Higher gregarine parasitism often in sibling species of host damselflies with smaller geographic distributions[1]

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ABSTRACT

1. This study investigated interspecific variation in parasitism by gregarines (Eugregarinorida: Actinocephalidae), among sibling species of damselflies (Odonata: Zygoptera), in relation to relative size of geographical ranges of host species.
2. Gregarines are considered generalist parasites, particularly for taxonomically related host species collected at the same sites or area. Prevalence and median intensity of gregarine parasitism was obtained for 1,338 adult damselflies, representing 14 species (seven sibling species pairs) across three families within the suborder Zygoptera. Damselflies were collected at six local sites in Southeastern Ontario, during same periods over the season.
3. Six of seven species pairs had significant differences in parasitism between sibling species. The less widespread host species was the more parasitized for three species pairs with significant differences in gregarine prevalence, and for three species pairs with differences in median intensity. The more widespread host had higher prevalence of infection as expected, in the fourth species pair..
4. Future studies on ecological determinants of parasitism among related species should examine robust measures of abundance of species and representation of species regionally.

 Keywords: geographic distribution, gregarines, parasitism, sibling species

INTRODUCTION

Similarity in traits between closely related species might be due to shared evolutionary history (Gaston, 1990; Blomberg *et al.*, 2003). In several host-parasite associations, parasitism demonstrates a phylogenetic signal (Poulin, 2005; Poulin, 2007; Muñoz *et al.*, 2007). For example, Krasnov *et al.* (2011) demonstrated how phylogenetic distance between two species was important with respect to the similarity of parasite faunas of rodents. Clopton and Gold (1996) showed that a species of gregarine can successfully parasitize five species of domiciliary cockroach, but mentioned that it is difficult for this species to infect other species, representing higher taxonomic levels. Even though closely related species can share parasites, there is often interspecific variation in prevalence or intensity of infections (e.g. Yourth et al. 2002).

Other traits that vary between related species are the sizes of their geographic ranges and their abundance. A positive relationship between geographic range and local abundance often has been presented (Hanski, 1982; Brown, 1984), even though there are exceptions (e.g. Päivinen et al. 2005). However, studies have shown that the larger the host species' geographic distribution, the more parasite species it will harbour (Dritschilo *et al.*, 1975; Price & Clancy, 1983; Gregory, 1990) and that prevalence of certain types of parasites can be correlated positively with host geographic distribution (Tella *et al.* 1999). Thus, species with larger geographic ranges are expected to have higher local abundance and also more parasite species and more parasites of a given species (*cf.* Brown, 1984; Arneberg *et al.*, 1988). More locally abundant species are perhaps larger targets of selection for parasites than are less abundant or rare species (Lajeunesse et al., 2002; Grant and Samways, 2011). As such, parasites are thought to be more capable of encountering and/or infecting more locally abundant species.

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Another trait that varies between host species is immune response to parasites (Forbes *et al.* 1999; Yourth *et al.* 2001). In four species of damselflies parasitized by a generalist ectoparasite, Yourth et al. (2001) concluded that temporal and spatial variation in abundance did not fully explain interspecific variation in immune response. However, immune response may be expected to correlate with geographic distribution (Thompson 2005). As a host's geographic range increases, the more parasite species it should encounter, which leads to that host species evolving stronger generalised immune responses (Thompson 2005). This could lead to observations where the more widespread and locally abundant host species are less parasitized.

Gregarine parasites are passively-transmitted, protozoan gut parasites of most invertebrates (Bush *et al.*, 2001). For odonate hosts, gregarines are ingested as oocysts by the host and then migrate to the mid-gut where they develop (Åbro, 1974). Once they reach sexual maturity, gregarines in the insect gut form gametocysts and are excreted by the host as oocysts (Bush *et al.*, 2001). The prevalence of gregarines in odonates is on average 20%, although there is considerable variation (Corbet, 1999). Abundance of infection has been recorded to vary between 1 and 900 gregarine individuals in samples of *Enallagma cyathigerum* Charpentier (Odonata: Coenagrionidae) (Åbro, 1974). Higher gregarine numbers were associated with lower survival, lower female egg loads, lower female mating success and shorter mate guarding in *Calopteryx haemorrhoidalis* Linden (Odonata: Calopterygidae) (Córdoba-Aguilar *et al.*, 2003), while increased parasitism by gregarines was associated with lower longevity and lower female egg loads in *Enallagma praevarum* Hagen (Odonata: Coenagrionidae) (Canales-Lazcano *et al.*, 2005; see also review by Forbes & Robb, 2008).

