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**Life history strategies of different developmental rates:
causes and consequences**

Thesis for the Degree of Doctor of Philosophy (PhD)

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Debrecen, 2020

Hereby I declare that I prepared this thesis within the Doctoral Council of Natural Sciences and Information Technology, Doctoral School of Juhász-Nagy Pál , University of Debrecen in order to obtain a PhD Degree in Natural Sciences at Debrecen University.

The results published in the thesis are not reported in any other PhD theses.

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Prof. Ferenc Samu

**Life history strategies of different developmental rates:
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Written by **Zoltán Rádai** certified Biologist

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"The Guide is definitive. Reality is frequently inaccurate."

Douglas Adams

(The Hitchhiker's Guide to the Galaxy)

CHAPTER I

In this chapter I present an overview of the scientific subjects and corresponding literature associated with the studies included in my dissertation. I will then describe the main study questions and results, then discuss the findings, before finishing this chapter with some concluding remarks.

I.1 GENERAL INTRODUCTION

I.1.1 Variation in life histories

Life history theory is one of the most important frameworks in biology, providing a theoretical perspective for studying the evolutionary causes and consequences of the astonishing diversity observed in today's biosphere (Flatt & Heyland, 2011; Stearns, 2000). As a sub-topic of evolutionary ecology, life history theory seeks to understand genetic and phenotypic variation in traits that has critical impact on organisms' survival and reproductive success (Roff, 2002). In this framework life history is defined as the characteristics and phenology of an organisms' life cycle, comprised of key life history events such as birth, maturation, first reproduction, and death. Notably, throughout this dissertation the term "life cycle" refers to the post-embryonic phase of an organisms life, i.e. birth to death. Central elements of life history are called life history traits, which are fitness components that fundamentally determine life table parameters, such as post-embryonic development, juvenile and adult survival, and number of offspring produced.

Traditionally, life history traits considered to be of key importance are age of maturity, amount and size of offspring, and age-specific mortality (Stearns, 1976). Additionally, lifespan and developmental rate, as well as much more complex traits, such as behavioural and physiological characteristics are considered to be crucially important in an organism's life history and are also often included in life history traits (Dammhahn, Dingemanse, Niemelä, & Réale, 2018; Ricklefs & Wikelski, 2002). Over the course of evolution these traits are

shaped by natural selection in response to external conditions in the given environment, by which organisms are adapted to the environment in which they reside, maximising lifetime reproductive output (Stearns, 2000). Intuitively one could argue that enhancing both survival and reproduction should be the “best” strategy (leading eventually to the rise of a “Darwinian demon”: Law, 1979). However, due to physiological and evolutionary constraints it is not possible to simultaneously maximise all aspects of an organism’s life history. The most important corner stones of these limitations are called trade-offs (Agrawal, Conner, & Rasmann, 2010), defined as the fitness cost paid when an advantageous change in a trait that affects fitness is associated with a disadvantageous change in another (Stearns, 1989). Such associations between life history traits often manifest as negative phenotypic correlations, and multiple mechanisms were proposed to contribute to their emergence.

One of these mechanisms stems from the allocation-conflict of traits between which limited resources should be partitioned, as proposed by the “acquisition-allocation” model or “Y-model” (Harshman & Zera, 2007; Zera & Harshman, 2001). This model states that a finite pool of resources will limit the possible investment into traits that rely on it. Consequently, organisms have to balance their limited resources between those traits that share this resource pool, and an increased investment into one trait will entail a decreased resource availability for another, leading to a negative phenotypic correlation between them. In my dissertation and in the studies included, trade-offs between life history traits are important elements, and I mainly discuss trade-offs from a resource allocation perspective.

Resource allocation trade-offs are integral parts of life history evolution as they constrain viable evolutionary trajectories (Stevens, Hansell, & Monaghan, 2000). Due to the limits of trait evolution optimal, co-evolved sets of traits emerge. These specific sets (or combinations) of traits or trait values are often referred to as life history strategies. Life history theory considers the evolution

of life histories and life history strategies as an optimisation problem¹ (Fabian & Flatt, 2012), with the ultimate aim of maximising long term (i.e. lineage) reproductive success in the given environment. Shaped by both abiotic and biotic factors, a multitude of solutions has risen over the course of evolution to this optimisation problem, resulting in the wide variety of life history strategies.

Among the most remarkably variable elements of life histories is the chronological age at maturation, shaped by characteristics of developmental trajectories. Such characteristics include length of post-embryonic life cycle and number of reproductions. Lifespans of organisms vary from a few days to more than a century across the animal kingdom. For example, mayflies and salmon species develop in their juvenile stages often for years, but shortly after maturation and reproduction they show a shockingly quick senescence and die soon after mating (Groot & Margolis, 1991; Kureck & Fontes, 1996; Tokeshi, 1985). Such singular reproductive event in the organism's lifetime is called semelparity (Young, 2010). Additional examples are males of a number of hymenopteran species, which also have a rather short adult life and an almost instantaneous death right after a successful reproduction (Gould & Gould, 1988; Hölldobler & Wilson, 1990). Among much smaller invertebrates the whole life cycle might last only a few days or weeks, such as in gastrotrichs (Balsamo & Todaro, 1987, 1988), or in *Daphnia* species (Ebert, 2005). While some animals live short lives, or are characterised by short adult lifespan and only one reproductive event within their life cycle, others might live for years or decades, and can reproduce multiply (called iteroparity). Iteroparity and relatively long lifespan are quite frequently observed in vertebrates. For example Laysan albatrosses live almost as long as humans do (e.g. a female, called Wisdom, is estimated to be ca. 68 years old as

1 The word “problem” here refers to a phenomenon of mathematical nature, in which an optimisation process comprises the maximisation of a parameter (fitness) by finding the best local solution in a parameter space (combinations of species trait values). Such mathematical approach to evolutionary biology was gaining considerable ground in understanding evolutionary processes during the 20th century, e.g. by Ronald Fisher, Sewall Wright, John B. S. Haldane, John Maynard-Smith, Ernst Mayr, and many others.

of 2019, and was observed to lay an egg in 2018; Riley, 2018). Greenland sharks may live up to centuries, with an observed maximum lifespan of 272 years (Constantini et al., 2017; MacNeil et al., 2012). More interestingly, there are a few examples of long living iteroparous animals among invertebrates too, for example the mygalomorph spider *Gaius villosus* may live for to decades, with one specimen observed to live for 43 years (Mason et al., 2018). One of the most intriguing invertebrate life cycle was observed in the cnidarian *Turritopsis dohrnii*, widely known as the “immortal jellyfish”, which is capable of complete reversion to the immature polyp stage after maturity, with the potential of “biological immortality” (Schmich et al., 2003).

Developmental characteristics and their associations with other life history traits have long been in the centre of research due to their fundamental effects on survival and reproductive success (Ricklefs & Wikelski, 2002; Stearns, 1982). Development can be defined as unidirectional² temporal changes in the phenotype that are shaped by both genetic programme and environmental stimuli. It can be divided into two major phases: embryonic and post-embryonic development, the latter being comprised of age-specific survival patterns, such as juvenile and adult survival rates. These parameters, together with adult reproductive performance will translate to individual fitness (Lindström, 1999). In the past the role of developmental characteristics in contributing to different life histories was extensively studied (Stearns, 1982 and references therein), as well as the proximate³ and ultimate⁴ mechanisms that might affect post-embryonic development and the evolution of developmental pathways (West-Eberhard, 2003).

2 Developmental change in anatomy and/or physiology over time generally follows a trajectory which is not reversible, with the unique exceptions among cnidarians such as in *Turritopsis dohrnii*.

3 Direct causes behind a biological phenomenon (manifested on individual level), e.g. hormone physiology.

4 Evolutionary processes acting on a biological function (manifested on population level), e.g. directional selection.

1.1.2 Variation in developmental rate and cohort splitting

As described above, there is huge variation in longevity and developmental rate across animal taxa, with life cycle lengths ranging from a few days to even a century. Although not as dramatic as on the interspecific level, individuals within a species, a population, or even a brood, may also exhibit considerable variation in their rate of development (progression in ontogeny, life stage transitions), and / or growth (building up somatic tissues). This variation may originate from various sources. For example, it could arise from between-population differences in environmental conditions (Carlson & Holsinger, 2012; de Jong & Bochdanovits, 2003; Schmidt & Paaby, 2008), or different life history strategies within a population (Kleinteich & Schneider, 2011; Metcalfe, Huntingford, Graham, Thorpe, & Vickerman, 1989; Smalås, Amundsen, & Knudsen, 2013). Also, mere individual differences in resource allocation patterns in result of between-individual variation in the experienced environmental factors may also contribute to such variation (Bochdanovits & Jong, 2003; Kaspi, Mossinson, Drezner, Kamensky, & Yuval, 2002). Given that post-embryonic development on its own has substantial costs and increasing the rate of development is also quite resource-demanding, variation in developmental rate among individuals of a population might correspond to differences in how individuals balance their resources between costly life history traits (Stevens et al., 2000). A higher rate of development is often negatively associated with other life history traits, such as size at maturation or longevity, often due to the presence of trade-offs from resource-based conflicts between traits (De Block, Campero, & Stoks, 2008; Ficetola & De Bernardi, 2006; Metcalfe & Monaghan, 2001). Notably, such negative associations between costly traits might not be so prominent when the resource allocation conflict between them could be mitigated by increased resource acquisition. As such, “good quality” individuals that are able to attain more resources (or are better able to utilize available resources) may be capable of meeting the

energy demands of multiple costly traits, leading to the phenomenon of “big house, big car” (Reznik et al., 2000; van Noordwijk & de Jong, 1986).

A mechanism by which variation in developmental characteristics may occur is developmental plasticity in response to environmental stimuli (West-Eberhard, 2005), due to which different developmental pathways can emerge in the population (Moczek et al., 2011). Within-population variation in developmental pathways may occur through a phenomenon called cohort splitting (Crowley & Hopper, 2015; Sunderland, Hassall, & Sutton, 1976). In cohort splitting, individuals belonging to the same age-group (i.e. cohort) show divergence in life histories, in which divergence may occur in three forms. First is called initiation asynchrony, when time differences in the onset of ontogeny lead to the presence of multiple developmental stages in the population, for example due to delayed egg laying by the mother, or delayed hatching of the offspring (Moreira & Peckarsky, 1994; Schultheis, Booth, Vinson, & Miller, 2008). The second type is called mid-point cueing, taking place when individual differences are present among the offspring in their condition, developmental stage, or assessment of environmental conditions. For instance, behaviour-mediated decrease in developmental rate may occur among the offspring due to the presence of predators, with some offspring slowing down their development in response to predation risk (Johansson, Stoks, Rowe, & Block, 2001; Kozáčeková, Figueroa, López-Rodríguez, Beracko, & Derka, 2009). In the third type, termed as diversified tracking⁵, a bet-hedging strategy of the mother yields alternative developmental phenotypes that simultaneously occur in the population. Such as in the scenario of genetic polymorphism of the offspring, resulting in genetically determined differences in developmental phenotype, or within-clutch variation of maternal investment into individual eggs (Kiss & Samu, 2005; Parichy & Kaplan, 1995; Sinervo, 1990; Watts & Thompson, 2012). Notably, cohort splitting generally results in developmental types that differ in their developmental times and lifespan. Although

⁵ In this context, the term “tracking” refers to developmental pathways, i.e. diversified tracking is a (random) diversification of developmental trajectories.

genetic polymorphism may also contribute to its occurrence (Watts & Thompson, 2012), environmental factors (e.g. population density, seasonal state, weather, food availability) also play a pivotal role in evoking cohort splitting (David & Geoffroy, 2011; Kozáčeková et al., 2009; Todd, 1996).

In the last decades arthropods proved to be quite useful as model organisms when studying characteristics of post-embryonic development (e.g. Blanckenhorn, 1998; De Block et al., 2008; Fischer, Zeilstra, Hetz, & Fiedler, 2005; Garrad, Booth, & Furlong, 2016), and cohort splitting (see Crowley & Hopper, 2015), from a life history theory perspective. However, our knowledge is still rather limited about the associations between cohort splitting, development rate and other life history traits. Instances for such traits are stage-specific mortality, condition, somatic maintenance, or reproductive success. Also, our understanding of what advantages and costs might be associated with the alternative developmental types arising from cohort splitting is still quite limited, as most studies concerned developmental and growth rates in uniform cohorts (Blanckenhorn, 1998; De Block et al., 2008; Johansson et al., 2001; Kaspi et al., 2002; Stevens et al., 2000).

In the wolf spider *Pardosa agrestis* (Westring, 1861) a rather intriguing case of cohort splitting was observed (Kiss & Samu, 2005). *P. agrestis* is a common epigeic⁶ spider species in Europe, predominantly inhabiting arable habitats and alkaline grass lands (Samu & Szinetár, 2002). The peculiarity of this species is that, unlike its congeners in Europe, it shows a bimodal yearly phenology which results from cohort splitting due to different developmental times within the first progeny generation (Kiss & Samu, 2002, 2005).

6 Epigeic: residing exclusively above soil surface (i.e. not being capable of burrowing, swimming, or flying).

The first adult peak of each year comprises those specimens that overwintered (in juvenile or subadult stages, but never as adults), and matured during spring and early summer (Fig. 1, I.). These adults reproduce, then perish during early summer, and their offspring start to develop throughout the year (Fig. 1, II.). Some of these spiderlings follow a developmental trajectory through which they only mature

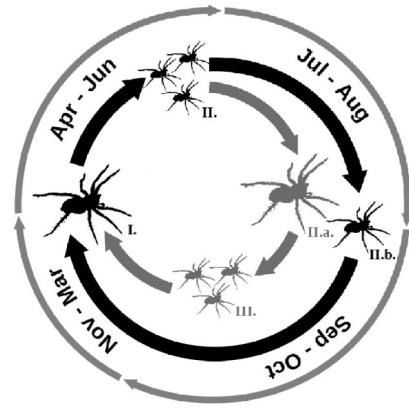


Figure 1: phenology of *P. agrestis*.
(Figure used from Rádai et al., 2020)

and reproduce in the next year, after overwintering (Fig. 1, II.b.). However, some of the spiderlings follow a much more rapid developmental pathway (Fig. 1, II.a.), and will mature, reproduce, and perish by late summer or autumn. These rapidly developing spiders represent the second adult peak of the given year. Intriguingly, these rapidly developing spiders generally occur within broods along with slowly developing ones, i.e. females that matured at early summer have slowly and rapidly developing offspring as well among their spiderlings (Kiss, 2003; Kiss & Samu, 2005). So, developmental asynchrony among the first offspring generation leads to cohort splitting, during which spiders of the same age will diverge in their developmental stage by late summer (Kiss & Samu, 2005). For this, rapidly developing spiders have to substantially increase both their developmental and growth rate, i.e. must achieve life stage transitions, and build up somatic and reproductive tissues, in a considerably shorter time. Notably, rather little is known about whether or not such cohort splitting takes place during late summer as well (Kiss, 2003).

1.1.3 Specific background and hypotheses

Up till now, there has been little known about the above described case of cohort splitting. Studies on insects (e.g. Clark & Platt, 1969; Hagstrum & Miliken, 1988; Saunders, 1983) and arachnids (e.g. Hardman & Rogers, 1991; Miyashita,

1988), and past observations of Kiss (2003) on laboratory-reared *P. agrestis* specimens suggest that environmental factors such as photoperiod and temperature may affect developmental rate, and potentially play a role in evoking cohort splitting in *P. agrestis*. For example, Kiss (2003) and Kiss & Samu (2002) showed that higher rearing temperatures were associated with higher occurrence of rapidly developing spiders. Ambient conditions such as temperature, humidity, or rainfall may have multifaceted effects that might either contribute to the occurrence of cohort splitting, and / or affect the fitness of the different developmental types. Indeed, temperature regimes were found to be associated with the dynamics of cohort splitting in other arthropods as well, such as in millipedes (David, Celerier, & Geoffroy, 1999), dipterans (Todd, 1996), and moths (Wipking, 1990). To address the question **how weather conditions might affect the presence of rapidly developing spiders in natural habitats**, we carried out non-parametric niche-modelling on long term collection data (**Study 1**).

Life history theory suggests that an increased developmental rate (e.g. observed in the rapidly developing sub-cohort of *P. agrestis*) would be expected to pair with an increased acquisition of resources. The reason of this is that a higher rate at which somatic tissues are built up has an increased demand for resources that can be utilized for such growth. Indeed, the pace-of-life syndrome hypothesis⁷ suggests that **an increased growth rate would be expected to be associated with increased foraging activity, aggression, and risk proneness**, which enable individuals to attain those resources needed to cover the energetic expenses of rapid growth (Dammhahn et al., 2018; Niemelä, Vainikka, Hedrick, & Kortet, 2012; Ricklefs & Wikelski, 2002; Niemelä, Dingemanse, Aloravainen, Vainikka, & Kortet, 2013; Réale et al., 2010). In order to assess this hypothesis, we

⁷ The pace-of-life syndrome (POLS) hypothesis states that organisms may be placed on a hypothetical one-dimensional axis based on their rate of living, and the organisms' position on that axis predicts the states of a number of correlated traits. On the fast end of this axis are organisms characterised by early maturation, high reactivity of the hypothalamic-pituitary-adrenal (HPA) axis, short lifespan, and high fecundity, etc., whereas on the slow end organisms show late maturation, low HPA reactivity, long lifespan, and low fecundity, etc.

used behavioural tests on *P. agrestis* individuals to quantify activity, voracity and boldness of spiders, and compared behavioural characteristics between slowly and rapidly developing individuals (**Study 2**).

Rapid development was shown to have multifaceted costs (Brommer, 2004; De Block et al., 2008; Ficetola & De Bernardi, 2006; Lindström, 1999; Rantala & Roff, 2005), these costs often manifested as trade-offs with other life history traits. However, whether or not negative covariance might occur between life history traits may not only depend simply on their corresponding costs. Costly traits are expected to interfere with one another, if they rely on the same limited resource pool. However, beside to adaptive resource allocation strategies, individual quality (i.e. the capacity to bear the above mentioned costs) might also play a role in how costly traits or processes are associated (Reznick, Nunney, & Tessier, 2000; van Noordwijk & de Jong, 1986). For example, a so-called good quality individual may invest, in absolute terms, the same energy into somatic tissues (such as skeletal muscles) than other specimens, but these costs may relatively be lower for this “good-quality” individual. An analogous phenomenon for this is the association of disposable income⁸ and car maintenance for humans: for the same amount of gas, two people will pay the same amount of money, but for the one who possesses a higher amount of disposable income, this price would appear relatively less. Consequently, while negative associations between costly life history traits might be expected, individual quality or adaptive plasticity might mask such relationships, by mitigating physiological costs (Engqvist, 2007).

Although quite a few studies showed that an enhanced rate of growth and / or development often entails considerable costs that manifest in trade-offs (e.g. Blanckenhorn, 1998; De Block et al., 2008; Dmitriew & Rowe, 2005; Ficetola & De Bernardi, 2006; Fischer et al., 2005; Gotthard, Nylin, & Wiklund, 1994; Met-

8 The part of regular currency income, which needs not to be invested into livelihood (like food, or rent).

calfe & Monaghan, 2001; Metcalfe & Monaghan, 2003; Metcalfe & Alonso-Alvarez, 2010), it is not clear whether one should expect such costs in an adaptive developmental plasticity scenario (such as cohort splitting) as well. However, we still know very little about this phenomenon, as empirical studies on the individual-level consequences of developmental differences due to cohort splitting are scarce (Khelifa, Zebba, Amari, Mellal, & Mahdjoub, 2019). In populations of *P. agrestis* cohort splitting leads to the emergence of developmental types that differ markedly in developmental rate, so this species appears to be an ideal model organism to test associations between developmental time and other, costly life history traits. Also, more intriguingly, we are able to compare costly traits between sub-cohorts, i.e. between the slowly and rapidly developing phenotypes. By doing so we might draw a more detailed picture on what consequences cohort splitting might have in physiological terms. *P. agrestis* is also ideal for such an approach because both the slowly and rapidly developing sub-cohorts are quite abundant in natural habitats. Additionally, this spider species is relatively easy to be handled and reared in laboratory, and, based on the work of Kiss (2003), developmental time of the individual spiders can be effectively manipulated by regulating the photoperiod during their rearing. To **identify physiological consequences of belonging to the rapidly developing sub-cohort** we collected spiders from their natural habitat, and we also reared spiderlings in the laboratory. We quantified a number of costly life history traits for each specimen (such as measures of condition and immunocompetence), and assessed juvenile mortality rates among slowly and rapidly developing spiderlings in the laboratory (**Study 3**).

Survival up till reproduction is an important element of individual fitness. Intrinsic survival has multiple components (Letcher, Rice, Crowder, & Rose, 1996), one of which is somatic maintenance that helps to uphold the integrity of the organisms (Flatt & Heyland, 2011). Somatic integrity may be compromised by a number of internal and external factors, an example for the latter being in-

vasion by parasites that attempt to take advantage of the hosts' resources in order to grow and reproduce (Zuk & Stoehr, 2002). Immunity, or the capacity to prevent, tolerate, and / or eliminate parasite invasion, is a crucially important element of somatic maintenance, and in the last few decades substantial effort was put into integrating immunity and mechanisms of the immune system into life history theory (Lee, 2006). As a result, a growing body of literature addresses what physiological costs immunocompetence have, and how the maintained capacity to fend off parasite attacks, and the efficiency of an elicited immune response of the immune system are correlated with other life history traits (Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996). A number of elements of maintained immune capacity and efficient immune responses were shown to be associated with developmental characteristics. For example, in the Mediterranean field cricket *Gryllus bimaculatus* it was found that the efficiency of encapsulating a foreign object within the body is negatively associated with the time of development, while bacterium lytic activity was positively correlated with it (Rantala & Roff, 2005). Although a substantial body of knowledge had been accumulating in the past few decades on the associations between developmental characteristics and given measurements of immunity (Tieleman, 2018), still rather little is known about how developmental stages themselves might affect elements of the immune system (Wilson-Rich, Dres, & Starks, 2008). The different life cycle stages may differ in their fitness objectives, e.g. adult individuals have to partition their resources between investment in survival and reproduction, whereas juveniles generally are not yet able to realise any reproductive effort. Similarly, developmental stage-dependent differences in the diversity or types of pathogens encountered might shape the stage-specific characteristics of the immune system, balancing the stage-specific costs and benefits of efficient immune defences (Shi & Sun, 2010; Wilson-Rich et al., 2008), ultimately contributing to developmental stage differences in the efficiency of given elements of the immune system (Giglio & Giulianini, 2013).

In *P. agrestis* juveniles and adults reside in the same habitat type, and have very similar life styles (Kiss, 2003). Owing to this one might expect that individuals of different developmental stages (such as juveniles, or adults) do not differ substantially in their encounters with a range of parasites (e.g. microbial pathogens). On this basis, developmental stage-differences would not be expected in the individuals' capacity to resist (or tolerate) such invaders. However, the developmental stages differ considerably in their fitness objectives, as juveniles and subadults predominantly have to focus on foraging and survival, whereas adults have to reproduce as quickly as possible. Because adults have only one reproductive period throughout their lives (i.e. are semelparous), they are not expected to invest as much into survival as juveniles, as opposed to organisms that can reproduce multiple times (i.e. are iteroparous) and can trade off current reproduction in favour of long term survival and future reproduction (Young, 2010). Consequently, individuals of a semelparous species such as *P. agrestis* might maximise their reproductive output by increasing investment into reproductive tissues, via a shift in prioritization of resource allocation between elements of survival (i.e. somatic maintenance, such as immunity) and reproduction. Therefore, one may hypothesise that, within sub-cohorts, baseline-level efficiency of **given immune mechanisms would decrease after sexual maturation, and / or reproduction**. We addressed this hypothesis by testing whether subadult and adult specimens of *P. agrestis* differ in two measures of humoral immune capacity (**Study 4**).

Prioritizing reproduction over survival in a semelparous species might be advantageous for both sexes. However, the costs of reproduction can differ substantially between the sexes, so there might be sex-specific differences in the physiological costs associated with investment into reproduction. Sex-differences in reproductive investment were proposed to shape differences in immunity between males and females (Vincent & Gwynne, 2014), but it is still not clear in what direction these differences should be expected. Studies on inverteb-

rates yielded mixed results in that matter, presenting examples for both female and male-biased immunity (e.g. Kelly, 2016; Kelly & Jennions, 2009; Lindsey & Altizer, 2009; Rantala & Roff, 2006; Schwarzenbach, Hosken, & Ward, 2005; Vincent & Gwynne, 2014). Nevertheless it was suggested that seasonality and effective time-window of breeding should have considerable impact on the direction of sex differences in immunity (Zuk, Simmons, Rotenberry, & Stoehr, 2004). When individuals have limited time and / or opportunities to reproduce, relatively higher male immune capacity becomes more prevalent, due to the higher costs of reproductive investment in females and the shorter time period during which individuals must concentrate their resources to reproduction. It is important to note that this is expected to be true when females do not gain considerable fitness benefits from longevity, i.e. investment into somatic maintenance, and hence survival, does not improve female reproductive output substantially (Stoehr & Kokko, 2006). In order to test **whether in an invertebrate species with one short reproductive period during its life cycle, females indeed show decreased immune capacity relative to males**, we applied the widely used encapsulation test on *P. agrestis* females and males within a sub-cohort, also controlling for developmental time and body size (**Study 5**).

I.2 OBJECTIVES AND RESULTS

Here I present the studies included in this thesis, describing the aims and major results of them.

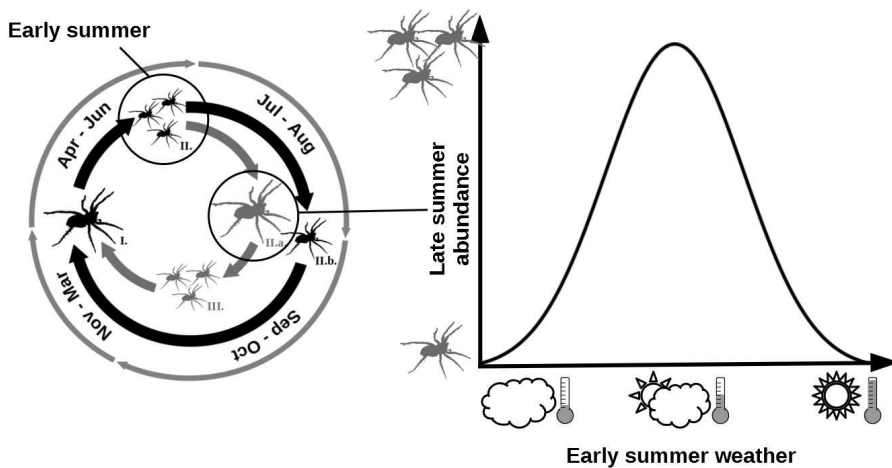
Study 1: *Effect of weather conditions on cohort splitting in a wolf spider species*

(i) Objectives

In their natural habitats, both slow and rapid developing specimens of *Pardosa agrestis* can occur, even originating from the same brood, due to cohort splitting among the early summer generation of spiderlings. However, it is largely unknown what factors play role in evoking this type of cohort splitting. Early laboratory rearing studies suggested that environmental factors, such as day length and temperature, can affect developmental rate in artificial conditions, but there is little information about what might contribute to the appearance of rapidly developing specimens in their natural habitats. Based on the observations that light cycle and ambient temperature can affect developmental rate and maturation in laboratory reared *P. agrestis* spiders, we hypothesised that the occurrence of rapidly developing phenotypes might result from a life history decision during early ontogeny, in response to external stimuli. In this study **we aimed to test how weather conditions might have influenced this ‘early choice’ between slow and rapid development**, by using long-term collection data from natural populations, weather data from the collection sites and dates, and non-parametric habitat modelling. Owing to the features of the collection data, only population-level informations of the abundance of rapidly developing spiders was available. We predicted that a range of favourable early weather conditions might act as cues promoting a life history decision on the part of newly hatched spiderlings to take on rapid development.

(ii) Results

Conforming to our prediction we found that a narrow range of early summer weather conditions exists which corresponds to the highest abundance of late summer second generation adults (i.e. rapidly developing spiders). We proposed that these conditions may represent an optimal range, promoting a life history decision on the part of spiderlings hatching in early summer to take on rapid development. Notably, these results correspond only to the population-level presence of rapidly developing spiders. Also, it still remains unclear if the weather has a direct (e.g. increased metabolic rate due to higher temperatures), or indirect (e.g. increased prey abundance) effect on late summer adult abundance, or both. We concluded that, while the results from this paper (and from other studies conducted on laboratory populations) are concordant with an ‘early choice’ hypothesis, effects weather has on survival rates might also have contributed to the observed abundance patterns. Indeed, in our analyses we only could test the overall presence of adult rapidly developing spiders. Notably, the abundance of rapidly developing adult spiders is likely downwardly biased, because we had no information about those individuals that engaged in rapid development, but did not mature (i.e. perished before maturing).



Study 2: Pace of life and behaviour: rapid development is linked with increased activity and voracity in the wolf spider *Pardosa agrestis*

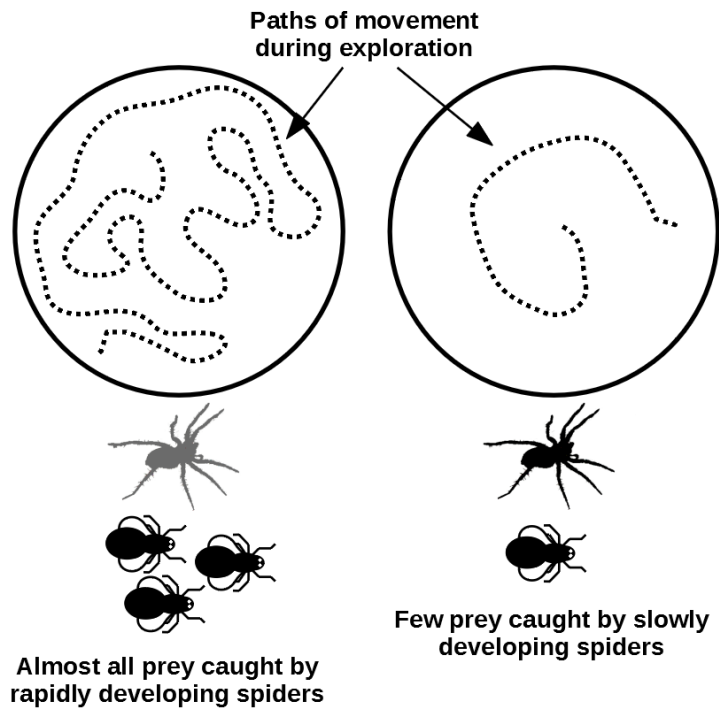
(i) Objectives

Until recently, a large number of studies assessed how the rate of living is associated with life history traits on interspecies and inter-population scales. However, few studies have focused on the within-population variation in the pace of life, and its relationship with other key life history traits. In the natural populations of *Pardosa agrestis* the two developmental forms (i.e. slowly and rapidly developing specimens) are quite different in their developmental rates, rendering this species ideal to test the influence of considerable variation in developmental rate on behavioural traits. Rapid post-embryonic development is energetically costly, and individuals that opt for an enhanced developmental rate must meet the associated resource demand. Consequently, **rapidly developing individuals would be expected to be more active and risk prone**, in order to be able to cover the energetic expenses of rapid growth. In this study we predicted that spiders of rapid developmental life history should be more active explorers in a novel environment, more voracious towards potential prey, and bolder in a shelter-use scenario.

