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

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2 **annual clutches**

3

4 **ABSTRACT:** Females of many iteroparous species face trade-offs between
5 producing one or multiple broods per reproductive season, and over fertilizing
6 broods with sperm from the same or different mates. Both trade-offs might be
7 affected by the availability of males (i.e. absence/presence of males) and the
8 timing and duration of male encounters.

9 Here, we experimentally manipulated the duration of mate availability at
10 the first brood and mate availability *per se* (i.e. absence/presence of mates) at
11 the second brood, and tested their effects on female and male reproductive
12 success, using the common lizard, *Zootoca vivipara*, as a model species. Females
13 were either exposed to males for a long time-span before their first annual
14 reproduction and they could re-mate before their second annual reproduction
15 (unrestricted treatment). Alternatively, they were exposed to males for a short
16 time-span before their first annual reproduction and they were not allowed to
17 re-mate (restricted treatment). Reproductive success of first clutches was not
18 directly affected by the duration of access to males. Re-mating positively affected
19 the probability of producing a second clutch, and the proportion of viable
20 offspring. Re-mating by females also affected the reproductive success of males:
21 fewer second-clutch eggs were fertilized with stored sperm in unrestricted
22 compared to restricted females. Sperm presence in males was high until the end
23 of the re-mating period.

24 Our results suggest a close coevolution between male and female
25 reproductive strategies and they point to facultative skipping of second broods

when fitness benefits are small. This shows that behavioural strategies are at least partially responsible for the production of multiple annual broods. The detected behavioural strategies are likely 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.

KEYWORDS: facultative multiple breeding, lizard, mate availability, polygamy, sperm storage, *Zootoca vivipara*

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INTRODUCTION

Females of iteroparous species face investment trade-offs between different reproductive events (Roff, 1992), i.e. trade-offs between current and future reproduction (Richner & Tripet, 1999). In species where 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 or not to produce an additional seasonal brood might be triggered 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

51 especially important if females cannot store sperm from a previous copulation,
52 or if stored sperm quality and/or quantity decline over time and thus
53 replenishment of the sperm stores is required to optimally reproduce (Birkhead
54 & Møller, 1993). Under suboptimal conditions a reproductive attempt may fail.
55 For example, it may fail due to a shortage of potential mates or successful
56 copulations (Courchamp, Clutton-Brock, & Grenfell, 1999), or due to incorrect
57 timing of encountering potential mates (Calabrese & Fagan, 2004). In such a
58 situation, reducing the energy allocated to a reproductive event (Roff, 1992), or
59 completely skipping the production of an additional seasonal brood (i.e.
60 facultative multiple breeding) may be the optimal reproductive strategy
61 (Verhulst et al., 1997). Nevertheless, whether facultative multiple breeding has
62 behavioural bases remains unclear.

63 Iteroparous females may furthermore face a trade-off between fertilizing
64 broods with the sperm of the partner(s) that fertilized the previous brood (e.g.
65 through stored sperm), or with the sperm of a new partner (e.g. through re-
66 mating), and strategic sperm utilization may exist (e.g. through cryptic female
67 choice; Eberhard, 1996). Fertilizing eggs with sperm of the same partner may be
68 beneficial if he provides good genes (Andersson, 1994), and could allow females
69 to avoid costs imposed by additional copulations (Bleu, Bessa-Gomes, & Laloi,
70 2012). On the other hand, re-mating may be necessary for a female to acquire
71 sufficient sperm (Anderson, 1974), and fertilizing eggs with sperm of new males
72 may increase the genetic diversity of the offspring, or guard against infertility or
73 genetic defects of mate partners (Jennions & Petrie, 2000; Wolff & Macdonald,
74 2004). However, patterns of sperm usage are not necessarily under female
75 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; Birkhead & Møller, 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). Much fewer studies have investigated effects on successive reproductive events within a single 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 et al., 1993). This is surprising, because a large variety of organisms can reproduce multiple times per season (see references above), and the frequency of multiple breeding importantly affects population dynamics (Bestion et al., 2015; Martin, 1995). Moreover, operational sex ratio, which is determined by the availability of mates ready to mate, can affect individual 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 ready to mate 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 were either submitted to restricted or unrestricted mate availability. Restricted females had short-term access to mates before producing the first clutch, and no access to mates after laying the first clutch, i.e. they were prevented from re-mating 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 re-mating and sperm storage, and to 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 et al., 2005), and females produce up to three annual clutches (Horváthová et al., 2013). Within a reproductive season, females can store sperm over longer time-periods, and can use sperm obtained previous to the production of the first annual clutch for the fertilization of the second annual 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 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 et al., 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 of 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 re-mating) 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 re-mating, 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 re-mating 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 will 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 re-mate with the same males.

