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RESEARCH ARTICLE

Is the local environment more important than within-host interactions in determining coinfection?

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Abstract

1. Host populations often vary in the magnitude of coinfection they experience across environmental gradients. Furthermore, coinfection often occurs sequentially, with a second parasite infecting the host after the first has established a primary infection. Because the local environment and interactions between coinfecting parasites can both drive patterns of coinfection, it is important to disentangle the relative contributions of environmental factors and within-host interactions to patterns of coinfection.
2. Here, we develop a conceptual framework and present an empirical case study to disentangle these facets of coinfection. Across multiple lakes, we surveyed populations of five damselfly (host) species and quantified primary parasitism by aquatic, ectoparasitic water mites and secondary parasitism by terrestrial, endoparasitic gregarines. We first asked if coinfection is predicted by abiotic and biotic factors within the local environment, finding that the probability of coinfection decreased for all host species as pH increased. We then asked if primary infection by aquatic water mites mediated the relationship between pH and secondary infection by terrestrial gregarines.
3. Contrary to our expectations, we found no evidence for a water mite-mediated relationship between pH and gregarines. Instead, the intensity of gregarine infection correlated solely with the local environment, with the magnitude and direction of these relationships varying among environmental predictors.
4. Our findings emphasize the role of the local environment in shaping infection dynamics that set the stage for coinfection. Although we did not detect within-host interactions, the approach herein can be applied to other systems to elucidate the nature of interactions between hosts and coinfecting parasites within complex ecological communities.

KEYWORDS
Arrenurus, coinfection, *Enallagma*, food web, gregarine, *Ischnura*, within-host interactions

1 | INTRODUCTION

Across many host–parasite systems, variation in coinfection patterns is often attributed to within-host interactions among coinfecting parasites (Budischak, Wiria, et al., 2018; Klemme et al., 2016; Telfer et al., 2010). Such within-host interactions often manifest as either facilitative or antagonistic interactions between parasites. In the former, infection with one parasite may facilitate the subsequent infection of a second parasite (Dallas et al., 2019; Klemme et al., 2016), such that increasing numbers of the primary parasite will result in an increase in number of the secondary parasite. Alternatively, parasites may exclude one another via priority effects (Hood, 2003; Marchetto & Power, 2018; Zilio & Koella, 2020) or competitive exclusion (Duncan et al., 2018; Gipson et al., 2019). In this case, increasing numbers of the first parasite will limit the second parasite. Within-host interactions between parasites have been examined in both theoretical (Clay et al., 2019; Jiao & Cortez, 2022; Milutinović et al., 2020) and empirical studies (Clerc et al., 2019; Garcia-Longoria et al., 2022; Ramsay & Rohr, 2023), yet it is important to note that relationships between coinfecting parasites within hosts may arise for reasons other than parasite–parasite interactions alone (Griffiths et al., 2014). Although prior studies have considered factors such as host age (Cattadori et al., 2008; Morrill et al., 2021) or the abundances of coinfecting parasites that hosts are exposed to (Johnson & Hoverman, 2012; Ramsay & Rohr, 2023), one key factor that can shape coinfection dynamics is the local environment (Bolnick et al., 2020; Rynkiewicz et al., 2019), with multiple potential pathways by which this could occur (Figure 1).

