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HOST-PARASITE INTERACTIONS IN DAMSELFLIES:
FROM INDIVIDUALS TO POPULATIONS

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Contents

ABSTRACT 4

TIIVISTELMÄ..... 5

LIST OF ORIGINAL PAPERS 6

1. INTRODUCTION..... 7

 1.1. Foreword..... 7

 1.2. Parasites are everywhere..... 7

 1.3. Host- parasite relationships in insects 8

 1.4. Immune response in insects 9

 1.5. Population structure and parasites 10

 1.6. Host’s genetic heterozygosity and parasites..... 11

 1.7. Parasite mediated competition..... 12

 1.8. Character displacement 13

 1.9. Aims of the thesis..... 14

2. MATERIALS AND METHODS 15

 2.1. Study species 15

 2.1.1. *The odonates* 15

 2.1.2. *The parasites of odonates and their occurrence* 18

 2.2. Description of study area and field work..... 20

 2.2.3. *Determining the immune response in damselflies*..... 22

 2.2.4. *Determining the parasite load* 24

 2.2.5. *Analyzing the inbreeding coefficient with AFLP* 24

 2.3. Data analyses 25

3. MAIN RESULTS AND DISCUSSION..... 28

 3.1. Infection rates of water mites and gregarines in damselflies (I-V) 28

 3.2. The effects of parasites on host’s immune response..... 30

 3.3. Population structure and parasite prevalence 31

 3.4. Genetic variation and parasite burden..... 33

 3.5. Effects of interspecific competition on parasite load..... 34

 3.6. Effects of parasites on character displacement..... 35

 3.7. Association of parasites and odonates – synthesis table..... 37

 3.8. Conclusions and future directions 38

ACKNOWLEDGEMENTS 39

REFERENCES..... 41

ORIGINAL PUBLICATIONS 46

ABSTRACT

The main goal of this thesis is to increase understanding on evolutionary and ecological factors that have contributed to differences in parasite numbers in insects. Furthermore, the thesis addresses the effects of parasites on their hosts.

The most important findings were:

The Northern damselfly's (*Coenagrion hastulatum*) immune response to artificial pathogen increased with increasing parasite numbers (Article I).

Marginal, more isolated *C. hastulatum* populations on the edge of distribution have fewer parasites when compared to distribution's core populations (Article II).

The Banded damselfly *Calopteryx splendens* individuals with higher homozygosity have more parasites, however, the rate of homozygosity did not differ between populations (Article III).

Parasite prevalence was affected by whether the host species occurred in allopatric or sympatric population: sympatric *C. splendens* populations with sister species the Beautiful damselfly *Calopteryx virgo* harbored more parasites (Article IV).

Parasites were associated with the wing spot size, an ornament under sexual selection, and thus may play an important role in character displacement, i.e. the size of the wing spot (Article V).

To conclude with, this thesis brings about new information on the parasite infection patterns in insects, proposing several factors to contribute to these patterns, as well as it addresses the effects of parasites on their hosts, from individual to population level.

TIIVISTELMÄ

Väitöskirjan päätavoitteena on tuottaa uutta tietoa hyönteisten loismääriin vaikuttavista evoluutiivisista ja ekologisista tekijöistä. Tämän lisäksi väitöskirjassa tutkitaan loisten aiheuttamia vaikutuksia isäntiinsä.

Väitöskirjan tärkeimmät tulokset voidaan tiivistää seuraavasti:

Mitä suurempi määrä loisia, sitä korkeampi immuunivaste isännällä oli (keihästytkorenolla, *Coenagrion hastulatum*) (Työ I).

Keihästytkorennon elinalueen uloimmilla reunoilla olevissa populaatioissa oli huomattavasti vähemmän loisia verrattuna elinalueen keskiosissa olevien populaatioiden loismääriin (Työ II).

Geneettisellä tasolla havaittiin, että korkean homotsygotian (lokuksen molemmat alleelit samoja) omaavilla yksilöillä oli merkittävästi enemmän loisia verrattuna heterozygoottisempiin, eli geneettisesti monimuotoisempiin yksilöihin (immenkorenolla *Calopteryx splendens*). Homotsygotian asteessa ei kuitenkaan ollut merkittävää eroa tutkimuspopulaatioiden välillä (Työ III).

Sympatrisissa ja allopatrisissa populaatioissa havaittiin huomattava ero loismäärissä kahden lähilajin välillä (immenkorento *C. splendens* ja neidonkorento *C. virgo*); sympatrisilla immenkorennoilla jotka esiintyivät yhdessä neidonkorentojen kanssa, oli enemmän loisia kuin allopatrisilla immenkorennoilla (Työ IV).

Loiset vaikuttivat myös seksuaalivalinnan alaisena olevan ornamentin (siipitäplän) kokoon immenkorennoilla. Tämä viittaa siihen, että loisilla on vaikutusta isäntälajin ominaisuudensiirtymään, eli tässä tapauksessa siipitäplän koon muutokseen (Työ V).

Tässä väitöskirjassa esitetään uutta tietoa loismäärien vaihteluista hyönteisillä ja tutkitaan niitä tekijöitä, jotka vaikuttavat näihin eroihin. Lisäksi siinä esitetään tapoja, joilla loiset vaikuttavat isäntiinsä yksilötasolta aina populaatiotasolle asti.

LIST OF ORIGINAL PAPERS

This thesis is based on the following publications and manuscripts which are referred to in the text by their Roman numerals:

- I** Kaunisto K. M., Suhonen J. (2013) Parasite burden and the insect immune response: interpopulation comparison. *Parasitology* – **140**: 87–94.
- II** Kaunisto K. M., Kaunisto P., Vahtera V., Suhonen J. Populations of the damselfly *Coenagrion hastulatum* have fewer parasites at the edge of the host range – *Resubmitted manuscript*.
- III** Kaunisto K. M., Viitaniemi H. M., Leder E. H., Suhonen J. (2013) Association between host's genetic diversity and parasite burden in damselflies. *Journal of Evolutionary Biology* – **26**: 1784–1789.
- IV** Ilvonen S., Ilvonen J. J., Kaunisto K. M., Krams I., Suhonen J. (2011) Can infection by eugregarine parasites mediate species coexistence in *Calopteryx* damselflies? *Ecological Entomology* – **36**: 582–587.
- V** Suhonen J., Ilvonen S., Dunn D. W., Dunn J., Härmä O., Ilvonen J., Kaunisto K. M., Krams I. Parasites, interspecific interactions, and character displacement of a sexually selected male ornament in male damselflies, *Calopteryx splendens*. *Resubmitted manuscript*.

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Article **III** License number: 3281220662660
Article **IV** License number: 3281231301973

1. INTRODUCTION

1.1. Foreword

The aim of ecology is to understand the factors that determine the distribution, abundance and diversity of living organisms, at different scales. Host-parasite relationships are an ideal area for exploring the factors that determine the occurrence of organisms: the coverage of these relationships ranges from the individual level to that of the population, and indeed further to large-scale patterns of parasite diversity and abundance, thus enabling multi-scale approaches in research (Poulin 2007).

Most previous host-parasite studies in insects have focused on single-host populations with a single parasite species (e.g. Cotter et al. 2003, Fedorka and Mousseau 2006). Differences *among* host populations, i.e. intra-species variation of the host, on the other hand, have remained nearly unexplored. In this thesis, I explore the factors affecting parasite loads from the individual to the interpopulational level, using two parasite groups with different life-history strategies and over a relatively large (roughly 880 km) geographical area. The thesis includes host interactions with parasites (both biotic and abiotic interactions), intraspecific selective constraints, and genetic processes, at the level of the individual, the population and the species. Ultimately, I seek in the thesis to understand the effects of parasites on their hosts, as well as how and why parasite numbers vary among both host individuals and host populations.

1.2. Parasites are everywhere

Parasites form a large proportion of the diversity of life. Nearly all organisms are parasitized; the tight coevolutionary processes between parasites and their hosts have resulted in high specificity, consequently leading to the speciation of myriad parasite species (Altizer et al. 2003). As a result of this coevolution, more than half of all animal

species are thought to be parasites, and to play a significant role throughout ecosystems (Toft 1986).

Evolutionary theory predicts that selection will favor the emergence of defenses against factors that lower the individual's fitness, whenever the benefits of these factors outweigh their costs (e.g. Schmid-Hempel 2005). This has led to the evolution of a highly specific immune system to counter the negative impacts caused by parasites and pathogens. This, however, has not led to the evolution of individuals with an insurmountable immune response, due to the presence of trade-offs between a costly immune system and other life history traits, such as number of offspring, which draw on limited resources (e.g. Folstad and Karter 1992, Zuk and Stoehr 2002). Consequently, a never-ending arms race prevails between parasites and their hosts. While parasites are constantly evolving novel ways for breaking through their host's defences, the hosts are in turn filtered through natural selection to keep up by adapting their immune defences in the process of evolution (Dawkins and Krebs 1979).

From a human aspect, parasites affect our everyday life in many ways. For example, raw or underdone pork has to be avoided for fear of trichinosis, caused by the *Trichinella spiralis* parasite. Billions are spent annually on vaccinations against parasitic infections around the world, while hundreds of millions of people suffer from the *Plasmodium* parasite, causing malaria. These are just a few examples of the effects that these ubiquitous life forms have on nearly all other organisms. It is thus extremely important to understand the factors that determine the existence and ecology of parasites.

1.3. Host- parasite relationships in insects

A parasite is defined as an organism that obtains nutrients from its host, normally causing harm but not immediate death (Price 1980). The term 'parasite' is derived from

the Greek words *para* meaning "beside" and *sitos* meaning "food". Parasites are usually classified by their interactions with their hosts: parasites that live *on* their hosts are called ectoparasites, those that live *inside* their hosts are endoparasites. Parasites that parasitize other parasites are called hyperparasites, while those whose *larvae* develop in or on the host, commonly resulting in the latter's death, are called parasitoids (Price 1980).

Parasites have many negative impacts on their hosts, and have been shown to reduce their host's fitness by decreasing survival and /or reproductive success (Konig and Schmid-Hempel 1995, Schmid-Hempel 1995, Sheldon and Verhulst 1996). For example, studies conducted with dragonflies and damselflies have shown that a parasite burden reduces the host's flight ability, fat content and muscle output, effectiveness in finding a mate, breeding success, female fertility and male condition, and increases the general mortality rate (e.g. Siva-Jothy and Plaistow 1999, Forbes et al. 2002, Marden and Cobb 2004, Schilder and Marden 2006, Rantala et al. 2010).

1.4. Immune response in insects

When a parasite succeeds in penetrating an insect's physical defence, for example the exoskeletal wall, the immune system is activated. The immune system of insects is based mainly on an innate immunity that relies on interactions between the hemocytes, the cellular component of arthropod blood, and the ability of soluble compounds in the humoral system to recognize and respond to a non-self (Gupta 2001). Thereafter, the presence of infectious particles results in melanin deposition around the damaged tissue or intruding object (Cerenius and Soderhall 2004). This melanin deposition is referred to as "encapsulation", the ability to form a hemocyte capsule around the parasite or pathogen. The invertebrate host's immune system then tries to inactivate the

encapsulated organism by releasing cytotoxic substances into the capsule, while the organism is isolated from nutrients (Lawniczak et al. 2007).

In addition to this innate immunity, invertebrates also possess certain immune mechanisms that could be classified as “induced” immunity, i.e. an immune response against certain parasites which is more protective upon secondary pathogen exposure (Little and Kraaijeveld 2004). However, the impact of this “immunological priming” on individual’s fitness seem to be fairly minor (e.g. Kurtz 2005).

Immune response is costly to the individual. These costs consist of the costs of evolving immunity and those associated with maintaining and deploying the immune system (Zuk and Stoehr 2002, Lawniczak et al. 2007). Several studies on insects have found trade-offs between immunity and survival, an increased risk of predation, and other life history traits (e.g. Zuk and Stoehr 2002, Hanssen et al. 2004, Rantala et al. 2010)

1.5. Population structure and parasites

The main demographic processes affecting population size are commonly divided into four classes: simply put, these are births, deaths, immigration and emigration. Parasites directly affect all of these major variables. First of all, parasites consume important energy resources from their hosts, which directly affects the birth-rate. Canales-Lazcano et al. (2005), for example, found that the damselfly *Enallagma praevarum* laid fewer eggs with an increasing number of endoparasitic gregarines. Leung et al. (2001) found parasites to increase mortality in damselflies, presumably due to the stress-reduced availability of energy for immune function. Suhonen et al. (2010) found that male *Calopteryx virgo* damselflies containing an artificial pathogen had higher dispersal rates and flew further than individuals without the pathogen. Parasites thus play an important role in shaping the population structure.

In addition to biotic factors like parasites, abiotic factors such as fragmentation also affect population structure by isolating populations (reviewed in Ewers and Didham 2006), which along with habitat loss affects population structure, dynamics, and consequently the existence of species (Hanski 2005). Since fragmentation leads to increased genetic drift, loss of gene flow and increased inbreeding, it is also suggested to have a negative effect on the ability of individuals to compensate for other threats, for example the impact of parasites (Young et al. 1996, Sork et al. 1999, Lowe et al. 2005).

1.6. Host's genetic heterozygosity and parasites

A few previous studies, mainly conducted with vertebrates, have revealed that low genetic variation in host individuals increases their susceptibility to parasite infection (e.g. Coltman et al. 1999, Isomursu et al. 2012). Genetic variation varies greatly in nature, mainly according to the level of inbreeding, which increases homozygosity: a particular gene has identical alleles of the gene present on both homologous chromosomes. Inbreeding occurs when individuals closely related by descent mate with one another; the resulting offspring may have lower fitness compared to the mean fitness of the population (Reed et al. 2003).

Inbreeding acts via associative overdominance, which is an apparent heterozygosity advantage due to correlations between heterozygosity at neutral loci and loci under selection (reviewed in Hansson and Westerberg 2002, Hawley et al. 2005). First, at loci involved in parasite defence, heterozygotes can present twice as many resistance variants as homozygotes (Coltman et al. 1999). Second, parasites may contribute to selection against less heterozygous individuals because of genome-wide effects, including homozygosity of deleterious recessive alleles and overdominance (Pearman and Garner 2005).

Animals choose their mates on a genetic basis, e.g. disease resistance, by scrutiny of characters whose full expression is dependent on health and vigor (Hamilton and Zuk 1982). This leads to selection against detrimental homozygosity.

1.7. Parasite mediated competition

The theory of parasite-mediated competition was introduced by Haldane (1949), who described non-specific parasites as “a powerful competitive weapon”. This means that a given species can have an advantage in competition by harboring and transmitting a parasite to its rival species. Further evidence of the importance of parasites in inter-species competition comes from studies of invasive species, in which invaders are shown to escape from parasites belonging to their native range (reviewed in Prenter et al. 2004). Release from natural parasites can subsequently aid the performance of an invader. For example, a detailed study of the invasive green crab *Carcinus maenas* found that invading individuals leave behind most of their parasites; this advantage allows invading *C. maenas* individuals to grow larger and more abundant (Torchin 2002).

Generalist parasites, which infect diverse host species, may also have an important effect on inter- and intraspecific competition. Parasite transmission and parasite-induced effects are invariably asymmetrical between competitive species; in other words, the effects of parasites may be severe for one host while being nearly neutral for another. Sensitive species are more likely to succumb to the pathogens that are maintained in abundance by tolerant species, sometimes even leading to the local extinction of these hosts. Thus apparent competition can act through parasitism, since levels of infection depend primarily on the rate at which the parasites flow from a tolerant species to a sensitive one, rather than on the density of the sensitive species. Furthermore, a study of the shared parasite *Heterakis gallinarum* in pheasants (*Phasianus colchicus*) and partridges (*Perdix perdix*) has shown that the fitness of a

single infective stage entering a pheasant is roughly 100 times greater than a similar stage entering a partridge (Tompkins et al. 2000). This is because the parasite is more likely to establish itself, grow larger and produce more infective stages in pheasants. Given that partridges suffer reduced fitness from parasites, the presence of pheasants can enhance infection in partridges, leading indirectly to their localized extirpation (reviewed in Hudson et al. 2006).

1.8. Character displacement

Character displacement was first explicitly explained by W. L. Brown and E. O. Wilson in 1956 as follows:

Two closely related species have overlapping ranges. In the parts of the ranges where one species occurs alone, the populations of that species are similar to the other species and may even be very difficult to distinguish from it. In the area of overlap, where the two species occur together, the populations are more divergent and easily distinguished, i.e., they "displace" one another in one or more characters. The characters involved can be morphological, ecological, behavioral, or physiological; they are assumed to be genetically based. (Brown and Wilson 1956)

Since then, character displacement has been studied in detail. Grether et al. (2009), for example defined the process of character displacement as follows: "When formerly allopatric species come into secondary contact, aggressive interactions between the species can cause selection on traits that affect interspecific encounter rates (e.g. habitat preferences, activity schedules), competitor recognition (e.g. coloration, song), and fighting ability (e.g. weaponry, body size)". They further defined character displacement as "the process of phenotypic evolution in a population caused by interference

competition with one or more sympatric species ... which results in shifts in traits that affect the rate, intensity or outcome of interspecific aggression" (Grether et al. 2009).

This divergent evolution in the characters of species with overlapping distribution, i.e. character displacement, is commonly thought to result mainly from selection against maladaptive hybridization (e.g. Waage 1975).

1.9. Aims of the thesis

The main purpose of this thesis is to increase our understanding of the evolutionary and ecological factors that have contributed to differences in parasite numbers in insects. A second aim is to address the effects of parasites on their hosts. Towards these goals, I conducted the following experiments and investigations:

I tested the effect of parasite infection rate on the average immune response of the host population (**I**).

I determined whether isolated marginal populations differ from core populations in their parasite burden (**II**)

I tested the heterozygosity-fitness correlation hypothesis with respect to parasites: do host individuals with higher genetic diversity harbor fewer parasites? (**III**)

I investigated the occurrence of parasite-mediated competition between two closely related damselfly species *Calopteryx splendens* and *Calopteryx virgo* (**IV**)

I investigated a possible association between, parasites and character displacement in a trait under multiple selection pressures (**V**)

In addition to the main results of this thesis, the underlying aim has been to contribute to our general knowledge of parasite patterns in insects, from the individual to the population level.

2. MATERIALS AND METHODS

2.1. Study species

Insects provide an excellent model for the study of immune function in an evolutionary context. They have a wide geographical range, and their survival depends to a large extent on effective immune function against miscellaneous pathogens and parasites (Frank 2000, Lawniczak et al. 2007). It is thus clear that insects have very efficient means of recognizing and defending themselves against potentially harmful microorganisms, and they consequently serve as excellent study organisms for host-parasite research.

In this thesis, I chose odonates, particularly damselflies, as study species because their basic biology is well known (e.g. Corbet 1999). Dragonflies and damselflies are also common around nearly all types of watersheds, they are effortless to observe because of their large body size, and are relatively easy to identify (Dijkstra et al. 2014). Finally, since the odonates commonly harbor considerable numbers of parasites (e.g. Åbro 1974), they are excellent model organisms for both immuno-ecological and host-parasite–interaction studies.

2.1.1. *The odonates*

The insect order Odonata is traditionally divided into two suborders, Zygoptera and Anisoptera. Zygoptera are commonly known as damselflies and Anisoptera as dragonflies.

Nearly all odonate larvae have an aquatic stage, and adults are consequently most often seen near water bodies. They exhibit incomplete metamorphosis, their life-cycle consisting of the egg and several larval stages followed by an adult stage. As adults, however, many species disperse far from water, and some dragonfly species have been

observed for example in Iceland, which has no native breeding species. Odonates are predators throughout their life, mostly feeding on smaller insects (Corbet 1999).

The species used in this thesis were the Northern Damselfly (*Coenagrion hastulatum*, Charpentier, 1825) (I-II) (Fig. 1), the Banded Demoiselle (*Calopteryx splendens*) (Harris, 1780) (III-V) (Fig. 2) and the Beautiful Demoiselle (*Calopteryx virgo*) (V) (Fig. 3).



Figure 1. *The Northern Damselfly (Coenagrion hastulatum).*



Figure 2. *The Banded Demoiselle (Calopteryx splendens).*



Figure 3. *The Beautiful Demoiselle (Calopteryx virgo).*

Coenagrion hastulatum was chosen as a study species for three reasons: (i) it is widely distributed in Finland (Dijkstra 2006) with a large geographical range; (ii) it harbours both ectoparasitic water mites and endoparasitic gregarines that are common across odonates (e.g. Morrill et al. 2013), providing simultaneous effects of infection; (iii), it is also abundant in scarce edge populations close to the northern extremity of its range (Kaunisto and Suhonen 2013), enabling sample collection even from marginal populations.

The other two study species, *C. splendens* and *C. virgo*, were chosen because they have both sympatric and allopatric populations (Corbet 1999). They also harbor large quantities of endoparasitic gregarines (while mainly lacking ectoparasitic water mites) (Åbro 1976) that vary considerably between populations (e.g. Kaunisto and Suhonen 2013). In addition they use multiple mating strategies, e.g. territoriality, associated by wing pigmentation (Córdoba-Aguilar 2009), making them unique model organisms for immuno-ecological studies.