With such substantial fitness-related effects, it is important to understand how and why gregarine infections vary between species. Within populations, variation between individuals appears to be the result of phenology, with changes in gregarine parasitism throughout the year. This can take the form of an increase during the season (Locklin & Vodopich, 2010) or a unimodal relationship with a peak in the middle of the season (Forbes *et al.*, 2012), which matches a pattern seen for ectoparasitic mites in a natural insect population (Hassall *et al.*, 2010). However, some species show no variation in gregarine parasitism over the season (Locklin & Vodopich, 2010). Other work suggests that the nature of the habitat may influence gregarine parasitism, with populations inhabiting artificial wetlands exhibiting lower levels of infection than populations in natural wetlands (Mlynarek *et al.*, 2011). While time of sampling and habitat are important moderator variables to control for, neither type of study sheds much light on interspecific variation in parasitism among related host species at specific sites.

In this study, we examine interspecific patterns in the prevalence and intensity of gregarine parasitism in 14 species of damselflies (Odonata: Zygoptera). The damselflies species form seven species pairs from three families (Coenagrionidae: *Argia* Rambur*, Enallagma Enallagma* May*, Enallagma Chromatallagma* May*, Ischnura* Charpentier*, Nehalennia* Selys; Calopterygidae: *Calopteryx* Leach; Lestidae: *Lestes* Leach). We chose host species that are phylogenetically related, to first assess whether sympatric sibling species differed significantly in gregarine prevalence or median intensity. The same gregarine species are expected to infect sibling host species in the same habitat because gregarine parasites have been shown to be generalist parasites, including gregarine parasites of odonates (Clopton, 2009). For example, Cielocha *et al.* (2011) collected *Nubenocephalus secundus* Cielocha, Cook & Clopton from at least seven hosts and *Nubenocephalus nebraskensis* Clopton, Percival & Janovy from two sympatric *Argia* hosts.

Our hypothesis was that the extent of the host distribution may explain interspecific variation in parasitism when sibling species with same evolutionary ages of association with parasites were compared. Our study controlled for locality and time of collection, thereby controlling for potential seasonal variability and latitudinal and longitudinal gradients in parasitism, which do appear to exist (e.g., Locklin & Vodopich, 2010).

There are two possible, but conflicting, predictions. The first prediction is that the host species with the larger distribution may have higher measures of parasitism than a sibling species with a smaller

geographic distribution. This prediction is based on the rationale that as a species distribution increases in size, they are believed to 'jack-of-all-trades' and therefore will not be able to build defences against parasites specialised in particular habitats (REF). The second prediction, that host species with the larger distribution may have lower measures of parasitism than a sibling species with a smaller geographic distribution. This prediction is based on the premise that with a larger distribution the individuals of a species will interact with more parasite species and evolutionarily developed higher general immunity (REF).

METHODS

Zygoptera collection

Adult female and male damselflies were sampled using aerial sweep nets between May 17 and July 15, 2010 and preserved in 95% ethanol. Species of species pairs were collected much closer together in time (see below). Damselflies were all processed and stored in separate vials with individual codes. Fourteen species of Zygoptera belonging to seven species pairs (*Argia, Calopteryx, Enallagma Enallagma* subgenus*, Enallagma Chromatallagma* subgenus*, Ischnura, Lestes,* and *Nehalennia*) were collected. Target species pairs within the suborder Zygoptera were chosen because they were sympatric at collecting sites and because there were reasonable differences in sizes of their geographic distributions (minimum of 200,000 km^2 difference).

