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Reproductive biology of ladybirds

Ph.D. Thesis

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Annotation

In this thesis I investigated the reproductive biology of the invasive alien population of one of the most important coccinellid species in Europe and north America, *Harmonia axyridis*, by demonstrating several aspects such as reproductive success of the hibernating females, verifying of the level of polyandry in the wild for the overwintering and summer populations and inspecting female choice and sperm competition phenomena.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, ...

Mona A. Awad

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Awad, M.**, Kalushkov P., Nedvědová T., Nedvěd, O., 2013. Fecundity and fertility of ladybird beetle *Harmonia axyridis* after a prolonged cold storage. Biocontrol DOI 10.1007/s10526-013-9512-4 (IF=1.9)
Mona A. Awad contributed to collecting the ladybirds from the hibernating sites, to breeding animals and writing the manuscript, and was responsible for protein extraction and literature survey.

II. **Awad, M.**, Laugier, G.J.M., Loiseau, A., Nedvěd O., submitted. Polyandry in wild-caught ladybirds *Harmonia axyridis* (Coleoptera: Coccinellidae). Submitted to Journal Ecology and Evolution
Mona A. Awad contributed to collecting the ladybirds from the hibernating sites, microsatellite analysis and other molecular methods and to the writing the manuscript, and was responsible for breeding animals and DNA extraction.

III. **Awad, M.**, Piálek, L., Krejčí, A., Laugier, G.J.M., Nedvěd O., submitted. Sperm competition following multiple mating in *Harmonia axyridis*. Submitted to Animal Behaviour
Mona A. Awad contributed to microsatellite analysis and to the writing the manuscript, and was responsible for breeding and observation of behavior of animals and for DNA extraction.

IV. **Awad, M.**, Nedvěd, O., 2013. Sperm competition following multiple mating in *Harmonia axyridis*. IOBC Bulletin, in press.
Mona A. Awad contributed to the writing the manuscript, and was responsible for breeding and observation of behavior of animals.

V. **Awad, M**., Kalushkov, P., Nedvědová, T., Nedvěd, O., 2013. Sperm survival in fertilized females of *Harmonia axyridis* during overwintering. IOBC Bulletin, in press.
Mona A. Awad contributed to collecting the ladybirds from the hibernating sites, their dissection and writing the manuscript, and was responsible for breeding animals.

VI. Nedvědová, T., **Awad, M.**, Ungerová, D., Nedvěd, O., 2013. Characteristics of ladybird *Harmonia axyridis* during autumn migration. IOBC Bulletin, in press.
Mona A. Awad contributed to collecting the ladybirds during migration, to their dissection, sexing and weighting the animals.

Contents

GENERAL INTRODUCTION

Biological control

Using of exotic natural enemies to control exotic pests one of classical biological control (**Caltagirone & Doutt 1989**). One of this exotic natural enemies is variously known as the Multicolored Asian lady beetle, Halloween beetle and Harlequin ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). It is native to China, Japan, Korea, Mongolia and Siberia (**Dobzhansky 1933**; **Kuznetsov 1997)**. it invaded many habitats, and in parts of both its native and invasive ranges has been recorded mainly on woody plants (**Vandereycken et al. 2012**), also in meadows, heathlands and riparian zones (**Adriaens et al. 2007**), reedbeds **(Ware et al. 2005; Brown et al. 2007**) and crop systems (**Colunga-Garcia & Gage 1998**). This species has a long history of use as a classical biological control agent of aphids and coccids in North America, where it was introduced in 1916 (**Gordon 1985**). Where it has been widely used for pest control in crops as diverse as pecans (**Tedders & Schaefer 1994**) and red pines (**McClure 1987**). As a biological control agent *H. axyridis* has succeeded to control aphid species on crops, including apples (**Brown & Miller 1998**) and citrus fruits (**Michaud 2002**).

Invasive alien species

The Convention on Biological Diversity and the 10th Conference of the Parties in Nagoya (2010) identified invasive alien species (IAS) as one of the five major pressures driving biodiversity loss and ultimately extinction of species (**Thomas et al. 2004**; **Hooper et al. 2005**; **Winter et al. 2009**). Invasive alien species (IAS) has a direct ecological effect on other species through a variety of mechanisms (**Parker et al. 1999**). In particular, invertebrate predators may displace native species by direct predation, exploitative competition for food or space, lower immunity to shared natural enemies, introduction of new pathogens or disrupted mating systems (**Snyder & Evans 2006**; **Kenis et al. 2009**). The need to limit the impact of non-indigenous species on natural communities has generated many recent experimental studies **(Kolar & Lodge 2001; Keane & Crawley 2002; Snyder et al. 2004**).