2.1.2. *The parasites of odonates and their occurrence*

Odonates are among the most parasitized of all insects, harboring both ecto- and endoparasites (Åbro 1976, Åbro 1986). The most abundant endo- and ectoparasites in these damselflies are gregarines (Protozoa: Apicomplexa) (Fig. 4) and water mites, (Acari: Hydrachnida) (Fig. 5) (Robinson 1983, Åbro 1990). Both parasite taxa are species-rich groups, with roughly 1700 gregarines (Rueckert et al. 2011) and 6000 water mites (Di Sabatino et al. 2002, Smith et al. 2010) described so far. A recent study of water mites, however, found only from one to a few water mite species per host damselfly species (Zawal and Szlauer-Lukaszewska 2012). Both parasite groups, water mites and gregarines, are extremely difficult to identify by their morphological features; in this thesis, both groups were therefore treated as an aggregate.

The aquatic, hexapod larvae of water mites attach phoretically to the last instar of odonate larvae, and during the emergence of the adult the mites move to the dragonfly's thorax or abdomen, where they remain attached feeding on the host's body fluids throughout their parasitic phase. After engorgement, the mite larvae drop from their host and start the aquatic phase of their life cycle (Corbet 1999). The ectoparasitic water mites drain substantial amounts of body fluids through the chitinous exoskeleton of the host damselfly; this is likely to reduce the damselfly's survivorship and lifetime reproductive success (Forbes and Baker 1991, Neubauer and Rehfeldt 1995, Leung et al. 2001).

The endoparasitic gregarines, on the other hand, are extracellular sporozoan parasites of invertebrates. Within the family of Actinocephalidae, the gregarines are cosmopolitan mid-gut endoparasites of damselflies (Åbro 1990, 1996, Ilvonen et al. 2011 **(IV)**, Kaunisto and Suhonen 2013 **(I)**). Eugregarine infection in damselflies occurs via the ingestion of oocysts attached to the legs of small flying prey organisms (e.g. flies) that adult damselflies use as food (Åbro 1976). Once ingested by a damselfly,

eugregarines attach to the posterior gut region of the adult, where they develop and reproduce (Åbro 1974, Taylor and Merriam 1996, Siva-Jothy and Plaistow 1999). Gregarines may have a negative impact on host fitness by impairing the survival and mating success of the male host (e.g. Åbro 1990, Åbro 1996, Forbes et al. 1999, Cordoba-Aguilar et al. 2003, Marden and Cobb 2004). Gregarines reduce the energy absorbed by the host from its food (Siva-Jothy and Plaistow 1999). Individuals with a high burden of trophozoites also often have a ruptured and/or blocked mid-gut, which is likely to reduce the host's longevity (Siva-Jothy and Plaistow 1999). Moreover, as longevity is a major determinant of fitness in many odonates (Åbro 1990), and energy resources are vital for the individual's immune response and reproduction (e.g. Sokolovska et al. 2000), gregarines are very likely to have a dramatic impact on host fitness.

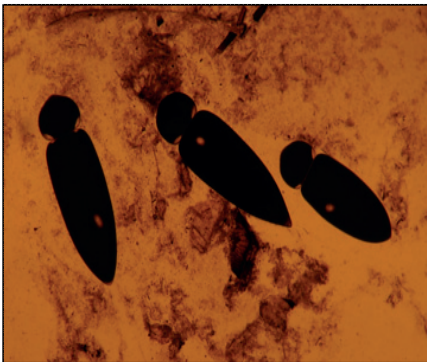


Figure 4. *Endoparasitic gregarines inside the damselfly's gut.*

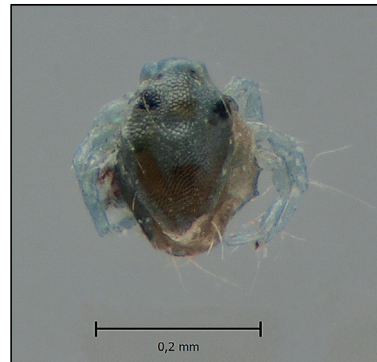


Figure 5. *Ectoparasitic water mite from above.*

The distribution of parasites is rarely random. More commonly, a majority of hosts in a population harbor a few parasites or none, while a few hosts harbor many parasites; in other words, parasite distribution is generally aggregated, right-skewed. The rate of parasite infection is usually described in terms of intensity, prevalence, and abundance. *Intensity* is defined as the total number of parasites of a particular species found in a sample, divided by the number of hosts. *Prevalence* is defined as the proportion of

infected hosts among the study population. *Abundance* is the total number of individuals of a particular parasite species in a sample of a particular host species, divided by the total number of hosts of that species examined, including both infected and uninfected hosts (Zar 1999).

2.2. Description of study area and field work

The data for *Coenagrion hastulatum* populations was collected between Kemiö (60° 5'N, 22° 4'E) and Sodankylä (67° 31'N, 26° 4'E) in northern Finland from thirteen independent populations during 20–27 June 2009 (I). Samples were collected from a latitudinal gradient of approximately 880 km, consisting of virtually the whole area of continuous distribution of *C. hastulatum*, the Northern Damselfly, in Finland (Dijkstra 2006), thus providing a large geographical gradient with varying photoperiod and mean temperature. According to the Finnish Meteorological Institute, the mean temperature varies by approximately 6 °C between the southernmost and the northernmost sample sites (colder in the north), and odonates develop more rapidly in warmer conditions, similarly to larvae in temperate regions (Karjalainen 2010, Dijkstra et al. 2014). It was thus assumed that northern individuals would emerge somewhat later in Finland, and the samples from northern sites were therefore generally collected a few days later to compensate for the slight delay in emergence. In Finland, the whole flight period of the study organism, *C. hastulatum*, lasts roughly three and half months out of the year. Our samples were collected over eight days per year, using individuals as close as possible to the same age in order to deal with individuals in the same part of the flight period. All the sample sites were freshwater watersheds. Sample site location was determined to the nearest fifty meters on a grid of the sample collection area with a GPS device. From each population, twelve *C. hastulatum* males and twelve females were captured, except

for Vehniä (fourteen males, eight females) and Kuusamo (eleven males, twelve females), giving a total of 309 individuals.

Along with the data collected in 2009 we collected additional samples in 2010 and 2012 (**II**), in order to acquire the desired quantity of data for all populations and to deal with variation in parasite abundances between the years. The distribution of *C. hastulatum* was derived from the database of the Finnish Museum of Natural History (Fig. 6). In order to determine the south-north cline in the general distribution of *C. hastulatum*, we divided the number of all 10*10 kilometers quadrats (excluding those on open water) by the number of quadrats in which the damselfly *C. hastulatum* was observed.

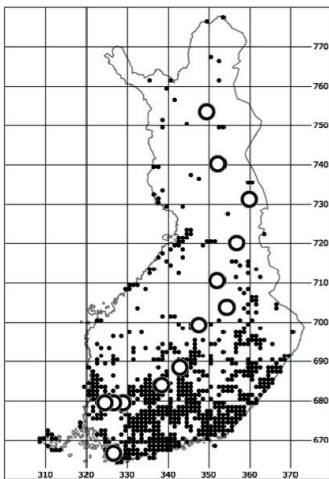


Figure 6. Distribution of *Coenagrion hastulatum* in Finland, according to insect database of Finnish Museum of Natural History. Land area divided into 10*10 kilometer quadrats. Black dots represent observations of *C. hastulatum*, larger open dots represent approximate locations of sample populations in (**I**) and (**II**).

Samples of *Calopteryx splendens* (**III, IV, V**), and *Calopteryx virgo* (**IV**) populations were collected during 2004–2009 (**III, IV** and **V**). In all of the sampled sites, individuals were collected from an area of roughly 100 meters along the river bank. All samples were collected between 10:00 and 15:00 hrs, which is the peak of *Calopteryx* species activity

(Corbet 1999). The fieldwork was carried out from mid-June to mid-July. The field samples were collected as soon as the daily temperature reached about 18°C. As with all the studies in this thesis, only adult damselflies of roughly the same age were collected, as gregarine levels tend to increase with age (Ward and Mill 2005). We were able to separate adult damselflies from juveniles by the stiffness of the wings, as described e.g. in (Cordoba-Aguilar et al. 2003), and by the coloration of the body. Samples were kept in a small, cylindrical 48 ml plastic container until all the samples had been netted. Each container had a piece of dampened paper towel, to keep the specimen from drying out.

In addition, we measured to the nearest 0.01 mm the total length of each hind wing, and the length of the spot on the same wing, with digital Vernier calipers, in order to estimate the effect of body size on wing spot and parasite load.

2.2.3. Determining the immune response in damselflies

The immune response of damselflies was determined (I) in order to uncover the association between the parasite burden and resource allocation by the host to costly immune response (e.g. Zuk and Stoehr 2002). In the wild, multicellular pathogens activate an encapsulation reaction by the damselfly immune system (Forbes 1991). This relatively simple yet efficient immune response can also be activated by artificial “parasites”, such as nylon inserts, and its efficiency can be analyzed precisely (Cordoba-Aguilar et al. 2006). To challenge the damselfly’s immune system, we inserted an alcohol-sterilized nylon filament, 2.0 mm long and 0.18 mm in diameter (Fig. 7) through the third abdominal pleura on the dorsal side of the sternal tergal margin, in order to mimic the natural penetration of the parasite through the exoskeleton. The immune system of the damselflies was then allowed to respond to this implant for exactly 720 minutes (12 hours). This duration was chosen on the basis of preliminary studies, which had shown that it delivers the largest differences between immune responses. During

incubation, all samples were kept in individual plastic containers in an incubator, model RS-IF-202, at a constant temperature of 21 ± 0.5 °C and at a standardized humidity. After incubation, the implant was gently removed and air-dried. The inserts were then stored within 12 hours at -12° C; finally, after all the samples had been collected, the inserts were freeze-dried at -27 °C.

The removed inserts were photographed in the laboratory through a stereomicroscope from two opposite directions: they were first placed vertically toward the camera, and then rotated 180° before the second photo. All the inserts were knotted so as to have the loose end bent. Using this bent end as a support enabled precise rotation. All the photographs of the inserts were taken in a standardized set-up, with identical lighting, camera equipment and settings. The photographs were then analyzed with the Image J computer program in order to measure the optical density of the capsulation film which had formed. Individual values thus consist of a combination of the thickness of the cell layer and the darkness of the cells, caused by a melanization reaction. The higher the value, the more efficient the immune response is assumed to be. This method, combining cell layer thickness and the cell melanization reaction, has been used successfully in previous studies and has proved to be associated with the individual's ability to react against natural pathogens (e.g. Rantala and Roff 2007).

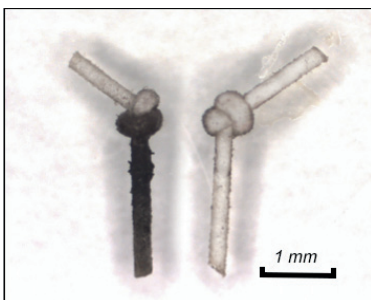


Figure 7. Two nylon filaments used in determining the immune response, showing the considerable variation in individual encapsulation ability. The filaments had been subjected for an equal response time, 12 hours, to the immune response of the study species *C. hastulatum*.

2.2.4. Determining the parasite load

In most parasite studies, quantifying the rate of parasite infection is extremely challenging to conduct accurately; parasites are commonly so small that they are hard to detect, or so numerous that they are too laborious to calculate. However, the most common macroparasites of odonates, the water mites and gregarines, are relatively easy to detect because of their large body size; they are also easy to sum up to precise numbers, as odonates are relatively small and consequently cannot harbor myriads of parasite for sheer lack of resources.

In this thesis, the number of parasites was calculated in the laboratory (I-V), with each specimen being investigated with a stereomicroscope under identical conditions and by the same person. Each damselfly's exoskeleton was thoroughly checked for water mite parasites (I-II); clear scars of fallen mites were included in the final number.

The gregarine parasite investigation was conducted by dissecting the abdomen of the sample longitudinally to expose the attached gregarines, which were then enumerated (I-V). Unfortunately there is no existing literature for the identification of either gregarines or water mite larvae to species level. We therefore restricted the investigation to a higher taxonomic level.

2.2.5. Analyzing the inbreeding coefficient with AFLP

An individual's heterozygosity can be defined via the inbreeding coefficient as the probability that two alleles at a locus will be identical by descent (Dasmahapatra et al. 2008). More inbred individuals therefore exhibit increased homozygosity. At a dominant Amplified Fragment Length Polymorphism (AFLP) locus, absence of a band is deemed to indicate homozygosity for the null allele; the presence of a band, on the other hand, can indicate either heterozygosity for the null allele or homozygosity for the "presence"

allele, and thus cannot be assigned a single state. As homozygosity increases through inbreeding, the number of bands carried by an individual decreases, and consequently the number of absent vs. true homozygous genotypes, thus increases. We therefore used the method described by (Dasmahapatra et al. 2008) to estimate f_{AFLP} , an inbreeding coefficient, based on AFLP markers, which has been found to correlate strongly with pedigree-based inbreeding coefficients Dasmahapatra et al. (2008).

2.3. Data analyses

To explain the variance in encapsulation response between populations, we used multiple linear regression analysis (**I**). In this model, the mean population encapsulation response was used as an dependent variable. Explanatory variables were the prevalence of (i) water mites and (ii) gregarines; (iii) mean size of the individual (as wing length to the nearest 0.1 mm); and (iv) latitude (in km) as the measure of day length. Latitude was measured to the nearest 50 meters of the sampling site's center using GPS. The multiple regression model was constructed with stepwise selection. All thirteen study populations were considered as independent observations.

Model selection based on Akaike's Information Criteria (AIC) was used in **I** to investigate which hypothesis best explained the results. The best data fit was provided by the model with the lowest AIC value.

To study the effect on the occurrence probability of parasites of distance from the southernmost edge of the distribution range in Finland (**II**), we used Generalized Linear Models with type III errors. The link function was set to "logit" in testing the relationship between the distance from the southernmost range edge in Finland and host occurrence along latitude strips of 100 kilometres, and the probability of infection by mites or gregarine parasites (probability distribution binomial). We used the scaled deviance method and "events per trial" option, where the number of infected individuals was

treated as an events variable and the number of examined individuals from each population or number of occupied square measuring 10*10 kilometres as the trial variable. In this model, distance from the southern range edge in Finland was used as the covariate.

Differences in the f_{AFLP} 's between the eleven study populations were tested with Analysis of Variance (ANOVA) (III). We used generalized linear models with type III errors in testing the relationship between number of parasites and f_{AFLP} . Numbers of parasites were negative binomial distributed and link functions were logarithmic. In the first model, number of parasites was used as a dependent variable, population as a factor (random variable), and f_{AFLP} as a covariate. The interaction between population and f_{AFLP} was not significant ($\text{Wald}_8 = 9.25, P = 0.321$); we therefore removed interaction from the final model for simplification. In the final model, the number of parasites was used as a dependent variable, population as a random factor and f_{AFLP} as a covariate.

In the study (IV), we first tested whether or not the allopatric and sympatric *C. virgo* and *C. splendens* populations were infected by eugregarine parasites, using the Fisher's exact test. We defined a population as infected if at least one damselfly was infected with at least one gregarine. Otherwise the population was not infected. We could not use logistic regression because all allopatric *C. virgo* populations but only one *C. splendens* population were infected, meaning that there was no variation. We calculated the prevalence (Zar 1999) of gregarine parasites for each population and both species. The prevalence was the proportion of damselflies infected among all the damselflies examined. We calculated 95% confidence intervals for the prevalence of gregarine parasites and for populations infected by them (Zar 1999). Differences in parasite prevalence between populations and species were tested using Mann-Whitney U-tests; differences in parasite prevalence in sympatric populations were tested using

Wilcoxon's matched pairs tests. Finally, we tested the association between the parasite prevalence of the two damselfly species in sympatric populations, using Spearman's rank correlation.

To study parasite mediated competition (**IV**), we tested the effects of eugregarine parasitism and population structure (allopatry versus sympatry) on mean *C. splendens* wing spot size with Generalized Linear Models (GLM) and included an identity link function. As predictors, parasitism rate and population mean wing length (to control for body size) were used as covariates, with population structure (allopatric or sympatric) as a fixed factor. Since most allopatric populations were found to be eugregarine-free, we did not include interaction between population structure and parasitism rate. Instead, we performed a separate analysis using only sympatric populations. We also excluded from the analysis population structure as a predictor variable. Traits of individuals from populations closer to each other are expected to be more similar than those from populations located further apart. We therefore measured distances in km (± 0.5 km) between populations, using national coordinate systems from maps; we then performed two Mantel tests, one for absolute wing spot size and another for relative wing spot size (as estimated from the stored residuals of a regression of wing spot size on wing length), to calculate correlation coefficients between the matrices of distances and the differences in mean wing spot sizes. Probability estimates were based on 1000 perturbations. Throughout the thesis, all statistical analyses were performed with IBM-SPSS Statistics 20.

3. MAIN RESULTS AND DISCUSSION

3.1. Infection rates of water mites and gregarines in damselflies (I-V)

During the three years of data collection on *Coenagrion hastulatum* (I & II), the combined data showed that on average 71.7 % (SD =23.1, n=13) of individuals in each population of *C. hastulatum* were infected by water mites, and correspondingly 54.0 % (SD = 26.3, n=13) by gregarines. On average, we found 22.9 gregarine parasites (SD = 56.0; ranging from 0 to 430), and 16.7 water mites (SD = 27.7; ranging from 0 to 190), in a total of 778 damselflies sampled across Finland. At the population level, the prevalence of gregarines increased with increasing mite prevalence ($r = 0.72$, $n = 13$, $P = 0.005$), i.e. populations harboring one parasite taxon had higher rates of the other taxon as well (Fig. 8). The prevalence of water mites was higher than that of gregarines (Paired t-test, $t = 3.56$, $df = 12$, $P = 0.004$) (Fig. 8).

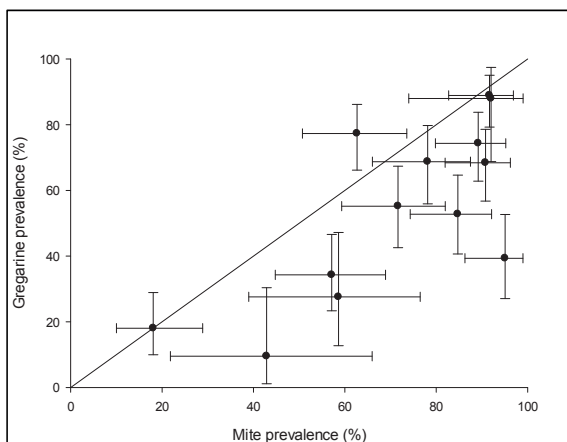


Figure 8. Prevalence of gregarines (in %) in 13 Finnish *Coenagrion hastulatum* populations in relation to prevalence of water mites, with 95 % confidence intervals. The continuous line indicates the value that the gregarine and water mite prevalence was the same in each population.

Gregarine parasite number in *C. splendens* varied between 0 – 47 gregarines per individual, (mean \pm 2 SE, 3.4 ± 1.3 , $n = 199$) (III). In this study, 20 % of the most parasitized *C. splendens* individuals harbored 94 % of all parasites. In all our sample

populations, 5 – 92 % of *C. splendens* individuals were parasitized by endoparasitic gregarines. The mean prevalence, i.e. the proportion of parasitized individuals per population, was $33\% \pm 19$ ($n = 11$).

Gregarine parasites were found in both *Calopteryx* damselfly species, with the prevalence varying from 0 to 80% in *C. splendens* populations and from 0 to 100% in *C. virgo* (IV). Parasite intensity, i.e. the number of parasites found in an infected individual, varied from one to 312 gregarines in *C. virgo* and from one to 64 in *C. splendens*. Gregarine parasites were found in 69% of individuals in allopatric *C. virgo* populations, but in only 0.9% of individuals in allopatric *C. splendens* populations. In sympatric populations, 45% of *C. virgo* and 19% of *C. splendens* individuals had gregarine parasites. The parasite prevalence was found to be higher in sympatric than allopatric *C. splendens* populations (Mann–Whitney U-test, $U = 35$, $n_1 = 13$, $n_2 = 12$, $P = 0.005$; Fig. 9), but there was no difference between allopatric and sympatric *C. virgo* populations ($U = 19.5$, $n_1 = 11$, $n_2 = 5$, $P = 0.364$; Fig. 9).

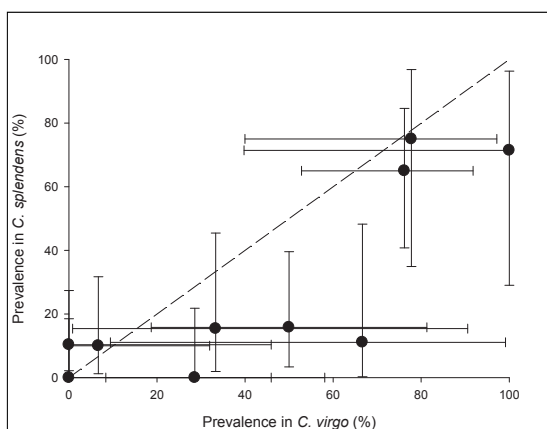


Figure 9. Prevalence and 95% confidence intervals of gregarine parasites (%) in sympatric *Calopteryx virgo* and *C. splendens* populations in Finland. Filled circles indicate prevalence of both species in sympatric population. Dashed line represents the situation where both species have the same prevalence of gregarine parasites in the sympatric population. If a filled circle lies below the dashed line, *C. splendens* has a lower prevalence of gregarine parasites than *C. virgo* in the same population and vice versa.