We differentiated between the two *Enallagma* species pairs because, from current phylogenetic analyses, they are in distinct subgenera (May, 2002). Each of the species pairs were collected at different sites, but at sites as close as possible to each other. The *Calopteryx* species pair was collected on July 2nd and 6th, 2010 at Brassett Creek (45º01'09.20"N, 75º50'08.77"W), the *Nehalennia* species pair was collected at Hebert's Bog (44º29'54.69"N, 76º24'53.66"W) on June 7th and 30th , *Enallagma Chromatallagma* and *Argia* species pairs were collected at the edge of Lake Opinicon June 30th-July 3rd and from July 3rd-10th, respectively (44º33'56.32"N, 76º19'26.46"W), the species of the *Enallagma Enallagma* clade were collected at Barb's Marsh (44º31'27.54"N, 76º22'25.89"W) and Jack's Marsh (44º32'1.82"N, 76º22'35.31"W) on May 25th and from June 7th-10th, *Ischnura* species pair was collected at the slow stream by Osprey Marsh (44º30'43.74"N, 76º23'39.32"W) on July 4th and 10th, and the *Lestes* were collected at Yzerinac Pond (44º32'12.82"N, 76º22'58.29"W) on July 7th and 12th.

We did not measure abundance of those species *per se*. Testing for a linkage between geographical distribution and local abundance requires many replicate samples over time taken within each replicate site for each species; something beyond the logistical scope of this study. Rather, we spent our time at sites trying to collect opportunistically over short time periods (median sample size across host species was 88; IQR 68-94; Table 1). Our sampling at single sites was intensive to obtain reliable estimates of parasitism, but was often confined to one or a few days (up to a week), barely enough to also account for seasonal variation in abundance of species within species pairs. This schedule of sampling allowed us to include more species pairs and more sites in our study, which was related to our main objective.

Although we did not measure which species was most abundant in each species pair for this study, we do note that various types of studies in our study area have been done over the years. Those studies requiring sufficient samples sizes have concentrated on the following damselfly (Odonata: Zygoptera) species that are relatively abundant compared to congeners, *Enallagma boreale* Selys (Hecker et al. 2002); *Calopteryx maculata* Beauvois (Fosythe and Montgomerie 1987); *Nehalennia irene* Hagen (Van Gossum et al. 2007); *Lestes forcipatus* Rambur (Yourth et al 2002); *Argia moesta* Hagen (M.R. Forbes, unpubl. study); and, *Ischnura verticalis* Say (Mlynarek et al. 2011). When this coarse index of relative abundance is considered, it appears the most abundant species based on researchers' preferences was also the most widespread species in five of six instances where this comparison could be undertaken (Table 1). No such studies have been done on either of the *Chromatallagma* species.

Gregarine collection

Presence or absence of gregarine infection was determined by dissecting the abdomen of each damselfly. Dissections were done by cutting the pleural membrane between the tergites and sternites and exposing the gut. The entire gut was removed, although gregarine parasitism was always restricted to the mid-gut. The gregarine trophozoites (feeding stage) were subsequently counted. The quantity of gregarine individuals within a host appears to be an important factor in determining effects on hosts (Åbro, 1971). Throughout this study, gregarine species diversity was not considered (gregarines are treated as one "morphospecies" among species in species pairs), because there are studies that demonstrate the same species of gregarine infecting several species within a habitat. Detwiler and Janovy (2008) demonstrated that gregarine species can infect related *Tribolium* (Coleoptera: Tenebrionidae) host species (J.J. Mylnarek, unpubl. data). Cielocha *et al.* (2011) additionally showed that the same species of gregarine, *Nubenocephalus secundus* infects related host species of *Argia* within a habitat. Based on morphometric observations, the *Calopteryx* species pair had distinct gregarines from the other host species. Otherwise, there was overlap in the characters in the gregarine fauna in the remaining host species; therefore, we were unable to differentiate morphospecies. We make the assumption that that gregarine species are the same between host species within species pairs (*cf.* Clopton, 2009).

Zygoptera distribution

All the host species used in this analysis are restricted to the Nearctic region. The size of the geographic distribution was assessed using the Odonata Central database (Abbott, 2007). The database is actively managed and an expert verifies all records. A 2° latitude-longitude grid was superimposed on the geographic data and the presence-absence was calculated for each cell. Once the presence was determined within a cell, the area of the species distribution was determined by multiplying the area of the cells by the area in km that each cell represents (see Hassall, 2011 for details).