(ii) Results

We found that (1) spiders of rapid development were consistently more active throughout exploration tests, i.e. covered larger distances and spent more time moving than slowly developing spiders. Also, (2) rapidly developing spiders were more likely to attack both artificial and living prey, and they showed shorter latencies before attacking living prey in comparison to slowly developing specimens. Furthermore, (3) slowly developing spiders showed longer latencies prior to emerging from a shelter, although this was only apparent in the first test repetition, but not in the second. Overall, these results imply that spiders engaging in rapid development are generally characterised by more motile, voracious

and risk prone behaviour, which are argued to be necessary to achieve and maintain increased growth rate, for example by the way of increased hunting success and hence resource acquisition.



Study 3: Consequences of rapid development owing to cohort splitting: just how costly is to hurry?

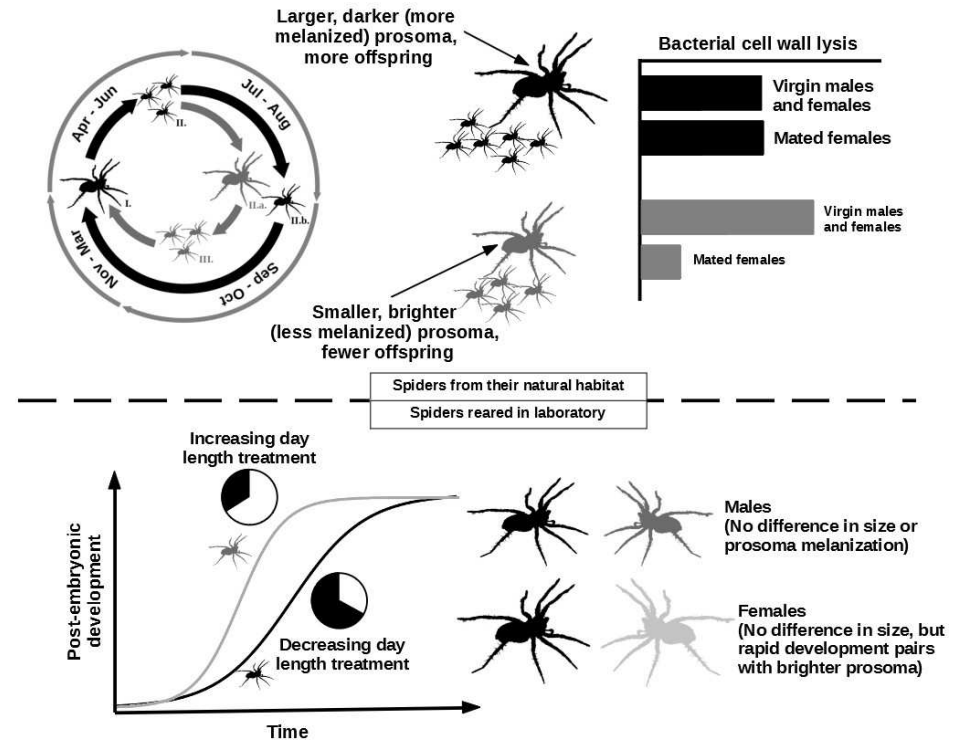
(i) Objectives

Although there are some studies on the background of, or potential costs linked to cohort splitting, complex studies on the individual-level consequences of following alternative developmental pathways are still lacking. Consequently, our understanding of the potential costs and benefits for the specimens in a given cohort splitting scenario is rather limited. In this study **our goal was to assess important life history components related to condition, somatic maintenance and fecundity in females, and compare them between sub-cohorts to identify what physiological characteristics might be linked with rapid development**. To do so we collected spiders from their natural habitat from both slow and rapid sub-cohorts, and also reared slowly and rapidly developing spiderlings in the laboratory. Upon maturation of the spiders we measured body size, fat stores, prosoma melanisation, levels of antimicrobial peptides, and capacity to inhibit bacterial growth. During analyses we tested whether the assessed traits differ between sub-cohorts, and how growth rate itself is associated with the assessed traits. Furthermore, we assessed juvenile mortality among laboratory-reared spiders.

(ii) Results

Spiders of rapid development collected from natural habitat were smaller, had fewer fat reserves, and had less melanised prosoma cuticles than slowly developing ones. However, most of these differences were not apparent among laboratory reared spiders, indicating that physiological costs of rapid development might be mitigated to a considerable extent by increased resource acquisition (given the *ad libitum* food availability under laboratory rearing). Also, no clear trend was apparent in terms of immunity costs of rapid development, apart from cell wall lytic activity among mated females, hinting at possibly higher costs of

reproductive investment in rapidly developing females. Notably, rapidly developing females were characterised by much lower fecundity in comparison to slowly developing ones. This might be due to decreased investment into reproductive tissues, owing to higher relative pre-adult investment into somatic maintenance. Overall, our findings appear to imply that rapid development might pose some physiological costs on individual spiders, but these costs can be mitigated to a considerable extent, indicating that so-called “good quality” rapid individuals may achieve similar physiological status to slow spiders.



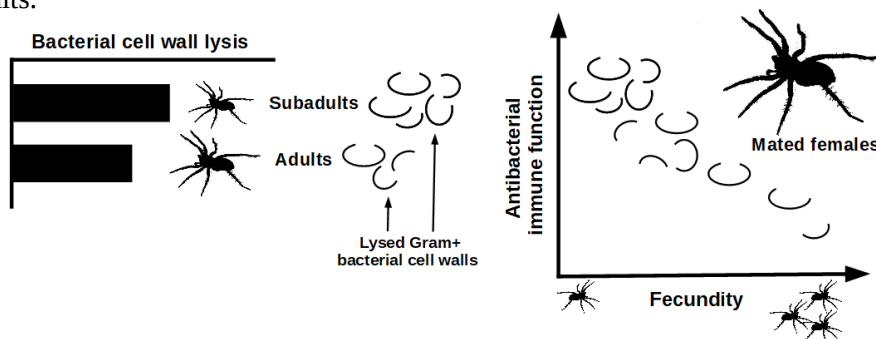
Study 4: *Antibacterial immune functions of subadults and adults in a semelparous spider*

(i) Objectives

Ontogenetic dynamics of immune functions are still poorly understood. Although life stage-dependent changes in the types and prevalence of pathogens (that might contribute to the evolution of stage-differences in immunity) are frequently studied, the role of life history characteristics in shaping the extent and direction of temporal changes in immunocompetence over the course of post-embryonic development are rarely assessed. In this study **we investigated how growth rate during ontogeny, and maturation *per se* are associated with elements of humoral immunity in *Pardosa agrestis***. We predicted that in a semelparous species adult (sexually mature) specimens would be expected to have decreased immunocompetence in comparison to subadults, due to the interplay of (1) maintenance costs of immunocompetence, (2) costs of maturation, such as gamete production, and (3) the absence of the trade-off between current and future reproduction in adults. We used the bacterial growth inhibition and cell wall lytic activity assays, in which biological samples from the spiders produce so called inhibition and clearing zones, of which the areas are proportional to the antimicrobial peptide and enzyme contents, while controlling for growth rate of the spiders. Measures from the immune assays are thought to inform us about the maintained capacity to mount an immediate humoral immune response in spiders. Because in our predictions we presumed that the decrease in immunocompetence should be expected to be a result (at least in part) of the resource allocation-conflict between investment into somatic maintenance and reproduction, we also tested how the described immune measures are associated with the occurrence of mating, and with fecundity in females as well.

(ii) Results

Results showed that (1) subadult spiders had a stronger cell wall lytic capacity than adults, but ontogenetic stages did not differ in their bacterial growth inhibition power. (2) Growth rate did not correlate with either of the assessed immune measures, and (3) there was no substantial co-variation between bacterial growth inhibition and cell wall lytic capacities. Furthermore, (4) mated females showed depressed capacity of bacterial growth inhibition and cell wall lysis in comparison to virgin ones, although the latter was only significant among late summer (i.e. rapidly developing) females. Also, among mated females, (5) those with higher fecundity tended to have lower immune measures. Overall these results suggest that in *Pardosa agrestis* the costs of gamete production and reproduction can interfere with some elements of humoral immunity. These costs appear to be dependent on other life history characteristics too, as for example, our results hint at a three way interaction between developmental type, immunocompetence, and reproduction. Surprisingly, while developmental type (being a slowly or rapidly developing individual, i.e. maturing early or late summer, respectively) appears to affect the tested immune markers and their relationship with reproduction, growth rate *per se* was independent of them, implying that these elements of humoral immunity are not in an energetic conflict with post-embryonic development itself. Notably, though, *ad libitum* food availability during laboratory rearing might have mitigated the association between them, masking the resource allocation conflict-based negative phenotypic correlation of these costly traits.



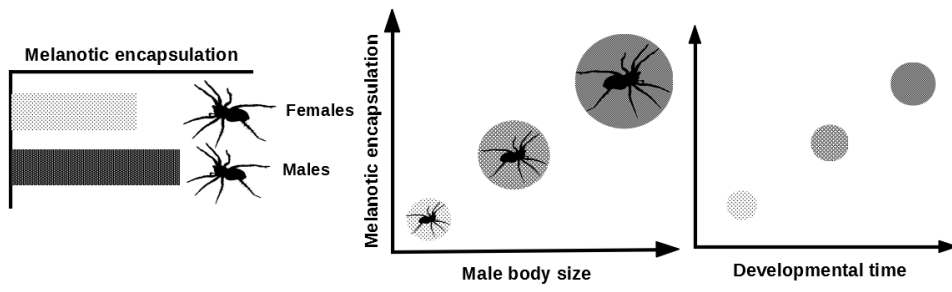
Study 5: Sex-dependent immune response in a semelparous spider

(i) Objectives

Activation of the immune system is known to have multifaceted costs, which might interfere with a number of other life history traits. Due to differences in how they are able to maximise their fitness, sexes might differ in their ability to mitigate, or cope with such costs. Different costs of reproduction may be manifested differently in the sexes, leading to sex-specific trade-offs with other life history traits. In this study **we aimed to assess the efficiency of an immune response associated with deployment costs** (i.e. physiological costs that are realised during immune activation) **in both sexes**, by using the so-called encapsulation test, a widely used method for measuring immunocompetence in arthropods. We predicted that in *Pardosa agrestis*, the interplay of high costs of egg maturation in females, limited time available for mating (i.e. seasonal breeding) and the absence of a trade-off between current and future reproduction (i.e. semelparity) should contribute to relatively lower competence to mount a costly encapsulation response. We also wanted to see how condition (assessed as prosoma size) and developmental time are related to the efficiency of encapsulation in the two sexes.

(ii) Results

We found that (1) on average, males were able to mount a stronger encapsulation response against the inserted monofilaments than females, supporting the hypothesis tested in this study. (2) Level of encapsulation was positively associated with body size in males, but not in females, suggesting that this immune parameter might be much more tightly linked with individual condition in males. (3) Developmental time did not show correlation with adult body size in either of the sexes, but (4) it was positively associated with encapsulation both in males and females. Altogether these results highlight the importance of life history characteristics and sex in shaping trade-offs between important life history traits.



I.3 GENERAL DISCUSSION

We found that during early summer weather conditions that newly hatched spiderlings experienced correlates with the abundance of rapidly developing adults during late summer. Indeed, a narrow range of early summer weather conditions (such as average daily temperature or rainfall) coincided with the highest rapidly developing adult abundance in the natural habitats (**Study 1**). Although the literature on the effects of weather conditions on cohort splitting is scarce, ambient temperature and temperature regimes were found to have a significant effect on not only developmental rate, but cohort splitting dynamics as well (Crowley & Hopper, 2015; David et al., 1999; Todd, 1996). Weather conditions may have multifaceted effects on development, for example serving as direct cues based on which individuals might make a life history decision whether or not to engage in rapid development (Danks, 1994). However, weather conditions are more likely to have indirect influence on the early development of spiderlings, for example by affecting prey availability (Hayashi, 1988), metabolic rate (Gillooly, Brown, West, Savage, & Charnov, 2001), foraging activity (Ford, 1978), and survival in general (Dondale & Binns, 1977). In the frames of **Study 1**, it is not possible to disentangle these potential mechanisms, but it is apparent that late summer abundance of the rapidly developing phenotype is significantly correlated by early summer weather conditions. Notably, in the rearing studies of Kiss (2003) it was found that ambient temperature conditions had quite considerable impact both on developmental time and maturation-propensity of spiders. Effects of rearing regimes shown in **Study 3** and by Kiss (2003) indicate that photoperiod also has considerable influence on the occurrence of rapidly developing phenotype, or more strictly, on the probability of spiderlings to mature and reproduce without overwintering. Although abiotic environmental factors such as weather conditions likely have various indirect effects on the survival and success of developmental types, these observations suggest that they might also

play a role as stimuli which might contribute to a life history decision on the part of spiderlings at their early ontogeny.

Notably, an “advantageous” set of weather conditions might positively affect prey availability for spiders (Romero & Harwood, 2010). High prey availability at the onset of development could help to maintain a relatively high rate of growth and development in the rapid sub-cohort via increased resource acquisition. Indeed, spiders following rapid development were found to be more voracious predators (**Study 2**), and the positive feedback between higher voracity and increased resource income presumably helps in maintaining the increased growth and developmental rate in the spiders (Luttbeg & Sih, 2010). Owing to that rapidly developing spiders exhibited higher activity and voracity consistently throughout their ontogeny, it appears to be likely that increased resource acquisition is not the cause, but rather a consequence of rapid development. Note that all spiders were reared in the same laboratory conditions, and were provided food during the rearing period *ad libitum*. Based on the results of our first two studies (and studies carried out by Kiss (2003)) it seems plausible that spiderlings take on slow or rapid development quite early in their ontogeny, and an increased resource demand of higher growth and developmental rate results in higher foraging activity, in order to meet this demand. Indeed, in the past it was suggested that consistent behavioural differences between individuals likely reflect differences in key life history traits, such as growth rate or fecundity (Biro & Stamps, 2008).

An increased effort for resource acquisition might not only enable higher growth and developmental rates, but also might help to mitigate physiological costs associated with them (Dmitriew & Rowe, 2005). Although among spiders collected from their natural habitat rapidly developing specimens were significantly smaller than slowly developing ones, such size difference was not apparent in the spiders reared in the laboratory (**Study 3**). Notably, there can be year-to-year variation in the size difference between slow and rapid sub-cohorts, as suggested

by the results of body size measurements of slowly and rapidly developing spiders from natural habitat in three consecutive years, by Kiss (2003). In fact, in one of the years, females of the slow and rapid sub-cohort were even of the same size on average. These observations seem to indicate that the increased growth and developmental rate have considerable costs, e.g. in the form of increased resource income demand, limiting the growth rate and hence adult body size when resources are limited. However, these costs might be “buffered” by increased resource acquisition, i.e. when resource acquisition is less constrained, spiders might be able to meet the energetic demands of high growth rate, much higher than in slowly developing spiders, or than in rapidly developing spiders with limited access to resources.

Resource limitation might also help explain why rapidly developing spiders from natural habitat have lower amounts of fat reserves in comparison to slowly developing ones, while among the laboratory reared spiders fat contents were higher among specimens in the rapid treatment group. Due to the limited availability of resources in the natural habitat, building up fat reserves is likely challenging, because the acquired food would be used mainly for basic metabolic processes, growth, or in adults, to investment into reproductive tissues (Boggs & Freeman, 2005). However, the pattern of resource allocation is less restricted when food is unlimited. Therefore, spiders in the laboratory might have been able to maintain an enhanced growth and developmental rate, and when growth stopped (i.e. after the final moult to maturity), a large proportion of the (more voraciously) acquired resources could have been allocated into fat reserves in rapidly developing spiders.

While spiders in the natural rapid sub-cohort were smaller and had less fat reserves, their capacity to eliminate gram positive bacteria was found to be higher than in the slow sub-cohort. Interestingly, life history theory predicts considerable physiological costs of rapid development that are expected to be traded off against elements of somatic maintenance such as immunity (Ricklefs & Wikel-

ski, 2002; Zera & Harshman, 2001). For example, in the moth species *Spodoptera littoralis* some elements of immunity were found to be negatively correlated with larval development rate (Cotter, Kruuk, & Wilson, 2004; Cotter, Myatt, Benskin, & Wilson, 2008). In the laboratory, slow and rapid treatment groups did not differ in any of the tested immune parameters, and growth rate did not correlate with them either (**Study 3 & 4**). Relatively higher bacteria-killing efficiency in rapid sub-cohort spiders collected from natural habitat might imply that those spiders able to mature before winter are also able to invest relatively more into the tested element of humoral immunity. This might be due to their higher resource acquisition rate in comparison to slowly developing spiders (**Study 2**), if during resource allocation elements of somatic maintenance have higher priority than growth rate, hence the smaller adult sizes but higher humoral immune capacity. The absence of significant differences in the tested immune parameters between slow and rapid development groups among the laboratory reared spiders could result from the unconstrained availability of food, similarly to what was observed in the case of body size. Indeed, resource limited environments were shown to affect the trade-offs themselves between immunity and other life history traits, and unlimited food availability throughout larval development may dampen the energetic conflicts between them (Fellous & Lazzaro, 2010; McKean, Yourth, Lazzaro, & Clark, 2008). Unfortunately we can only speculate on the effect of food availability on the phenotypic associations between the assessed life history traits. In future studies that manipulate food availability under the different photoperiod regimes would likely help us clarify its role in shaping life history traits and the correlations between them.

In **Study 5** we found that developmental time was positively correlated with another element of immunity, namely melanotic encapsulation efficiency. However, it also should be noted that testing encapsulation informs us about a rather different element of immunity, with quite different physiological costs associated with it. Specifically, while the tests on antibacterial capacity against gram posit-

ive bacteria rely solely on the humoral immunity of the spiders, the process of encapsulation is mainly a cellular response to parasites invading the soma. As of their costs, the baseline level of humoral immune capacity to eliminate bacteria has so-called maintenance costs, whereas encapsulation (being an active response to parasitic presence) is associated with deployment costs of the immune system as well (Sadd & Schmid-Hempel, 2008; Schmid-Hempel, 2003). Overall, while an immune response with deployment costs was found to be correlated with developmental time, our findings appear to hint at no substantial developmental costs on the assessed elements of humoral immunity in *P. agrestis*. Resource allocation pattern differences that likely contribute to the developmental type differences in antibacterial capacity in natural spiders might be more balanced when food availability is less limited. Notably, our results might also suggest developmental type-differences in how developmental rate is associated with elements of immunity with maintenance costs, and with other elements incurring deployment costs, and indeed, with immune activation *per se* in living spiders as well. Future studies testing immune activation in both slow and rapid sub-cohorts will hopefully help to clarify these associations (e.g. whether immune activation has different costs in the sub-cohorts).

Surprisingly, virgin females of the rapid sub-cohort (collected from nature) maintained a stronger antibacterial immune capacity than spiders of the slow sub-cohort, but mating and egg laying appeared to compromise the cell wall lytic (although not bacterial growth inhibition) capacity of rapidly developing females (**Study 3 & 4**). This result appears to hint at a three-way interaction between developmental rate, investment into cell wall lytic capacity, and reproduction, meaning that investment into producing offspring might incur different costs in rapidly developing females than in slowly developing ones. Notably, though, an alternative explanation might be that the observed decrease in cell wall lytic capacity in mated rapidly developing females was due to increased rate of immunosenescence (Stanley, 2012). Considering that rapidly developing spiders com-

plete their life cycles 3-4 times faster than slowly developing ones, decreased lytic activity might occur sooner and / or more prominently than in slowly developing females. Future studies should investigate the potential effects of immunosenescence on different immune parameters in both developmental types to gain better understanding whether this phenomenon plays a significant role in this spider species.

The costs of reproductive investment were apparent in both developmental types, as in mated females fecundity was negatively associated with bacterial growth inhibition and cell wall lytic capacity as well. Similar costs were also found to affect hemolymph lytic activity in other arthropods as well (Calbacho-Rosa, Moreno-García, Lanz-Mendoza, Peretti, & Córdoba-Aguilar, 2012; Fedorka, Zuk, Mousseau, & Tregenza, 2004), pointing at a trade-off between investment into humoral immunity and reproduction. Additionally, mated females of *P. agrestis* were characterised by significantly lower fat reserves than virgin females, indicating substantial resource expenditure on egg production in both sub-cohorts (Arrese & Soulages, 2010). Although the dramatic drop in bacterial cell wall lytic capacity in rapidly developing females after egg laying might suggest increased relative reproductive investment in comparison to slowly developing ones, we found no developmental type differences in the effect of mated status on fat reserves, or in the associations between antibacterial capacity and fecundity. In other terms, there were no slope differences between slowly and rapidly developing females in the models assessing the associations of immune parameters with fecundity. This means that the decrease in fat reserves in mated females was equivalent in the slow and rapid developmental sub-cohorts. These seem to imply no relative differences in the costs of reproductive investment between females. It remains an open question whether the offspring from the different sub-cohorts differ in size and / or reserves, and how these are related to maternal condition, which could clarify the association between offspring size and number within sub-cohorts, and maternal investment into individual eggs

(Lim, Senior, & Nakagawa, 2014). Unfortunately in our studies we did not measure either body sizes of newly hatched spiderlings, or sizes of eggs, therefore we cannot infer on the potential association between offspring size and number. In future studies it would be very important to assess offspring sizes (directly after hatching) and their associations with maternal condition and with the spiderlings' developmental characteristics.

In an adaptive developmental plasticity scenario, such as the studied case of cohort splitting, a decreased investment into fecundity might not pose serious disadvantages at all, even if it is not linked with increased investment into individual eggs. While developmental costs could also contribute to decreased fecundity via decreased allocation into reproductive tissues (Khelifa et al., 2019), consistently lower investment into reproductive tissues might also help to mitigate developmental costs on other traits due to altered resource allocation patterns. The absence of developmental type differences in relative fecundity costs (described previously) seems to be in accordance with this. Also, ongoing theoretical investigations seem to imply that expected reproductive success of slowly and rapidly developing females might not differ even in the face of the marked difference in fecundity. In a stochastically alternating good *versus* bad year scenario differential survival of the developmental types in the different environmental states (good or bad) might translate to differences in mortality patterns in good and bad years between the sub-cohorts. Specifically, in good years lifetime mortality of rapidly developing spiders is expected to be lower than that of slowly developing ones (chiefly because of their shorter lifespan), while in bad years the opposite would be true. This scenario appears to be in agreement with some conclusions from **Study 1**, namely that advantageous environmental conditions (good years) might contribute to higher survival in rapidly developing spiders, while adverse conditions (bad years) likely incur substantially increased mortality in them.

Notably, when mortality patterns are the same for the developmental types up till winter, spiders of the rapid sub-cohort will be characterised by a lower lifetime mortality rate than slow spiders. This is due to that rapidly developing spiders do not have to engage in overwintering, which otherwise imposes high mortality on spiders (Kiss & Samu, 2002; Schaefer, 1977). Consequently, rapidly developing spiders will be more likely to reach maturity and reproduce even if there are no sub-cohort differences in juvenile mortality rates. Even more intriguingly, in our laboratory survival test (where conditions likely resembled that of a “good year”) we found that rapidly developing spiders were less likely to perish as juveniles than slowly developing ones (**Study 3**). Additionally, not only rapidly developing spiders may be more likely to mature and reproduce than slowly developing ones in good years, but at the point when their offspring hatch, the probability of rapidly developing spiders to have reproducing offspring is likely to be also higher than the probability of a slowly developing spider reproducing. This is owing to the fact that the probability of all offspring from a rapidly developing specimen perishing decreases geometrically with one unit increase in fecundity. Although we have no direct evidence either *pro* or *contra* for equal overwintering mortality within slowly developing spiders and the offspring of rapidly developing spiders, rearing studies of Kiss (2003) suggest that ontogenetic stage of the spiderlings is not associated with their overwintering success. If there are no substantial differences in overwintering survival, the expected number of offspring will be higher for rapidly developing spiders, in comparison to their slow sub-cohort siblings. On the other hand, during bad years rapidly developing spiders would not be likely to even mature, so slowly developing spiders would be expected to have higher fitness due to their relatively higher survival, and considerably higher fecundity (**Study 3**).

Variation in offspring traits (such as developmental type) within broods was suggested to be adaptive in environments where the future quality of the habitat shows considerable and unpredictable variation, as it decreases variance in the

short term fitness of the mother (Crean & Dustin, 2009; Marshall, Bonduriansky, & Bussière, 2008). Producing mixed broods in which different offspring phenotypes have differential survival and reproductive potentials, depending on the environmental state in which the offspring will develop, increases the probability that at least some of the offspring will mature and reproduce. In other terms, diversifying offspring phenotypes decreases the likelihood of total reproductive failure of the given lineage. Such risk spreading over the progeny may result in an adaptive bet-hedging strategy, characterised by decreased short term (arithmetic mean) fitness, but increased long term (geometric mean) fitness (Haaland, Wright, Tufto, & Ratikainen, 2018; Starrfelt & Kokko, 2012). That is, the number of reproducing offspring may be lower on average compared to a non-mixed brood (i.e. where only one developmental type occurs), but, owing to lower variation in the number of reproducing offspring, long term reproductive fitness will be higher. Simply put: decreased variation in reproductive success promotes low-variation (low-risk) strategies in a stochastically changing environment. *P. agrestis* mainly inhabits arable fields, alkaline grass lands and -marshes (Samu & Szinetár, 2002), which habitats are generally characterised by substantial variation in quality across different years, and it was hypothesised that this spider's unique phenology may have enabled it to become dominant in these habitat types (Kiss & Samu, 2005; Rádai, Kiss, & Samu, 2017). Taken together the results of our empirical studies, and the preliminary results of theoretical analyses, it appears to be likely that cohort splitting in *P. agrestis* might be a diversified bet-hedging strategy on the part of early summer females. Notably, this diversification in developmental phenotype shows a substantial and consistent tendency to take place in response to specific environmental stimuli (**Study 1-3**). Such plasticity among the offspring might still be a bet-hedging strategy of the females from which the offspring originate, as it was shown that more dynamic cases of bet-hedging can evolve in temporally variable environments (Frankenhuis, Panchanathan, & Belsky, 2016). In such scenarios, the extent of phenotypic plasticity itself is varying among the offspring. This concept of a “plastic bet-

hedging” is receiving increased attention (Frankenhuis et al., 2016; Grantham, Antonio, O’Neil, Zhan, & Brisson, 2016; Sadeh, Guterman, Gersani, & Ovadia, 2009), and might be a valuable perspective in understanding cohort splitting in *P. agrestis*. Indeed, data from **Study 3** suggest that there is considerable among-brood variance in the propensity of spiderlings to take on rapid development. This was apparent in the low variance between mothers in the average time of development among their offspring, whereas mothers varied substantially in how their offspring increased their developmental rate in response to rapid development treatment (i.e. day length dynamics). This might suggest that spiderlings have an inherent (and likely heritable; Frankenhuis et al., 2016) propensity to engage in rapid development under given circumstances (e.g. increasing day length, indicating the start of the season). In the future theoretical analyses may help to predict whether or not plastic bet-hedging might be a likely explanation for cohort splitting in *P. agrestis*. Also, more elaborate rearing studies should help us to identify additive genetic and maternal components that might be associated with variation in offspring developmental rate, and the strength of genotype-by-environment interactions affecting it.

I.4 CONCLUSIONS

Using the wolf spider *Pardosa agrestis* as a model organism, we implemented various tests and experimental designs to assess what causes and consequences might cohort splitting have, and what life history trait-associations are present within sub-cohorts. By studying life history characteristics of spiders (both laboratory reared and field collected) within and between sub-cohorts we not only acquired knowledge on this unique species, but also gained important insights on cohort splitting and the consequences of an enhanced pace of life on the within-population level. Our results highlight that physiological costs and trade-offs between life history traits might not be present in adaptive plasticity scenarios. The role of resource availability and developmental phenotype-specific allocation patterns of acquired resources was emphasised, in influencing not only life history traits themselves, but phenotypic correlations between them as well. A better understanding of how organisms may mitigate hypothesised costs of rapid development would help us draw a more detailed picture in the future on how the pace of life shapes life history traits and associations between them, on both between and within species levels. Additionally, ongoing and future studies on *P. agrestis* will hopefully help us determine whether or not cohort splitting might be a bet-hedging mechanism on the part of females, providing rare insight into plastic bet-hedging scenarios in the animal kingdom.

I.5 KEY FINDINGS

- Abiotic conditions, such as weather, experienced by early summer *Paradosa agrestis* spiderlings affect the abundance of the rapid developmental phenotype in natural populations, either by their effects as cues based on which an “early choice” between developmental trajectories can be made, and / or through their effects on survival.
- Spiders of rapid development exhibit more proactive behaviour than slowly developing ones in a repeatable manner, providing evidence for consistent behavioural differences between individuals in accordance with their life history (pace of life).
- Rapid development imposes considerable physiological costs on spiders in their natural habitat.
- Physiological costs associated with rapid development can be mitigated by high resource availability, lending support for that these costs are relative to individual quality (“big house, big car”).
- Bacterial growth inhibition, cell wall lytic activity, and levels of antimicrobial peptides (the baseline levels of which all being associated with so-called maintenance costs) are not correlated with growth rate *pre se*.
- When assessed within sub-cohorts, bacterial cell wall lytic activity is weaker in adults in comparison with subadults.
- In mated females fecundity was negatively associated with antibacterial immune capacity.
- Efficiency of melanotic encapsulation (an immune process associated with both maintenance and deployment costs) is correlated with developmental rate.
- Females exhibit weaker melanotic encapsulation response than males.
- The strength of melanotic encapsulation immune response is correlated with body size in males, but not in females.

I.6 ÖSSZEFOGLALÁS

A pusztai farkaspókot (*Pardosa agrestis*) alkalmazva modellszervezetként számos vizsgálatban mértük fel a kohorsz (korcsoport) szétválás egyedszintű kiváltó okait és következményeit, valamint azt, milyen módon kapcsolódnak egyes életmenet jellegek egymással al-kohorszok közt és azokon belül. A vizsgálatok során nem csupán ezen faj életmenetének jellemzőiről szereztünk újszerű ismereteket, hanem jobb rálátást nyertünk a kohorsz szétválás és az azzal kapcsolt fokozott egyedfejlődési sebesség populáción belüli következményeire is. Eredményeink alapján a fiziológiai költségek és az életmenet jellegek közti negatív csereviszony jelenléte nem törvényszerű adaptív fejlődési plaszticitás esetében. Ebben kiemelt szerepe van a táplálékélérhetőségnek és az adott fejlődési fenotípushoz kötött forrás szerzési és allokációs mintázatoknak, melyek nem csupán az életmenet jellegekre, de azok kapcsoltságára is hatással vannak. Annak megértése, hogy miként csökkenthetők a fokozott egyedfejlődés költségei segíthet megértenünk miként befolyásolja az életmenet üteme az életmenet jellegeket és azok egymás közti kapcsolatát, mind populációs, mind pedig faji léptékben. Ezek mellett jelenleg és a jövőben zajló további kutatások remélhetőleg segítenek annak tisztázásában, hogy a kohorsz szétválás ezen esete vajon egy evolúciós kockázat megosztó stratégia-e a populációkban, és vajon annak egy klasszikus, vagy egy jóval dinamikusabb esete, mely utóbbi értékes bepillantást biztosítana a kevésbé ismert “plasztikus bet-hedging” jelenségébe.

