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A MATTER OF TIME!

TIME DEPENDENT MATING DECISIONS IN THE COMMON LIZARD, *ZOOTOCA VIVIPARA*

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To my family

TABLE OF CONTENTS

GENERAL INTRODUCTION & OBJECTIVES

GENERAL METHODS

RESULTS

GENERAL DISCUSSION & CONCLUSIONS

SUMMARY

Life-history theory predicts that reproductive traits and behavioral strategies displayed by an organism should vary strategically with respect to the intrinsic and extrinsic conditions it is exposed to. Condition-dependent strategies may allow an individual to enhance its reproductive success, a crucial component of Darwinian fitness. Such strategies are predicted to vary with mate availability and the time of mate encounter, which may determine Darwinian fitness, especially in species where reproduction is restricted to annually defined seasons. Moreover, an individual's intrinsic conditions (e.g., its reproductive stage) at the time of encounter, may determine its mating propensity. It may thus be beneficial to adjust mating decisions, including mate acceptance thresholds and the amount of resources invested into an accepted mate, in response to when mates are encountered.

Studies that determine how the timing of mate encounter affects individual mating decisions are currently scarce. In this thesis we experimentally investigate whether and how mating decisions are affected by the time of mate encounter, and determine the consequences of potential strategies on reproductive success, using the common lizard, *Zootoca vivipara*, as a model species. Effects of the time of mate encounter on mating propensity, the degree of mate choice expressed, investment during mating, and the production of multiple seasonal broods are demonstrated. The results point to strategic, time dependent, mating decisions, which can importantly affect an individual's reproductive success. Moreover, they provide evidence that the detected mating decisions may mitigate predicted negative effects of reduced mate encounter on reproduction and, ultimately, on population viability.

The findings demonstrate that time dependent strategies may allow individuals to increase reproductive success and they might be more important in low-density populations. Processes such as habitat fragmentation and climate change affect population density, and thus can have negative effects on reproductive success and population viability. Moreover, temporal variation in mate availability exists in a wide range of species. Therefore, the results presented in this thesis may contribute to improving predictions of how species may respond to changes in population demography and climate change, and could provide crucial insight for conservation efforts.

PRESENTACIÓN

La teoría de las estrategias vitales predice que los caracteres reproductivos y los comportamientos relacionados con la reproducción exhibidos por un organismo deberían variar en consonancia con las condiciones intrínsecas y extrínsecas a las que está expuesto el organismo. Las estrategias vitales dependientes de la condición pueden permitir a un individuo mejorar su éxito reproductivo, un elemento crucial de la eficacia darwiniana (el «fitness»). En este contexto, se predice que estas estrategias varían en función de la disponibilidad de pareja y del momento en el que se produce el encuentro macho-hembra; dos situaciones que podrían determinar la eficacia darwiniana, especialmente en aquellas especies cuya reproducción está restringida a una época concreta del año. Es más, las condiciones intrínsecas de un individuo (por ejemplo, su estadio reproductivo) en el momento del encuentro con la pareja puede determinar su tasa de acoplamiento. Teniendo todo esto en cuenta, regular las decisiones de emparejamiento, incluyendo los niveles de aceptación de pareja y la cantidad de recursos invertidos en una pareja aceptada, puede ser beneficioso en respuesta a cuando se produce el encuentro macho- hembra.

Los estudios que examinan cómo el momento del encuentro macho-hembra afecta a las decisiones reproductivas de los individuos son aún escasos. La presente tesis doctoral aborda de forma experimental el estudio de si las decisiones de emparejamiento están influidas por el momento del encuentro macho-hembra y cómo estas decisiones se ven afectadas por este encuentro, y determina las consecuencias de la existencia de potenciales estrategias en el éxito reproductivo, empleando como especie modelo la lagartija de turbera, *Zootoca vivipara.* A lo largo de los capítulos que constituyen esta tesis se demuestra el efecto del momento del encuentro macho-hembra en la tasa de acoplamiento, el grado de selección de pareja expresado, la inversión durante el emparejamiento y la producción de múltiples puestas a lo largo de la época de reproducción. Los resultados señalan la existencia de decisiones estratégicas de emparejamiento dependientes del momento en el que se produce el encuentro macho-hembra, que pueden afectar de manera significativa al éxito reproductivo del individuo. Es más, proporcionan indicios a cerca de que las decisiones de emparejamiento detectadas pueden reducir los efectos negativos predichos debido a una baja tasa de encuentro de pareja en la reproducción y, en último lugar, en la viabilidad poblacional.

Los descubrimientos presentados en esta tesis demuestran que las estrategias dependientes del tiempo pueden permitir a los individuos aumentar su éxito reproductivo y que estas podrían ser de gran importancia en poblaciones con una baja densidad de individuos. Procesos como la fragmentación del hábitat y el cambio climático pueden afectar a la densidad poblacional y, por tanto, estos pueden ejercer un efecto negativo tanto en el éxito reproductivo como en la viabilidad poblacional. Además, se ha descrito la existencia de una variación temporal de la disponibilidad de pareja en un gran número de especies. Por tanto, los resultados presentados en esta tesis pueden contribuir a mejorar las predicciones de cómo las especies pueden responder a cambios demográficos en sus poblaciones y a la amenaza del cambio climático y podrían proporcionar conocimientos de gran valor para los esfuerzos de conservación.

• CHAPTER 1 •

GENERAL INTRODUCTION

Life-history theory is concerned with an organism's strategic decisions over the course of its lifetime (Stearns 1992). Since Darwin's description of evolution through natural selection[1] (Darwin 1859), biologists recognize that selection favors the evolution of traits that increase an individual's fitness[2], and thereby an individual's genetic contribution to future generations. Specifically, fitness measures the reproductive success of an individual or genotype relative to the reproductive success of other individuals or genotypes in the same or in different populations (Emlen and Oring 1977). Understanding how animals optimize reproductive success is thus central to life-history theory. Moreover, it is a main concern of sexual selection[3] studies (Andersson 1994).

Since an organism's optimum actions can vary under different conditions (e.g., environmental or individual physiological conditions), fitness could be maximized if individuals exhibit flexible traits, i.e., condition-dependent life-history strategies (McNamara & Houston 1996). Theory predicts that condition-dependent reproductive strategies, including behavioral, morphological, or physiological traits displayed by an individual, should follow optimum reactions norms with respect to the extrinsic and intrinsic condition of an individual, a process known as individual optimization[4] (Perrins and Moss 1975; Pettifor et al. 1988; Kisdi et al. 1998). Studies have demonstrated patterns that are in line with these predictions. For example, collared flycatchers, *Ficedula albicollis*, in better pre-breeding nutritional state have been shown to migrate to breeding areas and initiate reproduction earlier, leading to a higher reproductive success as a result of the more favorable breeding conditions early in the season (Andersson and Gustafsson 1995). In kestrels, *Falco tinnunculus*, males that catch prey at higher rates are fathers of clutches that are laid earlier, have

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[1] Natural selection (Stearns 1992): "The process that takes place in a population of entities that vary among themselves with respect to reproduction and survival, whereby those with greater differential reproductive success have higher expected representation in future generations."

[2] Fitness (Stearns 1992): "The expected contribution of an allele, genotype, or phenotype to future generations. The fitness of genes and organisms is always relative to the other genes and organisms that are present in the population. It is also a function of the environment in which it is measured."

[3] Sexual selection (Arnqvist & Rowe 2005): "Sexual selection describes variance in mating/fertilization success that is attributable to variation among individuals in some underlying metric trait (behavioural, physiological, or morphological), while natural selection describes variance in all other fitness components. Thus sexual selection can be viewed as the subset of natural selection that concerns variance in mating/fertilization success."

[4] Individual Optimization Hypothesis (Pettifor et al. 1988): "Individuals lay that number of eggs which will maximize the number of recruits produced from a single season; individual differences in the ability to rear offspring mean that the optimum clutch differs between individuals."

more eggs, and more surviving offspring (Daan et al. 1990). And female Colombian ground squirrels, *Spermophilus columbianus*, may adjust litter size in relation to food abundance (Risch et al. 1995). Note that two individuals that follow the same condition dependent strategy can thus show different state-dependent traits. This is especially evident in coho salmon, *Oncorhynchus kisutch*, where growth rate during maturation determines which of two distinct reproductive behaviors adult males (of the same genotype) adopt; as "hooknoses" they fight for access to females and as "jacks" they sneak matings (Gross 1996).

In many animals reproduction depends on the direct sexual encounter between a male and a female, i.e., on mating. The probability of encounter between mates can be highly variable both in space and time. Primarily, both the spatial and temporal aspects of mate encounter depend on demographic factors such as population size and density, operational sex ratio[5], and connectivity between mates (Emlen and Oring 1977; Shuster and Wade 2003; Kokko and Rankin 2006). The temporal aspect of mate encounter furthermore depends on species-specific reproductive characteristics, including seasonal migration to reproductive areas, the length of the reproductive season, and the timing of male and female reproductive maturation (Calabrese and Fagan 2004; Gowaty and Hubbell 2005; Shuster 2009; Fagan et al. 2010), which determine the temporal overlap between receptive mates. Therefore, variation in the time of mate encounter may be especially pronounced in species that live in seasonal environments where mating occurs annually during defined reproductive periods, and where the availability of potential mates can show high variability (Calabrese and Fagan 2004; Shuster 2009). In addition, processes such as dispersal, mate searching strategies, and mate preferences, can influence the probability and time of mate encounter (Real 1990; Arnold and Duvall 1994; Gowaty and Hubbell 2005; Kokko and Mappes 2005; Kokko and Wong 2007; Robinet et al. 2008). Such variation in mate encounter can lead to differences in the costs and benefits of mating, as well as in the fitness payoffs of the energy invested (e.g., in terms of time or resources) into an already acquired mate (Burley 1986; Real 1990; Sheldon 2000; Reinhold et al. 2002; Gowaty and Hubbell 2009). As a result, individuals may show variation in mating decisions with respect to mate encounter (Crowley et al. 1991; Shelly and Bailey 1992; Simmons et al. 1992; Gowaty and Hubbell 2009; Dreiss et al. 2010).

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[5] Operational sex ratio (Emlen 1976): "The ratio of potentially receptive males to receptive females at any time."

The spatial effects of mate encounter on mating decisions and reproductive success have been extensively studied (e.g., Wells, Wells & Cook 1990; Veit & Lewis 1996; Boukal & Berec 2002; Kokko & Rankin 2006; Bleu, Bessa-Gomes & Laloi 2012a). For example, in high-density populations where a large number of potential mates are available, individuals are predicted to show higher mate acceptance thresholds. This is supported by studies that demonstrate an increased expression of mate preferences, i.e., choosiness, in high compared to low-density populations (e.g., Palokangas et al. 1992; Shelly and Bailey 1992; Berglund 1995; Jones et al. 2004; Lehmann 2012). At low densities the risk of remaining unmated exists, and individuals may not be able to afford rejecting un-preferred mates. At high densities, however, strong choosiness can be beneficial and it can result in a higher degree of selection on preferred traits in mates, i.e., intersexual selection (Andersson 1994). This can ultimately lead to the evolution of sexually dimorphic characteristics (traits that are preferred by/attractive to the opposite sex) that provide reproductive advantages, but not necessarily survival advantages. They may even be deleterious to survival, as highlighted by the classical example of the peacock's tail (Zahavi 1975).

Effects of temporal variation in mate encounter on mating decisions and reproductive success are also expected to exist (e.g., Parker 1970a; Crowley et al. 1991; Grant, Bryant & Soos 1995; Calabrese & Fagan 2004; Gowaty & Hubbell 2005; Løvlie & Pizzari 2007; Kasumovic et al. 2008), but have generally received far more limited attention. First of all, the probability of acceptance of potential mates, as well as the probability that mating leads to fertilization, should depend on whether mate encounter happens at the right moment in time during the male and female reproductive cycle, i.e., when they are in the mating pool and thus available and ready to mate (Kokko et al. 2012). For instance, for encounter to result in the acceptance of a mate it should occur during female receptivity, and for mating to result in fertilization it should happen during male fertility. By contrast, the incorrect timing of mate encounter could have negative fitness consequences. For example, in *Parnassius* butterflies, reproductive asynchrony between males and females (i.e., a difference in their time of reproductive maturation), resulting from differences in their time of arrival to the breeding site, can lead to reproductive failure in females (Calabrese et al. 2008). Moreover, the timing of reproduction can be a critical determinant for its success (Einum and Fleming 2000; Thomas et al. 2001). For example, in jacky dragon lizards, *Amphibolurus muricatus*, the offspring of early reproducing parents has a higher change of surviving than late offspring (Warner and Shine 2007). This highlights the existence of time constraints on reproduction, that result from the approaching end of the mating season or the time that offspring must be produced for assuring their survival, and hence for assuring parental reproductive fitness (Perrins 1970; Real 1990; Jennions and Petrie 1997). As a result of these time constraints, the time of mate encounter may drive variation in mating decisions (Gowaty and Hubbell 2009). For instance, a decline in the number of receptive mates with advancing reproductive seasons should lead to an increased mating propensity or to a lower expression of mate choice, late in the season. Some studies have shown patterns that are in line with these predictions. For example, female three-spined sticklebacks, *Gasterosteus aculeatus*, lower their acceptance thresholds when they approach their inevitable latest spawning time (Bakker and Milinski 1991); female crickets, *Gryllus lineaticeps*, lower their mate acceptance threshold when they have not heard attractive male song types recently (Beckers and Wagner Jr 2011); and in the solitary bee, *Nomadopsis puellae*, copulations between mates that encounter each other early in the daily mating period are brief, suggesting low reproductive investment, while the duration of copulation between mates that initiate copulation late in the mating period are long, suggesting high investment (Rutowski and Alcock 1980). Similarly, in species that can produce multiple broods per reproductive season, the encounter of mates after producing a first brood may importantly affect whether an individual invests in a subsequent brood or not.

Despite the potential effects of temporal variation in mate encounter on mating decisions and reproductive success, experimental studies that manipulate the timing of mate encounter and examine the effects on mating decisions are currently rare. In this thesis, the effects of temporal variation in mate encounter on male and female mating decisions and reproductive success are experimentally determined, using the common lizard, *Zootoca vivipara*, as a model species. The common lizard is a temperate species that has annual reproductive seasons, during which most females mate with multiple males, i.e., polyandry (Laloi et al. 2004), and males mate with multiple females, i.e., polygyny (Fitze et al. 2005). In the past, its distribution has rapidly expanded, and nowadays ranges from Ireland to Japan and from the Balkans to Finland (Surget-Groba et al. 2006). There exists high variation in population density and structure (e.g., Pilorge 1987; Strijbosch & Creemers 1988; Cavin 1993; Heulin, Osenegg-Leconte & Michel 1997). Moreover, studies have revealed a high degree of geographic variation in life-history traits (Sorci et al. 1996; Sorci and Clobert 1999; Lorenzon et al. 2001; Horváthová et al. 2013; Roitberg et al. 2013). Within populations, (moderate) fluctuations in population densities also exist (Bauwens et al. 1986; Pilorge 1987; Heulin et al. 1997), and life-history traits show density dependence (Massot et al. 1992; Mugabo et al. 2013). Moreover, the exact timing of the reproductive season and the phenology of reproductive cycles vary within and between populations, as a result of annual variation in climatic conditions and the species' large geographical range respectively (Saveliev et al. 2006; Roitberg et al. 2013). The within-population heterogeneity in population densities and reproductive phenology may have selected for flexibility in reproductive decisions with respect to the timing of encounter of mates. For example, in years where population density is high and mates are encountered rapidly, being choosy may be beneficial, while in years with low population density the same level of choosiness could hinder or even impede reproduction, since a suitable partner might be found very late or not at all. Therefore, if the expression of mate choice would be flexible, for instance, if individuals exhibit stronger choosiness during periods of high density and weaker choosiness during periods of low density, this could allow them to optimize reproductive success.

The broad objective of this thesis is therefore to determine the effects of variation in the time of mate encounter on mating decisions. For this purpose, various temporal aspects of mate encounter were experimentally manipulated (i.e., time of mate encounter; Fig. 1.1), including the time of first mate encounter, the time interval

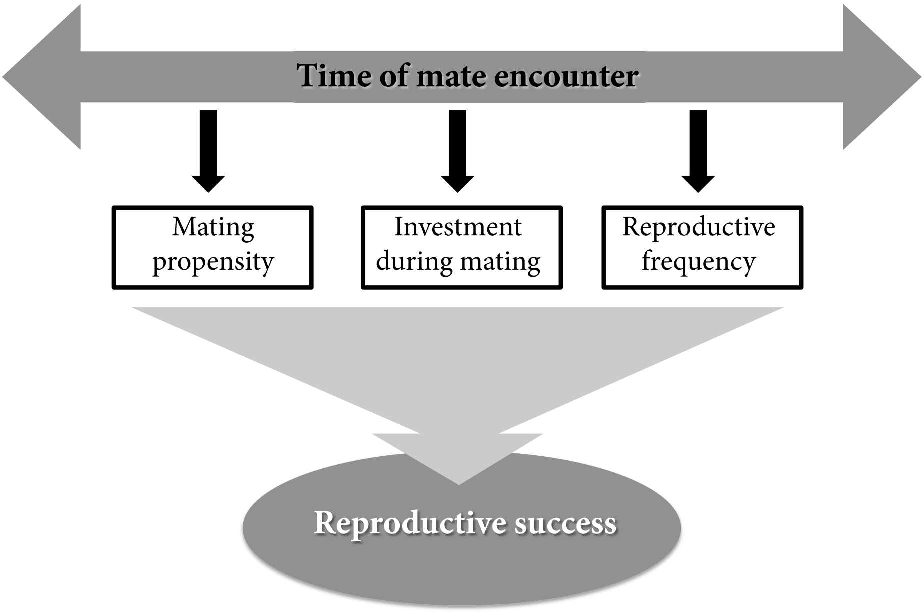

Fig. 1.1 Schematic diagram of the main components of reproductive success studied in this thesis, in response to experimental manipulations of various temporal aspects of mate encounter

between encounters, and the encounter of mates after the production of first clutches. Treatment effects on components of reproductive success, including mating propensity, investment during mating, and reproductive frequency, were determined, in order to investigate whether mating decisions vary with respect to the time of mate encounter and to identify potential flexible reproductive strategies.

In the following sections, we discuss how mating decisions of individuals could change through time due to underlying changes in their reproductive stage and their mating history. As a result, the time of mate encounter may drive variation in mating propensity (section 1.1) and investment during mating, i.e., reproductive allocation (section 1.2). In addition, we discuss how variation in reproductive frequency, i.e., the number of seasonal broods produced by an individual, may exist in response to the presence or absence of mates after the production of first broods (section 1.3). Subsequently, the specific objectives of this thesis are presented (page 39).

1.1 TEMPORAL VARIATION IN MATING PROPENSITY

1.1.1 EFFECTS OF REPRODUCTIVE STAGE

Temporal variation in mating propensity, in other words the decision of an individual to accept an encountered potential mate or not, can result from differences in an individual's reproductive stage at the time of mate encounter (Lea et al. 2000; Shuster and Wade 2003; Lynch et al. 2005). With reproductive stage, we refer to an individual's endogenous maturation stage in the reproductive cycle. This stage may determine whether an individual is fertile and/or sexually receptive at the time of mate encounter.

In females of many species, ovulation, and hence the time of female fertility, is under hormonal control and occurs spontaneously (i.e., regardless of whether mating occurs or not), and sexual receptivity is a transient state that occurs in association with ovulation (Huck et al. 1989; Becker et al. 2002). This positive relationship between receptive behavior and ovulation is known as associated reproduction (see box 1; Whittier & Crews 1987; Whittier & Tokarz 1992). Therefore, a female that encounters a potential mate around her time of ovulation might be more inclined to accept him than a female that encounters a potential mate when she is not ovulating. In line with this, the time of copulation coincides with the time of

BOX 1 | REPRODUCTIVE PATTERNS OF VERTEBRATES

Based on the classical framework established by Whittier and Crews (1987), vertebrate reproductive patterns fall into three generalized categories, which vary with respect to the degree of association between gametogenesis and the expression of mating behavior (Fig. B1; Whittier and Tokarz 1992). In the **associated reproductive pattern**, mating behavior is temporally associated with gonadal activity, i.e., copulation directly follows or coincides with maximal sex steroid secretion and gamete production. For instance, in females mating coincides with maximal follicular size, elevated sex steroid levels, ovulation, and hence fertilization. This pattern is common in species with regular and prolonged reproductive seasons and is exhibited by the majority of vertebrates (including most lizards; Lovern 2011). In the **dissociated reproductive pattern**, mating behavior is unrelated to gonadal activity, i.e., copulation is temporally dissociated from maximal sex steroid secretion and gamete production. This pattern exists in some species with regular but short reproductive seasons. For example, in female freshwater turtles, *Sternotherus odoratus,* ovulation occurs in spring, months after copulation, and fertilization is achieved through the storage of sperm in the female tract (Gist and Congdon 1998). Finally, in species exhibiting a **constant reproductive pattern**, gonads are maintained at or near maximal development during prolonged periods, and mating is initiated whenever the appropriate environmental conditions arise. Species exhibiting this pattern live in unpredictable and aseasonal environments, where favorable breeding conditions are usually very brief. Breeding must therefore occur opportunistically whenever favorable conditions arise. For instance, desert dwelling Australian zebra finches, *Taeniopygia guttata*, initiate mating within minutes of the onset of rainfall, regardless of the time of year or of hormone levels (Kabelik et al. 2010). **Note** that these reproductive patterns are represented as three mutually exclusive extremes for the sake of clarity only, as intermediate forms of these reproductive patterns are known to exist. Moreover, males and females of the same species may express different reproductive patterns. For example, in the viviparous Tasmanian skink, *Niveoscincus ocellatus*, mating occurs in the fall and is associated with spermatogenesis and plasma testosterone concentrations in males, but dissociated from ovulation, fertilization, and pregnancy in females (which occur the following spring; Jones, Wapstra & Swain 1997).

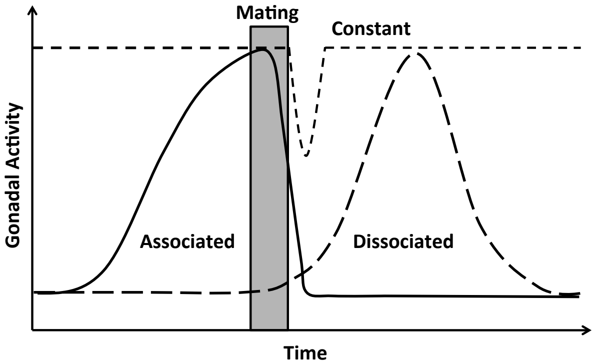

Fig. B1 Schematic representation of an associated (solid line), a dissociated (dashed line), and a constant (hatched line) reproductive pattern, modified from Whittier and Crews (1987) ovulation in many species (e.g., in mammals, birds, reptiles; Whittier & Tokarz 1992; Eberhard 1996; Becker 2002). However, in other species, such as those exhibiting dissociated or constant reproductive patterns (see box 1.1), there is no relation between ovulation and mating probability, or in other words, there is a temporal uncoupling of sexual behavior and gonadal development (Whittier and Crews 1987; Whittier and Tokarz 1992). In fact, mating may even occur long before ovulation, and hence fertilization, in species with female sperm storage (see section 1.1.2; Birkhead & Møller 1993). Besides the stage of the reproductive cycle, receptivity is sometimes affected by external stimuli, including climatic and social factors. Moreover, in some species environmental, behavioral, and physiological factors control female sexual receptivity (Crews 1975; Crews and Moore 1986; Whittier and Tokarz 1992). As a result of the cyclic nature of a female's internal reproductive stage, as well as the potential influence of external stimuli on receptivity, finite time windows of sexual receptivity, i.e., mating windows, often exist, and female mating propensity and mate acceptance thresholds may vary with respect to when mates are encountered relative to this window (Real 1990; Lynch et al. 2005; Kokko and Wong 2007).

From the male's perspective, mating behavior is, like in females, either temporally associated or not to gametogenesis (box 1.1; Whittier & Crews 1987; Whittier & Tokarz 1992). In the associated reproductive pattern, male mating behavior coincides with the time of the year that gonads are enlarged and actively producing gametes, while in the dissociated reproductive pattern, spermatogenesis occurs (long) before mating and gametes are stored until the mating period, e.g., in epididymes or spermatophores (Whittier and Crews 1987; Moore and Lindzey 1992). Furthermore, male mating propensity may or may not be affected by whether or not a male indeed carries fertile sperm. For instance, males in some species have been shown to decline potential mates or decrease the expression of mating behavior when they have depleted sperm reserves (Halliday 1976; Sæther et al. 2001). In many species, however, males will mate independent of whether they can fertilize or not (e.g., Sheldon 1994; Olsson and Madsen 1996; García-González 2004; Uller and Olsson 2005; Gomendio et al. 2007).

Protandry, defined as the earlier arrival of males to breeding areas than females (Morbey and Ydenberg 2001), is common in seasonal breeders. Among others, earlier arrival of males than females can refer to their earlier emergence from hibernation (e.g., Gregory 1982; Michener 1983; Olsson & Madsen 1996). One hypothesis for protandry is that early male arrival allows males to complete reproductive maturation before females arrive and mating starts. Therefore, whether a male carries fertile sperm at the moment of mate encounter (i.e., whether or not he is functionally fertile; Sheldon 1994) may depend on the time a male has had for sperm maturation before female arrival to breeding areas (Olsson and Madsen 1996; Olsson et al. 1999). Consequently, variation in the degree of protandry, in other words the difference in arrival time between an individual male and its female mate partner, could directly affect the fitness payoffs of mating for males and females (Morbey and Ydenberg 2001). On the one hand, males that arrive early may be better prepared than males that arrive late. This could lead to higher fitness payoffs of mating for early arriving males, as well as for the females that mate with these early arriving males (Olsson and Madsen 1996; Olsson et al. 1999; Møller et al. 2009; Canal et al. 2012). On the other hand, if males arrive late and mating occurs too soon, it may not result in fertilization due to the lack of mature sperm. The latter could result in males and females paying the costs of mating while low fitness returns are expected. Therefore, variation in mating decisions with respect to the degree of protandry could allow individuals to avoid the costs of mating during infertility. Avoidance of these costs could be achieved if, for instance, males or females would not mate unless males are fertile at the time of encounter.

1.1.2 EFFECTS OF MATING HISTORY

Besides the potential effects of an individual's reproductive stage, temporal variation in mating propensity could result from differences in an individual's mating history at the time of mate encounter (Sauter and Brown 2001; Kokko and Mappes 2005; Peretti and Carrera 2005). Mating history may refer to whether or not an individual has already mated (i.e., whether it's mating status is mated or virgin). For instance, compared to an already mated individual or an unmated individual that encounters its first potential mate early in the season (signaling high mate availability), a virgin that encounters its first potential mate late in the season (signaling low mate availability) should show a higher mating propensity, as a result of the time constraints on reproduction. Moreover, in choosy individuals, these time constraints could result in lower mate acceptance thresholds (Real 1990; Backwell and Passmore 1996; Moore and Moore 2001; Kokko and Mappes 2005); but for reverse pattern see: (Crowley et al. 1991; Qvarnström et al. 2000). Therefore, in females that have not yet mated, an increased mating propensity as well as a decreased acceptance threshold of encountered mates (i.e., decreased choosiness) with time is expected, in order to assure the acquisition of sperm before it is too late. On the other hand, females that have already mated, and thus are no longer under time pressure to fertilize their eggs, may be able to afford declining subsequently encountered mates.