While the host can represent a complex and diverse series of habitats that the parasite exploits (Pedersen & Fenton, 2007; Rynkiewicz et al., 2015), host–parasite interactions and within-host interactions between coinfecting parasites, all occur amidst complex environments within ecological communities. Abiotic factors within these communities such as temperature, pH and precipitation are known to explain spatial variation in parasite prevalence (proportion of the population parasitized) and intensity (average number of parasites per infected host) among host populations (Hasik & Siepielski, 2022; LoScerbo et al., 2020; Preisser, 2019). Furthermore, biotic interactions, both direct and indirect, are also known to affect host–parasite relationships. For example, non-consumptive effects of predators can indirectly reduce (Navarro et al., 2004) or increase (Duong & McCauley, 2016) infection by shaping immune function, and immune function itself is often resource limited (Budischak, Hansen, et al., 2018; Campero et al., 2008; Hasik, Tye, et al., 2021). Due to their connections with host immune function, both predators and prey availability therefore have the potential to indirectly impact a host's relationship with its parasites, which may then affect coinfection patterns across environmental gradients. Such a scenario could manifest as within-host interactions, but would instead be due to the relationship(s) between the local environment and one (or both) coinfecting parasites (Figure 1a). Conversely, the local environment may only affect one parasite, but a second coinfecting parasite responds to variation in infection by the first parasite (Figure 1b). In this case, although infection by the second parasite is decoupled from the local environment, environmental variation remains a key driver of coinfection patterns via its effects on the first parasite. Finally, it may be that within-host interactions are the sole driver of coinfection (Figure 1c). Infection by both parasites would therefore be decoupled from the local environment, and the second parasite would only respond to variation in infection by the first parasite. Discerning among these possibilities is challenging, but doing so is important because it will offer a more representative picture of the dynamics connecting host–parasite relationships to (and their myriad effects on, Hasik, de Angeli Dutra, et al., 2023) the other species interactions embedded within communities.

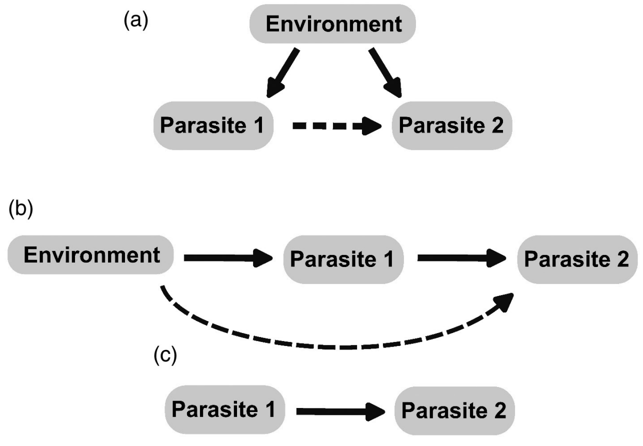

FIGURE 1 Conceptual diagram of the hypothesized relationships between the local environment and two coinfecting parasites. (a) The case in which both parasites respond similar to a shared, but potentially unknown environmental variable. In this case, not accounting for this environmental variation gives the false impression of within-host interactions (dashed line). (b) The case in which parasite 1 responds to a given environmental variable, and parasite 2 subsequently responds to variation in parasite 1. In this situation, within-host interactions do occur, but they are mediated by the local environment. Furthermore, not accounting for the influence of parasite 1 would give the impression that there is a direct relationship between the local environment and parasite 2 (dashed line). (c) The case in which parasite 2 responds directly to variation in parasite 1, without any influence from the local environment.

If such factors within the local environment affect either host–parasite dynamics (or the parasite itself) for one or more parasites, then a deterministic structure of coinfection may emerge, wherein a given environmental factor (or factors) drives a predictable pattern of coinfection across abiotic and/or biotic gradients (Johnson et al., 2015; Rynkiewicz et al., 2019). We therefore hypothesize that the local environment sets the stage for coinfection, via a mix of direct effects on one parasite and indirect, parasite-mediated effects on the second parasite (Figure 1b).

To test this hypothesis, we utilized standardized field observations of multiple damselfly species (Odonata: Zygoptera) and two of their most common parasites: ectoparasitic water mites (*Arrenurus* spp.) and endoparasitic gregarines (Apicomplexa, see Figure 2 for a