I.7 JELENTŐSEBB EREDMÉNYEK

- A kora nyári pusztai farkaspók generációban egyes abiotikus körülmények, mint például egyes időjárási tényezők, jelentős mértékben befolyásolják a gyorsan fejlődő fenotípus jelenlétét természetes populációkban, feltehetően direkt környezeti információként, és/vagy a gyorsan fejlődő pókok túlélésének befolyásolása révén.
- Gyors egyedfejlődésű egyedek, összhangban életmenetük ütemével, táplálékszerző viselkedésükben konzisztensen aktívabbak lassan fejlődő társaiknál.
- Természetes populációkban a fokozott egyedfejlődési sebességnek jelentős fiziológiai költségei figyelhetők meg.
- A fokozott egyedfejlődési sebesség fiziológiai költségei lényeges mértékben csökkenthetők növekedett táplálékélérhetőség mellett, amely ezen költségek egyedszintű minőség-kapcsoltságára utalnak (“nagy ház, nagy autó” hipotézis).
- Baktérium növekedés gátlás, bakteriális sejtfal bontás, valamint antimikrobiális peptidek szintje (melyek a humorális immunrendszer fenntartási költségeihez járulnak hozzá) nincsenek összefüggésben magával a növekedési rátával a vizsgált fajban.
- Al-kohorszokon belül a bakteriális sejtfal bontási aktivitás gyengébb ivarérett egyedekben a szub-adult egyedekhez viszonyítva.
- Párosodott nőstényeknél az antibakteriális immunitás hatékonysága negatív összefüggésben áll a nőstények utódszámával.
- Az ún. melanotikus betokozódásos immunválasz (mely fenntartási és aktiválási költségekkel is jár) pozitív összefüggésben áll az egyedfejlődési idővel.
- A nőstények átlagosan gyengébb betokozódásos immunválaszt mutatnak, mint a hímek.
- A hímek esetében a betokozódásos immunválasz pozitív összefüggésben áll a testmérettel, ám ezen kapcsolat nem figyelhető meg nőstényeknél.

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CHAPTER II

In this chapter I list published articles presented in my dissertation and additional publications as well, and I also include the research items themselves introduced previously.

II.1 LIST OF PUBLISHED RESEARCH ARTICLES

Published forms of articles are adapted with permission.

II.1.1 Publications relevant for the dissertation

Study 1 – Rádai, Z., Kiss, B., & Samu, F. (2017). Effect of weather conditions on cohort splitting in a wolf spider species. *The Journal of Arachnology*, 45(3), 444-448. <https://doi.org/10.1636/JoA-S-17-008.1>

Study 2 – Rádai, Z., Kiss, B., & Barta, Z. (2017). Pace of life and behaviour: rapid development is linked with increased activity and voracity in the wolf spider *Pardosa agrestis*. *Animal Behaviour*, 126, 145-151. <https://doi.org/10.1016/j.anbehav.2017.02.004>

Study 3 – Rádai, Z., Kiss, J., Babczyńska, A., Kardos, G., Báthori, F., Samu, F., Barta, Z. (2020). Consequences of rapid development owing to cohort splitting: just how costly is to hurry? *Journal of Experimental Biology*, 223(6). <https://doi.org/10.1242/jeb.219659>

Study 4 – Rádai, Z., Kiss, P., Nagy, D., & Barta, Z. (2019). Antibacterial immune functions of subadults and adults in a semelparous spider. *PeerJ*, 7, e7475. <https://doi.org/10.7717/peerj.7475>

Study 5 – Rádai, Z., Németh, Z., & Barta, Z. (2018). Sex-dependent immune response in a semelparous spider. *The Science of Nature*, 105(39), 7. <https://doi.org/10.1007/s00114-018-1568-7>

II.1.2 Additional publications

Báthori, F., **Rádai, Z.**, & Tartally, A. (2017). The effect of *Rickia wasmannii* (Ascomycota, Laboulbeniales) on the aggression and boldness of *Myrmica scabrinodis* (Hymenoptera, Formicidae). *Journal of Hymenoptera Research*, 58, 41.

Rádai, Z., Popovici, O., Vas, Z., & Fusu, L. (2018). First record of the parasitoid *Idris flavicornis* (Hymenoptera: Scelionidae) from eggs of the wolf spider *Parosa agrestis* (Araneae: Lycosidae). *Folia Entomologica Hungarica*, 79, 101-106.

Báthori, F., Pfliegler, W. P., **Rádai, Z.**, & Tartally, A. (2018). Host age determines parasite load of Laboulbeniales fungi infecting ants: Implications for host-parasite relationship and fungal life history. *Mycoscience*, 59(2), 166-171.

Haelewaters, D., Boer, P., Báthori, F., **Rádai, Z.**, Reboleira, A. S. P., Tartally, A., ... & Nedvěd, O. (2019). Studies of Laboulbeniales on *Myrmica* ants (IV): host-related diversity and thallus distribution patterns of *Rickia wasmannii*. *Parsite*, 26.

Szentiványi, T., Haelewaters, D., **Rádai, Z.**, Mizsei, E., Pfliegler, W. P., Báthori, F., ... & Glaizot, O. (2019). Climatic effects on the distribution of ant-and bat fly-associated fungal ectoparasites (Ascomycota, Laboulbeniales). *Fungal Ecology*, 39, 371-379.

Imre, A., Rácz, H. V., Antunovics, Z., **Rádai, Z.**, Kovács, R., Lopandic, K., ... & Pfliegler, W. P. (2019). A new, rapid multiplex PCR method identifies frequent probiotic origin among clinical *Saccharomyces* isolates. *Microbiological research*, 227, 126298.

Kiss, B., **Rádai, Z.**, Toft, S., & Samu, F. (2019). Sperm competition tactics shape paternity: adaptive role of extremely long copulations in a wolf spider. *Animal Behaviour*, 156, 121-128.

Kiss, J., **Rádai, Z.**, Rosa, M. E., Kosztolányi, A., & Barta, Z. (2020). Seasonal changes in immune response and reproductive investment in a biparental beetle. *Journal of Insect Physiology*, 121, 104000.

II.2 RESEARCH ITEMS

SHORT COMMUNICATION

Effect of weather conditions on cohort splitting in a wolf spider species

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Abstract. Cohort splitting has been described as differences in time until maturation and / or life span in the same age group. Cohort splitting generally occurs when individuals of a cohort originating from the same season experience different environmental conditions, such as in early and late progenies. However, in the wolf spider *Pardosa agrestis* (Westring, 1861) spiderlings of the same clutch may follow either slow or rapid development, leading to a second adult peak within a year comprised of the rapidly developing individuals. We hypothesized that weather conditions experienced by the spiderlings in their early ontogeny may contribute to a life history decision between slow and rapid development. To test this hypothesis, we have used long term collection data and non-parametric habitat modeling. We found that highest abundance of the rapidly developing phenotype was correlated with a narrow range of early weather conditions. This result is in accordance with our early choice hypothesis, although the possibility remains that differential survival of the developmental morphs also contributes to the observed pattern.

Keywords: Lycosidae, habitat modeling, long term sampling, development, ontogeny

Cohort splitting can be defined as a life history scenario in which individuals within a cohort exhibit variation in developmental rates, maturation time and / or life span, leading to a divergence of individual ontogenies within an age group (Danks 1992; Watts & Thompson 2012; Crowley & Hopper 2015). This phenomenon is well described in several arthropod groups (Sunderland et al. 1976; David et al. 1993; Moreira & Peckarsky 1994; Townsend & Pritchard 1998; David et al. 1999; Bonte & Maelfait 2001; Johansson et al. 2001; Gonçalves et al. 2005; Kozáčeková et al. 2009), as well as in some vertebrates (Newman 1989, 1992; Post et al. 1997; Callihan et al. 2008).

Different factors may play a role in orchestrating cohort splitting, such as genetic polymorphism, maternal effects and environmental cues (reviewed in Crowley & Hopper 2015). Cases of environmentally induced differences in developmental trajectories are well described in animals, for example in relation to population density (Post et al. 1997), prey availability (Pickup & Thompson 1990), ambient temperature (Callihan et al. 2008) or photoperiod (Kozáčeková et al. 2009). Such changes in developmental trajectories due to environmental factors are often regarded as bet-hedging strategies in unpredictable environments (Stearns & Crandall 1981; Tuljapurkar 1994; Watts & Thompson 2012), and can have considerable impact on the fitness of the individuals (Hopper 1999).

An interesting case of cohort splitting was described in populations of the wolf spider *Pardosa agrestis* (Westring, 1861), an epigeic spider species, dominant in agricultural habitats of Central Europe (Samu & Szinetár 2002). Unlike the typical univoltine *Pardosa* life cycles in the temperate regions—where generally one sexually mature cohort exists in each year (see Schaefer 1977)—this semelparous species exhibits a phenology with two adult peaks, and hence two reproductive periods in a year (Samu et al. 1998). As Kiss and Samu (2002, 2005) have shown, this phenological pattern arises from developmental asynchrony in the early summer progeny. Some of the spiderlings, hatching from the first adult generation, between May and June, are only able to reach maturity in the next spring or summer, after overwintering. Others, even from the same brood, are capable of reaching adulthood and reproduce in about three months, thus comprise a second generation within a year (Fig. 1).

The background of this case of cohort splitting is largely unknown, although previous studies suggested that photoperiod and ambient temperature might be key factors, as spiderlings experiencing long day-length and high (but not stressful) temperatures matured sooner and in higher proportions compared to spiderlings reared in short day-length and lower temperatures (Kiss & Samu 2002; Rádai, unpublished results). In artificial rearing experiments, the ratio of slowly and rapidly developing spiderlings varies among clutches (i.e., among mothers) which might indicate genetic involvement in the rapid development (Kiss 2003; Rádai et al.; unpublished data). Still, the strong influence of ambient conditions suggests that cohort splitting may be the result of a decision on the part of the offspring, in an early time window of their ontogeny.

In this study we propose an early choice hypothesis, stating that weather conditions experienced by the early summer progeny of *P. agrestis* contribute to a decision between slow and rapid developmental life histories. Based on the results presented by Kiss and Samu (2002) and Kiss (2003), in an early choice scenario we predict to see that under a narrow range of favourable early weather conditions more spiderlings will choose rapid development. Hence, in years when early weather conditions fall in this narrow range, we would expect to see higher abundances of second generation adults during late summer in natural *P. agrestis* populations. To test this hypothesis, we have used long term collection data from the natural habitats of this spider species in Hungary (Samu 1999), and long term weather data provided by the CarpatClim project (Szalai et al. 2012).

Our working data table consisted of 535 samples (with a total of 5284 collected adult *P. agrestis* specimens), recorded between 1993 and 2002 in the months from August to November, from the vicinity of 13 settlements across Hungary. For each sample we defined sampling success as the number of adult spiders collected with a given sampling method, at the sampling site and date. Sampling method was either suction sampling or using pitfall traps. Because suction sampling and pitfall traps might differ in how they relate sampling success to density and activity of the animals, our records of sampling successes reflected a combination of both abundance and activity during sampling. In all cases, sampling effort was specified as the number of pitfall traps used, or number of sampling transects of identical lengths (10 meters) for suction sampling. For early weather

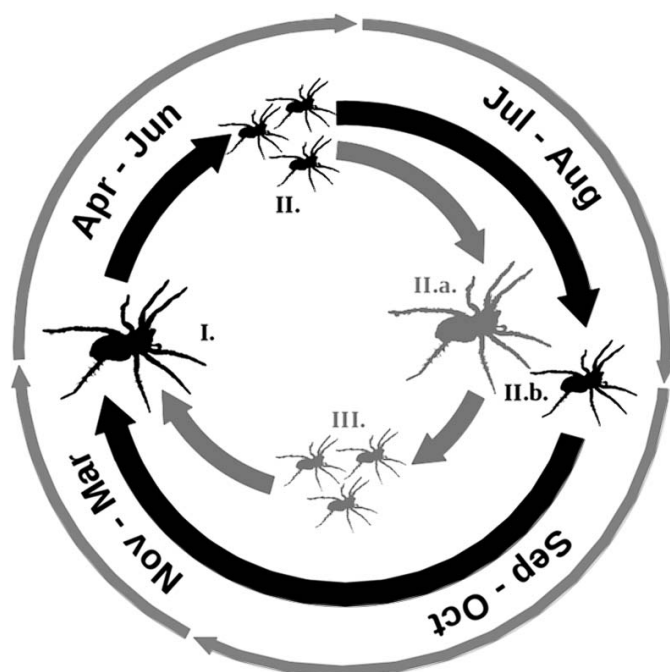


Figure 1.—Phenology of *P. agrestis*; the first adult generation matures between the end of March and April (I.), after which the first wave of progeny hatches in May and June (II.). Some of the spiderlings follow slow development (II.b.) reaching maturity only in the next year, while others develop rapidly, mature and reproduce between August and September (II.a.), giving rise to a new cohort of progeny (III.).

conditions, principal components (PCs) were calculated for May. We also calculated PCs for the given month of sampling. In the PC analyses, we used six weather parameters for each month, measured at the settlement of the sampling location. The spatial detail of available weather data was 10×10 km, therefore we had unique weather data for settlements being at least 10 km apart from one another. The weather parameters were minimum, maximum and mean monthly temperature (Celsius), amount of precipitation (mm), monthly mean of relative humidity and total of sunshine hours in the given month. From the calculated PCA axes, we have used only those with eigenvalues of 1 or higher (Kaiser criterion: Kaiser 1960), which yielded two PCs for May (hereafter, PC1 and PC2 of May) and two for the sampling dates (hereafter PC1 and PC2 of sampling date). The proportions of variance represented by PC1 and PC2 of May were ca. 74 and 17%, respectively. Also, the proportions of variance represented by PC1 and PC2 of sampling-date were ca. 60 and 20%, respectively. PC1 of both May and the sampling date was strongly correlated with all six weather parameters, and PC2 of both May and the sampling date were at least moderately correlated with all weather parameters (see supplementary material for details, online <http://dx.doi.org/10.1636/JoA-S-17-008R2.s1>).

To test, how the calculated PCs for May and for the sampling dates are related to the sampling success of second generation adults in the months between August and November, we have applied non-parametric habitat modeling using HyperNiche v2.30 (McCune & Mefford 2009). In the models, we defined sampling success as response variable, while predictor variables were the calculated principal components for the weather of May and sampling date weather. As additional predictors we also used habitat type (categorical, with levels: arable field, wet meadow, alkaline grassland, rock grassland, disturbed grassy habitat), sampling method (categorical, with levels: pitfall traps or suction sampling) and sampling effort.

Models were acquired by running three model-searching sessions of Local Linear Estimator, using conservative, medium and aggressive over-fitting settings, respectively (3% allowable missing estimates in each). Each session yielded a high number of models, from which the one with the highest cross-validated R^2 value was selected as the best fit, so finally we had one model from each session (i.e., three models in total). To test whether the fitted models are better than could be obtained by chance alone, we have used randomization tests (integrated in HyperNiche) with 100 runs, comparing each model to 100 models with randomly generated predictor values.

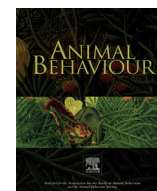
In all three best fit models, sampling method and PC1 of May were retained as predictor variables, indicating that out of the tested variables, these consistently explained the variance in sampling success best. Additionally, in the models using medium and aggressive over-fitting settings, PC1 of the sampling date was also retained. All three models explained sampling success significantly better than did randomized predictor values ($P=0.01$ in case of all models). Based on the models, highest second generation adult abundances were related to a narrow range of early weather conditions in May (see Fig. 2). Similarly, in the models using medium and aggressive over-fitting, a narrow range of sampling date weather conditions was also apparent, at which sampling success peaked (Fig. 2). Regarding the sampling methods, on average pitfall traps collected more spiders per sample compared to suction sampling; estimated sampling success of pitfall traps and suction sampling were 12.41 ± 22.97 and 1.98 ± 1.58 (mean $\pm$ standard deviation), respectively.

The selected models' consistent retainment of the PC representing weather conditions in May indicates that early conditions strongly influence the abundance of the second adult generation. The relatively narrow range in the values of May PC1 corresponding to high second generation abundance can be regarded as an optimal set of weather conditions, which enable or contribute to the appearance and high abundance of rapidly developing individuals in the populations.

Although our results support that weather conditions during early larval stages contribute significantly to the abundance of rapidly developing spiders, the mechanism in the background remains unclear. Environmental factors may affect postembryonic development directly, for example through providing cues based on which life history decisions can be made (reviewed in Danks 1994). However, they can act indirectly as well, for example due to their effects on prey availability (Hayashi 1988) or metabolism (Gillooly et al. 2001). In years of drought, low prey abundance might limit the opportunities of the spiders because limited prey availability possibly cannot provide sufficient quantity and / or quality of resources for rapid growth. On the other hand, low temperatures during the first larval stages could lengthen developmental time either by reducing the efficiency of different metabolic processes (Brown et al. 2004; Irlich et al. 2009), or simply by decreasing locomotory activity of the animals (Ford 1978), leading to reduced hunting virtue.

Furthermore, differential survival of spiderlings of alternate developmental trajectories could also contribute to the correlation between late summer adult abundance and weather conditions in May. More precisely, it might be possible that the ratio of slowly and rapidly developing spiders (not assessed in this study) is not as strongly related to environmental cues, e.g., due to genetic polymorphism, and the abundance of late summer adults is affected also by their survival throughout the first larval stages. For instance, spiderlings engaging in rapid development have probably higher demand for energy and nutrients, which, if not met, could lead to increased mortality among rapidly developing spiderlings when food is scarce. Therefore, we cannot rule out that late summer abundance of adults, at least in part, is shaped by the differential survival of the two developmental phenotypes during their early ontogeny. However, considering the results of past rearing studies, in which ambient conditions were shown to increase moulting frequency and probability of moulting to adulthood (Kiss & Samu 2002; Kiss 2003), it seems

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Pace of life and behaviour: rapid development is linked with increased activity and voracity in the wolf spider *Pardosa agrestis*



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Modern life history theory hypothesizes that pace of life is a strong predictor of life history traits. Recently, the notion that life history studies should integrate animal behaviour has emerged, because between-individual differences in behaviour are often coupled with fitness differences. So far, studies have mainly focused on interspecies or interpopulation perspectives, and research on the effects of life history differences on individual behaviour remain scarce. In the present study we aimed to contribute to the understanding of how pace of life is related to consistent individual behaviour. We investigated the relationship between developmental speed and consistent behaviour of the field wolf spider, *Pardosa agrestis*. In this species, individuals originating from the same clutch can typically follow either a slow or a rapid developmental pathway, characterized by a developmental time of about 10 or 3 months, respectively. We found that spiders, regardless of their developmental speed, behaved consistently in most of the tests. Our results also show that individuals developing rapidly were significantly more active during exploration and more successful in prey-catching tests than slowly developing spiders. Although rapidly developing spiders were bolder in one of the tests, this difference did not persist over the repeated measurements. Our work seems to support the notion that pace of life and animal personality are correlated, and pace of life might predict the behavioural types of individuals.

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In recent decades, studies of animal personality have shown that individuals are consistent in their behaviour across time, and in many cases behaviour in one context or situation may predict the behaviour in another (Bell & Stamps, 2004; Brodie III & Russell, 1999; Dingemanse & de Goede, 2004; Gyuris, Feró, & Barta, 2012; Gyuris, Feró, Tartally, & Barta, 2011; Johnson & Sih, 2007; Stamps & Groothuis, 2010; Verbeek, Drent, & Wiepkema, 1994). Interindividual differences in behaviour can result in a wide range of behavioural types in a population. Wolf, Doorn, Leimar, and Weissing (2007) were among the first to propose that this variation in behaviour can be the result of trade-offs between different life history traits (e.g. longevity versus fecundity), or the timing of key life history events (such as current versus future reproduction). Although the importance of the connection between life history and consistent behaviour is becoming more recognized (Biro, Post, & Abrahams, 2005; Biro & Stamps, 2008; Nakayama, Rapp, &

Arlinghaus, 2016; Réale, Martin, Coltman, Poissant, & Festa-Bianchet, 2009; Wang, Kruger, & Wilke, 2009), it is still not entirely clear how these are related. For example, Clark (1994) and Wolf et al. (2007) suggested a negative feedback between future fitness expectations and risk-taking propensity, which might maintain behavioural differences in the short term, but leads to convergence of behavioural types in the long term (McElreath, Luttbegg, Fogarty, Brodin, & Sih, 2007). An alternative scenario has also been put forward, that the long-term persistence of different personalities and behavioural types could rather be maintained by a positive feedback between boldness/aggression and gaining more assets, causing 'good-quality' individuals to be more willing to take risks, making them more capable of coping with hazardous situations (Luttbegg & Sih, 2010; McElreath et al., 2007). The intrinsic potential of individuals to cope with environmental challenges such as parasite attacks has also been proposed to be a major factor in the occurrence and persistence of personalities that are stable within, while diverse between, individuals (Kortet, Hedrick, & Vainikka, 2010). Recent theories with special emphasis on the relevance of feedback loops between behaviour and state variables suggest that interindividual variation in consistent behaviour might result from state and condition dependence, leading to the

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emergence and persistence of behavioural syndromes, although a unifying theoretical framework still seems elusive (Dingemanse & Wolf, 2010; Sih, 2011; Wolf & Weissing, 2010). However, little is known about how interindividual variation in behaviour covaries with different life history traits.

A general theoretical framework for understanding the high degree of variation in life history traits is the pace-of-life syndrome (POLS) hypothesis (Ricklefs & Wikelski, 2002). This hypothesis defines a slow–fast life history continuum, and suggests that closely related species (or populations of a species) along this continuum should differ in a specific manner regarding a suite of physiological and life history characteristics, in accordance with their environment and evolutionary history. Tropical versus temperate birds are a well-known example of this phenomenon (McNamara, Barta, Wikelski, & Houston, 2008; Wiersma, Muñoz-García, Walker, & Williams, 2007). Tropical birds live at a relatively slower pace (e.g. reach maturity later, live longer and have lower resting metabolic rate) than their close relatives in the temperate regions. While many studies have looked at the variation in life histories between species and populations (e.g. Adelman, Bentley, Wingfield, Martin, & Hau, 2010; Cardillo, 2002; Martin, Hasselquist, & Wikelski, 2006; Sandercock, Martin, & Hannon, 2005; Von Merten & Siemers, 2012; Wikelski, Spinney, Schelsky, Scheuerlein, & Gwinner, 2003), our knowledge of the consequences of within-population variation in life history traits regarding the predictions of the POLS hypothesis is still limited (Koolhaas, 2008; Koolhaas et al., 1999; Sgoifo, Coe, Parmigiani, & Koolhaas, 2005; Sih & Bell, 2008). Nevertheless, some studies suggest that life history characteristics can correlate with animal behaviour and personality. For example, individuals with a relatively fast pace of life should show increased activity (as argued in Réale et al., 2010) and propensity to take risks, compared to individuals with slow life histories (Ackerman, Eadie, & Moore, 2006; Réale et al., 2009; but see Niemelä, Dingemanse, Alioravainen, Vainikka, & Kortet, 2013).

To investigate whether individual differences in pace of life are coupled with behavioural differences, we studied the field wolf spider, *Pardosa agrestis*. This semelparous spider species exhibits a unique phenology: while its close relatives (e.g. *Pardosa saxatilis* and *Pardosa minutus*, see Dondale, 1976; *Pardosa hortensis*, discussed in Kiss & Samu, 2002) have only one sexually mature cohort in each reproductive season, in *P. agrestis* populations, a facultative second generation might arise because of differences in the developmental speed of spiderlings hatching in early summer (Kiss & Samu, 2002, 2005; Samu et al., 1998). Some spiderlings become mature in the next spring or summer (i.e. after overwintering), while others (often from the same brood) reach adulthood and reproduce in about 3 months, in the same season they hatched. Hence, these latter, rapidly developing individuals represent a cohort with a considerably faster pace of life in the population. This case of cohort splitting is unique, because generally cohort splitting occurs when the offspring from early and late clutches follow different developmental pathways, which are strongly determined by environmental factors (e.g. seasonal or maternal effects). In *P. agrestis*, however, cohort splitting also occurs within and not only between clutches (Kiss & Samu, 2005).

Because of within-clutch cohort splitting, *P. agrestis* seems to be an ideal model organism to study the behavioural correlates of alternative life histories along the pace-of-life continuum within the same population. In our study, we assessed the consistency of individual behaviour of *P. agrestis* individuals and, following Réale et al. (2010), we tested the predictions of the POLS hypothesis regarding behaviour, namely that rapidly developing individuals should (1) be more active in a novel environment, (2) catch a potential prey more readily and (3) be bolder than those developing more slowly.

METHODS

Test Specimens

Two groups of specimens were reared under controlled laboratory conditions and tested in two separate test sessions. Specimens of both groups originated from cocoon-carrying female spiders collected in early summer of 2014 (first group) and 2015 (second group), at an uncultivated plot next to a maize field between Hajdúszoboszló and Nádudvar, Hungary (47°26'57.49"N; 21°18'01.96"E). After hatching from the cocoons, each spiderling was kept separately in a plastic cup with a floor area of 25.5 cm². These cups contained ad libitum water (wet cotton wool) and food (tropical Collembolae specimens for young spiderlings from commercially available cultures, and *Drosophila* sp. specimens reared in our own laboratory, for aged spiders after several moults). With artificial lighting, we applied increasing day length until mid-July, followed by decreasing day length until the end of the tests, mimicking natural day:night lengths at the collection site. Spiders reaching maturity (i.e. possessing fully developed genitals) by 1 September were considered as rapidly developing.

The first group of spiders (*N* = 24, of which 12 showed rapid development) hatched in June 2014. They originated from two females: 11 from one female, of which five (46%) developed rapidly, and 13 from the other, of which seven (54%) developed rapidly. The second group of spiders (*N* = 74, of which 59 developed rapidly) originated from eight females and hatched in May 2015. In this latter group, the number of spiders from the different females varied from two to 20 (9.25 ± 6.5, mean ± SD), with 50–100% (75.3 ± 22.5, mean ± SD) of spiders within clutches developing rapidly.

Identification of sex was only possible for adult individuals, and during (and following) the tests not all the spiders matured; therefore, we could record sex only for some specimens. Because of this and of the main aim of our study (comparing individuals with slow and rapid development), we decided not to include sex in the later analyses.

Behavioural Tests

We performed two behavioural test sessions (Table 1). Within each session, multiple repetitions of several behavioural tests were carried out, at random times of day, and all spiders were tested in a random order within test rounds.

The first test session was carried out on the first group of spiders, in July and August 2014. In this session, we used two types of tests in four rounds for each individual to assess willingness to catch a potential prey and activity in a novel area. On average, there was 1 week between consecutive test rounds and about 1 month between the first and last test rounds. To control for the hunger level of the spiders (which might affect their behaviour), each spider received three fruit flies, 2 days before the tests, and then

Table 1
Set-up of the two test sessions

Session	Test
First (<i>N</i> = 24)	Willingness to attack artificial prey (small)
	Willingness to attack artificial prey (large)
Second (<i>N</i> = 74)	Activity in a novel area
	Emergence from shelter
	Activity in a novel area
	Willingness to catch living prey

The order of test methods represents their order of application in the tests. The first test session comprised four test rounds and the second two test rounds.

they did not get additional food until the tests. In most cases, all three flies were consumed by the beginning of the tests, although we did not record whether all the flies were eaten.

When testing the spiders' willingness to attack a potential prey (i.e. the hunting motivation of the spiders), we placed an individual in a plastic box with a floor area of 200 cm². After 1 min acclimatization time, an artificial prey consisting of a compressed and black-painted cotton wool ball glued to a fishing line was presented to the spider. We used two sizes of balls (ca. 1 mm and 3 mm in diameter, respectively), the larger being about the same size as the spiders. Balls were presented to the spiders visually by hanging them about 1 cm in front of the spiders, for a maximum of 10 s. Following the visual presentation with the small cotton wool ball, the test was repeated with the larger ball. In each case, the reaction of the spiders was recorded as a binomial variable (1 = spider attacked the object, 0 = no attack). During this test session, the latencies before attacking were not recorded. After each test, the plastic box was thoroughly cleaned.

When testing activity, we placed the spiders individually in a glass petri dish 20 cm in diameter. The inner side of the petri dish was treated with Fluon (AGC Chemicals Europe, Ltd., Thornton-Cleveleys, U.K.), preventing the spiders from escaping. The movement in the dish of each specimen was recorded on video for 5 min. Following each recording, the petri dish was cleaned, eliminating contamination that might have affected the behaviour of the next spider. The videos were later analysed by converting them into a series of images, 1/s, then using ImageJ (version 1.46a, [Schneider, Rasband, & Eliceiri, 2012](#)) to assess the coordinates along the spider's path. In the first test session, we did not record the time of the tests (i.e. the hour of the day) or the number of consecutive moults, and therefore could not control for these possible confounding effects in later analyses.

The second test session was carried out on the second group of spiders, in June and July 2015. We used three types of tests in two repeats for each individual to measure boldness, willingness to catch living prey and activity. We performed the second repeat of tests 4 days later than the first, and the whole test session took 7 days. To control for hunger level, the spiders were fed with three fruit flies 2 days before each test round. To test boldness, spiders were put in a 1.5 ml microtube (its sides covered with black duct tape, one end left open), and this tube was placed in the middle of a glass petri dish (20 cm in diameter). Spiders were given a maximum of 300 s to emerge from the tube. They were considered as 'emerged' if their front two pairs of legs were outside the tube. If they emerged, we recorded the latency before emergence, as the time elapsed from the placement of the tube into the petri dish. When spiders did not emerge, we assigned 310 s to the latency variable. By doing so we were able to define a binary variable of emergence with levels of 'emerged' (latency $\leq$ 300 s) and 'not emerged' (latency > 300 s) in later analyses. When an individual did not emerge from the darkened tube, we moved on to the next test, namely the activity test. If a spider left the microtube, we put it back in the tube prior to the activity test.

In the activity test, the individual to be tested was released from the tube in the middle of the same petri dish, and its movement was video recorded for 5 min. The spider was then put in a plastic cup with a floor area of about 4 cm² and the inner sides lubricated with Fluon. After a 1 min acclimatization period, one living, flightless fruit fly was introduced. All spiders had a maximum of 120 s to catch their prey. Captured flies were taken from the spiders (to control for feeding frequency) and the latency of the catch was recorded. If a spider did not catch the fruit fly, we recorded latency as 130 s. From these latencies we were able to construct a binary prey catch variable with levels of 'caught' and 'not caught'. Following each test, the plastic cup was thoroughly cleaned to

prevent contamination which might have altered the next spider's behaviour. Footages from the activity tests were then analysed with the software Tracker (version 4.87, [Brown, 2009](#)), because it provided a quicker way to deal with the recordings.

In both test sessions, the measure of activity was a score calculated from three, highly correlated movement parameters, by averaging the rescaled variables of (1) total time spent moving, (2) total walking distance and (3) mean movement speed within individuals. For rescaling, the variables (i.e. all values across individuals) were divided by twice the standard deviation of the given variable ([Gelman, 2008](#)).

Statistical Analyses

Statistical analyses were performed using the R statistical software (version 3.0.2, [R Core Team, 2013](#)). Data was mainly analysed using linear mixed-effects (LMM) and generalized linear mixed-effects (GLMM) models of the R package 'lme4' ([Bates, Maechler, Bolker, & Walker, 2014](#)), because these methods enable to control for both pseudoreplication (i.e. repeated measures of individuals) and confounding effects. Also, GLMMs offer flexible ways to handle non-Gaussian error distributions. In addition, mixed-effects Cox models of the R package 'coxme' ([Therneau, 2012](#)) were also used. In all the fitted models (LMMs, GLMMs and mixed-effects Cox models), individual ID nested within mother ID were set as random factors. To assess conditional R^2 values of the fitted linear models (only for LMMs and GLMMs) we used pseudo-r-squared for generalized mixed-effects models from the R package 'MuMIn' ([Bartoń, 2013](#)).