Obviously, mating status has profound effects in species where individuals mate with one mate only, i.e., monogamy, since mated individuals will simply not re-mate. But also in multiple mating[6] or polygamous[7] species, mating status can affect the likelihood of copulation with sequentially encountered potential mates. For instance, mated females may change their mate acceptance thresholds at subsequent mate encounters (Lea et al. 2000; Kokko and Mappes 2005; Fitze et al. 2010), and mated females of some species may mate only with males of higher quality than their previous mate, i.e., trade-up choice strategies (Gabor and Halliday 1997; Jennions and Petrie 2000; Bateman et al. 2001; Pitcher et al. 2003; Bleu et al. 2012). Specifically, in species exhibiting trade-up choice strategies, virgin females are expected to be relatively indiscriminate in order to assure the acquisition of sperm, and once they have mated, females are expected to become increasingly choosy during successive mating opportunities. Overall, it is important to note that the costs of mating can importantly determine the strength of choosiness and the existence of rejection behavior (Kokko and Monaghan 2001; Bleu et al. 2012; Kokko and Mappes 2013).

In males, the optimum number of mate partners is usually higher than in females, so that mate rejection by males is less common (Bateman 1948; Trivers 1972). However, it is now widely recognized that male choice does exist in a variety of species (Engqvist and Sauer 2001; Edward and Chapman 2011). This may result from the costs involved in sperm production (Dewsbury 1982; Wedell et al. 2002). For instance, if a trade-off exists between the acceptance of current and future encountered mates as a result of finite sperm reserves, virgin males, like virgin females, may easily accept a mate in order to assure reproductive success, while mated males may show an increased acceptance threshold of subsequent potential mates. Moreover, besides discriminating among females as mates, males may vary the amount of resources invested in a mate, e.g., through sperm allocation strategies (see section 1.2.2), in response to their own or their mate's mating status (Simmons et al. 1994; Kelly and Jennions 2011).

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[6] Multiple mating (Shuker & Simmons 2014): "Multiple mating describes cases in which individuals of at least one sex have several mates."

[7] Polygamy (Shuker & Simmons 2014): "If both sexes mate with multiple partners, the mating system is classified as polygamous."

In multiple mating species, mating history can furthermore refer to how long ago an already acquired mating took place. For instance if re-mating results in fertilization only within a certain time interval from an initial mating, the time interval between sequential mate encounters could drive variation in mating propensity. In fact, females may become unreceptive after mating and the termination of a female's window of receptivity may occur sooner if mating occurs than when it does not (Becker et al. 2002). Therefore, a female might be willing to re-mate only if a subsequent mate is encountered before the end of her window of receptivity. On the other hand, a male should only mate with an already mated female if he indeed still has a chance to fertilize her eggs. Additionally, a male himself may be willing to re-mate only if he still has (enough) sperm. Moreover, like the time of first mate encounter, the interval between subsequent mate encounters may signal the availability of mates (Shelly and Bailey 1992; Simmons et al. 1992), and thus potentially affects mate acceptance thresholds.

Finally, the effect of mating history on mating decisions can be importantly influenced by the ability of females to store sperm. Female sperm storage exists in various species, including birds, mammals, and reptiles (Birkhead and Møller 1993). The duration of sperm storage is highly variable, being shortest in mammals, where sperm is stored typically less than 24 hours, and longest in reptiles, where females of some species may store sperm for up to seven years (Birkhead and Møller 1993). Sperm storage can lead to a longer availability of sperm in females, thereby allowing copulation and fertilization to occur at separate moments in time (Birkhead and Møller 1993). It furthermore allows females to fertilize eggs when mates are scarce, and is crucial for reproduction in species where sperm production, mating, and ovulation, do not occur at the same time, i.e., dissociated reproduction (Birkhead and Møller 1993; Sever and Hamlett 2002). Moreover, female sperm storage may enhance sperm competition by extending the time period over which ejaculates from different males overlap in the female reproductive tract (see section 1.2.2; Birkhead & Møller 1993). Therefore, in females that can store sperm, the propensity of accepting an encountered potential mate may vary with respect to the constraints arising from sperm storage. For instance, the time passed since a female has previously mated could determine whether she still has sperm available in her reproductive tract for the fertilization of her eggs, and therefore whether she should re-mate. In addition, females may prefer to utilize stored sperm over sperm obtained through re-mating, for instance if mating is costly, or vice versa, and plastic sperm utilization strategies may exist (see section 1.3).

1.2 TEMPORAL VARIATION IN REPRODUCTIVE ALLOCATION

In the previous section we have seen that the fitness payoffs of accepting a potential mate can vary in a time dependent manner, due to underlying variation in the reproductive stage and mating history of males and females, and that this may lead to differences in mating propensity as well as in the expression of mate preferences. After the decision to accept an encountered mate, mating decisions can continue in the form of variation in the amount of resources invested during mating, i.e., differential allocation (Burley 1986; Sheldon 2000; Bonduriansky 2001; Reinhold et al. 2002). For example, males may invest more or less sperm in a female, i.e., strategic sperm allocation (delBarco-Trillo and Ferkin 2004; Parker and Pizzari 2010), and females may eject sperm after a copulation (Birkhead 1998a; Pizzari and Birkhead 2000). Similarly, in species where sperm is transferred continuously during copulation, males may control copulation duration in order to benefit the transfer of more or less sperm (Lorch et al. 1993; Engqvist and Sauer 2001), and females may cooperate or resist during copulation or terminate mating before insemination or complete insemination occurs (Thornhill 1983; Eberhard 1998; Andrés and Cordero-Rivera 2000; Engqvist and Sauer 2003; Pilastro et al. 2007).

1.2.1 FEMALE ALLOCATION

A female's investment during mating may be directly linked to her willingness to mate at the time of encounter, and thus differences in female investment should be in line with predictions derived about female mating propensity (section 1.1). For example, if a female that is close to ovulation at the time of mate encounter is more inclined to mate, she may subsequently also spend more time in copulation, in order to assure that she acquires a sufficient amount of sperm for the fertilization of all of her eggs. On the other hand, a female that is under no time constraints may spend less time copulating with an acquired mate. Similarly, a previously mated female may show a decreased investment during re-mating with time passed since her fist mate, given that she has already obtained sperm and given that fertilization of her eggs is becoming less likely due to reproductive constraints (e.g., mating windows).

Besides the possibility of copulation duration being a simple reflection of a female's willingness to mate, female control of copulation duration could also constitute a form of cryptic female choice[8] (Eberhard 1996). Cryptic choice mechanisms exhibited by females take place after a male has been accepted as a partner in copulation, i.e., after genitalic coupling (Eberhard 1996). They allow females to influence their mates' chances of fathering their offspring, for instance, to selectively favor paternity by a male with a particular trait. A variety of cryptic female choice mechanisms exist that occur either during or after copulation, including the prevention of ejaculation, premature interruption of copulation, variation in the degree of cooperation, biased use of stored sperm, discarding of sperm, lack of ovulation or oviposition, re-mating, variation of investment in offspring, and more (Eberhard 1996). In other words, if these processes bias the probability that a particular type of male sires the offspring in females that mate with multiple males, females are exercising cryptic choice (Eberhard 1996). Evidence for the existence of cryptic female choice is growing and is changing traditional views of predominantly male control (e.g., in reptiles, insects, birds, and fish; (Olsson et al. 1996; Wilson et al. 1997; Andrés and Cordero-Rivera 2000; Evans et al. 2003; Calsbeek and Bonneaud 2008; Rosengrave et al. 2008; Alcaide et al. 2012; Mongue et al. 2014).

1.2.2 MALE ALLOCATION

Male strategic allocation of sperm may occur with respect to the predicted level of sperm competition[9] (Parker 1970b), a phenomenon widely recognized as a major driving force in evolution (Birkhead and Møller 1998). In species with internal fertilization, sperm competition occurs when ejaculates from two or more males overlap in the female tract, and the degree of competition will determine the proportion of eggs fertilized by rival males (Parker 1970b). Generally, a higher investment of sperm is expected to give males an advantage. However, sperm expenditure theory predicts that the costs involved in ejaculate production (Dewsbury 1982; Olsson et al. 1997) should drive males to strategically utilize sperm reserves, though adjustment of ejaculate size or the number of sperm transferred, in order to optimize their resources in relation to the predicted fertilization success (Parker 1990; Parker 1998; Parker and Pizzari 2010). In general, males are predicted to invest more sperm when an increased risk of sperm competition exists (Parker et al.

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[8] Cryptic female choice (Eberhard 1996): "Postcopulatory ability of females to favour the sperm of one conspecific male over another."

[9] Sperm competition (Parker 1998): "Competition between the sperm from two or more males for the fertilization of a given set of ova."

1997). However, in situations with many competitors or reduced female fertility, e.g., fewer remaining fertilizable eggs, release of additional sperm should be avoided to minimize expenditure (Parker 1998; Pilastro et al. 2002).

The degree of sperm competition may critically depend on the timing of copulations. This is because the latter is predicted to affect the relative number, and/or fertilization ability, of sperm from each male in the female tract, at the moment of fertilization (Parker 1990; Parker 1998). For example, ejaculates acquired earlier may have sustained a greater number of sperm losses in the female tract at the moment of fertilization (Parker 1990). Alternatively, sperm acquired longer before fertilization might have a higher fertilization efficiency in species where sperm maturation is completed within the female tract, a process known in mammals as capacitation[10] (Parker 1990; Olsson and Madsen 1998; Johnson 2012). As a result, fertilization success can be affected by, for instance, the time interval between competitors' matings (Dziuk 1965; Dewsbury 1985; Huck et al. 1985; Lessells and Birkhead 1990; Schwagmeyer and Foltz 1990; Birkhead 1998b; Moreira et al. 2007; Pizzari et al. 2008). Therefore, time-dependent male allocation strategies may exist in response to when mates are encountered (Simmons et al. 1992; Simmons 1995; Reinhold and von Helversen 1997; Lehmann and Lehmann 2000). Other factors, such as the number of rival males, a female's mating status (e.g., virgin or previously mated), and a male's mating order (i.e., first or second to mate), can furthermore contribute to variation in the risk of sperm competition (Parker and Pizzari 2010), and various studies have shown that males indeed adjust ejaculate expenditure in response to these factors (e.g., Suter 1990; Simmons et al. 1993; Shapiro et al. 1994; Cook and Wedell 1996; Gage and Barnard 1996; Wedell 1998; delBarco-Trillo and Ferkin 2004; Olsson et al. 2004; Cornwallis and Birkhead 2006; Ramm and Stockley 2014).

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[10] Capacitation (Johnson 2012): "The process by which the attainment of a full fertilizing capacity [of sperm] is achieved within the female tract."

1.3 EFFECTS OF MATE ENCOUNTER ON REPRODUCTIVE FREQUENCY

Besides the previously discussed effects on mate acceptance (section 1.1.) and investment during mating (section 1.2), the time of mate encounter may also affect the frequency of breeding. For example, females of some iteroparous[11] species face a conflict over producing one or multiple broods during a reproductive season (Cree and Guillette 1995; Verhulst et al. 1997). In these species, producing more than one brood can be facultative decision, and adjusted in response to current conditions, including environmental conditions and the time in the reproductive season (Roff 1992). For instance, there will be some point in the season at which the production of another clutch will contribute very little to parental fitness, because proximity to the unfavorable season may not allow time for eggs to hatch or young to reach a critical size (Tinkle 1969). Under such suboptimal current conditions, reducing the investment in a current breeding event, or skipping the production of a second brood, can allow individuals to preserve energy for future reproduction (Clutton-Brock 1984). However, under optimal environmental conditions and if the length of the remaining reproductive season allows it, producing a second brood can enhance fitness.

Since mates are a critical resource for reproduction in most species, their availability after the production of the first brood may similarly contribute to the optimality of conditions for the production of second broods. For example, females that can re-mate after producing their first brood may invest in a second brood, while females that do not encounter new mates may be unable to produce additional broods. Alternatively, in species that exhibit sperm storage (see section 1.1.2), females may be able to store sperm from initial matings and produce an additional brood without the necessity of re-mating. Moreover, in these species eggs of different broods may be fertilized with the sperm from either the same or from a different partner, and plastic sperm utilization strategies may exist (Parker 1970b; Walker 1980; Eberhard 1996; Gist and Congdon 1998; Pearse et al. 2001; Simmons 2001; Pearse et al. 2002; Singh et al. 2002). On the one hand, fertilizing second broods with sperm stored from initial matings may be beneficial if sperm originates from a male with "good genes"[12]. On the other hand, fertilizing second broods with new sperm acquired through re-mating, may increase the genetic diversity of the offspring or protect females from

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[11] Iteroparity (Stearns 1992): "More than one reproductive event per lifetime."

[12] Good genes (Jennions & Petrie 2000): "These genes may enhance the viability of offspring and/or increase sexual attractiveness (Fisherian traits)."

sterility or genetic defects in mate partners (Thornhill and Alcock 1983; Wolff and Macdonald 2004). However, it has also been shown that sperm from first males might simply be displaced by sperm of second males (Halpert et al. 1982; Manier et al. 2010), suggesting that the use of old sperm may principally depend on whether a female remates (i.e., on whether fresh sperm is acquired). Therefore, depending on the existence of female sperm storage, the presence of mates after the production of a first annual brood may be a more or less important determinant for the production more than one seasonal brood.

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OBJECTIVES

In contrast to the spatial effects of mate encounter, limited evidence about strategic mating decisions with respect to the temporal pattern of the encounter of mates exists. The general objective of this thesis is to fill this knowledge gap by providing new insight into how mating decisions are affected by the time of mate encounter, and to determine the consequences of potential strategies on reproductive success, using the common lizard, *Zootoca vivipara*, as a model species.

In the introduction we discussed potential underlying factors - such as an individual's reproductive stage and mating history, and the degree of protandry - that may cause variation in mating decisions - including mating propensity, the degree of choosiness, the amount of energy invested in a mate partner, and the production of second broods - with respect to the time of mate encounter. With this broad framework in mind, the specific objectives of this thesis are as follows.

Previous studies have shown that emergence from hibernation and the initiation of female ovarian activity are triggered by environmental factors in the common lizard. Thereafter, the female reproductive cycle is under hormonal control and ovulation occurs spontaneously (i.e., independent of mating). The time of mate encounter could thus crucially affect a female's mating propensity. However, the mechanisms controlling the initiation and duration of the female period of receptivity (i.e., the female mating window) are unknown. For instance, receptivity could be initiated spontaneously (e.g., after a fixed time period from emergence) or initiated when first mates are encountered. Therefore, in **Chapter 3**, the time of first mate encounter with respect to female emergence date, and the time of second mate encounter with respect to first mate encounter, are manipulated. The effects on female mating propensity and choosiness are investigated. This will allow determining the mechanism of initiation and the duration of the female mating window (**objective 1**), and will reveal whether or not female choice strategies exist in response to when mates are encountered (**objective 2**).

Male common lizards emerge several weeks before females (i.e., protandry) in order to undergo reproductive maturation (i.e., spermiogenesis) before the mating period starts. The degree of protandry could thus crucially determine whether a male is fertile at the time of mate encounter, with potential fitness consequences for both males and females. However, the direct fitness benefits of protandry have not been demonstrated experimentally in this or in other species. Therefore, in **Chapter 4**, the emergence date of males with respect to females is manipulated. We determine the relationship between male emergence date and the time of reproductive maturation, and investigate whether the time of first mate encounter (i.e., the degree of protandry) affects male mating propensity. This will allow us to investigate whether direct fitness consequences of protandry for males and females exist (**objective 3**).

Copulation duration is positively associated with the amount of sperm transferred and thus a proxy for resource investment during mating. Evidence for investment strategies in common lizards do not exist, and experimental evidence of the effects of the timing of sequential mate encounters on investment during mating is scarce in general. Therefore, in **Chapter 5**, we investigate the effects of the temporal manipulation of mate encounter performed in Chapter 2, on copulation duration and fertilization success of mating individuals. Specifically, we determine whether copulation duration and fertilization success depend on the time delay until a female's first mating, and/or on the length of the interval between her first and her second mating. This will allow us to determine whether male or female investment strategies during mating exist with respect to the timing of sequential mating events (**objective 4**), and whether potential strategies affect fertilization success (**objective 5**).

Previous work has shown that female common lizards cannot store sperm from one reproductive season to the next (i.e., during hibernation), and that females that are not able to mate at the start of a reproductive season nevertheless produce unfertilized clutches, indicating that ovulation is spontaneous and hence that first clutch production is not facultative. It is also known that common lizards are able to produce more than one seasonal clutch and facultative responses to the altitude and temperature of populations in the production of second clutches have been shown to exist. However, the importance of mate availability later in the season (i.e., after first clutch production) for the production of second clutches is unknown. Therefore, in **Chapter 6**, the availability of mates after the production of first clutches is manipulated. We investigate the effects of the presence or absence of mates after the production of first clutches on the production of second clutches. We additionally investigate the temporal pattern of sperm presence in males. This will allow us to determine whether encountering mates for re-mating after first clutch production is important for the production and viability of second clutches, i.e., for female reproductive success (**objective 6**); whether males are fertile during the potential re-mating period of females (**objective 7**); whether females use sperm obtained from initial mates or from mates with which they re-mated, for the fertilization of second clutches (**objective 8**); and ultimately whether re-mating after first clutch production is important for male reproductive success (**objective 9**).

• CHAPTER 2 •

GENERAL METHODS

Lizards in general have been an important model group for studying the evolution of reproductive traits, because they show pronounced temporal and geographic variation in their reproductive biology, both between species (Tinkle 1969; Tinkle et al. 1970; Ballinger 1973; Vitt and Congdon 1978; Dunham and Miles 1985; Lovern 2011) and within species (e.g., Pianka 1970; Tinkle & Ballinger 1972; Horváthová et al. 2013; Roitberg et al. 2013). This variation evolved in response to, among other factors, the environmental conditions of the habitat that each species inhabits, including altitude, latitude, temperature, resource availability, and population density (e.g., Massot et al. 1992; Van Damme et al. 1992; Adolph & Porter 1993; Sorci et al. 1996; Olsson & Shine 1997; Braña & Ji 2007; Warner, Lovern & Shine 2007; Yajuan et al. 2011). Within species, plastic responses to proximate factors (i.e., phenotypic plasticity) can produce variation in reproductive traits (Bauwens 1999). Such variation might be most pronounced in species with annually defined activity seasons (e.g., lacertid lizards), where the time available for reproduction is restricted to periods of the year that provide the environmental conditions (e.g., sunlight, heat, moisture, food availability) necessary for offspring production and survival (Tinkle 1969; Whittier and Crews 1987). For instance, inter-annual variation in the length of the reproductive season (including incubation, hatching of eggs, and hatchling growth), can determine the number of clutches a female produces during a single season (Tinkle and Ballinger 1972). Moreover, reproductive traits can vary in response to changes in population density (Massot et al. 1992), potentially indicating plasticity. Such density dependent effects may be more easily detectable in lizards than in other vertebrates (Massot et al. 1992; Clobert et al. 1994), since fluctuations in size of lizard populations are thought to be moderate compared to other vertebrates (Schoener 1985; Bauwens et al. 1986). Finally, lizards generally lack parental care (Shine 2005), facilitating the estimation of reproductive investment, as it is completed when eggs are laid (at least in oviparous species).

2.1 MODEL SPECIES

2.1.1 GENERAL DESCRIPTION AND DISTRIBUTION

The common lizard (Fig. 2.1), *Zootoca vivipara* (Lichtenstein, 1823), is a small, ground-dwelling lizard that belongs to the family Lacertidae (Sauropsida, Squamata). It has the largest geographic distribution of any terrestrial reptile, and can be found from Europe to northern Asia and Japan (Fig. 2.2.a; Surget-Groba *et al.* 2006). It lives in cool, temperate climates, and typical habitats include peat bogs and humid heath land (Fig. 2.2.b-c; Strijbosch and Creemers 1988; Massot et al. 1992). The common lizard shows reproductive bimodality, including both oviparous and viviparous populations, a phenomenon that has been documented in two other lizard species only (*Lerista bougainvillii* and *Saiphos equalis*; Qualls & Shine 1995; Smith & Shine 1997). Populations have been attributed to six different clades, of which four clades exhibit viviparous reproduction and two clades are oviparous (Surget-Groba et al. 2006). The individuals used in the present study originated from oviparous populations in the central Spanish Pyrenees, located at the southern end of *Z. vivipara´s* distribution range. These populations belong to the western oviparous clade (Surget-Groba et al. 2006), and specifically, to the subclade of north-eastern Spain (Pyrenees; Milá et al. 2013).

Fig 2.1 Illustration of a male common lizard, *Zootoca vivipara*

Studies have revealed geographic variation in reproductive traits of *Z. vivipara*, including in size at maturity of females, clutch size, and hatchling mass, in response to climate (Horváthová et al. 2013; Roitberg et al. 2013). Studies have also revealed plastic responses to variation in physical environments (Sorci et al. 1996; Sorci and Clobert 1999; Lorenzon et al. 2001; Roitberg et al. 2013). For instance, growth rate varies in response to altitude and water availability (Sorci et al. 1996; Lorenzon et al. 1999), and clutch size and dispersal vary in response to population density (Massot et al. 1992; Clobert et al. 1994; Mugabo et al. 2013).

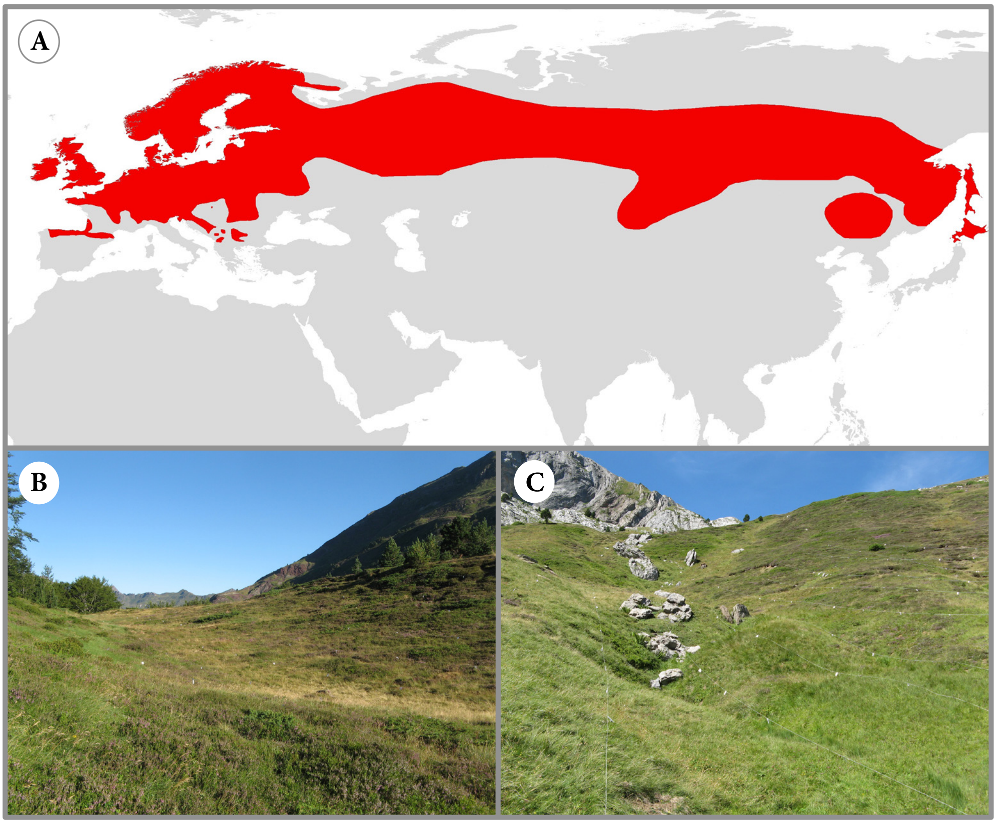

Fig 2.2 Geographic distribution (A) and two typical habitats (B, C) of the common lizard in the Central Pyrenees, close to (A) Somport and (B) Candanchú (Huesca, Spain)

2.1.2 REPRODUCTIVE SEASON

Z. vivipara has annual reproductive seasons (Fig. 2.3) that start after springtime emergence from hibernation, which occurs between February and May, depending on the altitude and latitude of the population (Heulin et al. 1994; Roig et al. 2000; Horváthová et al. 2013). Females emerge 2 to 4 weeks after males, after which mating starts (Bauwens and Verheyen 1985; Roig et al. 2000). Adult females are larger than adult males (Braña 1996), and become sexually mature upon attaining a minimum body size (e.g., snout-vent length (SVL) 43-45 mm; Bauwens & Verheyen 1987), usually during their second or third activity season (Bauwens and Verheyen 1987; Bauwens 1999; Carretero 2006).

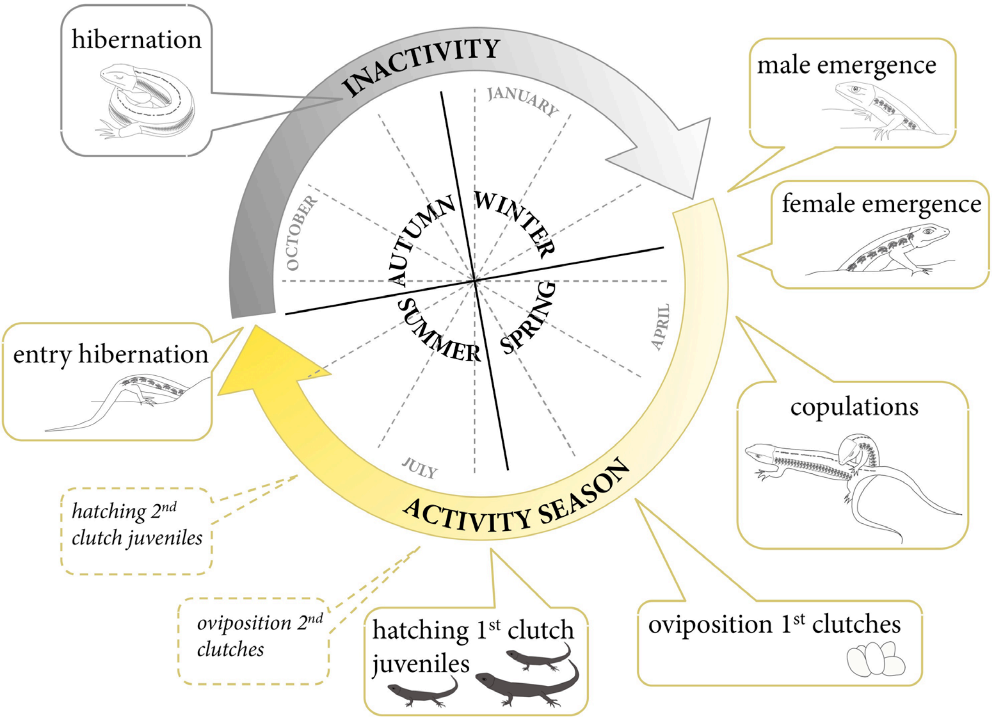

Fig 2.3 Generalized schematic overview of the annual cycle of the oviparous common lizard. **Note** that the approximated timing of specific events represented can vary between populations and years. The approximate timing of oviposition and hatching of potential second annual clutches are included and depicted in *italics* (for details see the main text)

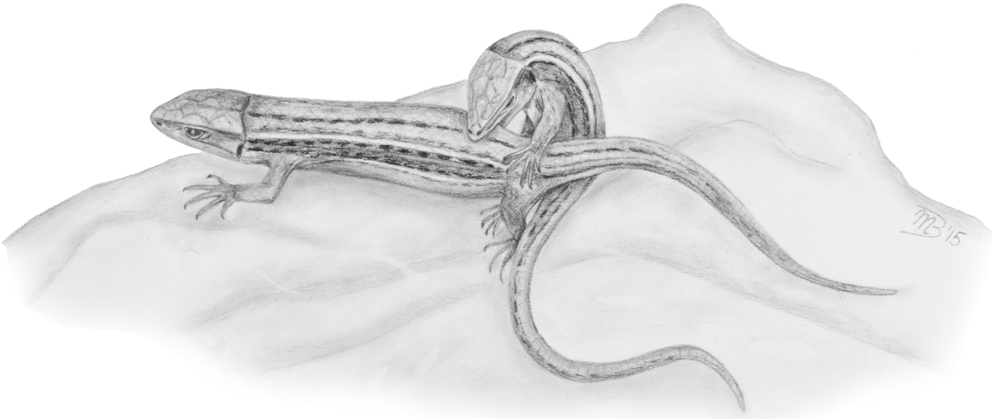

Fig 2.4 Illustration of copulation in the common lizard

The mating system is polygynandrous[1] (Fitze et al. 2005; Fitze et al. 2010); females lay clutches that are fathered by 1 to 5 different males and on average by 2 males, and males sire clutches of 1 to 14 different females and on average of 4 females (Fitze et al. 2005). Males, which are non-territorial, move around in search of females, which are sedentary (Uller and Olsson 2005). During copulation, a male first holds a female at the posterior abdomen with his mouth, twists his body around the female, and inserts one hemipenis[2] into the female's cloaca (Fig. 2.4). Ejaculation is non-instantaneous, i.e., sperm is transferred over longer time periods during copulation (Olsson and Madsen 1998). Copulation duration is a proxy for the amount of transferred sperm and can last from a few minutes up to several hours (Heulin 1988b; Olsson and Madsen 1998; Olsson et al. 2004). Due to the male's grip, the female's belly shows a typical U-shaped mating scar after copulation (Bauwens and Verheyen 1985; Fitze and Le Galliard 2008).