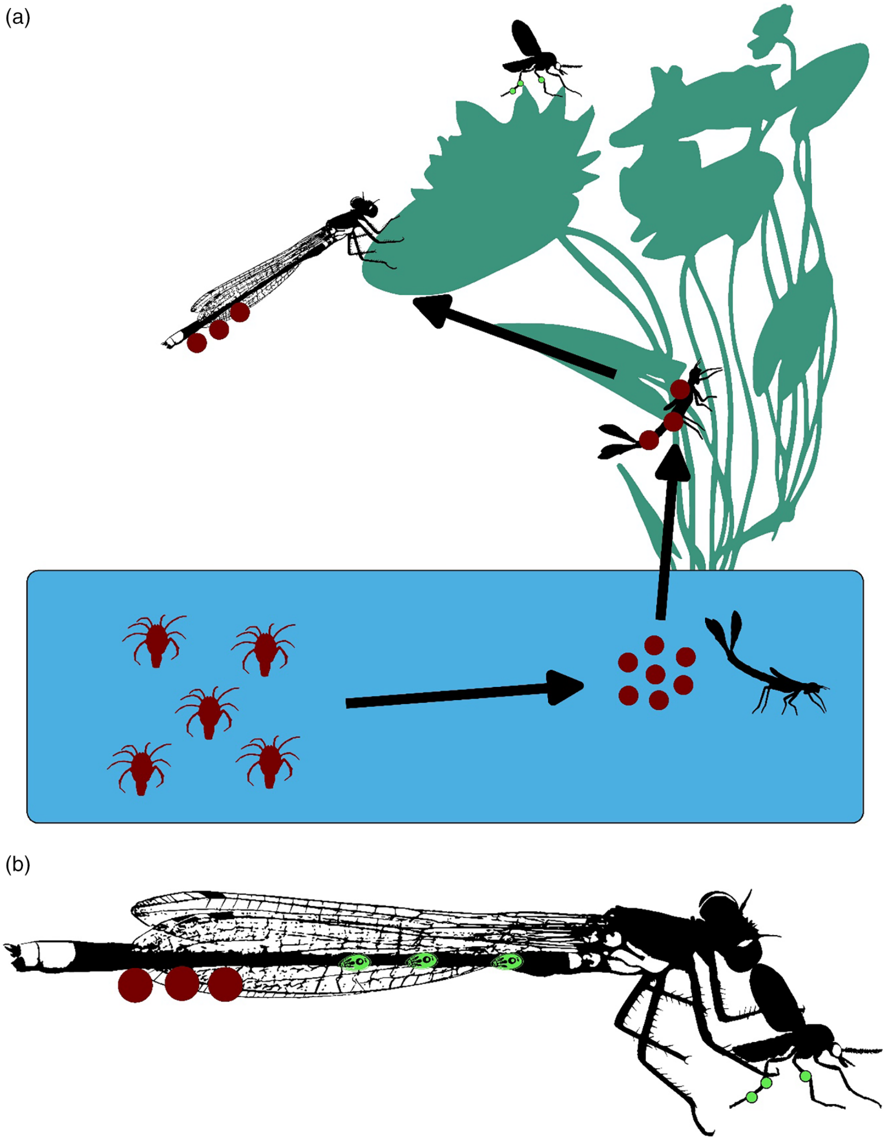

FIGURE 2 Conceptual diagram of the infection dynamics in the odonate-water mite-gregarine host–parasite system. Adult water mites are predators in the water column and lay eggs which hatch into parasitic larvae that seek out aquatic larval odonates (a). The larval mites are phoretic on the larval odonate as the latter crawls out of the water onto emergent macrophytes to complete its transition to the adult stage. Before the cuticle of the newly emerged adult odonate hardens the larval mites pierce it with a feeding tube to engorge on haemolymph. Shown on top of the emergent macrophyte is a small dipteran with infective gregarine oocytes on its legs (green circles). Adult odonates hunt down and consume these dipterans, ingesting the infective oocytes (b). Once consumed, the oocytes mature and consume the host's nutrients (shown in green inside the abdomen of the adult odonate). Plant, mite, larval damselfly, adult damselfly and dipteran images courtesy of Guillaume Dera (CC0 1.0), Rachel Sloop (CC0 1.0), Adam M. Siepielski, Maxime Dahirel (CC BY 3.0) and Nathan Jay Barker (CC0 1.0) respectively.

conceptual diagram of the infection dynamics). Although experimentally manipulating infection would be an ideal test of the hypothesis, it is logistically infeasible in this system as this would require rearing of the mites, gregarines and each of the host species, and then performing infection experiments, as well as manipulating environmental conditions. In place of an experimental approach, our use of multiple hosts from a well-understood study system provides a comparative, multispecies approach that strengthens our inferences.