The repeatability of activity was assessed using the R package 'rptR' ([Schielzeth & Nakagawa, 2013](#)), applying LMM-based repeatability estimation with Gaussian error structure for activity and the log-transformed variables of latency before prey-catch and emergence. GLMM-based repeatability estimation was used for binary variables of artificial and living prey catching behaviour and emergence from shelter. In both LMM- and GLMM-based estimations, models were fitted by restricted maximum likelihood. In these fitted models, we used 100 permutations to get more reliable asymptotic P values ([Schielzeth & Nakagawa, 2013](#)). In case of repeatability fitted with Gaussian error-structure, we controlled for confounding effects in the specified models, namely to developmental strategy (as a factor) and number of test rounds (also as factor). Because of convergence errors, we could not specify confounding effects for the repeatability estimation of binary variables (i.e. catch and emergence). In order to test whether activity is rank order repeatable, we used Kendall's coefficient of concordance (also known as Kendall's W) as well. The reason of this was that by doing so one might see if individuals are consistent in their activity even if there is shift in their mean behaviour over ontogeny ([Gyuris et al., 2012](#)).

When testing the differences in activity between slowly and rapidly developing individuals, LMMs were fitted with activity as response variable. Developmental speed was specified as a predictor factor variable (with levels of fast and slow), with control for the number of actual test round (also as factor, comparing all levels to the first round), and in the case of the second test session for the ontogenetic stage (factor with levels of adult and not-adult), time of day at the test (a continuous variable) and number of successive moults (as factor, comparing other moult stages to the lowest moult-number stage, which was 3; the highest value was 6) as well. With controlling for the number of successive moults, we controlled indirectly for size. We note that measurements of the prosome would have been more accurate for this purpose, however it also would have posed the risk of injuring the spiders.

To test whether slowly and rapidly developing spiders differ in their probability to catch an artificial or living prey, or emerging

from shelter, we applied binomial GLMMs, in which developmental speed was entered as predictor, while number of test rounds, and in the case of second test session, ontogenetic stage, time of day and number of moults were also controlled for.

When testing for the difference between slowly and rapidly developing individuals in their latency before prey-catch, or before emergence from shelter, we fitted mixed-effects Cox models. In these models we defined the latency before catch, and (in a different model) latency before emergence as the 'follow up' time, while the status indicator event was successful prey-catch in the former, and emergence for the latter model. In the mixed-effects Cox models, we also controlled for the ontogenetic stage, number of test round, time of day and number of moults as confounding effects.

For predictors, the significance level for each variable was assessed by comparing the full model (containing all predictors) to a model without the given variable, using log-likelihood ratio tests (LRT). In the 'Results', the P values and χ^2 values from the LRTs are shown.

Ethical Note

Because *P. agrestis* is a spider species not protected by natural conservation laws in Hungary, no ethics approval was required either for collecting, rearing and handling them. During rearing and behavioural tests, we tried to minimize unnecessary disturbance to the spiders.

RESULTS

Repeatability of Measured Behaviours

Activity in the first test session was not significantly repeatable based on the LMM estimation (Table 2); however, it was rank order repeatable (Table 2). In the second test session, activity proved to be significantly repeatable by LMM and Kendall's W (Table 2). In the first test session, attacking artificial prey was not significantly repeatable in the case of small objects, but was significant for the larger ones (Table 2). In the second test session, both probability of catching prey and probability of emergence from shelter were significantly repeatable (Table 2). The repeatability of latency before catching a prey was not significantly repeatable nor was the latency before emerging from shelter (Table 2).

Behavioural Differences of Slowly and Rapidly Developing Spiders

Spiders of slow development were significantly less active in the novel environment than those of rapid development in both the

first ($R^2 = 0.35$, $\chi^2_1 = 9.8$, $P = 0.002$) and second ($R^2 = 0.48$, $\chi^2_1 = 6.6$, $P = 0.011$) test sessions (Fig. 1). In the first test session, we found that the activity significantly increased with the number of test rounds ($\chi^2_3 = 20.1$, $P < 0.001$). In the second test session, the time of day had a significant effect on the spiders' activity, as in later hours of the day the individuals showed increased activity in the novel area ($\chi^2_1 = 5.5$, $P = 0.019$). Ontogenetic stage ($\chi^2_1 = 0.2$, $P = 0.677$), the number of moults ($\chi^2_3 = 6.3$, $P = 0.101$) and the number of test rounds ($\chi^2_1 = 0.2$, $P = 0.678$) did not have a significant effect on activity in the second test session.

In the first test session, the probability of attacking both the small and large cotton wool balls was significantly lower for slowly developing individuals than for rapidly developing ones (small: $R^2 = 0.51$, $\chi^2_1 = 11.8$, $P < 0.001$; large: $R^2 = 0.38$, $\chi^2_1 = 10.9$, $P = 0.001$). The number of test rounds did not have a significant effect on the probability of attacking the small ($\chi^2_3 = 6.2$, $P = 0.102$) or the large ($\chi^2_3 = 2.2$, $P = 0.527$) artificial prey.

In the second test session, slowly developing spiders had a lower probability of catching prey than rapidly developing ones ($R^2 = 0.47$, $\chi^2_1 = 8.7$, $P = 0.003$). Spiders exhibited a significant decrease in their probability of catching prey in the second test round compared to the first ($\chi^2_1 = 10.5$, $P = 0.001$). Ontogenetic stage ($\chi^2_1 = 0.5$, $P = 0.475$) and the time of day ($\chi^2_1 = 0.6$, $P = 0.430$) did not affect prey-catching probability significantly, but individuals that moulted six times before their test showed a marginally significant increase in the probability of catching the living prey ($\chi^2_3 = 6.5$, $P = 0.089$). Based on the mixed-effects Cox model, slowly developing individuals showed higher latencies until catching the prey than rapidly developing ones ($\chi^2_1 = 14.1$, $P < 0.001$; Fig. 2). Compared to the first test round, spiders in the second test round exhibited lower latencies before catching the living prey ($\chi^2_1 = 16.5$, $P < 0.001$). The ontogenetic stage ($\chi^2_1 = 0.09$, $P = 0.760$), time of day ($\chi^2_1 = 2.5$, $P = 0.114$) and the moult number ($\chi^2_3 = 3.7$, $P = 0.295$) did not influence the latency before catching the prey.

We found no significant difference between slowly and rapidly developing spiders in their probability of emerging from shelter ($R^2 = 0.31$, $\chi^2_1 = 0.9$, $P = 0.328$), and there was no influence of ontogenetic stage ($\chi^2_1 = 0.04$, $P = 0.836$), number of test rounds ($\chi^2_1 = 0.01$, $P = 0.937$), time of day ($\chi^2_1 = 0.001$, $P = 0.979$) or number of moults prior to the tests ($\chi^2_3 = 2.2$, $P = 0.534$). In the mixed-effects Cox model, slowly and rapidly developing individuals also did not differ in their latency before emergence ($\chi^2_1 = 0.8$, $P = 0.363$; Fig. 3). Furthermore, the ontogenetic stage ($\chi^2_1 = 0.3$, $P = 0.560$), number of test rounds ($\chi^2_1 = 0.02$, $P = 0.895$), time of day ($\chi^2_1 = 0.03$, $P = 0.856$) and number of moults ($\chi^2_3 = 0.6$, $P = 0.899$) did not have a significant effect on the latency before emergence. Based on the cumulative incidence plot (Fig. 3), in the first test round there seemed to be some difference between slowly and rapidly developing spiders regarding the emergence latency. To see whether this difference was significant, we refitted the Cox model with an additional interaction term between developmental strategy and number of test rounds, enabling the calculation of different responses for the two test rounds, for both slowly and rapidly developing spiders. In this Cox model, we found that slowly developing individuals showed higher latencies before emergence, which was marginally significant ($\chi^2_1 = 3.6$, $P = 0.057$), and the interaction between developmental speed and test rounds was also marginally significant ($\chi^2_1 = 3.6$, $P = 0.057$). The effects of ontogenetic stage ($\chi^2_1 = 0.6$, $P = 0.448$), number of test rounds ($\chi^2_3 = 3.6$, $P = 0.163$), time of day ($\chi^2_1 = 0.23$, $P = 0.632$) and number of moults ($\chi^2_3 = 0.4$, $P = 0.941$) on latency, similarly to the model fitted on the full data, were not significant. When we fitted a third emergence latency Cox model, in which observations from the second test round were excluded, the difference between the latencies of

Table 2
Repeatabilities of tested behavioural variables

Test session	Variable	Repeatability	
First	Probability of attacking small artificial prey	$R = 0.03$, $P = 0.930$	NR
	Probability of attacking large artificial prey	$R = 0.3$, $P = 0.010$	SR
	Activity	$R = 0.1$, $P = 0.130$	NR, RR
		$W = 0.43$, $P = 0.006$	
Second	Probability of catching prey	$R = 0.2$, $P = 0.010$	SR
	Probability of emergence	$R = 0.25$, $P = 0.020$	SR
	Latency to catch prey	$R = 0.27$, $P = 1$	NR
	Latency to emerge	$R = 0.23$, $P = 1$	NR
	Activity	$R = 0.4$, $P = 0.010$	SR, RR
		$W = 0.69$, $P = 0.003$	

R and W denote repeatability (estimated with rptR) and Kendall's coefficient of concordance, respectively. NR, SR and RR stand for 'not significantly repeatable with rptR', 'significantly repeatable with rptR' and 'rank order repeatable', respectively.

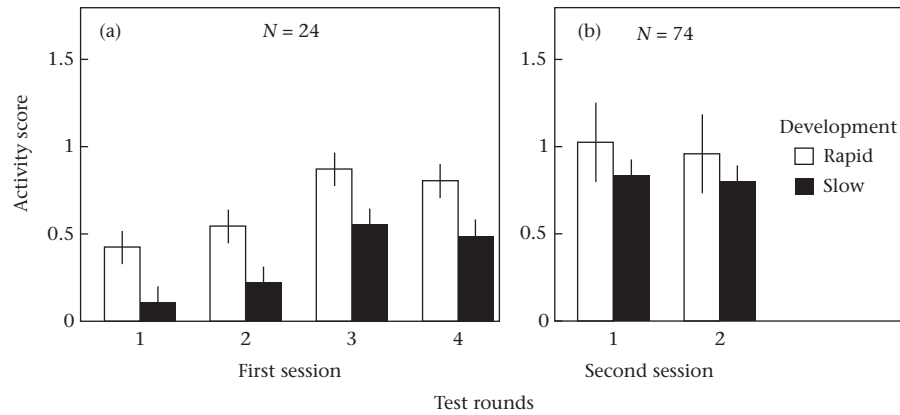


Figure 1. Activity during exploration from the fitted models in (a) the first test session, over four test rounds, and in (b) the second test session, over two test rounds (mean ± SE).

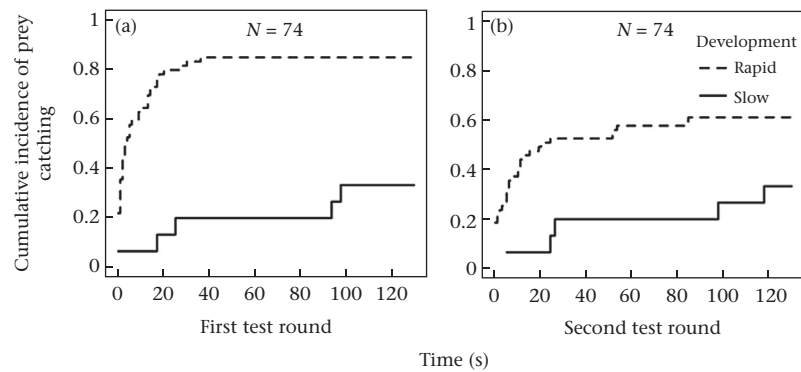


Figure 2. Cumulative incidence of prey catching in the second test session, in (a) the first and (b) the second test rounds.

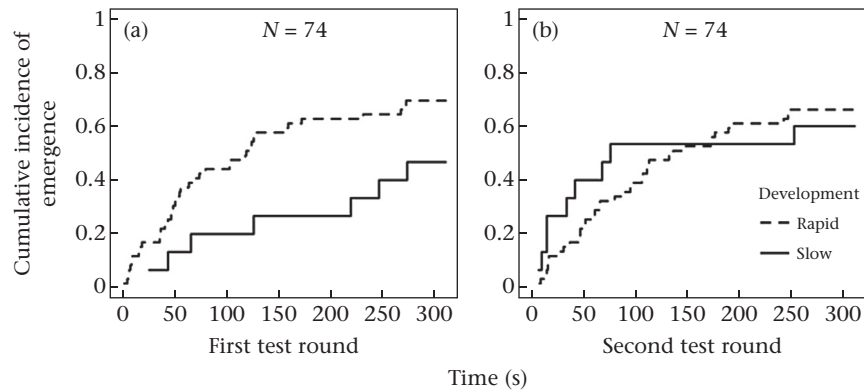


Figure 3. Cumulative incidence of emergence from shelter during the boldness test of the second test session, in (a) the first and (b) the second test rounds.

slowly and rapidly developing spiders was significant ($\chi^2_1 = 5.8$, $P = 0.016$), as slowly developing specimens emerged later than rapidly developing ones. The effects of ontogenetic stage ($\chi^2_1 = 0.3$, $P = 0.611$), time of day ($\chi^2_1 = 1.6$, $P = 0.212$) and number of moults ($\chi^2_3 = 3.1$, $P = 0.384$) on latency, similarly to the model fitted on the full data, were not significant.

DISCUSSION

Our results are in line with our predictions, that is spiders following a fast pace of life showed increased activity and prey-catching motivation, but the results for boldness were mixed. Also, spiders were consistent in their behaviour across time in

many of the behaviours. The exceptions were activity in the first test session, willingness to attack small artificial prey (also in the first test session), and in the second test session the latencies before catching prey and emergence from shelter. For activity in the first test session we got contradictory results between rank- and LMM-based analyses. Activity was repeatable according to the Kendall's coefficient of concordance, but not the LMM, probably because of the small sample size. As for the latencies before catching prey and emerging from shelter, it seems that those individuals that caught prey or emerged made their decisions in the first few seconds of the tests, which resulted in relatively large within-individual variances in the latency values, and thus statistically nonrepeatable measures of behaviour. Although individuals were consistent in their

willingness to attack large artificial prey (in the first test session) and living prey (in the second test session), the probability of attacking small artificial prey (first test session) did not show significant repeatability. However, in this latter case attacks occurred in only 13 of 96 observations. It is possible that spiders generally did not consider the small object appealing enough to prey on it.

Spiders of rapid development showed significantly higher activity, higher propensity to catch (artificial or living) prey and shorter latencies before catching a potential prey. Increased prey-catching propensity of rapidly developing spiders might be the result of increased growth and metabolic rate, since the more rapid construction of the soma demands higher resource intake (Biro & Stamps, 2008; Stamps, 2007). The higher level of activity is also expected, as higher resource demand should result in increased locomotion (e.g. as prey-searching behaviour) in a species that hunts actively.

In the activity tests, rapidly developing spiders might have been more active because they were larger (e.g. in the same time interval spent moving larger spiders probably cover greater distances on average). However, we controlled for the number of successive moults for each spider as a proxy for size (although only in the second test session), which did not have a significant effect on activity. Therefore, we argue that size-based differences in activity are unlikely to contribute considerably to the difference between slowly and rapidly developing spiders. Increased hunting motivation might also be correlated with larger body size, as the size of prey was more or less constant, so their relative size decreased with the growth of the spiders. Indeed, spiders with six moults were more willing to catch a potential prey.

The results that individuals characterized by higher developmental rate and shorter life span exhibited higher activity and willingness to catch a potential prey appear to be in concordance with the predictions of the pace-of-life hypothesis. Also, the significant difference in the boldness of spiders in the first round of the second test session (where rapidly developing specimens emerged from the shelter sooner than did slowly developing individuals) is in accordance with the predictions, as individuals with higher developmental costs in a given time window should be more risk prone during foraging in order to be able to maintain faster growth. These results could be explained by the positive feedback between voracity (or boldness during foraging) and gaining resources, suggested by Luttbegg and Sih (2010), as inherently bolder individuals would be expected to gather more resources, i.e. catch more prey and increase their rate of development, which in turn requires more resources.

While the results discussed above indicate a clear connection between activity and developmental speed, and between hunting motivation and developmental speed, the association between boldness and life history is not as clear. In our results, slowly developing spiders emerged from the darkened microtube later than their rapidly developing conspecifics, but only in the first test round. Also, slowly and rapidly developing spiders did not differ in the probability of emergence per se in either test round. Stamps (2007) and Biro and Stamps (2008) also proposed that increased growth rate should be coupled with higher boldness levels. However, other studies suggested that growth rate and boldness might not as firmly correlated as previously thought (Laakkonen & Hirvonen, 2007; Niemelä, Vainikka, Hedrick, & Kortet, 2012). The background of these mixed results, at least for now, remains largely obscure. Presumably, however, the artificial rearing conditions (especially ad libitum food) could have dampened the differences in boldness between slowly and rapidly developing individuals. It is also possible that, owing to the highly favourable conditions (high prey abundance, low mortality risk), boldness differences were not maintained during development, as Luttbegg and Sih (2010)

suggested. Notably, the disappearance (and in other studies, the absence) of the differences in boldness might be a result of habituation to the test set-up as well, although, in our case, two repeats were probably insufficient for such habituation, as the mean time before emergence from shelter did not decrease in the second test round.

The role and possible advantages of being a rapidly developing individual in *P. agrestis* populations are unclear, although by maturing before winter, rapidly developing spiders could be spared the high mortality risk of overwintering. It is not known, however, whether the overwintering mortalities of slowly developing spiders and the offspring of rapidly developing spiders differ. On the other hand, the potential disadvantages are not clear either, although it seems that there is a marked discrepancy in fecundity of slowly and rapidly developing spiders, as rapidly developing females produce fewer offspring (B. Kiss & Z. Rádai, personal observations). Kiss and Samu (2005) found that mean prosome width was significantly smaller in rapidly than slowly developing adults collected in the same year, suggesting the presence of a negative correlation between growth rate and size at maturation, a well-known trade-off (Stearns, 1989), highly, although not exclusively, characteristic of arthropods (Davidowitz & Nijhout, 2004; Shingleton, Das, Vinicius, & Stern, 2005). Furthermore, our preliminary results suggest that rapidly developing spiders are less efficient at encapsulating plastic implants than slowly developing ones (Rádai & Barta, 2017). These findings point to intriguing questions regarding the potential physiological costs of the rapid somatic development. However, still little is known about such consequences of this life history strategy, and practically nothing is known about its proximate background.

In conclusion, our results show that slowly and rapidly developing individuals of the spider *P. agrestis* exhibit consistent differences in their behaviour, in accordance with their pace of life. We emphasize that life history theory would most certainly benefit from the integration of within-population studies regarding life history characteristics and animal behaviour, as has been noted in several other studies (Careau, Bininda-Emonds, Thomas, Réale & Humphries, 2009; Niemelä et al., 2013; Réale et al., 2010). By shedding light on correlations between life history traits in more detail, we will have a better basis for studies exploring causal connections and environmental and evolutionary constraints that drive the evolution of life histories. We think that *P. agrestis* could be an excellent model organism in such studies.

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RESEARCH ARTICLE

Consequences of rapid development owing to cohort splitting: just how costly is it to hurry?

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ABSTRACT

In cohort splitting, diverging sub-cohorts may show substantial differences in their growth and developmental rates. Although in the past, causes and adaptive value of cohort splitting were studied in detail, individual-level consequences of cohort splitting are still rather overlooked. Life history theory predicts that considerably increased growth and developmental rates should be traded off against other costly life history traits. However, it is not clear whether one should expect such associations in adaptive developmental plasticity scenarios, because natural selection might have promoted genotypes that mitigate those potential costs of rapid development. To address these contrasting propositions, we assessed life history traits in the wolf spider *Pardosa agrestis*, both collected from natural habitat and reared in laboratory. We found that some traits are negatively associated with developmental rates in spiders collected from the wild, but these associations were relaxed to a considerable extent in laboratory-reared specimens. In general, we observed no consistent trend for the presence of developmental costs, although some results might suggest higher relative fecundity costs in rapidly developing females. Our study provides a detailed approach to the understanding of individual-level consequences of cohort splitting, and to the associations between key life history traits in adaptive developmental plasticity scenarios.

KEY WORDS: Costs, Developmental plasticity, Life history evolution, Physiology, Trade-offs

INTRODUCTION

There is an immense diversity in the rate of post-embryonic development (the process during which individuals go through different stages of ontogeny from birth to maturation and reproduction) across the animal kingdom, and variation in developmental characteristics often occurs even within species (West-Eberhard, 2003). In the last two decades, it was established that developmental processes can show a high level of plasticity in response to environmental stimuli, and that this adaptive plasticity can contribute to the evolution of alternative developmental pathways (Moczek et al., 2011; Nettle and Bateson, 2015; West-Eberhard, 2003,

2005). One intriguing instance for adaptive developmental plasticity is when individuals belonging to the same age group within a population follow different trajectories of post-embryonic development, a phenomenon termed cohort splitting (Crowley and Hopper, 2015). Cohort splitting was observed in a number of species (for an extensive list, see Crowley and Hopper, 2015), and was described as a bifurcation of life histories, by which alternative developmental pathways of different durations emerge in the population. In past studies, the adaptive value of cohort splitting was mostly assessed either specifically for the species in which it occurred, or with respect to environmental characteristics more generally, such as owing to stochastic changes in environment quality (Crowley and Hopper, 2015). Although individual decision-making was proposed to be likely an important element of cohort splitting scenarios, empirical studies on the individual-level consequences of following either of the alternative developmental pathways arising from cohort splitting are still largely overlooked (Khelifa et al., 2019).

Alternative developmental pathways may entail different costs, arising from differences in how individuals balance their limited resources between life history traits in order to maximise their reproductive success. These costs manifest through the organisms' inability to simultaneously improve all their life history traits, leading to trade-offs between traits (e.g. investment to growth rate versus energy storages) that compete for the same pool of resources (Fischer et al., 2005; Stoks et al., 2006). Resource expenditure costs as such are the basis for allocation-based trade-offs central in life history theory (Agrawal et al., 2010; Stearns, 2000), and physiological mechanisms behind trade-offs in resource investment into different aspects of life history (e.g. survival versus reproduction) play crucial roles in shaping resource allocation patterns themselves (Cox et al., 2010). Notably, such costs ultimately translate into fitness costs, driving life history evolution by promoting resource allocation patterns that yield the highest lifetime fitness (English and Bonsall, 2019; Smith and French, 2017).

In cohort splitting, individuals of alternative cohorts might show different rates of development (life stage transitions, i.e. processes that entail differentiation) and/or growth (increase in size or number of somatic cells in a time unit) (David and Geoffroy, 2011; Martin et al., 1991; Watts and Thompson, 2012). Growth and development are resource-demanding processes, and their rates often show negative associations (trade-offs) with other costly traits that utilise the same resource pool, such as size at maturation, condition or efficiency of somatic maintenance (Bayne, 2000; De Block et al., 2008; Ficetola and De Bernardi, 2006; Peterson et al., 1999; Yearsley et al., 2004). An intriguing case of cohort splitting was observed in the wolf spider *Pardosa agrestis* (Westring 1861), in which the diverging sub-cohorts show considerable differences in developmental rate (Kiss and Samu, 2005). *Pardosa agrestis* is a semelparous epigeic (i.e. soil surface-residing) spider, common in Central and Eastern Europe, predominantly residing in arable

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habitats, alkaline grasslands and marshes (Samu and Szinetár, 2002). It shows a yearly phenology in which individuals (after overwintering in juvenile or subadult stage) mature and reproduce during spring and early summer (Fig. 1, I). Cohort splitting takes place among the progeny originating from the early summer adults (Fig. 1, II), via asynchronous development of spiderlings, even within the same broods. Consequently, this case of cohort splitting results in the emergence of slowly (Fig. 1, IIb) and rapidly (Fig. 1, IIa) developing phenotypes in the population. Slowly developing spiderlings develop throughout the year, overwinter in juvenile or subadult stage, and mature and reproduce during spring/early summer of the next year. Rapidly developing spiderlings, however, have a much shorter life cycle, reaching maturity, producing offspring and perishing by late summer of the same year they hatched (Kiss, 2003; Kiss and Samu, 2002; Rádai et al., 2017b). The offspring of rapidly developing spiders develop alongside the slowly developing spiders, i.e. they overwinter, mature and reproduce during early summer of next year. Although not much is known about the proximate background of this case of cohort splitting as of now, past studies both on laboratory-reared spiders and spiders collected from natural habitats throughout the yearly phenology strongly indicate that day-length dynamics and ambient conditions (e.g. temperature) play important roles in evoking cohort splitting among the newly hatched spiderlings (Kiss, 2003; Kiss and Samu, 2002), which was proposed to pre-adapt *P. agrestis* into changeable arable habitats (Kiss and Samu, 2005).

Rapidly developing spiders finish their life cycles in a much shorter span (approximately one-third) of time than slowly developing ones. Based on past studies, one might hypothesise that such an increased rate of development is traded off with other life history traits, because of their competing resource demands (Zera and Harshman, 2001). For example, in damselflies, increased

growth rates were found to negatively affect symmetric somatic development and dry mass build-up (De Block et al., 2008; Dmitriew and Rowe, 2005). Indeed, one might also argue that certain costs would be expected to be associated with rapid development, because if increased developmental and/or growth rate were to have no costs at all (but nevertheless would provide fitness advantages), they should have been fixed in the population. Furthermore, an enhanced pace of life was predicted to be associated with a specific ‘syndrome’ of life history characteristics, being associated with high metabolic and growth rate, precocious reproduction and low investment into somatic maintenance (Réale et al., 2010). Although between-species observations appear to generally support such trends, within-species interpretations of trait associations in light of organisms’ pace of life remain ambiguous (Royauté et al., 2018), presumably owing to species-specific characteristics of natural history, sexual selection and/or inter- and intra-sexual competition for resources and mating opportunities (Dammhahn et al., 2018; Mathot and Frankenhuis, 2018). Notably, however, some studies suggest that such negative phenotypic correlations might not be apparent in ‘good quality’ individuals that can meet the energetic demands of the competing traits – the so-called ‘big house, big car’ effect (McNamara and Simmons, 2017; van Noordwijk and de Jong, 1986). For example, increased resource acquisition might mitigate the resource-allocation conflicts between the traits, hence masking the negative phenotypic association between them (Reznick et al., 2000). Indeed, increased resource acquisition might buffer developmental costs to other traits (Engqvist, 2007), and in adaptive developmental scenarios one might expect that the different developmental pathways have similarly efficient ways to balance their resources between costly traits. Also, theory predicts that individuals characterised by high metabolic rate and rapid development and growth may have a competitive edge over their ‘slower’ conspecifics when resources are less limiting (Careau et al., 2008; Salzman et al., 2018). As so, when food availability is not restricted, individuals of enhanced metabolism and growth rate may gain substantial fitness advantages, whereas in times of low food availability, they would be expected to show higher mortality if they were not able to meet the resource demands of a high metabolic rate (Biro and Stamps, 2010; Burton et al., 2011; Montiglio et al., 2018). So altogether it is still rather unclear what the individual-level consequences of rapid development might be, especially in an adaptive developmental plasticity scenario. For example, do rapidly developing individuals face such costs that must be paid in trade-offs against other, costly traits? Should we expect to see the predicted trait-association syndromes in specimens of *P. agrestis* in accordance with their (slow or rapid) life history?

To test the somewhat contradicting predictions of different aspects of life history theory, we conducted a study using the wolf spider *P. agrestis* as model organism by assessing a number of physiologically costly traits associated with condition and efficiency of somatic maintenance, and investigated whether the slow and rapid developmental types differ in these respects in the ways predicted by life history theory and the pace-of-life syndrome (POLS) hypothesis. We collected spiders from a natural habitat at both the early and late summer adult peaks, i.e. from slowly and rapidly developing sub-cohorts, respectively. To be able to test how developmental characteristics and life history traits are associated with one another within controlled conditions, we also reared spiderlings in the laboratory under two light cycle regimes that strongly affected developmental time and the occurrence of the rapidly developing phenotype itself. In both the wild-caught and

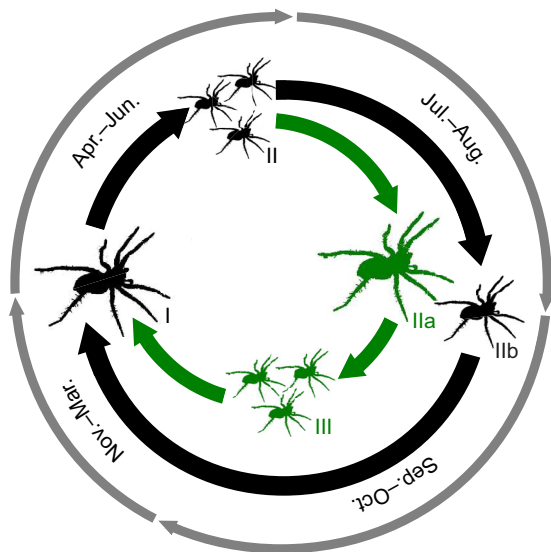


Fig. 1. Diagram depicting the yearly two-adult-peak phenology of *Pardosa agrestis*. The first adult peak (I) appears at spring/early summer, which produces the early summer (i.e. first) offspring generation (II). Some of the spiderlings from the early summer generation (II) will show rapid post-embryonic development and will mature during late summer (IIa), comprising a second peak of adults in the given year. These adults will reproduce and die before winter, but their offspring (III) will develop, overwinter and then mature in the next year. Meanwhile, the other group of spiderlings (IIb) will overwinter and mature in the next year. Figure modified from Rádai et al. (2017a,b).

laboratory-reared spiders, we assessed a number of important life history traits, namely adult size, fat reserves, cuticle melanisation, fecundity and immune parameters (see details in Materials and Methods, ‘Tested traits’). Until now, studies using a composite approach to the individual-level consequences of cohort splitting have been rather scarce, and utilising both wild-caught and laboratory-reared individuals is arguably necessary for a more detailed understanding of this topic. Here, we aim to advance our understanding of the consequences of an enhanced developmental rate owing to cohort splitting by comparing several physiological and life history traits between spiders of slowly and rapidly developing sub-cohorts. Doing so, we wanted to test the hypotheses of high physiological costs of rapid development manifested as negative phenotypic associations between traits versus a ‘big house, big car’ scenario in which physiological costs (and hence negative phenotypic correlations) might be masked by good individual condition. Based on life history theory and the POLS hypothesis, we predicted that an enhanced developmental rate would bear substantial costs, manifested in decreased investment into somatic build-up and maintenance in rapidly developing spiders in comparison with slowly developing ones.

MATERIALS AND METHODS

Collection of spiders

Spiders were collected on multiple occasions, but always from the same population, in an uncultivated plot between Hajdúszoboszló and Nádudvar, Hungary (47°26′57.49″N, 21°18′01.96″E). Firstly, we collected 128 subadult individuals (penultimate larvae; 66 females and 62 males) between 21 March and 4 April 2017, and reared them until maturation in the laboratory. On 2 May 2017, we collected 111 cocoon-carrying females (i.e. females that mated, and carried the fertilised eggs in an egg sac). Later on we refer to specimens collected in March, April and May as slowly developing (spring) spiders. From the cocoon-carrying females collected in May, we randomly selected 12, the offspring of which we used in the first session of laboratory rearing (see ‘Laboratory rearing sessions’).

On 24 and 26 July 2017, we collected 43 subadult males and 47 subadult females, and reared them until maturity in the laboratory. We also collected a total of 92 cocoon-carrying females. In subsequent sections of this paper, we refer to spiders collected in July as rapidly developing (late summer) spiders. From the cocoon-carrying females, 19 spiders were randomly selected to provide spiderlings for the second laboratory rearing session (see ‘Laboratory rearing sessions’). Over the course of our study we worked with a total of 902 spiders.