In the oviparous populations, clutches consisting of 2 to 9 calcified eggs are laid after 14 to 30 days of gestation (Heulin et al. 1991; Horváthová et al. 2013). Clutch size is positively related to female body size (Horváthová et al. 2013; Roitberg et al. 2013). No parental care exists after egg laying and autonomous juveniles hatch after 30 to 50 days of incubation (Heulin et al. 1991; Heulin et al. 1994). Females can produce a

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[1] Polygynandry (Shuker & Simmons 2014): "Greater variability in male than female mating success [in a polygamous mating system]."

[2] Hemipenis (Tokarz 1988): "Unlike other amniote vertebrates, squamate reptiles (lizards and snakes) possess two independent intromittant organs termed hemipenes. Only one hemipenis is intromitted into the female's cloaca during copulation."

second and sometimes even a third annual clutch, especially large females that initiate reproductive activities early in the season, and females from low altitude populations (Heulin et al. 1991; Heulin et al. 1994; Roig et al. 2000).

At the end of the activity season, between September and October, lizards gradually enter hibernation, where they remain until the following spring emergence (Salvador 2014). Females and males have been observed to live for up to 13 and 4 years, respectively (Massot et al. 2011).

2.1.3 REPRODUCTIVE CYCLES

Annual gonadal cycles of *Z. vivipara* are under thermoperiodic regulation (Licht 1972; Gavaud 1991a; Gavaud 1991b).

Spermatogenesis starts after the mating period, develops during summer, and is inhibited by hibernation (Gavaud 1991b). The last step of spermatogenesis, i.e., spermiogenesis, is completed after emergence from hibernation, and depends on heat input during daily basking (Gavaud 1991b). Spermatozoa are therefore already present in the testis upon emergence from hibernation, however they are not mature until they pass to the epididymis, a few weeks after emergence, when males become fully fertile (Courty and Dufaure 1980; Depeiges and Dacheux 1984; Gavaud 1991b; Roig et al. 2000). Plasma steroid levels are associated with breeding (Courty and Dufaure 1979; Courty and Dufaure 1980; Courty and Dufaure 1982). Males remain fertile for about 2 months, and involution occurs in June with the elimination of remaining germ cells, a drop in plasma testosterone levels, and the lysis of epithelial cells in the epididymes (Gavaud 1991b).

Males undergo their first annual molt after emergence from hibernation and it has been suggested that its completion is a prerequisite for male mating behavior (Bauwens et al. 1989).

The ovarian cycle is induced by the change in thermal conditions following springtime emergence from hibernation (Gavaud 1983). It ends with the ovulation of matured oocytes about one month after emergence from hibernation (Gavaud 1983; Heulin et al. 2008). Plasma steroid levels are higher during the late stages of vitellogenesis[3] than after ovulation (Heulin et al. 2008). Experimental evidence

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[3] Vitellogenesis (Jones 2011):"In reptiles, follicular growth and maturation typically involve the deposition of large amounts of yolk. The bulk of that yolk is derived from the precursor molecule Vtg, a large molecular weight lipophosphoglycoprotein that is synthesized in the liver. Vitellogenesis is the process of hepatic synthesis of Vtg and its transport to the oocytes."

indicates that in first clutches ovulation is spontaneous and copulation does not affect clutch size (Bleu et al. 2011). Effects on multiple clutches have not been investigated.

Mating behavior in males and females is positively related to gametogenesis and sex steroid production, i.e., associated reproduction (Courty and Dufaure 1982; Uller and Olsson 2006; Heulin et al. 2008). Observations of mating scars indicate that most females mate within a few days from emergence (Bauwens and Verheyen 1985; Fitze et al. 2010). A study on the behavior of three females suggests that shortly after copulation females resist copulation attempts by novel males (Heulin 1988b). This fast initiation of mating in emerged (virgin) females and rapid resistance to additional copulation attempts in mated females suggests that females have a short time window during which they are receptive.

Common lizards exhibit no sperm storage in the female tract during hibernation, i.e., from one reproductive season to the next (Bleu et al. 2011). Moreover, specialized structures for long-term sperm storage, found in many other lizards (Sever and Hamlett 2002), have not been reported in common lizards. Evidence from one single female indicates that sperm from initial copulations can potentially be stored for the fertilization of eggs during a second clutch (Heulin 1988a), but the generality of this finding is unknown.

2.1.4 MATING STRATEGIES

Females can resist male copulation attempts and exhibit mate choice, the strength of which depends on reproductive costs, population sex ratio, and mating history (Fitze et al. 2005; Fitze and Le Galliard 2008; Fitze et al. 2010). Copulating males exhibit larger SVL, longer tails, and better body condition than males that do not copulate (Fitze et al. 2005; Fitze et al. 2010; Huyghe et al. 2013).

Males compete for access to females by aggressively chasing away other males, and they exhibit best-of-bad-job mating strategies; unattractive (low-quality) males - towards which females show a reduced interest and increased aggression - compensate by exhibiting an increased interest in females and performing more copulation attempts (Heulin 1988b; Richard et al. 2005; Gonzalez-Jimena and Fitze 2012).

No evidence exists for male or female allocation strategies during copulation, neither that fertilization success can be biased by post-copulatory strategies such as mate guarding, mating plugs, the induction of female non-receptivity through copulation (Parker, 1998), or cryptic female choice (Thornhill 1983; Parker 1998).

2.2 LABORATORY CONDITIONS

All experimental lizards were captured by hand and brought to the Instituto de Pirenaico de Ecología (Jaca, Huesca, Spain). In the laboratory, the sex of each lizard was determined, and measurements of weight (to the nearest milligram), snout-to-vent length (SVL; to the nearest millimeter), and tail length were taken. Each lizard was marked by toe-clipping for individual identification and a tissue sample was collected from the tail tip for genotyping.

In the laboratory, lizards were housed in individual terrariums (25 × 15 × 15 cm) containing a shelter, a drinking pond, and a floor substrate, and terrariums were covered with a wire mesh. Lizards were provided with water ad libitum and with food every 3 days (moth larvae; *Galleria mellonella*, Pyralidae). Light and heat were provided by a 40-W light bulb between 9:00 a.m. and 6:00 p.m., and ultraviolet (UV)-B light was provided between 1:00 p.m. and 3:00 p.m. Humidity was maintained high by spraying each terrarium with water twice every day. Males and females were housed on separate shelves and within sexes lizard traits were randomly distributed among shelves.

Lizards not housed in the laboratory were maintained in 100 m^2 semi-natural enclosures, at the research station 'El Boalar' (42°33′N, 0°37′W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología. The enclosures contained natural vegetation, hides, rocks, logs, and water ponds, and were surrounded by escape proof walls and covered with nets to prevent predation (Fitze et al. 2008; San-Jose et al. 2014).

2.3 GENERAL EXPERIMENTAL PROCEDURES

2.3.1 MATING TRIALS

Mating trials were always performed between 9:00 a.m. and 6:00 p.m. During the trials, individuals were introduced in escape-proof wooden boxes (2500 cm^2), equipped with cardboard shelters, a heating rock, a drinking pond, and light (Fitze et al. 2010). Lizards were observed from a concealed location and mating trials were in addition videotaped.

2.3.2 SPERM EXAMINATION

Sperm presence in males was determined by gently pressing on the sides of the cloaca to extract a droplet of seminal fluid, and by subsequently performing microscopic analysis of the extracted droplet. 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 recorded.

2.3.3 LABORATORY HIBERNATION

Prior to hibernation in the laboratory, individuals were fasted in the laboratory for 1 week, and acclimatized under dark and cool (15°C) conditions. Food was provided last on the morning of the first fast day, and animals were provided with heating (warm cable) for 4.5 hours on the first three days of the fasting week. This allowed animals to complete digestion and empty digestive tracts prior to hibernation, taking into account the natural gut passage time in *Z. vivipara* (Van Damme et al. 1991). On their day of entry into hibernation, individuals were given a warm water bath and an abdominal massage to expel any feces remaining in the digestive system. Animals were then placed into an incubator at a temperature of 4°C, in individual hibernation cups containing humid soil and air holes. At the end of their hibernation period, animals were removed from the incubator and returned to standardized lab conditions.

Further, chapter-specific information about experimental designs and procedures are provided in chapters 3 to 6.

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• CHAPTER 3 •

A MATTER OF TIME: DELAYED MATE ENCOUNTER POSTPONES MATING WINDOW INITIATION AND REDUCES THE STRENGTH OF FEMALE CHOOSINESS

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ABSTRACT

Reproductive success is determined by the presence and timing of encounter of mates. The latter depends on species-specific reproductive characteristics (e.g., initiation/duration of the mating window), season, and reproductive strategies (e.g., intensity of choosiness) that may potentially mitigate constraints imposed by mating windows. Despite their potentially crucial role for fitness and population dynamics, limited evidence exists about mating window initiation, duration, and reproductive strategies. Here, we experimentally tested the mechanisms of initiation and the duration of the common lizard's *Zootoca vivipara* mating window by manipulating the timing of mate encounter and analyzing its effect on (re-)mating probability. We furthermore tested treatment effects on female reproductive strategies by measuring female choosiness. The timing of mate encounter and season did not significantly affect mating probability. However, a longer delay until mate encounter reduced female choosiness. Re-mating probability decreased with re-mating delay and was independent of mating delay. This indicates that mating window initiation depends on mate encounter, that its duration is fixed, and that plastic reproductive strategies exist. These findings contrast with previous beliefs and shows that mating windows per se may not necessarily constrain reproductive success, which is congruent with rapid range expansion and absence of positive density effects on reproductive success (Allee effects). In summary, our results show that predicting the effect of mating windows on reproduction is complex and that experimental evidence is essential for evaluating their effect on reproduction and reproductive strategies, both being important determinants of population dynamics and the colonization of new habitats.

KEYWORDS

Mate choice • Population dynamics • Reproductive constraints • Reproductive strategies • Reproductive success • *Zootoca vivipara*

INTRODUCTION

Mating windows, defined as the time an individual spends in mating activities, can crucially affect behavioral strategies and reproductive success of females, especially of females living in low-density populations (Kokko and Mappes 2005; Kokko and Wong 2007). The magnitude of the effect of mating windows depends on the mechanisms triggering their initiation and on their duration. However, despite their importance for reproductive success and the likely consequences for population dynamics, Allee effects, and the colonization of new habitats, the study of the duration and the mechanisms initiating mating windows, as well as the behavioral adaptations related to mating windows, has attracted relatively little research.

In sexually reproducing organisms, Allee effects can constrain the persistence of low-density populations and the colonization of new habitats (Allee 1931). For instance, low individual density can reduce mate encounter (Kokko and Mappes 2005; Gascoigne et al. 2009) and thereby reproductive success (Courchamp et al. 1999). Mate encounter is also affected by, among others, protandry, reproductive asynchrony, sexual selection, and mating windows (Møller and Legendre 2001; Calabrese and Fagan 2004; Kokko and Wong 2007). Reduced mate encounter may have strongest effects in species with delimited annual reproductive windows (Crews and Moore 1986; Lovern 2011) since the synchronization between mate encounter and the short mating window needs to be high. The strength of the constraint imposed by mating windows on mate encounter, and consequently on reproductive success, depends on the mechanism of their initiation and on their duration. Long mating windows are predicted to have a smaller effect on reproduction than short mating windows since in the former more time is available for mate encounter (Calabrese and Fagan 2004). For example, lemurs are receptive to mating only once a year during a very short period, and females may miss reproduction if no male is available at the start of the mating window (Dunham and Rudolf 2009). In contrast, in species with long mating windows, reproduction will not be compromised if a female does not encounter a mate partner at the start of the mating window, given that a later encounter may result in fertilization. Furthermore, mating window initiation may be triggered by male presence or copulation in line with mechanisms of male-induced ovulation (Brockway 1965; Crews 1975; Leboucher et al. 1998; DeNardo and Autumn 2001). In this case, the constraint on reproductive success is predicted to be smaller compared to initiation triggered endogenously (Short 1984) or through other exogenous determinants. For example, if female receptivity is induced at emergence from hibernation, females of annual breeders face the risk of no reproduction if they do not find a mate partner before the end of the mating window. On the other hand, species with monthly ovulation, long mating windows, and mating windows induced by the presence of mates may simply delay reproduction, thereby facing a lower risk of no annual reproduction.

However, short mating windows do not only have negative effects but may also entail advantages that outbalance their negative effects. Mating windows may maximize reproductive prospects by coordinating offspring production and development with the time of year that provides suitable conditions for their survival (Perrins 1970; Lovern 2011). Furthermore, the constraints of mating windows on mate encounter may be mitigated by adaptations such as sperm storage (Sever and Hamlett 2002) and phenotypic plasticity of female reproductive strategies (Jennions and Petrie 1997). Specifically, sperm storage can assure fertilization in the absence of males so that mate encounter does not need to be synchronized with ovulation and female choosiness may be plastic. Choosiness may depend on, for instance, mate availability (Kokko and Rankin 2006; Bleu et al. 2012), season (Lynch et al. 2005), or female reproductive stage (Teuschl and Blanckenhorn 2007). For example, in the presence of many males, females can afford to be choosy since finding a suitable male will not require a lot of time. Similarly, early in the season or at the start of the mating window, females can afford expressing strong choosiness since there is time left to encounter a suitable male. In contrast, in the presence of a single male, females cannot afford to be choosy and will mate even with a nonpreferred male in order to assure fertilization. Restricted mate availability may thus favor trade-up strategies (Jennions and Petrie 2000), during which females may not be choosy when encountering the first mate (to ensure fertilization) but may express choice when re-mating (e.g., Gabor and Halliday 1997; Fitze et al. 2010; Laloi et al. 2011). On the other hand, late in the season or close to the end of the mating window, when the time to find a preferred mating partner is limited, strong choosiness may compromise reproductive success, especially in low-density populations.

In summary, behavioral strategies depend on the mechanisms of initiation and on the duration of the mating window. However, despite the importance of mating windows for reproductive success and the likely consequences for population dynamics and the colonization of new habitats, the study of their duration and the mechanisms initiating them has attracted relatively little research, and almost no experimental evidence exists. This lack of knowledge crucially limits the understanding of the behavioral adaptations of females, of the evolution of reproductive behavior in general, and, ultimately, of Allee effects.

Here, we experimentally investigate the mechanism triggering the initiation and the duration of the common lizard's (*Zootoca vivipara*; Jacquin 1787) mating window as well as the existence of behavioral adaptations in females. *Z. vivipara* is a seasonal breeder whose reproductive season starts directly after female emergence from hibernation (Bauwens and Verheyen 1985; Heulin 1988; Fitze et al. 2010; Bleu et al. 2011). Common lizards exhibit no sperm storage during hibernation (Bleu et al. 2011). Environmental factors trigger the endogenous program of its reproductive cycle (Gavaud 1983, 1991a), leading to ovulation and fertilization several weeks after the mating period (Gavaud 1983; Bauwens and Verheyen 1985). Behavioral studies suggest that females have a short mating window and that this window starts directly after emergence from hibernation (Bauwens and Verheyen 1985; Heulin 1988; Fitze et al. 2010). These characteristics suggest that the mating window may constrain common lizard reproduction, potentially explaining the formerly observed Allee effects at low densities in this species (Mugabo et al. 2013). In conclusion, important parts of the common lizard's life history are known, and indirect evidence about its mating window exists. However, no robust evidence that allows understanding what determines the initiation and duration of the mating window in this and other species exists, and thus it is not clear whether the mating window could lead to the observed Allee effects.

To determine whether the mating window can compromise reproductive success and thereby lead to the observed Allee effects at low densities (Mugabo et al. 2013), we hibernated lizards under standardized conditions in semi-natural outdoor enclosures and determined the exact date of emergence from hibernation for each female (hereafter referred to as emergence date). Subsequently, we manipulated the timing of first and second mate encounter using a crossed two-factorial design (hereafter referred to as mating and re-mating delay). We quantified treatment and emergence date effects on mating and re-mating probability to determine the mechanism of initiation and the duration of the mating window and understand the importance of male presence therein. We furthermore linked climatic parameters with the date of emergence from hibernation to understand how climate triggers emergence and whether the initiation and duration of the mating window are directly linked to climatically triggered emergence. This experimental design therefore allowed understanding the importance of various exogenous parameters for the initiation and duration of the mating window.

We predicted a link between emergence date and female mating probability if the initiation of the mating window is triggered by emergence from hibernation. If male presence triggers initiation, we predicted no effect of mating delay on female mating probability. The manipulation of re-mating delay allowed us to investigate whether the duration of the mating window is fixed (e.g., endogenously determined) or flexible. If the duration of the mating window is fixed, we predicted, in the case of its initiation at emergence from hibernation, that the re-mating probability depends on the time since emergence from hibernation and that there exists a significant interaction between mating and re-mating delay. In other words, we predicted no effects of mating and re-mating delay per se, but we predicted that in lizards with longer mating delays the re-mating probability will decrease with increasing re-mating delay. In the case that initiation of the mating window is triggered by mate presence or copulation, we predicted a significant effect of re-mating delay on the re-mating probability, but no significant interaction between mating and re-mating delay.

To test whether females show plastic reproductive strategies that may mitigate potential negative effects of mating windows, we analyzed the effects of mating and re-mating delay on female choosiness (Fitze et al. 2010). Short delays simulate situations with many mate partners, where encountering a mate partner will be easy and thus fast, while long delays simulate situations with few mate partners, where mate encountering will be difficult and thus slow. We predicted that females with short mating delays are choosier than females with long mating delays, given that early-mating females may have more time to find suitable partners compared to females encountering males late. In contrast, in the absence of plastic female strategies, we predicted no treatment effects on female choosiness. Moreover, if the mating window is initiated by mate presence, we predicted no treatment effects on choosiness at the first encounter, but significant effects of re-mating delay on choosiness at the second encounter.

MATERIALS AND METHODS

SPECIES DESCRIPTION

Z. vivipara is a small ground-dwelling Lacertidae that is widely distributed throughout Eurasia, where it inhabits habitats like peat bogs and moist heath land (Massot et al. 1992; Clobert et al. 1994). It is a spontaneous ovulator (Bleu et al. 2011) that exhibits annual or biannual breeding (Roig et al. 2000), and males generally emerge from hibernation up to 1 month earlier than females (van Nuland and Strijbosch 1981). Mating starts right after female emergence from hibernation (Bauwens et al. 1989); females mate multiply and lay clutches of three to eight eggs (Roig et al. 2000) that are fathered by one to five males (Laloi et al. 2004; Fitze et al. 2005). Females exhibit mate choice, and the choosiness depends on reproductive costs, population sex ratio, and mating history (Fitze et al. 2005, 2010; Fitze and Le Galliard 2008). Copulating males exhibit larger snout-to-vent length (SVL) and better body condition than males that do not copulate (Fitze et al. 2005, 2010), and SVL is the most important predictor of sexual selection in *Z. vivipara* (Fitze and Le Galliard 2008; Fitze et al. 2010). A study on the behavior of three females suggests that females are resistant to male copulation attempts shortly after mating (Heulin 1988), and copulations start directly after the emergence of the females (Bauwens and Verheyen 1985; Heulin 1988; Fitze et al. 2010). This suggests that females may have a short period of receptivity (Bauwens and Verheyen 1985; Heulin 1988; Fitze et al. 2010). Ovulation and fertilization occur several weeks after the mating period, and the former is endogenously determined (Gavaud 1983; Bauwens and Verheyen 1985).

PRE-EXPERIMENTAL CONDITIONS

Adult lizards were captured at Roncesvalles (Spain), and 37 individuals were released prior to hibernation (September 2010) in each of four semi-natural enclosures (100m^2) located at the research station 'El Boalar' (42°33′ N, 0°37′ W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología. The enclosures contained natural vegetation, hides, rocks, logs, and water ponds and were surrounded by escape proof walls and covered with nets to prevent predation (Fitze et al. 2008). Males and females were kept in different enclosures to prevent uncontrolled mating. After the first male emerged from hibernation (March 2011), female enclosures were searched daily and intensively during the hours of lizard activity, i.e., from 9:00 a.m. to 5:00 p.m. (Van Damme et al. 1987). Females were captured upon detection and males shortly prior to experimentation. Limited size of the enclosures, and knowledge of the residing lizards and preferred sites, guaranteed captures at first sight and maximum precision in the determination of the emergence date. Searches were conducted until the 10th of April, at which point no females had been detected for five full days. Subsequent inspections of the enclosures confirmed that all remaining females had been captured. Captured animals were transported to the laboratory, measured for body size (SVL), and housed in individual terrariums. Lizards were randomly distributed among shelves (all *P*>0.5). Females and males had no visual contact and were maintained on separate shelves. Food was provided every 3 days (*Galleria mellonella*, Pyralidae), and water was available 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 supplied. All individuals of the same sex used for the experiment were handled in the same way.

MATING EXPERIMENTS

We simulated differences in timing of mate encounter by presenting females (*N*=26) with males (*N*=80) either 2 or 9 days after emergence from hibernation (mating delay). All mating trials were performed between the 20th of March and the 7th of April (2011), and between 9:00 a.m. and 6:00 p.m., in escape-proof wooden boxes (2500 cm^2), equipped with shelters, a heating rock, a drinking pond, and light (Fitze et al. 2010). Each female was released into a separate clean box, and a randomly selected male was introduced. In the absence of mating, males were removed after 1 h and replaced with a new randomly selected male, respecting the average rate of natural mating attempts (1.1±0.9 SE/h; Heulin 1988). If mating did occur, both individuals were removed after 1 h. A maximum of nine males were presented to a female since in a previous experiment 94 % of the females copulated with one of the first nine males (Fitze et al. 2010).

Mated females (*N*=23) were thereafter assigned to re-mating trials that occurred 2, 6, or 10 days after the mating trials (re-mating delay) using a 2×3 design (mating × re-mating treatment). All re-mating trials were performed between the 22nd of March and the 19th of April, and the same above-described procedures were applied. Females could thus mate with maximally two males, corresponding to the average number of males fertilizing a clutch (2.05±0.09 SE; Fitze et al. 2005). There were no significant differences among mating and re-mating delay treatments in emergence date and SVL of females (ANOVA, all *P*>0.4). Males were released in the enclosures where they were captured immediately after a (re-)mating trial. They were used not more than two times throughout the experiment (presentation interval ≥ 2 days), and they were never presented twice to the same female. At the mating and re-mating trials, females thus had the same likelihood to face males that had not yet been presented to a female, and male mating status (previously mated or not mated) of the presented males did not depend on season, time spent in the laboratory, or time passed since first capture (GLMM with binomial error, logit link and male identity as random effect, all *P*>0.2).

STATISTICAL ANALYSIS

Mating probability and re-mating probability were analyzed using generalized linear models with a binomial error term and a logit link. Mating and re-mating delay were modeled as factors, with female emergence date and SVL as covariates. All calculable double interactions with mating delay and re-mating delay were included. Final models were obtained by backward elimination of non-significant terms (*P*>0.1). An additional model with *N* days since emergence as a covariate was run to test whether re-mating probability depended on time since female emergence.

To test the relative importance of calendar day and climatic conditions on date of emergence, we analyzed the probability that at least one female emerged on a given calendar day (hereafter referred to as day in the season) and five daily climatic predictors. Meteorological data were obtained from a station of the Spanish Meteorological Service (AEMET, Agencia Estatal de Meteorología), 7 km northeast of the study site (see supplementary material S1, page 77). Female emergence was coded as a binomial dependent variable, where 1 and 0 indicate that at least one female or not one single female emerged on a given day, respectively. We included all calendar days from the day on which the first female emerged until the day when the last female emerged. We fitted all possible models, including double and triple interactions. Model selection was based on the Akaike Information Criterion, and significant differences between models were confirmed using likelihood ratio tests.

To test for plasticity of reproductive strategies of females, we used the number of males presented to a female until copulation occurred as a proxy for female choosiness. Treatment effects on female choosiness were assessed in mating and re-mating trials using generalized linear models with a Poisson error and a log link. Models were tested for over-dispersion. In the existence of over-dispersion, we used quasi-Poisson models. Mating and re-mating delay were modeled as factors, with female emergence date and SVL as covariates. All calculable double interactions with mating and re-mating delay were included. Final models were obtained by backward elimination of non-significant terms (*P*>0.1).

Data were analyzed using R 3.0.0 (R Core Team 2013) and JMP 8.0.2 (SAS Institute 2009).

RESULTS

MATING AND RE-MATING PROBABILITY

Mating probability was not significantly affected by mating delay (χ^2_1=1.227, *P*=0.268). The minimal adequate model for re-mating probability consisted of re-mating delay only (χ^2_2=8.251, *P*= 0.016, 46.3 % of variance explained; Fig. 3.1). When re-mating delay was 10 days, re-mating probability was significantly lower than when it was 2 or 6 days (χ^2_1=5.451, *P*=0.020), and re-mating probability was not significantly different between the latter two (χ^2_1=0, *P*=1; estimates ± SE for 2-, 6-, and 10-day re-mating delay: 1±0, 1±0, 0.57± 0.20). Re-mating probability was not significantly affected by mating delay (χ^2_1=0.197, *P*=0.658), and the interaction between mating and re-mating delay was not significant (*P*=1). Female emergence date, SVL, and the interactions were not significant (all *P*≥0.3).