The establishment of invasive alien population *H. axyridis* is associated with the decline of native coccinellid populations in urban, agricultural, and natural habitats in Europe **(Adriaens et al. 2010; Brown et al. 2011a**) and in North America **(e.g., Michaud 2002; Alyokhin & Sewell 2004**). Part of invasion success as an invader has been attributed to its aggressive behaviour towards potential prey (including other predators). **(Roy et al. 2012)** mentioned substantial declines attributable to the arrival of *H. axyridis*. Indeed, the two-spot ladybird, *Adalia bipunctata*, declined by 30% (Belgium) and 44% (Britain) over 5 years after the arrival of *H. axyridis*. Trends in ladybird abundance revealed similar patterns of declines across three European countries.

In a risk assessment of 31 exotic natural enemies of pest species used in biological control in Europe, *H. axyridis* had the second highest environmental risk index. This was based on its wide host range (i.e. multiple prey species), ability to establish and disperse, and direct and indirect effects on non-target species (**van Lenteren et al. 2003**). **Van Lenteren et al. 2007** concluded that there are no easy ways to mitigate or reduce the risk of *H. axyridis* and that it should not have been released in northwest Europe. The harlequin ladybird beetle *H. axyridis* has emerged as a model species in invasion biology because of its strong resistance against pathogens, high reproductive rate, wide host range, competitive advantage and phenotypic plasticity (the ability of an organism to change its phenotype in response to changes in the environment). The invasive success of the species may reflect its well-adapted immune system. Recent studies have shown that it is better protected against intra-guild predation than native ladybird species.

Invasive success of *Harmonia axyridis*

The studies on coccinellids have naturally concentrated on their relation to prey: prey specificity, quantitative aspects, prey foraging behaviour and impact. The development of gonads and mating has been less studied, although they also have some practical relevance, particularly when coccinellids are reared as predators to be used in biological control. Among biological and environmental factors that contribute to the invasiveness of species (**Marco et al. 2002**), life-history traits like increased developmental rate, fecundity and fertility are important for successful invasion and establishment **(Sakai et al. 2001; Shea & Chesson 2002; Lanzoniet**

al. 2004) and their knowledge are of basic importance to develop a better understanding of interspecific interactions among exotic and native species.

Harmonia axyridis dominates aphidophagous guilds in some agricultural ecosystems in part due to high fecundity **(Iablokoff-Khnzorian 1982),** large body size (**Michaud 2002**), polyphagous feeding (**Koch 2003**), aggressive behavior (**Cottrell & Yeargan 1998**; **Yasuda & Ohnuma 1999**; **Yasuda et al. 2001**; **Cottrell 2004**; **Felix and Soares 2004**; **Snyder et al. 2004**), high mobility (**Osawa 2000**; **With et al. 2002**) and low susceptibility to pathogens (**Hoogendoorn & Heimpel 2002**; **Cottrell & Shapiro-Ilan 2003**; **Firlej et al. 2005**). Rapid juvenile development of *H. axyridis* relative to indigenous species could be another characteristic of invasiveness of this species (**Lanzoni et al. 2004**).

Reproductive biology of coccinellids

Multiple-mating

Multiple mating or specifically female re-mating occurs frequently in insects **(Arnqvist & Nilsson 2000)** despite their numerous costs **(Daly 1978).** They are not only time and energy consuming **(Thornhill & Alcock 1983),** but they also increase risk of predation **(Arnqvist 1989)** and disease transmission **(Hurst et al. 1995).** On the other hand, the adaptive significance of multiple mating has been extensively debated and the general idea is that, to be adaptive, the costs of multiple mating must be offset by benefits that enhance female fitness (defined as a measure of the number of individuals that are propagated into the following generations). Two types of benefits are commonly used to explain the adaptive value of multiple mating: non-genetic benefits and genetic benefits. Direct benefits (non-genetic benefits) derive from the quality of the sperm of certain males that may increase female fecundity and longevity. Additionally, if males transfer insufficient sperm, females may mate multiply to ensure all eggs are fertilized, hence obtaining fertility benefits. Indirect benefits (genetic benefits) are achieved through an increase in fertility in the next generation and variability of offspring ensuring success in variable environment. **(Barbosa et al. 2012)** showed strong evidence that multiple mating is advantageous to females, as manifested in an increase in female fecundity. Females produce substantially more grand-offspring than singly mated females even when males contribute only sperm. Increase in fecundity in the first generation is involved rather than other fitness correlates such as size at birth, growth rate, time to sexual maturation and survival.