It should be noted that in the present study we termed spring adults as slowly developing spiders, although the whole spring/early summer adult generation is presumably a mixture of slowly developing spiders and the offspring of rapidly developing spiders, both from the previous year (see Introduction). In other words, it is possible that early summer spiderlings are composed of two types of spiders from the last year. To see whether this is so, we utilised model-based clustering using parametrised finite Gaussian mixture models (see Appendix), which revealed no bimodal distributions in prosoma size and melanisation (the two traits most influenced by developmental type; see Results) among the tested early summer spiders. This appears to indicate that either (i) there are no large, consistent differences in these traits between slowly developing spiders and the offspring of rapidly developing spiders, and/or (ii) owing to the early collection dates, mainly (or entirely) slowly developing spiders were collected.

Tested traits

The traits on which the effect of developmental rate was assessed in our study were adult body size, level of fat reserves, cuticular melanisation, levels of antimicrobial peptides and immunocompetence against gram-positive bacteria. Adult body size and fat content are generally considered as proxies of individual quality, especially in arthropods, where body size is less flexible owing to the rigid exoskeleton (Aisenberg and Peretti, 2011; Jakob et al., 1996; Kelly et al., 2014), and most often they are both positively correlated with survival and fecundity (Contreras-Garduño et al., 2006; Ellers and Alphen, 1997; Knapp and Uhná, 2014; Krams et al., 2011). Cuticular darkness, i.e. the extent of melanin incorporation in the cuticle, is an important characteristic in arthropods as well, found to be associated with developmental rate (Ma et al., 2008), fecundity (Ma et al., 2008; Roff and Fairbairn, 2013), and a number of immune-related traits (Bailey, 2011; Cotter et al., 2008; Rolff et al., 2005). Also, immunocompetence, as a vital part of somatic maintenance in multicellular organisms with the main role of upholding the somatic integrity of organisms, is firmly associated with fitness (Lee, 2006; Schmid-Hempel, 2003), and maintaining an efficient immune system bears considerable costs (Lochmiller and Deerenberg, 2000; Rauw, 2012; Sheldon and Verhulst, 1996). By measuring antimicrobial peptide levels (specifically cecropins and defensins, which are known to have important roles in invertebrate immunity; Hancock et al., 2006; Wu et al., 2018) and antibacterial activity against gram-positive bacteria, we wanted to assess the maintained level of immunocompetence, as we hypothesised that the costs of increased developmental rate in spiders of the rapidly developing cohort might interfere with maintenance costs of the humoral immune system (McKean and Lazzaro, 2011).

Laboratory rearing sessions

There were two laboratory rearing sessions, and in both of them we designed rearing conditions to match natural conditions as closely as possible, based on the methods described in previous publications (Kiss and Samu, 2002, 2005). In both rearing sessions, spiders were placed individually in plastic cups (ca. 25.5 cm² floor area, 4.5 cm wall height). Plastic cups (each containing only 1 specimen) were arranged in six large plastic boxes (60×40×20 cm in length, width and height). These large boxes were covered by non-transparent lids, and equipped with LED strips (Global JS-3528-30, 3000 K warm white, DC12V, 1000 mA) to provide light during the set daytime. In both rearing sessions we used two light-cycle regimes as treatments. In one of them, the length of the day cycle increased from 11 to 16 h over 11 weeks (mimicking early summer day length dynamics), while in the other group the day length decreased from 16 to 11 h (mimicking late summer day lengths). By using these treatment groups we could manipulate the rate of the spiders’ development, as it was shown previously that long day length promotes maturation among the spiders, while short day lengths appear to inhibit maturation (Kiss, 2003). Henceforward, we refer to the group receiving the increasing day length treatment as the ‘rapid treatment group’, and the spiders receiving the decreasing day length treatment as the ‘slow treatment group’.

Each rearing session was divided into two phases. In the first phase, we started with 11 and 16 h day lengths for the rapid and slow treatment groups, respectively. One week after the start of the rearing experiment, we changed day lengths of the treatment groups once every week, increasing or decreasing day length by 30 min, according to the treatment. The first phase lasted for 11 weeks, and the day lengths converged to 16 and 11 h in the rapid and slow treatment groups, respectively. The second phase started 1 week

after the last day length change of the first phase. During the second phase, we started to increase the day lengths in the slow treatment group by 30 min every week, increasing day length to 16 h, over 11 weeks. We did not alter the day length in the rapid group (i.e. day lengths were held constant at 16 h during the second phase). Applying the two rearing phases was necessary to evoke a developmental time difference (i.e. the appearance of slowly and rapidly developing spiders) in the laboratory.

All along both rearing sessions, spiders were provided water (wet cotton wool pieces) and food (springtails to first and second instar larvae, flightless fruit flies to larger spiders) *ad libitum*. Every 2 days we checked the spiderlings for exuvia (moult), during which we remoisturized the cotton wool, removed exuvia and dead prey, and delivered living prey for the spiders.

In the first session of laboratory rearing, we randomly selected 12 mated (i.e. cocoon carrying) spring females (from those collected in May 2017; see 'Collection of spiders'). Subsequently to the hatching of their offspring, we separated 24 randomly selected spiderlings from each female, i.e. we started the rearing with 288 spiderlings. Out of the 288 spiderlings, we randomly assigned 144 to the rapid treatment group, and the other 144 to the slow treatment group. From each mother, 12 offspring were assigned to each treatment group. Prior to all subsequent tests, we randomly assigned 72 spiders from each treatment group to the fat extraction treatment, and another 72 spiders from each treatment group to immune measurements. From the first day when a spider moulted to maturation, we collected all adult spiders that matured within a week, and used them for the designated tests (see 'Fat extraction' and 'Homogenate preparation' sections). The first rearing session lasted from 10 May to 25 November 2017. Henceforward we refer to this laboratory rearing session as the 'physiological test session', because spiders from this session were used for assessing physiological tests (see subsequent subsections). In the rearing laboratory, the temperature was $25.7 \pm 0.5^\circ\text{C}$ (mean $\pm$ s.d.) during the first rearing session.

The second rearing session was conducted to acquire precise data on the survival rate of spiderlings belonging to the different treatment groups, as we did not keep specific record of whether and when spiderlings perished throughout the physiological test session. In the following, we refer to the second laboratory rearing session as the 'survival test session', because spiders belonging to this session were reared to assess pre-maturation mortality patterns. From each of the 19 collected mated females, we selected an arbitrary number of spiderlings (15.6 ± 7.4 , mean $\pm$ s.d., range: 4–33), resulting in 297 spiderlings. These spiderlings were randomly assigned to increasing and decreasing day length treatment groups, in a way so that from each mother, half of the selected spiderlings were assigned to the increasing, and the second half to the decreasing day length rearing group. The second rearing session lasted from 30 July 2018 to 8 January 2019. The rearing room temperature was $24.5 \pm 2.0^\circ\text{C}$ (mean $\pm$ s.d.) during the survival test session.

Fat extraction

Before fat extraction, the body mass of the living spiders was measured with accuracy to the tenth of a milligram. Subsequently, spiders were euthanised in -20°C . Fat extraction was performed according to the methodology of Aisenberg and Peretti (2011). Briefly, spiders were placed in 1.5 ml microtubes individually, and dried in a drying box at 60°C for 24 h. After that, we weighed the dried spiders (first dry mass), then pipetted 1 ml of chloroform on them, in which they were suspended for another 24 h. Following the chloroform soak, we disposed of the used chloroform and dried the spiders again for 24 h at 60°C . Finally, we measured again the dry

mass of the spiders (second dry mass). For later analyses, we calculated the fat content of the body as the difference between the two dry masses, divided by the living mass. When fat extraction was complete, we put spiders in 0.5 ml 96% ethanol and stored them in -20°C for later measurements.

Homogenate preparation

For immune measurements, spiders were euthanised in -20°C , and their opisthosomas were homogenised in 50 μl of sterile, ice-cold 0.1 mol l^{-1} Sørensen buffer (pH 7.4). Spider prosomas were stored in 0.5 ml 96% ethanol, at -20°C , for later measurements. We stored homogenates on ice until centrifugation, and we centrifuged samples in 4°C at 10,000 *g* for 10 min. Subsequently, $2 \times 20\text{ }\mu\text{l}$ supernatant was pipetted into separate microtubes: one for ELISA, and another for bacterial growth inhibition and cell wall lysis assays. Samples were stored at -70°C until tests.

Prosoma measurements

Prior to measurements, we dried prosomas on filter paper and gently removed the legs in order to make positioning easier and repeatable across specimens. Then, we placed the prosomas separately on a millimetre grid, next to a white standard photo paper and photographed them using a light box to ensure standard and repeatable light conditions. We used a Canon EOS 550D, with a Tamron 90 mm f/2.8 macro lens. On the pictures, we measured prosoma width as the widest segment along the dextro-sinister axis, using ImageJ (version 1.46a; Schneider et al., 2012). The extent of prosoma cuticular melanisation of the dorsal prosoma was evaluated by measuring the median grey value of the dorsal surface of the prosoma. In later analyses, we used a calculated estimate of cuticular darkness, with a range from 0 to 1, as:

$$1 - \frac{(G - G_{\min})}{(G_{\max} - G_{\min})}, \quad (1)$$

where G is the median of grey pixel values of a given spider (ranging between 0 for black and 255 for white), and $G_{\min}$ and $G_{\max}$ are the smallest (minimum) and largest (maximum) of these medians across all individuals measured, respectively. In the analyses and results, we refer to this measure as the prosoma darkness, and it reflects the relative prosoma darkness of individuals compared with the most melanised specimen (having a prosoma darkness value equal to 1): values close to 1 indicate dark cuticle, while values close to 0 indicate brighter cuticle.

To make sure that all of the photos from all spider groups (spring, late summer, laboratory-reared increasing and decreasing day length group) are indeed comparable in their brightness, we randomly chose 10 photos from each group, measured the median grey value of a rectangle on the white standard paper (each rectangle was approximately the same size as the prosoma of a spider), and used the Conover–Iman test (<https://cran.r-project.org/web/packages/conover.test/index.html>) with Bonferroni's P -value correction to determine whether they vary substantially. We did not find significant differences between the white standard measures across the four photographing occasions ($P > 0.127$ for all group-by-group comparisons).

Immune measures: cecropin B and β -defensin content

For cecropin B and β -defensin content, an indirect ELISA technique was applied, according to standard protocol (Crowther, 2009). Because of the small volume of some samples, we were not able to measure both proteins from all of the samples; therefore, for some

spiders the measurements of β -defensin were not feasible. Briefly, the wells of 96-well transparent flat-bottom plates (Corning; a separate plate for each antigen) were filled with 100 μ l of supernatant samples of known protein concentration and incubated overnight. The potentially uncoated areas of the plates were then blocked with 1% bovine serum albumin (Sigma-Aldrich; 100 μ l 1 h, 37°C). Anti-cecropin B and anti- β -defensin primary antibodies (anti-cecropin-B produced in rabbit, ab27571, Abcam; anti- β -defensin 3 produced in rabbit, D2444, Sigma-Aldrich; 100 μ l, 1:1000, 2 h at 37°C) and then secondary antibody (goat anti-rabbit IgG polyclonal antibody, alkaline phosphatase conjugate, ADI-SAB-301-J, Enzo; 100 μ l, 1:1000; 1 h at 37°C) were subsequently used. Between each application, the wells were washed four times with 0.05% Tween-20 detergent in Soerensen buffer, pH 7.4. Finally, after washing, the secondary antibody was replaced by 100 μ l para-nitrophenylphosphate (Sigma-Aldrich) solution in 10 mmol l⁻¹ diethanolamine buffer (pH 9.5, 0.5 h, room temperature). Primary and secondary antibodies as well as original homogenates were dissolved in protease inhibitor buffer (0.1 mol l⁻¹ Sørensen buffer, pH 7.4; 100 ml buffer containing 32.5 mg sodium-azide, 140 μ l β -mercaptoethanol, 4 mg phenylmethylsulfonyl fluoride). The contents of cecropin B and β -defensin were assessed spectrophotometrically at 405 nm using a Tecan Infinite M200 microplate reader. The contents of the detected proteins were expressed as absorbance values, which are proportional to the antigen contents in the samples. The comparability of the results was guaranteed by the application of the same total protein content per well for each sample and each antigen. For measuring total protein concentration of original samples, we used the modified Bradford method described by Ernst and Zor (2010).

Immune measures

Bacterial growth inhibition assay

A bacterial growth inhibition assay was used based on the methodology used by Castella et al. (2010). Briefly, we prepared a medium containing 1 g of bacto-tryptone, 1 g NaCl, 0.5 g yeast extract and 1 g agar in 100 ml distilled water. Prior to pouring the agar into Petri dishes, 50 μ l from a 2.6×10^8 cells ml⁻¹ suspension of *Micrococcus luteus* (obtained from the stock culture of the Department of Microbial Biotechnology, University of Debrecen) was added and mixed carefully. We used *M. luteus* because of its availability, and because it is frequently present in soil (Biskupiak et al., 1988; Sims et al., 1986); as *P. agrestis* is an epigeic species, it may often encounter *M. luteus* strains. We pipetted 2 μ l from each spider's homogenate sample on the surface of the thin agar layer (using 6 ml of agar in a Petri dish of 100 mm diameter), in duplicates from each spider. Duplicates were placed in the same Petri dish in the case of all samples. Following the application of samples on the Petri dishes, they were incubated at 30°C for 24 h. Samples containing antimicrobial peptides inhibit bacterial growth; therefore, after an incubation period, when bacteria grows homogeneously on the surface of the agar, empty zones (so called inhibition zones) will persist where antimicrobial peptide containing samples were applied. The area of inhibition zones is proportional to the antimicrobial peptide content of the samples (Castella et al., 2010). Following the incubation, all Petri dishes were photographed (Figs S1–S4), and the areas of inhibition zones were measured using ImageJ.

We quantified the bacterial growth inhibition capacity for the analyses as follows. After measuring inhibition zone areas, we also measured the area of the given Petri dish. This was necessary because photographing was done from slightly different distances with respect to the Petri dishes, i.e. Petri dish areas covered different

numbers of pixels, hence inhibition zones were not directly comparable. To correct for this, we divided inhibition zones (number of pixels within the zone) by the area of the given Petri dish (number of pixels within the whole Petri dish; Petri dish area was measured separately for each Petri dish photo, and the Petri dishes themselves were of the same size). Because all spider opisthosomas were homogenised in the same volume of buffer solution (i.e. larger spiders yielded more concentrated samples), this quantity was divided by the living mass of the spider which the given inhibition zone belonged to. This calculation resulted in a variable that is likely closely proportional to the bacterial growth inhibition power per milligram body mass. Finally, we centred this variable at zero by subtracting the mean of the variable from all values, and we scaled values by dividing them by the standard deviation of the variable (i.e. we performed mean centring and SD scaling). In later analyses, we used this variable as a quantification of bacterial growth inhibition power.

Cell wall lysis assay

Bacterial cell wall lysis assay was done based on the methodology described in Castella et al. (2010). We prepared a cell wall containing agar medium (1 g agar in 100 ml distilled water) with 5 mg ml⁻¹ *Micrococcus lysodeikticus* (lyophilised *M. lysodeikticus*, ATCC no. 4698, Sigma-Aldrich), and 0.6 mg ml⁻¹ ampicillin sodium. In this method, cell wall residues are homogeneously distributed in the agar, creating a thin, opaque layer. Samples containing lysozyme produce transparent clearing zones on the agar, and the area of clearing zones is proportional to the lysozyme content of the sample (Castella et al., 2010). We poured 5 ml of this medium into each Petri dish (100 mm in diameter) and applied 2 μ l from each homogenate sample on the surface of the cell wall agar, in duplicates from each spider. Duplicates were placed in the same Petri dish in the case of each sample. Petri dishes were then incubated at 30°C for 24 h. Following the incubation, all Petri dishes were photographed, and the areas of clearing zones were measured using ImageJ. In later analyses we used a variable derived from the clearing zone the same way as in the case of bacterial growth inhibition (see above).

Statistical analyses

Statistical analyses were performed using R statistical software (version 3.5.2, <https://www.r-project.org/>), mainly with linear regression modelling. Some parameter estimates for variables in interaction that were not directly available from model result summaries were obtained by calculating estimated marginal means (EMMs) with the R package emmeans (<https://cran.r-project.org/web/packages/emmeans/index.html>). Non-significant interactions ($P \geq 0.100$) were removed from the final models, and the reported estimates in the Results come from the reduced final models.

Prior to statistical tests, we visually checked grouped data and associations to be tested in order to identify potential outliers or anomalies. There was one anomalous data point in the bacterial growth inhibition measurements (value more than four standard deviations from the median) among the spiders from natural habitat (causing downward bias in effect size of male group, and upward bias in the standard error estimation for this group effect, rendering it non-significant), which was excluded from the analyses (Ruan et al., 2005).

We analysed data from natural and laboratory-reared spiders separately, mainly using linear regression models (LMs). In the analyses of spiders collected from the field, the used response variables were size (as prosoma width), fat content (proportion of

fat in living mass), prosoma darkness (level of melanisation), levels of cecropin B and β -defensin, bacterial growth inhibition and cell wall lytic activity. In the models, we used season (spring or late summer) and status (male, virgin female or mated female) as explanatory (predictor) variables, except in the models on size and prosoma melanisation, in which we simply used sex instead of status (because female reproductive status is neither expected nor found to affect these traits; for all virgin versus mated female EMM, $P>0.110$). The interaction between the predictors was also included in the models. In an additional model, we tested how season affects female fecundity (number of offspring) using season and size as predictors. Season strongly affected size (see Results), but as a result, the interaction between season and size was not significant (i.e. regression slopes of size on fecundity did not differ between seasons); this case of correlation between predictors does not bias the results, nor does it cause interpretational problems. To confirm whether a fecundity difference is simply due to body size differences between slowly and rapidly developing females, we estimated their fecundity per size unit (i.e. we divided the number of offspring by body size), and used this as response variable in a simple linear regression model (LM), with season as a predictor.

In the analyses of spiders reared for physiological tests, we used linear mixed-effects models (LMMs; Kuznetsova et al., 2017) to control for kinship between spiders by defining the ID of mother spiders as a random effect. Firstly, we tested the effect of rearing treatment group on developmental time (specified as days from hatching to maturation) by fitting a model with developmental time as the response variable, and rearing group (slow or rapid treatment), sex (female or male) and their interaction as explanatory variables. Subsequently, we fitted an LMM in which growth rate (specified as adult size divided by number of moults, representing average increase in size per moulting) was the dependent variable, and treatment group, sex and their interaction were explanatory variables. In addition, we used a generalized LMM (GLMM; Bates et al., 2015) with Poisson error distribution to test whether spiders in the rapid and slow treatment groups differ in the number of moults (i.e. number of moults was the response variable, and rearing treatment was the explanatory variable), also controlling for sex, and the interaction of sex and developmental treatment.

Secondly, we fitted separate mixed-effects models using prosoma size, fat content, prosoma darkness, levels of cecropin B and β -defensin, bacterial growth inhibition and cell wall lytic activity as response variables. In the model on prosoma size, explanatory variables were rearing treatment, developmental time and sex, and the two-way interactions between the predictors. Because developmental time and rearing treatment were strongly correlated, we re-centred developmental time at zero within treatment groups by subtracting the mean developmental time of groups from all values within treatment groups, to eliminate the correlation between the explanatory variables. Developmental time was used in the linear regression on prosoma size instead of growth rate, because growth rate itself is a variable derived from prosoma size. In the other models, explanatory variables were rearing treatment, growth rate (re-centred within treatment groups) and sex. In these models we also included the interaction terms between rearing treatment and sex, rearing treatment and growth rate, and growth rate and sex.

Using the data from the survival test session, we tested how rearing treatment affected maturation incidence and mortality, by fitting mixed-effects Cox regression models (<https://cran.r-project.org/web/packages/coxme/index.html>) (using mother spiders' ID variable as random effect). We chose Cox regression instead of

linear regression because (unlike among the spiders in the physiological test session) there were observations from non-mature specimens as well, and proportional hazards models can handle data in which some events and corresponding times (maturation and developmental time, respectively) are not observed. In order to be able to differentiate between juvenile and adult mortalities, also we fitted two additional binomial GLMMs, testing whether developmental treatment group affected the probability of dying before maturation: in the first binomial GLMM, we used the total mortality data from the survival test session, while in the second we used a subset, including only those spiders that died over the course of the rearing (i.e. as juveniles). Sex was not included as a co-factor either in the Cox models or in the GLMMs because identification of sex was not possible for juveniles.

RESULTS

Spiders collected from natural habitat

We found that slowly developing spiders are larger and have darker prosoma (although this difference was smaller, yet still significant, among males) and, although only among females (both virgin and mated), have more fat reserves (Table 1, Fig. 2A–C). Also, slowly developing females had much higher fecundity in comparison to rapidly developing females, having approximately 1.7 times more offspring on average [39.0 ± 9.2 and 22.9 ± 8.5 (mean $\pm$ s.d.) offspring, for average sized slowly and rapidly developing females, respectively; Table 1, Fig. 2D]. Fecundity per body size unit was also significantly higher in slowly developing females than in rapidly developing females (LM: $\beta=6.380$, s.e.=0.964, $t=6.62$, $P<0.001$), indicating that developmental phenotype difference in fecundity was not exclusively due to differences in body size.

Table 1. Results from linear regression models on condition and fecundity, fitted using data from the spiders collected from natural habitat

Explanatory variables	Coefficients	s.e.	<i>t</i>	<i>P</i>
Size (prosoma width), <i>n</i> =385				
Intercept (slow development, female)	2.116	0.009	235.86	<0.001*
Developmental type (rapid)	−0.243	0.013	−18.35	<0.001*
Sex (male)	−0.100	0.014	−6.90	<0.001*
Fat content, <i>n</i> =97				
Intercept (slow development, virgin female)	0.085	0.005	18.77	<0.001*
Developmental type (rapid)	−0.015	0.006	−2.44	0.016*
Status (mated female)	−0.026	0.006	−4.23	<0.001*
Status (male)	−0.047	0.007	−7.07	<0.001*
Developmental type $\times$ Status (rapid, mated female)	−0.002	0.008	−0.20	0.841
Developmental type $\times$ Status (rapid, male)	−0.017	0.009	−1.84	0.068(*)
Prosoma darkness, <i>n</i> =385				
Intercept (slow development, female)	0.779	0.007	113.83	<0.001*
Developmental type (rapid)	−0.382	0.011	−33.87	<0.001*
Sex (male)	0.391	0.017	23.63	<0.001*
Developmental type $\times$ Sex (rapid, male)	−0.257	0.021	−12.06	<0.001*
Fecundity, <i>n</i> =80				
Intercept (slow development)	−52.859	15.307	−3.45	<0.001*
Developmental type (rapid)	−6.466	2.357	−2.74	0.008*
Size	43.772	7.262	6.03	<0.001*

Asterisks mark statistically significant effects ($P<0.05$); asterisks in parentheses mark marginally significant effects ($0.05<P<0.10$).

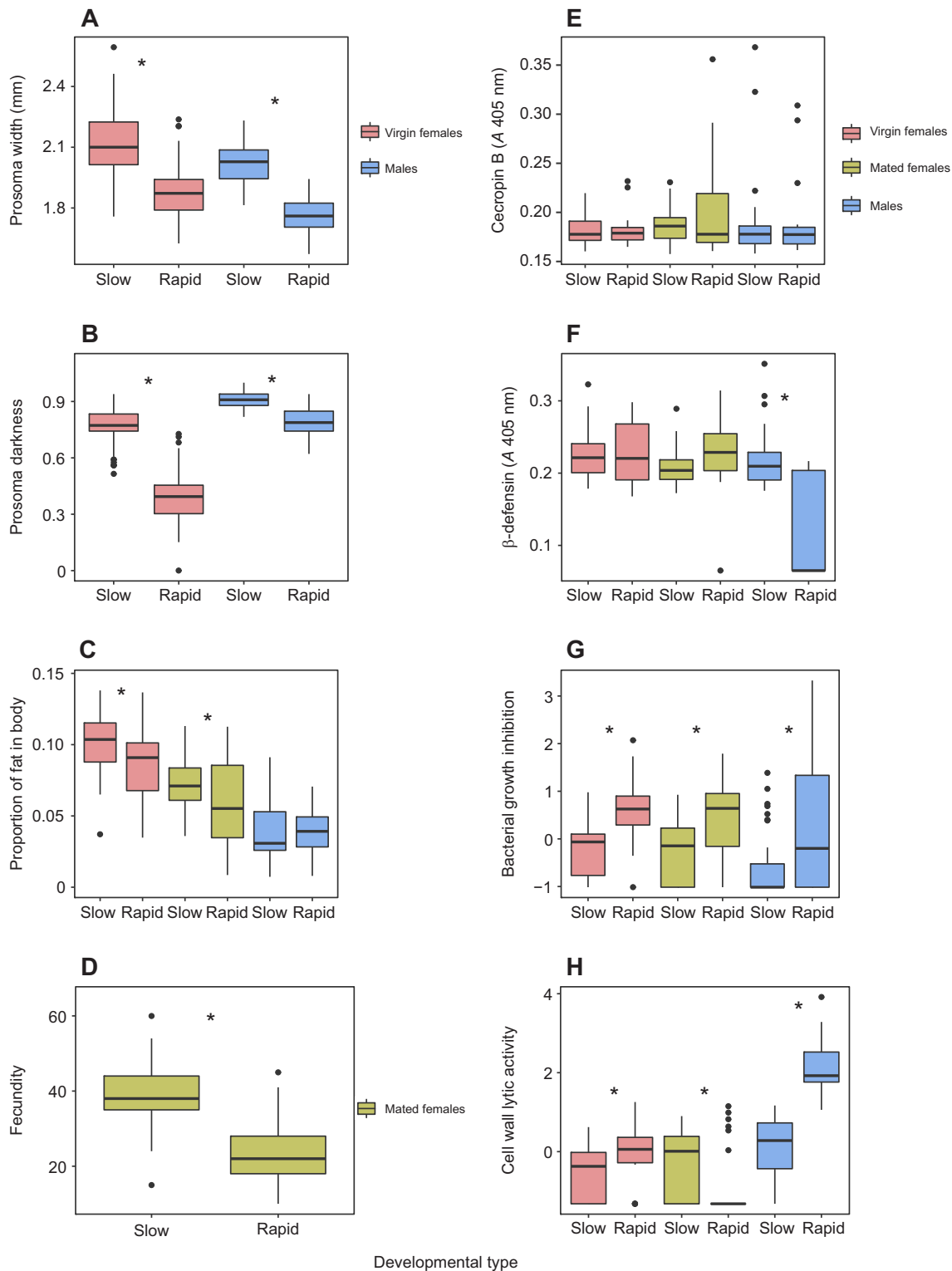


Fig. 2. Box plots visualising distributions of observed values for the assessed traits in slowly and rapidly developing spiders, collected from the natural habitat. Assessed traits, arranged from top-left to bottom-right, are (A) prosoma width, (B) extent of prosoma melanisation (prosoma darkness), (C) percentage of fat in the body, (D) fecundity, (E) level of cecropin B, (F) level of β -defensin, (G) bacterial growth inhibition and (H) cell wall lytic activity. Asterisks mark significant differences between developmental types within sexes ($P < 0.05$). Vertical whiskers show minimum and maximum ranges, boxes represent the interquartile range between the first and third quartiles, horizontal solid lines show the median of the given value distribution, and individual dots represent outlier values.

There were no significant differences in cecropin B levels between sub-cohorts (Table 2, Fig. 2E). In males, rapidly developing spiders showed lower β -defensin levels compared with

slow ones (EMM: $\beta = -0.096$, s.e. = 0.018, $t = -5.32$, $P < 0.001$), but we found no such difference in females (Fig. 2F). Unexpectedly, slowly developing spiders had weaker bacterial growth inhibition

Table 2. Results from linear regression models on immune parameters, fitted using data from the spiders collected from the natural habitat

Explanatory variables	Coefficients	s.e.	t	P
Cecropin B, n=215				
Intercept (slow development, virgin female)	0.179	0.004	44.43	<0.001*
Developmental type (rapid)	0.007	0.004	1.53	0.129
Status (mated female)	0.008	0.005	1.74	0.083(*)
Status (male)	0.007	0.005	1.25	0.212
β-defensin, n=181				
Intercept (slow development, virgin female)	0.227	0.006	38.33	<0.001*
Developmental type (rapid)	-0.001	0.010	-0.06	0.949
Status (mated female)	-0.020	0.008	-2.69	0.008*
Status (male)	-0.009	0.009	-0.94	0.348
Developmental type $\times$ Status (rapid, mated female)	0.022	0.014	1.62	0.108
Developmental type $\times$ Status (rapid, male)	-0.095	0.021	-4.57	<0.001*
Bacterial growth inhibition, n=210				
Intercept (slow development, virgin female)	-0.229	0.112	-2.05	0.041*
Developmental type (rapid)	0.801	0.116	6.90	<0.001*
Status (mated female)	-0.106	0.132	-0.80	0.423
Status (male)	-0.350	0.147	-2.38	0.019*
Cell wall lytic activity, n=211				
Intercept (slow development, virgin female)	-0.517	0.126	-4.10	<0.001*
Developmental type (rapid)	0.483	0.204	2.37	0.019*
Status (mated female)	0.239	0.158	1.51	0.132
Status (male)	0.563	0.180	3.13	0.002*
Developmental type $\times$ Status (rapid, mated female)	-1.094	0.268	-4.08	<0.001*
Developmental type $\times$ Status (rapid, male)	1.616	0.294	5.50	<0.001*

Asterisks mark statistically significant effects ($P<0.05$); asterisks in parentheses mark marginally significant effects ($0.05<P<0.10$).

than rapidly developing spiders (Table 2, Fig. 2G). The effect of developmental type and sex was a bit more complex in cell wall lytic activity, as males and virgin females of the slowly developing sub-cohort were characterised by smaller values than in the rapid sub-cohort, while cell wall lytic activity of rapidly developing mated females was dramatically lower than in slowly developing mated females (and, in fact, in any other group; Fig. 2H).

Laboratory-reared spiders

Physiological test session

In total, 218 spiders matured in the first laboratory rearing session (119 and 99 spiders in the rapid and slow treatment groups, respectively). Rearing photoperiod treatments had strong impact on both developmental time and growth rate: spiders reared in rapid treatment group matured in 84.0 ± 25.2 days (mean $\pm$ s.d.) on average, while spiders in the slow treatment group reached adulthood in 125.7 ± 20.3 days (mean $\pm$ s.d.), an almost 50% increase (Table 3, Fig. 3D). By the end of the first phase of the rearing, 4% of spiders in the slow group matured ($n=4$), while 62% of the spiders in the rapid group reached maturity ($n=74$). Treatment group did not affect significantly the number of moulting, and sexes did not differ in the mean number of moults (Table 3). Males were smaller, were characterised by much darker prosomas and had lower fat content than females (Fig. 3A–C, Table 3). Additionally, males matured sooner than females in the slow treatment group (EMM: $\beta=12.021$, s.e.=4.250, $t=2.83$, $P=0.005$), but not in the rapid treatment group (EMM: $\beta=0.756$, s.e.=3.850, $t=0.20$, $P=0.845$).