*N*days since emergence from hibernation did not significantly affect the re-mating probability (χ^2_1=1.688, *P*=0.194). In fact, only 50 % of the females re-mated when they were 12 days out of hibernation (mating delay 2 days and re-mating delay 10 days), while 100 % of females re-mated when they were 15 days out of hibernation (mating delay 9 days and re-mating delay 6 days).

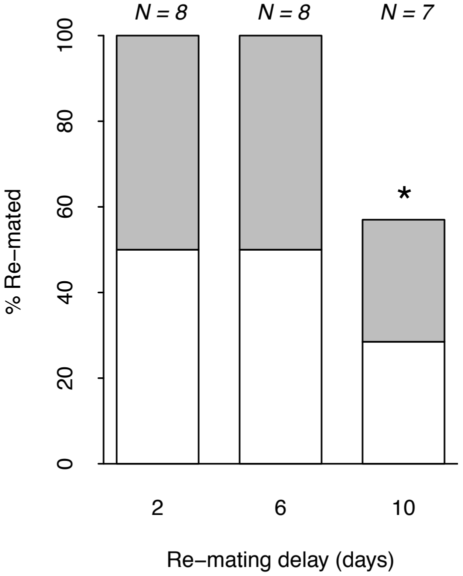

Fig. 3.1 Percentage of re-mated females in response to re-mating delay. *Gray shading* of the bars represents females with a mating delay of 9 days and *white shading* those with a mating delay of 2 days. Significant posthoc tests among the re-mating delay levels are depicted with an *asterisk*, and sample sizes are given *above* each bar

DETERMINANTS OF EMERGENCE FROM HIBERNATION

All females (*N*=26) emerged from hibernation within 19 calendar days, between the 18th of March and the 5th of April. The minimal adequate model for predicting emergence contained daily maximum temperature, daily minimum temperature, and their interaction (model 3 in supplementary material, Table S1; Fig. 3.2). The minimal adequate model was selected from the three highest-ranking models (models 1–3)

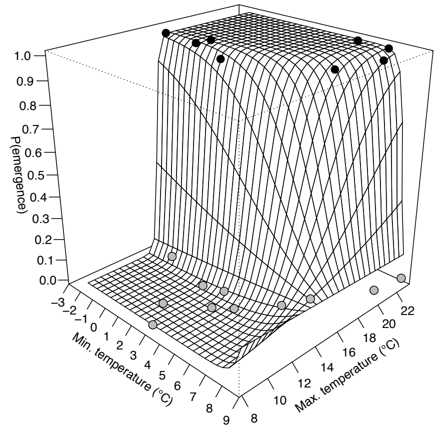

Fig. 3.2 Probability (*P*) that at least one female emerged from hibernation on a given calendar day in relation to maximum and minimum temperature (Table S1; model 3). All calendar days from the day of first female emergence (18th of March) until the day of last female emergence (5th of April) were included in the analyses. Plotted are model predictions and raw data (*dots*). Days on which emergence took place are depicted with *black dots*, and days on which emergence did not take place are depicted with *gray dots*

because all lower-ranking models (model ≥ 4) had significantly worse AICs than model 1. The highest-ranking model (model 1) included daily maximum temperature, minimum temperature, their interaction, and mean maximum wind velocity. The second highest-ranking model (model 2) included the same terms as model 1 plus the interaction between maximum temperature and mean maximum wind velocity. Removal of mean maximum wind velocity from model 1 (i.e., resulting in model 3) and removal of the interaction between mean maximum wind velocity and maximum temperature from model 2 (i.e., resulting in model 1) did no result in significantly higher AIC (all ΔAIC < 2). These latter terms are thus uninformative (Arnold 2010), indicating that model 3 is the most parsimonious fit. Model 4, which included the same terms as model 3 plus day, was not significantly different from model 3, while all other models (model number ≥ 5) were significantly worse than model 3 (all ΔAIC > 2). Removal of day from model 4 resulted in model 3 (ΔAIC < 2), showing that day is also uninformative. Moreover, removal of the interaction between maximum and minimum temperature from model 1 (i.e., resulting in model 20), 2 (i.e., resulting in model 35), 3 (i.e., resulting in model 10), or 4 (i.e., resulting in model 21) resulted in significantly worse AIC (all ΔAIC ≥ 4.63), as well as subsequent removal of maximum or minimum temperature (i.e., removal from model 20 resulting in model 83 or 41 or removal from model 10 resulting in model 57 or 29) resulted in a significantly worse AIC (ΔAIC = 6.59, 2.51, 5.93, 2.56; respectively), indicating that these terms are important and thus confirming that model 3 is the minimal adequate model.

The most important predictor of emergence was daily maximum temperature. It explained the highest proportion of variance in model 3 (estimate ± SE = 1.99±1.21, χ^2_1=14.56, P<0.001, 30.6 % of variance explained) and was present in the first 50 models ordered by increasing AIC. Moreover, its backward elimination always resulted in a significant and large increase in AIC. Minimum temperature (estimate ± SE = −0.88±0.63, χ^2_1=5.53, P=0.0187) and the interaction between minimum and maximum temperature (estimate ± SE= −0.43±0.26, χ^2_1=6.64, P=0.01; Fig. 3.2) explained 17.6 and 25.7 % of variance in model 3, respectively. The range of daily maximum temperatures when females emerged from hibernation was 14.4–22.9 °C, while the range of daily minimum temperatures was −2.1–7.9 °C. Figure 3.2 shows that the probability of emergence rapidly increased from maximum temperatures between 14 and 16 °C and thereafter leveled off to a probability of 1. Minimum temperatures above 7 °C rapidly decreased emergence probability towards 0.

NUMBER OF MALES PRESENTED UNTIL COPULATION

The minimal adequate model for the number of males presented until copulation in the mating trials contained mating delay, female SVL, and their significant interaction ($F_{1,19}$= 4.523, P=0.047, 36.6 % of variance explained; Fig. 3.3). The number of males presented to a female significantly increased with SVL when females mated 2 days after emergence (posthoc test: estimate ± SE = 0.17±0.05, $F_{1,10}$=11.342, P=0.007). There was no significant relationship when females mated 9 days after emergence (estimate ± SE=0±0.07, $F_{1,9}$= 0.004, P=0.952). Female emergence date and all interactions were not significant (all P≥0.1).

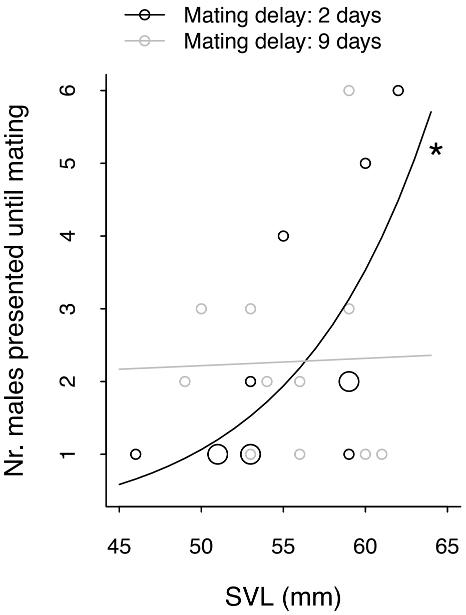

Fig. 3.3 Number of males presented to females until copulation occurred during the mating trials in response to female body size (SVL). Dot size of raw data is proportional to sample size; *large dots* represent N=2 females, while *small dots* represent N=1 female. Significant post-hoc tests of linear predictions are depicted with an *asterisk*

In the re-mating trials, the number of males presented to a female was not significantly affected by mating delay, re-mating delay, female emergence date, SVL, and the interactions (all P≥0.1).

DISCUSSION

Mating windows can compromise mate encounter, reproductive success, and thereby population dynamics and colonization (Berec et al. 2001; Kokko and Rankin 2006; Gascoigne et al. 2009). The strength of the constraint imposed by mating windows depends on the mechanisms triggering their initiation, their duration, the capacity of sperm storage, and the existence of reproductive adaptations or plastic behavioral strategies mitigating their effect. For example, in species with absence of sperm storage, successful reproduction requires that mate encounter is synchronized with the mating window, if mating window initiation is determined endogenously or triggered by emergence from hibernation or climatic conditions. On the contrary, asynchronous mate encounter leads to reduced reproduction, which can result in the Allee effects observed in low-density populations. However, few studies have tested how mating windows are determined and whether plastic reproductive strategies that mitigate their effects exist. This lack of knowledge compromises the understanding of observed behavior, behavioral strategies, and population dynamics. Here we experimentally manipulated the timing of mate encounter in *Z. vivipara*, a species with an endogenously determined reproductive cycle (Gavaud 1983, 1991a), recognized Allee effects in low-density populations (Mugabo et al. 2013), and a suggested short mating window that starts after emergence from hibernation (Bauwens and Verheyen 1985; Heulin 1988; Fitze et al. 2010). Our experiment demonstrates that neither the timing of first mate encounter (mating delay) nor emergence date affected the probability of mating. The probability of re-mating decreased with increasing re-mating delay and mating delay, emergence date, and the interaction between mating and re-mating delay were not significant. The latter shows that the duration of the mating window was similar in all groups of the mating delay treatment.

The absence of an effect of mating delay and emergence date on copulation probability and the presence of a negative effect of re-mating delay on re-mating probability indicate that the initiation of the mating window depends on mate encounter or copulation. Moreover, it indicates that the mating window is of fixed duration.

Emergence from hibernation could be predicted by climatic variables but had no effect on mating and re-mating probability. Specifically, emergence was triggered by daily maximum temperatures above 14 °C, which is in line with previous studies (van Nuland and Strijbosch 1981), and it was hindered by daily minimum temperatures above 7 °C (i.e., by warm nights). This negative effect might be related to sexual functions, e.g., vitellogenesis, whose onset requires low temperatures (Gavaud 1983). More generally, it might be the consequence of a reduced thermal range since regulation of gonadal cycles depends on high as well as low temperatures (Whittier and Tokarz 1992). The fact that emergence is determined by climatic parameters is in line with the general consensus that thermal factors initiate the endogenously controlled components of the reproductive cycle of reptiles (Licht 1972) (in *Z. vivipara* vitellogenesis and sperm maturation; Gavaud 1991a, b), as well as in other species (e.g., mammals: Bronson 1989; birds: Wingfield et al. 1992; amphibians: Duellman and Trueb 1986). The finding that *Z. vivipara* females produce eggs in the absence of copulation and fertilization and that copulation does not affect parturition date (Bleu et al. 2011) provides support for the previously observed endogenous control of the female reproductive cycle (Gavaud 1991b). The initiation of the female's mating window triggered by mate encounter together with the endogenous regulation of the gonadal cycle suggests that mating window initiation is flexible as long as it occurs within the limits imposed by the underlying endogenous program (Gavaud 1991a; Bleu et al. 2011).

Given these constraints imposed by the mating window, plastic reproductive strategies of females may have evolved. There was a significant interaction between mating delay and female SVL on the number of males that had to be presented until the first copulation. Large early-mating females (mating delay 2 days; Fig. 3.3) were choosier than late-mating females and small early-mating females. This finding is in line with previous studies suggesting that larger and older females can exert more intense sexual selection (Richard et al. 2009) because of their superiority in competitive interactions, resulting from large body size, and their greater sexual attractiveness (Fitze et al. 2010) due to the larger number of fertilizable eggs (Bauwens and Verheyen 1987). The fact that no size-dependent effects existed in the late group (mating delay 9 days) indicates that in a situation where mate partners are a scarce resource, females plastically adapt their mating strategy, i.e., exhibit reduced mate choice, to ensure the fertilization of their eggs (Bleu et al. 2011). This in line with findings in *Sepsis* flies where females with no ripe eggs are not choosy, and female mate choice is exerted when flies are not in need of sperm (Teuschl and Blanckenhorn 2007). During re-mating, neither mating nor re-mating delay affected female choosiness, and SVL was not significant either. This is in line with a previous study (Fitze et al. 2010), and it indicates that re-mating delay does not affect choosiness, most likely because fertilization is ensured.

In conclusion, our study provides evidence for a mating window that is initiated at first copulation and that exhibits a fixed duration, as well as for plastic female strategies, which potentially mitigate the negative effects imposed by the window's fixed duration. Initiation of the mating window determined by copulation and the relatively long period where copulations were possible (≥20 days) indicate that the mating window may not impose strong reproductive constraints. Nevertheless, the constraints may be strong enough to favor the evolution of plastic reproductive strategies in females. Both flexible initiation of the mating window and plastic reproductive strategies may be especially beneficial in low density populations submitted to the Allee effect (Allee 1931) and potentially explain why in experimental studies population density affected the timing of reproduction, but not reproduction per se (Mugabo et al. 2013). These results are furthermore in line with the common lizard's rapid range expansion since the Quaternary glaciations (Surget-Groba et al. 2006), and they do not support previous beliefs about the initiation and duration of the mating window (i.e., that the mating window may impose important constraints on reproduction). This illustrates that experimental knowledge of the mechanisms initiating the mating window, of its duration, and of plastic reproductive strategies of females is crucial for understanding whether and how mating windows affect reproduction, population dynamics, and the colonization of new habitats, and, more generally, for understanding past and current range expansions.

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ETHICAL STANDARDS The study conducted complies with the current Spanish laws and with ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research. Capture and handling of lizards was conducted under licenses provided by the Instituto Aragonés de Gestión Ambiental (Gobierno de Aragón).

CONFLICT OF INTEREST The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

S1 MODELS OF EMERGENCE PROBABILITY IN RELATION TO CLIMATIC PREDICTORS

Six daily climatic predictors were initially used to model the probability that females emerged from hibernation on a given day: maximum temperature (°C; max T), mean temperature (°C; mean T), minimum temperature (°C; min T), daily cumulative precipitation (mm), daily maximum wind velocity (km/hr; max wind), and mean maximum wind velocity (km/hr; max mean wind). Mean temperature was strongly correlated with minimum and maximum temperature ($P < .0001$) and had a very low tolerance (0.01). The tolerances of the five remaining predictor variables were ≥ 0.27, indicating no risk of collinearity (Quinn and Keough 2002). Mean temperature was therefore not used as a predictor variable.

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Table S1 Probability of female emergence from hibernation as a function of calendar day, temperature, wind, and precipitation. Shown are the 10 models with lowest Information Criterion (AIC) and seven additional models (depicted in italics) for model comparison. AIC values and ΔAIC with respect to model 1 are given

Model	Main effects			Interactions		AIC	ΔAIC
1	max T	min T	mean max wind	max T × min T		17.47	0.00
2	max T	min T	mean max wind	max T × min T	max T × mean max wind	18.88	1.41
3	max T	min T		max T × min T		19.08	1.60
4	max T	min T	day	max T × min T		20.95	3.47
5	max T	day	mean max wind	max T × day		22.14	4.67
6	max T	min T	day	min T × day		22.32	4.84
7	max T	day	mean max wind	max T × mean max wind	day × mean max wind	22.38	4.90
8	max T	day	mean max wind			22.44	4.97
9	max T	day	mean max wind	max T × day	day × mean max wind	23.22	5.75
10	max T	min T				23.71	6.24
20	*max T*	*min T*	*mean max wind*			*25.02*	*7.55*
21	*max T*	*min T*	*day*			*25.13*	*7.66*
29	*max T*					*26.27*	*8.80*
35	*max T*	*min T*	*mean max wind*		*max T × mean max wind*	*27.00*	*9.53*
41	*max T*	*mean max wind*				*27.53*	*10.06*
57	*min T*					*29.64*	*12.16*
83	*min T*	*mean max wind*				*31.61*	*14.14*

• CHAPTER 4 •

EXPERIMENTAL EVIDENCE THAT PROTANDRY PROVIDES DIRECT FITNESS ADVANTAGES IN MALES AND FEMALES

MEREL C. BREEDVELD • PATRICK S. FITZE. SUBMITTED.

ABSTRACT

Protandry, i.e., the earlier arrival to breeding areas of males than females, has attracted a lot of scientific attention. However, evidence for the mechanisms leading to its evolution is surprisingly scarce. Here, we experimentally manipulate protandry in the common lizard, *Zootoca vivipara*, to disentangle among postulated hypotheses and to unravel the mechanisms leading to its evolution. Our results experimentally demonstrate that the degree of protandry affected sperm presence, but not the probability of copulating. Early emergence from hibernation of males thus increases the chance of fertilizing eggs and later emergence of females reduces the female's reproductive costs and the probability of mating with infertile males. Protandry thus might be the result of multiple selective forces operating simultaneously and independently in each sex. This, together with evidence that copulations with infertile males exist in most taxa exhibiting protandry, suggests that direct benefits may have led to protandry in many species.

KEYWORDS

Mating probability • Molting • Infertility • Sperm maturation • Waiting costs • *Zootoca vivipara*

INTRODUCTION

Protandry, defined as the earlier arrival of males to breeding areas than females, is common in species with restricted breeding seasons. It exists in plants, arthropods, fish, birds, mammals, amphibians and reptiles (Morbey and Ydenberg 2001). For example, in birds, males frequently come back earlier from migration than females (Alatalo et al. 1986; Møller 2004; Rubolini et al. 2004; Morbey et al. 2012) and male fish appear earlier on spawning grounds than females (Morbey 2000). Similarly, in many insects and spiders, males eclose or molt into adulthood (i.e., become sexually mature) earlier than females (Wiklund and Fagerström 1977; Bulmer 1983; Gunnarsson and Johnsson 1990; Wang et al. 1990; Zonneveld 1996; Lehmann 2012); in amphibians, reptiles, and mammals, males frequently emerge earlier from hibernation than females (Michener 1983; Semlitsch et al. 1993; Olsson et al. 1999); and in various angiosperms, males flower earlier than females (Stanton 1994; Forrest 2014). At least seven different hypotheses explaining the occurrence of protandry have been proposed (Morbey and Ydenberg 2001). They can be grouped into indirect and direct selection hypotheses. Direct selection hypotheses include the mate opportunity, the waiting cost, the mate choice and the outbreeding hypothesis, and assume that the degree of protandry (i.e., the magnitude of the difference in arrival of males and females) has direct fitness consequences for males and females (Morbey and Ydenberg 2001). More specifically, they assume that the direct selection benefits of protandry maintain the difference in arrival timing between males and females (Morbey and Ydenberg 2001). For example, the mate opportunity hypothesis posits that selection acts on males to arrive earlier than females, given that early arrival maximizes mating opportunities and thus provides higher reproductive success (e.g., Kokko et al. 2006). Similarly, the waiting cost hypothesis posits that selection acts on females to arrive later than males, given that late arrival minimizes the female's time spent waiting for males to complete their physiological or behavioral preparations required for reproduction (Morbey and Ydenberg 2001). Such preparations include reproductive maturation, establishment of territories, and establishment of dominance hierarchies (Morbey and Ydenberg 2001) and theory suggests that later arrival is safer and energetically cheaper than waiting at the breeding area until males are ready. For example, in some species protandry reduces the risk that females mate with infertile males (Olsson and Madsen 1996; Uller and Olsson 2005), and it reduces the costs of mating and male harassment. In contrast, the indirect selection hypotheses suggest that protandry provides indirect fitness advantages. For example, the rank advantage hypothesis states that selection acts on the territorial sex to be earlier than other individuals of the same sex in order to improve an individual's rank. Other indirect selection hypotheses suggest that protandry arises because sexes differ in their susceptibility to adverse environmental conditions (susceptibility hypothesis), or that selection acts on a trait correlated with arrival that differs among sexes (constraint hypothesis; Morbey & Ydenberg 2001).

Although protandry has received a lot of scientific attention, to date experimental studies are very rare and surprisingly little evidence exists for its selective advantages (Morbey et al. 2012; Morbey 2013). Comparative and non-manipulative studies do not allow disentangling among different proposed mechanisms responsible for the existence and maintenance of protandry (Morbey et al. 2012). To identify the selective factors that shape differential timing of arrival, individual-based intraspecific experiments need to compare the relative fitness among males and females in response to the degree of protandry, i.e., the length of the delay between the arrival of individuals relative to their mates (Morbey et al. 2012).

Furthermore, experimental and non-experimental results are not concordant and experimental studies cannot exclude alternative hypotheses (Morbey and Ydenberg 2001; Morbey et al. 2012). For example, Olsson & Madsen (1996) experimentally manipulated protandry by emerging males sooner or later. Females presented to early emerged males became receptive sooner and had a higher fertilization success. This finding can be explained by two alternatives: 1) that the benefits were due to protandry, i.e., due to the positive effects of early emergence, or 2) due to the costs of prolonged hibernation, given that later emerged males were kept in hibernation for longer. Moreover, no knowledge about the mechanisms leading to the observed differences are provided. Thus, the selective advantages of protandry are not clear.

Here we experimentally disentangle among hypotheses explaining the evolution of protandry. We experimentally manipulated protandry by entering males into hibernation at three different dates using the common lizard, *Zootoca vivipara* as a model species. In early spring males were emerged after the exact same number of days in hibernation. Protandry treatment effects (early, intermediate, and late emerged) were tested on male physiological state (molting and sperm maturation), on mating probability, and on male infertility during copulation.

Protandry has been documented in northern and southern populations of common lizards; males emerge from hibernation in spring and females emerge from several days up to several weeks later, marking the onset of the mating season (van Nuland and Strijbosch 1981; Bauwens and Verheyen 1985; Van Damme et al. 1987; Roig et al. 2000). Sperm maturation and molting start after emergence, and an association between the completion of the first annual molt cycle and the onset of male mating behavior has been suggested (Bauwens et al. 1989; Gavaud 1991b; Roig et al. 2000). This suggests that the temporal difference in emergence gives males time to undergo physiological preparations (sperm maturation, molt completion) before females arrive and at the same time allows females to avoid waiting for males (waiting cost hypothesis).

In line with the mate opportunity and the waiting cost hypothesis we predicted a directional effect of the protandry treatment on male physiological state, namely 1) earlier molt completion in early emerging males, intermediate molt completion in intermediate emerging males, and latest molt completion in late emerging males, given that molting starts after emergence from hibernation (Bauwens et al. 1989). For the same reason, we predicted 2) earlier presence of sperm in early emerging males, later presence in intermediate emerging males, and latest presence of sperm in late emerging males. Under the mate opportunity hypothesis, we furthermore predicted that 3) earlier males copulate with a higher probability and with more females than late males. Under the waiting cost hypotheses, and given the suggested association between molting and mating propensity (Bauwens et al. 1989), we also predicted 4) treatment effects on the day of first mating. More specifically, we predicted that early emerged males mated earlier than intermediate males and that late emerged males mated latest. Alternatively, if mating propensity is driven by sperm maturation, we predicted 5) an association between sperm presence and mating, independent of treatment or the day of molt completion. Moreover, if protandry evolved due to waiting costs, we predicted that 6) mating costs due to male infertility will be reduced for females that were presented to males emerging early.

MATERIALS AND METHODS

SPECIES DESCRIPTION

The common lizard, *Z. vivipara* (Lichtenstein 1823), is a small ground-dwelling Lacertidae that occurs in cool, temperate climates, and is widely distributed throughout Eurasia, where it inhabits habitats like peat bogs and moist heath land (Massot et al. 1992). In natural populations hibernation starts between late September and late October, depending on meteorology.

Males emerge from hibernation around February-March, and females two to three weeks later (Bauwens and Verheyen 1985; Roig et al. 2000; Breedveld and Fitze 2015). The first annual molt of males takes place after emergence and subsequent molts happen in ca. 1 month intervals, and it has been suggested that completion of the first annual molt is a prerequisite for exhibiting mating activities (Bauwens et al. 1989).

Annual gonadal cycles of *Z. vivipara* are under thermoperiodic regulation (Licht 1972; Gavaud 1991a; Gavaud 1991b). Spermatogenesis starts after the mating period, develops during summer, and is inhibited by hibernation (Gavaud 1991b). The last step of spermatogenesis, spermiogenesis, is completed after emergence from hibernation, and depends on heat input during daily basking (Gavaud 1991b). Spermatozoa are therefore already present in the testis upon emergence from hibernation, however they are not mature until they pass to the epididymis, about two weeks after emergence, when males become fully fertile (Courty and Dufaure 1980; Gavaud 1991b). The ovarian cycle is induced by the change in thermal conditions from hibernation to spring, i.e., following female emergence (Gavaud 1991a). The ovulation of matured oocytes occurs about 3-4 weeks after mating (Bauwens and Verheyen 1985).

HIBERNATION

Adult males (*N*=54) and females (*N*=28) were captured in the Pyrenees in northern Spain during late September and early October of 2009, close to the end of their activity period. All captured lizards were transported to the laboratory (Instituto Pirenaico de Ecología, Jaca, Huesca), where body mass, body size (SVL: snout-to-vent-length), and tail length were measured to the nearest millimeter. Lizards were housed in individual terrariums (25×15×15 cm), males and females assigned to separate shelves, and randomly distributed with respect to the previously measured traits (all *P*>0.5). Lizards were kept under standardized heat, light, water, and food conditions (Fitze et al. 2010). They were fed every 3 days with moth larvae (*Galleria mellonella*, Pyralidae) and provided with water ad libitum. Light and heat was 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. light was provided by an ultraviolet (UV)-B light.

Males were randomly assigned to three protandry treatments: “early emerging males” were emerged on the 11th of March, “intermediate emerging males” were emerged 9 days later, and “late emerging males” were emerged 13 days after "early emerging males". There were no differences in male traits between treatments (all *P*>0.3). Date of entry into hibernation was adjusted to ensure that all males were hibernated for exactly 148 days. All females entered into hibernation on the same day as the early emerging males, and were emerged on the same day as the late emerging males.

All individuals were prepared for controlled hibernation in the laboratory, by fasting them for 1 week and gradually acclimatizing them to dark and cool (15°C) conditions. Food was last provided on the first morning of the acclimatizing week, and heating (by means of a heating cable) was provided for 4.5 hrs during the first three days. This duration corresponds to *Z. vivipara*'s gut passage time (Van Damme et al. 1991), and allowed them to complete digestion and empty the digestive tract prior to hibernation.

On the day of entry into hibernation, individuals were given a warm water bath and an abdominal massage to expel any feces remaining in the digestive system. They were then placed in individual hibernation containers containing humid soil and air holes, which were introduced into a dark incubator where they were maintained at a temperature of 4°C throughout hibernation. Hibernating animals were revised every few days for survival. At the end of the hibernation period, lizards were removed from the incubator, returned to standardized lab conditions, and body mass, SVL, and tail length, were measured. In summary, all lizards were hibernated under exactly the same conditions and only emergence date varied between treatment groups.

MOLT COMPLETION

To determine when males completed their first annual molt cycle (Bauwens et al. 1989), males were continuously examined from emergence until the end of June. The end of molt (hereafter referred to as day of molt completion) was determined when a male had recently shed all large skin flakes of the old epidermal layer, at most small skin flakes remained on extremities of the body, and a bright and shiny coloration of the new epidermal layer could be detected.

SPERM PRESENCE

As soon as all males were emerged from hibernation, completion of sperm maturation was determined by the presence of spermatozoa in the distal region of the epididymis (Courty and Dufaure 1980; Depeiges and Dacheux 1985). For this purpose, sperm presence was determined every week during a period of 1 month, and one and two months after the mating trials had ended. Sperm presence was determined by gently pressing on the sides of the cloaca, to extract a droplet that was microscopically analyzed. 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 to avoid sacrificing animals. Its accuracy has been validated in 12 non-experimental male *Z. vivipara*. In each of these 12 males, the non-invasive method was first performed, and directly afterwards the male was sacrificed, and presence/absence of sperm in the epididymis determined following dissection. A highly significant positive correlation between sperm presence/absence as determined through the two methods existed (r_s=0.837, $P \leq 0.001$; M. Breedveld, unpublished result). The day of first sperm presence was defined as the first day on which sperm presence was detected. The duration of sperm presence was defined as the number of days passed since sperm was first detected until the last time sperm was detected.