Statistical analyses

Hecker et al. (2002) determined that, overall, there was a significant host sex bias in gregarine parasitism levels in *Enallagma Enallagma boreale,* but this bias was diminished or lost when samples from the same site and same date were compared. Furthermore, Mlynarek *et al.* (2011) and Forbes *et al.* (2012) reported no sex bias in *Ischnura verticalis* or in *Nehalennia irene* respectively. In the present study, we found no sex bias differences in any species apart from *E. boreale* (the same species studied in Hecker *et al.* (2002))*.* However, as a precaution, we include "sex" as a random factor in our models.

Prevalence was defined as the proportion of hosts infected with at least one gregarine parasite (Bush *et al.*, 1997). Clopper-Pearson 95% confidence intervals (Zar, 1996) were calculated for prevalence estimates and are included. To test for differences between species in the intensity and prevalence of parasitism while taking into account potential confounding variables, we used generalised linear mixed effects models (GLMMs) constructed separately for each species pair and implemented using the lmer function in the lme4 library (Bates et al., 2011) in R (R Development Core Team, 2012). In one group of models, prevalence was specified as the response variable with logistic error distribution, the host species as a fixed effect, and sex and date of collection as random effects. In the second group of models, parasite abundance in the host was specified as the response with Poisson error distribution, the host species as a fixed effect, and sex and date of collection as random effects. This gives a total of 14 models.

To test for effects of geographical distribution, we compared the parasite prevalence of the geographically restricted species against that of the geographically widespread species for each species pair. We first asked whether there were significant variation in either prevalence or intensity of gregarine infection between species in species pairs and then whether any such differences were explained as the more widely distributed host having the higher prevalence or intensity.

RESULTS

A total of 1,338 damselflies were collected for this study (Table 1). There was considerable variation between host species in gregarine parasitism. Gregarine prevalence ranged from 1.1 % (0.03-5.8% Clopper-Pearson 95% CI) in *Argia violaceae* Hagen, to 67.9% (56.7-79.8% 95% CI) in *E. boreale* (Table 1). Median intensity was also variable ranging from 1 (1-2 95% CI) in *Enallagma Chromatallagma vesperum* Calvert to 17.5 (9-25 95% CI) in *Calopteryx aequabilis.*

Using GLMMs controlling for the effects of host sex and sampling date, we found that three out of seven species pairs (*Calopteryx, Enallagma Chromatallagma* subgenus and *Ischnura*) showed significant differences in prevalence between the species in the species pairs. Five of seven species pairs (*Calopteryx, Enallagma Enallagma, Ischnura*, *Lestes* and *Nehalennia*) also had statistically significant different median intensities between the two species. Thus in six of the seven species pairs, there were significant or near significant differences between species members in one or more measures of parasitism.

When we plotted differences in prevalence between species within species pairs, we found no evidence that species with the larger geographical distribution had higher prevalence (Fig. 1). In fact, for all three of the species pairs with significant differences in gregarine prevalence, it was the less widespread host species that was most often parasitized (see parameter estimates in Table 2). The remaining four species pairs did not show significant differences in prevalence between their sibling species, despite the fact that one of the species pairs (*Enallagma Enallagma*) had quite disparate geographical distributions. A similar pattern was seen in the intensity of infection, with three species pairs (*Calopteryx*, *Ischnura* and *Lestes*) showing significantly lower intensity of infection in the more widespread species (Table 2). In contrast, the *Enallagma Enallagma* species pair showed a smaller, though still highly significant, effect of greater intensity in the more widespread species.

Discussion

Our study was based on 14 associations between parasites thought to be generalists (gregarines), and sibling species of their damselfly hosts. We found that in several species pairs, one species often had a higher measure(s) of parasitism; however, this was most often the less widely distributed host species of a species pair. This pattern certainly occurred for *Calopteryx, Enallagma Chromatallagma, Ischnura* and *Lestes* species No pattern was evident for *Argia* species and the opposite pattern was present only for the *Nehalennia* and *Enallagma Enallagma* species pairs, similar to what was observed for *Calopteryx* species in Finland (*cf.* Ilvonen et al. 2011).