Post copulatory sexual selection can select for compatible genes (alleles) (**Zeh & Zeh 2003**) thus reducing inbreeding depression (**Lukasz et al. 2011**) and leading to the production of offspring of higher quality. Alternatively, post mating sexual selection may also favour males with competitive ejaculates to sire more competitive offspring (i.e., sperm competition and cryptic choice;**Yasui 1997**).

Timing of mating

Female ladybirds mate quite often and for a long time and change partners **(Nedvěd & Honěk, 2012)** Sperm transfer is a component of sexual activity and directly associated with mating. **(Omkar et al, 2006)** find out that there is a positive correlation between duration of mating and fecundity and fertility where the while fecundity and fertility increased with increase in mating duration in two aphidophagous ladybirds, *Cheilomenes sexmaculata* and *Coelophora saucia.* The mating duration was recorded as 54 minutes in *Coccinella septempunctata* **(Rana & Kakker, 2000)** However, if the mating interrupted after 30 min this resulted by unfertilized eggs in *H. axyridis* **(Obata 1987),** this could indicate that sperm is not transferred during the early phase of mating (complete mating lasting 119.08 ± 16.82 min) in this species (**Obata & Johki 1991).** Likely, large ejaculate size in prolonged mating appears to fertilize greater quantity of eggs and increases the fertility.

Female ladybird beetles are highly polygamous and storage sperm in a spermatheca **(Hodek, 1973).** However, females of *Adalia bipunctata* (L.) mate about 10 times more than needed for fertilization of all their eggs **(Majerus 2009).** Such behaviour may represent a bet-hedging strategy and provides conditions for sperm competition. The bet-hedging strategy overcomes risks from the unpredictability of the environment in which progeny will live. Females produce genetically diverse offspring due to mating with genetically different males. Some of the progeny may then have genes suitable to unknown future conditions.

The details of some traits about differential sperm storage between diapausing and non-diapausing ladybird females has been examined in a few ladybird species, in the USA, North Carolina the overwintering females of *H. axyridis* from 12 to

41% **(Nalepa et al. 1996)** and 20 to 60% of the autumn females in the Czech Republic **(Nedvědová et al. 2013)** were mated and had a full spermatheca. Individuals of a number of other ladybird species also mate before diapause in autumn but do not lay eggs until the next spring: 28 to 47% of *Coccinella septempunctata* L. females had sperm in their spermatheca at overwintering site **(Ceryngier *et al.* 1992; Ceryngier *et al.* 2004; Hodek & Ceryngier 2000)**. In *Coccinella quinquepunctata* L., the proportion of fertilized females was low (10%) in September, but high (80%) in January **(Hodek and Ceryngier 2000)**, indicating that females mated at the overwintering sites. Two of 15 diapausing females of *Adalia bipunctata* in Belgium had stored sperm **(Arnaud et al. 2003)**. In *Ceratomegilla undecimnotata* (Schneider), no spermatheca contained any sperm **(Ceryngier et al. 2004)**. This sperm can be used for egg fertilization after the end of diapause and eliminate the need for females to copulate in spring to start reproduction. This can considered as key biological characteristic of *H. axyridis* with consequences for population dynamics and interspecific competition: the speed with which it can start reproducing after winter dormancy.

Sperm competition and paternity

Repeated mating by females occurs in many insects, giving possibility for sperm competition (**Parker 1970**; **Ridley 1989**). The primary function of mating is transfer of sperm to females. Since each mating offers an opportunity to father offspring, males can generally increase their fitness by mating with many mates and high mating rate are thus typically associated with high male reproductive success. Females, in contrast, maximize reproductive success by maximizing the number of viable eggs produced. This competition among the sperm from two or more males for the fertilization of a given set of oocytes within a female (**Parker 1970**) has given rise to a wide array of adaptations and counter-adaptations within males that enhance the chance of a male's own sperm fertilizing the eggs or that reduce the chance of another male's sperm.