There was considerable variation in the rate of gregarine parasitism among populations, ranging from 0 to 100 % (mean \pm SD = 15.9 \pm 31.2 %, N = 26) (Fig. 9). All but one allopatric *C. splendens* population (12 out of 13, 92.3%) were gregarine-free, whereas gregarines were present in most sympatric populations (8 out of 13; 61.5%; Fisher's exact test, P = 0.011). *Calopteryx splendens* males from populations sympatric with *C. virgo* (mean \pm SD = 48.5 \pm 60.0%) had higher rates of gregarine parasitism than those from allopatric populations (3.2 \pm 11.7; Mann-Whitney U-test, Z = 2.51, P = 0.039).

3.2. The effects of parasites on host's immune response (I)

We found a strong inter-population level association between the prevalence of water mite infection and the average immune response in *C. hastulatum* damselfly populations. In other words, more parasitized populations had a higher average immune response to the "artificial pathogen" with which their immune system was challenged (Fig. 10). In

the case of gregarine parasites, on the other hand, parasite prevalence did not explain inter-population variation in the immune response of *C. hastulatum*.

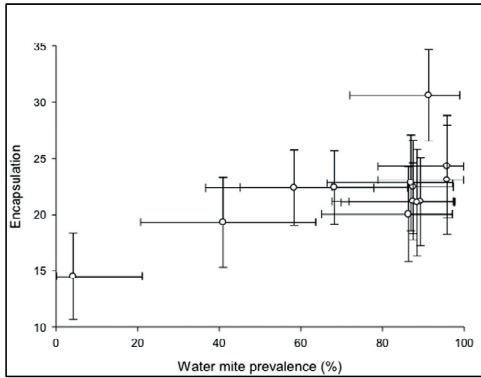


Figure 10. Prevalence and 95 % confidence intervals of water mites (%) in relation to the encapsulation response. Open dots represent the mean values of sample populations ($n=13$).

This positive association between the immune response and the rate of water mite infection may indicate (i) local adaptation to chronic parasite stress, (ii) effective “induced” immune response against parasites, or (iii) a combined effect of both.

3.3. Population structure and parasite prevalence (II)

We found that the prevalence of two different parasite taxa of *Coenagrion hastulatum* decreased simultaneously with the shift from the more continuous distribution area in southern Finland towards the more fragmented northern populations. In other words, the prevalence of both gregarines (Wald = 8.70, df = 1, $P = 0.003$; Fig. 11 a) and water mites (Wald = 4.72, df = 1, $P = 0.030$, Fig. 11 b) decreased with increasing distance from southernmost range edge in Finland and increased isolation. Neither taxon, however, showed a uniformly low prevalence further away from the southernmost range edge, reflecting a single local peak in parasite prevalence in the northern populations (Fig. 11 a, b).

In a previous study, (Locklin and Vodopich 2010) found that gregarine prevalence increased with increasing population density, which is consistent with our results. This is also predicted by metapopulation theory, according to which the more distant a population is from other populations, the more challenging it will be for a parasite to colonize the population (Hanski 1999). Moreover, as local extinction is suggested to be more likely at edge populations than at the center of distribution (Channell and Lomolino 2000), host population dynamics are likely to play an important role in marginal populations, by way of varying parasite recolonization speed. In addition, Channell and Lomolino (2000) presented that introduced species have fewer parasites, which may indicate similar factors constraining parasite prevalence differently in more distant populations than in those closer to each other.

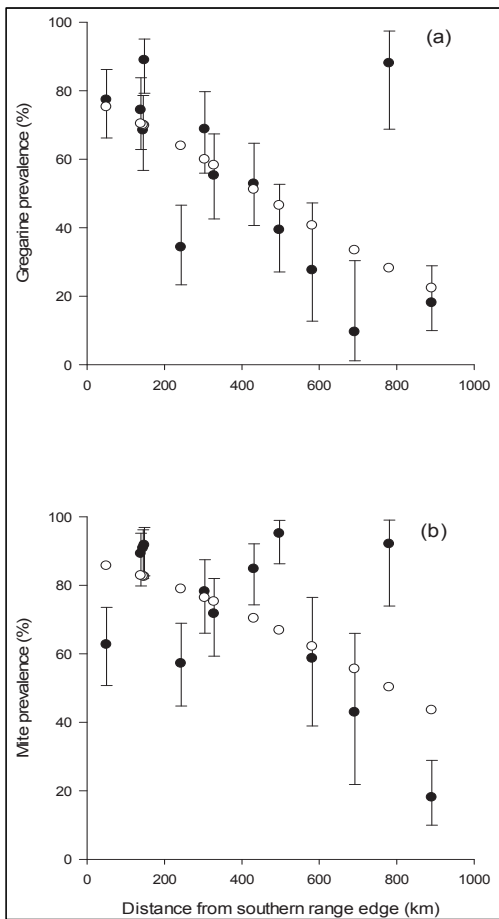


Figure 11. Relationship between prevalence of gregarines (a) and water mites (b) in 13 *Coenagrion hastulatum* populations and distance from southernmost range edge in Finland. Filled dots represent observed prevalence and open dots predicted prevalence. Error bars showing 95 % confidence intervals.

3.4. Genetic variation and parasite burden (III)

Gregarine parasite abundance was positively associated with an individual's f_{AFLP} level (Fig. 12). In other words, the more homozygous the individual, the more parasites it harbored. However, mean individual f_{AFLP} did not differ among populations. The positive association between f_{AFLP} and parasite abundance suggests that heterozygosity is linked with an individual's condition and consequently with that individual's susceptibility to

parasitism. The positive relationship between homozygosity and parasite abundance is consistent with the heterozygosity–fitness correlation hypothesis (Coltman et al. 1999).

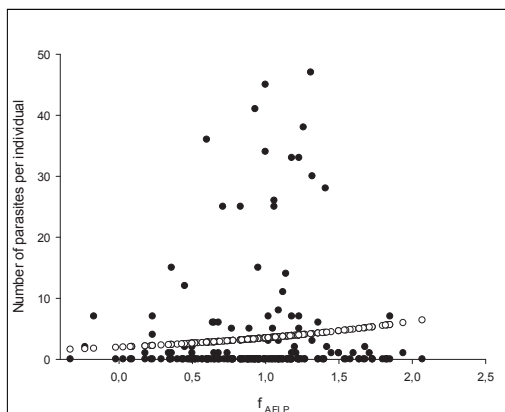


Figure 12. Relationship between number of parasites and Amplified Fragment Length Polymorphism–based inbreeding coefficient f_{AFLP} . Filled dots represent *Calopteryx splendens* individual’s parasite number in relation to each individual’s inbreeding coefficient. Open dots represent predicted values for each individual, based on negative binomial distribution.

The results imply that because of parasites’ negative impact on their hosts, parasites may in turn provide selection against homozygosity, thereby contributing to the maintenance of genetic variation of the host species.

3.5. Effects of interspecific competition on parasite load (IV)

We studied parasite-mediated competition between two closely related *Calopteryx* damselflies, *C. virgo* and *C. splendens*. We investigated a total of 31 populations, including eighteen allopatric and thirteen sympatric populations. We measured the occurrence of gregarines, a gut parasite.

We found that gregarine prevalence was higher in *C. virgo* than in *C. splendens*. On average, more than half of *C. virgo* individuals were infected by eugregarines in both allopatric and sympatric populations. However, hardly any allopatric *C. splendens*

populations had gregarines, while most sympatric populations had infected individuals (Fig. 13).

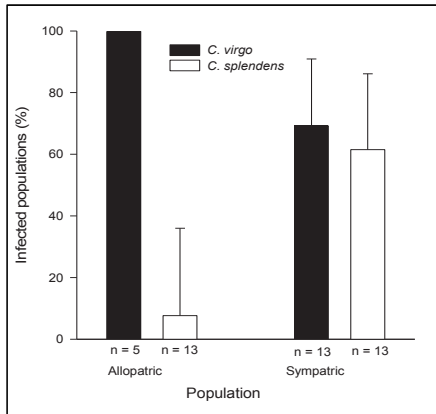


Figure 13. Percent of populations with gregarine parasites and in allopatric and sympatric *C. virgo* (black) and *C. splendens* (white) populations in Finland. *n* denotes the number of populations studied. Whiskers denote upper limit of 95 % confidence interval.

According to our results, the co-existence of host species affects the likelihood of the subordinate species, *C. splendens* to show higher levels of parasitism. We propose interspecific aggression, lower species genetic heterozygosity, and a difference in host species immunity as possible explanations for greater parasite burdens in *C. splendens* at sympatric sites.

3.6. Effects of parasites on character displacement (V)

Here we studied the damselfly *C. splendens*, whose males exhibit melanized wing spots which serve as ornaments. Males with large wing spots acquire more matings, but they also have an increased likelihood of becoming involved in aggressive encounters with male *C. virgo*, a common sympatric competitor. In addition to producing wing spots, melanin is linked to immune response, since it is also used in insect immune defence against parasites such as the gregarines.

In this study, we tested the association of four factors with wing spot size: population structure (*C. virgo* in sympatry), latitude, individual size, and the presence of parasites. *C. splendens* with fewer gregarines had larger wing spots (Fig. 14). This effect was independent of individual size, population structure, or latitude, in sympatric populations with *C. virgo*.

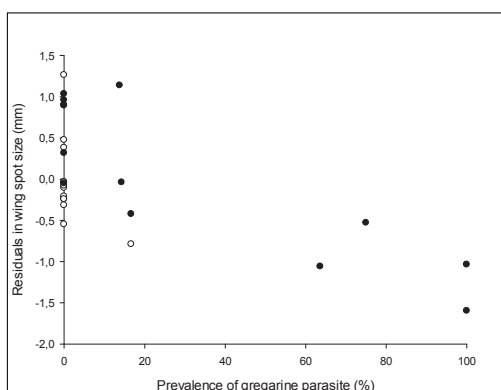


Figure 14. Relationship between residuals of wing spot size (mm) and prevalence of eugregarine parasites (%) in 26 *C. splendens* populations in Finland (24) and Latvia (2). Residuals were calculated from a linear regression, with wing spot size as the dependent variable and total wing length as the predictor. Open dots represent allopatric populations and filled dots sympatric populations with *C. virgo*.

In other words, our results indicate a possible interplay between parasites, driving character divergence in a sexual trait, the wing spot. We suggest that differences in the relative costs and benefits of different sized wing spots within and between populations may help to maintain variation of these sexually selected traits.

3.7. Association of parasites and odonates – synthesis table

ASSOCIATION OF PARASITES AND ODONATES			
Variable	Number of parasites	Level of association	Reference
<i>Genetic variation</i>	Increase	Individual	Kaunisto et al. 2013 (II)
<i>Food stress</i>	Increase	Individual	De Block & Stoks 2008
<i>Fluctuating asymmetry</i>	Increase	Individual	Bonn et al. 1996
<i>Gender, male vs. female</i>	Increase	Individual	Córdoba-Aguilar 2009
<i>Individual age, young vs. old</i>	Increase	Individual	Siva-Jothy & Plaistow 1999
<i>Date of emergence, early vs. late</i>	Increase	Individual	Nagel L. et al. 2009
<i>Androchromy in females</i>	Increase	Individual	Sanchez-Guillen et al. 2013
<i>Sexual ornament heterogeneity</i>	Increase	Individual	Siva-Jothy 2000
<i>Fat content and muscle power output</i>	Decrease	Individual	Marden & Cobb 2004
<i>Stronger mating ability</i>	Decrease	Individual	Forbes 1991
<i>Part of flight season, early vs. late</i>	Decrease	Individual	Forbes et al. 2012
<i>Abundance of other parasite</i>	Decrease	Individual	Smith & Cook 1991
<i>Immune response to artificial pathogen</i>	Increase	Population	Kaunisto & Suhonen 2013 (I)
<i>Marginal population vs. core population</i>	Decrease	Populations	Kaunisto et al. (III)
<i>Coexistence with other damselfly-species</i>	Increase	Population	Ilvonen, et al. 2011 (IV)
<i>Size of sexual ornament</i>	Increase	Population	Suhonen, et al. (V)
<i>Higher rate of fragmentation of habitat</i>	Decrease	Population	Taylor & Merriam 1996
<i>Host species rarity</i>	Decrease	Species	Grant & Samways 2007
<i>Larger size of distribution in sibling species</i>	Decrease	Species	Mlynarek et al. 2012
<i>Aquatic oviposition</i>	Decrease	Species	Rolf 1997

3.8. Conclusions and future directions

Presumably the most important finding was that: the damselfly *C. hastulatum*'s immune response to artificial pathogen increased with a higher number of parasites. Moreover, the parasite numbers decreased considerably towards northern populations of *C. hastulatum* (I). Marginal, more isolated *C. hastulatum* populations at the edge of distribution have fewer parasites compared to core populations (II). Damselfly *C. splendens* individuals with higher homozygosity have more parasites, however the rate of homozygosity did not differ between populations (III). Parasite prevalence was affected by whether the host species occurred in allopatric or sympatric population: sympatric *C. splendens* populations with sister species *C. virgo* harbored more parasites (IV). Parasites were associated with the wing spot, an ornament under sexual selection, and thus may play an important role in character displacement, i.e. size of the wing spot (V).

This thesis places intra-species variation of the host, e.g. varying immune response and population structure, in the context of host-parasite relationships; this has been a relatively understudied aspect in much of previous research.

In future studies, identifying the particular genes involved in the immune response of invertebrates and their effect on host fitness will be a critical next step. In order to further explore the aggregation patterns of parasites, the estimation of host density would also cast new light on the matter. Furthermore, it is important to study the association between the genetic background and isolation of populations. Since isolation is suggested to weaken genetic quality, it is likely to have a considerably effect on parasite prevalence. Finally, identifying all parasites to species level for instance with molecular methods, as well as supplying information on host and parasites phylogeny, could contribute new information on parasite dynamics and its effect on the hosts.

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Original Communications

Parasite burden and the insect immune response: interpopulation comparison

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SUMMARY

The immune response affects host's survival and reproductive success. Insurmountable immune function has not evolved because it is costly and there is a trade-off between other life-history traits. In previous studies several factors such as diet and temperature have been proposed to cause interpopulation differences in immune response. Moreover, the insect immune system may be functionally more protective upon secondary exposure, thus infection history may associate with the immune response. Here we measured how geographical location and parasite burden is related to variation in immune response between populations. We included 13 populations of the Northern Damsel fly *Coenagrion hastulatum* (Odonata: Coenagrionidae) in Finland over a latitudinal range of 880 km to this study. We found that water mites associated strongly with the immune response at interpopulation level: the more the mites, the higher the immune response. Also, in an alternative model based on AIC, latitude and individual size associated with the immune response. In turn, endoparasitic gregarines did not affect the immune response. To conclude, a positive interpopulation association between the immune response and the rate of water mite infection may indicate (i) local adaptation to chronic parasite stress, (ii) effective 'induced' immune response against parasites, or (iii) a combined effect of both of these.

Key words: Odonata, encapsulation, gregarine, water mite, *Coenagrion*.

INTRODUCTION

Nearly all organisms are parasitized, but there are substantial differences in the rate of parasite exposure between individuals and also populations. Parasites reduce their host's fitness by decreasing survival and/or reproductive success (e.g. Köning and Schmid-Hempel, 1995; Sheldon and Verhulst, 1996). Immune function has evolved to counter these negative impacts. Individuals with an insurmountable immune response do not exist due to trade-offs between a costly immune system, and other life-history traits e.g. the number of offspring, as resources for these traits are limited (Zuk and Stoehr, 2002). The costs of immune function result from evolving immunity (antagonistic pleiotropy) and from physiological costs of maintaining and utilizing the immune system against pathogens. Even if the highly effective immune function would appear to be the norm, the interpopulation variation in the immune response is unexpectedly high (e.g. De Block *et al.* 2008a).

Insects are excellent models for studying immune function in an evolutionary context. Their wide dispersal and survival depend to a large extent on effective immune function against miscellaneous microorganisms and parasites (Frank, 2000; Suhonen *et al.* 2010). Thus, it is clear that invertebrates must

have very efficient means of recognizing and defending against potentially harmful microorganisms (Söderhäll and Cerenius, 1998). The immune system of insects is based mainly on innate immunity that relies on the interactions between haemocytes, i.e. the cellular component of arthropod blood and the ability of soluble compounds in the humoral system to recognize, and respond to non-self. The main immune response of invertebrates is encapsulation, i.e. the capacity to form a capsule of haemocytes, around a multicellular pathogen. Encapsulated pathogens are thought to be killed by a combination of isolation from nutrients and the active release of cytotoxic molecules into the capsule by host cells (reviewed by Lawniczak and Barnes, 2007).

Our study taxon, odonates, is one of the most parasitized of all insects, harbouring both ecto- and endoparasites (Åbro, 1974, 1986). Although individuals are likely to be simultaneously infected by various parasite species, and the response to different parasites may vary, most former studies have concentrated on the effects of a single parasite taxon (e.g. Reinhardt, 1996; Andres and Cordero, 1998; Siva-Jothy, 2000, but see Honkavaara *et al.* 2009). This study was conducted with the Northern Damsel fly (*Coenagrion hastulatum*, Charpentier, 1825), which has probably the widest distribution of odonates in Finland (Dijkstra, 2006; Karjalainen, 2010) and is commonly infected by water mites and gregarines (Corbet, 1999). Both parasite taxa are spatially variable and might produce distinct patterns

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of resistance among damselfly host populations. Owing to the wide distribution of damselfly *C. hastulatum*, they encounter a diverse set of environments. Both temperature and length of photoperiod vary across the *C. hastulatum* range and are important regulators of the invertebrate host immune response (e.g. De Block *et al.* 2008b; Corby-Harris and Promislow, 2008). Across spatial scales, such environmental variation could cause spatial patterns in the ability of the host to resist parasites. In this study, we chose *C. hastulatum* sample sites from a wide geographical gradient because, according to the geographical mosaic theory of co-evolution, selection pressures are likely to vary between northern and southern sample sites with, for example, substantially different photoperiod mean temperature and level of parasitism (Thompson, 1994). This can result in significant genetic differences between subpopulations of a species. These differences may include pathogen defence levels.

The most abundant endo- and ectoparasites in these damselflies are water mites, (Acari: Hydrachnida) and gregarines (Protozoa) (Robinson, 1983; Åbro, 1990). Ectoparasitic water mites drain substantial amounts of body fluids through the chitinous exoskeleton of the host damselfly, which is likely to reduce the damselfly's survivorship and lifetime reproductive success (Forbes and Baker, 1991; Neubauer and Rehfeldt, 1995; Leung and Forbes, 1997). In the damselflies, the immunological response to water mite infection is melanotic encapsulation of the mite's feeding tube, the stylostome (Forbes *et al.* 1999). Other parasites, the endoparasitic gregarines are extracellular sporozoan parasites of invertebrates. Within the family of Actinocephalidae, the gregarines are cosmopolitan mid-gut endoparasites of damselflies (e.g. Åbro, 1990, 1996; Ilvonen *et al.* 2011). Gregarines reduce the energy absorbed from the food by its host (Siva-Jothy and Plaistow, 1999). Also, individuals that have a high burden of trophozoites often have ruptured and/or blocked mid-guts, which are likely to reduce the host's longevity (Åbro, 1990, 1996). Moreover, as longevity is a major determinant of fitness in many odonates (e.g. Sokolovska *et al.* 2000), and energy resources are vital for an individual's immune response and reproduction (Mesterton-Gibbons *et al.* 1996), gregarines are very likely to have dramatic impacts on host fitness.

In the previous studies in odonates, it has been shown that water mites and gregarine parasite burden reduces flight ability, fat content and muscle output, effectiveness of finding mate, breeding success, female fertility, male condition, and to increase mortality rate (Siva-Jothy and Plaistow, 1999; Forbes *et al.* 2002; Marden and Cobb, 2004; Schilder and Marden, 2006; Forbes and Robb, 2008; Rantala *et al.* 2010). Furthermore, the length of the photoperiod has been shown to affect both phenoloxidase, the main component of the

invertebrate immune response, and fat content, which are tightly linked to fitness in damselflies (Plaistow and Siva-Jothy, 1996; Rolff and Siva-Jothy, 2004; De Block *et al.* 2008a). On the other hand, fat content has been shown to also affect individual size in invertebrates (Boggs and Freeman, 2005). Finally, the size of an individual may play a role in parasite infection rate, so that larger individuals may harbour more parasites, for example because of the enhanced carrying capacity, or because a larger size may represent a greater resource for more individual parasites (Bush *et al.* 2001).

Most previous damselfly studies were conducted at an individual level in one population, but inter-population level studies are particularly scarce (but see De Block *et al.* 2008b) and most studies have focused only on a single parasite taxon while ignoring other possible infection agents (but see Honkavaara *et al.* 2009). In this study our main goal was to untangle the relationship between variation in parasite burden and variation in immune response across different insect populations. We measured immune responses in the *C. hastulatum* damselfly to see whether these are related to natural ecto- and endoparasite infection levels, the size of the individual measured by length of the wing, and length of day (latitude).