MATING EXPERIMENT

To determine the effect of protandry on mating, 30 males were distributed into 10 triplets consisting of one early, one intermediate, and one late emerged male. A subset of randomly selected males was used in the mating trials, given that not all females survived hibernation. Males of the subset did not differ from the not included males, in any of the measured traits (P>0.2). Within triplets, males of the 3 protandry treatments differed only in the time of emergence from hibernation, but not in other traits (triplet modeled as random effect, all treatment effects P>0.3). 20 females were distributed into 10 female pairs, and within pairs females were of similar SVL (average range (biggest to smallest) = 1.5 mm; minimum = 0 mm; and maximum = 3 mm). Female pairs were then assigned to a male triplet size-assortatively (r_s= 0.976, P<0.001; big females were presented to big males, and small females to small males; Heulin 1988). Staged mating trials started one day after the emergence of females, which corresponds to the timing of copulation observed in natura (Fitze et al. 2010). Mating trials started one day after emergence of late emerging males, 5 days after emergence of intermediate emerging males, and 14 days after emergence of early emerging males. Mating trials were conducted every 5 days (25 March, 30 March, 4 April, 9 April, 14 April, 19 April, 24 April, 29 April; 2010), between 9:00 a.m. and 4:00 p.m. During each mating trial, males of a triplet were presented sequentially to one single female. At the first mating trial, the female was randomly selected, and thereafter females were presented alternately. Triplets were assigned to one of the six possible male presentation orders (e.g., early, intermediate, and late emerged), and each male presentation order was used for one (*N*=2) or two (*N*=4) triplets. Within triplets, the male presentation order and the time of presentation were the same in all mating trials and each triplet was presented to both females four times, i.e., within 36 days 8 mating trials were conducted. Mating trials thus spanned the pre-ovulatory, ovulatory, and post-ovulatory period (Bauwens and Verheyen 1985), and the whole period during which maturation and completion of sperm maturation should happen in late emerging males.

All mating trials were performed in escape proof wooden boxes (2500 cm^2), equipped with two cardboard shelters, a heating rock, a small drinking pond, and light (Fitze et al. 2010). Females were released into the box and thereafter one of the three males was presented. Males were allowed to interact during one hour, and if lizards were not mating, they were replaced with the subsequent male, respecting the average rate of natural mating attempts (1.1 ± 0.9 h^{-1}; Heulin 1988). If individuals were still mating after 1 hr, they were allowed to complete copulation, and removed 5 minutes after copulation ended. Video cameras allowed constant observation without disturbing the lizards and detection of all copulations. All copulations were recorded and the following variables derived. To analyze protandry effects on mating opportunities, the day of first mating and the number of days copulating were determined. The former corresponds to the day when a male's first copulation happened and the latter to the number of different days on which a given male was copulating. To analyze potential mating costs for females, sperm presence during copulation and protandry effects on sperm presence were analyzed. Sperm presence and sperm absence during copulation were defined as sperm presence before and after the copulation, and sperm absence before and/or after the copulation, respectively.

STATISTICAL ANALYSIS

The day of molt completion, the day of first sperm presence, the day of first mating, and the number of days a male was copulating were analyzed using generalized linear models (GLMs). Day 1 was defined as the day on which the first lizards emerged (11th of March 2010 = day 1). One male did not molt during the entire experiment and was therefore excluded from the analyses. The four models were fitted with a Poisson error and a log link function, and a quasi-Poisson GLM was used in the presence of over-dispersion. Treatment was modeled as a factor and day of molt completion as a covariate. For the analysis of day of first mating, the day of sperm presence was additionally included as a covariate. All two-way interactions between treatment and covariates were included in the initial models.

Ordered Heterogeneity (OH) analyses (Rice and Gaines 1994) were performed to test the predicted directional hypotheses; namely, that early emerging males were earliest, intermediate males later, and late emerging males latest.

The probability of mating (i.e., whether a male did or did not mate at least once) was analyzed using a generalized linear model with a binomial error and a logit link function. Treatment was included as a factor, and day of first sperm presence and day of molt completion were included as covariates. Interactions between treatment and the covariates were also tested. The number of days copulating was analyzed using a generalized linear model with quasi-Poisson family. Final models were selected using backward elimination of non-significant terms ($P>0.05$) and model assumptions were verified in all cases.

Data was analyzed in R 3.0.0 (R Core Team 2013).

RESULTS

MOLT COMPLETION

The day of molt completion differed significantly among treatments ($F_{2,40}$= 8.620, $P<0.001$, Fig. 4.1a) and it significantly followed the predicted order (OH: $r_sP_c=0.999$, $P<0.001$). Early emerging males molted earlier than intermediate emerging males and late emerging males molted latest (Fig. 4.1a). Differences were significant between late and intermediate (individual contrast: $P=0.012$) and late and early emerging males ($P<0.001$). The time from emergence from hibernation until molt completion did not significantly differ among treatment groups ($F_{2,40}=2.008$, $P=0.148$).

SPERM PRESENCE

The day of first sperm presence differed significantly among treatments ($F_{2,34}$= 4.841, $P=0.014$, Fig. 4.1b) and it significantly followed the predicted order (OH: $r_sP_c=0.986$, P<0.010). In early emerging males the day when sperm was present for the first time was earlier than in intermediate emerging males and late emerging males had sperm present latest (Fig. 4.1b). In late emerging males, the day of first sperm presence was significantly later than in early emerging males ($P=0.010$) and it tended to be later than

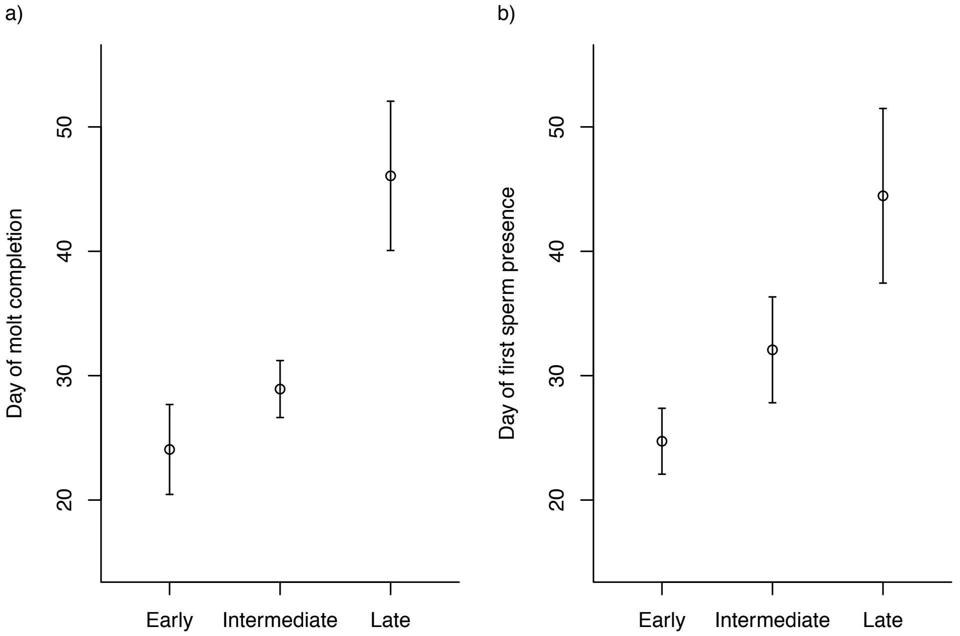

Fig. 4.1 a) Average ± SE day of molt completion and **b)** average ± SE day of first sperm presence for each of the three treatment groups. Day 1 refers to March 11th

in intermediate emerging males (P= 0.092). There was no significant effect of the day of molt completion ($F_{1,33}$= 0.891, P=0.352) and no significant interaction between treatment and the day of molt completion ($F_{2,31}$=0.221, P=0.803). The time from emergence from hibernation until the day of first sperm presence did not significantly differ among treatment groups ($F_{2,33}$=0.430, P= 0.654), and day of molt completion and its interaction with treatment were not significant (P≥0.2).

On the day the males were presented to a female for the first time, the probability of sperm presence was significantly affected by treatment (χ^2_2= 6.211, P=0.045). 100 % of the early males had sperm present, while 40 % of the intermediate and 20 % of the late males had sperm present. This order significantly followed the predicted order (OH: r_sP_c= 0.955, P<0.010).

The duration of sperm presence was not significantly affected by treatment ($F_{2,34}$=1.382, P=0.265), or the day of molt completion ($F_{1,33}$=2.260, P=0.142), and there was no significant interaction ($F_{2,31}$=1.291, P=0.289).

MATING

The probability of mating was significantly and negatively affected by the day of molt completion (χ^2_1=4.618, P=0.032, Fig. 4.2). Males that molted early mated with females, while those molting very late did not copulate. Treatment (χ^2_2= 4.234, P=0.120), the day of first sperm presence (χ^2_1=0.257, P=0.612), and the interactions (P>0.2) were not significant.

The day of first mating was not significantly affected by treatment ($F_{2,8}$= 0.336, P=0.724), the day of molt completion ($F_{1,10}$= 0.018, P=0.897), or the day of first sperm presence ($F_{1,11}$=2.376, P= 0.152). The time from emergence from hibernation until the day of first mating was not significantly affected by treatment ($F_{2,10}$= 2.896, P=0.102), the day of molt completion ($F_{1,8}$=0.331, P=0.581), or the day of sperm presence ($F_{1,9}$=3.159, P=0.109).

31 % of males copulated before they completed molt. The probability of mating before molt completion was positively related with the day of molt completion (χ^2_1=12.229, P=0.001, Fig. 4.3). Early emerging males did not copulate before molt completion, while males that completed molt late copulated with the females, independent of molt completion. Treatment and the interaction with day of molt completion were not significant (P≥0.9).

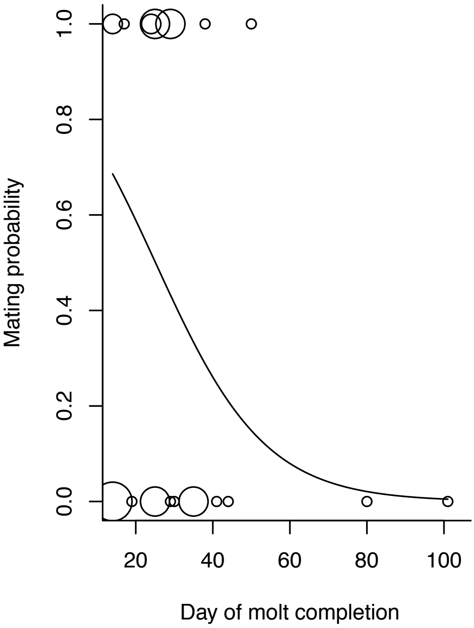

Fig. 4.2 Probability of mating in relation to the day of molt completion. The line corresponds to the model prediction and dot size corresponds to sample size. Day 1 refers to March 11th

70 % of males copulated before they first had sperm present. The probability of copulating before sperm presence was independent of treatment, day of molt completion, or their interaction (P≥0.1).

The number of days on which a male was copulating did not significantly differ between treatment groups ($F_{2,9}$= 1.794, P=0.221), and day of molt completion and the interaction were not significant (P≥0.5).

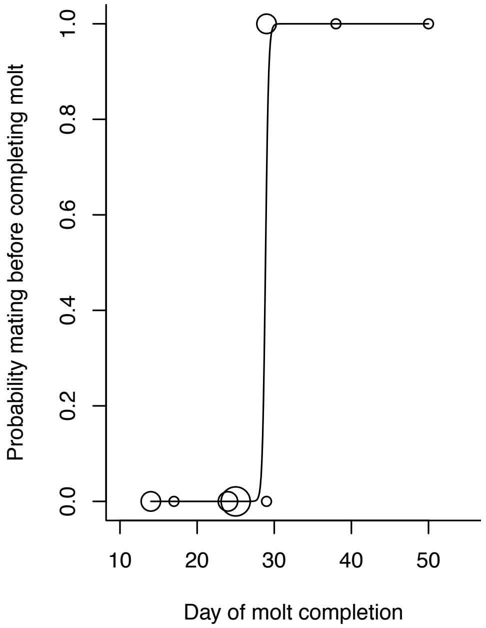

Fig. 4.3 Probability of mating before molt completion in relation to the day of molt completion. The line corresponds to the model prediction and dot size corresponds to sample size. Day 1 refers to March 11th

DISCUSSION

Protandry is very common and has been observed in a wide range of species covering large parts of the tree of life (reviewed in: Morbey and Ydenberg 2001). As a result it has attracted a lot of interest and theoretical models have tackled its evolution (e.g., Wiklund & Fagerström 1977; Bulmer 1983; Iwasa et al. 1983; Parker & Courtney 1983; Zonneveld & Metz 1991; Morbey 2002; Rubolini et al. 2004; Kokko et al. 2006; Spottiswoode, Tøttrup & Coppack 2006). Several hypotheses exist and direct selective advantages have been put forward, although they have been difficult to demonstrate conclusively (Morbey and Ydenberg 2001; Morbey et al. 2012). Here we provide experimental evidence for direct selective advantages of protandry. Our experiment shows that protandry affected the date of molt completion and the date of first sperm presence. In early males molt was completed first and sperm was present first, and in late males molt was completed last and sperm was present last (Figure 4.1). These findings are in line with the directional predictions that molting (prediction 1) and sperm maturation (prediction 2) are temporally related to the date of emergence from hibernation. Moreover, there was no significant difference between protandry treatments in the time from emergence from hibernation until molt completion and in the time from emergence until first sperm presence, consistent with the presence of an endogenous program that determines molt and sperm maturation, which is initiated at emergence (Gavaud 1991b). This result and the fact that 100 % of the early males, 40 % of the intermediate, and 20 % of the late males had sperm present when presented to females for the first time, show that the earlier males emerge from hibernation the more likely they are physiologically prepared for mating when females emerge from hibernation and when mating starts. This physiological advantage may result in a higher reproductive success of early compared to late males, given that the latter will have reduced access to females due to both the female's short mating window (Breedveld and Fitze 2015) and the increased number of males with sperm present. The negative effect of the day of molt completion on the probability of mating indeed shows that late molting males had reduced reproductive success (Figure 4.2), and is in line with the observed association between molt completion and mating (Bauwens, Van Damme & Verheyen 1989) and thus with prediction 3.

Interestingly, the probability of mating, as well as the day that a male first mated, were independent of sperm presence. This and the absence of a treatment effect on the time interval between emergence from hibernation until first copulation is in line with the occurrence of mating without functional sperm. Indeed, 40 % of the copulating males of the late, 60 % of the intermediate, and 0 % of the males of the early group had no sperm present during at least one copulation. These findings support the waiting cost hypothesis, since they show that early emerging females (i.e., females emerging at the same time as males) would have to wait until males have mature sperm (prediction 6). Moreover, the fact that males mate without having mature sperm may pose additional costs through the exposure of females to prolonged copulation attempts, or male sexual harassment (Fitze et al. 2005; Le Galliard et al. 2005). The finding that the day of first mating was neither predicted by the day of first sperm presence, nor by the day of molt completion, contradicts prediction 5, i.e., that an association between sperm presence and mating exists. This indicates that females either cannot detect male fertility or that they cannot resist copulation attempts of males with infertile sperm, showing that females are exposed to the risk of mating with infertile males, in accordance with the hypothesis that multiple partner mating is driven by the benefits derived from mating with multiple partners. More specifically, our results suggest that multiple partner mating ensures fertilization and evolved to bet hedge against infertile males (e.g., Jennions & Petrie 2000; Fitze et al. 2005; Uller & Olsson 2005).

An association between the completion of the first annual molt and the onset of male mating behavior has been suggested, potentially existing due to the time needed for physiological preparations (sperm maturation, molt completion) after emergence from hibernation (Bauwens et al. 1989; Gavaud 1991b). Our results show that both, molt completion and sperm maturation, need time, but no association between them was found. Moreover, the probability of mating before molt completion depended on the date when molt was completed (Figure 4.3). Males that completed molt early did not copulate before molt completion and almost 100 % of the males that completed molt late copulated before completing molt. This shows that molt completion is neither a requirement for copulation nor for sperm maturation, pointing to independent mechanisms being initiated at emergence from hibernation.

The here presented results provide experimental evidence for direct fitness consequences of protandry in males and in females. On one hand, early male emergence, relative to female emergence, can be beneficial to males. This is because early-emerged males that mate can benefit from an advanced stage of sperm maturation, resulting from an earlier onset of spermiogenesis (Olsson et al. 1999). Earlier emergence thereby increases the probability of fathering offspring. On the other hand, late female emergence, relative to male emergence, can be beneficial to females since the probability of mating with infertile males will be smaller in late emerging females (Olsson and Madsen 1996), and thus mating costs through the physical act of copulation or sexual harassment will be reduced, which is in line with the waiting cost hypothesis.

In summary, our results suggests that protandry may have evolved through direct selection on male and female emergence time, and more specifically due to constraints imposed by sperm maturation. Reduced male fertility or temporal infertility, and evidence for the occurrence of mating with infertile males, exists in most taxa exhibiting protandry (e.g., birds (Sheldon 1994), mammals (Gomendio et al. 2007), reptiles (Olsson and Shine 1997), insects (García-González 2004)). Moreover, in a species where sperm production starts and copulations happen months after emergence, the Tasmanian snow skink, *Niveoscincus microlepidotum*, no protandry has been detected (Olsson et al. 1999). This suggests that in many species protandry may have evolved due to constraints imposed by sperm maturation, and thus due to direct selection, which is in line with claims of observational studies (for instance, in birds, fish, amphibians, mammals, reptiles, and arthropods; Morbey & Ydenberg 2001). This result, together with temperature-dependence of sperm maturation (Licht 1972), suggests that higher temperatures, e.g., due to climate change, may accelerate sperm maturation and thereby affect the degree of protandry. However, our study also points to the existence of directional selection on both sexes, suggesting that multiple selective forces may operate simultaneously and independently in each sex and that adaptation to higher temperatures, e.g., due to climate change, may be followed by fast changes in the degree of protandry.

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ETHICAL STANDARDS The conducted study complies with current Spanish laws and with ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research.

CONFLICT OF INTEREST The authors declare that they have no conflict of interest.

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• CHAPTER 5 •

THE TIMING OF MATE ENCOUNTER AFFECTS INVESTMENT DURING MATING: EVIDENCE FOR FLEXIBLE REPRODUCTIVE STRATEGIES OPTIMIZING DARWINIAN FITNESS

MEREL C. BREEDVELD • PATRICK S. FITZE. SUBMITTED.

ABSTRACT

The benefits obtained from mating are usually condition-dependent, which may favor the evolution of flexible investment strategies, e.g., through variation in the strength of mate choice or the duration of copulation. Flexible investment strategies are predicted to depend on the likelihood with which alternative mates can be acquired and thus they should depend on the timing of mate encounter, however to date scarce experimental evidence for this hypothesis exists. Here we manipulated the length of the delay until first mating and the interval between first and second mating in the common lizard, *Zootoca vivipara*, and determined their effects on copulation duration, a proxy of the amount of sperm transferred, and fertilization success. Copulation duration of second mates depended on the inter-mating interval and on the fertilization success of the first mate, providing evidence for flexible mating strategies. Those were most likely the consequence of time constraints imposed by female reproductive stage and they were incongruent with predictions derived from male strategies. Fertilization success of first mates increased with increasing inter-mating interval and it was higher in females that were closer to ovulation, showing that flexible mating strategies significantly affected a close correlate of Darwinian fitness. This indicates that reproductive strategies are time dependent. Increased success of first mates in the absence of a rapidly following subsequent copulation, points to a mechanism assuring fertilization of the eggs, and thus to a mechanism potentially mitigating demographic effects on reproductive success, e.g., Allee effects.

KEYWORDS

Copulation duration • Fertilization success • Mating interval • Reproductive investment • *Zootoca vivipara*

INTRODUCTION

Mating decisions are predominantly based on the potential benefits of mating with a particular partner (Andersson 1994). These benefits may depend on extrinsic and intrinsic factors, such as environmental or social conditions and an organism's physiological state (McNamara and Houston 1996; Jennions and Petrie 1997). Thus, condition-dependent, i.e., flexible reproductive strategies may have been favored by evolution, allowing individuals to optimize reproductive success (Bull and Shine 1979; Parker and Simmons 1994; Gage 1995; Roff 2002). For example, strong choosiness exerted by females may be beneficial in high-density populations where many males are available. However, in low-density populations, or during the colonization of new habitat, strong choosiness may lead to no reproduction, given that a suitable male (exhibiting the preferred traits) may never be encountered, potentially potentiating Allee effects (Calabrese and Fagan 2004; Kokko and Mappes 2005; Bleu et al. 2012; Larsen et al. 2013). Flexible strategies, e.g., strong expression of mate preferences in the presence of many males and no mate choice in the presence of a single male, may thus optimize reproductive success and thereby guarantee reproduction in low-density populations or during the colonization of new habitat. Similarly, the amount of resources invested during mating might be adjusted with respect to the expected benefits, i.e., differential allocation (Burley 1986; Sheldon 2000; Bonduriansky 2001; Reinhold et al. 2002). If benefits are high, a lot of resources should be invested, however, if they are low, few resources should be invested (e.g., Fitze and Le Galliard 2008). Such resource adjustment may be especially easy in males, since males may invest more or less sperm in a female, i.e., strategic sperm investment (Lorch et al. 1993; Olsson and Madsen 1998; Engqvist and Sauer 2001; delBarco-Trillo and Ferkin 2004; Parker and Pizzari 2010). Similarly, females may control the transferred sperm quantity. For example, in species where sperm is transferred continuously during copulation, females may control the duration of copulation and thereby the amount of transferred sperm by cooperating or terminating copulation (Thornhill 1983; Eberhard 1998; Andrés and Cordero-Rivera 2000; Engqvist and Sauer 2003; Pilastro et al. 2007), and females of instantaneous ejaculating species may simply eject sperm after the copulation (e.g., in birds and mammals; Birkhead 1998a; Pizzari and Birkhead 2000).

Such flexible strategies should depend on the likelihood with which alternative mates can be acquired and thus they should depend on the rate of mate encounter (Emlen and Oring 1977;

Shuster and Wade 2003; Kokko and Rankin 2006; Kokko et al. 2012). It has been shown that the availability of mates affects mating strategies (e.g., Veit and Lewis 1996; Boukal and Berec 2002; Cornwallis and Birkhead 2006; Mugabo et al. 2013). Similarly, mating strategies can be affected by the temporal pattern of mate encounter (e.g., Rutowski and Alcock 1980; Crowley et al. 1991; Calabrese and Fagan 2004; Gowaty and Hubbell 2005). For instance, in some multiple mating species it has been shown that males strategically allocate sperm under sequential mate encounter (e.g., with respect to female novelty, the risk of sperm competition, and female quality; Engqvist and Sauer 2001; Pizzari et al. 2003; Lüpold et al. 2011; Ramm and Stockley 2014). Moreover, the time interval between sequential mates can affect fertilization success (Parker 1990). This suggests that investment strategies in response to the interval between matings could be beneficial. However, besides a few experiments in insects (Simmons et al. 1992; Simmons 1995; Reinhold and von Helversen 1997; Lehmann and Lehmann 2000), experimental studies that manipulate the time interval between successive matings with the aim of determining the effects on investment during mating are, to our knowledge, lacking.

The time until mate encounter and the time interval between a first and second mate encounter could indeed drive variation in reproductive investment of females because it signals the rate of mate encounter and thus the likelihood with which additional copulations may be obtained. Due to the limited amount of time during which eggs can be fertilized, the availability of mates can change a female's benefits obtained through additional mating (Real 1990; Jennions and Petrie 1997), and the time of encounter may thus affect mate choice and investment (Lea et al. 2000; Qvarnström et al. 2000; Lynch et al. 2005; Uetz and Norton 2007; Hoffer et al. 2012; Tinghitella et al. 2013). For example, if the time interval between the encounter of first and second mates is short, signaling high mate availability, females may afford to be choosy and/or resist copulation attempts. Thus lower investment in mating is predicted. In contrast, when the delay between first and second mate encounter is long, signaling low mate availability and a low probability of encountering additional mates, females might be more interested and more willing to copulate, given the low chances of encountering another male. Alternatively, increased intervals could also lead to decreased female interest, given that a female has already obtained sperm from a first mate, and given that fertilization of the eggs is becoming less probable due to reproductive constraints (e.g., mating windows; Breedveld and Fitze 2015). This alternative becomes more likely if a female's mating window is of temporary duration, as in most sexually reproducing species, and is initiated during first mating (i.e., female receptivity decreases with time since a first mate), or if increased intervals lead to higher choosiness due to trade-up strategies (Gabor and Halliday 1997; Jennions and Petrie 2000; Bleu et al. 2012).

Similarly, since the time interval between a first and second mate can affect a male's likelihood of fertilizing eggs, i.e., it can determine the outcome of sperm competition (Dziuk 1965; Huck et al. 1985; Lessells and Birkhead 1990; Schwagmeyer and Foltz 1990; Birkhead 1998b; Moreira et al. 2007; Pizzari et al. 2008), males may adjust ejaculate expenditure in response to the length of the interval (Parker 1990; Ball and Parker 2000). Theoretical models of optimum sperm investment assume the existence of a trade-off between current ejaculate expenditure and the amount of sperm that can be invested in subsequent mates, imposing important constraints on male sperm investment (Parker and Pizzari 2010). In general, males are predicted to invest more sperm when an increased risk of sperm competition exists (Parker et al. 1997). The presence of rival males, a female's mating status (e.g., virgin or previously mated) and a male's mating order (i.e., first or second to mate), among other factors, can contribute to variation in the risk of sperm competition (Parker and Pizzari 2010), and various studies have shown that males indeed adjust ejaculate expenditure in response to these factors (Linley and Hinds 1975; Bellis et al. 1990; Suter 1990; Gage 1991; Gage and Baker 1991; Simmons et al. 1993; Shapiro et al. 1994; Cook and Wedell 1996; Gage and Barnard 1996; Wedell 1998; Olsson et al. 2004; Cornwallis and Birkhead 2006; Ramm and Stockley 2014). Thus, if fertilization rewards decline with the time passed since a female's first copulation (Huck et al. 1985; Schwagmeyer and Foltz 1990), second males may adjust sperm investment and invest less with longer intervals.

In summary, it has been shown that flexible mating strategies, including differential reproductive investment, exist in males and females, and that mate encounter per se can affect them. However, there is, to our knowledge, scarce experimental evidence of investment strategies in response the sequential timing of mate encounter. Considering that sequential mate encounter is common in natural populations; that processes such as climate change and habitat fragmentation can drastically affect the temporal pattern of mate encounter (Møller 2004; Lane et al. 2011; Morbey et al. 2012); and that the latter can negatively impact fitness and population viability, e.g., through mating failure and Allee effects (Calabrese and Fagan 2004; Saino et al.

2011; Larsen et al. 2013); lack of evidence for the generality of this hypothesis can compromise predictions of the effects of variation in population demography on reproductive success and population dynamics.