Asian populations of *H. axyridis* are highly polygamous and also show a high level of genetic polymorphism, that is, multiple genotypes and resulting phenotypes occur within a population (**Komai and Hosino 1951**). Genetic polymorphism is expressed not only by intra-specific variation in body size and shape (**Soares et al.**

2001; **Soares et al. 2003**), but also by striking intraspecific variation in colour morphs (**Komai 1956**). Phenotypes are classified into two groups, the *succinea* group, which is characterized by a light reddish or brownish background colour, with or without black spots, and the melanic group, which includes the *conspicua*, *spectabilis*, *axyridis*, *equicolor*, *aulica* and other morphs. The frequency of color morphs varies according to geographical location of the population, as well as over time. Macrogeographical variations in Asian populations of *H. axyridis* are related mainly to climatic factors, because light and melanic phenotypes have different physiological responses to them (**Komai 1956**). The relative frequency of elytral pattern phenotypes also varies with the host plants (microgeographical variation) that support aphid prey (**Komai & Hosino 1951, Belyakova 2013**). Seasonal variation in the proportion of dark and light forms in Japanese populations was observed by **(Osawa & Nishida 1992)**, who reported a significant increase in light phenotypes during the spring and early summer.

Females of the ladybird *A. bipunctata* (L.) may mate many times during the spring reproductive period which lasts several weeks. About 23% of the adult population were in copula during any observation in the field in the Netherlands **(Brakefield 1984a).** *A. bipunctata* also possess genetic polymorphism for melanism: non-melanics are red with two black spots while melanics, which are genetically dominant **(Lus 1928, 1932)**, are black with red spots. Field data have provided evidence for different forms of sexual selection associated with the different colour morphs: (i) a female preference for melanic males has been described in some British populations **(Majerus et al. 1982; O'Donald & Majerus 1988; Kearns et al. 1990, 1992);** (ii) a frequency-dependent mating advantage for melanics has been recorded **(Muggleton 1979; Kearns et al. 1990);** (iii) a general mating advantage to melanics over non-melanics was found in The Netherlands **(Brakefield 1984b).** An understanding of the extent of sperm competition in this species is necessary to interpret the consequences of any deviation from random mating. If, for example, melanic beetles tend to mate more frequently or earlier than non-melanics, as in the Dutch study, then the genetic consequences will depend on the pattern of sperm precedence and the timing of egg laying. The mating advantage of melanics will only increase their fitness relative to non-melanics when eggs are laid at the beginning of the season or when sperm transferred in early mating can fertilize eggs laid after later mating.

Sloggett and Honěk (2012) provided a review of genetic studies using colour polymorphism and molecular methods to show female choice and sperm competition. **De Jong et al. (1998)** studied the effects of two different mating regimes on sperm precedence in the two-spot ladybird, *Adalia bipunctata,* by using the polymorphic gene for melanism as a marker for paternity. Virgin non-melanic females (homozygous recessive) were mated to non-melanic male(s) and then, after laying fertilized eggs, were mated to a melanic and then, by rearing the larvae to adulthood and scoring their colour pattern, found a greater variation in the percentage of eggs fertilized by a melanic male, both when the female had previously been singly or multiply mated to non-melanic males. This was supported by evidence from the field, which showed that sperm competition also occurs under natural conditions between heterozygote melanic and non-melanic individuals.

The availability of molecular methods makes it much easier to extend studies to coccinellids that are not polymorphic for colour pattern; thus in the future the generality of instantaneous mixing during sperm displacement across the Coccinellidae can be established. Because microsatellites provide a unique genetic fingerprint for each individual studied, they possess great potential for investigating multiple mating in coccinellids and to compare levels of promiscuity across individuals, populations and species. They are particularly promising markers for studies of populations from the field (**Haddrill et al. 2008**). In **2008, Haddrill et al.** provide the first direct evidence that female two-spot ladybirds are highly polyandrous in the wild by using three microsatellite loci with high variable. They find out that female of the two-spot ladybird, *Adalia bipunctata* are highly polyandrous in the wild, both in terms of population mating rates (~20% of the population found *in copula*) and the number of males siring offspring in a single clutch (three to four males, on average).

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AIMS OF THE THESIS

Several mechanisms contribute to the rapid spread of *H. axyridis* and its effects on native species. One of these mechanisms could be its **high fecundity and fertility**. The first aim of our study was to describe the reproductive biology of hibernating females of the invasive alien population *H. axyridis* in Europe by recording their fecundity after returning to suitable conditions and investigate the presence and absence of the yolk protein – a major component of oogenesis. The post-hibernation onset of female reproduction is a key characteristic influencing population dynamics and competition with related species. The second mechanism of success of the model species could be high genetic diversity of progeny through **multiple mating and polyandry.** The level of polyandry is an important variable for understanding the role of females in mating competition, including post-mating, pre-fertilization competition **(Eberhard 1996).** After having evidence about multiple mating and fertilization in the wild, we might continue with the investigation of female choice and sperm competition phenomena.