MATERIALS AND METHODS

Description of study areas and fieldwork

The data were collected in Finland between Kemiö (60°5'N, 22°4'E) and Sodankylä (67°31'N, 26°4'E), during 20–27 of June in 2009 from 13 different populations (Fig. 1). Samples were collected across an approximately 880 km latitudinal gradient, consisting of virtually the whole area of continuous distribution of *C. hastulatum* in Finland (Dijkstra, 2006; Karjalainen, 2010), thus providing a large geographical gradient with varying photoperiod and mean temperature. According to the Finnish Meteorological Institute, the mean temperature varies approximately 6 °C between the most southern and most northern sample sites (colder in north), and odonates develop faster as larvae in warmer conditions in temperate regions (e.g. Corbet, 1999; Johansson *et al.* 2010; Karjalainen, 2010). Thus, it was assumed that northern individuals emerge somewhat later in Finland and therefore the samples from the northern sites were collected generally a few days later to compensate for the slight delay in emergence. In Finland, the whole flight period of the study organism, *C. hastulatum*, lasts for roughly 3–5 months a year. Our samples were collected during 8 days, using as many same aged individuals as possible, in order to deal with the individuals in the same part of the flight period. All sample sites are freshwater watersheds. Sample site location was determined to

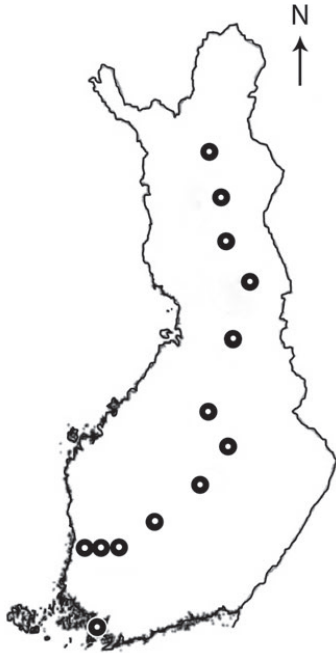


Fig. 1. Locations of the study populations of *Coenagrion hastulatum* in Finland are indicated with filled dots ($n=13$).

the nearest 50 metres grid of sample collection area by use of a GPS-device. From each population, 12 males and 12 females (except from Vehniä: 14 males and 8 females, and from Kuusamo: 11 males and 12 females) of *C. hastulatum* were captured; in total 309 individuals.

When samples were collected, each individual's age was determined by stiffness of the wings, as described by Plaistow and Siva-Jothy (1996) for *Calopteryx* damselflies, and by colouration of the body. Only sexually mature, i.e. 'adult coloured' and stiff-winged individuals, were accepted for this study. Samples were kept in a small, cylindrical 48 ml plastic container, until the rest of the samples were netted. Each container had a piece of wet towel in order to avoid drying of the specimen.

Activation of the immune system of damselfly

Multicellular pathogens activate an encapsulation reaction of the immune system of damselflies in nature. This relatively simple, yet efficient immune response can be activated also by artificial 'pathogens' or rather 'foreign body', such as nylon inserts, and its efficiency can be analysed precisely (e.g. Rantala and Roff, 2007; Cordoba-Aguilar *et al.* 2009; González-Santoyo *et al.* 2010). To challenge the damselfly's immune system, we inserted a 2.0 mm long piece of nylon (diameter 0.18 mm) 'implant' that had been

sterilized with alcohol, through the third abdominal pleura on the dorsal side of the sternal tergal margin in order to mimic the natural penetration of the parasite through the exoskeleton. Thereafter, the immune system of the damselflies was allowed to respond to this implant for exactly 720 min (12 h). This time was chosen because our preliminary studies have shown that it delivers the largest differences between immune responses of *C. hastulatum*. During the incubation, all samples were kept in their individual plastic containers, at constant temperature (21 ± 0.5 °C) and standardized humidity in an RS-IF-202 incubator. After incubation, the implant was gently removed and air-dried. Thereafter, inserts were stored within 12 h at -12 °C and finally, after all the samples were collected, inserts were freeze-dried at -27 °C. Odonate samples were stored in 70% ethanol for further analysis in the laboratory.

Removed inserts were photographed in the laboratory through a stereomicroscope from two opposite directions, so that inserts were placed vertically towards the camera, and rotated 180° before the second photograph. All the inserts were knotted in order to have the loose end bent. By using this bent end as a support, rotation was precise. All photographs of the inserts were taken in a standardized setup, i.e. identical lighting, camera equipment and settings. Thereafter photographs were analysed using the Image J computer program in order to measure the optical density of the formed capsulation film. Hence, individual values consist of a combination of the thickness of cell layer and the darkness of the cells, caused by a melanization reaction. The higher the value, the more efficient the immune response is assumed to be. This method, comparing cell layer thickness and melanization reaction of cells, has been used successfully in previous studies and has been proven to associate with an individual's ability to react against natural pathogens (e.g. Rantala and Roff, 2007).

Wings were cut off the body of odonates and placed between 2 thin microscope slides in order to prevent bending of the wing and other disturbing factors. Both hind wings of individuals were measured to the nearest 0.01 mm from the second antenodal crossvein with a caliper and the average of the wings was calculated. This measuring method is extremely accurate, as described by Tynkkynen *et al.* (2004). As in many other insects, wing length can be used as a standard estimate of body size in odonates (Forbes and Baker, 1991; Corbet, 1999; Koskimäki *et al.* 2009). The size was measured for the reason that the larger individuals may harbour more parasites, for example because of the enhanced carrying capacity, or because a larger size may represent a greater resource for more individual parasites (Bush *et al.* 2001). Numbers of water mites were calculated from the surface of damselflies with a stereomicroscope. In addition, few recently fallen mites (altogether

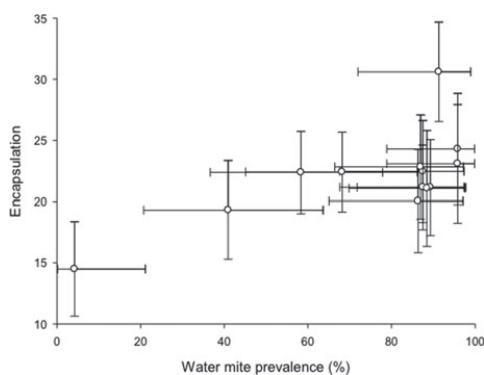


Fig. 2. Prevalence and 95% confidence intervals of water mites (%) in relation to the encapsulation response. A dot represents the mean values of sample populations ($n = 13$).

14 scars out of 6347 mites), recorded from the scars on damselflies, were included into prevalence and intensity estimates. Afterwards specimens were dissected with a fine surgical blade and numbers of gregarines were calculated from the interior of the individuals.

Statistics

We calculated the prevalence of water mites and gregarine parasites for each population, as the total number of parasitized individuals, divided by the number of all individuals in the population (Bush *et al.* 1997). Prevalence has been shown to be a relatively stable measure of infection of parasites (e.g. Hassall *et al.* 2010, but see Nagel *et al.* 2010) over the years. The prevalences of both water mites and gregarines were arcsin square transformed to satisfy assumption of normality. After transformation both variables fulfilled normal distribution ($z = 1.19$, $n = 13$, $P = 0.119$ and $z = 0.71$, $n = 13$, $P = 0.703$, respectively). Thereafter 95% confidence intervals were calculated for the prevalence and mean encapsulation rate of the populations (Fig. 2) (see Zar, 1999).

To explain variance in encapsulation response between populations, we used multiple linear regression analysis. In this model, the population mean encapsulation response was used as an independent variable, and (i) prevalence of both water mites and (ii) gregarines, (iii) the mean size of the individual (as length of the wing to nearest 0.1 mm) and (iv) the latitude as the measure of length of day (in km) were used as explanatory variables (Table 1). The latitude was measured to the nearest 50 metres of the sampling site's centre by using GPS. The multiple regression model was performed with stepwise selection. All 13 study populations were considered as independent observations. Analyses were conducted with PASW Statistics 18 software.

Table 1. Result from multiple regression analysis

(The mean immune response of population was used as an independent variable and the prevalence of water mites, prevalence of gregarines, the mean size of individual measured by length of hind wing (mm) and the latitude (km), to the nearest 50 m of the sampling site's centre by using GPS, were used as explanatory variables for *Coenagrion hastulatum*. The model was performed with stepwise selection. No other variable except water mite prevalence met the significance level of 0.15 for entry into the model.)

Variable in model	Coefficient	S.E.	<i>t</i>	<i>P</i>
Intercept	13.80	2.65	5.20	0.001
Mite	7.56	2.36	320	0.008

The final model: $F_{1,11} = 10.24$, $P = 0.008$, $r^2 = 0.44$.

Variables excluded from model	<i>t</i>	<i>P</i>
Latitude	1.26	0.237
Size	0.38	0.711
Gregarine	0.004	0.997

Model selection based on Akaike's Information Criteria (Anderson *et al.* 2000) was used to find out which of the alternative hypotheses best explain interpopulation variation in the immune response. Model selection can be used only if the data remain the same in all models tested. Altogether 4 main effects referring to the hypotheses (water mite, gregarine, latitude and individual size) were entered in computing alternative models (Table 2). The best model was selected based on Akaike's Information Criteria for small samples (AICc) (Andersson *et al.* 2000) where the model with the lowest AICc-value fits best to the data.

RESULTS

Overall, water mite and gregarine prevalence was high (grand mean from population was 76.3% (s.d. = 27.0) and 56.7%, (s.d. = 24.8), respectively). Further, 9 populations out of 13 had water mite prevalence greater than 80% (Fig. 1). The multiple regression model showed that interpopulation variation in immune response increased with increasing prevalence of water mites (Tables 1 and 2, Fig. 2). On the contrary, we did not find a statistically significant association between the immune response and the prevalence of gregarines (Tables 1 and 2). To further describe the mean number of parasites found in the infected hosts (the zeros of uninfected hosts were excluded), we calculated the mean intensity for both water mites and gregarines. The mean intensity of water mites was 27.3 ± 32.8 (mean \pm s.d.), range between 1 and 190 (infected individuals $n = 240$ out of 309). For gregarines, the mean intensity was 47.0 ± 79.1 , range between 1 and 430 (infected

Table 2. Individual model results of top models (with a Δ AICc <4) explaining the immune response in *Coenagrion hastulatum* populations in Finland

(Adjusted r^2 gives the percentage of variance explained by the model (from multiple regression analysis), estimated regression coefficient for the variable. t -statistics and P values indicate statistical significance of each variable in the model. The immune response was used as an independent variable and the prevalence of water mites (M), prevalence of gregarines (G), size of individual (S) and the latitude (L), were used as explanatory variables.)

Model	Δ AICc	Adjusted r^2	Parameter	S.E.	t	P
M	0.00	0.435	7.56	2.36	3.20	0.008
M+	0.09	0.463	9.87	2.95	3.35	0.007
L			0.004	0.004	1.26	0.237
M+	2.00	0.378	7.55	2.85	2.65	0.024
G			0.01	3.44	0.004	0.997
M+	2.05	0.406	10.15	3.52	2.88	0.018
L+			0.005	0.004	1.21	0.258
G			-0.574	3.40	-0.17	0.869
M+	4.05	0.331	10.21	4.12	2.48	0.038
L+			0.005	0.004	1.07	0.314
G+			-0.62	3.82	-0.16	0.875
S			-0.16	4.52	-0.04	0.973

individuals $n=178$ out of 309) in the sample populations.

DISCUSSION

In this study, we found a strong interpopulation level association between the prevalence of water mite infection and the average immune response in *C. hastulatum* damselfly populations. In other words, more parasitized populations had an increased average immune response to 'artificial pathogen' with which their immune system was challenged. In addition, an alternative model from AIC included also the latitude and individual size. However, the prevalence of gregarine parasites did not explain the interpopulation variation in immune response of *C. hastulatum*.

In previous studies, the interpopulation difference in the immune response has been explained for example, by the differences in the length of the larval period (De Block *et al.* 2008b). In this study, the mean temperature varied between sample sites by roughly 6 °C and the maximum length of the day from 18 to 24 h, which is likely to have effects, for example, on the length of the growth period, as the damselfly larvae do not grow substantially in water below a temperature of 10 °C (e.g. Karjalainen, 2010). In addition, diet, temperature and reproductive behaviour have been shown to affect the immune response in insects (Braune and Rolff, 2001; Schmid-Hempel, 2004; De Block *et al.* 2008b). Here we found a fundamental complementary factor to explain the detected difference in the immune response, as there was a strong interpopulation level association between the immune response of the *C. hastulatum* and the water mites. An explanation for our results may be that *C. hastulatum* populations are (i) locally adapted to chronic water mite infection,

(ii) individual damselflies have an effective, 'induced' immune response against parasites, or (iii) a combined effect of both of these effects. One possible explanation for our results may be the geographical mosaic theory of co-evolution (Thompson, 1994), in which selection pressures vary between local populations with e.g. a substantially different level of parasitism, photoperiod and/or mean temperature (here latitude). This can result in significant immunity differences between *C. hastulatum* populations. The positive association between the water mite burden and the immune response may be due to local adaptation to chronic stress of parasite burden, so that the immune response is initially higher in heavily parasitized populations. If local population was adapted to local water mite burden, infection rate between years has to be stable and/or dispersal between populations must be low. If dispersal between populations is high and/or infection rate between nearest populations varied considerably, the selection may break down the local adaptation process. In previous studies, the prevalence of water mites was fairly constant between the years (Hassall *et al.* 2010, but see Nagel *et al.* 2011). Also, it has been shown that distance is a main restricting factor for dispersal of damselflies and populations seem to be essentially restricted to their natal pond, especially small species such as Coenagrionidae damselflies (e.g. Angelibert and Giani, 2003). Furthermore, *Coenagrion mercuriale* was found to be extremely sedentary, with dispersal limited to an area of contiguous habitat. The median net lifetime movement was 31.9 m and 66% of individuals moved less than 50 m in their lifetime. Movements of greater than 500 m were rare and the longest recorded movement was 1.79 km (Rouquette and Thompson, 2007). *Coenagrion mercuriale* is presumably the most sedentary of its genus, but other *Coenagrion* species

do also have weak dispersal abilities. Thus, according to these studies and personal observations from *C. hastulatum* dispersal abilities, we assume that dispersal between our study sites with tens of kilometers distance is fairly insignificant. Consequently, we consider local adaptation as a highly possible explanation for the strong association between the immune response and the water mite burden.

In this study, the other parasite taxon, endoparasitic gregarines, did not affect the interpopulation level immune response. This is consistent with several previous studies, which failed to find evidence for gregarine parasite infection and immunity in damselflies (Cordoba-Aguilar *et al.* 2006; Contreras-Garduno *et al.* 2008; Honkavaara *et al.* 2009). However, gregarine parasites have other fitness costs, for example they absorb energy from their host (Siva-Jothy and Plaistow, 1999). Also, individuals that have a high burden of trophozoites often have ruptured and/or blocked mid-guts, which are likely to reduce the host's longevity (Åbro, 1990, 1996). Moreover, as longevity is a major determinant of fitness in many odonates (e.g. Sokolovska *et al.* 2000), and energy resources are vital for an individual's immune response and reproduction (Mesterton-Gibbons *et al.* 1996), gregarines are very likely to have a negative impact on host fitness.

An alternative model, based on Akaike's Information Criteria, which included either the prevalence of water mites, the latitude, or the individual size, fitted only slightly less well than the models with only prevalence of water mites. The role of individual size may originate from the fact that the larger individuals may physically harbour more parasites, e.g. because of the enhanced carrying capacity, or because a larger size may represent a greater resource for more individual parasites (Bush *et al.* 2001). Latitude, in turn, has effects on temperature, as well as the length of the photoperiod. According to long-term data from the Finnish Meteorological Institute, the average temperature varies by approximately 6 °C between the southernmost and the northernmost sample sites during one year. Winters in southern Finland (average day-time temperature is below 0 °C/32 °F) are usually 4 months long and in northern Finland, nearly 7 months long, affecting e.g. freezing of the water body. In addition, the maximum length of the day varies from roughly 18 to 24 h, from south to north, during the main flight period of *C. hastulatum*. In previous studies, the length of the photoperiod has been shown to affect PO, the main component of the immune system activity in damselfly species (De Block *et al.* 2008b). Moreover, the length of the photoperiod has been shown to affect fat content in damselflies (De Block *et al.* 2008b), and fat content in turn may be linked to individual size (Plaistow and Siva-Jothy, 1996; Boggs and Freeman, 2005; Koskimäki *et al.* 2009). Both fat content and

PO are tightly linked to fitness in damselfies (Plaistow and Siva-Jothy, 1996; Siva-Jothy and Plaistow, 1999; Rolf and Siva-Jothy, 2004). As these traits seem to be somewhat linked, there is likely to be some compensatory effects between them, which may conceal some part of the association. Thus further studies are needed, ideally with environmental variables, to see how the environment affects these evolutionary responses. Moreover, in order to create a causative link between the immune response and the effects of parasites, traits should be examined in a variety of genetic backgrounds and under controlled conditions.

In addition to the innate immunity, recent studies have shown that invertebrates do also have certain immune mechanisms that could be classified as 'induced' immunity, thus being functionally more protective upon secondary pathogen exposure (Arala-Chaves and Sequeira, 2000; Schmid-Hempel, 2005; Sadd and Schmid-Hempel, 2006). This may be an alternative explanation for our results. It may be that the effective response to high infection rate by water mites has raised the immune response for 'artificial pathogen', with which we measured the immune response. In a previous study in *Lestes* damselfies Nagel *et al.* (2010) found that the level of parasite infection and immune response varied interannually, a pattern that could not be explained by local adaptation to chronic parasite stress. Furthermore it is important to bear in mind that these explanations do not exclude each other, so further studies are required to resolve the potential role for induced immunity effects to operate in different populations.

To conclude, *C. hastulatum* showed a positive association between the immune response and water mite prevalence. In addition, individual size and latitude also associated with the immune response. This is to say that when determining associations between the immune response and other traits, it seems to be crucially important to take both the parasite history of a population and the individual size and latitude into account.

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RUNNING HEAD: Host distribution and parasite burden

Populations of the damselfly *Coenagrion hastulatum* have fewer parasites at the edge of the host range

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Abstract We studied variation in population-level prevalence of two parasite taxa across the geographical range of their host, from an area with more continuous host populations in the south of Finland towards the northern edge of the host distribution characterized by more isolated populations. Prevalence of both ectoparasitic water mites and endoparasitic gregarines decreased with increases in latitude and decreases in continuity of host populations toward the edge of the distribution of the host damselfly, *Coenagrion hastulatum*. Prevalence of the two parasite groups showed a positive correlation, i.e., host populations parasitized by one parasite group showed a higher prevalence of the other parasite group as well. Our results are consistent with the metapopulation theory, which predicts that parasites will be more challenged to recolonize more distant populations after local extinction.

Key words Odonata Parasite Range edge

INTRODUCTION

Population density of a species is commonly thought to be highest in the centre of the range and lowest in more peripheral parts areas (Brown 1984, but see Sagarin and Gaines 2002). This is based the idea that species should experience less favourable biotic and abiotic conditions as the distance from the centre of the distribution increases (Channell and Lomolino 2000). Several factors have been proposed to decrease species abundances towards the edges of the distribution. Among these, parasites are thought to ubiquitous factors that reduce host fitness (Price 1980). Moreover, parasites can affect their hosts differently in edge populations as compared effects near the centre of the distribution area (Hochberg and Ives 1999). Nonetheless, studies of differences in parasite dynamics across the host distributions remain few and far between.

Intensity of parasitism in populations is affected by various factors. For example, abundant host species with large geographical distributions may harbour more parasitic species and higher parasite numbers than rare host species with more limited distributions (Dritschilo, Cornell, Nafus and Oconnor 1975, Grant and Samways 2007, Gregory 1990). Additionally, temperature and photoperiod affects the immune response of invertebrate hosts, thus indirectly affecting parasite load (De Block, McPeck and Stoks 2008). Also, host vulnerability may be affected by parasites already present (Kraaijeveld and Godfray 2008, Poitrineau, Brown and Hochberg 2003). For example, birds with endoparasites harbour more ectoparasites, possibly because of the reduced energy available for preening (Holmstad, Jensen and Skorpning 2008). Although an increasing encounter rate with a second parasite is normally thought to decrease investment in defence against the first parasite, some results indicate that it may favour resistance to the first enemy in some situations (e.g. Poitrineau, Brown and Hochberg 2003). Even though individuals are likely to be simultaneously infected by several parasite species, and

their responses to different parasites may vary, most studies have concentrated on effects of a single parasite taxon on the host (e.g. Andres and Cordero 1998, Canales-Lazcano, Contreras-Garduno and Cordoba-Aguilar 2005, but see Honkavaara, Rantala and Suhonen 2009, Morrill, Mlynarek and Forbes 2013).

Parasite prevalence and abundance may also be affected by spatial factors that vary across host range. For example, host may become too scarce towards its range limits for viable population of the parasites to persist (e.g. Radomski et al. 1991; Brawer and Gaston 2003). Consequently, it is unlikely that the host will come extinct before the parasite will come to extinct itself or parasite species switch to use an alternative, more abundant host species. Further evidence of varying parasite burden across the distribution of the host comes from studies of invasive species, in which invaders are shown to escape from their parasites of their native range (reviewed in Prenter et al. 2004). Release from natural parasites can subsequently aid the performance of an invader. For example, a detailed study on invasive green crab *Carcinus maenas* proposed that invading individuals leave behind most of its parasites, and by this benefit they grow larger and become more abundant (Torchin 2002). On the other hand, at the edge of host geographic range, the stresses caused by abiotic factors can be seen as potentially reducing host body condition and increasing its vulnerability and susceptibility to parasites. For example, a study has been conducted on a freshwater snail (*Lymnaea stagnalis*) in which parasite prevalence was significantly higher close to the range limit than in the more central parts of its geographical range (Briers 2003).