METHODS

Species description

Z. vivipara (Lichtenstein, 1823) is a small, ground-dwelling Lacertid lizard that is widely distributed throughout Eurasia, and a large degree of variation in population density and structure exists (e.g. Heulin, Osenegg-Leconte, & Michel, 1997). The average lifespan of *Z. vivipara* is 2.8 years (Strijbosch & Creemers, 1988). The maximum recorded lifespan is 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 two to three 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 et al., 2005). Mating scars initially exhibit a light blue colour, which subsequently becomes dark blue, black, and thereafter gradually fades, until disappearing completely (results herein).

A female's mating window initiates upon copulation, after which females

remain receptive for up to 20 days (Breedveld & Fitze, 2015). Females lay 1 to 3 clutches per year, and clutch size, which is positively related to female body size, ranges from 2 to 9 eggs (4.65 ± 0.3 ; mean $\pm$ SE; Horváthová et al., 2013). The mating system is polygynandrous, and eggs are fathered by 1 to 5 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 inter-population 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 et al., 2013), the encounter of mates outside a female's period of receptivity (Breedveld & Fitze, 2015), or the encounter of males that do yet not have mature sperm (Breedveld & Fitze, 2016b).

Experimental procedures

Here, we studied oviparous individuals from the Spanish Pyrenees (Milá et al., 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 a.s.l.) and Somport (42°47'N, 0°31'W, 1640 m a.s.l.). In May 2009, adult individuals 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; in mm) and body mass (in mg) were measured, and lizards were housed in individual terraria (25×15×15 cm) 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. females that did not yet copulate 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 6 different males (i.e. 6 trials) over a time-span of two days. The same male sextet was only presented to one single female. Standardized mating trials were performed between 9:00 a.m. and 6:00 p.m. in escape-proof wooden boxes (2500 cm²; Fitze et al., 2010). In each of the 6 trials, one male was presented to the female and after one hour he was removed and replaced with a new male, except when copulation was in progress. In this case, the male was replaced 5 minutes after the end of the copulation. After the mating trials, females were released in 2 semi-natural 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 6 mixed-sex semi-natural enclosures (average: $N = 5$ females per enclosure) directly after measurement, and had access to 9 or 10 males during the whole reproductive season, i.e. they were allowed to re-mate after first clutch production. Females of

both treatments thus had access to 6 to 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 post-oviposition body mass; Shine 1992). Clutches were incubated in the laboratory under standardized conditions, at 21°C during the day (9:00 a.m. - 9:00 p.m.) and 19°C at night (for details San-Jose et al., 2014).

While in the laboratory, all lizards were provided with food every three days (*Galleria mellonella*, Pyralidae), and with water *ad libitum*. Light and heat were provided by a 40-W light bulb between 9:00 a.m. and 6:00 p.m., and between 1:00 p.m. and 3:00 p.m. ultraviolet (UV)-B light was provided. Terrariums were sprayed with water twice a day for continuous humidity. Semi-natural enclosures were located at the research station 'El Boalar' (40°33'N, 0°37'W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología. Each enclosure (100 m²) contained natural vegetation and prey, hides, rocks, logs, and water ponds, and was surrounded by escape proof walls and covered with nets to prevent predation (San-Jose et al., 2014).

