restricted females, i.e. females with short exposure to males before producing the first clutch and no access to mates before producing the second clutch. White bars represent clutches of unrestricted females, i.e. females with long exposure to males before producing the first clutch and unrestricted mate availability before producing the second clutch. An asterisk indicates a significant contrast.

DISCUSSION

Behavioural reproductive strategies in response to the availability of mates can be of great relevance for an individual's reproductive success (Kvarnemo & Ahnesjö, 1996). However, evidence is scarce for flexible (facultative) reproductive strategies within reproductive seasons and with respect to mate availability, and experiments unravelling the determinants of producing an additional seasonal brood are very limited (Verhulst et al., 1997). Here, we experimentally manipulated the duration of mate availability before the first annual reproductive event, and mate availability per se (i.e. absence/presence of mates) before the second annual reproductive event, and tested their effects on multiple breeding strategies and reproductive success, using the common lizard as a model species.

Treatment did not significantly affect the probability of producing a first clutch and clutch size, and restricted females exhibited fewer mating scars than unrestricted females (prediction 1). The latter result shows that reduced mate availability led to significantly fewer copulations. Moreover, clutch viability, the

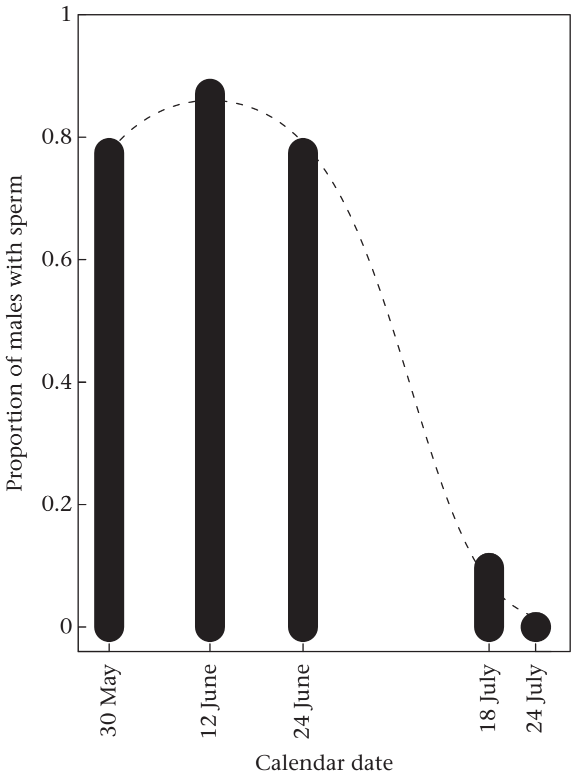

Figure 3. Sperm presence measured in males throughout the reproductive season. Black bars represent raw data and the dashed line corresponds to the model prediction.

number of sires and maternal investment did not differ between restricted and unrestricted females. This indicates that females of both treatments received enough sperm to fertilize their eggs, and thus that the duration of the availability of males ready to mate did not directly affect the success of first clutches. It also suggests that the number of males ready to mate is of reduced importance for female reproductive success as long as one male fertilizes the eggs. The increased number of copulations in females with prolonged exposure to males (unrestricted females), and thus prolonged exposure to intersexual competition, and the absence of fitness benefits (i.e. no difference between restricted and unrestricted females) are therefore in line with an intersexual conflict over mating rates, where the female's optimal number of copulations is lower than that of males (Arnqvist & Rowe, 2005). The reproductive success of males exposed to restricted and unrestricted females did not differ, despite the differences in intrasexual competition among males (males presented to restricted females faced fewer potential mates than those exposed to unrestricted females), which contrasts with prediction 2. Thus, the duration of mate availability had no direct effects on female or male reproduction during the first seasonal clutch. However, the duration of mate availability did affect oviposition date (Fig. 1). Specifically, in the unrestricted treatment there was a significant and negative association between oviposition date and female SVL, i.e. larger females laid clutches earlier, while no significant relation existed in restricted females. After copulating for the first time, female *Z. vivipara* are receptive for a confined period (Breedveld & Fitze, 2015) and oviposition date is tightly linked with the date of a female's first annual copulation. This points to earlier mating of larger females and thus to size-dependent intrasexual competition among females, which is in line with their greater sexual attractiveness to males (i.e. due to higher fecundity; Andersson, 1994; Shine, 1988), their higher competitiveness in intrasexual competition and their bigger fat stores upon emergence from hibernation (Avery, 1974; Bauwens & Verheyen, 1985). Delayed oviposition can entail negative fitness consequences through reduced survival of later-hatched young caused, for example, by increased competition with other hatchlings or reduced time left to prepare for hibernation (Olsson & Shine, 1997b). This suggests that the duration of access to mates may indirectly affect Darwinian fitness.