Widespread species are often more numerous (Hanski, 1982; Brown, 1984). Intuitively, more numerous host species should be larger targets of selection on parasites; therefore, it is expected that parasites should be more prevalent or numerous in widespread hosts (Morand et al. 2010). Stated another way, the parasite is expected to be better adapted to the widespread and more abundant host (Price et al. 1988; Forbes et al., 1999; Ilvonen et al., 2011). There is also the possibility that widespread hosts bring their parasites with them into regions occupied by less widespread species (Durrer and Schmid-Hempel 1995). Such secondary contact could mean that one species of the species pair had associations with the parasite for a shorter period of evolutionary time.

In most cases in this study, the widely distributed host species was thought to be more locally abundant based on previous work, although this was not known *a priori* for *Enallagma Chromatallagma* spp. However for *Lestes* spp., the host species that was expected to be more locally abundant had higher parasitism, a similar result as found for *Nehalennia* in which the widespread host is also more locally abundant (Van Gossum et al. 2007). Our results leave two important issues unresolved. The first is why some species in species pairs show differences in parasitism, whereas others do not; and, second, why *Nehalennia* and *Enallagma Enallagma* species appear to be an exception to the rule of less widely dispersed species having higher parasitism, when such species differences in parasitism exist. We can only suggest that *Nehalennia* and *Enallagma Enallagma* show the opposite pattern of parasitism is

because of the differences in habitat preference of the two sibling host species of the pair. *Nehalennia irene* and *E. ebrium* can occur in water bodies with well-developed fish communities whereas *N. gracilis* and *E. boreale* have a clear preference for fishless water bodies (McPeek 1990). The remaining five species pairs have similar habitat affinities and therefore are present within the same parasite communities.

The first issue identified above is perhaps the most perplexing. That is, why do species in some species pairs show differences in measures of parasitism, whereas this is not universally true? Although sibling species share age of associations with their generalist parasites, different host species pairs might have diverged for different periods of evolutionary time. Such differences might allow there to be time for differences in degree of parasitism to evolve in some species pairs, but not others. In other words, species within *Enallagma* and *Argia* pairs might have had less time for divergence from one another. However, such explanations cannot explain why the less widespread species often tend to be more parasitized (but see Ilvonen et al. 2011) or why *Nehalennia* species are an exception in this study.

In the *Nehalennia* species pair, the more widespread and more locally abundant species is under higher parasite pressure. In fact, this is also seen for ectoparasitic mites on these two host species, but here the more widespread host species (*N. irene*) is susceptible to a mite species to which the less abundant, and less widespread, *N. gracilis* is resistant (J.J. Mlynarek, unpubl. data). As mentioned, these patterns of parasitism do not occur for species in other species pairs. The *Nehalennia* species pair is very different from the other species pairs, because *N. gracilis* is highly specialised and restricted to bogs. In contrast to *Nehalennia*, the sibling species of *Calopteryx*, *Enallagma Chromatallagma* and *Ischnura* are ecologically similar within the species pairs; the *Calopteryx* species have evolved at creek edges, *Enallagma Chromatallagma* are lake damselflies and *Ischnura* species are usually found around ponds, marshes and slow moving streams (Walker, 1953). If there was a species pair where secondary contact was likely, it might well be *Nehalennia*.

We note that an additional analysis could have been done to see if difference in distribution between species in species pairs related to the magnitude of difference in measures of parasitism. While possible, such analyses are not practical; there is a low sample size of only seven comparisons and an analysis of this type will compare larger species like *Calopteryx,* which have morphologically distinct gregarines, with smaller species like *Nehalennia*. Other factors, such as whether certain gregarine parasites exist at higher prevalence on average than other gregarine species, will determine the magnitude of difference we might expect for comparisons within host species pairs. We already know from our analysis that a general pattern of increasing difference in parasitism with increasing difference in geographical distribution is not evident. Species pairs in Fig. 1 were arranged along a gradient of increasing difference in geographical distribution and no gradient in differences in prevalence was evident.