Here we experimentally test, using the polygynandrous common lizard *Zootoca vivipara* as a model species, whether the timing of mate encounter affects mating strategies and Darwinian fitness. We experimentally manipulated the length of the interval between two successive mating events of females (hereafter referred to as re-mating delay) and analyzed its effect on copulation duration and fertilization success. The common lizard exhibits spontaneous ovulation (Bleu et al. 2011) and is a non-instantaneous ejaculator (i.e., sperm is transferred over longer time periods). Copulation duration is a proxy for the amount of transferred sperm (Olsson and Madsen 1998; Shine et al. 2000; Olsson 2001; Olsson et al. 2004) and thus for the investment in a specific mate. In order to disentangle whether potential investment strategies are driven by cues about sperm competition or by female reproductive stage, we also manipulated the time delay between female emergence from hibernation and the day of first mating (hereafter referred to as mating delay). Given the spontaneous ovulation, differences in the mating delay result in differences in the time left until ovulation (Bleu et al. 2011). Finally, we analyzed treatment effects on male and female fertilization success, to understand effects of copulation duration on reproductive success.

If re-mating delay drives differential reproductive investment, we predicted that 1) copulation duration with second mates depends on the length of the re-mating delay. If re-mating delay affects copulation duration, we further predicted 2) an effect of re-mating delay on the fertilization success of first and second mates. If copulation duration is due to male strategy in response to cues signaling the risk of sperm competition, we predicted 3) no effects of mating delay on copulation duration of first mates, since first males always mated with virgins. We further predicted differences in the copulation duration between first and second mating males, since second, but not first males were exposed to potential sperm competition. If copulation duration is driven by a female's reproductive stage, we predicted that 4) copulation duration increases with larger mating delay since in spontaneous ovulating species (Bleu et al. 2011) later mating females are further advanced in their reproductive cycle and thus under stronger time constraints to assure fertilization.

MATERIALS AND METHODS

SPECIES DESCRIPTION

The common lizard, *Zootoca vivipara* (Lichtenstein 1823), is a small ground-dwelling Lacertidae that is widely distributed throughout Eurasia, where it inhabits habitats like peat bogs and moist heath land (Massot et al. 1992; Clobert et al. 1994). Males emerge from hibernation in spring (around February-March), after which they complete spermiogenesis to become fertile (Roig et al. 2000). Females emerge several weeks after males, initiating the mating season (Bauwens et al. 1989; Roig et al. 2000).

The common lizard exhibits a polygynandrous mating system, in which most females mate with multiple males (1 to 5; Laloi et al. 2004) and males mate with multiple females (1-14; Fitze et al. 2005). During copulation, a male first holds a female at the posterior abdomen with his mouth, twists his body around the female, and inserts his hemipenis in the female's cloaca. Thereafter sperm is transferred continuously until the end of the copulation (Olsson and Madsen 1998). Copulation duration is positively related to ejaculate volume (Shine et al. 2000; Olsson and Madsen 2001; Olsson et al. 2004), and therefore an indicator of the number of spermatozoa transferred (Olsson and Madsen 1998). Copulation can last from a few minutes up to several hours (Heulin 1988; Olsson and Madsen 1998). Mating is costly for females, since the male's grip produces scars and mating can lead to the death of the female (Fitze and Le Galliard 2008). Females exert pre-copulatory mate choice, by accepting males or fending them off (Fitze et al. 2010). Moreover, they exhibit choice strategies with respect to the time of first mate encounter (Breedveld and Fitze 2015) as well as trade-up strategies (Fitze et al. 2010; Laloi et al. 2011). Males exhibit best-of-bad-job strategies (Gonzalez-Jimena and Fitze 2012).

In common lizards, the onset of ovulation, and hence fertilization, occurs spontaneously about one month after emergence from hibernation (Gavaud 1983; Heulin et al. 2008), which is typically 3-4 weeks after mating (Bauwens and Verheyen 1985; Bleu et al. 2011). The relatively long time interval between copulation and fertilization suggests prolonged sperm survival within the female's reproductive tract and possible overlap of ejaculates provided on different days, increasing the potential for sperm competition (Olsson and Madsen 1998; Girling 2002). Moreover, eggs belonging to the same clutch are usually fertilized by several males (Laloi et al. 2004; Fitze et al. 2005), indicating that sperm competition indeed exists (Girling 2002). However, specialized structures for long-term sperm storage, found in many lizards (Sever and

Hamlett 2002), have not been reported in common lizards. Furthermore, to date, neither evidence for cryptic female mate choice in the common lizard exists, nor have other mechanisms potentially biasing fertilization success, e.g., mate guarding, copulatory plugs, and induction of female non-receptivity through copulation (Parker 1998), been observed.

PRE-EXPERIMENTAL CONDITIONS

All lizards were captured from a population in Roncesvalles in the Spanish Pyrenees. Prior to hibernation (September 2010), they were released in 100 m^2 semi-natural enclosures, at the research station 'El Boalar' (42°33′N, 0°37′W, 700 m a.s.l.) of the Instituto Pirenaico de Ecología, Jaca, Spain. The enclosures contained natural vegetation, hides, rocks, logs, and water ponds, and were surrounded by escape proof walls and covered with nets to prevent predation (Fitze et al. 2008). Males and females were kept in different enclosures to assure that no individual mated prior to the experiment. After the first males emerged from hibernation (March 2011), female enclosures were searched daily and intensively between 9:00 a.m. and 5:00 p.m. Females were captured upon detection and males shortly prior to experimentation.

Captured animals were transported to the laboratory and measured for body mass (to the nearest milligram), body size (SVL; snout-to-vent-length), and tail length (to the nearest millimeter), and housed in individual terrariums (25×15×15 cm) under standardized conditions (Fitze et al. 2010). Within sexes, lizards were randomly distributed among shelves (body size, body mass, body condition, tail condition, coloration; all $P>0.5$), and males and females were housed on separate shelves, to avoid the perception of between-sex olfactory cues. Lizards were fed every 3 days with moth larvae (*Galleria mellonella*, Pyralidae) and provided with water ad libitum. Light and heat was 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. light was provided by an ultraviolet (UV)-B light.

MATING EXPERIMENTS

All females (N=26) were allowed to mate with two different males (N=80). "First mates" were presented to females that had emerged from hibernation either 2 or 9 days ago (mating delay = 2 or 9), and "second mates" were presented to females either 2, 6, or 10 days after first mates (re-mating delay = 2, 6, or 10), using a 2×3 experimental design (see Breedveld and Fitze (2015) for details).

All mating trials were performed between the 20th of March and the 19th of April, and between 9:00 a.m. and 6:00 p.m., in escape proof wooden boxes (2500 cm^2), equipped with cardboard shelters, a heating rock, a drinking pond, and light (Fitze et al. 2010). Each female was released into a separate and clean box and thereafter a randomly selected male was introduced. Lizards were observed from a concealed location every 5 minutes and all copulations taking place were recorded. If no mating occurred within 1 hour, males were removed and replaced with a new randomly selected male, respecting the average rate of natural mating attempts (1.1 ± 0.9 $hour^{-1}$; Heulin 1988). If mating did occur, both individuals were removed after 1 hour, or, if they were still mating, they were removed 5 minutes after the copulation ended. Mating trials were terminated as soon as a female completed copulation with one male. After a mating trial, males were immediately released in their outdoor enclosure of origin. Repeated mating by the same males was avoided throughout the experiment. Only 14 % of males copulated twice, but never with the same female, and with a minimum of two days between copulations. Females were released back into the enclosures 20 days after their capture. Thereafter they were recaptured on a weekly basis, and the status of egg development determined by abdominal palpation. Gravid females carrying eggs that approached size at oviposition were maintained in the laboratory under standardized conditions, and all other females were released immediately after recapture. Shortly after oviposition, clutch size was determined and 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 see: San-Jose et al. 2014).

There were no significant differences between first and second mates in SVL, body condition, tail length, and color (all $P \geq 0.1$). Moreover, there were no significant differences in the number of days passed since mates were first captured in the enclosures and copulation; in whether or not they had been previously presented to another female; and in whether or not they had previously mated (all $P \geq 0.1$). There were no initial differences in female emergence date, SVL, body condition, or tail length between females presented at different mating and re-mating delays, and the interaction between mating delay and re-mating delay was not significant either (all $P \geq 0.1$).

PATERNITY ASSIGNMENT

Tissue samples were collected from all mothers, putative fathers, juveniles, and undeveloped embryos, 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 (Marshall et al. 1998; Fitze et al. 2005). Detailed methods of DNA extraction, polymerase chain reaction, and allele size determination are described elsewhere (Laloi et al. 2004).

STATISTICAL ANALYSIS

Effects of mating and re-mating delay on copulation duration and fertilization success were analyzed, using all females that mated, re-mated, and laid fertilized eggs.

Copulation duration was measured with a precision of 5 minutes and square root transformed to meet the normality assumption. Copulation duration of first mates was analyzed using a generalized linear model with Gaussian family and identity link and with mating delay as a factor. Copulation duration of second mates was analyzed using a generalized linear model with Gaussian family and identity link and with mating delay and fertilization success of the first mate (i.e., whether or not at least one egg was fertilized by the first mate) as factors, re-mating delay (2, 6, or 10 days) as a covariate, and all two-way interactions. To test for differences in copulation duration between first and second mates, a generalized linear model with Gaussian family and identity link and was run with copulation duration as dependent variable and mate order (first or second mate) as a factor.

The number of eggs laid by each female was analyzed using a generalized linear model with quasi-Poisson family (to account for over-dispersion) and log link and mating and re-mating delay as factors. The proportion of fertilized eggs was analyzed using a generalized linear model with quasi-Binomial family and logit link and mating and re-mating delay as factors.

Fertilization success of either mate was defined as the number of eggs he fertilized, and was analyzed using a generalized linear mixed model with Poisson error and log link. Factors mating delay, mate order, the covariate re-mating delay, and up to two-way interactions were tested. Female was included as a random effect.

In all of the analyses, model selection was performed using likelihood ratio tests. Post-hoc tests were performed using individual contrasts whenever interactions between factors existed and *P* values adjusted using Bonferroni procedures.

Analyses were performed using R 3.0.0 (R Core Team 2013).

RESULTS

COPULATION DURATION

Copulation duration (√minute) with first mates was significantly longer when mating delay was 9 days (43.6 ± 7.6 minutes; mean ± SE) than when it was 2 days (21.0 ± 4.8 minutes; χ^2_1=5.926, *P*=0.015). Copulation duration with second mates was significantly shorter when mating delay (i.e., with first mates) was 9 days (36.4 ± 4.3 minutes) than when it was 2 days (43.0 ± 5.6 minutes; χ^2_1=7.337, *P*=0.007) and the interaction between re-mating delay and fertilization success of the first mate was significant (χ^2_1=4.182, *P*=0.041; Fig. 5.1).

Post-hoc analyses showed that when the first mates fertilized at least one egg, copulation duration with the second mate decreased significantly with increasing re-mating delay (χ^2_1=10.816, *P*=0.002, estimate = -0.3), while no significant relationship existed when first mates did not fertilize any eggs (χ^2_1= 0.558, *P*=0.455, estimate = -0.1; Fig. 5.1). There were no other significant interactions (all *P*≥0.2) and there was no significant difference in copulation duration between first or second mates (χ^2_1=1.116, *P*=0.291).

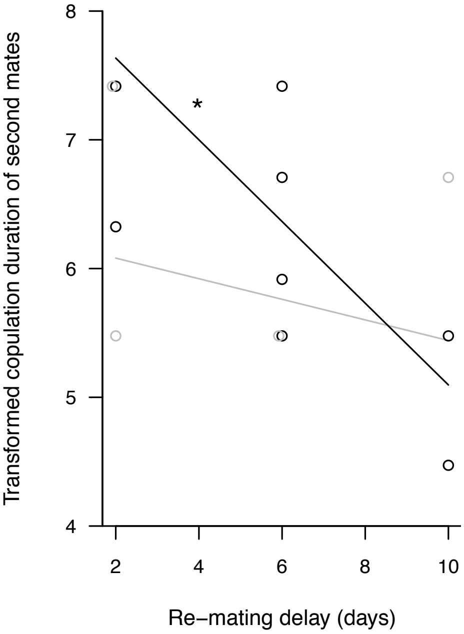

Fig. 5.1 Copulation duration of second mates in response to the interaction between re-mating delay and the fertilization success of first mates. The Y-axis corresponds to square-root transformed copulation duration. Original data and model predictions are shown for cases where no eggs (*grey*) or at least one egg (*black*) were fertilized by the first mate. Significant slope is depicted with an *asterisk*

FERTILIZATION SUCCESS

There were no significant effects of mating and re-mating delay on clutch size, number of eggs laid, or on the proportion of fertilized eggs (all *P*≥0.4). The number of eggs fertilized by either male was significantly affected by an interaction between mate order and mating delay (χ^2_1= 4.405, *P*=0.036; Fig. 5.2a). Post hoc contrasts showed that first mates fertilized significantly more eggs than second mates when mating

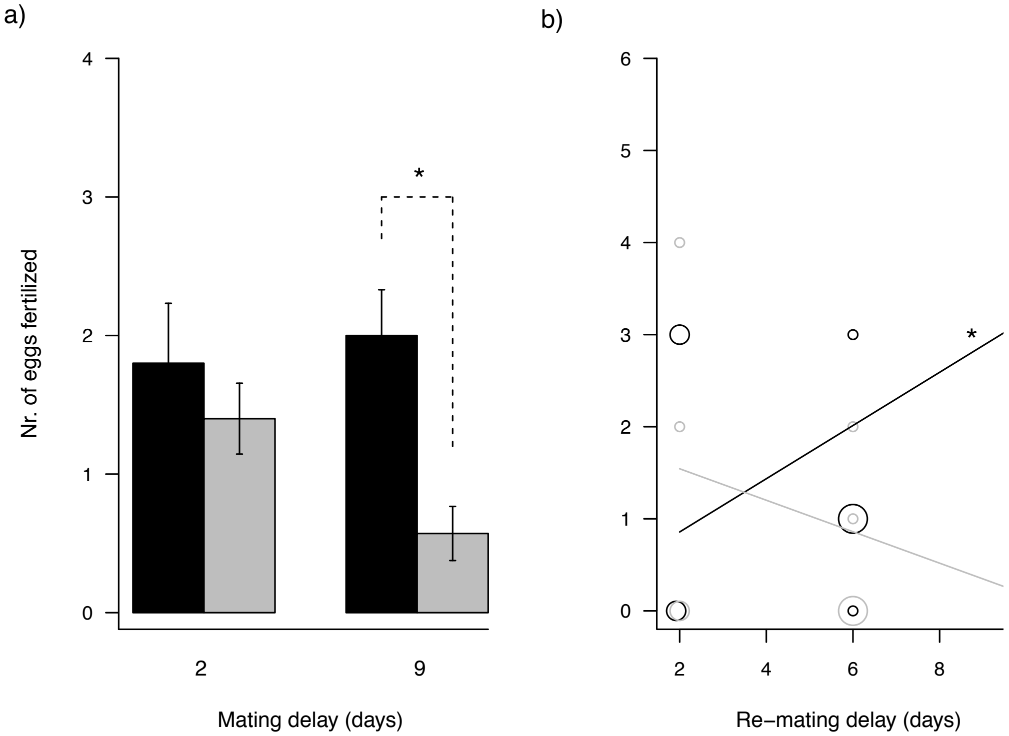

Fig. 5.2 Effects of mate order, mating delay, and re-mating delay on fertilization success. **a)** Interaction between male order and mating delay, and **b)** male order and re-mating delay on the number of fertilized eggs. Means ± SE (a) and original data and linear predictions (b) are shown. Dot size is proportional to sample size. Depicted in *black* and *grey* are first and second mates, respectively. Significant contrasts or significant slopes are depicted with an *asterisk*

delay was 9 days (*z*=2.672, *P*=0.032, estimate = 1.669) but not when mating delay was 2 days (*z*=0.081, *P*=0.935, estimate = 0.0436). The number of fertilized eggs was also affected by a significant interaction between mate order and re-mating delay (χ^2_1= 6.988, *P*=0.008; Fig. 5.2b).

Post hoc tests showed that the number of eggs fertilized by first mates significantly increased with increasing re-mating delay (χ^2_1=4.360, *P*=0.037, estimate = 0.153), while a marginally significant negative relationship existed in second mates (χ^2_1=3.117, *P*=0.077, estimate = -0.187). All other interactions were not significant (all *P*≥0.4).

DISCUSSION

Flexible mating strategies with respect to the timing of mate encounter may be an elegant way of optimizing reproductive investment and they may be one reason why low density populations can be evolutionary stable (Hissmann 1990; Calabrese and Fagan 2004; Gowaty and Hubbell 2005; Gascoigne et al. 2009; Fagan et al. 2010) and why the colonization of empty habitats can be successful. Here we experimentally manipulated the timing of mate encounter and analyzed its effect on copulation duration, fertilization and reproductive success of *Z. vivipara*. Our study reveals the existence of reproductive investment strategies in response to the length of the time interval between two sequential copulations. Copulation duration with second mates significantly decreased with increasing re-mating delay when first mates fathered eggs. This pattern is in line with prediction 1, i.e., that copulation duration with second mates depends on the length of the re-mating delay, and it points to the existence of reproductive investment strategies. However, copulation duration did not depend on the length of the re-mating delay when first mates did not fertilize any eggs in the female's clutch. This suggests that adjustment of copulation duration with second mates did not occur with respect to whether a female had previously mated or not. In contrast, it depended on whether a female had acquired sperm from a previous mate or not, which is in line with previous observations where males copulated independent of whether they carried sperm (M.C. Breedveld, personal observation). This finding is also congruent with bet-hedging strategies against sterility or genetic defects (Thornhill and Alcock 1983; Wolff and Macdonald 2004). First copulations that occurred 9 days after female emergence lasted significantly longer than first copulations that occurred 2 days after female emergence. This finding and the fact that copulation duration did not significantly differ between first and second mates (i.e., between mating with virgin or mated females) is in contrast to the predictions derived from strategic male investment in response to the risk of sperm competition (prediction 3). The findings are however in line with the adjustment of copulation duration with respect to female reproductive stage (prediction 4). Virgin females that emerged 9 days before the first copulation are closer to ovulation than virgin females that emerged 2 days before the first copulation, and thus they are under stronger pressure to assure fertilization, potentially explaining why they exhibited longer copulations. Similarly, the decreased copulation duration after longer re-mating delays by females that already obtained sperm from first mates may be caused by females that reduce their reproductive investment in order to conserve energy or avoid unnecessary mating costs, when smaller fitness returns are predicted (i.e., inseminations closer to ovulation might be less successful). This finding is thus in line with declining willingness to re-mate and with trade-up strategies (Fitze et al. 2010; Laloi et al. 2011; Bleu et al. 2012). The fact that clutch size and fertilization success were not significantly affected by mating and re-mating delay, suggests that females were not sperm limited and that trade-up strategies did not have negative effects on female reproduction.

In line with prediction 2, re-mating delay positively and significantly affected the number of eggs fertilized by first mates. Moreover, the number of eggs fertilized by second mates tended to decrease with re-mating delay, which is in line with the prediction, however this effect was only marginally significant. These results show that the length of the time interval between successive matings is an important determinant of a male's fertilization success, which is in accordance with previous studies of time-dependent sperm competition patterns (Schwagmeyer and Foltz 1990; Pizzari et al. 2008).

The fact that fertilization was influenced by mating delay, may be explained by the length of the time interval between insemination and ovulation (Olsson and Madsen 1998). Females presented with males later after emergence from hibernation may have been at a more advanced reproductive stage at the moment of first mating (Gavaud 1991), potentially leading to a higher probability of fertilization by first mates. However, even though first mates fertilized significantly more eggs than second mates when mating delay was 9 days, copulation duration with first mates was not longer than with second mates when mating delay was 9 days (estimate = 0.502, z=0.785, P= 0.678). Moreover, there was no difference in fertilization success when mating delay was 2 days, although copulation duration with first mates was significantly shorter than with second mates (estimate = -2.086, z=-2.754, P= 0.012). Therefore, we cannot confirm that copulation duration is a good predictor of fertilization success in common lizards, as is the case in other lizards (Olsson et al. 2004), at least not when two males copulate with the same female on different days. This underlines the previous findings suggesting that the observed plastic investment strategies with respect to the time of mate encounter are most likely not driven by male strategy but rather by female strategies.

Overall, the findings provide experimental evidence that the timing of successive mate encounters can be an important determinant in reproductive investment strategies and they confirm its effect on fertilization success and thus Darwinian fitness. These flexible strategies could mitigate the potential negative effects of reduced mate encounter, strong choosiness, and male sterility on a female's reproductive success (e.g., through adjusting the strength of mate choice and/or mating duration) and population dynamics, in line with observed rapid post-glacial population expansions (Surget-Groba et al. 2002). Since sequential mating is the norm (at least in internal fertilizers) and since sequential mate encounter is common in natural populations of most species, investment strategies with respect to inter-mating intervals might be widespread, but are unappreciated in most studies. Understanding how individuals adjust reproductive strategies in response to temporal variation in mate encounter is important for predicting how individuals, and ultimately populations, will respond to changes in population demography. If individuals' investment strategies are flexible, the effect of variation in mate encounter on the dynamics of populations as a whole might be small, while inflexible responses to variation in the timing of mate encounter can negatively impact fitness and population viability, e.g., through mating failure and Allee effects (Calabrese and Fagan 2004; Saino et al. 2011; Larsen et al. 2013).

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ETHICAL STANDARDS The conducted study complies with current Spanish laws and with ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research.

CONFLICT OF INTEREST The authors declare that they have no conflict of interest.

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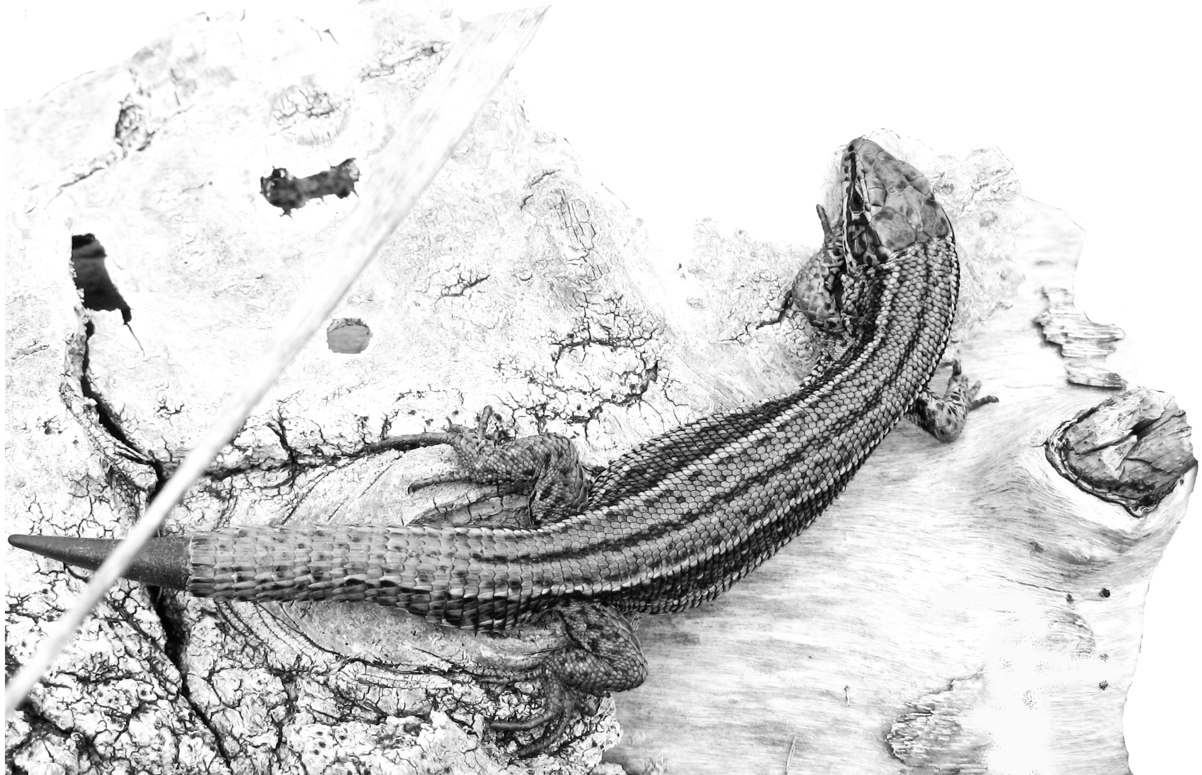
• CHAPTER 6 •



EFFECTS OF MATE AVAILABILITY ON THE PRODUCTION OF SECOND CLUTCHES: SPERM STORAGE VERSUS RE-MATING

MEREL C. BREEDVELD • LUIS M. SAN-JOSE • CRISTINA ROMERO-DIAZ • EDUARDO R.S. ROLDAN • PATRICK S. FITZE. IN PREPARATION.

ABSTRACT

Females of iteroparous species face conflicts over producing one or multiple broods per reproductive season, and over the fertilization of different broods with sperm from the same or different mates. These conflicts might be affected by the temporal availability of males and may affect the reproductive success of both sexes.

Here, we experimentally manipulated male availability at the first and second brood and test its effect on reproductive strategies and success of females and males, using the oviparous common lizard, *Zootoca vivipara*, as a model species. "Restricted" individuals could mate with a limited number of partners and only at the start of the season (i.e., before first clutch production), and "unrestricted" individuals could mate and re-mate freely throughout the reproductive season (i.e., before and after first clutch production). First clutch characteristics were not significantly affected by treatment. However, restricted females produced fewer second clutches and their fertilization success was lower, demonstrating that re-mating is crucial for female reproductive success. In unrestricted females, significantly fewer eggs were fertilized by stored sperm, demonstrating that re-mating by females also affects male reproductive success. Sperm presence in males was high until the end of the re-mating period, suggesting a close co-evolution between male and female strategies.

Re-mating thus affects the production, success, and fertilization patterns of second seasonal reproductive events. This highlights the importance of a close synchronization of sperm production and ovulation for egg fertilization and reproductive success, both affecting population dynamics and conservation efforts.

KEYWORDS

Fertilization success • Multiple mating • Multiple broods • Mate availability • Sperm storage • *Zootoca vivipara*

INTRODUCTION

Females and males of most species face a conflict over the number of mates (Rowe et al. 1994; Chapman et al. 2003). This conflict is mediated by the costs of mating and the benefits of mating with multiple partners. The former includes the costs of mating per se, as well as the costs of sexually transmitted disease, of mate searching, and of increased predation (Jennions and Petrie 2000). In contrast to males, females of iteroparous species face at least two additional conflicts. First, they face a conflict between producing one or multiple broods per reproductive season (Verhulst et al. 1997). This conflict usually depends on the optimality of the expected breeding conditions, and thus, among others on climatic conditions, advancement of the reproductive season, and mate availability (Emlen and Oring 1977; Bronson 1985; Adolph and Porter 1993; Dunn 2004). Mate availability is especially important if females cannot store sperm from a previous copulation or require a replenishment of their sperm stores (Anderson 1974; Birkhead and Møller 1993). In the presence of mates, fertilization of the eggs will be assured, while in the absence, a reproductive event may fail (Calabrese and Fagan 2004; Rhainds 2010; Bleu et al. 2011). Producing one or multiple broods per reproductive season directly affects the trade-off between current and future reproduction (Schaffer 1974; Roff 1992; Richner and Tripet 1999), which is mediated by the current reproductive value, the survival costs, and the future reproductive value. If survival costs are low, producing a second brood can enhance fitness under optimal conditions (e.g., in the presence of potential mates), while under suboptimal conditions (e.g., in the absence of potential mates), reducing the investment or skipping a breeding event, allows preserving energy for future reproduction (Bull and Shine 1979; Clutton-Brock 1984). In contrast, if survival costs are high a second brood might be produced anyway (despite a low fertilization rate), given the low survival prospects and thus the low probability of reproducing in the future (Roff 1992).