To disentangle between sperm storage and re-mating with the same male in the unrestricted treatment, we performed a confirmatory experiment in 2011. In this experiment, females were submitted to the same conditions as unrestricted females until oviposition, and thereafter released in a new

population containing only unknown males. The duration of the exposure to males did thus not differ from unrestricted females. If re-mating 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 laying the first clutch (2nd and 3rd week of May), and before laying 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 registered. 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 (note: this corresponds 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 mates ready to mate 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 CaCl₂, 1 mM MgCl₂, 12 mM NaHCO₃ 0.4 mM NaH₂PO₄, 5.5 mM glucose). The suspension was gently mixed, aliquots were examined under a microscope, and sperm presence or absence was recorded. This non-invasive method was used, since alternative methods would have required sacrificing lizards, and since the method's accuracy has been previously validated (Breedveld & Fitze, 2016a).

Paternity assignment

We collected tail tips (ca. 2 mm) from mothers, putative fathers and 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 4-6 polymorphic microsatellite DNA loci (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, and Lv-4-115; Boudjemadi et al., 1999). Genetic fathers were attributed using Cervus 3.0, and attributions were controlled one by one (Fitze et al., 2005; Marshall et al., 1998). Detailed methods of DNA extraction, polymerase chain reaction, and allele size determination are described elsewhere (Laloi et al., 2004).

Ethical note

The work complies with Spanish laws and was performed according to the ABS/ASAB guidelines for ethical treatment of animals and under licenses 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 semi-natural 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 vs. 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. ≥ 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 in case of overdispersion). Clutch size and oviposition date were analysed using GLMs with Poisson family and a log link (quasi-Poisson in case of 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 non-significant terms were backward eliminated ($P > 0.05$). Data were analysed using R 3.0.0 (R Core Team, 2013).

RESULTS

Female reproductive success

93% and 98% of the unrestricted and restricted females produced a first clutch. Their clutches contained 3.04 ± 0.15 (mean $\pm$ SE) and 3.66 ± 0.17 eggs. 75% and 86% of the clutches contained viable eggs; and the proportion of viable eggs (per clutch) was $63 \pm 8\%$ and $80 \pm 5\%$ in unrestricted and restricted females, 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 $\pm$ 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).

30% and 16% of the unrestricted and restricted females produced a second clutch, and clutch size of second clutches was on average 3.22 ± 0.40 and 3.86 ± 0.41 eggs. 89% and 43% of the second clutches contained viable eggs, and the proportion of viable eggs was $79 \pm 12\%$ and $34 \pm 18\%$ in unrestricted and restricted females, respectively. The probability of laying a second clutch, and the probability that second clutches contained viable eggs, were significantly higher in females from the unrestricted than from the restricted treatment, 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 June $8^{\text{th}} \pm 1.3$ days (range: 19th May – 27th 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 July $8^{\text{th}} \pm 1.9$ days (range: 20th June – 23rd July), and there were no significant differences between restricted and unrestricted females ($F_{1,14} = 0.055$, $P = 0.818$; interaction SVL $\times$ 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 significantly increased 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 ($F \leq 1.3$, $P \geq 0.27$).

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 $\pm$ 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 one hour 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 two 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 $1.4 \pm$

0.2, respectively. In both clutches, the number of sires did not significantly differ among 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 sired by males that sired eggs in 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 proportion of eggs fertilized by the main sire of the first clutch was smaller 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 vs. 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 compared to 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 re-mating of unrestricted females. In the confirmatory experiment, where females were able to re-mate but with different males than before producing the first clutch, 96% of the 23 second clutches contained eggs fertilized by sperm obtained from re-mating, 87% of the second clutches were exclusively fertilized by sperm obtained from re-mating, and 96% of the 75 second clutch eggs were fertilized by sperm obtained from re-mating. Thus, in the confirmatory experiment only 4% of the second clutch eggs were fertilized with sperm obtained previous to the production of the first clutch (i.e. stored sperm), while in restricted females, sperm obtained previous 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 (30th May – 18th 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 June 24th (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 significantly differ among 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 significantly differ 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 $\pm$ SE for the former and the latter group, respectively: $7.0 \pm 1.6, 2.2 \pm 0.3$).