If the parasites are not generalists, then our results still leave open the question of why some associations should be marked by higher measures of parasitism and why the balance seems to be that less widespread hosts (which also are expected to be less locally abundant on average) have higher measures of parasitism. It is possible that our less widespread species were actually more regionally widespread; however, when we chose the target host species we determined this not to be the case (Walker 1953). Another possible explanation would be that the more geographically restricted host species has not developed strong innate defences because of possible low encounter rates with parasites generally. It is believed that larger geographic distributions lead to increased levels of interactions (Pielou, 1974), which might mean higher innate immune defences. This possibility can be resolved with ecological immunology studies.

In summary, we are suggesting that two factors might both influence the degree to which related host species are parasitized. The first is relative investment in innate immune defences, which might be lower on average for less widespread species: a proposition, which could be tested. The second is time in association with the host whereby parasites might evolve to be more effective generalists. If a less

widespread host species has had a recent contact, it might show lowered measures of parasitism even if it is not particularly well defended, because the parasite has not yet evolved to exploit that species effectively. Less widespread species with longer contact times with parasites are expected to show higher levels of parasitism than their widely distributed sibling species. Studies comparing exposure of sibling species to infective stage of parasites would also prove useful in testing these ideas.

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Table 1 – Details of parasitism in 14 species of damselflies sampled at 6 sites. N represents the number of individuals of each species sampled. Geographic range of the host is provided. Relative abundance represents the species of a species pair that has received more attention from researchers in our study area, because of the need for large samples (*Chromatallagma* not known). Confidence limits for prevalence and median intensity are shown respectively.

Host species	Location	N	Geographic range (10^6 Km^2)	Relative abundance	Prevalence % (95% CI)	Median (95%CI)
Argia moesta	Lake Opinicon	88	4.10	>	1.1 (0.03-6.2)	1 (n/a)
Argia violacea	Lake Opinicon	94	3.98		1.1 (0.03-5.8)	8 (n/a)
Calopteryx aequabilis	Brasset Creek	62	3.55		54.8 (41.7-67.5)	17.5 (9-25)
Calopteryx maculata	Brasset Creek	68	3.77	>	25.0 (15.3-37.0)	1 (1-4)
Enallagma (*Enallagma*) *boreale*	Jack's Marsh	68	6.53	>	69.1 (56.7-79.8)	6 (4-10)
Enallagma (*Enallagma*) *ebrium*	Jack's Marsh	94	4.35		67.9 (47.6-84.1)	6 (4-10)
Enallagma (*Chromatallagma*) *signatum*	Lake Opinicon	120	3.23		5.0 (1.9-10.6)	1 (1-18)
Enallagma (*Chromatallagma*) *vesperum*	Lake Opinicon	98	2.6		16.3 (9.6-25.2)	1 (1-2)
Ischnura posita	Osprey Marsh	75	3.13		46.7 (35.1-58.6)	3.5 (2-6)
Ischnura verticalis	Osprey Marsh	23	4.36	>	21.7 (7.5-43.7)	2 (1-22)
Lestes forcipatus	Yzerinac Pond	182	3.19	>	25.8 (19.6-32.8)	2 (2-3)
Lestes congener	Yzerinac Pond	73	5.99		17.8 (9.8-28.5)	2 (1-2)
Nehalennia gracilis	Herbert's Bog	88	1.51		40.9 (30.5-51.9)	5 (2-7)
Nehalennia irene	Herbert's Bog	205	4.03	>	55.1 (48.0-62.1)	5 (4-6)

Table 2 – Results of generalised linear mixed effects models (GLMMs) comparing the prevalence and intensity of parasitic infection within the seven Zygoptera species pairs. "Species" columns give parameter estimate for the fixed effect term denoting the difference between species within each pair. Species are compared such that a positive parameter indicates greater prevalence or intensity in the more widespread host. "Sex" and "Date" columns give χ^2 statistics for a likelihood ratio test comparing full models and reduced models lacking the focal term to evaluate the significance of the random effects. *** = p<0.001, ** = p<0.01, * = p<0.05.