In line with prediction 3, the probability of laying a second clutch and the proportion of clutches with viable eggs were significantly lower in restricted than unrestricted females. In the former, 43% of second clutches contained viable eggs, confirming that females can store sperm for at least 2.4 months. However, the important reduction in reproductive success of restricted relative to unrestricted females (clutches containing viable eggs: −46%; viable eggs per clutch: −45%) indicates that not enough sperm could be stored, that not enough sperm survived until fertilization or that not all females were able to store sperm. In fact, 77% of the unrestricted (and none of the restricted) females exhibited black or grey mating scars, suggesting that most of the unrestricted females remated before producing a second clutch. Since the recapture before laying the second clutch occurred approximately 56 days after the first annual copulation, the observed scars did not originate from the start of the season (scars disappear 30–60 days after mating). Moreover, the time between female release (after laying the first clutch) and recapture was 16 ± 1 days, and thus scars originating from remating would indeed still be black or grey (scars become grey 16–38 days after mating).

The observed mating scars are thus in line with remating by unrestricted females. The results from the confirmatory experiment are fully congruent with this conclusion, since 96% of the females remated after laying their first clutch. Only 4% of the second clutch eggs were fertilized by stored sperm, which is in line with prediction 5, i.e. that second clutches are more likely to be fertilized with fresh sperm. Restricted females, which had no access to mate partners after first clutch oviposition, were less likely to produce a second clutch, and those that did so paid the costs of reduced clutch viability. This shows that producing a second brood without remating is suboptimal, pointing to a trade-off between producing one or multiple broods per reproductive season (Roff, 1992) that will depend on the availability of males ready to mate. The fact that restricted females were less likely to produce a second clutch also points to the existence of facultative alternative reproductive strategies (Verhulst et al., 1997). Besides mate availability, female body size positively predicted the production of a second clutch (Table 2). Larger females generally have more resources to allocate (Avery, 1975; Bleu et al., 2013) and are more efficient and more dominant foragers (González-Suárez et al., 2011), potentially explaining why they were more likely to produce a second clutch. Alternatively, larger and hence older females are more likely to die and they may thus carry out terminal investment (Clutton-Brock, 1984). Interestingly, female body size did not affect the size or viability of second clutches (Table 2), suggesting that larger and hence older females may maximize their fitness by increasing their reproductive effort before death.

In restricted females, the main sire of the first clutch sired eggs in 100% of the fertilized second clutches, and on average 92% of the fertilized eggs (range 75–100%), a similar percentage as in the first clutch (Fig. 2). This indicates that females may have preferred sperm from the same male, or that the most abundant or most competitive sperm fertilized the eggs of both clutches. In unrestricted females, the main sire of the first clutch sired eggs in 37.5% of the fertilized second clutches, and on average only 28% of the fertilized eggs (range 0–100%; Fig. 2). Moreover, in the confirmatory experiment only 4% of all second clutch eggs were fertilized with stored sperm, and the proportion of eggs per clutch fertilized by stored sperm was significantly lower than in restricted females. These results are in line with predictions 5 and 6; fresh sperm is more likely to fertilize eggs of second clutches and remating reduces the reproductive success of first mates, the latter indicating that remating by females is costly for males (Birkhead & Møller, 1993; Price et al., 1999). The fact that 28% of the unrestricted females' second clutch eggs were fertilized by males that fertilized eggs in her first clutch, and only 4% of the confirmatory females' second clutch eggs were fertilized with stored sperm, suggests that up to 37.5% of the unrestricted females remated with the main sire of the first clutch and thus that remating at the second clutch generally occurs with a different male. In addition, males that fertilized eggs of an unrestricted female's first and second clutch, fertilized significantly more eggs than those fertilizing eggs in one clutch only. This indicates that remating by males can increase male fitness, potentially favouring male strategies that increase their probability and success of remating. Sperm presence was high in males until the end of June (Fig. 3), when most females had already laid their first clutch (mean oviposition date 8 June), and rapidly declined thereafter. Remating thus happened when males still had sperm, in line with male strategies maximizing the success during remating and the coevolution of male and female reproductive strategies. Interestingly, even during the early mating season around 20% of the males had no sperm (Fig. 3), suggesting that multiple mating by females may have evolved to guard against male infertility (Olsson & Shine, 1997a).

In summary, our results show that the production of one or multiple broods per season is affected by mate availability. More *Z. vivipara* females skipped the production of a second brood when access to mates was restricted, pointing to the existence of facultative multiple breeding (Verhulst et al., 1997). Facultative skipping of a second brood may allow individual females to preserve energy for future reproduction (Le Galliard, Cote, & Fitze, 2008; Roff, 1992) when the reproductive benefit is low. Moreover, remating by females crucially improved their reproductive success, indicating that a lack of mates entails negative fitness consequences not only in first (Bleu et al., 2011), but also in second clutches. Species of many taxa produce several annual broods, including birds, reptiles, mammals, fish and insects (Verhulst et al., 1997), and isolation between mates is increasingly observed (e.g. due to anthropogenic influences or climatic conditions; Lane, Forrest, & Willis, 2011). Our results suggest that females exhibit alternative mating strategies, since the production of multiple annual broods is, at least to a certain extent, facultative. These findings point to a sophisticated behavioural repertoire that may have evolved as an adaptation to variable breeding conditions, including variable climatic conditions and mate availability. The facts that many iteroparous species can reproduce several times per year, that many species produce multiple annual broods in some but not in all years and that not all individuals produce multiple annual broods suggest that the alternative reproductive strategies detected here may be much more widespread than previously suggested.

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