In this study we used the damselfly *Coenagrion hastulatum* as our study object. The damselfly *C. hastulatum* has probably the widest distribution of odonates in Finland (Dijkstra 2006; Karjalainen 2010) and northern parts of Finland is considered to be the northern edge of

the total range of this species, while the southern boundary of Finland may be relatively closer to the distribution centre (Askew 1988). This damselfly is very suitable for host-parasite studies because it is commonly infected by water mites and gregarines (Kaunisto and Suhonen 2013). In the present study, we explored how population level prevalence on the same damselfly host of two parasite groups with different life strategies (ecto- and endoparasites), varied over a relatively large (880 km) spatial scale from distribution centre to northern edge of this species. In order to assess how parasite prevalence varies with distance between populations in Finland, we compared samples from areas in the southern parts of the distribution range with those from more continuous populations with those from more isolated northern populations.

MATERIALS AND METHODS

Study species

We studied parasitism in the northern damselfly *Coenagrion hastulatum* (Odonata, Zygoptera), an insect with a flying, terrestrial adults and aquatic larvae. Damselfly *C. hastulatum* occurs in both running and standing waters and it forms a meta-population structure, in which populations suffer from local extinctions and recolonizations (Suhonen et al. 2010, 2014). The species is suitable for our the study in Finland as (i) it is widely distributed (Dijkstra 2006), (ii) it harbours various parasites, including ectoparasitic water mites and endoparasitic gregarines throughout its whole distribution (Kaunisto and Suhonen 2013), (iii) the population structure of the species varies from more continuous in the south to more isolated in the north (Askew 1988; Dijkstra 2006) (Fig. 1).

Damselflies are regularly parasitized by water mites (Acari: Hydrachnida) and gregarines (Protozoa) (Åbro 1974, Åbro 1986). Water mites are ectoparasites (e.g. Corbet 1999), whereas the endoparasitic gregarines are extracellular parasites occurring generally in the digestive system of invertebrates (e.g. Siva-Jothy and Plaistow 1999). Both parasites belong to species-rich groups; currently some 6,000 water mite species (Di Sabatino, Martin, Gerecke and Cicolani 2002, Smith, Cook and Smith 2010) and 1,700 gregarine species (Rueckert, Simdyanov, Aleoshin and Leander 2011) have been described. However, recent study on water mites parasitizing the odonates found only from one to a few water mite species per host damselfly species (Zawal and Szlauer-Lukaszewska 2012). Individuals of both of these parasite groups are extremely difficult to identify, and therefore we treated them at these two higher taxonomic levels.

Water mites and gregarines reduce host fitness in multiple ways, e.g. by reducing the host's energy resources (Åbro 1976, Siva-Jothy and Plaistow 1999). Individuals with high burdens of gregarines often have ruptured and/or blocked mid-guts, which is likely to reduce the individual's longevity (Åbro 1996). Consequently, as adult longevity is a major determinant of fitness in many odonates (for meta-analysis, see Sokolovska, Rowe and Johansson 2000), and as energy resources are vital to support an immune responses and reproduction (Mesterton-Gibbons, Marden and Dugatkin 1996), gregarines and water mites are likely to have considerably impact on host fitness.

Description of the study area and sample preparation

During the years 2009, 2010 and 2012 we sampled thirteen *C. hastulatum* populations from freshwater ponds and lakes across Finland (Table 1; Fig 1). Focal populations ranged from near the southern border of Finland, (Kemiö, 60° 5'N, 22° 4'E) to above the Arctic Circle in the north (Sodankylä, 67° 31'N, 26° 4'E), spanning a latitudinal gradient of roughly 880 km (Fig. 1, data drawn from the database at the Finnish Museum of Natural History). The northern sample sites are at the northern edge of the distribution of *C. hastulatum* (Dijkstra 2006, Karjalainen 2010). In order to discern the general distribution pattern, we divided the number of all 10 sq. km quadrats (excluding quadrats on open water) by the number of quadrats in which *C. hastulatum* was observed, separately for every 100-km band of latitude (Fig. 2).

Our data were collected so that the timing of samples with respect to host flight season was roughly equivalent between the years. Flight times were estimated by analysing the age of individuals captured during preliminary visits to the southern sample sites. Ages were determined by stiffness of the wings (Plaistow and Siva-Jothy (1996) and in relation to colouration of the body in order to exclude bland coloured teneral and discoloured old individuals. Only sexually mature individuals with full adult-colour and stiff-wings were included in the study to reduce potential correlation between age and parasite load (Siva-Jothy and Plaistow 1999).

Sample site location was determined to the nearest 50-metres grid of sample collection area by means of a GPS device and all the samples were collected from roughly 100 meter area. We aimed to collect 12 males and 12 females by netting them during the day from each population each year. However, on several occasions all 24 individuals could not be collected,

mainly due to harsh, rapidly-changing weather conditions in the northern areas. We examined, on average, 59.8 (SD = 20.4; range 21 to 76) host individuals from each of the populations over the three of sampled years (Table 1.). During fieldwork individuals were kept alive in small, cylindrical 48 ml plastic containers with a piece of wet towel until the rest of the samples were netted. The samples were stored in 70% ethanol in the field for further counting water mites and gregarine parasites in the laboratory.

Number of water mites on the surface of each damselfly was enumerated using an Olympus MVX 10 stereomicroscope and number of attachment scars on the exoskeleton (e.g. Nagel, Zanuttig and Forbes 2011) was used to include recently detached mites in the prevalence counts. After mite counts, the specimen was dissected with fine surgical blades to determine number of gregarines in the gut of each specimen (See Table 1).

Data analysis

Because parasite prevalence varied considerably from year to year (Table 1), we pooled our data across years to more a reasonably estimate average parasite prevalence. We estimated prevalence of water mites and gregarines separately for each population by dividing the number of infected individuals by the total number of individuals examined (Bush, Lafferty, Lotz and Shostak 1997). The previous studies showed that this prevalence of infection by parasites is relatively over the years in damselflies (Hassall, Lowe, Harvey, Watts and Thompson 2010, but see Nagel, Zanuttig and Forbes 2010). Additionally, the intensity of parasitism was calculated for both water mites and gregarines as the mean number of parasites found in hosts infected by at least one individual (Bush et al. 1997).

We tested the effect of distance from southernmost distribution range edge in Finland on

the occurrence probability of parasites using Generalized Linear Models with type III errors. The link function was set to “logit” when testing the relationship between the distance from the southernmost range edge in Finland and the host occurrence along latitude stripes of 100 kilometres, and the probability of infection by mites or gregarine parasites (probability distribution binomial). In the model, we used the scaled deviance method and “events per trial” option, where the number of infected individuals was treated as events variable and the number of examined individuals from each population or number of occupied square measuring 10*10 kilometres were treated as the trial variable. In this model, the distance from the southern range edge in Finland was used as the covariate.

The Pearson correlation coefficient was used to test the associations between the prevalence of water mites and gregarines. Additionally, the paired t-test was used to compare the means of water mites and the gregarines, with the population as the independent variable.

All analyses were performed using the IBM-SPSS statistical package, version 20.

RESULTS

The host damselfly *C. hastulatum* has a more continuous distribution in southern Finland than in northern part of the distribution range (Fig 2). The probability that the damselfly *C. hastulatum* was collected in a 10*10 km square in a 100 km band of latitude decreased with increasing distance from the southern distribution range edge in Finland (Wald = 428.7, df = 1, $P < 0.001$; Fig. 2).

On average, 71.9 % (SD =23.6) and 54.3 % (SD = 26.8) of the individuals in each population were infected by water mites and gregarines (Fig. 3), respectively. We found means of 22.9 gregarines (SD = 56.0; range 0-430) and 16.7 water mite larvae (SD = 27.7; range 0-190)

for the 778 damselflies in the total sample. The observed prevalence of gregarines increased with increasing mite prevalence ($r = 0.75$, $n = 13$, $P = 0.003$; Fig. 3); i.e. populations harbouring kind of parasite also generally had the other kind. The overall prevalence of water mites was higher than that of gregarines (Paired t-test, $t = 3.51$, $df = 12$, $P = 0.004$; Fig. 3). Finally, the average intensity of water mite parasitism was 20.7, ranging from 1.2- 68.7 (SD= 16.56), and intensity of gregarines was 42.9, ranging from 6.3- 91.5 (SD= 26.7).

Southern *C. hastulatum* populations had more parasites than the more isolated northern populations. The prevalence of gregarines (Wald = 8.28, $df = 1$, $P = 0.004$; Fig. 4a) and the prevalence of water mites (Wald = 4.54, $df = 1$, $P = 0.033$, Fig. 4b) decreased with increasing distance from the southernmost range of damselfly host in Finland. Prevalence of either parasite group did not show the expected pattern of becoming steadily lower with distance from southern Finland. Furthermore, there was a local peak (Table 1) in parasite frequency in one northern population approximately 800 km from the southern range edge (Fig. 4, Table 1).

DISCUSSION

We found that the prevalence of parasites in two groups decreased in the direction of more isolated northern populations. We also found that the water mites and gregarines prevalences were positively correlated; i.e., populations already parasitized by one parasite group had higher numbers of other parasites as well. Radomski et al (1991) have proposed three non-mutually exclusive hypotheses why host species have fewer parasites at the periphery of their geographic range: (i) unsuitable host hypothesis, (ii) geographical barrier hypothesis and (iii) physiological barrier hypothesis.

Unsuitable host hypothesis, i.e. the absence of alternative host from the northern part of the *C. hastulatum* range could be one explanation for lower number of infected individual with water mites or gregarine parasites, since it is proposed that parasites of damselflies can successfully parasitize a few host species (Morrill, Mlynarek and Forbes 2013). Furthermore, previous study with holly leaf-miner *Phytomyza ilicis* found that absence of alternative host for parasitoid (*Chrysocharis gemma*) may explain lower larval parasitism at edge population (Brewer and Gaston 2003). However, the absence of alternative host species did not convincingly explain our results. Four out of six closely-related *Coenagrion* species occurring in Finland that could presumably serve as substitute hosts, *C. johanssoni*, *C. armatum*, *C. lunulatum*, occur nearly throughout the distribution range of *C. hastulatum*, (Dijkstra 2006, Karjalainen 2010). Thus, even the northernmost populations of *C. hastulatum* are likely to have alternative hosts, suitable for both water mites and gregarines. Additionally, the cline in parasite prevalence does not follow the occurrence of any of these alternative host damselfly species' distributions range. Therefore, it seems unlikely that our results in parasite patterns could result from the absence of other substitute host damselflies.

According to the geographical barrier hypothesis (Radomski et al 1991), the host individuals which emigrated from infected population do not have sufficient number of parasites and/or to maintain parasite population in new habitat patches near the edge of host geographical range. Support for this hypothesis is derived e.g. from habitat fragmentation studies. For example, habitat fragmentation has been found to associate with the rate of parasitism and to be a potential factor in decreasing parasite prevalence the closer the population is to species' distribution edge (e.g. Lloyd, Martin, Redmond, Langner and Hart 2005). However, the geographical barrier hypotheses or habitat fragmentation did not directly explain our results, as

all of our study populations harboured parasites, and human land use and/or freshwater management is by far more intense in the southern parts of Finland with more continuous population structure of *C. hastulatum* (for land use in Finland, see e.g. Lappalainen 1993).

Physiological barrier hypothesis states that some unique physiological features of the host may prevent establishment, growth, and/or reproduction of parasites (Radomski et al 1991). This may partly explain our results as we have shown in previous study that *C. hastulatum* populations vary considerably in their immune response to parasites (Kaunisto and Suhonen 2012). However, physiological barrier hypothesis is unlikely to explain our results completely, for the reason that even the northern populations with fewer parasites had strong immune response (Kaunisto and Suhonen 2013).

As is usual with large-scale observational data, multiple factors may affect the results. For example, in the present study, the host distribution map (Fig. 1) is likely to suffer from collecting bias, as doubtlessly more intensive field work has been done in southern Finland, resulting in fewer observations in more remote and less colonized northern parts of the country. However, odonate observations have been reported from most 10x10 km terrestrial quadrats in northern Finland (Finnish insect database <http://hyonteiset.luomus.fi>), suggesting that northern Finland has been reasonably well surveyed for odonates.

It was predicted that increasing abiotic stress reduces host body condition and increasing its vulnerability and susceptibility to parasites (Morand 2010). In our study, mean temperature varies approximately 6 °C across the range of latitudes and colder mean temperature may be associated with lower parasite numbers because of low tolerance of colder temperatures (Kaltz and Shykoff 1998). The temperature differences between northern and southern Finland may

partly have affected our results because we found relatively high numbers of parasites in one northern population, suggesting that low temperature suppress host damselflies. However, abiotic stress due to varying temperatures should be higher towards colder northern populations that contradictorily had less parasites in our study.

Finally, simple local abundance of the host species is likely to affect parasite abundance. Unfortunately, we do not have accurate data about the local abundance of individuals in our study populations through the years. However, *C. hastulatum* has been generally abundant even in the northernmost study population throughout the sampling years (pers. obs. Kaunisto) and therefore it seems unlikely to directly explain the decrease in parasite prevalences.

Our results from host – parasite interaction from centre to northern distribution range of host species are consistent with the metapopulation theory, which predicts that the more distant a population is from other populations, the more challenging it should be for the parasite to recolonize the host population after a local extinction (Hanski 1999). Moreover, as local extinction is proposed to be more likely at edge populations rather than in the centre of the distribution (e.g. Channell and Lomolino 2000), host population dynamics are likely to play even more important role in marginal populations by influencing the parasite recolonization speed. In addition, Torchin, Lafferty, Dobson, McKenzie and Kuris (2003) found that introduced species have fewer parasites, which may indicate similar factors constraining parasite prevalence differently in more distant populations than in populations closer to each other.

In conclusion, our results show that isolated edge populations harbour fewer parasites in *C. hastulatum*. This may increase the probability of persistence in marginal populations under harsher conditions at the extremities of their distribution range (e.g. Radomski et al 1991; Brewer

and Gaston 2003). Moreover, our results provide new information for host and parasite population dynamics at the edge of host species distribution range (Gaston 2003). However, long-term data sets from larger geographical areas are required to give more insight into the differences between both temporal and spatial variance in parasitism rates and to produce new information on parasite dynamics among host and parasite populations.

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Table 1. The study populations and their locations in Finland. Gregarine prevalence (%), water mite prevalence (%), and sample size for *Coenagrion hastulatum* for each study population and each study year.

Population	Location	Gregarine prevalence			Mite prevalence			Sample size		
		2009	2011	2012	2009	2011	2012	2009	2011	2012
Kemiö	60°5'N, 22°34'E	78	61	96	87	57	46	23	28	24
Elijärvi	60°52'N, 22°20'E	65	63	96	89	92	88	26	24	24
Vaalijärvi	60°55'N, 22°4'E	57	63	88	89	92	92	28	24	24
Koskeljärvi	60°58'N, 22°9'E	75	92	100	96	96	83	24	24	24
Jämsä	61°53'N, 25°17'E	18	17	67	86	46	49	22	24	24
Vehniä	62°26'N, 25°41'E	64	78	67	68	78	88	22	18	24
Suonenjoki	62°39'N, 27°20'E	71	75	11	88	54	74	24	24	19
Iisalmi	63°35'N, 27°14'E	67	67	25	88	96	71	24	24	24
Kajaani	64°10'N, 27°33'E	57	46	0	91	100	93	23	24	14
Suomussalmi	64°53'N, 28°55'E	29		20	58		60	24		5
Kuusamo	65°54'N, 29°12'E	9			41			22		
Kemijärvi	66°43'N, 27°18'E	92			96			24		
Sodankylä	67°43'N, 26°45'E	50	0	4	4	8	42	24	24	24
Total		57	56	58	76	71	70	310	238	230

Legends for the figures

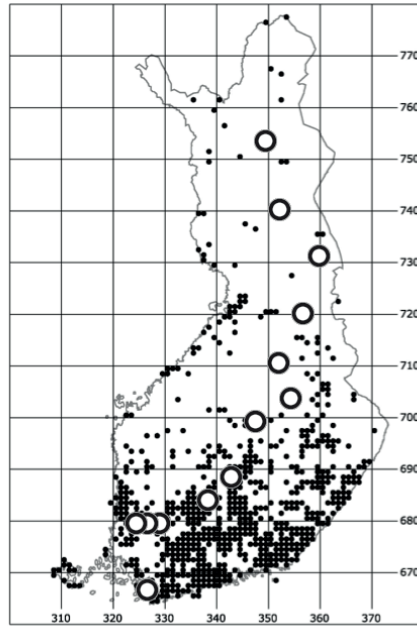
Figure 1. Distribution of *C. hastulatum* in Finland according to the insect database at the Finnish Museum of Natural History. The area is divided into quadrates measuring 10*10 kilometres, the solid dots represent observations of *C. hastulatum*, and the larger open dots represents roughly the locations of the sample populations.

Figure 2. Distribution of *C. hastulatum* in Finland. The number of inhabited squares measuring 10*10 kilometres in relation to uninhabited squares, and the distance from the southernmost range edge in Finland. The solid dots represent the observed probability and the open dots represent the predicted probability of a generalized linear model with a binomial distribution. The more northern the location, the less inhabited squares there are.

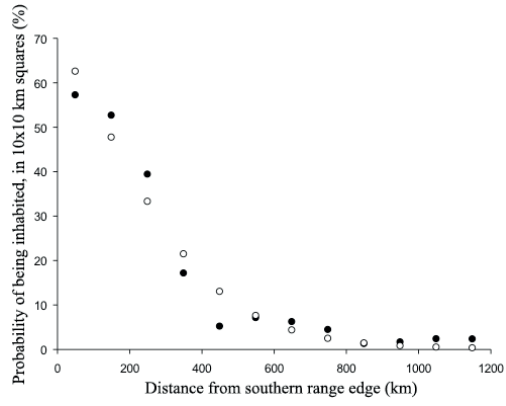
Figure 3. The prevalence of gregarines (%) within the thirteen *C. hastulatum* populations in relation to the prevalence of water mites in Finland, with 95 % confidence intervals. The continuous line indicates that the prevalence of gregarines and water mites was the same in each population.

Figure 4. The relationship between the prevalence of gregarines (a) and water mites (b) in thirteen populations of *C. hastulatum* and the distance from the southernmost range edge in Finland. The solid dots represent the observed prevalence and the open dots represent the predicted prevalence of a generalized linear model with a binomial distribution. The error bars show 95 % confidence intervals.

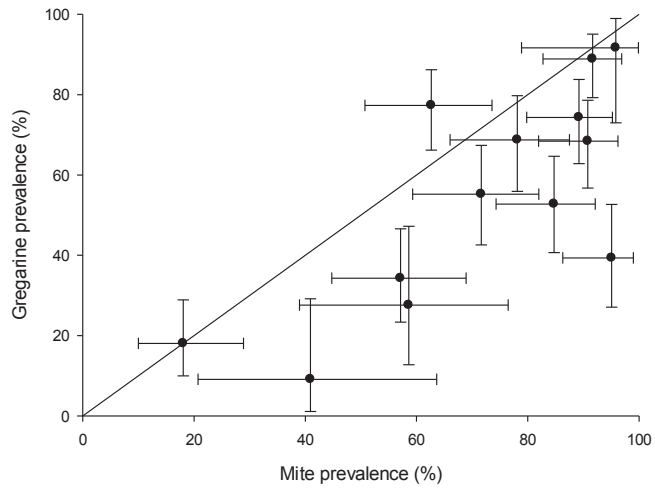
Kaunisto et al. 2013 Figure 1



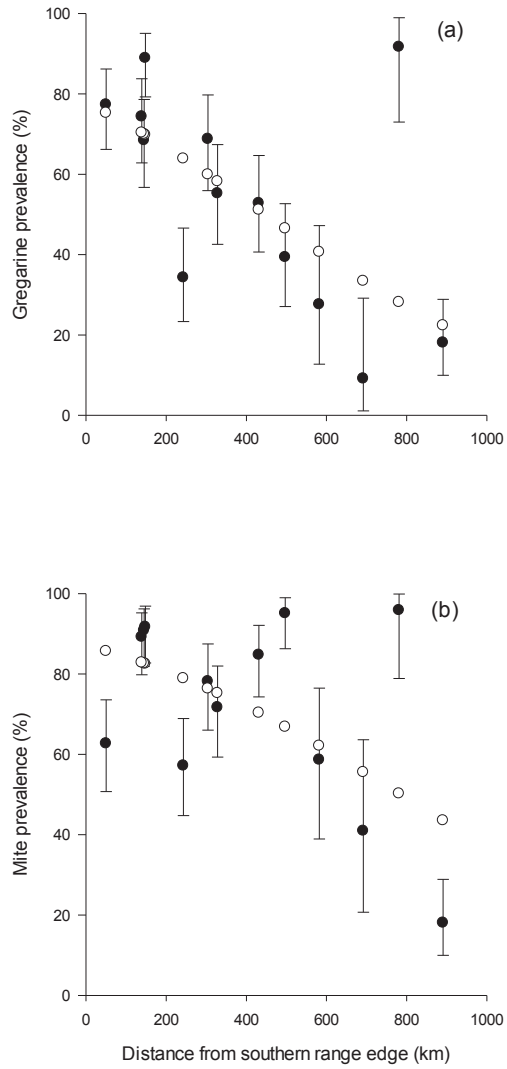
Kaunisto et al. 2013 Figure 2



Kaunisto et al. 2013 Figure 3



Kaunisto et al. 2013 Figure 4



Association between host's genetic diversity and parasite burden in damselflies

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Abstract

Recent research indicates that low genetic variation in individuals can increase susceptibility to parasite infection, yet evidence from natural invertebrate populations remains scarce. Here, we studied the relationship between genetic heterozygosity, measured as AFLP-based inbreeding coefficient f_{AFLP} , and gregarine parasite burden from eleven damselfly, *Calopteryx splendens*, populations. We found that in the studied populations, 5–92% of males were parasitized by endoparasitic gregarines (Apicomplexa: Actinoccephalidae). Number of parasites ranged from none to 47 parasites per male, and parasites were highly aggregated in a few hosts. Mean individual f_{AFLP} did not differ between populations. Moreover, we found a positive association between individual's inbreeding coefficient and parasite burden. In other words, the more homozygous the individual, the more parasites it harbours. Thus, parasites are likely to pose strong selection pressure against inbreeding and homozygosity. Our results support the heterozygosity–fitness correlation hypothesis, which suggests the importance of heterozygosity for an individual's pathogen resistance.