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 for flexible (facultative) reproductive strategies within reproductive seasons and with respect to mate availability is

451 scarce, and experiments unravelling the determinants of producing or not
452 producing an additional seasonal brood are very limited (Verhulst et al., 1997).
453 Here, we experimentally manipulated the duration of mate availability before the
454 first annual reproductive event, and mate availability *per se* (i.e.
455 absence/presence of mates) before the second annual reproductive event, and
456 tested their effects on multiple breeding strategies and reproductive success,
457 using the common lizard as a model species.

458 Treatment did not significantly affect the probability of producing a first
459 clutch and clutch size, and restricted females exhibited fewer mating scars than
460 unrestricted females (prediction 1). The latter shows that shorter mate
461 availability led to significantly fewer copulations. Moreover, clutch viability, the
462 number of sires, or maternal investment did not differ among restricted and
463 unrestricted females. This indicates that females of both treatments received
464 enough sperm to fertilize their eggs, and thus, that the duration of the availability
465 of males ready to mate did not directly affect the success of first clutches. It also
466 suggests that the number of males ready to mate is of reduced importance for
467 female reproductive success, as long as one male fertilizes the eggs. The
468 increased number of copulations in females with prolonged exposure to males
469 (unrestricted females) and thus, prolonged exposure to inter-sexual competition,
470 and the absence of fitness benefits (i.e. no difference among restricted and
471 unrestricted females) is therefore in line with an inter-sexual conflict over
472 mating rates, where the female's optimal number of copulations is lower than
473 that of males (Arnqvist & Rowe, 2005). The reproductive success of males
474 exposed to restricted and unrestricted females did not differ, despite the
475 differences in intra-sexual competition among males (males presented to

restricted females faced less potential mates than those exposed to unrestricted females), which contrasts to prediction 2. Thus, the duration of mate availability had no direct effects on female nor 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 time 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 intra-sexual 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 intra-sexual 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, for example, as a consequence of increased competition with other hatchlings, or of 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. 43% of the restricted females' 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 (-46% in the proportion of clutches containing viable eggs and -45% in the proportion of viable eggs per clutch) 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 restricted) females exhibited black or grey mating scars, suggesting that most of the unrestricted females re-mated 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 passed from female release (after laying the first clutch) until recapture was 16 ± 1 days, and thus scars originating from re-mating would indeed still be black or grey (note: scars become grey 16 to 38 days after mating).

The observed mating scars are thus in line with re-mating by unrestricted females. The results from the confirmatory experiment are fully congruent with this conclusion, since 96% of the females re-mated 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 nevertheless produced a second clutch paid the costs of reduced clutch viability. This shows that producing a second brood without re-mating 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 re-mating reduces the reproductive success of first mates, the latter indicating that re-mating 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 re-mated with the main sire of the first clutch and thus, that re-mating 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 re-mating by males can increase male fitness, potentially favouring male strategies that enhance their probability and success of re-mating. 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: 8th of June), and rapidly declined thereafter. Re-mating thus happened when males still exhibited sperm presence, in line with male strategies maximizing the success during re-mating 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, re-mating 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 data 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 variable mate availability. The fact 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, suggests that the here detected alternative reproductive strategies may be much more widespread than previously suggested.

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FIGURE CAPTIONS

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.

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 delimitate 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 delimitate 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. Significant contrasts are depicted with an asterisk.

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.

TABLES

Table 1.

Reproductive success during first clutches.

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

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.

851 **Table 2.**

852 Reproductive success during second clutches.

Trait	Treatment	SVL	Treatment × SVL
Pr of producing eggs	$\chi^2_1 = 6.151, P = \mathbf{0.013}$	$\chi^2_1 = 5.545, P = \mathbf{0.019}$	$\chi^2_1 = 0.473, P = 0.492$
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	$\chi^2_1 = 4.035, P = \mathbf{0.045}$	$\chi^2_1 = 0.035, P = 0.851$	$\chi^2_1 = 0.499, P = 0.480$
Proportion of viable eggs	$F_{1,14} = 4.319, P = 0.057$	$F_{1,13} = 0.645, P = 0.436$	$F_{1,12} = 0.008, P = 0.931$

853 Reproductive success with respect to treatment and snout-to-vent length (SVL).

854 Probabilities (Pr), clutch size, and proportion were modelled as dependent variables.

855 Significant effects are depicted in bold