In Papers I and V, the three objectives of the study were **i)** to demonstrate whether the sperm in ladybird females fertilized in autumn can effectively survive to spring after prolonged cold exposure (hatching rate), **ii)** to investigate whether a single fertilized female may then establish a new large colony, which would partially explain the invasive success of the species studied (fecundity), and **iii)** to assess the potential application of ladybirds as biocontrol agents after long storage (changes in time of fecundity, fertility and protein content). To achieve these objectives, we measured the reproductive parameters of females of the European invasive population of *H. axyridis* after prolonged cold storage during winter by recording their fecundity after their return to suitable conditions. We also measured body soluble protein content and characterized the main protein type changes during overwintering. Furthermore, we wanted to record the level of vitality of sperm after the onset of reproduction when females were deprived of any further mating. We measured this vitality as egg hatching rate.

In Paper II, we tried to determine the prevalence, and therefore biological relevance, of polyandry in natural populations, important for explaining and predicting the success of an invasive species such as *H. axyridis*. A key parameter in this regard is the estimate of frequency of mixed paternity broods and the number of fathers contributing to mixed paternity broods. Such data provide information on the opportunity for post-copulatory sexual selection in natural populations. Since the multiple mating rates in laboratory cultures of beetles does not necessarily reflect situation relevant in the field conditions, we estimated the level of polyandry of the ladybird *H. axyridis* in overwintering and summer samples using microsatellite markers. Microsatellites with their high allelic diversity within wild populations are particularly useful markers for studies of multiple mating and levels of promiscuity.

Papers III and IV addressed the question, what characterise the winners of the sperm competition in H. axyridis. We tested the following alternative hypotheses: 1) Advantage by order. To find whether the first or the last among several males mated with a single female has higher chance to sire progeny. 2) Genetic quality. To find whether the genotype of a male determines his success in paternity regardless of order. 3) Quantity of sperm. We will measure the variation between males in the sperm amount (number of sperm cells determined by real time PCR).

Paper VI. The aims of this study were to compare body mass, sex ratio, percentage of colour morphs, and percentage of fertilized females of the invasive alien ladybird beetle *Harmonia axyridis* sampled during autumn migration on different dates and daytime. These characteristics may influence survival over winter and subsequent reproduction in the next season.

Results – articles and manuscripts

Paper I.

Fecundity and fertility of ladybird beetle *Harmonia axyridis* after a prolonged cold storage

Mona Awad, Plamen Kalushkov, Tereza Nedvědová and Oldřich Nedvěd. (2013) Biocontrol DOI 10.1007/s10526-013-9512-4

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Fecundity and fertility of ladybird beetle *Harmonia axyridis* after prolonged cold storage

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Abstract Some species of ladybird beetles (Coleoptera: Coccinellidae) mate both before and after overwintering. The purpose of the pre-diapause mating was studied in the alien invasive ladybird *Harmonia axyridis* (Pallas 1773). Our study demonstrates the persistence of high fecundity (daily oviposition rate of 21 eggs per fertilized female during the first month of reproduction) and fertility (85 % of eggs hatching) of females of *H. axyridis* after long storage (up to eight months) at low temperature (6 °C). The females were not mated after activation in spring and had to rely on the sperm supply maintained from the pre-winter period (58 % of females were fertilized). Unfertilized females also laid eggs but in low numbers (an average of 345 eggs by virgin females during an individual's lifetime, 1,174 eggs by females fertilized before winter) and after a longer pre-oviposition period (2–5 weeks in comparison to 7–8 days for fertilized females). We show that the unfertilised eggs were not trophic eggs. The high sperm survival ability observed questions the need for the high levels of sexual activity generally observed in Coccinellidae. Fertilized females of *H. axyridis* may found large colonies after dispersal to new areas even without males, which contributes to the striking invasive ability of this species.

Keywords Ladybird · Hatching rate · Overwintering · Reproduction · Protein · Mating

Introduction

The life cycle of ladybird beetles (Coleoptera: Coccinellidae) in temperate regions includes one or more generations of development from egg to adult during spring and summer (Nedvěd and Honěk 2012) and adult diapause during winter. This adaptive state has two main functions: (1) to synchronize the occurrence of feeding stages with favourable seasons by preventing reproduction, (2) to increase the chance of survival during the harsh season by lowering metabolic rate, and accumulating energetic reserves and substances increasing cold hardiness (Hodek 2012).