Secondly, females that mate with multiple partners also face a conflict between fertilizing the eggs of different broods with the sperm from the same or a different partner, and it has been suggested that plastic sperm utilization strategies may exist, e.g., through cryptic female choice (Parker 1970; Walker 1980; Eberhard 1996; Gist and Congdon 1998; Pearse et al. 2001; Simmons 2001; Singh et al. 2002). Fertilizing the eggs with sperm from the same partner may be beneficial if the male provides good genes. On the other hand, fertilizing eggs with sperm of different males may increase the genetic diversity of the offspring, or hedge against sterility or genetic defects of the mate partners (Thornhill and Alcock 1983; Wolff and Macdonald 2004). Furthermore, sperm of first males may be physically displaced by sperm of second males (Halpert et al. 1982; Manier et al. 2010), and thus the use of old sperm may be principally dependent on mate availability (i.e., dependent on whether fresh sperm is acquired).

The first conflict has attracted a lot of research. For example, many studies have examined how multiple partner mating (e.g., monandry versus polyandry or the degree of multiple partner mating) affects female and male reproductive success (e.g., Tregenza and Wedell 1998; Yasui 1998; Fitze et al. 2005). However, few studies about the conflict between producing one or multiple broods per reproductive season exist and studies that investigate how mate availability affects this conflict are restricted to a few insect species only (Wiklund et al. 1993; Morse 1994; Reinhardt et al. 1999; Wang and Davis 2006), even though many other species, including birds, mammals, reptiles, fish, and mollusks, are iteroparous.

Here, we experimentally tested whether mate availability (before the first clutch and after producing the first clutch) affects the production and timing of multiple clutches per reproductive season. To this end we manipulated mate availability by submitting female common lizards, *Zootoca vivipara*, to either a restricted or an unrestricted mating treatment. In the former, females were able to mate once with up to 6 different males before producing the first clutch, while in the latter, they were allowed to copulate unrestrictedly throughout the reproductive season. Effects of mate availability on mating propensity, reproductive investment, fertilization patterns, reproductive success, and more generally on parameters related to female and male fitness were quantified for first and second clutches. Additionally, female re-mating propensity and the seasonal pattern of sperm presence in males were examined, to understand the importance of re-mating after first clutch production, whether males are fertile during the re-mating period, and whether re-mating affects fertilization patterns in second clutches.

The common lizard is an ideal species 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). Clutch size is big enough to detect multiple paternity and not too big, allowing for exact genetic paternity analyses (Fitze et al. 2005), i.e., for precise quantification of fertilization patterns, and thus potential sperm utilization strategies. Females exhibit mate choice and the degree of choosiness depends on reproductive costs, population sex ratio, and mating history (Fitze et al. 2005; Fitze and Le Galliard 2008; Fitze et al. 2010). Moreover, males exhibit best-of-bad-job strategies (Gonzalez-Jimena and Fitze 2012), indicating that both sexes exhibit plastic mate choice strategies. There exists experimental evidence that first clutch production is independent of copulation (Bleu et al. 2011), but no evidence exists for effects of mate availability on the production of multiple broods or the existence of sperm utilization strategies.

If mate availability in terms of a female's number of mate partners is important for female and male reproductive success, we predicted that restricted females should exhibit reduced mating propensity, reproductive success and maternal investment during their first annual clutch (prediction 1). Furthermore, males mating with restricted females may exhibit higher reproductive success (prediction 2), since on average more eggs can be fertilized by a single male (i.e., lower competition for fertilizing eggs). If mate availability is important for producing multiple broods, we predicted that restricted females exhibit a lower probability of producing a second clutch (prediction 3). However, if sperm can be stored or if sperm replenishment through re-mating is not necessary, we predicted no differences between restricted and unrestricted females in the probability of producing a second clutch (prediction 4). If fertilization patterns depend on mate availability and if fresh sperm physically displaces old sperm, we predicted that the restricted females' second clutch will be fertilized with stored sperm, while the unrestricted females' second clutch will be fertilized with fresh sperm (prediction 5). Moreover, if fertilization mainly depends on sperm displacement and not on female choice, we predicted that the main sire of the restricted female's first and second clutch will be the same male, while this will not be the case in the unrestricted females (prediction 6). More generally, and according to the displacement hypothesis, males fertilizing eggs in the female's first clutch will fertilize few eggs in the second clutch given the physical displacement of their sperm (prediction 7). As a consequence, we also predicted that males copulating with females before the production of the first clutch will have higher reproductive success when mating with a restricted compared to an unrestricted female (prediction 8).

MATERIALS AND METHODS

SPECIES DESCRIPTION

Z. vivipara (Lichtenstein 1823), is a small, ground-dwelling Lacertid lizard that is widely distributed throughout Eurasia. It inhabits cool, temperate climates and prefers peat bogs and moist heath land (Massot et al. 1992; Clobert et al. 1994). Its reproductive mode is viviparous over the biggest part of its geographic distribution and in the south (on the Balkan, in Italy, and in Spain) the reproductive mode is oviparous (Surget Groba et al. 2001). Here, were studied oviparous individuals from the Spanish Pyrenees (Milá et al. 2013). In the study populations, males emerge from hibernation around February-April, and females two to three weeks later. The mating season starts after the females emerge from hibernation (Bauwens and Verheyen 1985; Roig et al. 2000; Fitze et al. 2010). The common lizard exhibits a polygynandrous mating system (Laloi et al. 2004; Fitze et al. 2005). During copulation, a male first holds a female at the posterior abdomen with his mouth, twists his body around the female, and inserts his hemipenis in the female´s cloaca. Due the male's grip, the female's belly shows a typical U-shaped mating scar after copulation (Bauwens and Verheyen 1985; Fitze and Le Galliard 2008). Mating scars are an indicator of the number of mate partners and thus of mating propensity (Fitze et al. 2005; Le Galliard et al. 2005). Mating scars initially exhibit a light blue color, which subsequently becomes dark blue, black and thereafter gradually fades (L.M. San-Jose, unpublished data/results herein).

A female's mating window is activated by copulation (Breedveld and Fitze 2015) and its length is independent of re-mating. Females lay 1 to 3 clutches per year (Horváthová et al. 2013), and primarily large females, that initiate reproductive activities early in the season, and females from low altitude populations, where warmer climate results in longer reproductive seasons, produce more than one clutch per reproductive season (Heulin et al. 1994; Roig et al. 2000). Clutch size ranges from 2 to 9 (4.65 ± 0.3) and it is positively related to female body size (Horváthová et al. 2013; Roitberg et al. 2013). Eggs are fathered by 1 to 5 different males and on average by 2 males (Fitze et al. 2005).

The ovarian cycle starts after females emerge from hibernation and ends about 1 month later with the ovulation and fertilization of matured oocytes (Gavaud 1983; Heulin et al. 2008), which is typically 3-4 weeks after first mating (Bauwens and Verheyen 1985). Maternal reproductive investment is completed when eggs are laid (after 14-30 days of gestation; Horváthová et al. 2013) since no parental care is exhibited after egg laying. No evidence exists that sperm can be stored in the female tract from one reproductive season to the next (Bleu et al. 2011). Evidence from one single female suggests that sperm from initial copulations can potentially be stored for the fertilization of eggs during a second clutch (Heulin 1988), but the generality of this finding is unknown. Experimental evidence has shown that in first clutches ovulation is spontaneous and mating does not affect litter size (Bleu et al.

2011), but effects on multiple clutches have not been investigated.

EXPERIMENTAL PROCEDURES

All lizards used in this study originated from two natural populations belonging to the same phylogenetic linage (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 the Spanish Pyrenees. In May 2009, adult individuals were captured and transported to the laboratory at the Instituto Pirenaico de Ecología (Jaca, Huesca, Spain). Initial SVL (snout-to-vent length) and body mass were measured, and lizards housed in individual terrariums (25×15×15 cm), under standardized conditions (see below).

2 days after capture, virgin females and males of the "restricted" treatment (*N*=45 and *N*=92) were submitted to mating trials, in which they had restricted access to mate partners. Females were allowed to mate with up to six males and males had the chance to mate with 2 different females. Thereafter females were released in semi-natural populations (see below) containing only females, where they could not mate with additional males during the rest of the reproductive season. Females and males of the "unrestricted" treatment (*N*=30 and *N*=56) were released in six mixed-sex semi-natural populations directly after measurement, where females had access to 9 or 10 males (average: 9.33 males) and males had the chance to mate with an average of 5.2 females (range: 3-7).

Semi-natural populations 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. They contained natural vegetation, hides, rocks, logs, and water ponds, and were surrounded by escape proof walls and covered with nets to prevent predation (Fitze et al. 2008). Released individuals were feeding on naturally occurring prey. Mating propensity was measured by counting the number of mating scars on each female's belly (Fitze et al. 2005) during the 2nd and 3rd week of May and in the unrestricted treatment also during the 4th week of June. All females were recaptured on a weekly basis to determine gravidity via abdominal palpation. Gravid females whose egg size was close to the oviposition egg size were brought to the laboratory, and all other females were released. In the laboratory, females were maintained under standardized conditions until egg laying, measured for post-oviposition body mass, and thereafter returned to their semi-natural population. Clutch mass and size (i.e., number of eggs) were measured and reproductive 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 see: San-Jose et al. 2014).

While residing in the laboratory, 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. an ultraviolet (UV)-B light was provided.

To understand whether scar presence and the number and color of the scars can reveal re-mating propensity, the persistence of fresh mating scars and their color change was examined. For this purpose an experiment was run previously, in 2007. In this experiment 22 females were mated to different males under controlled conditions (further methodological details see above) and the mating date and number of mates were registered. Females were thereafter examined every 1-2 weeks and the scar color classified as light blue, dark blue, black, grey, and light grey (note: the order of the colors corresponds to the natural color change of the mating scars observed in *Z. vivipara*; personal observation).

DETERMINATION OF SPERM PRESENCE

The seasonal pattern of sperm presence in males was analyzed to understand whether re-mating provides females with sperm and thus, whether mate availability after the production of the first clutch may affect the production of a second clutch. To this end, thirty-one adult male common lizards (SVL ≥ 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 throughout the reproductive season (Courty and Dufaure 1980). Sperm presence was determined 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 to avoid sacrificing animals. Its accuracy has been validated using 12 additional male *Z. vivipara*. In each of these males, the non-invasive method was first performed, and directly afterwards the male was sacrificed, and presence/absence of sperm in the epididymis determined following dissection. There was a highly significant and positive correlation between sperm presence/absence determined by the two methods (r_s=0.837, P≤0.001; M. C. Breedveld, unpublished result).

PATERNITY ASSIGNMENT

Tissue samples were collected from all mothers, putative fathers, juveniles, and undeveloped embryos, 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 (Marshall et al. 1998; Fitze et al. 2005). Detailed methods of DNA extraction, polymerase chain reaction, and allele size determination are described elsewhere (Laloi et al. 2004).

STATISTICAL ANALYSIS

Initial models of female reproductive traits contained treatment as a factor, SVL as a covariate, and their interaction. Final models were obtained using backward elimination of non-significant terms. The probability of producing a clutch, the probability of producing a second clutch, and the probability that a male fertilized at least one egg were analyzed using generalized linear models with binomial error and logit link. Clutch size was analyzed for first and second clutches using generalized linear models with quasi-Poisson family (to account for over-dispersion) and log link. The proportion of clutches containing viable eggs (i.e., the proportion of clutches containing successfully hatched and alive juveniles), and the proportion of viable eggs within a clutch, were analyzed for first and second clutches using generalized linear models with logit link and binomial or quasi-binomial family respectively. Reproductive investment was analyzed for first and second clutches using linear models.

Date of oviposition was analyzed for first and second clutches using generalized linear models with quasi-Poisson family and log link.

To understand the reliability of scar counts as a proxy of mating propensity, the determinants of scar persistence were analyzed. To this end, the date on which scars were grey for the first time (i.e., no longer new) was analyzed using a generalized linear model with Poisson family and log link. Time since last copulation, the number of different males a female mated with, the number of times she mated, and the total duration of copulation were included as covariates.

The number of mating scars present before laying the first clutch, the number of females with which a male had offspring, and the total number of eggs fertilized by a male were analyzed using generalized linear models with quasi-Poisson family, a log link and treatment as a factor. Models on the number of mating scars of restricted females included number of mates, date of mating, and time passed between mating and oviposition as covariates.

The proportion of eggs sired in the first and second clutch by the main sire of the first clutch was analyzed using a generalized linear mixed model 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 analyzed using a linear model with treatment as a factor.

The seasonal pattern of sperm presence was analyzed using a generalized linear mixed model with the proportion of males with sperm present as the dependent variable, a binomial error and a logit link. Day and day squared were included as covariates, and male as a random effect.

Data was analyzed in R 3.0.0 (R Core Team 2013).

RESULTS

REPRODUCTIVE SUCCESS OF FEMALES

72 females produced a total of 88 clutches (total number of eggs = 302). 93 % of the females of the unrestricted treatment and 98 % of the females of the restricted treatment produced a clutch. The probability of producing a clutch was not significantly affected by treatment (χ^2_1=0.231, P=0.631), or SVL (χ^2_1=1.362, P=0.243), and no significant interaction existed (χ^2_1=0.777, P=0.378). 30 % and 16 % of the females of the unrestricted and restricted treatment laid two clutches, respectively. The probability of laying a second clutch was significantly higher in females from the unrestricted than from the restricted treatment (χ^2_1=6.151, P=0.013; mean ± SE for unrestricted and restricted females respectively: 0.32 ± 0.03, 0.16 ± 0.01). It was significantly and positively related to female SVL (χ^2_1=5.545, P=0.019, estimate: 0.186 ± 0.085), and no significant interaction existed (χ^2_1=0.473, P=0.492).

Clutch size of the first clutch did not significantly differ among treatments ($F_{1,69}$=0.402, P=0.528) and it significantly increased with SVL ($F_{1,70}$=34.020, P< 0.001, estimate: 0.041 ± 0.007). There was no significant interaction ($F_{1,68}$=3.066, P=0.084). Treatment, SVL, and the interaction did not significantly affect the clutch size of the second clutch (F≤ 2.448, P≥0.14).

In the unrestricted group, 75 % of first and 89 % of second clutches contained viable eggs, and in the restricted group, 86 % of first and 43 % of second clutches contained viable eggs. Treatment did not significantly affect the probability that first clutches contained viable eggs (χ^2_1=0.044, P=0.833) and second clutches of restricted females contained significantly less viable eggs (χ^2_1=4.035, P=0.045). The probability that clutches contained viable eggs increased significantly with SVL in first (χ^2_1=11.488, P<0.001, estimate: 0.266 ± 0.089), but not in second clutches (χ^2_1=0.035, P=0.851) and no significant interactions existed (all P>0.1).

In the unrestricted group, 63 ± 8 % of first clutch eggs and 79 ± 12 % of second clutch eggs were viable, and in the restricted group, 80 ± 5 % of first clutch eggs and 34 ± 18 % of second clutch eggs were viable. The proportion of viable eggs of the first clutch did not significantly differ among treatments ($F_{1,69}$=0.045, P=0.834) and it increased with maternal SVL ($F_{1,70}$=21.286, P< 0.001, estimate: 0.275 ± 0.068). However, treatment had a nearly significant effect on the proportion of viable eggs of the second clutch ($F_{1,14}$=4.319, P=0.057; mean ± SE for unrestricted and restricted females respectively: 0.79 ± 0.12, 0.34 ± 0.18), and SVL and the interactions were not significant (all P>0.1).

TIMING OF REPRODUCTION AND REPRODUCTIVE INVESTMENT

The mean oviposition date of the first clutches was the 8th of June ± 1.3 days (range: 19 May – 27 July). Oviposition date was significantly affected by an interaction between treatment and SVL ($F_{1,68}$=16.098, P<0.001; Fig. 6.1). In unrestricted females the day of oviposition was negatively correlated with SVL ($F_{1,68}$=27.479, P_{adj}<0.001) and in the restricted treatment no significant correlation existed ($F_{1,68}$=0.689, P_{adj}=0.819; Fig. 6.1). The mean oviposition date of second clutches was the 8th of July ± 1.9 days (range: 20 June – 23 July) and oviposition date did not significantly differ between treatments ($F_{1,14}$=0.055, P=0.818; interaction SVL * treatment: $F_{1,12}$=0.218, P=0.649).

The reproductive investment during the first clutch significantly increased with female SVL ($F_{1,69}$=10.565, P=0.002, estimate: 0.009 ± 0.003). Treatment and the interaction with SVL were not significant (all P>0.1). Treatment, SVL, and the interaction did not significantly affect the reproductive investment during the second clutch ($F \leq 1.3$, $P \geq 0.27$).

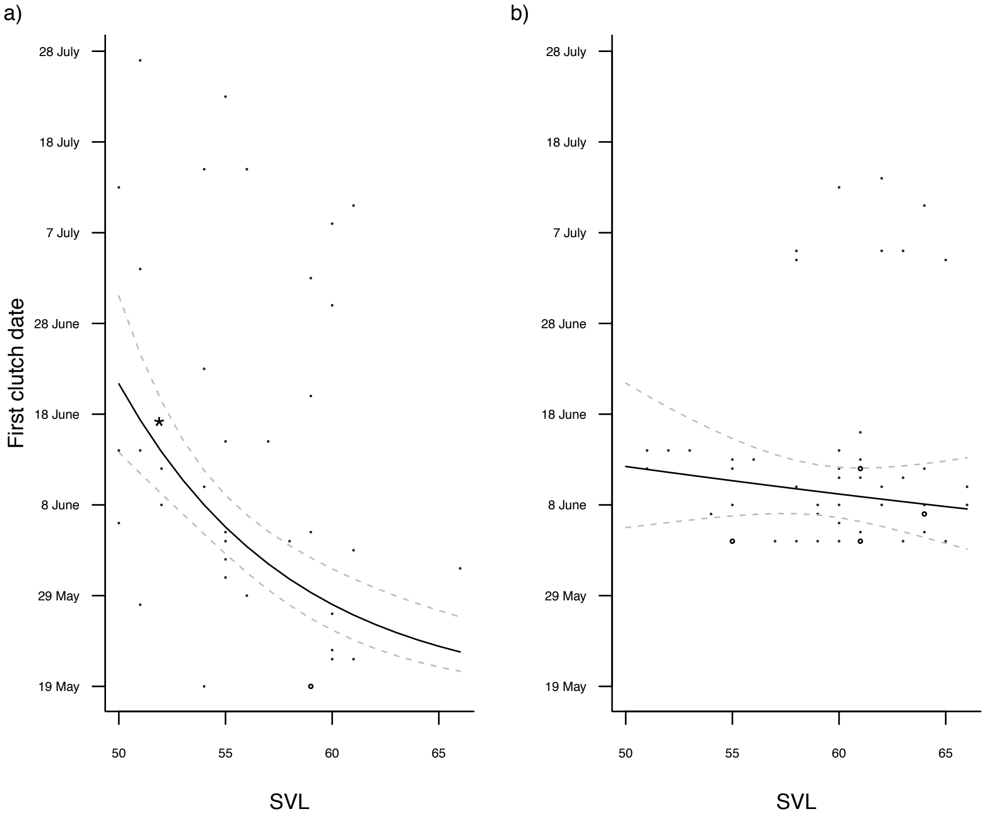


Fig. 6.1 Date of oviposition of the first clutch in relation to female SVL for **a)** unrestricted and **b)** restricted females. Raw data (*dots*) and the model predictions (*solid lines*) are shown. Dot size of raw data is proportional to sample size, and *dashed lines* delimitate 95 % confidence intervals. Significant post-hoc tests of linear predictions are depicted with an *asterisk*

MATING PROPENSITY

A few minutes up to one hour after mating, light blue mating scars appeared on the female's belly. These mating scars changed to dark blue within 0-6 days and from dark blue to black within 8-14 days from mating. Thus, mating scars can change from light blue to black within one week. Thereafter black scars started to fade and became grey and light grey before they disappeared. The grey color was observed on average 21.5 ± 1.2 days after the last mating. The date on which scars exhibited grey coloration for the first time, depended on the time since the last copulation (χ^2_1=7.721, P=0.005, estimate: 0.072 ± 0.026). The number of times a female mated, the number of different males with which she mated, and the copulation duration were not significant (all $P>0.1$). 30 to 60 days after the last mating, all scars were light grey and most of them very hard to spot. Scars thus persisted for up to 2 months.

The number of mating scars exhibited by females before laying the first annual clutch was significantly higher in unrestricted than restricted 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). In the restricted treatment, where the exact number of mates was known, the number of mating scars before laying the first annual clutch significantly increased with the number of mates ($F_{1,40}=10.136$, $P=0.003$, estimate = 0.148 ± 0.047) and it did not depend on mating date and the time passed between mating and oviposition (all $P>0.1$). Before laying the second annual clutch, no light blue or blue (i.e., fresh) mating scars were observed in the unrestricted and the restricted treatment. 77 % of the unrestricted and none of the restricted females exhibited black or grey mating scars.

SIRING SUCCESS

The mean number of sires in the first and second clutch was 1.5 ± 0.1 and 1.4 ± 0.2 respectively, and was independent of treatment (all $P>0.1$). In females from the unrestricted treatment 37.5 % of the fertilized second clutches contained sires that also fathered eggs of the first clutch and in females from the restricted treatment all fertilized second clutches were fathered by males that mated with the female before producing the first clutch. The proportion of fertilized eggs sired in the first and second clutch by the main sire of the first clutch depended on an interaction between treatment and clutch number ($\chi^2_1=5.561$, $P=0.018$, Fig. 6.2). In the first clutch, the proportion of eggs sired by the main sire did not differ between treatments ($\chi^2_1=0.008$, $P_{adj}=1$). In contrast, there was a significant difference in the second clutch ($\chi^2_1=12.047$,

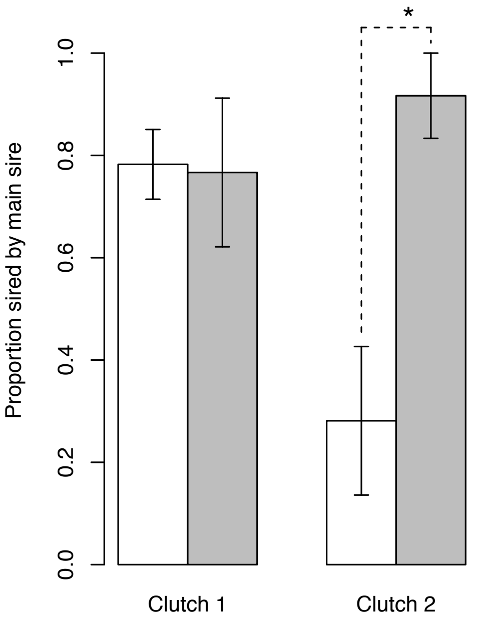


Fig. 6.2 Proportion (mean ± SE) of clutches sired by the main sire of the first clutch in unrestricted (*white shading*) and restricted (*gray shading*) females. Significant contrasts are depicted with an *asterisk*

$P_{\mathrm{adj}}=0.001$), where the main sire of the first clutch fertilized a smaller proportion of eggs in the unrestricted compared to the restricted treatment. Moreover, the proportion of eggs sired in the second clutch by males siring eggs in the first clutch was significantly lower in the unrestricted compared to the restricted treatment ($F_{1,9}=6.491$, $P=0.031$).

MALE SPERM PRESENCE AND MALE REPRODUCTIVE SUCCESS

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. 6.3). Sperm was on average present in 81 % of the males until the 24th of June (range: 77 - 87 %) and thereafter declined. By the end of July none of the males had sperm.

38 % of the restricted males and 46 % of the unrestricted males fertilized at least one egg and there was no significant difference among treatment groups ($\chi^2_1=$ 1.114, $P=0.291$). Similarly, there were no significant differences among treatments in the number of mate partners ($\chi^2_1=2.608$, $P=0.116$), the number of fertilized eggs ($\chi^2_1=0.026$, $P=$ 0.872), and the average number of eggs fertilized per mate partner ($\chi^2_1=2.241$, $P=0.140$) of reproducing males. Males of the unrestricted treatment that fertilized eggs in first and second clutches (suggesting re-mating), fertilized significantly more eggs than males that fertilized eggs in the first or second clutch only ($\chi^2_1=27.912$, $P<0.001$; mean ± SE for the former and the latter group respectively: 7.0 ± 1.6, 2.2 ± 0.3).

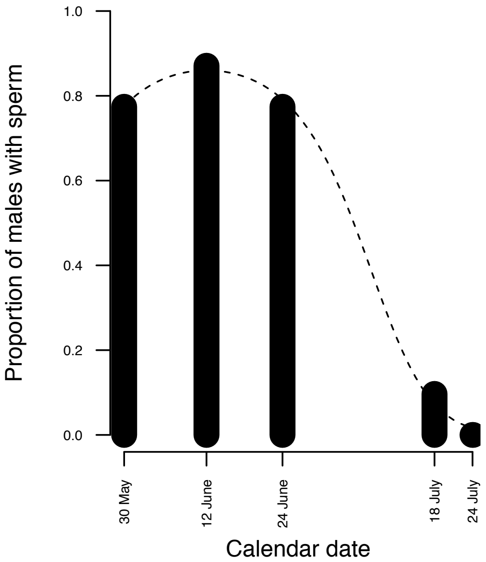


Fig. 6.3 Sperm presence throughout the reproductive season. Raw data is presented as *black bars* and the *dashed line* corresponds to the model prediction

DISCUSSION

Reproductive strategies of males and females are of great relevance for population dynamics and particularly for the colonization of new habitats (e.g., Allee effects; Kokko and Rankin 2006). While presence and absence of mate partners has been shown to importantly affect reproductive success (Veit and Lewis 1996; Kramer et al. 2008; Gascoigne et al. 2009; Mugabo et al.

2013), effects of variation in mate availability throughout the reproductive season have rarely been investigated. Here we experimentally manipulate mate availability before and after first clutch production and test its effect on reproductive strategies and reproductive success of males and females.

As predicted, the number of mates available before the production of the first clutch significantly affected a female's mating propensity (prediction 1). Restricted females exhibited less mating scars than unrestricted females, indicating that less copulations occurred in the former group (Fitze et al. 2005; Le Galliard et al. 2005). In contrast to prediction 1, in first clutches treatment did not significantly affect clutch size, the proportion of viable clutches and eggs, the number of sires, and maternal investment. This shows that treatment did not affect the reproductive success of females during first clutches, indicating that females of both treatments received enough sperm to fertilize their eggs. The higher number of mating scars in the unrestricted females (i.e., in the females with augmented sexual conflict) together with the absence of treatment effects on reproduction are in line with an intersexual conflict over mating rates, resulting in above optimal mating frequencies of females (Rowe et al. 1994; Chapman et al. 2003). In the absence of mates, adult *Z. vivipara* females make a reproductive investment and lay unfertilized clutches (Bleu et al. 2011), showing that they cannot store sperm over the winter. This, together with the lack of effect of male availability on reproductive success found in this study, indicates that the presence of mates per se crucially determines female reproductive success, but that the number of potential mate partners is of reduced importance.