Introduction

Despite that the theory of host genetic variation mediating pathogen susceptibility originated already decades ago (Haldane, 1949), evidence from natural populations still remains scarce (e.g. Frankham, 2002; Hawley *et al.*, 2005). Heterozygosity varies greatly in nature, mainly according to the level of inbreeding, which increases homozygosity (Crow, 1948). When individuals that are closely related by descent mate with one another, resulting offspring may have lower fitness compared with the mean fitness of the population. This phenomenon is known as inbreeding depression (e.g. Reed *et al.*, 2002). Positive associations between genetic heterozygosity and fitness, commonly known as heterozygosity–fitness correlation hypothesis (reviewed in Chapman *et al.*, 2009), have been recognized in organisms such

as plants, molluscs and vertebrates (Ledig, 1986; Mitton & Grant, 1984; Zouros, 1987; Danzmann *et al.*, 1988).

Recent studies have revealed parasite–host relationships, in which low genetic variation in individuals can increase susceptibility to parasite infection (e.g. Coltman *et al.*, 1999; Isomursu *et al.*, 2012). For example, in vertebrates, California sea lion (*Zalophus californianus*) pups with relatively low genetic heterozygosity, presumably due to inbreeding, are less able to prevent attachment of hookworms to the intestinal mucosa and expel them. This is possibly due to a weakened immune response (Acevedo-Whitehouse *et al.*, 2006). Furthermore, it has been shown in social bumblebees, living in isolated island populations, that the prevalence of the gut parasite *Crithidia bombi* was higher in populations with lower genetic diversity (Whitehorn *et al.*, 2011). These associations on individual and population (e.g. Meagher, 1999) level between host's genetic variation and pathogen prevalence provide evidence that genetically less diverse hosts are more susceptible to pathogens. Conversely, multiple matings with different partners can increase allelic diversity, which has been

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demonstrated to improve resistance to infection in ants (Denny *et al.*, 2004).

For investigating the association between individual heterozygosity and parasite burden in natural insect populations, we chose Odonata species, the banded damselfly (Odonata, Zygoptera: *Calopteryx splendens*, Harris 1780). Odonates are one of the most parasitized insect groups: only the group Orthoptera is proposed to have more parasites (Åbro, 1976). In addition, *Calopteryx* damselflies are used as a model organism for ecological, genetic and evolutionary studies due to their well-characterized ecology and behaviour (reviewed in Cordoba-Aguilar & Cordero-Rivera, 2005).

In this study, we focused on the link between an individual's parasite burden and heterozygosity. In the earlier studies on odonates, it has been shown that parasite burden reduces flight ability, fat content and muscle output, effectiveness of mate search, breeding success and female's fertility and increases mortality rate (reviewed in Forbes & Robb, 2008). Furthermore, individuals that have high burden of gregarine parasites often have ruptured and/or blocked midguts, which are likely to reduce host's longevity (Åbro, 1990). To summarize, as the longevity is a major determinant of fitness in many odonates (e.g. Sokolovska *et al.*, 2000), and energy resources are vital for individual's immune response and reproduction (Mesterton-Gibbons *et al.*, 1996), gregarines are very likely to have impact on their host's fitness. In this study, our main goal was to investigate how parasite burden associates with f_{AFLP} .

Materials and methods

Description of study area and fieldwork

We included samples from 11 different parasitized *C. splendens* populations from Finland, containing altogether 199 individuals (Table 1). All sampled individuals were males. Moreover, all populations were known to be sympatric with another closely related damselfly species, *C. virgo*. Sympatric *C. splendens* populations have been found to be more parasitized with gregarines than allopatric ones (Ilvonen *et al.*, 2011) and tend to have lower genetic variation (Viitaniemi, 2009).

In all of the sampled sites, individuals were collected from area of roughly 100 m along the river bank. Moreover, all samples were collected between 10–00 and 15–00 h, which is the peak of *Calopteryx* species activity. Fieldwork was carried out from mid-June to mid-July. The field samples were collected as soon as the daily temperature rose to about 18 °C. Only adult damselflies of roughly the same age were collected, because gregarine levels tend to increase with age (e.g. Cordoba-Aguilar *et al.*, 2003). We were able to separate adult damselflies from juveniles by the stiffness of the wings, as described in Plaistow & Siva-Jothy (1996).

Table 1 Study populations and their locations in Finland. Sample size for infected and noninfected *Calopteryx splendens* males for each study populations and total sample size.

Population	Location	Sample size		
		Infected	Not infected	Total
Pohja	60°6'N, 23°33'E	1	20	21
Espoonjoki	60°11'N, 24°36'E	1	15	16
Keravanjoki	60°16'N, 24°59'E	2	11	13
Pusulanjoki	60°28'N, 23°58'E	2	16	18
Kiesilänjoki	61°16'N, 27°24'E	3	3	6
Pääskylänjoki	61°39'N, 24°46'E	2	10	12
Kiteenjoki	61°57'N, 30°19'E	10	2	12
Kymäkoski	61°9'N, 23°40'E	2	29	31
Tiekoski	62°00'N, 30°15'E	11	1	12
Neulajoki	62°15'N, 25°30'E	20	18	38
Niemenjoki	62°16'N, 26°19'E	6	14	20
Total		60	139	199

Study species

This study was conducted with damselfly *C. splendens* and its common gut parasite gregarines. Males of our study species, *C. splendens*, establish a territory on a stream containing suitable vegetation patches for which the females can lay eggs (Siva-Jothy, 1999). Contest over patches is harsh, and only few males in a population obtain a high-quality territory.

Damselfly *C. splendens* has been reported to be generally a sedentary species (Tynkkynen *et al.*, 2005; Rantala *et al.*, 2010), which may induce reduced gene flow between populations (Svensson *et al.*, 2004; Chaput-Bardy *et al.*, 2008). Consequently, because of highly specialized mating strategies that favour only a few individuals and general sedentariness of species, nearly all descendants of one population may originate from a few individuals (e.g. Plaistow & Siva-Jothy, 1996), which may result in high levels of inbreeding.

Endoparasitic gregarines are extracellular sporozoan parasites of invertebrates. Gregarines are cosmopolitan midgut endoparasites of damselflies (Actinocephalidae) (e.g. Åbro, 1976, 1990). Infection occurs via the ingestion of oocysts attached mainly to the legs of the small flies on which the damselflies feed. The oocysts germinate and release the infective sporozoites, which then attach to the host's midgut epithelium and develop into trophozoites. The trophozoites develop inside the gut and finally fuse as a gametocyst, which may contain thousands of infective oocysts (Åbro, 1976).

Quantifying the parasite burden

In the field, all males used for parasite and DNA analysis were stored in 95% ethanol. In the laboratory, each damselfly was investigated with a stereomicroscope. During gregarine parasite investigation, the intestinal track of the damselfly was pulled out from the posterior

1786 K. M. KAUNISTO *ET AL.*

end of its abdomen with fine forceps and then dissected longitudinally to expose the attached gregarines, which were then counted. Unfortunately, there is no literature for gregarine species identification, and thus, we restricted the investigation to group level.

Genetic analysis

DNA was extracted from the head of 199 individuals using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). The quality of the DNA was assessed by running the DNA samples on 1% 1 × TBE-agarose gel prior to AFLP analysis. The AFLP protocol was modified from Bensch *et al.* (2002). Restriction digests were conducted using EcoRI and MseI in a final volume of 15 µl containing: 2.5 U EcoRI (New England Biolabs, Ipswich, MA, USA), 2.5 U MseI (New England Biolabs), 1 × Neb2 buffer (New England Biolabs), 1 µg BSA, 5 µl diluted DNA and ddH₂O. Reactions were incubated for 3 h at 37 °C. Adapters were ligated to the digested DNA by adding 4 µl of following reaction: 0.5 µM EcoRI adapter, 5 µM MseI adapter, 25 U T4 ligase (New England Biolabs) and 1 × ligation buffer (New England Biolabs). The reaction was then incubated for 16 h at 16 °C and diluted 8 times with 10 mM Tris–HCl.

The preselective PCR was carried out in final volume of 20 µl with 0.3 µM EcoRI-T, 0.3 µM MseI-C, 0.2 mM dNTPs, 2.5 mM MgCl₂ (Thermo Scientific, Waltham, MA, USA), 1 × PCR buffer (Thermo Scientific), 0.4 U *Taq* polymerase (Thermo Scientific) and 10 µl diluted ligation reaction. The preselective PCR was diluted 30 times with 10 mM Tris–HCl. The selective PCR was carried out in final volume of 10 µl with 0.25 µM EcoRI-sel (either FAM-TAG or NED-TAC), 0.25 µM MseI-sel (either-CAC or-CCG), 0.2 mM dNTPs, 2.5 mM MgCl₂ (Thermo Scientific), 1 × PCR buffer (Thermo Scientific), 0.4 U *Taq* polymerase (Thermo Scientific), 2.5 µl diluted preselective PCR and ddH₂O. Both pre-and selective PCR protocols were in accordance with Bensch *et al.* (2002). The adapters and primers used here were according to Svensson *et al.* (2004) in four combinations: FAM-TAG – CAC, FAM-TAG – CCG, NED-TAC – CAC and NED-TAC – CCG.

For capillary electrophoresis, 1 µl of diluted selective PCR was mixed with 10 µl of reaction mixture containing 170 µl formamide (ABI) and 2 µl GS-500LIZ (ABI) size standard. The reaction was denatured for 5 min at 95 °C. The fragments created by AFLP were visualized with ABI prism 3130xl (Applied Biosystems, Inchinnan Business Park, UK) sequencer and analysed for presence and absence using Genemapper v. 4.0 (ABI). Peaks were manually checked after analysis with Genemapper. All identified loci were checked for independence of markers (Gaudeul *et al.*, 2000), and nonindependent loci were excluded. A subset of 27 individuals was repeated to estimate the genotyping error rate. Loci with < 5 % variation across populations and genotyping error rate more than 10 % were excluded.

Estimating levels of heterozygosity using AFLP markers

An individual's heterozygosity can be defined via inbreeding coefficient as the probability that two alleles at a locus are identical by descent. More inbred individuals therefore exhibit increased homozygosity. At a dominant AFLP locus, absence of a band is deemed to indicate homozygosity for the null allele, whereas presence of a band can indicate heterozygosity for the null allele or homozygosity for the 'presence' allele and cannot be assigned a single state. Therefore, as homozygosity is increased through inbreeding, the number of bands carried by an individual decreases and, consequently, the number of absent true homozygous genotypes increases. Thus, the method described by Dasmahapatra *et al.* (2008) was used to estimate f_{AFLP} , an inbreeding coefficient, based on AFLP markers, which has been found to correlate strongly with pedigree-based inbreeding coefficients (Dasmahapatra *et al.*, 2008). The analogy of inbreeding is used inversely to measure heterozygosity. The higher the value of f_{AFLP} , the more homozygous the individual is in regard to the overall population, meaning the individual has more absent bands indicating homozygosity for the null allele. Due to the formula, negative values for f_{AFLP} are possible and indicate that, on average, an individual is more heterozygous than the population in general.

Statistical analyses

We used the Kolmogorov–Smirnov test to determine whether the frequency distribution of the number of gregarine parasites per single host damselfly *C. splendens* was randomly distributed. In other words, it was used to test the fit of the observed frequency distribution with a Poisson distribution. Differences in the f_{AFLP} 's between the eleven study populations were tested with analysis of variance (ANOVA). We used generalized linear models with type III errors when we tested the relationship between the number of parasites and f_{AFLP} . In the model, numbers of parasites were negative binomial distributed and link functions were logarithmic. In the first model, number of parasites was used as a dependent variable and population as a factor (random variable) and f_{AFLP} as a covariate. The interaction between population and f_{AFLP} was not significant (Wald₈ = 9.25, P = 0.321). Therefore, we removed interaction from the final model for simplification. In final model, the number of parasites was used as a dependent variable, population as random factor and f_{AFLP} as a covariate. All statistical analyses were conducted with IBM SPSS Statistics 20 software.

Results

Parasite number varied between 0 and 47 gregarines per individual (mean ± 2 SE, 3.4 ± 1.3, n = 199).

Number of parasite in *C. splendens* males did not follow a random (Poisson) distribution in our pooled data (Kolmogorov–Smirnov test, Poisson distribution, $D = 9.36$, $P < 0.001$). In other words, parasites were highly aggregated. In this study, 20% of the most parasitized *C. splendens* individuals harboured 94% of all parasites. In all of our sample populations, 5–92% of *C. splendens* individuals were parasitized by endoparasitic gregarines. Mean prevalence, that is, proportion of parasitized individuals per population, was $33\% \pm 19$ ($n = 11$).

Mean individual f_{AFLP} was 0.93 ± 0.06 ($n = 199$), and it ranged from -0.33 to 2.07 . f_{AFLP} did not differ among populations (ANOVA, $F_{10,188} = 1.26$, $P = 0.258$).

Number of parasites increased with increasing f_{AFLP} in *C. splendens* males (generalized linear models, $\text{Wald}_1 = 7.23$, $P < 0.007$, Fig. 1). However, in the model with population included, differences in parasite number between populations varied considerably ($\text{Wald}_{10} = 202.83$, $P < 0.001$; Table 1) and number of parasites showed tendency to increase with increasing f_{AFLP} ($\text{Wald}_1 = 3.30$, $P = 0.069$).

Discussion

In this study, gregarine parasite abundance was positively associated with an individual's f_{AFLP} level. In other words, the more homozygous the individual, the more parasites it has. However, mean individual f_{AFLP} did not differ among populations. Positive association between f_{AFLP} and parasite abundance suggests that heterozygosity is linked with an individual's condition and consequently with individual's susceptibility to parasitism (e.g. Morrill & Forbes, 2012). The positive

relationship between homozygosity and parasite abundance is consistent with heterozygosity–fitness correlation hypothesis (e.g. Coltman *et al.*, 1999; Acevedo-Whitehouse *et al.*, 2006; Hawley *et al.*, 2005; Isomursu *et al.*, 2012).

On one hand, part of the observed variation in parasite abundances, in addition to heterozygosity, originates from different exposures to parasites between populations (e.g. Kaunisto & Suhonen, 2013). On the other hand, some of the observed pattern is likely to originate from variation in hosts' conditions (Morrill & Forbes, 2012). Condition varies substantially between individuals, and it has been shown to be tightly linked with fat reserves, parasite burden and immunity (reviewed in Cordoba-Aguilar & Cordero-Rivera, 2005; Suhonen J. & Honkavaara, 2008). Furthermore, fat reserves are positively correlated with immunity, as was shown in closely related damselfly, *Calopteryx virgo* (e.g. Koskimäki *et al.*, 2004). In many of these *Calopteryx* species, male's condition is reflected with by their wing-spot size, which serves as sexual ornament; consequently, females are judging their partner's general condition via males' wing-spot size (Siva-Jothy, 1999; Rantala *et al.*, 2000). According to this, these condition-related traits are supposed to be under a strong selection (Rantala *et al.*, 2011).

High parasite burden has been shown to have a negative effect on fat content of *Calopteryx* damselflies (Marden & Cobb, 2004). Low fat content in turn has been shown to negatively affect territory holding ability of males (e.g. Plaistow & Siva-Jothy, 1996; Koskimäki *et al.*, 2004; reviewed in Suhonen J. & Honkavaara, 2008). Therefore, as territorial males acquire more offspring than nonterritorial ones, parasites indirectly may lower the number of offspring (e.g. Marden & Cobb, 2004). Consequently, low heterozygosity and increased susceptibility to parasites are likely to have impact on the fitness of these damselflies.

Inbreeding acts via associate overdominance, that is, apparent heterozygosity advantage due to correlations between heterozygosity at neutral loci and loci under selection (reviewed in Hansson & Westerberg, 2002; Hawley *et al.*, 2005). First, at loci involved in parasite defence, heterozygotes can present twice as many resistance variants as homozygotes (Coltman *et al.*, 1999). Second, parasites may contribute to selection against less heterozygous individuals because of genome-wide effects including homozygosity of deleterious recessive alleles and overdominance (Pearman & Garner, 2005). For example, Kekäläinen *et al.* (2009) found that the more heterozygous Arctic char (*Salvelinus alpinus*) individuals had fewer parasites and higher survival.

Calopteryx damselflies have life-history traits and behavioural differences that may partly account for these differences in heterozygosity levels and parasite numbers. For example, territorial males of odonate species *Libellula pulchella* have been shown to harbour less

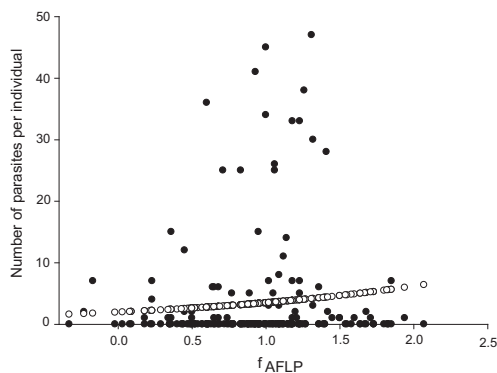


Fig. 1 Relationship between the number of parasites and AFLP-based inbreeding coefficient f_{AFLP} . Filled dots represent *C. splendens* individual's parasite number in relation to each individual inbreeding coefficient. Open dots represent predicted values for each individual, based on negative binomial distribution.

1788 K. M. KAUNISTO ET AL.

parasites (Marden & Cobb, 2004), which may also be the case with *C. splendens*. Furthermore, if *C. splendens* shared its breeding stream with another closely related damselfly *C. virgo*, *C. splendens* have higher parasite burden than in allopatric populations (Ilvonen *et al.*, 2011). Finally, as very few territorial individuals of *Calopteryx* damselflies gain considerably more matings than nonterritorial individuals (Plaistow & Siva-Jothy, 1996), offspring may inherit their genome mainly from a few males, which may have effect on heterozygosity in population.

In previous studies, it has been shown that parasites may provide a selective force capable of increasing the genetic variation in natural populations (e.g. Coltman *et al.*, 1999). This positive effect of parasites to its host's genetic variation is poorly studied in invertebrates. Our study is the first, to our knowledge, to show a positive association between homozygosity and parasite burden in a nonsocial insect (but see e.g. Whitehorn *et al.*, 2011 for social insects). Identifying the particular genes involved in immune response of invertebrates and how they affect fitness will be a critical next step.

To conclude, individual heterozygosity, measured as AFLP-based inbreeding coefficient f_{AFLP} , is associated with parasite burden in *C. splendens*. Despite parasite's negative impact on their hosts, they may in turn provide beneficial selection against homozygosity and therefore contribute to the maintenance of genetic variation.

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Can infection by eugregarine parasites mediate species coexistence in *Calopteryx* damselflies?

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Abstract. 1. Parasitism may be an important factor determining the coexistence of closely related species. Although host–parasite interactions can affect the ecology and distribution of the host species, virtually nothing is known about how other interspecific interactions affecting the host, such as competition or predation, relate to the parasite burden of the host.

2. We studied parasite-mediated competition between two closely related *Calopteryx* damselflies, *C. virgo* L. and *C. splendens* Harris. We investigated a total of 31 populations, including 18 allopatric and 13 sympatric populations. We measured the occurrence of gut parasites, eugregarines.

3. We found that the prevalence of gregarines was higher in *C. virgo* than in *C. splendens*. On average, more than half of the *C. virgo* individuals were infected by eugregarines both in allopatric and sympatric populations. However, hardly any allopatric *C. splendens* populations had gregarines, but most of sympatric populations had infected individuals.

4. According to our results, co-existence of the host species affects the likelihood of the subordinate species showing higher levels of parasitism. Interspecific aggression, lower species genetic heterozygosity, and the difference in host species immunity are proposed as possible explanations for greater parasite burdens in the inferior species at sympatric sites.

Key words. Calopterygidae, *Calopteryx splendens*, *Calopteryx virgo*, coexistence, gregarine parasites, interspecific interaction.

Introduction

Parasites and diseases have long been recognised as potentially important factors in shaping host species diversity and the structure of a host community (Haldane, 1949; Park, 1949; Holmes, 1979; Anderson & May, 1986; Dobson & Hudson, 1986; Hudson & Greenman, 1998). Most information about the role of parasites and diseases in determining a community structure comes from situations in which the introduction of alien parasites or pathogens caused a dramatic decline of a new host population or even an extinction of a population or a species (Warner, 1968; Anderson, 1981; Tompkins *et al.*, 2003; Bakke *et al.*, 2007).