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Paper II.

Polyandry in wild-caught ladybirds *Harmonia axyridis* (Coleoptera: Coccinellidae)

Mona Awad, Guillaume J. M. Laugier, Anne Loiseau and Oldřich Nedvěd. Submitted to Ecology and Evolution

Polyandry in wild-caught ladybirds *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Keywords: promiscuity, paternity, multiple mating, fertilization, overwintering, reproduction

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Abstract

Polyandry influences patterns of gene flow and levels of genetic diversity in animals and has implications for sexual conflict over mating. Despite this, surprisingly few studies of insects have quantitatively estimated levels of polyandry in the wild. Ladybird beetles are sexually very active and promiscuous, raising a question of the evolutionary importance of their multiple mating behaviour and mating before overwintering. We investigated the level of polyandry in a European invasive population of the ladybird *Harmonia axyridis* before and after overwintering. Our study using eight microsatellite loci provides the first direct evidence that females of *H. axyridis* are highly polyandrous in the wild and that they not only mate multiple times but also use the sperm from multiple males to fertilize their eggs. Three types of values (minimum number of males, estimated number of males and effective number of males contributing to progeny) were about two times higher in the spring sample (2.9, 5.2, 3.1), in the peak of their reproduction period, than in preceding autumn sample (1.6, 2.8, 1.6) of the same

generation in the Czech Republic. Values found in spring sample from southern France were smaller (1.8, 2.3, 1.5) probably due to lower population density in the front of invasion.

Introduction

Knowledge of the genealogical relationships among individuals in a population is important in many research areas, including in behavioural, ecological, and evolutionary genetics and in conservation biology. It is crucial in studying social behaviour, mating systems, and reproductive allocation in animals. Direct observation of female mating frequency in the wild is logistically difficult and sometimes severely underestimates true values. Reliance on field observations led to the suggestion that 93% of all passerine birds were monogamous (Lack 1968), whereas DNA fingerprinting revealed that 86% of all bird species are promiscuous to some degree (Griffith *et al.* 2002). Conversely, polyandry does not necessary imply multiple paternity. For instance, in the promiscuous green turtle, most clutches are actually sired by a single male (Fitzsimmons 1998). In addition to vertebrates, mating systems and mating behaviour in the wild are well studied in insects (Thornhill & Alcock 1983; Choe & Crespi 1997; Simmons 2001). As such, insects have played an important role in developing and testing ideas about sexual selection and sexual conflict (Arnqvist & Rowe 2005, Chapman 2006; Haapaniemi & Pamilo 2012).

In addition to natural selection, Darwin (1871) explained some characteristics of organisms as a result of sexual selection through male competition and female choice. The first species, in which heritable preference of some females for males of particular genotype (melanic colouration) was described, was the two spot ladybird (*Adalia bipunctata*) (Majerus 2009). Assortative mating was observed also in *H. axyridis* and it varied in seasonal cycles (Wang *et al.* 2009).

Multiple mating

One aspect of reproduction that has received a lot of attention in both insects and other taxa has been the number of times females mate, and whether those matings are with the same or different males (i.e. multiple mating vs. true polyandry). The level of polyandry is an important variable for understanding the

Paper III.

Sperm competition following multiple mating in *Harmonia axyridis*

Mona Awad, Lubomír Piálek, Alena Krejčí, Guillaume Laugier, Oldřich Nedvěd. *Submitted to Animal Behaviour*

Sperm competition following multiple mating in *Harmonia axyridis*

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ABSTRACT

We investigated factors which may determine realised paternity following multiple mating in a promiscuous insect species, ladybird *Harmonia axyridis*, namely the mating order and sperm quality. In experiments using males of different colour morphs, we found that the first male of the two and males of the morph *spectabilis* had a reproductive advantage over the second male and over males of the morph *axyridis*. When these two tendencies were synergistic, progeny ratio was 3:1. The advantage of the first male was slightly stronger than the advantage of the better genotype (*spectabilis* morph) in treatments with immediate re-mating, but weaker in treatments with second mating delayed by five days. Analysis of paternity using microsatellites, which avoids possible differential mortality of progeny, confirmed the precedence of the first male and higher paternity rate of colour morphs that are more frequent in the wild. Hatchability increased from 65% of eggs after the first mating to 85% after the second mating delayed by five days, but not after immediate second mating, suggesting positive influence of multiple but not too much frequent mating on female fitness. Lag time before starting mating was strongly skewed with overall median 7 min, average duration of mating was 157 min.