Spiders in the rapid treatment group did not differ in size from specimens in the slow treatment group (Fig. 3A). Females in the rapid treatment group were brighter (i.e. had less melanised cuticle) than females in the slow treatment group (EMM: $\beta=0.061$, s.e.=0.018, $t=3.31$, $P=0.001$), while there was no such difference in males (EMM: $\beta=0.009$, s.e.=0.019, $t=0.46$, $P=0.648$; Fig. 3B). Growth rate itself was positively associated with prosoma melanisation in both treatment groups (Table 3). In the rapid treatment group, spiders showed marginally significantly higher fat content than those in the slow treatment group (Fig. 3C). Also, growth rate was positively associated with fat content in both groups (Table 3).

No significant differences were found between treatment groups either in β -defensin levels (Fig. 3F) or in bacterial growth inhibition power and cell wall lytic activity (Table 4, Fig. 3G,H). However, the cecropin B levels in spiders reared in the rapid treatment group were significantly lower than in the slow treatment group (Table 4, Fig. 3E).

Survival test session

In total, 212 spiders matured in the second laboratory rearing session, 121 and 91 in the rapid and slow treatment groups, respectively. Throughout the entire rearing session, mortality among the rapid treatment group was higher than in the slow treatment group (Cox regression: $\beta=0.805$, s.e.=0.150, $z=5.36$, $P<0.001$), but this was due to the fact that spiders matured much sooner in this group (Cox regression: $\beta=1.149$, s.e.=0.143, $z=8.03$, $P<0.001$), and hence had shorter life cycles and perished as adults sooner than those developing slowly. Indeed, there was no difference between rearing treatment groups in likelihood of dying during the first rearing phase (Cox regression: $\beta=-0.204$, s.e.=0.345, $z=-0.59$, $P=0.560$). Even more surprisingly, based on the binomial GLMMs, probability of perishing before maturation was lower in the rapid treatment group than in the slow treatment group, both among the total reared population (binomial GLMM: $\beta=-0.809$, s.e.=0.277, $z=-2.92$, $P=0.004$) and among those that died during the rearing session (binomial GLMM: $\beta=-1.982$, s.e.=0.331, $z=-5.99$, $P<0.001$).

DISCUSSION

In our study, we quantified a number of important traits in slowly and rapidly developing sub-cohorts of *P. agrestis* to assess what physiological consequences might be associated with rapid post-embryonic development. Life history theory and the POLS hypothesis predicts that a substantially increased developmental (and growth) rate would incur high physiological costs, e.g. in the form of negative phenotypic covariance between costly traits utilising the same resource pool. It is also predicted by theory that these costs would translate into fitness costs (i.e. decreased survival or effective reproductive success), which appear to be rather rational assumptions, because if no costs were associated with such an enhanced developmental rate, it would be likely to invade, and become fixed in, the populations.

Our results are multifarious, as we have found some evidence implying physiological costs associated with the rapid developmental cohort, while in some traits we found no consistent effect of developmental type, or even found a positive link with rapid development. One of the most prominent differences between slowly and rapidly developing spiders was apparent in their cuticular darkness, as rapidly developing specimens had brighter prosomas, both among natural and laboratory-reared spiders (although in the latter the difference was milder, and statistically significant only in

Table 3. Results from linear regression models on development and condition, fitted using data from the physiological test session

Explanatory variables	Coefficients	s.e.	t	P
Developmental time, <i>n</i> =218				
Intercept (slow treatment, female)	130.798	4.208	31.08	<0.001*
Rearing treatment (rapid treatment)	−46.134	3.976	−11.60	<0.001*
Sex (male)	−12.021	4.242	−2.83	0.005*
Rearing group×Sex (rapid treatment, male)	11.265	5.733	1.97	0.050*
Growth rate, <i>n</i> =218				
Intercept (slow treatment, female)	0.321	0.006	57.23	<0.001*
Rearing treatment (rapid treatment)	0.013	0.006	2.32	0.022*
Sex (male)	−0.008	0.006	−1.48	0.140
Size (prosoma width), <i>n</i> =218				
Intercept (slow treatment, female)	2.182	0.0196	111.10	<0.001*
Rearing treatment (rapid treatment)	0.002	0.015	0.12	0.908
Developmental time (re-scaled within treatment groups)	0.001	0.0005	2.51	0.013*
Sex (male)	−0.181	0.015	−11.79	<0.001*
Sex×Developmental time (male)	−0.002	0.0007	−2.93	0.004*
Fat content, <i>n</i> =109				
Intercept (slow treatment, female)	0.084	0.004	21.65	<0.001*
Rearing treatment (rapid treatment)	0.007	0.004	1.82	0.072(*)
Growth rate (re-scaled within treatment groups)	0.100	0.041	2.43	0.017*
Sex (male)	−0.020	0.004	−5.49	<0.001*
Prosoma darkness, <i>n</i> =218				
Intercept (slow treatment, female)	0.708	0.021	33.05	<0.001*
Rearing treatment (rapid treatment)	−0.061	0.018	−3.31	0.001*
Growth rate (re-scaled within treatment groups)	0.434	0.165	2.63	0.009*
Sex (male)	0.104	0.019	5.35	<0.001*
Rearing treatment×sex (rapid treatment, male)	0.052	0.026	1.96	0.052(*)

Asterisks mark statistically significant effects ($P<0.05$); asterisks in parentheses mark marginally significant effects ($0.05<P<0.10$).

females). Melanin incorporation into the cuticle has multifaceted roles. For example, in arthropods, the level of cuticular melanisation is associated with the efficiency of thermoregulation and water retention (Rajpurohit et al., 2008; Watt, 1969). Also, thermal regime-dependent changes in cuticle melanisation are often associated with changes in the efficiency of melanin production-related elements of the immunity, hinting at a positive association between cuticular melanin content and pathogen resistance (Fedorka et al., 2013; Kutch et al., 2014). Indeed, in mealworm beetles, heavily melanised individuals were found to be able to mount a stronger melanotic encapsulation response upon repeated immune activation, and the simulated parasite attack had a much less detrimental impact on other life history traits, such as fecundity or longevity, implying a positive association between condition and cuticle melanisation (Krams et al., 2016). Brighter prosomas among rapidly developing spiders might indicate that the level of melanin production and incorporation in the cuticle is depressed in them. Melanin production was shown to have considerable costs in arthropods (Ethier et al., 2015; Hooper et al., 1999; Roff and Fairbairn, 2013; Siva-Jothy, 2000; True, 2003; but see Roulin, 2016), and negative phenotypic correlations between cuticular melanisation and developmental rate, along with other traits, were found by several studies in arthropods (Busso et al., 2017; Cotter et al., 2008; Ma et al., 2008). Lower melanin content of the prosomal cuticle could be a result of an energetic or physiological conflict between increased developmental rate and melanin production. Presumably, lower availability of precursor molecules, or lower expression and/or activity of contributing enzymes, such as phenoloxidase, might be at play behind this pattern (Armitage and Siva-Jothy, 2005; Bailey, 2011; Thompson et al., 2002). It also may be possible that in rapidly developing spiders, the timespan during which melanin is produced and incorporated into the new cuticle (prior to the moulting) is simply shorter, hence there is less time to build melanin into the cuticle. Interestingly, however, among laboratory-reared spiders, growth rate (increase in body size per

moult) was positively associated with prosoma melanisation, and in fact, the difference in melanisation between rapid and slow treatment group spiders was quite mild. These results might imply that an increased rate of building up the soma is associated with an increased rate of melanin-incorporation into the prosomal cuticle, but when resource availability is limited the production of melanin is more restricted, hence the prosoma darkness is substantially lower in rapidly developing spiders from the natural habitat. Notably, the thermal environment may also have contributed to the milder developmental group differences in prosoma melanisation among the laboratory-reared spiders. The hypothesis of thermal melanism predicts that melanin incorporation into the cuticle will increase in colder environments; therefore, a stable, warm environment might have reduced cuticle melanisation in laboratory-reared spiders (Fedorka et al., 2013). However, when comparing the prosoma darkness measurements between laboratory-reared and wild-caught spiders, we did not find generally lower melanisation among laboratory-reared spiders (Table A1).

Notably, in a previous study, we found evidence for a negative association between developmental rate and a melanin-based immune function in *P. agrestis*, as the time from hatching to maturation was positively correlated with the extent of melanotic encapsulation of a nylon monofilament (Rádai et al., 2018). Although we do not have information about how cuticular darkness and encapsulation efficiency are associated in *P. agrestis*, evidence from other arthropods suggests that they could be positively correlated (Bailey, 2011; Fedorka et al., 2013; Krams et al., 2016; Prokkola et al., 2013), providing further (although indirect) support for the common physiological mechanisms of cuticle melanisation and encapsulation, which may be in energetic conflict with developmental rate.

In other arthropods, not only cuticle melanisation, but also adult size was found to be negatively correlated with developmental rate (Ma et al., 2008; Windig, 1999). Similarly, we found that rapidly

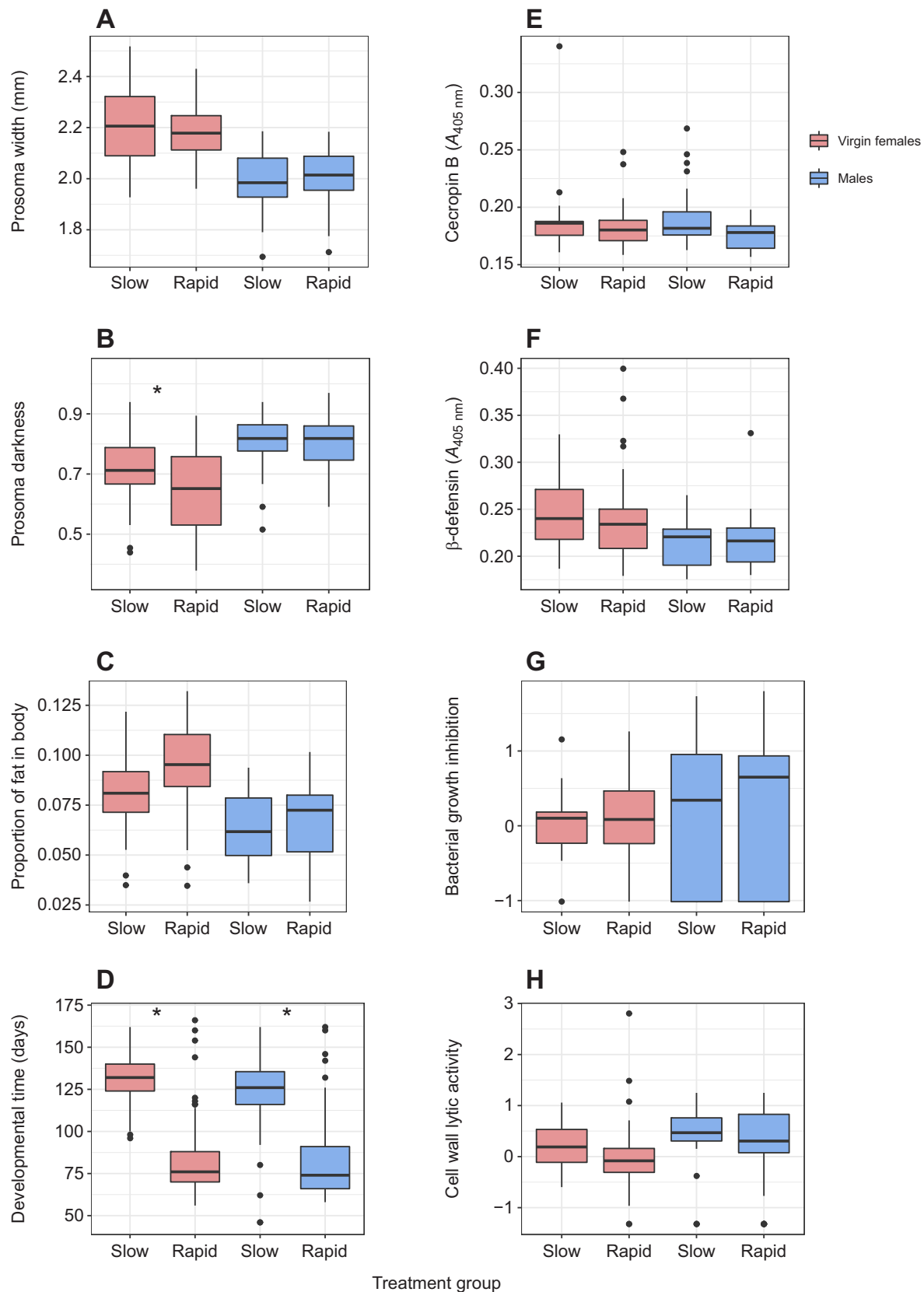


Fig. 3. Box plots visualising distributions of observed values for the assessed traits of spiders in the slow and rapid treatment groups, reared in laboratory (physiological test session). Assessed traits, arranged from top-left to bottom-right, are (A) prosoma width, (B) extent of prosoma melanisation (prosoma darkness), (C) percentage of fat in the body, (D) developmental time as days from hatching to maturation, (E) level of cecropin B, (F) level of β -defensin, (G) bacterial growth inhibition and (H) cell wall lytic activity. Asterisks mark significant differences between developmental treatment groups within sexes ($P < 0.05$). Vertical whiskers show minimum and maximum ranges, boxes represent the interquartile range between the first and third quartiles, horizontal solid lines show the median of the given value distribution, and individual dots represent outlier values.

Table 4. Results from linear regression models on immune parameters, fitted using data from the physiological test session

Explanatory variables	Coefficients	s.e.	t	P
Cecropin B, n=108				
Intercept (slow treatment, female)	0.194	0.005	42.08	<0.001*
Rearing treatment (rapid treatment)	-0.013	0.005	-2.62	0.010*
Growth rate (re-scaled within treatment groups)	-0.019	0.064	-0.30	0.767
Sex (male)	-0.005	0.005	-0.98	0.329
β -defensin, n=95				
Intercept (slow treatment, female)	0.244	0.008	29.97	<0.001*
Rearing treatment (rapid treatment)	-0.001	0.008	-0.15	0.885
Growth rate (re-scaled within treatment groups)	-0.078	0.115	-0.68	0.501
Sex (male)	-0.027	0.008	-3.27	0.002*
Bacterial growth inhibition, n=108				
Intercept (slow treatment, female)	0.110	0.173	0.64	0.528
Rearing treatment (rapid treatment)	-0.029	0.149	-0.20	0.844
Growth rate (re-scaled within treatment groups)	-2.662	2.099	-1.27	0.207
Sex (male)	0.067	0.148	0.45	0.651
Cell wall lytic activity, n=109				
Intercept (slow treatment, female)	0.168	0.143	1.18	0.242
Rearing treatment (rapid treatment)	-0.183	0.149	-1.23	0.221
Growth rate (re-scaled within treatment groups)	0.021	1.950	0.01	0.992
Sex (male)	0.185	0.147	1.26	0.211

Asterisks mark statistically significant effects ($P < 0.05$).

developing spiders collected during late summer had smaller prosomas than slowly developing spiders collected in spring. This result seems to imply that the costs of a higher developmental rate precluded building up the same body size as in slowly developing specimens. For rapidly developing specimens to achieve the same size at maturation as slowly developing ones, growth rate (i.e. somatic tissue built in a given time interval) has to be substantially increased (Dmitriew, 2011). Of course, increased growth rate may be apparent not just when rapidly and slowly developing spiders have the same adult size; in fact, rapidly developing spiders with relatively higher growth rate may still achieve smaller body size given that their time period of growth is considerably shorter. Increased developmental and growth rates are both often negatively associated with adult body size (Blanckenhorn, 1998; Gotthard et al., 1994), and energetic (i.e. resource allocation) limitations play a key role in these relationships (Fischer et al., 2005). In the case of spiders reared in the laboratory, we did not find differences in body size between spiders in the rapid and slow treatment groups, but growth rate was considerably higher in rapidly developing spiders than slow ones. This suggests that in the laboratory, spiders could

Table A1. Linear regression results, in which prosoma darkness is the response variable, while sex, developmental type (or treatment) and spider type (collected from nature, or reared in the laboratory) are predictors; all two- and three-way interactions between the predictors are also included

Contrasts (laboratory spiders – natural spiders)	Coefficient	s.e.	t	P
Rapid females	0.252	0.016	16.21	<0.001*
Slow females	-0.055	0.016	-3.47	0.003*
Rapid males	0.015	0.020	0.73	0.885
Slow males	-0.105	0.019	-5.62	<0.001*

Estimated marginal means for the contrasts between natural-collected and laboratory-reared spiders are shown. P -values are adjusted with Tukey's method. Asterisks mark statistically significant effects ($P < 0.05$).

reach high growth rates, by which they were able to attain adult sizes similar to those of slowly developing specimens. Because food availability was not limited during rearing, it seems to be plausible that rapidly developing laboratory-reared spiders might have been able to achieve adult body sizes similar to those of slowly developing spiders owing to increased acquisition of resources. Indeed, in a previous study, we found that rapidly developing spiders are more voracious foragers (Rádai et al., 2017a), which, combined with unconstrained food availability, might have helped rapidly developing spiders in the laboratory to grow to a similar body size as slowly developing specimens. When resources are less limited, enhancement of both developmental and growth rates may be possible without trading them off with adult size (Dmitriew and Rowe, 2005). This is a quite important result, because it reflects that rapidly developing spiders have the latent capacity to reach the same adult size as slowly developing ones, even at much higher rates of growth and development. As such, among females collected from their natural habitat, 23 out of the 103 rapidly developing spiders were as large or larger than the average female size among the slowly developing spiders. Being in accordance with the 'big house, big car' scenario (Reznick et al., 2000; van Noordwijk and de Jong, 1986), this shows that physiological and energetic costs linked with the rapid life history cohort can be mitigated to a considerable extent by increased resource acquisition. Also note that laboratory-reared rapidly developing spiders had more fat reserves than spiders in the slow treatment group, and growth rate was positively associated with fat reserves, suggesting that rapidly developing specimens are not only more voracious, but are also more prone to build up fat reserves when resources are not limiting. However, when resources are limited, fat reserve build-up might be halted, which would explain the result that in spiders collected from natural habitat, we observed lower fat reserve levels in rapidly developing spiders.

In the case of immune measures, although there were some differences between slowly and rapidly developing spiders collected from natural habitat, it is difficult to draw a general conclusion. Among spiders collected from nature, only in male β -defensin levels did we find lower values in rapidly developing specimens. However, rapidly developing spiders showed more efficient bacterial growth inhibition than slowly developing specimens. A similar pattern was observed in cell wall lytic power, although mated females of the rapidly developing cohort were characterised by extremely low values, lower than in any other group. Mated females had already laid eggs prior to the tests, so the prominent decrease in their cell wall lytic capacity might suggest the presence of an energetic conflict between this element of immunity and reproductive investment. Indeed, among rapidly developing mated females that did not produce measurable cell wall lytic zones, fecundity was higher, indicating a trade-off between investment into reproduction and investment into bacterial cell wall lytic capacity (Table A2). The observation that the dramatic decrease in cell wall lytic capacity was apparent only among rapidly developing females might hint at a three-way interaction between developmental rate, reproduction and antibacterial capacity in *P. agrestis* females, i.e. reproductive investment has higher relative immunity costs among rapid females. Additionally, fecundity of mated *P. agrestis* females was negatively associated with bacterial growth inhibition and cell wall lytic activity, although the latter was only marginally significant (Table A3). Such a negative association was observed in the cricket *Allonemobius socius*, and in the pholcid spider *Physocyclus dugesi* as well, as mated females showed considerable decrease in their hemolymph lytic activity (Calbacho-Rosa et al., 2012; Fedorka et al., 2004). Although there are fewer studies on the

Table A2. Results of the logistic regressions on the probability of not producing measurable bacterial growth inhibition and cell wall lysis, using fecundity and developmental type as explanatory variables; interaction of the explanatory variables was not significant in the model of bacterial growth inhibition and thus was excluded

Explanatory variables	Coefficients	s.e.	z	P
Bacterial growth inhibition				
Intercept (slow development)	2.479	1.237	2.00	0.045*
Fecundity	−0.044	0.029	−1.51	0.131
Developmental type (rapid)	−1.900	0.731	−2.60	0.009*
Cell wall lytic activity				
Intercept (slow development)	−2.121	1.985	−1.07	0.285
Fecundity	0.044	0.049	0.89	0.373
Developmental type (rapid)	2.774	2.418	1.15	0.251
Fecundity×Developmental type (rapid)	−0.183	0.090	−2.04	0.041*

Asterisks mark statistically significant effects (* $P < 0.05$).

trade-off between reproduction and immunity in spiders, this trade-off is rather well studied and known among insects (Schwenke et al., 2016).

Among the laboratory-reared spiders, we found no differences between slow and rapid group specimens either in β -defensin, bacterial growth inhibition or cell wall lytic activity, nor were these measures found to be associated with growth rate itself. Although we found lower cecropin B levels in rapid treatment spiders, this difference was quite small (see Fig. 3E), and no such difference was observed among spiders collected from the natural habitat. In contrast, higher antibacterial capacity in rapidly developing spiders from the natural habitat might suggest that those spiders able to mature before winter could also invest more into this element of immunity, possibly owing to their higher resource acquisition. Indeed, in some studies on arthropods, it appears that increased food availability might enhance the efficiency of some immune mechanisms (e.g. Simmons, 2012; but see also Kelly and Tawes, 2013). The absence of a significant difference between laboratory-reared slow and rapid groups in their bacterial growth inhibition power and cell wall lytic activity seems to suggest that when resources are not limited, the developmental types may achieve quite similar levels in the assessed immune elements. This result is quite similar to what was observed in the case of body size, and might hint at the important role of individual quality and resource acquisition in shaping these life history traits in such a cohort splitting scenario.

The result that adult body size of females was positively correlated with the number of their offspring is unsurprising. More interesting is the finding that besides variation owing to body size, developmental pathway also explained a significant amount of variation in fecundity. On average, slowly developing females had

Table A3. Results from the linear regression with prosoma size, bacterial growth inhibition, cell wall lytic activity and developmental type as explanatory (predictor) variables, and fecundity as response; interactions between the predictors were not significant, and therefore were excluded

Explanatory variables	Coefficients	s.e.	t	P
Intercept (slow inhibition)	36.501	1.711	21.34	<0.001*
Developmental type (rapid)	−13.081	2.024	−6.46	<0.001*
Bacterial growth inhibition	−3.313	1.310	−2.53	0.015*
Cell wall lytic activity	−2.101	1.221	−1.72	0.092(*)
Size (re-scaled)	4.968	0.813	6.11	<0.001*

Asterisks mark a statistically significant effect (* $P < 0.05$); asterisks in parentheses mark marginally significant effects ($0.05 < P < 0.10$).

1.5 times higher fecundity per body size unit than rapidly developing ones (LM parameter estimates were 12.11 and 18.49 offspring per millimetre prosoma width for rapid and slow females, respectively). These indicate that the difference in fecundity is not entirely due to the body size difference between slowly and rapidly developing females. One possibility is that physiological costs of rapid development impair the capacity of adult females to produce eggs. Notably, differences between slowly and rapidly developing females in egg sizes also might contribute to fewer offspring in rapid females, owing to the trade-off between egg number and size (Lim et al., 2014), meaning that rapidly developing females may invest relatively more into the size of eggs. Although it is not clear whether we should expect the same in *P. agrestis*, in the cricket *Gryllus firmus*, a significant negative association was observed between developmental time and egg size (Roff and Sokolovska, 2004). Unfortunately, currently we do not have any information about the egg (or hatching) sizes from either slowly or rapidly developing females, so this notion remains to be assessed in future studies. It is also worth noting that a decreased investment into fecundity may contribute to similar (or, apparently, even higher) levels of self-preservation in rapidly developing spiders as slowly developing ones, as a substantial proportion of resources that otherwise would be invested into reproductive tissues can be allocated into somatic maintenance. By doing so, rapidly developing spiders might be able to mitigate the costs of enhanced post-embryonic development to some extent, which would otherwise need to be paid in the currency of somatic maintenance.

The result that spiders in the rapid developmental treatment group exhibited higher overall mortality during the survival test may appear to hint at the survival costs of rapid development itself, but additional modelling results make it clear that this result emerges simply from their shorter life spans. Indeed, spiders in the rapid treatment group did not exhibit higher juvenile mortality than slowly developing spiders; quite the contrary. In other words, although longevity (life span) is shorter in rapidly developing spiders, we observed no survival cost related to the rapid development. Although the survival cost of rapid development might have been mitigated to some extent by the *ad libitum* food availability, past observations also did not show differences in mortality between slowly and rapidly developing spiders, neither in the laboratory nor in rearing conditions close to those in the natural habitat (Kiss, 2003). These observations may seem somewhat perplexing at first, as high growth rate was proposed (and, in a number of studies, found) to have considerable physiological costs that often manifest as increased mortality among rapidly developing individuals (reviewed by Dmitriew, 2011). Given that cohort splitting supposedly is an adaptive form of developmental plasticity in *P. agrestis*, it seems likely that juvenile survival costs of rapid development might have been decreased over the course of evolution in this species, promoting resource acquisition and allocation patterns that mitigate such costs. In future studies it will be quite important to assess metabolic rate measures of both slowly and rapidly developing spiders, how it relates to condition and survival, and how physiological costs (predicted to be associated with higher developmental and growth rates) are manifested under more resource-limiting treatment regimes.

Overall, our findings seem to indicate that the rapid post-embryonic development in the rapidly developing sub-cohort of *P. agrestis* spiders might not entail strict and general costs on the assessed life history characteristics, as opposed to some predictions of life history theory. Instead, individuals appear to change their resource allocation, and presumably even resource acquisition patterns as well, in accordance with their developmental pathway

after cohort splitting. As a likely explanation, we propose that, because cohort splitting is a result of adaptive developmental plasticity, *P. agrestis* adapted physiological and behavioural mechanisms helping to mitigate developmental costs to a considerable extent. Arguably, there is a large number of potential factors that may play a role in shaping life history traits and their associations with one another, and indeed the occurrence of cohort splitting itself, not assessed in the present study. In future studies, detailed assessments of the proximate and ultimate mechanisms evoking such adaptive developmental plasticity, and how they translate to individual differences in life history traits (and ultimately to fitness), will most certainly benefit from integrating genetic, epigenetic and ecological perspectives. For example, future studies should assess how food (or micro- and macro-nutrient) availability affects life history traits and trade-offs between them within sub-cohorts of different developmental rates, to determine to what extent high quantity and/or quality of food is able to mitigate resource allocation-based trade-offs predicted by theory. Intraguild and intraspecific competition may be important factors as well that may affect food availability, and hence potentially shape fitness consequences of taking on either of the developmental phenotypes. Also, a more detailed picture on how well slowly and rapidly developing spiders fare in different ecological conditions (e.g. with regards to predators or parasites) may help us better understand the individual-level consequences of an enhanced pace of life. Furthermore, a better understanding on the genetic components of cohort splitting would be quite important. Ongoing empirical and theoretical investigations suggest that engaging in rapid development by spiderlings in response to environmental stimuli varies consistently between mothers, hinting at genetically determined variation in how responsive spiderlings are to environmental cues, i.e. in how likely they will develop rapidly in the presence of rapid development-promoting stimuli. Intriguingly, such a mechanism could give way for a case of plastic bet-hedging on the part of early summer females from which cohort splitting spiderlings originate. Finally, the competing hypotheses on what drives the fecundity difference between slowly and rapidly developing spiders should be resolved in future studies in order to gain a better understanding of how cohort splitting affects fecundity (and, indeed, reproductive success) in *P. agrestis*, and to complement our general understanding on the physiological and fitness consequences of cohort splitting scenarios. We argue that this spider species will provide an excellent model organism for studies furthering our knowledge of life history evolution and the consequences of adaptive plasticity in development.

APPENDIX

Model-based clustering using parametrised finite Gaussian mixture models

Spring and early summer adults presumably are a mixture of two life history strategies from the previous year, namely (1) slowly developing spiders and (2) offspring of rapidly developing spiders. If differences in developmental times were to consistently affect prosoma size and/or melanisation, we might see bimodal distributions in these traits. We tested whether such bimodal distributions can be detected by model-based clustering (Scrucca et al., 2016) in prosoma size and melanisation. We ran models separately for males and females. For both male and female data subsets, models were run with 1 to 9 clusters (i.e. 9 models for each subset), and best models were selected based on Bayesian information criterion (BIC) scores; similarly to other information criteria (e.g. Akaike or deviance), models with small scores are

preferred. The best models were unimodal for both females ($n=66$, $BIC=186.31$, $\beta_{\text{size}}=2.165$, $\beta_{\text{darkness}}=0.777$) and males ($n=62$, $BIC=321.82$, $\beta_{\text{size}}=2.023$, $\beta_{\text{darkness}}=0.913$).

Prosoma melanisation

We did not find general support for the thermal melanism hypothesis (Table A1). Indeed, among rapidly developing females, laboratory-reared specimens had darker (i.e. more strongly melanised) cuticle in comparison to females collected from the natural habitat. This appears to support the resource-limited hypothesis of prosoma melanisation, and to contradict the thermal melanism hypothesis, because in the latter we would expect to see weaker melanisation among laboratory-reared individuals owing to the high and stable environmental temperature (Fedorka et al., 2013).

Fecundity and immune parameters

We assessed the association between fecundity and the two antibacterial immune measures in mated females, using data from mated females collected from natural habitat during early and late summer (i.e. slowly and rapidly developing females, respectively; $n=80$, see Table A2).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.R., J.K., A.B., G.K., F.B., F.S., Z.B.; Methodology: Z.R., J.K., A.B., G.K., F.B., Z.B.; Formal analysis: Z.R., J.K., A.B.; Investigation: Z.R., J.K., A.B., G.K., F.B., F.S., Z.B.; Resources: Z.B.; Data curation: Z.R.; Writing - original draft: Z.R., J.K., A.B., G.K., F.B., F.S., Z.B.; Writing - review & editing: Z.R., Z.B.; Visualization: Z.R.; Supervision: F.S., Z.B.; Funding acquisition: Z.B.

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Data availability

Datasets used in our analyses have been uploaded to figshare: <https://doi.org/10.6084/m9.figshare.9942533.v1>.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.219659.supplemental>

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Antibacterial immune functions of subadults and adults in a semelparous spider

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ABSTRACT

Although capacity to mount an efficient immune response plays a critical role in individuals' survival, its dynamics across ontogenetic stages is still largely unexplored. Life stage-dependent variation in the encountered diversity and prevalence of parasites were proposed to contribute to stage-dependent changes in immunity, but differences in life history objectives between developmental stages may also lead to stage-specific changes in efficiency of given immune mechanisms. The reason for this is that juveniles and subadults are unable to reproduce, therefore they invest resources mainly into survival, while adults have to partition their resources between survival and reproduction. The general trade-off between somatic maintenance and reproductive effort is expected to impair immune function. Especially so in semelparous organisms that only reproduce once throughout their lifetime, hence they do not face the trade-off between current and future reproduction. We hypothesised that in a semelparous species individuals would be characterised by decreased investment into somatic maintenance after maturation, in order to maximise their reproductive output. Accordingly, we predicted that (1) elements of somatic maintenance, such as immunity, should be relatively weaker in adults in comparison to subadults, and (2) increased reproductive investment in adults should be associated with lower immune efficiency. We quantified two markers of immunity in subadult and adult individuals of the semelparous wolf spider *Pardosa agrestis* (Westring, 1861), namely bacterial growth inhibition power and bacterial cell wall lytic activity. We found that subadults showed significantly higher cell wall lytic activity than adults, but the two life stages did not differ in their capacity to inhibit bacterial growth. Also, we found weaker immune measures in mated females compared to virgins. Furthermore, in mated females bacterial growth inhibition power was negatively associated with fecundity.