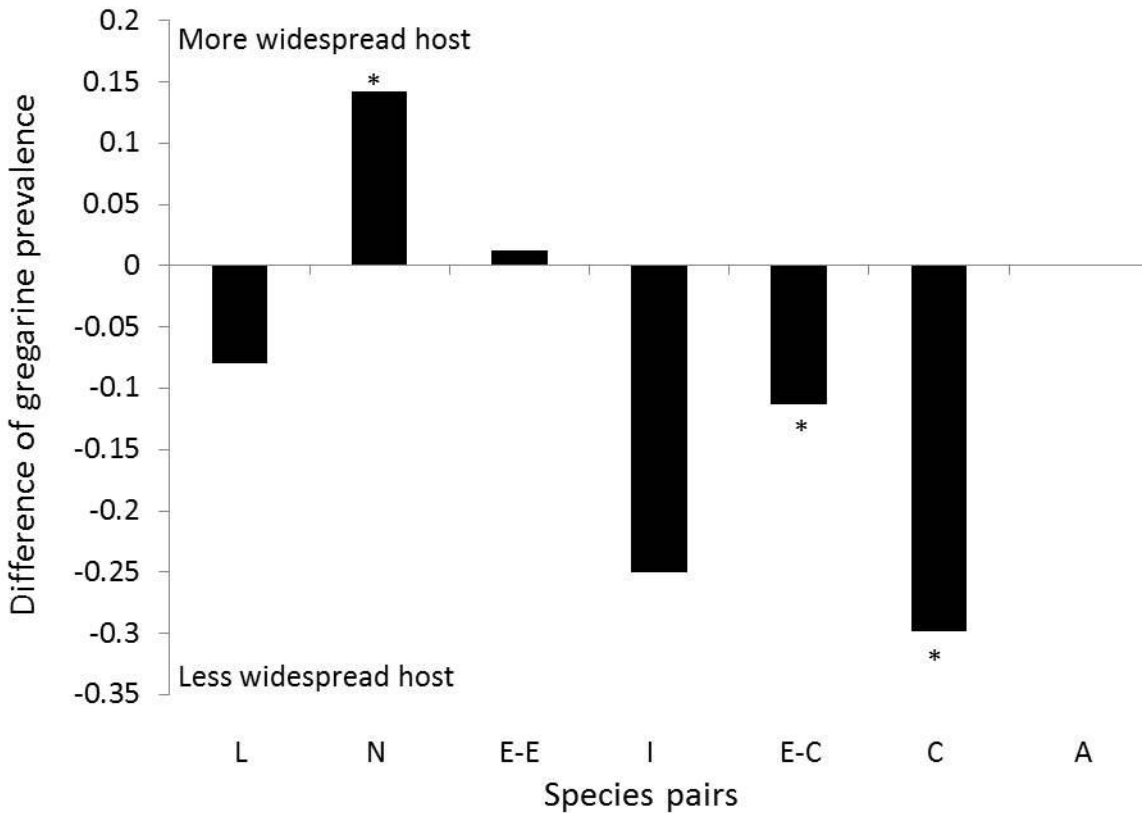| Species pair	Prevalence			Intensity		
	Species	Sex	Date	Species	Sex	Date
Argia	0.139	0.4	49.3***	-1.501	0.0	19.0***
Calopteryx	-1.293**	0.0	1913.6***	-2.323***	0.0	117.3***
Enallagma Enallagma	0.544	1.4	4089.8***	1.102***	0.0	376.3***
Enallagma Chromatallagma	-1.234*	0.0	222.7***	0.075	0.0	63.5***
Ischnura	-1.281*	0.0	1966.2***	-1.342***	0.0	140.4***
Lestes	-0.700	0.0	1041.6***	-1.396***	0.0	0.0
Nehalennia	0.401	1.8	9805.5***	0.706***	0.0	1707.5***

Figure 1: The difference in prevalence between species, within species pairs. If the difference is positive, then the more widespread species of the species pair has higher prevalence (from Table 1); if the difference is negative, then the species with the higher prevalence is the less widespread host. Significant differences in prevalence values are marked by * and can be seen in Table 2. Note that the Ischnura species pair has nearly significant difference in prevalence. Species pairs are organized from left to right as most different in geographical distribution to least different. Key: A= *Argia*, C= *Calopteryx*, E-C = *Enallagma Chromatallagma*, E-E = *Enallagma Enallagma*, I = *Ischnura*, N= *Nehalennia*, L=*Lestes*.