The theory of parasite-mediated competition was introduced by Haldane in 1949. He stated that ‘a non-specific parasite is

a powerful competitive weapon’. This means that one species can be a superior competitor by harbouring and transmitting a parasite to a more vulnerable species. Nowadays there are many studies showing that parasites do mediate competition between host species significantly affecting the coexistence of the host species (see, for example, reviews by Price *et al.*, 1988; Hudson & Greenman, 1998; Lefèvre *et al.*, 2008).

In this study, we tested the parasite-mediated competition hypothesis in two congeneric damselfly (Odonata: Zygoptera) species, *Calopteryx virgo* L. and *C. splendens* Harris (Misof *et al.*, 2000; Dumont *et al.*, 2005), which are parasitised by eugregarines in sympatric and allopatric populations (Åbro, 1996; Corbet, 1999; Siva-Jothy & Plaistow, 1999). *Calopteryx* species are common in northern Europe and the males defend territories that consist of patches of floating vegetation, which are used by females as oviposition sites in small streams and rivers (Siva-Jothy *et al.*, 1995; Tynkkynen *et al.*, 2006; Suhonen *et al.*, 2008). Territorial fights between males

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of *C. splendens* and *C. virgo* are common in localities in which both species are sympatric (Tynkkynen *et al.*, 2004, 2006). In a previous field experiment, *C. virgo* males won nine out of nine contests over territory competition against *C. splendens*, which indicates that *C. virgo* is better able to defend a territory during interspecific contests (Tynkkynen *et al.*, 2004). Moreover, this heterospecific aggression has important effects on territorial spacing of *C. splendens*: after *C. virgo* removal the number of territorial *C. splendens* males increased (Tynkkynen *et al.*, 2006). The larger species, *C. virgo* has also a larger geographical range in northern Europe than the slightly smaller *C. splendens* (Askew, 1988; Dijkstra, 2006).

Eugregarine infection in damselflies occurs via ingestion of oocysts attached to the legs of small flying prey items (i.e. flies) that adult damselflies use as food (Åbro, 1976). Once ingested by a damselfly, eugregarines attach to the posterior gut region of adults where they develop and reproduce (Åbro, 1971, 1974; Taylor & Merriam, 1996; Siva-Jothy & Plaistow, 1999). Gregarines may have a negative impact on host fitness (Åbro, 1987, 1990; Siva-Jothy & Plaistow, 1999) by decreasing survival and mating success of male hosts (Córdoba-Aguilar, 2002; Córdoba-Aguilar *et al.*, 2003; Marden & Cobb, 2004; Forbes & Robb, 2008).

If parasite-mediated competition can explain the coexistence of two competing host species, then the more superior competitor may be more vulnerable to the attacks of parasites than less competitive host species. Fighting or interference competition is known to be costly in terms of energy use, and *C. virgo* as the superior competitor is supposed to be more vulnerable to eugregarine parasites than *C. splendens*. Moreover, *C. virgo* may be affected by more eugregarine species within its larger distribution range than *C. splendens* (e.g. Price *et al.*, 1988). In the sympatric populations, we predict that *C. virgo* is more parasitised than *C. splendens*.

Material and methods

Description of study areas and field work

The study was carried out in Western and Southern Finland during the summer 2008 (62°31'N, 25°25'E to 62°6'N, 26°2'E). The study areas contained mainly open, shallow and quite fast flowing rivers and streams. Altogether data were collected from 31 populations: five of them were allopatric *C. virgo* populations, 13 allopatric *C. splendens* populations and 13 were sympatric populations. Our data included three populations from Latvia (55°86'N, 27°20'E to 55°88'N, 27°22'E): one allopatric *C. virgo*, one allopatric *C. splendens*, and one sympatric population. Latvian populations belong to the same continuous range of distribution as Finnish populations of both *Calopteryx* species (Dijkstra, 2006).

Damselflies were collected with a butterfly net between 10.00 and 15.00 hours, which is the peak of *Calopteryx* species activity (K.M. Kaunisto and J. Suhonen, pers. obs.). Field work was carried out from mid June to mid July, when both species were abundant on the streams in Finland (Askew, 1988; Dijkstra, 2006). The field samples were collected only

when the daily temperature rose to about 20 °C. We caught 3–29 individual damselflies of both species from each study population. Only adult damselflies were collected, because gregarine levels tend to increase with age (Córdoba-Aguilar *et al.*, 2003). We were able to separate adults from juveniles by the stiffness of the wings, as described in Plaistow and Siva-Jothy (1996) and by the coloration of the body. However, even though all the specimens were adults, it was not possible to determine the exact age of the individuals. For each population we pooled both sexes together to estimate the prevalence of gregarine parasites. To estimate the prevalence of gregarine parasites we included only populations from which we were able to collect at least three *C. virgo* individuals (mean = 9.7, SD = 5.8) and at least seven *C. splendens* individuals (mean = 16.2, SD = 5.3). This was done in order to achieve more accurate population estimates. In total we captured 555 individuals: 406 *C. splendens* and 149 *C. virgo*.

All collected individuals were placed individually into small plastic containers, which were then placed inside a cool box before being transported to the laboratory. This was done within 4 h of collection. In the laboratory each damselfly was killed by decapitation. Using a stereomicroscope, the intestinal tract of each damselfly was pulled out from the abdominal posterior part with fine forceps, and then dissected longitudinally to expose the attached gregarines, which were then counted. Unfortunately there is no explicit literature about gregarine identification and therefore species were impossible to distinguish from each other.

Statistics

First, we tested whether the allopatric and sympatric *C. virgo* and *C. splendens* populations were infected by eugregarine parasites or not by using the Fisher's exact test. We defined an infected population if at least one damselfly was infected with at least one gregarine. Otherwise the population was not infected. We could not use logistic regression because all allopatric *C. virgo* populations and only one *C. splendens* population was infected, meaning that there was a low amount of variation. We calculated the prevalence (Bush *et al.*, 1997) of gregarine parasites for each population and both species. The prevalence was the proportion of infected damselflies among all the damselflies examined. We calculated 95% confidence intervals for the prevalence of gregarine parasites and for populations that were infected by the gregarine parasites (see Zar, 1999). We could not calculate an accurate estimate of the intensity of parasites (Bush *et al.*, 1997) because most of the infected populations had only one or few parasitised individuals. The differences in parasite prevalence between populations and species were tested using Mann–Whitney *U*-tests and the differences in the parasite prevalence of sympatric populations were tested by using Wilcoxon's matched pairs tests. Finally, we tested an association between the parasite prevalence of the two damselfly species in sympatric populations using Spearman's rank correlation. All tests were performed with PASW for Windows (Version 18.0).

584 Sini Ilvonen et al.

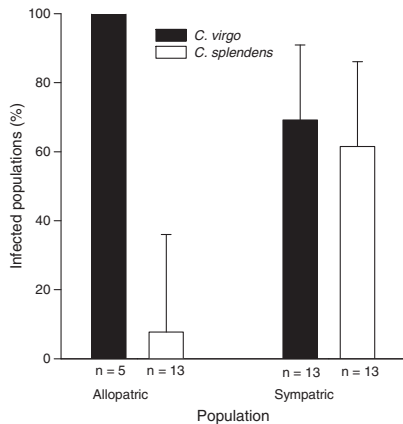


Fig. 1. Per cent of populations with gregarine parasites and in allopatric and sympatric *Calopteryx virgo* (black) and *Calopteryx splendens* (white) populations in Finland. *n* denotes the number of studied populations. Whiskers denote upper limit of 95% confidence interval.

Results

We found both infected and uninfected *C. virgo* and *C. splendens* populations (Fig. 1). We found parasites in every allopatric and 9 out of 13 sympatric *C. virgo* populations. Only one allopatric *C. splendens* population out of 13 had parasites compared to 8 out of 13 sympatric *C. splendens* populations (Fig. 1). There were more infected sympatric *C. splendens* populations than allopatric ones (Fisher's exact test, $P = 0.011$). However, the proportion of infected *C. virgo* and *C. splendens* populations was similar when both species occurred together (Fisher's exact test, $P = 1.00$).

Gregarine parasites were found in both *Calopteryx* damselfly species and the prevalence varied from 0 to 80% between populations (Fig. 2). The intensity of parasites, i.e. the number of parasites found in the infected damselfly, varied from 1 to 312 and 1 to 64 gregarines in *C. virgo* and *C. splendens*, respectively. In allopatric *C. virgo* populations, gregarine parasites were found in 69% of individuals, but only in 0.9% of individuals in allopatric *C. splendens* populations. In sympatric populations, 45% of *C. virgo* and 19% of *C. splendens* individuals had gregarine parasites (Fig. 2). The parasite prevalence was found to be higher in sympatric than allopatric *C. splendens* populations (Mann–Whitney *U*-test, $U = 35$, $n_1 = 13$, $n_2 = 12$, $P = 0.005$; Fig. 2), but there was no difference between allopatric and sympatric *C. virgo* populations ($U = 19.5$, $n_1 = 11$, $n_2 = 5$, $P = 0.364$; Fig. 2).

The parasite prevalence of gregarines in *C. splendens* correlated with the parasite prevalence of *C. virgo* in sympatric populations (Spearman's rank correlation, $r_s = 0.88$, $n = 10$, $P = 0.001$; Fig. 3), but the prevalence of gregarine parasites was lower in *C. splendens* ($27 \pm 30\%$, $n = 10$; Fig. 3) than in *C. virgo* ($44 \pm 36\%$, $n = 10$) (Wilcoxon test, $T = 40.00$, $P = 0.038$; Fig. 3).

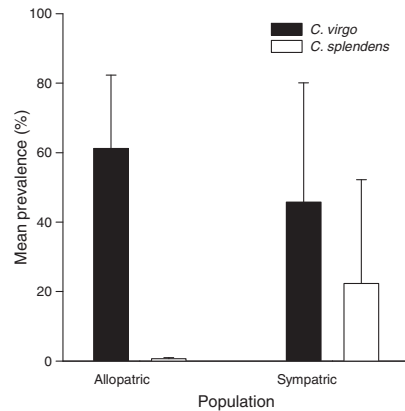


Fig. 2. Mean (+SD) prevalence of gregarine parasites (%) in allopatric and sympatric *Calopteryx virgo* (black) and *Calopteryx splendens* (white) populations in Finland.

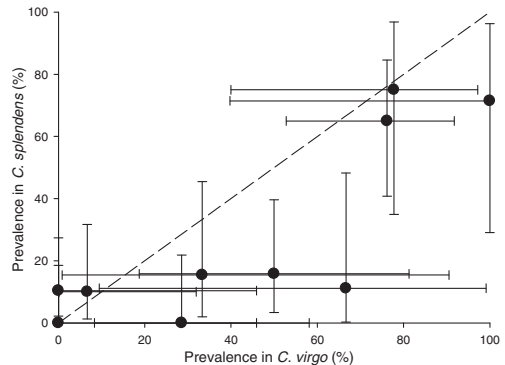


Fig. 3. Prevalence and 95% confidence intervals of gregarine parasites (%) in sympatric *Calopteryx virgo* and *Calopteryx splendens* populations in Finland. Filled circles indicate prevalence of both species in the sympatric population. The dashed line represents the situation where both species have the same prevalence of gregarine parasites in the sympatric population. If a filled circle is below the dashed line then *C. splendens* has lower prevalence of gregarine parasites than *C. virgo* in the same population and vice versa.

Discussion

In this study, we found four main results. First, a widespread damselfly, *C. virgo*, is more often parasitised than a less widespread damselfly, *C. splendens*. This is consistent with the geographic range hypothesis (Price *et al.*, 1988). Second, this pattern is especially true when comparing populations of both species that are allopatric (Fig. 1). Third, when sympatric, populations of both species appear often parasitised although *C. virgo* is still more often parasitised than *C. splendens* (except at two of three sympatric sites with highest prevalence;

see Fig. 3). Fourth, *C. splendens* is much more likely to be parasitised in sympatry than in allopatry.

Even though there is competition for territories between *C. splendens* and *C. virgo*, both species occur in sympatric populations. This coexistence of *C. splendens* and *C. virgo* can be explained by the parasite-mediated competition theory (e.g. Haldane, 1949; Barbehenn, 1969); two similar competing species can coexist if parasites affect the stronger species more negatively. The prevalence of gregarine parasites in *C. splendens* increased with the prevalence of gregarine parasites in *C. virgo*, but it was higher in *C. virgo* than in *C. splendens*. This supports the hypotheses of parasite-mediated competition. Several previous studies have also shown that parasites could mediate the outcome of interspecific competition. For example, Karns (1967) showed that moose *Alces alces* (Linnaeus, 1758) numbers were reduced by the effects of a nematode *Pneumostrongylus tenuis* (Dougherty, 1945) carried by the competitively inferior white-tailed deer *Odocoileus virginianus* (Zimmermann, 1780). Clearly, our results show how the superior competitor, *C. virgo*, has a higher infection rate and could mediate the transmission of gregarines to inferior competitor, *C. splendens*, in sympatric populations. Unfortunately we could not measure the direct effects that parasites have on its host's survival or reproductive success. Thus we cannot say whether the individuals of *C. virgo* were affected in a more harmful manner than their *C. splendens* counterparts.

According to our results, the occurrence of gregarine parasites was dependent on whether the host species lived in an allopatric or a sympatric population. However, we found that the prevalence of gregarine parasites in *C. virgo* did not differ between allopatric and sympatric populations. These results give support to the geographical range hypothesis (Price *et al.*, 1988), because *C. virgo* has a more widespread distribution in Finland than *C. splendens* (Askew, 1988; Dijkstra, 2006). On the other hand, in *C. splendens*, being the smaller species (Askew, 1988; Dijkstra, 2006), parasites occurred mainly in sympatric populations and only rarely in allopatric ones. Our results were opposite with the prediction of body-size hypothesis (see Price *et al.*, 1988).

Forbes *et al.* (1999) showed that when two species live in sympatry, one might have better immunity against parasites than the other. Their study of two dragonfly species suggests that *Sympetrum internum* (Montgomery, 1943), as the rarer host species when sympatric with *S. obtrusum* (Hagen, 1867), has developed a specific resistance against the mite species that infects both *Sympetrum* species. Forbes *et al.* (1999) suggested a pattern of coexistence in which one host species remains quite rare and resistant to parasites, whilst the other is more abundant but more susceptible to parasites with a significant effect on its fitness. *Calopteryx splendens*, as the weaker competitor of the two damselfly species, may thus have a better immunity against gregarines, giving it a competitive advantage against *C. virgo*. Better resistance could also explain the lower numbers of parasites in *C. splendens* in allopatric populations.

One potential mechanism mediating the function of parasites as a weapon of competition is interspecific aggression. Agonistic interspecific interactions can for its part explain why

C. splendens has parasites in sympatric populations but not in allopatric ones. *Calopteryx* males compete against each other for territories, potentially due to mistaken species recognition (Tynkkynen *et al.*, 2004). Thus in sympatric populations, the increased level of agonistic encounters may decrease condition, immune ability (Koskimäki *et al.*, 2004) and energy levels of males (Marden & Waage, 1990; Marden & Rollins, 1994; Plaistow & Siva-Jothy, 1996; Koskimäki *et al.*, 2004; Contreras-Garduño *et al.*, 2006, 2008). It seems that both the immunity and energy levels are important means against parasites in *Calopteryx* damselflies. A functional explanation for the more competitive species being more parasitised was found by Contreras-Garduño *et al.* (2009), who found a trade-off between immune ability mediated by a juvenile hormone and the resources spent on territorial fighting. Previous studies have found that *C. splendens* is a weaker competitor compared to *C. virgo* for territories (Tynkkynen *et al.*, 2004, 2006). Thus, the costs of interspecific aggression may increase the vulnerability of *C. splendens* males to parasite infection. Moreover, parasite load (Marden & Cobb, 2004) and immune activation (Rantala *et al.*, 2010) influence territory holding potential, which could benefit *C. splendens* against more infected and weakened *C. virgo*, and thus facilitate coexistence.

The dilution effect hypothesis (Ostfeld & Keesing, 2000; Keesing *et al.*, 2006) predicts lower infection rates in sympatric than in allopatric populations. There is an increasing amount of evidence that multiple host species within a community can decrease parasite transmissions (Johnson *et al.*, 2008). According to the dilution effect hypothesis, more diverse host communities inhibit parasite transmissions as a result of variation in host competency to support and transmit parasites (Ostfeld & Keesing, 2000; Keesing *et al.*, 2006; Johnson *et al.*, 2008). In our study, the prevalence of gregarines infecting *C. virgo* did not differ between allopatric and sympatric populations. Our results show that there were virtually no parasites in allopatric *C. splendens* populations but considerably in allopatric *C. virgo* populations, indicating that *C. virgo* may be a more suitable host for the gregarines. Thus, when the two damselfly species are sympatric, *C. splendens* may become more likely infected. Our results in sympatric *C. splendens* populations are in contradiction with the predictions of the dilution hypothesis. Even though *C. splendens* shares the parasite burden in sympatric populations, it does not significantly decrease the parasite prevalence of *C. virgo*. However, our data do show a trend towards a decrease in *C. virgo* parasite prevalence, so further studies are required to resolve the potential for the dilution effect to operate in the sympatric *C. virgo* populations. Still, we could not precisely determine the age of damselflies while gregarine number increases with the age of their hosts (Córdoba-Aguilar *et al.*, 2003). Finally, we did not estimate the population density of hosts in this study. These important individual and population parameters also should be taken into account in the future studies.

Finally, the quality of the habitat may play an important role in determining the rate of parasitism in damselflies (Forbes *et al.*, 1999). According to Taylor and Merriam's (1996) study, the prevalence and intensity of gregarine infection in *Calopteryx maculata* (Beauvois, 1805) is lower in open than

586 Sini Ilvonen et al.

in forested landscapes. They suggested that habitats may differ in their suitability for the development of gregarine parasites. Although it is known that *C. virgo* occurs in forested areas more often than *C. splendens* (Askew, 1988), there might be other habitat characters contributing to the trends found in our study. In order to explore this possibility, a detailed study of habitat properties in relation to parasitism in both species in sympatric and allopatric populations is needed (e.g. Córdoba-Aguilar & Contreras-Garduño, 2006).

In conclusion, the parasite prevalence was affected by whether the host species lived as an allopatric or sympatric population. The difference in the prevalence of eugregarine parasites in the *Calopteryx* damselflies could not alone be explained by the parasite-mediated competition theory or by the dilution hypothesis. Another potential explanation for *C. splendens* having parasites mainly in sympatric populations is that those populations have a lower genetic heterozygosity than allopatric ones. Previous studies have shown that the low genetic heterozygosity of a population increases individual's parasite susceptibility and these populations have more parasites than those with higher genetic variation (Coltman *et al.*, 1999; Puurtinen *et al.*, 2004; Rijks *et al.*, 2008). H. Viitaniemi, E. Leder, and J. Suhonen (unpublished) found that the more *C. virgo* individuals were present in a sympatric population, the lower the genetic heterozygosity of *C. splendens*. This supports the idea that the low genetic diversity of *C. splendens* could translate to high parasite infection rates in sympatric populations.

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Parasites, interspecific interactions, and character displacement of a sexually selected male ornament in male damselflies, *Calopteryx splendens*

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Abstract

Sexually selected ornaments in male animals are costly, with parasitism often affecting the degree to which they are expressed in hosts. In the damselfly *Calopteryx splendens*, males exhibit melanised ‘wing spots’. Males with large wing spots are favoured by females, but also have increased likelihood of becoming involved in aggressive encounters with male *Calopteryx virgo*, a common sympatric competitor. In allopatry, *C. splendens*’ wing spots are often larger than in populations sympatric with *C. virgo*, presumably due to reduced costs of inter-specific aggression. In addition to producing wing spots, melanin is also used in insect immune defence against parasites such as the eugregarines (Protozoa) that commonly infect damselflies. We studied 24 Finnish and two Latvian *C. splendens* populations, 13 of which were sympatric with *C. virgo*, and 13 being allopatric. Within our study populations, eugregarine infection rate correlated negatively with male wing spot size, and associated stronger with wing spot size than population structure (allopatric versus sympatric). Furthermore, we found that allopatric populations harbored considerably less eugregarine parasites than sympatric populations. Consequently, our results suggest that character displacement, i.e. change in wing spot size in *C. splendens* males, may reflect the effects on parasitism by eugregarines, inter-specific aggression, and thermoregulation. Our data suggest a possible interplay between different mechanisms, driving character displacement in a sexual trait. We suggest that differences in the relative costs and benefits of different sized wing spots within and between populations maintain size variation of this sexually selected trait.

Introduction

Parasites are ubiquitous in nature, and often act as agents of selection on the colour, morphology, physiology, behaviour and distribution of their hosts (e.g. Córdoba-Aguilar 2009). Parasites can play important roles in intra- and inter-specific interactions involving host species. For instance, parasites can alter costs and benefits to both partners in some mutualisms to promote system stability (Dunn et al. 2008), and can negatively affect the degree that male sexually selected traits are expressed (Hamilton and Zuk 1982). However, although the importance to ecological and evolutionary processes of intra- and inter-specific interactions have long been realised (Hoskin and Higgie 2010; Monroe 2012), their overall conjunctive effects with parasites on key host characters have often been neglected.