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INTRODUCTION

The capacity to effectively respond to antigens to minimize fitness costs of infection (Owens & Wilson, 1999) is a critically important element of somatic maintenance. Although its efficiency is highly correlated with fitness (Rolff, 2002; Viney, Riley &

Buchanan, 2005), considerable variation persists in the efficacy of immune functions in natural populations (*Brinkhof et al., 1999; Norris & Evans, 2000; Calsbeek, Bonneaud & Smith, 2008*). In the last two decades, immunoecology aimed at shedding light on the causes and consequences of such diversity. Until recently numerous studies were carried out in this subject (reviewed in: *Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Harshman & Zera, 2007; Cotter et al., 2008; Sadd & Schmid-Hempel, 2009; Crino et al., 2013; Palacios, Cunnick & Bronikowski, 2013; Park & Stanley, 2015*), mainly focusing on the variability of adult immunocompetence, and the effects of juvenile-experiences (such as immune-challenge or nutritional deficiency) on adult immunocompetence (*Norris & Evans, 2000; Ricklefs & Wikelski, 2002; Jacot et al., 2005; Stoehr & Kokko, 2006; Martin, Weil & Nelson, 2007; DeBlock & Stoks, 2008; Kriengwatana et al., 2013; Gilbert, Karp & Uetz, 2016*). However, still surprisingly little is known about how the baseline efficiency of the immune system changes through the different life stages.

Differences in the type or density of pathogens individuals might encounter throughout their life cycle were proposed to contribute to across-stage differences in immune function (*Wilson-Rich, Dres & Starks, 2008; Shi & Sun, 2010*). Also, expected reproductive value of different life history actions may change with the progression of ontogeny (e.g. investment in reproduction might be more valuable in mature than in immature individuals), so optimal investment into somatic maintenance, and hence into immunity, might also show stage-specific changes (*Shi & Sun, 2010; Giglio & Giulianini, 2013*). Indeed, juveniles and subadults can't reproduce, therefore they would be expected to invest resources mainly into somatic maintenance, while adults have to partition their resources between maintenance (and hence survival) and reproduction.

Stage-specific characteristics of organisms' life histories can strongly depend on their reproductive strategies. In semelparous organisms, individuals have only one chance to reproduce during their lifetime, and hence cannot trade off between current and future reproduction. Consequently, after maturation they would be expected to prioritise reproduction over somatic maintenance during the production of costly gametes, which starts shortly after maturation. We expect to see this prioritisation to be pronounced in semelparous species, because the production of gametes in sufficient amount (and quality) shortly after maturation has critical impact on lifetime reproductive success, as there will be no future reproductions during which an individual might increase its lifetime reproductive success. In such a scenario, we would expect to see that individuals of a semelparous species invest less into somatic maintenance (and hence into costly elements of immunity as well) in adulthood relative to the somatic maintenance during preadult development.

In this study, we evaluated two proxies of immune capacity in subadult and adult individuals of the wolf spider *Pardosa agrestis* (*Westring, 1861*), and quantified reproductive investment of females using the number of their offspring. Individuals of *P. agrestis* mature and reproduce during spring and early summer, and offspring either overwinter and reproduce in the next year, or during late summer and autumn, i.e. there are two separate adult peaks throughout the year (*Kiss & Samu, 2005; Rádai, Kiss & Samu, 2017*). In general, gamete production starts shortly after maturation in spider females

([Foelix, 2011](#)). Although little is known about sperm production in males, generally males also start gamete production shortly after the final moulting, and sperm is then excreted and stored in the pedipalps until mating. Adults reproduce shortly after maturation, not long after which males perish. Females usually lay one to three cocoons after mating, and carry the hatched spiderlings for a short period of time, then also perish. We chose *P. agrestis* as our model organism because it is a quite abundant semelparous spider in Europe, and is relatively easy to collect and study in laboratory ([Rádai, Kiss & Barta, 2017](#); [Rádai, Németh & Barta, 2018](#)). This semelparous spider species, by definition, reproduces once during its lifetime ([Kiss, 2003](#)). Therefore, based on the argument outlined above, one would expect individuals after maturation to invest less in somatic maintenance compared to premature individuals.

For proxies of immunocompetence we measured the efficiency of bacterial growth inhibition and cell wall lytic activity of tissue homogenates prepared from the spiders' abdomens. In the bacterial growth inhibition assay the availability of antimicrobial peptides is assessed, which are released in the presence of bacteria or fungi at the site of infection, as part of the spiders' humoral immune response ([Gao et al., 2005](#)). These molecules mainly compromise the integrity of cell wall and cell membrane of the invading microbes ([Broden, 2005](#)). Antimicrobial peptides are usually stored in granulocytes (specialised hemocytes), which are produced in the heart wall, from prohemocytes, but can be found in fat bodies as well ([Kuhn-Nentwig & Nentwig, 2013](#)). Lytic activity of lysozymes, is also important part of the humoral immune response. Being glycoside hydrolases, lysozymes conduct the lysis of the peptidoglycans in the bacterial cell wall ([Sharon, 1967](#)). Although it is unknown where lysozymes are produced and stored in spiders, the hemolymph contains them in considerable quantities ([Ahtiainen et al., 2005](#)). Therefore the homogenate sample of the spider abdomen is likely adequate to assess lysozyme activity, as the largest hemolymph vessel (i.e. heart) in the spiders' body is located in the abdomen. Because these biomolecules are not only produced during infection but are also present in the hemolymph and some cells of healthy individuals, measuring the growth inhibition and lytic activity of samples (which are proportional to the concentrations of corresponding molecules in the sample, see [Castella, Christe & Chapuisat, 2010](#)) informs us about the amount of the deployable molecules, and hence the level of investment into the maintenance of these immune functions ([McKean & Lazzaro, 2011](#)).

Although bacterial growth inhibition assay and lytic activity assay assess similar immunological functions (e.g. disruption of the cell wall integrity and production of bacteria), bacterial growth inhibition is a result of the effect of several types of peptides, with various mechanisms acting on a wide range of microbes, i.e. are expected to be effective against microbes in general when released simultaneously. Indeed, a number of bactericide and fungicide antimicrobial peptides were identified in arachnids, which collectively are thought to be efficient in the elimination of a wide range of microbes (see [Bednaski et al., 2015](#)). Notably, lytic activity strictly assesses the activity and availability of lysozymes, i.e. a type of enzymes with the sole function of disrupting the cell wall of Gram positive bacteria. Therefore, the former assay represents a more general assay on the potential capacity to prevent (or halt) microbial invasion, whereas the latter

informs about the potential capacity to neutralise Gram positive bacteria susceptible to lytic enzymes.

Using the above described assays we investigated whether subadult and adult individuals of *P. agrestis* differ in the bacterial growth inhibition power and cell wall lytic activity, i.e. in the maintained capacity to mount either a complex, or a simpler, immediate immune response against microbial infection. For these tests we used males and females as well, in order to see whether the costs of maturation interfere with adult immunity in both sexes. Sexes might differ in how they trade off key life history traits due to their differences in the ways they can maximise reproductive output (Lawniczak et al., 2007; Vincent & Gwynne, 2014). Additionally, in females collected from natural habitats we assessed how reproductive status (whether a female was virgin or not) and fecundity (number of offspring) were associated with the immune parameters we used. We predicted to see stronger immune parameters in subadult specimens, and expected mating and egg laying to negatively affect the tested females' immune measures. Similarly, due to the energetic trade-offs between somatic maintenance and reproduction, we expected to see negative associations between fecundity and the immune parameters.

MATERIALS AND METHODS

Spiders

We collected spiders on three occasions. Firstly, we have established a laboratory-reared population, in which specimens originated from nine cocoon carrying females. These females were collected in May of 2016, from two closely located habitats: an uncultivated plot next to a maize field (between Hajdúszoboszló and Nádudvar, Hungary; 47°26'56.3"N 21°18'00.2"E), and the edge of a maize field (between Hajdúszoboszló and Nagyhegyes, Hungary; 47°28'31.2"N 21°21'57.3"E). These habitats were quite close to one another (ca. five km linear distance), established on an alluvial plain, i.e. with minimal differences were expected in habitat characteristics. After spiderlings hatched in May and June, we separated 58 specimens, and placed each of them separately in plastic cups with a floor area of approx. 25.5 cm². Spiderlings were provided water (wet cotton wool) and food (springtails for the first few moults, then fruit flies for larger spiders) ad libitum. Cups were checked for exuvia (i.e. moulting) every 2 days, and the dates of hatching and moulting were recorded for each spider. Spiders were reared until December, 2016. Prior to the preparation of samples, each spider was categorised to be either as subadult (undeveloped, but recognisable genitals; observed in penultimate specimens one moult prior to maturation), or as adult (fully developed genitals). Henceforth, we refer to spiders that were one moult prior to maturation (i.e. penultimate) as 'subadult'. We chose to work with subadults rather than juvenile instar larvae because the small sizes of juveniles makes it rather difficult to use the established methods reliably. Following the rearing period, we were able to work with 38 spiders, of which 27 were subadults (11 females and 16 males) and 11 were adults (seven females and four males). All laboratory reared spiders used in the immune assays were virgins.

Secondly, we have collected subadult females and adult cocoon carrying females at spring and late summer in 2017 from the habitats described above. Subadult females were

reared until maturity in laboratory. This way we had virgin and mated females as well for our tests. In total we were able to work with 154 females, among which there were 37 virgin and 66 mated females from spring, and 23 virgin and 28 mated females from late summer. Because some of the cocoons from the mated females either enveloped dead embryos, were parasitized by *Idris flavicornis* (Hymenoptera: Scelionidae) wasps (see [Rádai et al., 2018](#)), or were used for other tests, we had fecundity data from 25 spring and 28 late summer females.

Sample preparation

Laboratory-reared spiders were used for sample preparation in December, 2016. Females collected from natural habitats were used for sample preparation in May and August, 2017 in the case of spring and late summer females, respectively.

Bacterial growth inhibition capacity and cell wall lytic activity assays were carried out according to [Castella, Christe & Chapuisat \(2010\)](#). For both methods the samples were prepared as follows. Spiders were killed at -20°C . The abdomen of the spiders was separated from the prosoma by cutting the pedicel. We decided to use full abdomens because the heart and fat bodies, which are known to have central role in the production of immune cells, can be found in the abdomen. Also, a large portion of hemolymph resides in the abdomen, that contains immune cells and lysozyme. Furthermore, the lung-openings and intestines are also found in the abdomen, which comprise a considerable contact surface for potential microbial invasion, so it would be expected that immune parameters of neighbouring tissues faithfully represent the spiders' readiness to keep microbes at bay.

Abdomens were homogenised on ice, in 50 μl PBS (ca. 4°C), and stored on ice until centrifugation. Centrifugation was carried out at $10,000\times g$, at 4°C for 10 min. After centrifugation, 20 μl from the supernatant per sample were collected separately for bacterial growth inhibition and cell wall lytic activity tests, then stored at -20°C until measurements. Prosomas were stored in 96% ethanol on -20°C for later size measurements, which was done with the prosomas photographed on millimetre graph paper, then measuring with ImageJ (version 1.46a, [Schneider, Rasband & Eliceiri, 2012](#)).

Samples from 2016 were tested in January, 2017, and samples from 2017 were tested in December, 2018. During the bacterial growth inhibition tests we always used freshly cultured bacteria (see below) from the same laboratory strain. Also, in the cell wall lysis tests we used the same type of commercially available cell wall residues (see below) in both test sessions.

Bacterial growth inhibition assay

We applied two μl from each tissue homogenate sample on the surface of a thin layer of agar-based medium (using six ml of agar in each petri dish), in duplicates from each spider. Duplicates were placed in the same petri dish in the case of all samples, and in one petri dish 10 samples (i.e. five duplicates) were applied. The medium contained one g of bacto-tryptone, one g NaCl, 0.5 g yeast extract and one g agar in 100 ml distilled water. Also, prior to pouring the agar into petri dishes, 50 μl from a 2.6×10^8 cells ml^{-1} suspension of *Micrococcus luteus* (obtained from Tamás Emri's lab culture at the Department of

Microbial Biotechnology, University of Debrecen) was added and mixed carefully. We used *Micrococcus luteus* because of its availability, and because it is frequently present in soil (Biskupiak et al., 1988; Sims, Sommers & Konopka, 1986); as *P. agrestis* is an epigeic species, it is expected to frequently encounter *Micrococcus luteus* strains. Petri dishes with the samples applied were incubated at 30 °C for 24 h. Samples containing antimicrobial peptides inhibit bacterial growth, therefore empty zones (so called inhibition zones) will persist on the bacterial culture where antimicrobial peptide containing samples were applied. The area of inhibition zones is proportional to the antimicrobial peptide content of the samples. Following the incubation, all petri dishes were photographed, and the areas of inhibition zones were measured using ImageJ (see below).

Bacterial cell wall lytic activity assay

We prepared a simple agar-based medium (one g agar in 100 ml distilled water) containing five mg ml⁻¹ *Micrococcus lysodeikticus* (lyophilized *Micrococcus lysodeikticus*, ATCC No. 4698, Sigma-Aldrich; note that *Micrococcus lysodeikticus* and *Micrococcus luteus* are considered to be the same group of bacteria, e.g. Litman, 1968) cell wall residues homogeneously distributed, and 0.6 mg ml⁻¹ ampicillin sodium. This strain was used because of its commercial availability. The basic principle of this method is that samples containing lysozyme will produce transparent clearing zones in the opaque layer of cell wall containing agar, and the area of clearing zones is proportional to the lysozyme concentration of the sample. We poured five ml of this medium in each petri dish and applied two µl from each sample on the surface of the cell wall agar, in duplicates from each spider. Duplicates were placed in the same petri dish in the case of each sample, and in one petri dish 10 samples (i.e. five duplicates) were applied. Petri dishes were then incubated at 30 °C for 24 h. Following the incubation, all petri dishes were photographed, and the areas of clearing zones were measured using ImageJ (see below).

Statistical analyses

Statistical analyses were performed using the R statistical software (version 3.0.2, R Development Core Team, 2014).

Because the cut-off point of rearing period was the same for all spiders, we checked whether there was a significant difference in the time interval from hatching to sample preparation between subadults and adults, by using Welch's *t*-test on developmental times (i.e. time from hatching to end of rearing period). A lack of significant difference would imply that spiders had approximately the same time to develop, therefore those specimens that reached maturity had a more rapid somatic development than those that couldn't reach maturity (i.e. remained subadults). Conversely, if adult spiders were characterised by significantly longer developmental time it would mean that they hatched more early and had more time to mature, hence their maturation success during the rearing period is not a direct result of faster development. Additionally, we used Chi-squared (χ^2) test to see if there are significant differences in the number of adults and subadults between mother spiders.

Quantifying bacterial growth inhibition and cell wall lytic power

Bacterial growth inhibition and cell wall lytic capacities were quantified as follows. Measured areas of inhibition- and clearing zones (for bacterial growth inhibition and cell wall lytic activity, respectively) were given in pixel count. Photographing the petri dishes was done at different times, hence on the distance between the camera and the petri dishes varied slightly between images. In order to render inhibition/clearing zone values comparable between petri dishes, we divided their area by the area of the given petri dish (all petri dishes were the same size). This resulted in values representing the relative size of inhibition and clearing zones, proportional to the total area of the given petri dish (hereafter relative zone areas). Relative zone areas were divided by the cube of prosoma width to calculate inhibition- and clearing zones corrected for body volume. As whole abdomens of different sized spiders were homogenized in the same volumes of buffer, the relative zones corrected with the cube of prosoma width estimated the inhibition- and lytic power of samples per mm^3 of body volume. In order to aid convergence of model fitting, and interpretation of results after analyses, the variables representing bacterial growth inhibition power and cell wall lytic power (i.e. size-corrected relative zone areas) were centred at zero (by subtracting the mean of the given variable from each value) and rescaled (by dividing all values by the standard deviation of the given variable). Because the response variables were divided by their standard deviation, regression coefficients coincide with the effect sizes (Cohen's d).

Developmental stage differences in immune markers

To quantify growth rate we used a variable calculated as dividing the cube of prosoma width by developmental time (i.e. number of days from hatching to the end of rearing). We analysed our data from an animal-model approach ([Wilson et al., 2010](#)) using the R-package 'MCMCglmm' ([Hadfield, 2010](#)), applying a multi-response mixed effects model. We chose this modelling approach because by fitting only one model we are able (a) to estimate regression coefficients (and the coefficients' credibility) for predictor variables on multiple response variables simultaneously, (b) to control for random effects (such as pseudo-replication from repeated measurements) and hence to estimate measurement repeatability, and (c) to estimate coefficients (and their credibility) representing association between the responses, while controlling for a number of fixed- and random-effects. In this specific model we used data only from laboratory-reared spiders.

The multi-response model was built as follows: bacterial growth inhibition power and cell wall lytic activity were response variables, and, for both response variables, life stage, sex and growth rate were predictors. We did not include interactions between predictors in order to avoid over-parameterization. In the model we used the ID number of spiders as random factor to control for repeated measurements, ID of petri dishes to control for between petri dish error, and the ID number of the spiders' mother to control for kinship between spiders. Variance structures of the model were defined to estimate random intercept- and residual covariance between responses. The former reflects the covariation between random intercepts estimated for the responses, practically

representing correlation between responses. A positive random intercept covariance indicates that those observations with higher-than-average values in the first response variable tend to have similarly higher-than-average values in the second response. Residual covariance represents the covariation between residuals of the responses. For example, a negative residual covariance might indicate the presence of a trade-off between the two responses, in the sense that those individuals investing too much into one trait will be characterised by lower values in the second response. Note that positive random intercept covariance and negative residual covariance can occur at the same time, hinting at that good quality specimens are able to invest more-than-average into both responses, but extremely high investment into the first will not enable similarly high investment to the second (e.g. due to the limited nature of used resources; see [Hadfield, 2018](#)).

The model was run for 115,000 iterations (out of which the first 15,000 was discarded as 'burn-in'), with a thinning interval of 20, resulting in a nominal sample size of 5,000 in the posterior distributions. Following model fitting we have estimated the precision of the used methods as the repeatability of the within-individual measurements, by calculating the proportion of between individual variance to the sum of between individual and residual variance. Low values of this measure indicate low precision, while high values indicate reliable measurements of high precision.

In the results we report parameter estimates (coefficients) as the modal (most frequent, i.e. most probable) values from the posterior distributions. Also, Bayesian credible intervals are presented as the lower (HPD_{lower}) and upper (HPD_{upper}) cut points of the 95 % highest posterior density intervals.

Association between reproductive status, fecundity and immune markers

We have fitted a multi-response model, using data from the females collected from the natural habitats in 2017. In this model the response variables were bacterial growth inhibition power and cell wall lytic activity, and predictors were reproductive status (virgin vs. mated), season of collection (spring vs. late summer), and the interaction between status and season. We defined ID number of spiders, and ID number of petri dishes as random effects to control for pseudo-replication and between-petri error. Similarly to the above described model, we fitted the model in a manner so that random intercept- and residual covariances were estimated. The model was run for 115,000 iterations (out of which the first 15,000 was discarded as 'burn-in'), with a thinning interval of 20, resulting in a nominal sample size of 5,000 in the posterior distributions. Repeatability of immune measurements was calculated the same way as for measurements on laboratory-reared spider samples.

To test how efficiency of bacterial growth inhibition and cell wall lytic activity were associated with fecundity in mated females, we fitted a classical (frequentist) linear regression model. In this model fecundity was the response variable, and the assessed immune measures were predictor variables. We also included season of spider collection as a confounding variable, and its interaction with both immune markers. Because a substantial number of samples produced no measurable inhibition and/or clearing zones,

we additionally fitted binomial generalised regression models, in order to test whether fecundity was associated with the probability of producing measurable (i.e. higher than zero) zones. In these models we specified binary variables as responses, indicating whether inhibition zones in bacterial growth inhibition assay, and clearing zones in cell wall lytic assay, were measurable or not. Predictor variables were fecundity, season of collection, and the interaction term between the two. Since in previous models season appeared to affect the response variables in the above described frequentist models, we estimated variance inflation factor (VIF) for the models, using the R-package ‘fmsb’ (Nakazawa, 2018); since in the frequentist models VIF values were low (below 2.5), we assessed that the correlating independent variables do not cause substantial multicollinearity. Additionally, because for each female we had two measurements of the immune markers in the data used by the afore described two frequentist models, but only had one fecundity value, we used the averaged bacterial growth inhibition and cell wall lytic values for each female.

In the results of Bayesian models we report P_{MCMC} -values as the proportion of MCMC samples not crossing zero in the posterior distribution. These values practically represent the probability that (based on the data) the posterior mean of the regression coefficient is zero. Using the P_{MCMC} values we specified the threshold for significance to be 0.05. Note that assessment of significance using this threshold coincides with the usage of 95% credible intervals, i.e. statistical significance can be assessed based on whether the credible intervals include zero. That being said, we decided to still report P_{MCMC} values to ease interpretation of results for those not familiar/comfortable with Bayesian statistics.

RESULTS

Developmental stage differences in immune markers

Spiders that reached maturity by the end of the rearing period on average hatched earlier than those that only reached subadult stage ($t_{31.6} = -4.38$, $P < 0.001$), indicating that individuals became adults because they had more time to develop rather than having much more rapid development. Also, the Chi-squared test showed no significant difference in the number of adults and subadults between mothers ($\chi^2_8 = 4.90$, $P = 0.768$).

There was no significant difference in bacterial growth inhibition power between subadult and adult spiders (Figs. 1A and 1B), and growth rate did not affect bacterial growth inhibition (Table 1). Males showed marginally significantly lower values of bacterial growth inhibition than females (Fig. 1B). Adults showed significantly lower cell wall lytic activity than subadults (Figs. 1C and 1D), but there was no difference between sexes, and growth rate did not have significant effect on cell wall lytic activity (Table 1). The estimated precision of the bacterial growth inhibition measurements was quite high ($R = 0.87$, $\text{HPD}_{\text{lower}} = 0.71$, $\text{HPD}_{\text{upper}} = 0.93$). Precision of cell wall lytic activity measurements was moderately good ($R = 0.63$, $\text{HPD}_{\text{lower}} = 0.39$, $\text{HPD}_{\text{upper}} = 0.80$).

We found no significant random intercept- (random intercept covariance = -0.06 , $\text{HPD}_{\text{lower}} = -0.22$, $\text{HPD}_{\text{upper}} = 0.13$, $P_{\text{MCMC}} = 0.672$) or residual covariance (residual covariance = 0.004 , $\text{HPD}_{\text{lower}} = -0.03$, $\text{HPD}_{\text{upper}} = 0.05$, $P_{\text{MCMC}} = 0.560$) between bacterial growth inhibition and cell wall lytic activity.

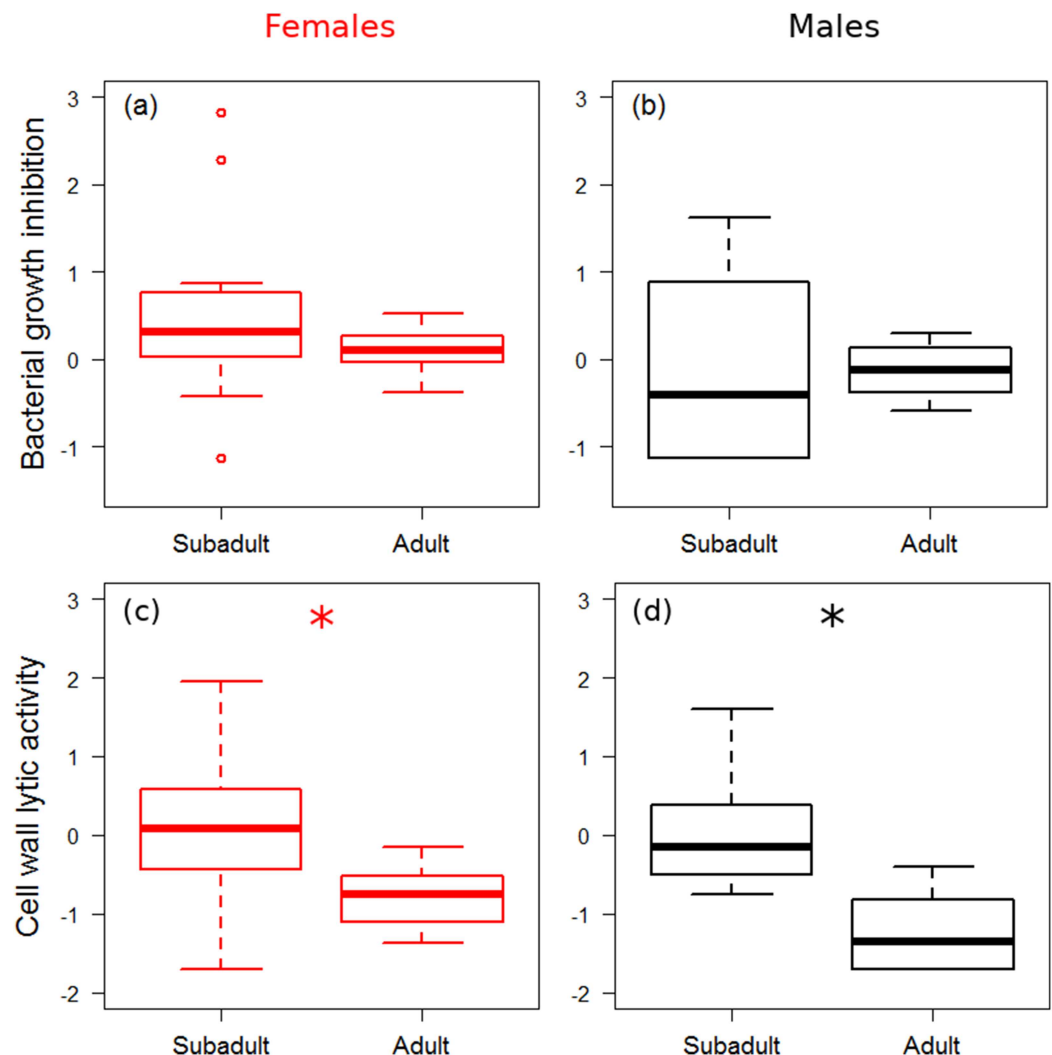


Figure 1 Immune function across ontogenetic stages. Box plot visualisation of bacterial growth inhibition (A and B) and cell wall lytic activity (C and D) of females (A and C) and males (B and D) of laboratory-reared spiders ($N = 38$). Whiskers visualise minimum and maximum ranges, within which boxes represent interquartile range between first and third quartiles; horizontal solid lines show median of the given value distribution; individual circles show outlier values. Asterisks mark significant effect or difference ($P \leq 0.05$).

Full-size [DOI: 10.7717/peerj.7475/fig-1](https://doi.org/10.7717/peerj.7475/fig-1)

Association between reproductive status, fecundity and immune markers

The interaction between season and reproductive status in bacterial growth inhibition power was not significant (Coefficient: 0.429, $\text{HPD}_{\text{lower}} = -0.298$, $\text{HPD}_{\text{upper}} = 1.121$, $P_{\text{MCMC}} = 0.245$), and mated females showed significantly lower levels of bacterial growth inhibition (Figs. 2A and 2B; Table 2). Females that were collected during spring showed significantly lower levels of bacterial growth inhibition than females from late summer (Fig. 2) (Coefficient: -1.220 , $\text{HPD}_{\text{lower}} = -1.784$, $\text{HPD}_{\text{upper}} = -0.681$, $P_{\text{MCMC}} < 0.001$).

The effect of interaction between reproductive status and season was significant on the cell wall lytic activity (Coefficient: 1.064, $\text{HPD}_{\text{lower}} = 0.412$, $\text{HPD}_{\text{upper}} = 1.757$,

Table 1 Fixed parameter estimates from the multi-response model on the effect of developmental stage and sex on immune markers. For categorical predictors (ontogenetic stage and sex) the contrast parameter is presented (i.e., average difference between two contrasted groups; subadult (S) to adult (A) in Stage, female (F) to male (M) in Sex). Asterisks mark significant effect or difference ($P \leq 0.05$).

Response	Predictors	Coefficient	HPD _{lower}	HPD _{upper}	P_{MCMC}
Bacterial growth inhibition	Stage (S-A)	0.27	-0.30	0.86	0.304
	Sex (F-M)	-0.50	-1.03	0.03	0.067
	Growth rate	0.71	-5.23	6.27	0.790
Cell wall lytic activity	Stage (S-A)	1.04	0.57	1.60	<0.001*
	Sex (F-M)	-0.17	-0.66	0.33	0.489
	Growth rate	1.46	-4.41	7.12	0.620

$P_{\text{MCMC}} = 0.004$); while there was no significant difference in cell wall lytic activity between virgin and mated females collected during spring (Coefficient: -0.216, HPD_{lower} = -0.621, HPD_{upper} = 0.219, $P_{\text{MCMC}} = 0.362$), among the females collected in late summer mated specimens showed significantly lower cell wall lytic activity than virgin females (Coefficient: -0.859, HPD_{lower} = -1.237, HPD_{upper} = -0.486, $P_{\text{MCMC}} < 0.001$; Figs. 2C and 2D).

The estimated precision of both the bacterial growth inhibition ($R = 0.84$, HPD_{lower} = 0.78, HPD_{upper} = 0.88) and cell wall lytic activity measurements were high ($R = 0.84$, HPD_{lower} = 0.77, HPD_{upper} = 0.88). There was no significant random intercept (random intercept covariance = 0.009, HPD_{lower} = -0.078, HPD_{upper} = 0.087, $P_{\text{MCMC}} = 0.918$) or residual covariance (residual covariance = -0.007, HPD_{lower} = -0.021, HPD_{upper} = 0.008, $P_{\text{MCMC}} = 0.297$) between bacterial growth inhibition and cell wall lytic activity, indicating no measurable association between them.

In the linear regression model, neither of the interaction terms were significant (between bacterial growth inhibition and season: Coefficient = -2.564, SE = 3.213, $t = -0.80$, $P = 0.429$; between cell wall lytic activity and season: Coefficient = -0.699, SE = 2.773, $t = -0.25$, $P = 0.802$), therefore we excluded them from the final model. Bacterial growth inhibition power showed significant negative association with fecundity in mated females (Fig. 3A), but cell wall lytic activity did not (Fig. 3B; Table 3). Also, females collected during spring were significantly more fecund than late summer females (Table 3).