Paper IV.

Sperm competition following multiple mating in *Harmonia axyridis*.

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Sperm competition following multiple mating in *Harmonia axyridis*

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Darwin (1859) made a clear distinction between natural and sexual selection. He argued that natural selection favoured traits in individuals that enhanced their viability, while sexual selection favoured traits that enhanced their success in reproduction. A prerequisite for sexual selection via sperm competition is multiple mating by females which is remarkably common in insects (Haddrill *et al.*, 2008). Multiple mating appears more adaptive for males than for females. Females can produce no more offspring than the number of their oocytes, and females in many species receive a lifetime supply of sperm from a single mating or a single mate (Thornhill & Alcock, 1983). It is, therefore, difficult to explain the adaptive significance of polyandry for females. In many cases, females seem to obtain some direct benefit from males (e.g. nutrients in the seminal fluid, protection against predators, paternal care of offspring). However, in species in which no direct benefit can be detected, the females have been supposed to obtain some "genetic benefit" from additional mating (Yasui, 1997).

Sperm competition is a term used to refer to the competition process between spermatozoa of two or more different males to fertilize an egg of a female. If males carrying different genetic markers are used in experiments, the offspring can then be genotyped to deduce which male is the father. There are several interrelated hypotheses explaining which male will be the father of most offspring: (1) by order – sperm of either the first or the second male, regardless of their genotype and

Paper V.

Sperm survival in fertilized females of *Harmonia axyridis* during overwintering.

Mona Awad, Plamen Kalushkov, Tereza Nedvědová and Oldřich Nedvěd. (2013). IOBC Bulletin, in press

Sperm survival in fertilized females of *Harmonia axyridis* during overwintering

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Ladybird beetles in temperate climatic zones spend winter in adult diapause. A proportion of the adults mate before winter and females store sperm in their spermathecae until spring. Previous studies showed that most female *Adalia bipunctata* (Arnaud *et al.*, 2003) and *Ceratomegilla undecimnotata* collected at dormancy sites had an empty spermatheca, while about half of diapausing females of *Coccinella septempunctata* were fertilized (Ceryngier *et al.*, 1992). In *Harmonia axyridis*, 12 to 41% of the overwintering females in North America had sperm in their spermatheca (Nalepa *et al.*, 1996), while 20 to 60% of females were fertilized in diverse autumn samples in Czech Republic (Nedvědová *et al.*, 2013). However, females mate again and repeatedly in spring, so that it is open to question whether the sperm from autumn mating can survive to spring and give rise to a full complement of viable offspring.

We collected migrating *H. axyridis* in October and stored them at 5°C. Each month from January to April and June we transferred 20 females individually to Petri dishes at 20°C, fed them with aphid *Acyrthosiphon pisum*, collected eggs and observed their hatch rate.

In subsamples inspected in January, February, March, April and June, respectively, there were 30%, 35%, 75%, 70% and 75% of the females fertilized. Fertilized females activated in January laid 21 eggs per day per female during the first month (Fig. 1). The hatch rate was 84% (Fig. 2). During the second month, the

Paper VI .

Characteristics of ladybird *Harmonia axyridis* during autumn migration

Tereza Nedvědová, Mona Awad, Dita Ungerová and Oldřich Nedvěd. (2013). IOBC Bulletin, in press

Characteristics of the ladybird *Harmonia axyridis* during autumn migration

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Abstract: Sampling of the migrating ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae) on building walls was conducted between 5th October and 17th November 2009 in České Budějovice, Czech Republic. Out of 1571 individuals, there were 845 females and 726 males. The sex ratio in particular samples was even more strongly biased, suggesting infection of the ladybird population by male-killing bacteria. Mean female fresh weight was 37.3mg, mean male weight was 30.8mg. Mean weight significantly decreased during autumn, suggesting shortage of nutrients in later developing beetles. There were 56 individuals (3.6%) of morph *conspicua* (mean weight 32.7mg), 156 individuals (9.9%) of morph *spectabilis* (mean weight 34.4mg), and 1359 individuals (86.5%) of morph *succinea* (mean weight 34.3mg). The proportion of melanic individuals was constant among samples. In later samples there were more individuals of the morph *succinea* that were heavily spotted. There were 46% of females fertilised before migration, regardless of colour morph. There was a slight decrease of fertilisation rate with later sampling date.