Mate availability significantly affected oviposition date in a size-dependent manner (Fig. 6.1). In the restricted treatment no significant correlation between oviposition date and female SVL existed, while in the unrestricted treatment a significant and negative correlation existed, i.e., larger females laid clutches earlier. Females of the unrestricted treatment were exposed to competition for mating, while females of the restricted treatment were individually presented to males and thus not exposed to competition. In *Z. vivipara* the amount of time an individual spends in mating activities is fixed (i.e., they exhibit a mating window of fixed and temporary duration; Breedveld and Fitze 2015), independent of SVL, and ovulation is spontaneous, indicating that oviposition is a proxy for when mating occurred. Thus, earlier egg laying by big females of the unrestricted treatment suggests that they mated earlier than smaller females. Earlier mating of big females is in line with greater sexual attractiveness to males due to the higher number of fertilizable eggs (Bauwens and Verheyen 1987; Shine 1988; Andersson 1994), with higher competitiveness in intra-sexual competition among females, and/or with size related differences in the amount of fat stored upon emergence from hibernation (Avery 1974; Bauwens and Verheyen 1985; Olsson and Shine 1997). Delayed oviposition can entail negative fitness effects through reduced survival of later hatched young, for example, as a consequence of increased competition with other hatchlings or the reduced time left to prepare for hibernation. This result indicates that competition for mate partners can indirectly affect Darwinian fitness of competitively inferior individuals, which is in line with previous findings showing that increased inter-sexual competition affects oviposition dates (Le Galliard et al. 2008).

Similar to the treatment effects on females, no significant differences existed between restricted and unrestricted males in the probability of fertilizing eggs during first clutches, and in the number of mate partners and fertilized eggs of reproducing males. This shows that mate availability had no direct effects on the female's and male's first annual reproduction, indicating that few mates are necessary to guarantee reproduction, and that mate selection may be relaxed when fewer mates are available (e.g., Shelly and Bailey 1992; Cornwallis and Birkhead 2006; Willis et al. 2011).

The lack of re-mating opportunities significantly affected the production and the success of a second annual clutch. As predicted, the probability of laying a second clutch and the proportion of clutches with viable eggs were significantly lower in restricted females than in unrestricted females (prediction 3). 43 % of the second clutches of the restricted females contained viable eggs, indicating that females can indeed store sperm over at least 2.4 months. However, the important reduction in reproductive success (-46 % in the proportion of clutches containing viable eggs; - 45 % in the proportion of viable eggs) 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, and it suggests that unrestricted females re-mated before producing a second clutch. 77 % of the females of the unrestricted and none of restricted treatment exhibited black or grey mating scars, pointing to re-mating in the former. Since the time past between mating and capture for laying the second clutch was approximately 56 days, scars originating from the start of the season were no longer visible, as confirmed in the restricted females (note: scars become grey ca. 22 days after mating, and disappear 30-60 days after mating). Moreover, the time past from release (after laying the first clutch) until recapture was 16 ± 1 days and thus, scars resulting from re-mating should still have been visible and should have exhibited black or grey coloration, as indeed observed in the females of the unrestricted treatment. These observations are thus in line with re-mating. However, the ultimate evidence for re-mating stems from an additional experiment, in which females were submitted to the above-described unrestricted mating conditions until oviposition and thereafter released in a new population containing only new unknown males. In 96 % (*N*=22) of the females laying a second clutch, offspring were fathered by males of the second population, and these males fertilized 96 % of the viable eggs (*N*= 72), while only 4 % (*N*=3) were fertilized by stored sperm. This demonstrates that females re-mate after laying the first clutch. Consequently, when mate partners are absent after oviposition, females are less likely to produce a second clutch and those that nevertheless produce a second clutch pay the costs of reduced clutch viability. These results are in line with the trade-off between producing one or multiple broods per reproductive season (Verhulst et al. 1997), which predicts that under optimal breeding conditions a second clutch should be produced, while under suboptimal conditions it should be skipped. Additional support for this trade-off is provided by the fact that second clutch production was affected by female body size. Large, but not small females produced second clutches. Since larger females generally have more resources to allocate (Avery 1975; Bleu et al. 2013) and since they are more efficient foragers (González-Suárez et al. 2011), they are in better condition and may afford producing a second clutch.

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 proportion as in the first clutch (Fig. 6.2). This suggests that either females may have preferred sperm from the same male, or that the most abundant or the most competitive sperm may have fertilized the eggs of both clutches. In unrestricted females, the main sire of the first clutch sired on average only 28 % of the fertilized eggs in second clutches (range: 0 - 100 %; Fig. 6.2). Interestingly, in second clutches of unrestricted females where the main sire of the first clutch did sire eggs (37.5 % of the clutches), he sired on average 75 % of the eggs, the latter being comparable with the siring success in first clutches. Unrestricted females were released in the same population and were therefore able to re-mate with the same males and those carrying eggs of the main sire of the first clutch also exhibited black or grey mating scars, indicating re-mating with the same male. The difference in the fertilization patterns thus suggest that at the second clutch females generally mate with a different male (the main sire of the first clutch did not sire eggs in 62.5 % of second clutches of the unrestricted females), and that old sperm may have been competitively inferior, deselected by females, or displaced by fresh sperm, the latter being in line with prediction 5. The latter hypotheses are strengthened by the results of the additional experiment, where females could re-mate but not with the same males. Stored sperm fertilized only 4 % of the eggs (3 out of 80 eggs), and the proportion of eggs fertilized by stored sperm was significantly lower than in restricted females ($F_{1,26}$=9.165, P=0.006). This indicates that new rather than old sperm fertilized the eggs. The low proportion of unrestricted females re-mating with the same male (only 37.5 %) is in line with the hypothesis that females mate multiply in order to increase genetic diversity or bet hedge against infertile sperm or sperm with genetic defects (Thornhill and Alcock 1983; Wolff and Macdonald 2004). Alternatively, mating with a given male may simply depend on the probability of encountering a male in the right moment, potentially explaining why few females re-mated with the main sire of the first clutch.

Predominant egg fertilization with sperm obtained through re-mating also shows that re-mating by females is costly for the males (Parker 1970; Birkhead and Møller 1998; Price et al. 1999). Re-mating by females actually led to a significant reduction of a male's reproductive success, given that the proportion of second clutch eggs fertilized by the main sire of the first clutch was significantly smaller in the additional experiment compared to the restricted group. This shows that re-mating leads to no or low reproductive success of first mating males in second clutches. Moreover, in the unrestricted group, males that re-mated with females fertilized significantly more eggs than those fertilizing eggs in one clutch only, showing that re-mating increases fitness, potentially favoring male strategies that enhance the probability and success of re-mating. Sperm presence was high until the end of June (Fig. 6.3), when most females had already oviposited their first clutch (mean oviposition date: 8th of June), and thereafter rapidly declined, which is in line with male strategies maximizing the success of re-mating. Re-mating thus happened when males still exhibited sperm presence, in line with the co-evolution of male and female reproductive strategies.

In summary, our results show that mating and re-mating per se crucially affect the reproductive success of females and males, and we found no evidence that the number of available mates affected a female's reproductive success. Re-mating males fertilized the vast majority of second clutch eggs and our analyses suggest that earlier acquired sperm was displaced. This suggests that females do not face a conflict over fertilizing eggs of different broods with the sperm of the same or a different partner, but that they face a trade-off between re-mating or not with males, which importantly affects the success of second clutches. Females that were unable to re-mate were less likely to produce a second clutch and obtained a lower proportion of fertilized eggs, providing evidence for sperm limitation and reduced reproductive success. Our results also show that the conflict between producing one or multiple broods per season was mediated by the optimality of the breeding conditions, namely, by mate availability and female resources. This suggests that *Z. vivipara* females may skip the production of second broods when resources are limited, pointing to facultative multiple breeding in a species without parental care (Verhulst et al. 1997), and a potential strategy that could allow females to preserve energy for future reproduction (Bull and Shine 1979; Roff 1992). Interestingly, the sperm presence patterns show that not all males had sperm present; around 20 % of the males had no sperm present even during the early mating season. This, together with the negative effects of not re-mating, shows that re-mating and mating with several males may have evolved to bet-hedge against infertile males, being in line with the evolution of multiple partner mating due to direct fitness benefits (Jennions and Petrie 2000).

Conflicts over producing one or multiple broods and over re-mating, are faced by many iteroparous species (Verhulst et al. (1997) and references therein). Here we experimentally show that this conflict is mediated by mate availability and female resources. We show that the lack of mates entails negative fitness consequences, both in the second and the first clutch (Bleu et al. 2013). Mate scarcity has become increasingly likely due to anthropogenic influences on the demography and phenology of natural populations. For instance, habitat fragmentation and climate change have resulted in the spatial and temporal isolation of mates in many species (Lane et al. (2011) and references therein). Our results show that isolation of mates can potentially have important and rapid negative fitness consequences for individuals, and ultimately population dynamics (Calabrese and Fagan 2004; Kokko and Rankin 2006). Therefore, knowledge on changes in mate availability are crucial for predicting the stability of populations and the outcome of conservation efforts.

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ETHICAL STANDARDS The conducted study complies with current Spanish laws and with ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research.

CONFLICT OF INTEREST The authors declare that they have no conflict of interest.

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• CHAPTER 7 •

GENERAL DISCUSSION

The broad objective of this thesis was to provide new insight into how mating decisions are affected by the time of mate encounter, and to determine the consequences of potential strategies on reproductive success, using the common lizard, *Zootoca vivipara*, as a model species. The reproductive traits that were studied are mating propensity, choosiness, investment during mating, and reproductive frequency, in relation to experimental manipulations of the time of mate encounter. The main hypothesis was that individual mating decisions should be plastic with respect to when mates are encountered, due to temporal variation in an individual's reproductive stage, mating history, and the expected fitness payoffs of mating at a specific time. In particular, we tested whether the time of mate encounter affects female mating propensity and choosiness as a result of her reproductive stage (Chapter 3); whether the time of mate encounter affects male mating propensity as a result of his stage of reproductive maturation (Chapter 4); whether the time of mate encounter affects male or female investment during mating (Chapter 5), and; whether the encounter of mates throughout the season is important for reproductive frequency (Chapter 6). Consequences of variation in these traits on reproductive success were examined. In this chapter, the main findings of this thesis are summarized and discussed in a more general framework and finally, the general conclusions of this thesis are presented (page 155).

The results presented in this thesis provide evidence for variation in mating decisions and reproductive success in response to various temporal aspects of mate encounter in the common lizard, *Z. vivipara*.

To begin with, the determination of female mating propensity (Chapter 3) has led to the identification of a finite time window of female receptivity, which is initiated at the time of first mate encounter. Based on observations that indicate that most females mate within a few days from emergence in the field (e.g., Fitze, Cote & Clobert 2010), and the endogenous determination of the reproductive cycle, it has been postulated that that the initiation of the female mating window may happen upon emergence from hibernation, or after a specific (short) time frame since emergence. Under this hypothesis, we predicted a lower mating propensity in females that experience a delay until they have access to their first mates. However, we found that mating probability did not depend on whether females encountered their first potential mates 2 or 9 days after emergence from hibernation. Moreover, a female's propensity to re-mate did not depend on when she had emerged, but did depend on when she had first acquired a mate; re-mating probability was significantly lower when females encountered their potential second mates 10 days after their first mate than when females encountered their potential second mates 2 or 6 days after their first mate. The findings thus provide evidence for mating window initiation when a first mate is encountered, and show that mating window duration is thereafter fixed, i.e., once a female has acquired a mate (and can fertilize her eggs), a fixed time period is available for re-mating. This mechanism may allow females to delay the onset of reproduction when mates are scarce and therefore not encountered rapidly upon emergence from hibernation.

We furthermore found that longer delays until a female's first encounter with potential mates (i.e., until the onset of her mating window) led to reduced levels of female choosiness (Chapter 3). Specifically, larger females, of the experimental group that encountered their first potential mates 2 days after emergence from hibernation, had to be presented with more males before copulation occurred than smaller females, i.e., larger females had higher mate acceptance thresholds. This relationship did not exist in females that encountered their first potential mates 9 days after emergence from hibernation. This result provides support for the existence of female choice strategies with respect to the time of mate encounter (which may signal the availability of mates), and substantiate previously determined flexibility in female mating decisions in response to current conditions, e.g., population sex ratio and mating history (Fitze et al. 2005; Fitze and Le Galliard 2008; Fitze et al. 2010). By lowering their degree of choosiness, i.e., reducing their mate acceptance threshold, when a delay until first mate encounter exists (signaling low mate availability), females may assure the acquisition of sperm when quick access to (preferred) mates is absent.

The timing of mate encounter in males did not significantly affect mating propensity (Chapter 4). However, sperm presence was temporally related to when males emerged from hibernation, i.e., the earlier a male emerged from hibernation the earlier he had sperm present. In other words, mating propensity was independent of whether males had completed sperm maturation or not. As a result, females that mated with males that emerged from hibernation recently (i.e., with a low degree of protandry), ran the risk of mating with infertile males, pointing to negative consequences on reproductive success. The existing protandry in *Z. vivipara* thus reduces the probability of mating during male infertility, thereby providing direct fitness benefits to males and females. Males that emerge from hibernation early have a higher chance of fertilizing eggs than males that emerge late, and later emergence of females reduces the female's chances of mating with infertile males. Later emerging females thus need to mate with fewer males to assure the fertilization of their eggs, reducing the female's mating costs imposed by males (Fitze and Le Galliard 2008). These results provide novel experimental evidence for the evolution of protandry - for which 7 distinct hypotheses currently exist - and demonstrate that protandry may have evolved due to the waiting cost hypothesis (Morbey and Ydenberg 2001; Morbey 2013).

In addition, earlier emerging males completed their first annual molt soonest, but no association between the time of molt completion and of reproductive maturation was found (Chapter 4). Only a few males that completed molt very late showed a reduced mating propensity. Moreover, males that molted early did not mate before molting, but males that molted late copulated independent of whether they had molted. This suggests that the described relationship between molting and the onset of mating behavior (Bauwens et al. 1989) may be correlative rather than causative, and may be valid for early but not for late molting males.

The timing of mate encounter also had significant effects on investment during mating (Chapter 5). Copulation duration between a female and her first mate partner was longer in females that emerged 9 days before copulation than in females that emerged 2 days prior to copulation. Copulation duration with second mates decreased with longer time intervals from first mates, when first mates fertilized eggs in the female's clutch. These results point to the existence of investment strategies in relation to the time and interval of sequential copulations in *Z. vivipara*. Even though sequential mate encounter is common in natural populations of many species, further evidence for effects of mating intervals on investment is currently very scarce and restricted to a few insect species only (Simmons et al. 1992; Simmons 1995; Reinhold and von Helversen 1997; Lehmann and Lehmann 2000). Since females that first mate after longer delays should be closer to ovulation, we believe that the here observed investment strategies occurred in relation to a female's reproductive stage. Moreover, since a female's mating status (i.e., whether a female was a virgin or had previously mated), did not affect copulation duration (as expected under male strategies in response to the risk of sperm competition), we believe that strategies were female driven. A female that is more proximate to ovulation may spend more time and energy on an acquired mate to assure the acquisition of a sufficient amount of sperm for the fertilization of all of her eggs.

Moreover, investment during mating was closely related to the reproductive success achieved by males (Chapter 5). First mates of females that emerged 9 days before copulation fertilized more eggs than first mates of females that emerged 2 days before copulation, in line with the longer copulation duration observed in the former. Furthermore, first mates of females that re-mated after a longer interval fertilized more eggs than first mates of females that re-mated after short intervals, confirming that the time interval between successive matings is an important determinant in a male's fertilization success (Lessells and Birkhead 1990; Parker 1990; Schwagmeyer and Foltz 1990; Pizzari et al. 2008).

Finally, the encounter of mates after the production of first clutches determined brood frequency and reproductive success (Chapter 6). In females that had no access to mates after laying their first clutch, evidence for sperm storage from initial matings was found, in line with a single previous observation in *Z. vivipara* (Heulin 1988). However, the production and viability of second clutches was reduced in these females compared to females that had continuous access to mates. This indicates that access to mates for re-mating, i.e., for the acquisition of new sperm, is important for female reproductive success. In line with this, sperm presence was high in males until the end of June, which exceeds the mean laying date of first clutches (8th of June). Males are thus fertile during the re-mating period of females, pointing to a close co-evolution of male and female reproductive traits.

On the basis of mating scars and paternity data we could establish that the prevalence of re-mating was indeed high in females that had access to mates after producing their first clutch (Chapter 6). New mating scars were detected in most of the females that could re-mate, and only a very small proportion of second clutch eggs was fertilized by stored sperm in females that could re-mate. The vast majority of eggs was fertilized by sperm acquired through re-mating. Moreover, main sires of first clutches sired significantly fewer second clutch eggs when females could re-mate than when females could not re-mate, and a reduced reproductive success of initial mates of re-mating females existed in general. This suggests that new sperm might displace stored sperm (Halpert et al. 1982; Manier et al. 2010), that females might exhibit preferences for new sperm, or that new sperm might have higher competitive ability (Eberhard 1998; Parker 1998). Finally, males that could re-mate and fertilized eggs during first and second clutches of females, fertilized significantly more eggs than males that fertilized during first or second clutches only. This indicates that re-mating with females after the production of first clutches is important for male reproductive success as well.

The traits and strategies identified in this thesis, and their consequences on male and female reproductive success, allow us to conclude that the timing of mate encounter is an important determinant for reproduction in *Z. vivipara*. Moreover, they may have mitigated potential negative effects of reduced mate encounter on reproductive success and population dynamics (Mugabo et al. 2013), in line with observed rapid post-glacial population expansions in this species (Surget-Groba et al. 2002).The fact that the timing of mate encounter may show variation in many species, highlights that its study could enhance knowledge about the mechanisms of reproduction and the processes underlying sexual selection in general. Moreover, the time of mate encounter may be affected in many species as a result of anthropogenic effects, such as habitat fragmentation and climate change (Lane et al. 2011; Saino et al. 2011; Morbey et al. 2012). The negative impacts of reduced mate encounter, for instance through Allee effects, and of changes in the time of mate encounter, have already been demonstrated in populations of many species (e.g., Courchamp, Clutton-Brock & Grenfell 1999; Saino et al. 2011; Larsen et al. 2013). However, if individuals of a species could respond to changes in the time of mate encounter, by adjusting their strategies flexibly, negative effects of reduced access to mates on reproduction and population viability might be avoided. Thus, flexible mating strategies with respect to the timing of mate encounter may contribute to the stability of low density populations (Calabrese and Fagan 2004; Gowaty and Hubbell 2005; Kokko and Rankin 2006; Gascoigne et al. 2009; Fagan et al. 2010) and may mitigate constraints of low density following the colonization of new habitats (Kramer et al. 2008). Therefore, understanding whether individuals adjust mating decisions in response to temporal variation in mate encounter may not only enhance knowledge for biological theory in general, but may also improve predictions of how species may respond to changes in population demography, potentially providing crucial information for conservation efforts.

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CONCLUSIONS

1. **The mating window of female *Zootoca vivipara* is initiated upon first mating and its duration is fixed.** Mating probability did not depend on whether females encountered their first potential mates 2 or 9 days after emergence from hibernation. Re-mating probability was significantly lower when females encountered their potential second mates 10 days after their first mate than when females encountered their potential second mates 2 or 6 days after their first mate.

2. **Female *Z. vivipara* exhibit mate choice strategies with respect to when mates are first encountered.** Of those females that encountered their first potential mates 2 days after emergence from hibernation, larger females had higher mate acceptance thresholds than smaller females. This relationship did not exist in females that encountered their first potential mates 9 days after emergence from hibernation.

3. **Direct fitness benefits of protandry exist in male and female *Z. vivipara*.** Male reproductive maturation was temporally related to emergence date. Mating propensity was independent of male emergence date and the completion of sperm maturation. Protandry thus reduces the probability of mating during male infertility. It allows females to avoid costs of mating and increases the probability that a male fertilizes eggs.

4. **Investment strategies with respect to the timing of sequential mating events exist, and these are most likely female driven in *Z. vivipara*.** Copulation duration between a female and her first mate partner was longer in females that emerged 9 days before copulation (and thus were closer to ovulation) than in females that emerged 2 days prior to copulation. Copulation duration between a female and her second mate decreased with longer time intervals between first and second mates, when first mates fertilized eggs in the female's clutch. Copulation duration was independent of female mating status (i.e., of whether a female was virgin or not), decreasing the likelihood that observed patterns were male driven.

5. **Investment strategies significantly affect fertilization success in *Z. vivipara*.** First mates of females that emerged 9 days before copulation fertilized more eggs than first mates of females that emerged 2 days before copulation. First mates of females that re-mated after longer intervals fertilized more eggs than first mates of females that re-mated after shorter intervals.

6. **Even though females can potentially store sperm from initial mates, mate encounter after the production of first clutches is important for female reproductive success in *Z. vivipara*.** Evidence for sperm storage from initial matings was found in the second clutches of females that had no access to mates after laying their first clutch. However, the production and viability of second clutches was much higher in females that could re-mate than in females could not.

7. **Male *Z. vivipara* are fertile until after first clutches are laid, and thus during the re-mating period of females.** Sperm presence was high in males until the end of June and the mean laying date of first clutches was the 8th of June, indicating a close co-evolution of male and female reproductive traits.

8. **Re-mating after the production of first clutches is important for the fitness of female *Z. vivipara* and sperm obtained through re-mating fertilizes most of the second clutch eggs.** The prevalence of re-mating was high in females that had access to mates after producing their first clutch. Only a very small proportion of second clutch eggs was fertilized by stored sperm in females that could re-mate, while the vast majority of eggs was fertilized by sperm acquired through re-mating. Moreover, main sires of first clutches sired significantly fewer eggs during second clutches when females could re-mate than when females could not re-mate, and a reduced reproductive success of initial mates of re-mating females existed in general. This suggests that stored sperm was displaced by new sperm in females that could re-mate.

9. **Re-mating after the production of first clutches is important for male reproductive success in *Z. vivipara*.** Males that could re-mate and that fertilized eggs during first and second clutches, fertilized significantly more eggs than males that fertilized during first or second clutches only.

CONCLUSIONES

1. **La ventana reproductiva en las hembras de *Zootoca vivipara* se inicia a raíz del primer acoplamiento y su duración es fija.** La probabilidad de acoplamiento fue independiente de si las hembras encontraron su primera pareja potencial 2 o 9 días después de salir de hibernación. Cuando las hembras encontraron su segunda pareja potencial 10 días después de su primer emparejamiento, la probabilidad de reacoplamiento fue significativamente menor que cuando las hembras encontraron su segunda pareja potencial 2 o 6 días después de su primer emparejamiento.

2. **Las hembras de *Z. vivipara* exhiben estrategias de selección de pareja con respecto al momento en el que se produce el encuentro con la primera pareja.** En aquellas hembras que encontraron su primera pareja potencial 2 días después de salir de hibernación, las hembras más grandes presentaron un mayor umbral de aceptación de pareja que las hembras más pequeñas. Esta relación no apareció en las hembras que encontraron su primera pareja potencial 9 días después de salir de hibernación.

3. **Existen beneficios directos de la protandria para la eficacia biológica de machos y de hembras de *Z. vivipara*.** La maduración reproductiva de los machos estaba correlacionada temporalmente con la fecha de salida de hibernación. La tasa de acoplamiento fue independiente tanto de la fecha de salida de hibernación como de la finalización de la maduración del esperma de los machos. Por tanto, la protandria reduce la probabilidad de acoplamiento durante el periodo en el que los machos no son fértiles. Esto permite a las hembras evitar costes derivados del emparejamiento y aumentar la probabilidad de que un macho fertilice los huevos.

4. **En *Z. vivipara* existen estrategias de inversión reproductiva con respecto al momento en el que se producen eventos secuenciales de emparejamiento y estas están probablemente determinadas por las hembras.** La duración de la cópula entre una hembra y su primera pareja fue mayor en las hembras que salieron de hibernación 9 días antes del emparejamiento (y, por tanto, estas estaban más próximas a comenzar la ovulación), que en las hembras que salieron de hibernación 2 días antes del emparejamiento. Cuando la primera pareja fertilizó al menos uno de los huevos de la puesta, la duración de la cópula entre una hembra y su segunda pareja disminuyó conforme el intervalo de tiempo entre el

encuentro con el primer macho y el encuentro con el segundo macho era mayor. La duración de la cópula fue independiente de la historia reproductiva de la hembra (es decir, de si la hembra era virgen o no), disminuyendo la probabilidad de que los machos sean los responsables de los patrones observados.

5. **Las estrategias de inversión reproductiva afectan significativamente al éxito de la fertilización en *Z. vivipara*.** Los machos que se acoplaron con las hembras que salieron de hibernación 9 días antes del emparejamiento fertilizaron más huevos que los machos que se acoplaron con las hembras que salieron de hibernación 2 días antes del emparejamiento. La primera pareja de las hembras que se reacoplaron tras un mayor intervalo de tiempo entre emparejamientos fertilizó más huevos que la primera pareja de las hembras que se acoplaron tras un menor intervalo de tiempo entre emparejamientos.

6. **Aunque potencialmente las hembras pueden almacenar esperma de los machos con los que se acoplan en primer lugar, el encuentro de pareja tras la producción de la primera puesta es importante para el éxito reproductivo de las hembras en *Z. vivipara*.** Se encontraron indicios del almacenamiento de esperma de la primera pareja reproductora en la segunda puesta de las hembras que no tuvieron acceso a los machos tras la ovoposición de la primera puesta. Sin embargo, la producción y la viabilidad de la segunda puesta fue mucho mayor en las hembras que pudieron reacoplarse que en las hembras que no pudieron volver a emparejarse.

7. **Los machos de *Z. vivipara* son fértiles hasta después de la ovoposición de las primeras puestas y, por tanto, durante el periodo de reacoplamiento de las hembras.** Se detectó una elevada presencia de esperma hasta finales de junio y la media de la fecha de ovoposición de la primeras puesta fue el 8 de junio, señalando una coevolución de las estrategias reproductivas de machos y hembras.

8. **El reacoplamiento tras la producción de la primera puesta es importante para el éxito biológico de las hembras de *Z. vivipara* y el esperma obtenido por medio del reacoplamiento fertiliza la mayoría de los huevos en la segunda puesta**. La prevalencia del reacoplamiento fue alta en aquellas hembras que tuvieron acceso a machos tras la producción de la primera puesta. En la segunda puesta de las hembras que pudieron reacoplarse, solo una pequeña proporción de los huevos fue fertilizada por el esperma almacenado, mientras que la gran mayoría de los huevos fue fertilizada por el esperma de los machos con los que se reacoplaron.

Es más, cuando las hembras pudieron reacoplarse, los machos responsables de fertilizar la mayoría de los huevos en la primera puesta fertilizaron menos huevos en la segunda puesta que cuando las hembras no pudieron volver a emparejarse y, en general, los machos que se acoplaron en primer lugar con las hembras reacopladas presentaron un menor éxito reproductivo. Esto sugiere que el esperma almacenado fue desplazado por el nuevo esperma adquirido por las hembras que podían reacoplarse.

9. **El reacoplamiento después de la producción de la primera puesta es importante para el éxito reproductivo de los machos en *Z. vivipara*.** Los machos que pudieron reacoplarse y que fertilizaron huevos tanto en la primera como en la segunda puesta fertilizaron significativamente más huevos que los machos que solo fertilizaron huevos en la primera o en la segunda puesta.

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