Females have been shown to select their mates on the basis of the size or intensity of sexual ornaments. These traits are thought to be reliable indicators of male quality because the costs of these traits prevent cheating (e.g. Verhulst et al. 1999). As the immunocompetence handicap hypothesis propose, males carry ornaments at the expense of their resistance to disease and parasites (e.g. Folstad & Karter 1992).

Male damselflies *Calopteryx splendens* have dark wing patches ('wing spots'), the sizes of which are under positive sexual selection via female choice (Siva-Jothy 1999). Wing spot size in male *C. splendens* can also be under negative selection because smaller spotted males are harassed less by male *Calopteryx virgo*, a dominant and aggressive competitor (Tynkkynen et al. 2004), and are more visible to predatory birds (Svensson and Friberg 2007). Reduced costs of inter-specific aggression thus contributes to character displacement in *C. splendens* wing spot size, with males in populations sympatric with *C. virgo* often having smaller spots than those in allopatry (Honkavaara et al. 2011; Tynkkynen et al. 2004). Importantly, these population differences in *C. splendens* wing spot size also reflect differences in the rates of

infestation by eugregarine gut parasites; in populations sympatric with *C. virgo*, *C. splendens* male parasitism rates are higher than in allopatric populations (Ilvonen et al. 2011). Wing spot pigments consist of melanin, which is used by insects in their immune response to parasites (Rantala et al. 2000; Siva-Jothy 2000) and is costly to produce. Due to the likely trade-off between the costs of implementing the immune system, in part via melanin production costs (Gillespie et al. 1997; Talloen et al. 2004), and investing in sexual ornamentation, wing spots in *C. splendens* populations in which parasitism rates are high may be smaller than in populations in which parasites are absent. Parasitism may thus act as an agent of character displacement, and result in changes in the wing spot sizes of *C. splendens* in addition to the effects of *C. virgo* aggression.

We tested the hypothesis that variation in *C. splendens* male wing spot size differs between populations in which eugregarine infection is present or absent, and its effects are independent of the presence of *C. virgo*. Because dispersal rates between populations of this rather sedentary species are unlikely to be high enough to counter the effects of selection (Viitaniemi 2009), we expected smaller wing spots in parasitised populations sympatric with *C. virgo*. We also predicted that parasite load, and the presence of *C. virgo* (Honkavaara et al. 2011; Tynkkynen et al. 2004; Tynkkynen et al. 2005), would reduce the wing spot size of *C. splendens* males relative to those in parasite-free allopatric populations.

Material and methods

Study species

Calopteryx splendens is common across Northern Europe (Askew 1988). Males defend territories along the banks of small watercourses that contain patches of floating vegetation, which are used by females as oviposition sites (Tynkkynen et al. 2006). *Calopteryx splendens*

often occurs sympatrically with *C. virgo*, where inter-specific territorial aggression between males is common (Tynkkynen et al. 2004; Tynkkynen et al. 2006). *Calopteryx virgo* males usually initiate and then win these contests (Tynkkynen et al. 2004), which are probably the result of *C. virgo* males misidentifying individual male *C. splendens* for conspecific males (Tynkkynen et al. 2004; Tynkkynen et al. 2005; Tynkkynen et al. 2006).

Adult male *C. splendens* have characteristic melanised wing spots on otherwise clear wings, whereas in *C. virgo* male wings are almost entirely melanised (Askew 1988). After emergence, pre-reproductive imagos forage and accumulate body fat, which is vital to adult males for use during intra- and inter-specific territorial contests (Marden and Waage 1990). In addition, *C. splendens* male wing spots begin to appear soon after emergence, and become fixed in size and colour after a few days (Siva-Jothy 1999).

Eugregarines are protozoan (Apicomplexa) parasites of invertebrates (Smyth 1976), and are often found in the mid-guts of damselflies (Odonata: Zygoptera) (Åbro 1974). Infection usually occurs when adult damselflies ingest eugregarine oocysts attached to insect prey (e.g. small Diptera) (Criado-Fernelio et al. 2013; Åbro 1976), although infected larvae have also been reported (Siva-Jothy and Plaistow 1999). Once ingested, eugregarines attach to the posterior of the host mid-gut where they develop and reproduce (Criado-Fernelio et al. 2013). The host main immune response against eugregarines, encapsulation, depends on the deposition of melanin via the prophenoloxidase (PO) cascade, which also uses other limited somatic resources such as tyrosine (Hooper et al. 1999; Siva-Jothy 2000; True 2003; Wilson et al. 2001).

Data collection

Data were collected from a total of 26 sites in western and southern Finland (24 sites), and in Latvia (two sites), from mid-June to mid-July 2008. Each site contained mainly open, shallow, and moderately fast flowing rivers and/or streams. Thirteen sites supported allopatric *C. splendens* populations, with the remaining 13 containing both *C. splendens* and *C. virgo* in sympatry. Furthermore, one Latvian site supported only *C. splendens* and the other both *C. virgo* and *splendens*. All 26 sites reside within a single continuous range of distribution for both *Calopteryx* species in Europe (Askew 1988).

At each site male damselflies were caught individually with a butterfly net between 10:00 and 15:00 hrs, the peak of *Calopteryx* damselfly activity (Ward and Mill 2006). Collections were made only when the air temperature exceeded 20°C. At each site, we caught from two to 29 *C. splendens* males (a total of 261 individuals). To determine the relative abundance of *C. virgo* males, two researchers sampled a ~100-m section of the river and counted all *C. splendens* and *C. virgo* males at each river site during 30 minutes searching. Male *C. splendens* were then collected. Immediately after collection, each damselfly was placed individually into a small plastic container, which was then placed inside a cool box prior to transportation within four hours to a laboratory. Each damselfly was removed from its container and then killed by decapitation. With the aid of a stereomicroscope and fine forceps, the intestinal tract was removed from the posterior part of the abdomen. Each intestine was then slit longitudinally to expose any attached eugregarines within, which were counted (Ilvonen et al. 2011). In addition, we measured to the nearest 0.01 mm the total length of each hind wing, and the length of the spot on the same wing, with digital Vernier callipers (Tynkkynen et al. 2004). This was conducted to estimate the effect of body size on wing spot and parasite load. We calculated the prevalence (Bush et al. 1997) of eugregarine parasites for

each population as the proportion of infected individuals of all the damselflies examined for that site. With the exception of one infected population (two infected *C. splendens* males caught), we estimated parasite presence and wing spot size from at least five *C. splendens* males (mean = 10.0, SD = 5.1) per population. Male *C. virgo* relative abundance was calculated by dividing the total number of *C. virgo* males by the total number of males of both species (Tynkkynen et al. 2004).

Statistical analyses

Both prevalence and the relative abundance of *C. virgo* were arcsine square-root transformed to normalise the error variances to enable the data to fulfil the criteria for parametric analysis. We tested the effects of eugregarine parasitism and population structure (allopatry versus sympatry) on mean *C. splendens* wing spot size with generalized linear models and included an identity link function. As predictors, parasitism rate and population mean wing length (to control for body size) were used as covariates, with population structure (allopatric or sympatric) being a fixed factor. Because most allopatric populations were found to be eugregarine-free, we did not include the interaction between population structure and parasitism rate. Instead, we performed a separate analysis using only sympatric populations (which also did not include population structure as a predictor variable). Because the size of melanised wing spots may also affect the efficiency of insect thermoregulation (Clusella Trullas et al. 2007; Svensson 2012; Wellenreuther et al. 2012), we also included the latitude of each site as an additional covariate to account for variation in photoperiod and temperature amongst sites. We measured latitude as distance to the north in 100 km from the most southern population (in Latvia). The total distance from the most southerly to the most northerly population sampled was 723 km. To test differences in parasite prevalence between

sympatric and allopatric populations, we used a Mann-Whitney U-test (for parasitism rate) and a Fisher's exact test (for eugregarine presence *per se*). For the latter test, we defined a population as having eugregarines present if at least one damselfly was infected (for more details (see Ilvonen et al. 2011)).

Traits of individuals from populations closer to each are expected to be more similar than from populations spaced further apart. We therefore measured using national coordinate systems from maps the distances in km ($\pm 0.5\text{km}$) between populations, and then performed two Mantel-tests, one for absolute wing spot size and another for relative wing spot size (as estimated from the stored residuals of a regression of wing spot size on wing length), to calculate the correlation coefficients between the matrices of distances and the differences in mean wing spot sizes. Probability estimates were based on 1000 perturbations. All statistical analyses were performed with IBM-SPSS Statistics 20, except spatial autocorrelation which was tested with Mantel tests in R with the add-on package 'Vegan'.

Results

Male *C. splendens* wing spot sizes ranged from 9.9 to 19.3 mm. There was also considerable variation among populations in mean wing spot size (range 14.4 - 17.3 mm, mean \pm S.D. = 15.6 ± 0.9 mm, N = 26). Larger damselflies had larger wing spots (Table 1). On average, male *C. splendens* with fewer eugregarines had larger wing spots (Table 1.; Fig. 1.). This effect was independent of body size, population structure, and latitude, all three factors of which significantly or marginally non-significantly affected the mean wing spot size (Table 1.). Contrary to our predictions, male *C. splendens* sympatric with *C. virgo* had significantly

larger wing spots than those in allopatry. Male *C. splendens* in more northerly populations tended to have the largest wing spots (Table 1.; Fig. 1.). All these trends remained when we removed from the dataset measurements from populations with few sampled males (< six) and then repeated the analyses, although latitude no longer predicted wing spot size ($P > 0.10$).

There was considerable variation in the rate of eugregarine parasitism among populations, which ranged from 0 to 100 % (mean \pm SD = 15.9 ± 31.2 %, N = 26). All but one allopatric *C. splendens* population (12 out of 13, 92.3%) was eugregarine free, whereas eugregarines were present in most sympatric populations (8 out of 13; 61.5%; Fisher's exact test, $P = 0.011$; Fig 1). *Calopteryx splendens* males from populations sympatric with *C. virgo* (mean \pm SD = 48.5 ± 60.0 %) had higher rates of eugregarine parasitism than those from allopatric populations (3.2 ± 11.7 ; Mann-Whitney U-test, $Z = 2.51$, $P = 0.039$; Fig. 1).

Geographical distance between populations did not significantly affect absolute (Mantel test $r = 0.02$, $P = 0.34$) or relative (Mantel test $r = 0.15$, $P = 0.13$) wing spot size.

Discussion

Our data show that the infection rate of eugregarines in a *C. splendens* population is a stronger predictor of wing spot size than the presence of *C. virgo*. The trends in *C. splendens* wing spot size we detected may thus be partly due to costs of parasitism affecting the degree to which this sexually selected trait is expressed. Our data compliment previous findings that *C. virgo* is more likely to be parasitised by eugregarines in allopatric populations than those that are in sympatry with *C. splendens* (Ilvonen et al. 2011), suggesting that *C. virgo* is more

prone to eugregarine infection than *C. splendens*. We also found that the size of melanised wing spots slightly increased with increasing latitude to the north, which may be due to increased thermoregulation efficiency (Clusella Trullas et al. 2007; Svensson 2012; Wellenreuther et al. 2012).

Sympatric and allopatric populations differ in costs of producing and maintaining large wing spots. In the sympatric *C. splendens* populations potential costs are diverse, and would include increased energy demands of performing defensive aerial manoeuvres (Marden and Waage 1990; Marden and Rollins 1994; Koskimäki et al. 2004; Marden and Cobb 2004), increased allocation of melanin through immune defence (Wilson et al. 2001; True 2003), and reduced digestive efficiency (Forbes and Robb 2008).

In addition, individual's heterozygosity has been proposed to affect wing spot size. For example, smaller wing spots of *C. splendens* in parasitised populations may be due to those populations having lower overall levels of genetic heterozygosity than those in which we failed to detect any eugregarines. Stronger selection pressure would reduce genetic variation in the short term, which would also include the effects of increased parasitism/interspecific aggression in determining wing spot size. For instance, we have previously shown that genetic variation is low in *C. splendens* males in populations sympatric with *C. virgo* (Viitaniemi 2009). Moreover, loss of genetic variation on loci associated with immune function may also increase susceptibility to parasitism at the population level (Spielman et al. 2004). Additionally, in *C. splendens* males homozygosity may be associated with high rates of individual parasitism when sympatric with *C. virgo* (Kaunisto et al. 2013), and *C. splendens* males with many eugregarine parasites have low genetic diversity (Kaunisto et al. 2013). These findings concur with our new data showing the size of *C. splendens*' wing spots

in parasitised populations is reduced. However, more detailed investigations into the genetic diversity of populations in relation to wing spot size is required.

Furthermore, in previous studies parasites have been shown to play an important role in determining wing spot size. The negative relationship in our study between the size of wing spots and parasite infection rate in male *C. splendens* is consistent with earlier studies (Siva-Jothy 1999, Siva-Jothy 2000; Cordoba-Aguilar 2002). Moreover, the presence of *C. virgo* may be a prerequisite to maintain a viable eugregarine population in a particular locality, that in turn may increase the likelihood of infection of alternative host species such as *C. splendens*. This may explain why *C. splendens* males were more often infected in sympatric than in allopatric populations. In sympatric populations *C. splendens* males may use more of their fat reserves, e.g. in interspecific contests, than those in allopatric populations. Consequently, eugregarine infection rates may be high if foraging rates increase as a result and higher numbers of small Diptera are consumed, because eugregarine oocysts are often attached to the legs of these prey items. The consequences of gregarine infection may be to reduce survival and reproductive success of small spotted males. In a previous study it was found that infected males were seldom territorial (Marden and Cobb 2004). On the other hand, interspecific aggression by *C. virgo* males against *C. splendens* males with large wing spots (Tynkkynen et al. 2004) reduces the survival of the targeted males (Tynkkynen et al. 2005), reduces their likelihood of establishing a high quality territory (Tynkkynen et al. 2006); *C. splendens* males with large wing spots in populations sympatric with *C. virgo* also do not necessarily have higher mating success than those with small spots (Kuitunen et al. 2011). It is thus feasible that in sympatric populations overall selection on wing spot size may be stabilising due to the combined effects of parasitism and interspecific aggression.

In this study we found that allopatric populations harbored considerably less eugregarine parasites than sympatric populations. This may, at least in part, be due to higher average genetic heterozygosity than in populations sympatric with *C. virgo* (Viitaniemi 2009). Previous studies have shown that low genetic heterozygosity within a population increases parasite susceptibility, and individuals within these populations overall have higher rates of parasitism than those with higher genetic variation (Coltman et al. 1999; Puurtinen et al. 2004; Rijks et al. 2008). In allopatric, eugregarine-free populations, male *C. splendens*' wing spot size may thus be under positive directional selection. The lack of parasites may mean that individual males that invest more in sexual ornaments have higher fitness than individuals that invest more in melanin based immune function. Some previous studies support this scenario. For example, male *C. splendens* with large wing spots have been found to be more often territorial (Rantala et al. 2010) and had higher mating success than males with small spots (Plaistow and Siva-Jothy 1996; Siva-Jothy 1999). Allopatric male *C. splendens* may thus be able to afford to express to a greater extent costly sexual ornaments such as wing spots (Contreras-Garduno et al. 2008; Grether and Grey 1996) than those sympatric with *C. virgo* (Suhonen et al. 2008). However, we found the reverse with males in the allopatric populations, in most of which evidence of eugregarine presence was absent, having the smallest wing spots. This suggests that the physiological costs of inter-specific territorial fights and their associated increased nutritional demands, do not affect the ability of male *C. splendens* to exhibit large wing spots as much as does infestation by eugregarine parasites. Alternatively, large wing spots may reflect individuals in good condition, that are able to cope in localities in which parasitism by eugregarines and the likelihood of harassment by male *C. virgo* is high. In other words, *C. splendens* males in poorer condition, i.e. those with smaller wing spots, are likely to have higher mortality rates (Tynkkynen et al. 2005) or may be forced

to emigrate from preferred localities with high quality microhabitats (Tynkkynen et al. 2006) that are more likely to support both species.

In addition to heterozygosity and parasites, interspecific competition has been proposed to affect character displacement. Previous studies have suggested that costs inflicted by *C. virgo* males in sympatric populations results in selection for character displacement in the mean size of *C. splendens* wing spots (Honkavaara et al. 2011; Tynkkynen et al. 2004; Tynkkynen et al. 2006). Our data therefore suggest that eugregarine parasitism may play a previously unreported role in driving wing spot character displacement in European *C. splendens* (Tynkkynen et al. 2004; Tynkkynen et al. 2006; Honkavaara et al. 2011). Similar patterns in character displacement have also be found in north American *Calopteryx* (Waage 1975; Waage 1979; Iyengar et al. 2014) and *Hetaerina* damselflies (Grether et al. 2009; Anderson and Grether 2010; Anderson and Grether 2011). However, these effects may be indirect because the host immune response to parasitic eugregarines residing in the gut may require less melanin than the direct encapsulation of, for instance, an ectoparasite or parasitoid (Wilson et al. 2001; True 2003). A recent cross population study of damselflies *Coenagrion hastulatum* in Finland, infected with both eugregarines and ectoparasitic water mites, found that on average host encapsulation responses to experimental immune challenges were significantly positively correlated with the infection rates of water mites but not eugregarines (Kaunisto and Suhonen 2013). Water mites feed by penetrating their host's cuticle with their feeding tubes. These feeding tubes are then often directly encapsulated by the host's immune system. *Coenagrion hastulatum* males lack wing spots, but their heads, thoraxes and abdomens are coloured black and blue (Askew 1988). The effects of different parasites on the expression of melanin in damselfly host pigments of different body parts (wing spots, thorax, abdomen) are unknown, but immune system challenges may affect

different host species differently, e.g. those with and without wing spots. This also requires further investigation.

In sympatric populations, costs of agonistic encounters are likely to decrease the general condition, and hence possibly also the overall immunocompetence, of individual male *C. splendens* (Marden and Waage 1990; Marden and Rollins 1994; Plaistow and Siva-Jothy 1996; Koskimäki et al. 2004; Contreras-Garduno et al. 2006; Contreras-Garduno et al. 2008). This may have reduced the resources early in adulthood that could have been attributed to producing large wing spots directly, or indirectly via making individuals more prone to parasitism. Increased parasitism and the resulting activation of the immune system, also reduces the ability of *C. splendens* males to acquire and defend territories (Marden and Cobb 2004; Rantala et al. 2010). However, in some populations, damselfly males with highly pigmented wings have the fewest eugregarine loads (Siva-Jothy 2000; Cordoba-Aguilar 2002), and thus may be those most likely to thrive even if infected.

The wing spots of *C. splendens* males were on average approximately 1 mm larger in northern populations than in more southerly populations, an effect that may reflect increased thermoregulation efficiency (Clusella Trullas et al. 2007; Svensson 2012; Wellenreuther et al. 2012). For every 100 km increase in latitude, wing spots get about 0.14 mm longer (Table 1). The behavioural and ecological performance of ectotherms depends largely on their body temperature. Winged insects in arctic and temperate regions often require body temperatures exceeding those of the surrounding environment for flight (Dreisig 1995; Van Dyck and Matthysen 1998). Larger wing spots in more northerly latitudes may thus be influenced by positive directional selection due to physiological benefits associated with thermoregulation.

In conclusion, our data provide evidence that both parasites and the presence of a congeneric competitor species, and possibly thermoregulation, all contribute to population differences in the expression of a sexually selected ornament in male *C. splendens* damselflies, wing spots. Our finding that eugregarine parasitism is likely to be an additional factor affecting *C. splendens* wing spot size, demonstrates a need for additional studies examining inter-population variation in these sexual ornaments in this species.

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Table 1. Results of three generalised linear models predicting mean wing spot size in male *C. splendens*. Mean wing length, parasite prevalence (proportion, arcsin transformed), relative abundance (proportion, arcsin transformed) of *C. virgo* and the latitude of the population (distance from the most southern population in 100 km) were continuous covariates in each model. Population structure (allopatric or sympatric) was a fixed factor in the model that used data from all populations. Each model assumed normal errors and used an identity link function.

Source of variation	B ± s.e.	Wald	df	P
<i>All data</i>				
Intercept	2.76 ± 2.99	0.69	1	0.36
Population structure	-0.67 ± 0.19	12.72	1	<0.001
Wing length	0.44 ± 0.10	19.46	1	<0.001
Parasite load	-1.41 ± 0.21	49.05	1	<0.001
Latitude	0.10 ± 0.05	3.65	1	0.056
<i>Only sympatric populations</i>				
<i>Without C. virgo abundance</i>				
Intercept	5.87 ± 3.22	3.33	1	0.068
Wing length	0.33 ± 0.11	9.38	1	0.002
Parasite load	-1.44 ± 0.18	66.83	1	<0.001
Latitude	0.014 ± 0.06	5.65	1	0.017
<i>Including C. virgo abundance</i>				
Intercept	5.87 ± 3.22	3.33	1	0.068
<i>C. virgo</i> abundance	0.013 ± 0.53	0.001	1	0.980
Wing length	0.33 ± 0.11	9.18	1	0.002
Parasite load	-1.44 ± 0.24	37.30	1	<0.001
Latitude	0.14 ± 0.06	5.35	1	0.021

Figure legend

Figure 1. The relationship between residual wing spot size (mm) and the prevalence of eugregarine parasites (%) in 26 *C. splendens* populations in Finland/Latvia. Residuals were calculated from a linear regression in which wing spot size was the dependent variable and total wing length the predictor. Open dots represent the allopatric populations and filled dots populations sympatric with *C. virgo*.

Suhonen et al. Figure 1