Key words: body mass, colour morph, overwintering, sex ratio

SUMMARY OF RESULTS AND DISCUSSION

We found that autumn samples of migrating ladybird beetle *Harmonia axyridis* had sex ratio in particular samples strongly biased, suggesting infection of the ladybird population by male-killing bacteria. Mean female fresh weight was 37.3 mg, mean male weight was 30.8 mg. Mean weight significantly decreased during autumn suggesting shortage of nutrients in later developing beetles.

There were 3.6% of the morph *conspicua*, 9.9% of morph *spectabilis*, and 86.5% of morph *succinea*. Proportion of melanic individuals was constant among samples. There were more individuals of the morph *succinea* that were heavily spotted in later samples. There were 46% females fertilised before migration, regardless of colour morph. There was a slight decrease of fertilisation rate with later date of sampling (Paper VI).

Subsequently, we sampled these females migrating to overwintering sites to measure their reproductive performance. Our study demonstrates persistence of high fecundity (21 eggs per day, 1200 over lifetime) and fertility (82% of egg laid during the first month) of females of the ladybird *H. axyridis* over a long storage (up to 8 months) at low temperature even when they were not mated after activation in suitable conditions and had to rely for the rest of their lives on the sperm supply stored through the winter period. Fecundity decreases faster than fertility. Protein content decreases over the long storage period but vitellogenine necessary for egg production is present even after long time. High sperm survival questions the presumed need for multiple, frequent and long mating that has generally been observed in Coccinellidae. A single female of *H. axyridis* with such high fertility even without access to repetitive mating may found a large new colony. Reproductive biology thus contributes to the striking invasive ability of this species (Papers I and V).

In the next step, we compared level of polyandry between the above mentioned overwintering and summer field samples. Proportion of females in the overwintering sample that were fertilized was 50%, all summer females laid fertilized eggs. Most fertilized females of the overwintering sample laid eggs that were fertilized by one or two males. A few females showed higher level of polyandry, maximum estimated number of males was 8; average estimated number of fathers was 2.8. Most females of the summer sample laid eggs that were fertilized by three different males. The values ranged from 1 to 10 males, average was 5.2 fathers. French sample of the summer population showed lower level of polyandry (2.3), probably due to low population density in the front of invasion.

The overwintering sample, collected during autumn migration, may contain both young females that hatched from pupa a short time (less than one month) before migration which didn't mate or mate once or twice and older females of the preceding generation which already regularly reproduced and mated multiple times. Proportion of females observed in copula in summer sample was 12.5%, which means probable frequence of remating each eighth day. The estimated time spent in the mating pool to achieve the mean number of 5.2 matings was 6 weeks, half of which might have been achieved already before overwintering. (Paper II).

In a parallel study, we estimated the conditions influencing paternity in a laboratory experiment. Preliminary results on the sperm competition gave a clear trend towards the males of the morph *spectabilis* having higher fertility than *axyridis*, but this occurred strongly enough only if *spectabilis* was in the same time the first male. If he were the second one, his quality was strong enough to compete with the first male (*axyridis*) and get about half of the progeny, but not strong enough to get significantly more progeny than the first one. This was supported by counting the sperm by using Real time PCR, which showed no visible differences between the fertilizing efficiency of the ejaculates males of the form *spectabilis* and *axyridis*. (Paper IV).

More comprehensive study on paternity showed that the first male of the two and males of the morph *spectabilis* had a reproductive advantage over the second male and over males of the morph *axyridis*. When these two tendencies were synergistic, progeny ratio was 3:1. The advantage of the first male was slightly stronger than the advantage of the better genotype (*spectabilis* morph) in treatment with immediate re-mating, but weaker in treatment with second mating delayed by five days. (Paper III).

GENERAL CONCLUSIONS

Undoubtedly, the investigation of the reproductive biology of one of the most important coccinellid species, *Harmonia axyridis*, can open the door to understand the rapid spread of its invasive alien population through Europe.

Our study demonstrates persistence of high fecundity and fertility of the hibernating females of *H. axyridis* over a long storage (up to 8 months) at low temperature even when they were not mated after activation in suitable conditions and had to rely for the rest of their lives on the sperm supply stored through the winter period.

We provided direct evidence that females of *H. axyridis* in two populations in the wild (overwintering samples and summer samples) are highly polyandrous and that they not only mate multiple times but also use the sperm from multiple males to fertilize their eggs. We found that among these multiple males, sperm competition gives a reproductive advantage to the first male and to males of the morph more common in the wild.

Hatchability increased from 65% of eggs after the first mating to 85% after the second delayed mating, suggesting positive influence of multiple but not too much frequent mating on female fitness.

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