In the binomial regression model on bacterial growth inhibition the interaction between fecundity and season was not significant (Coefficient = 0.102, SE = 0.085, $t = 1.20$, $P = 0.229$), and season did not significantly affect the probability of producing a measurable inhibition zone (Coefficient = -2.503, SE = 2.834, $t = -0.88$, $P = 0.377$). Fecundity was negatively associated with this probability, meaning that samples from more fecund females were more likely to produce no bacterial growth inhibition at all (Coefficient = -0.148, SE = 0.067, $t = -2.20$, $P = 0.028$). In the binomial regression model on cell wall lytic activity the interaction between fecundity and seasonality was significant (Coefficient = 0.281, SE = 0.128, $t = 2.20$, $P = 0.028$), showing that fecundity negatively affected the probability of producing measurable clearing zones only among late summer females (Coefficient = -0.238, SE = 0.118, $t = -2.02$, $P = 0.044$). Probability of producing

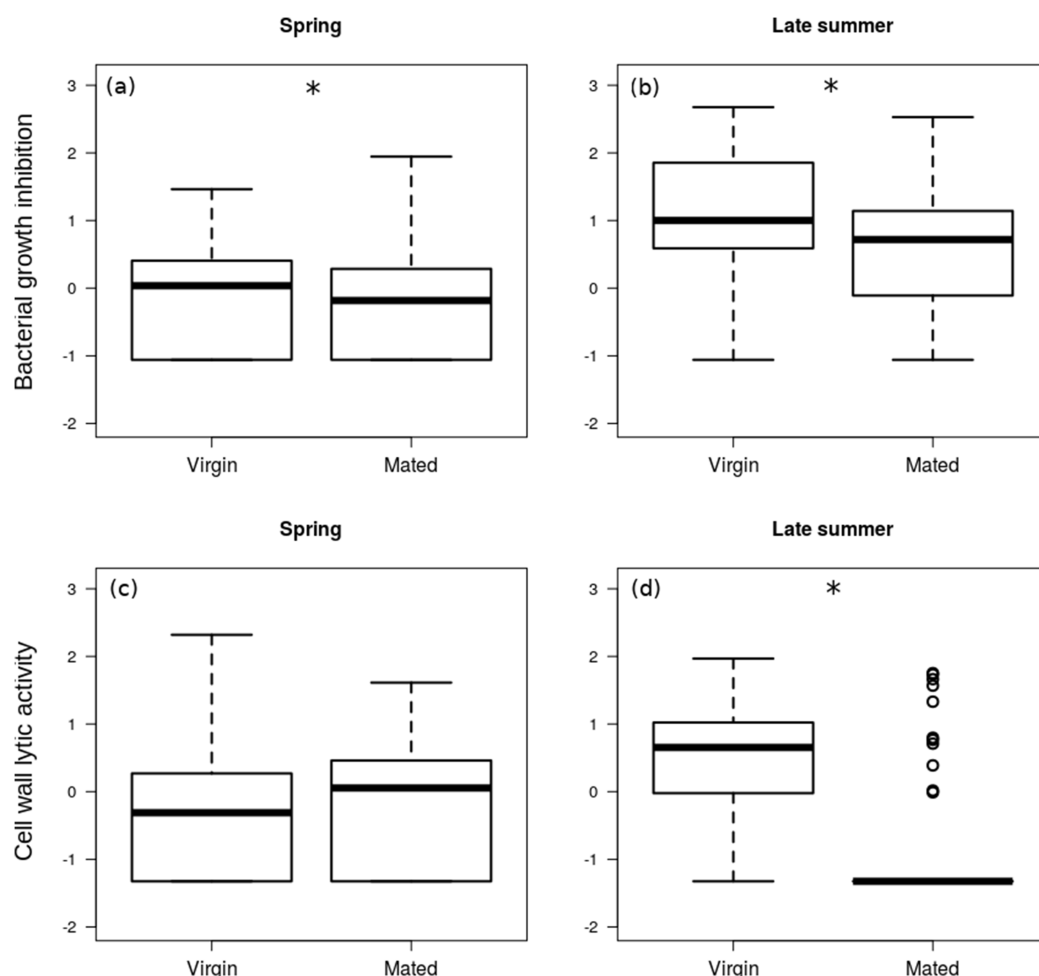


Figure 2 Immune function in females. Box plot visualisation of bacterial growth inhibition power (A and B) and cell wall lytic activity (C and D) of virgin vs. mated females, collected during spring (A and C; $n = 108$) and late summer (B and D; $n = 51$). Whiskers visualise minimum and maximum ranges, within which boxes represent interquartile range between first and third quartiles; horizontal solid lines show median of the given value distribution; individual circles show outlier values. Asterisks mark significant effect or difference ($P \leq 0.05$). [Full-size](#) DOI: 10.7717/peerj.7475/fig-2

Table 2 Contrasts acquired from the multi-response model on the effect of reproductive status and season on females' immune markers. In order to make results easier to comprehend and interpret, contrasts for the given group-interactions are presented, instead of individual parameter estimates directly from the fitted model. Asterisks mark significant difference ($P \leq 0.05$).

Response	Contrast	Coefficient	HPD _{lower}	HPD _{upper}	P_{MCMC}
Bacterial growth inhibition	Spring: virgin-mated	0.51	0.11	1.22	0.030*
	Late summer: virgin-mated	0.60	0.13	1.24	0.019*
Cell wall lytic activity	Spring: virgin-mated	-0.16	-0.52	0.45	0.759
	Late summer: virgin-mated	1.24	0.64	1.88	<0.001*

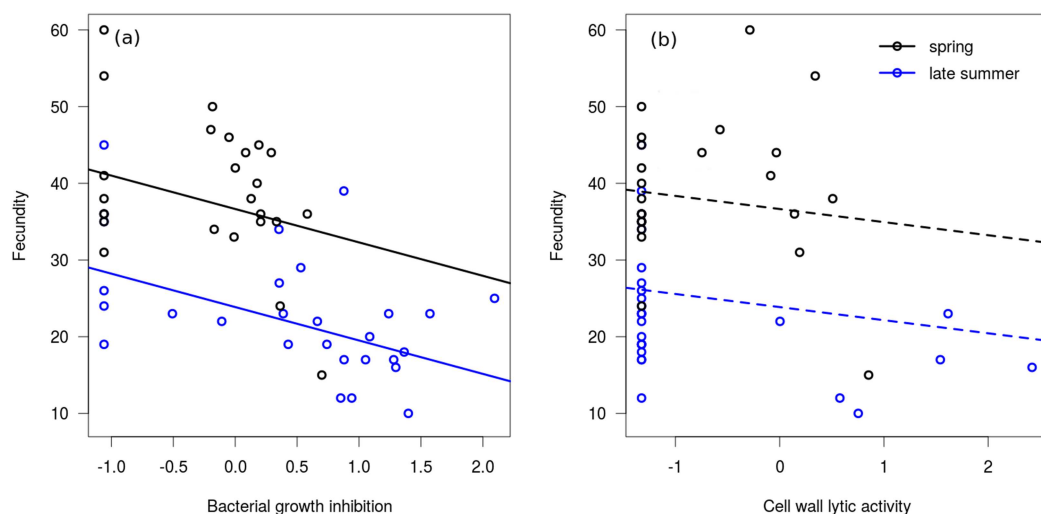


Figure 3 Immune function and fecundity. Association of bacterial growth inhibition (A) and cell wall lytic activity (B) with fecundity in mated females collected in spring (black circles; $n = 25$) and autumn (blue circles; $n = 28$). Lines show regression lines from the fitted linear model: solid and dashed lines represent significant ($P \leq 0.05$) and non-significant ($P > 0.05$) associations, respectively.

Full-size [DOI: 10.7717/peerj.7475/fig-3](https://doi.org/10.7717/peerj.7475/fig-3)

Table 3 Fixed parameter estimates from the linear regression model on the association between immune markers and season with fecundity in mated females. For the predictor variable Season the contrast coefficient is given, representing the difference of spring specimens from late summer specimens. Asterisks mark significant effect ($P \leq 0.05$).

Predictors	Coefficient	SE	t-value	P
Bacterial growth inhibition	-4.15	1.31	-3.16	0.003*
Cell wall lytic activity	-1.67	1.15	-1.45	0.153
Season (spring)	12.79	2.36	5.41	<0.001*

measurable clearing zone was only marginally significantly lower in spring females (Coefficient = -5.595, SE = 2.986, $t = -1.87$, $P = 0.061$).

DISCUSSION

Subadult *P. agrestis* specimens had stronger lytic activity against bacterial cell wall compared to adult individuals. This result indicates that tissue homogenate samples from subadult spiders contained higher amount of lysozyme per unit body volume, than did samples from adults. This is in accordance with our prediction based on life history theory, namely that in a semelparous species one would expect to see relatively lower efficiency of somatic maintenance in adults compared to subadults. We propose that this difference is likely due to a shift in resource allocation patterns, altering the prioritisation between somatic maintenance and reproduction. Subadults do not have fully developed, functional reproductive organs yet (Foelix, 2011), so their reproductive tissues are likely to have lower demand of resource income in comparison to adults. Mature individuals, however, have to invest resources into gonad development and gamete production. Consequently, a higher proportion of the acquired energy and nutrients can be assigned to somatic tissues

and to elements of somatic maintenance in subadults, while these limited resources will be partitioned into reproductive tissues and gamete production as well in adults. Producing gametes has considerable costs ([Hayward & Gillooly, 2011](#); [Edward & Chapman, 2011](#); [Michalik & Rittschhof, 2011](#)), so by paying the costs of reproduction, adults are presumably able to assign fewer resources to somatic maintenance.

The decreased lytic activity in adults in comparison to subadults may be associated with lower resource allocation into this particular element of immunity, which would be in accordance with our predictions. In *Meligethes aeneus* it was shown that out of 10 screened lysozymes 4 were expressed in higher levels in juveniles in comparison to adults, although in the case of 3 lysozymes, expression levels were higher in adults ([Vogel et al., 2014](#)). In another study on white shrimps (*Litopenaeus vannamei*) it was found that expression level of the screened lysozyme was elevated in most of (albeit not in the last few) post-larval stages compared to adults ([Gollas-Galvan et al., 2017](#)), indicating a dynamic change in lysozyme availability, with a peak during the subadult stages. Notably, energetic costs of reproduction were found to be negatively linked to lytic activity in the carabid beetle *Carabus lefebvrei*, where mated individuals showed dramatically decreased lytic activity in comparison to virgin individuals ([Giglio et al., 2016](#)), showing that not only maturation, but reproduction too can deteriorate the efficiency of this immune function. Indeed, we found that in mated females collected during late summer, bacterial growth inhibition power and cell wall lytic activity were lower than in virgin females, indicating that the cost of reproduction is relatively higher (or, at least, more pronounced) in late summer females. Females from spring and late summer belong to two separate generations of different life cycles ([Kiss & Samu, 2005](#); [Rádai, Kiss & Samu, 2017](#)), and it is not clear how external seasonal conditions and the spiders' intrinsic physiological characteristics contribute to the observed patterns. Albeit we acknowledge the potential importance of this aspect of the life cycle of *P. agrestis* spiders, reconciling the mechanism behind this seasonal difference in lytic activity is beyond the scope of this study.

In past studies, immune measures in subadults were found to show comparable, or even higher levels than in adults in a handful of invertebrate species. For example, although not assessed explicitly, in the study of [Vogelweith et al. \(2017\)](#) earwig (*Forficula auricularia*) juveniles seemed to have hemocyte concentrations in their hemolymph similar to that of adults, and 4th instar (penultimate) larvae even had higher hemocyte counts than adults. Also, 4th instar larvae appeared to have slightly higher total phenoloxidase activity compared to adult males, but not to females. [Piñera et al. \(2013\)](#) found that 8th and 9th instar larvae of the cricket *Acheta domesticus* had hemocyte loads and encapsulation efficiency similar to, or in some cases higher than, adults. However, juveniles had considerably lower phenoloxidase activity compared to adults. In the cricket *Gryllus texensis* males were shown to have lower phenoloxidase activity than nymphs, and their phenoloxidase activity considerably declined with age after maturation ([Adamo, Jensen & Younger, 2001](#)).

Surprisingly, subadults did not seem to differ from adults in bacterial growth inhibition power against *Micrococcus luteus*. This result suggests no change in the overall availability of antimicrobial peptides due to maturation. Considering studies similar to ours, however,

it seems unlikely that antimicrobial peptide levels do not change at all throughout the life cycle. For example, expression levels of antimicrobial peptides were shown to change over ontogeny in the pollen beetle *Meligethes aeneus* (Vogel *et al.*, 2014). Notably, only some of the screened antimicrobial peptide transcripts were observed to decrease after maturation, indicating that not only the overall availability of these biomolecules might show ontogenetic stage-dependent shifts, but also the composition of them. How changes in the composition of available antimicrobial peptides affect the animals' capacity to overcome pathogen invasion, is a question to be considered in future studies. Notably, the absence of significant difference in bacterial growth inhibition between subadults and adults could be a result of depressed antimicrobial protein concentration in both tested life stages, in comparison to juveniles. Unfortunately, in the current study design we cannot confirm whether this is the case or not.

We saw similar patterns in males and females regarding the effect of maturation on the assessed immune markers. Although males showed somewhat lower capacity for bacterial growth inhibition compared to females, this difference was only marginally significant, and there was no sex difference in cell wall lytic capacity. While gamete production is expected to be more costly in females, our observations do not show stronger effect of maturation in females in either of the immune measures. In the past, costs of sperm production in males was thought to be relatively 'cheap', but recent studies show that males also pay considerable energetic costs for gamete production and storage (Michalik & Rittschof, 2011), which costs might have contributed to similar energetic conflicts between the tested immune markers and reproductive investment during maturation in the sexes. Notably, due to the low final sample sizes of data on the laboratory reared spiders drawing general conclusions was made with caution, as some ambiguities in our results may have resulted from the low sample size, such as the absence of significant sex-differences in the immune measures, or their association with life stage. Nevertheless, the similar decrease in lytic activity in both sexes might suggest that not only gamete production, but maturation itself also has considerable energetic costs that might mask sex-differences in the changes in immunity associated with the life stage transition.

While mated females collected in late summer showed a much more prominent decrease in cell wall lytic activity than in bacterial growth inhibition, and subadults and adults only differed in cell wall lytic activity, we found no significant linear association between cell wall lysis and fecundity. It should be noted, however, that a considerable number of samples from mated females failed to induce measurable clearing zones on the cell wall agar. Consequently, the number of observations in which we could regress fecundity on non-zero values of cell wall lytic activity was quite low, rendering it difficult to identify a credible linear association between these variables. Based on the binomial models, females with higher fecundity were more likely to have undetectable lytic activity, although this observation was significant only in late summer females. Bacterial growth inhibition showed no stage-differences, and was only slightly decreased in mated females. Note, however, that bacterial growth inhibition showed considerable negative correlation with fecundity, both in spring and late summer females. While this correlation was not significant in the case of lytic activity, more fecund late summer females were still more

likely to exhibit zero cell wall lytic competence. Altogether, these observations seem to support that physiological costs of production and storage of the wide variety of antimicrobial biomolecules may interfere with elements of reproduction. Indeed, reproduction (and reproductive effort) is often shown to be negatively associated with elements of immunocompetence ([Nordling et al., 1998](#); [Schwenke, Lazzaro & Wolfner, 2016](#)).

We propose that lower adult capacity of bacterial cell wall lysis, and decreased capacity to inhibit bacterial growth and to break down cell wall in mated females characterised by high fecundity, may be due to that adults have only one short time period for reproduction. As such, the trade-off between immunity and fecundity might be expected to be quite substantial in species that reproduce only once throughout their lifetime. The main argument for this is that they have to invest as much as possible into the first reproduction in order to maximise reproductive output, because in semelparous organisms there are no future reproductions, i.e. the first reproduction is also the last. As such, this may lead to a 'quasi-terminal investment' into reproduction, in which an individual might take the risk of having some elements of immunocompetence at a decreased efficiency due to decreased investment into somatic maintenance, for the prospect of increased reproductive success. The negative correlation between bacterial growth inhibition power and fecundity, and the decreased capacity of producing measurable bacterial growth inhibition and cell wall lysis in more fecund females appear to support this hypothesis, as those females that 'gave up' more capacity in the assessed immune measures tended to have a larger number of offspring. Maximising reproductive output through a terminal investment-like scenario would be expected to be favoured by natural selection in environments where adult future mortality rate is high (assuming that this high mortality is not due to pathogens), which is known to be characteristic to populations of semelparous species ([Goldstein, Merrick & Koprowski, 2017](#); [Sæther, 1988](#); [Young, 2010](#)).

Differences in expected pathogen diversity or prevalence were also proposed to drive the evolution of life stage differences in immune function ([Wilson-Rich, Dres & Starks, 2008](#); [Shi & Sun, 2010](#)). We, however, find this proposition unlikely to be the main cause of the difference in lytic activity between subadults and adults in *P. agrestis*, because they reside in the same habitat and are quite the same in their foraging habits and preys ([Nyffeler & Benz, 1988](#); [Samu & Szinetár, 2002](#); Z. Rádai, 2016, personal observations).

Because both the maintenance of an efficient immune system and a higher rate of post-embryonic development are considered to be costly, one might expect the presence of a negative correlation between them ([Modak et al., 2009](#); but see [Rantala & Roff, 2006](#)). However, we found no significant trend regarding the relationship between growth rate and bacterial growth inhibition or cell wall lytic activity in the laboratory-reared spiders. Notably, ad libitum availability of food might have mitigated the energetic conflict between these traits leading to the observed results ([Brzek & Konarzewski, 2007](#); [Mangel & Stamps, 2001](#)). In a previous study we have found a positive relationship between age at maturation and encapsulation efficiency in *P. agrestis* adults ([Rádai, Németh & Barta,](#)

2018), meaning that individuals characterised by faster development showed weaker encapsulation response. In the cricket *Gryllus bimaculatus*, developmental time was found to be positively correlated with lytic activity of the hemolymph, but was negatively associated with encapsulation rate (Rantala & Roff, 2005).

The result that no significant relationship was found between bacterial growth inhibition power and cell wall lytic activity either suggests that there is little, or no energetic conflict between these two immune parameters, or that other, in physiological sense costly, traits obscure this conflict between the two assessed elements of immunity. A potential energetic conflict may also have been masked by the unrestricted food availability of the laboratory reared specimens. Also, the absence of a positive association implies that individuals that invest relatively more in bacterial growth inhibition are not necessarily able (or prone to) invest similarly high amount of resources into lysozyme production (and vice versa). It should be noted, though, that while cell wall lytic activity is mainly dependent on lysozymes, bacterial growth inhibition results from the presence of many types of peptides and proteins, which lysozyme is also a part of. Hence, the failure to detect a significant relationship between bacterial growth inhibition and cell wall lytic activity may be due to the sheer number of biomolecules with a role in bacterial growth inhibition that can have diverse associations with lysozymes, rendering it difficult to identify a consistent association between them. It is also noteworthy that in the case of other elements of immunity it is often found that there is no strict association between assessed immune parameters (Fedorka, Zuk & Mousseau, 2004; Gershman et al., 2010; Kortet, Rantala & Hedrick, 2007; Piñera et al., 2013).

CONCLUSIONS

Overall, our results show that in a semelparous species adults are characterised by lower cell wall lytic activity in comparison with subadults, but we found no evidence for subadults and adults to differ in their bacterial growth inhibition power. We argue that a likely explanation for relatively higher subadult lytic activity may be associated with an energetic conflict leading to a shift in prioritisation between somatic and reproductive tissues in adults. This conflict is expected on the basis that adults of a semelparous species don't face the trade-off between current and future reproduction therefore maximal reproductive success may be achieved by allocating more resources into reproductive tissues, rather than in somatic maintenance and long-term survival. Also, bacterial growth inhibition was negatively associated with fecundity in mated females, hinting at the presence of a trade-off between investments into reproduction vs. somatic maintenance. Indeed, among late summer specimens mated females had much weaker bacterial growth inhibition power and cell wall lytic activity than virgins. Admittedly, in future studies higher sample sizes and better balanced study designs will be needed in order to clarify the remaining ambiguities of our results. Also, comparative studies on both semelparous and iteroparous species would undoubtedly help to reconcile the role of reproductive strategies in shaping trade-offs between key life history traits. We argue that *P. agrestis* would be an excellent model organism to study both the intrinsic and extrinsic factors that might contribute to these elements of life history evolution.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Zoltán Rádai conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Péter Kiss performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Dávid Nagy performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Zoltán Barta contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

Raw data are available in the [Supplemental Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.7475#supplemental-information>.

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Sex-dependent immune response in a semelparous spider

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Abstract

Previous studies on arthropods showed that seasonality and parity in breeding considerably impact the direction of sex differences in immunocompetence, and it has been suggested that life span and the time window available for breeding play key roles in shaping sex-differences in immunity. One proposed mechanism behind this phenomenon is differential investment into life history traits in sexes. Here, we tested whether in a seasonally breeding semelparous arthropod sexes differ in their immunocompetence, predicting that females would show weaker immune response than males. We compared encapsulation efficiency (a well-established and widely used method for assessing immunocompetence) of freshly matured, virgin males and females of the lycosid spider *Pardosa agrestis* (Westring, 1861). On average, males mounted stronger immune response than females and the extent of encapsulation was positively associated with prosoma length in males, but not in females. Also, time until maturation was positively related to the extent of encapsulation in both sexes, but did not significantly affect adult prosoma length. We propose that sex-difference in encapsulation is likely shaped by combined effects of relatively higher costs of reproduction in females, narrow time window of reproductive activity, and the absence of trade-off between current and future reproduction.

Keywords Sex-bias · Immunity · Reproductive strategy · Semelparity

Introduction

Immunocompetence often varies between sexes (Aisenberg and Peretti 2011; Fargallo et al. 2002; Schwarzenbach et al. 2005; Zuk et al. 2004). This has been suggested to arise from differential reproductive investment by the sexes (Vincent and Gwynne 2014), differentially affecting immune function (French et al. 2007; Hayward and Gillooly 2011). As of the direction of sex-differences in immunity, studies on invertebrates yielded mixed results, with observations in comparable numbers for both male- and female-biased immune-efficiency (Kelly 2016; Schwarzenbach et al. 2005; Vincent and Gwynne 2014; Zuk et al. 2004). When considering the same immunological processes across different species, this diversity is likely explained by the larger between-species differ-

ences in breeding characteristics and fitness-limiting resources in invertebrates (Lin et al. 2016; Vincent and Gwynne 2014).

Studies carried out on seasonally breeding semelparous arthropods have shown that males often have significantly more efficient immune function compared to females (e.g. Cappa et al. 2015; Zuk et al. 2004). However, in arthropods breeding continuously (or, relative to their life span, have more time to reproduce), females appeared to exhibit stronger immunocompetence than males (McKean and Nunney 2005; Zuk et al. 2004). These findings suggest that the direction of sex-difference in immunocompetence is considerably affected by life span and length of reproductive activity. Zuk et al. (2004) argued that in species with short reproductive period individuals have to concentrate their resources on reproduction more strictly, and higher proportion of limited resources must be invested in reproduction in order to maximise reproductive success. As females invest relatively more energy in the development of reproductive tissues than males, the trade-off between reproductive effort and somatic maintenance should be more pronounced in females, resulting in relatively lower immunocompetence compared to males.

Based on Zuk et al. (2004), we predicted that females of a seasonally breeding semelparous species show lower immunocompetence relative to males. In our study, we assessed the

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encapsulation efficiency in the semelparous *Pardosa agrestis* (Westring 1861). During encapsulation, a foreign object is recognised and enveloped by melanized haemocytes, in order to isolate and neutralise it (Lackie 1980; Söderhäll and Cerenius 1998). Encapsulation has considerable deployment costs, meaning its physiological costs are realised during immune challenge, e.g. due to resource allocation into production and transportation of melanized haemocytes (see Schmid-Hempel 2003). Because of this, assessing the extent of encapsulation is thought to reflect the amount of available resources a given individual is able to allocate into the immune response.

Methods

Eight cocoon carrying females were collected between Hajdúszoboszló and Nádudvar (Hungary) in August, 2015. Spiderlings ($N=61$) were reared individually in plastic cups (28 cm² floor area) in standard laboratory conditions, provided food (springtails for small, fruit flies for larger spiders) and water ad libitum. By the end of the experiment, 42 specimens from 7 females matured (17 males, 25 females). Number of maturing spiders from females varied between 1 and 9 (6 individuals per female on average). Spiders matured in 74 ± 27 days (mean $\pm$ SD) and were tested 2–4 days after final moult, based on the methodology described by Ahtiainen et al. (2005). Briefly: spiders were anaesthetized at -20°C , then taped laterally on glass slides. Immediately after that, a sterile nylon monofilament (1 mm long, 0.12 mm diameter) was implanted in the dorsal haemocoel, which was removed after 3 h, then dried at 5°C for a day. Dried implants were photographed from two angles next to a clean control filament. Images were analysed using ImageJ (Schneider et al. 2012). We quantified encapsulation as the difference in modal grey value between clean and used filaments. Since each implant was photographed twice, we used the mean value of the two measurements as encapsulation score for each spider. After the tests, the spiders were photographed on millimetre graph paper and prosoma length of each spider was measured with ImageJ.

Statistical analysis was done using R (version 3.0.2, Team RC 2014). We fitted linear mixed-effects models (LMMs) with the R-package “lme4” (Bates et al. 2015). Encapsulation score was response variable and sex, prosoma length (mm), interaction between them, and number of days to maturation were predictors. To achieve good model convergence, we rescaled the values of encapsulation score, by subtracting the mean encapsulation score from each value, then dividing them by two standard deviations (Gelman 2008). To obtain sex-specific estimates of mean encapsulation values directly from the model, we have centered the prosoma length variable at zero, by subtracting the mean prosoma

length from each value. We similarly centered maturation time at zero.

To see whether time until maturation affects prosoma length, another LMM was fitted, in which prosoma length was response variable, while maturation time, sex, and their interaction were predictors. To control for relatedness among spiders, ID of mother spiders was included as random variable in both LMMs.

Results and discussion

On average, males showed stronger encapsulation than females ($t_{36.65} = 2.97$, $P = 0.005$). Prosoma length was positively correlated with the encapsulation score in males ($t_{36.39} = 3.41$, $P = 0.002$) but not in females ($t_{36.93} = -0.30$, $P = 0.769$; Fig. 1). In both sexes, number of days until maturation showed significant positive association with the extent of encapsulation ($t_{36.22} = 3.13$, $P = 0.003$; Fig. 2). In the second LMM, interaction term between maturation time and sex was not significant ($t_{38} = -1.34$, $P = 0.187$) therefore, we excluded it from the final model. Time until maturation did not have significant effect on prosoma size ($t_{39} = 1.19$, $P = 0.242$). Also, males tended to be smaller than females, although this size difference was not significant at the 0.05 threshold ($t_{39} = -1.85$, $P = 0.072$).

Our results show that on average encapsulation response of virgin *Pardosa agrestis* adults is stronger in males than in females. Because individuals of *P. agrestis* do not invest in long-term survival and future reproduction, individual fitness may be maximised by allocating resources in current

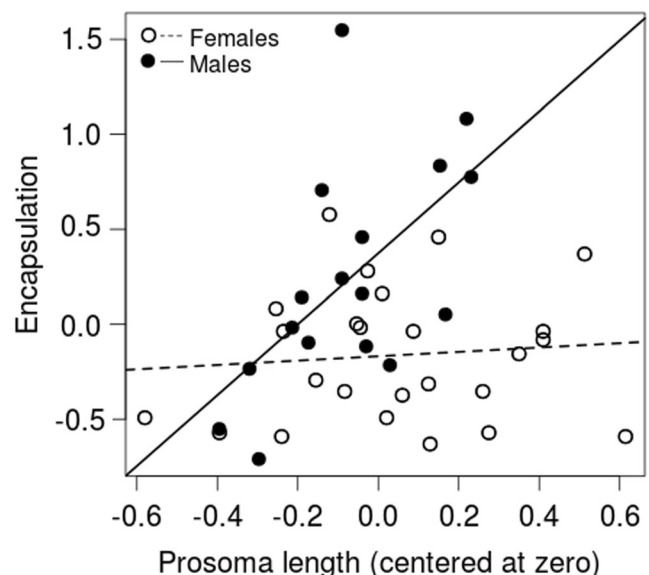


Fig. 1 The relationship between prosoma length, sex and the extent of encapsulation in *Pardosa agrestis*. Open circles (and dashed line) represent females, while filled circles (and solid line) represent males

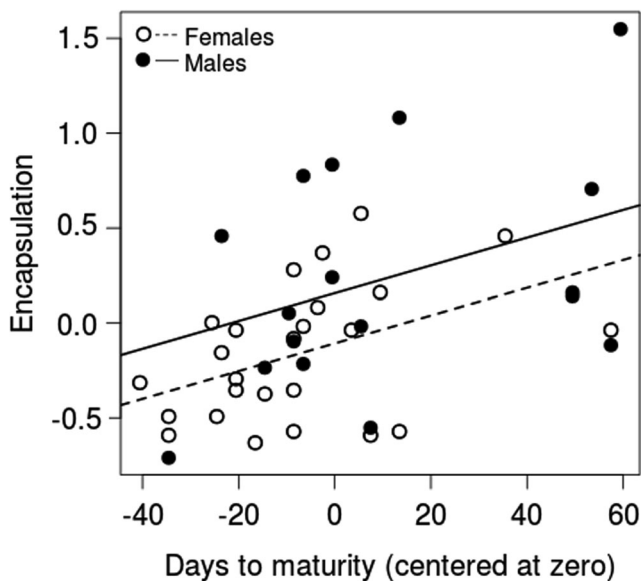


Fig. 2 The relationship between developmental time, sex and the extent of encapsulation in *Pardosa agrestis*. Open circles (and dashed line) represent females, while filled circles (and solid line) represent males

reproduction at the expense of somatic maintenance. Since the tested spiders matured few days before encapsulation tests, it is likely that the difference in encapsulation efficiency between the sexes resulted from the demand of resources for egg production in females, which starts shortly after maturation (Foelix 2011). Resource demand for egg production is assumed to be higher than for sperm production (Hayward and Gillooly 2011). Besides the high costs of egg production, the short time period of reproductive activity is likely to have contributed to this discrepancy. As discussed by Zuk et al. (2004), in species characterised by short breeding periods, individuals are limited not only by resources, but by time as well. Consequently, females of such species must sequester available resources for egg production in a narrow time window, during which reproductive output can be increased by trading off somatic maintenance for investment into offspring. However, due to our study design, we obtained only a snapshot of immunocompetence in the adult life of virgin spiders; thus, we cannot exclude the potential for temporal changes in encapsulation efficiency. In future studies, it should be investigated whether there are temporal changes in the encapsulation efficacy in adults.

The result that prosoma length and encapsulation efficiency were positively associated only in males suggests different selection pressures acting on male and female immunity and condition. One explanation might be that male body size is an honest signal, based on which females might be able to assess male “quality”. In other lycosid spiders, different signalling traits were shown to be positively correlated with encapsulation efficiency, and it was suggested that females might obtain fitness benefits by choosing better quality males, and producing offspring with better immune function (Ahtiainen et al.

2004, 2005; Uetz et al. 2002). Unpublished results from laboratory experiments also suggest a preference for larger males on the part of females in *P. agrestis* (Kiss, personal communication), although this preference depends on the reproductive status of females, a phenomenon observed in other spider species as well (e.g. Maklakov et al. 2004).

Finally, relatively rapidly developing individuals exhibited lower encapsulation efficiency than slower developing ones, which is consistent with results from past studies on other invertebrates (e.g. Niemelä et al. 2013), and with the predictions of the pace-of-life syndrome hypothesis (Ricklefs and Wikelski 2002), indicating that increased developmental rate bears considerable physiological costs in spiders as well.

In conclusion, we found that in a seasonally breeding semelparous invertebrate species, mature males have stronger encapsulation response than females. This difference likely results from the interplay of relatively higher costs of reproduction in females, limited time available for reproduction and absence of trade-off between current and future reproduction. These results highlight the importance of sex, parity and seasonality in shaping immune function in this, and likely other invertebrate species.

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List of publications related to the dissertation

Foreign language scientific articles in international journals (5)

1. **Rádai, Z.**, Kiss, J., Babczyńska, A., Kardos, G., Báthori, F., Samu, F., Barta, Z.: Consequences of rapid development owing to cohort splitting: just how costly is it to hurry?
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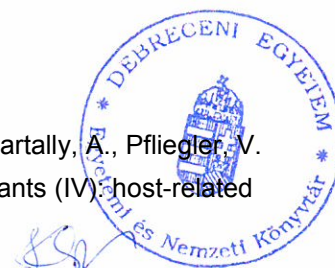
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6. **Rádai, Z.**, Popovici, O., Vas, Z., Fusu, L.: First record of the parasitoid *Idris fl avicornis*(Hymenoptera: Scelionidae) from eggs of the wolf spider *Pardosa agrestis*(Araneae: Lycosidae).
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7. Kiss, J., **Rádai, Z.**, Rosa, M. E., Kosztolányi, A., Barta, Z.: Seasonal changes in immune response and reproductive investment in a biparental beetle.
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