Damselflies are aquatic predators for most of their life cycle, emerging from the water as aerial adults to reproduce and complete their life cycle. During this transition, they are attacked by *Arrenurus* mites (Hasik, Ilvonen, et al., 2023; Smith et al., 2010), which are generalist parasites on multiple host species (Morrill et al., 2013; Worthen & Turner, 2015). Damselflies acquire gregarines during the aerial adult stage by ingesting infective oocysts from prey items, such as dipterans (Åbro, 1976; Hasik, Ilvonen, et al., 2023; Locklin & Vodopich, 2010). This system is useful for testing hypotheses about within-host interactions and environmental variation driving coinfection, as both water mite (LoScerbo et al., 2020; Mlynarek et al., 2015) and gregarine prevalence (Ilvonen et al., 2018; Locklin & Vodopich, 2010) are not only frequently high but also vary within and among species and across environmental gradients (da Silva et al., 2021; Hasik & Siepielski, 2022; Ilvonen & Suhonen, 2016; Locklin & Vodopich, 2010; LoScerbo et al., 2020; Mlynarek et al., 2011). Critically, beyond their frequent co-occurrence across habitats, there is potential for interactions between the ectoparasitic water mites and endoparasitic gregarines via multiple indirect mechanisms such as impacts on flight ability (Reinhardt, 1996) or immune defences (Forbes et al., 1999), which we detail in the next section. Local damselfly diversity is also often high (e.g. 5–12 co-occurring species in eastern North American fish ponds and lakes; McPeek, 1989, 1990b, 1998; Siepielski et al., 2010), allowing for comparisons among multiple host species. Moreover, the sequential nature of the timing of infection by ectoparasitic mites (larval to adult host transition) and endoparasitic gregarines (adult stage; Figure 2) make them well suited for detecting either priority effects or parasite-mediated facilitation (Hasik, Ilvonen, et al., 2023). Below we detail our predictions for coinfection patterns in this system.

1.1 | Specific predictions within this study system

Coinfection patterns are likely to vary among host species, as previous studies have shown that both ecto- and endoparasitism vary dramatically among host species (Hasik & Siepielski, 2022; Ilvonen et al., 2018; Morrill et al., 2013; Worthen & Turner, 2015). To some extent, differences in the abundances of parasites within lakes may shape the likelihood of host infection, as might dispersal propensity by either the host or parasites. We lack information on parasite abundances (independent of those observed infecting hosts), and while the parasites also likely vary in their dispersal ability both mites (Bohonak et al., 2004) and gregarines (Cielocha et al., 2011) depend on their hosts to disperse between habitats, and the damselflies themselves are known to be poor dispersers (typical dispersal <1 km, McPeek, 1989). However, it is unclear which specific environmental factors are expected to affect coinfection in this system. Water mites are only parasitic as larvae and become predators as adults (Smith et al., 2010), with larvae existing solely within the water column prior to infecting odonate hosts, while gregarines are acquired in the terrestrial environment via consuming prey covered in oocysts (Figure 2). Therefore, the effects of the aquatic environment should be decoupled from terrestrial parasitism. However, an indirect relationship may provide the answer if within-host interactions, either antagonistic or facilitative, are the driving force behind coinfection in this system. That is, because water mites infect the odonate host upon the latter's emergence to the adult stage, they have the potential to either inhibit or enhance subsequent infection by endoparasitic gregarines. Specifically, infection with water mites is known to reduce body condition (Forbes & Baker, 1991) and flight ability (Baines et al., 2020; Reinhardt, 1996), which could lead to a reduction in gregarine infection as the host will be less able to hunt down and consume the prey vectors of the gregarine infective stages (Hasik, Ilvonen, et al., 2023). Additionally, infection with water mites stimulates the phenoloxidase (PO) cascade (Forbes et al., 1999; Forbes & Baker, 1990; Mlynarek et al., 2014). Such stimulation could result in increased infection by gregarines due to the depletion of PO, since the PO cascade is a key immune response to infection as the activation of PO is a precursor to the production of melanin as an end product, which encapsulates and kills parasites (González-Santoyo & Córdoba-Aguilar, 2012). Indeed, interactions between ecto- and endoparasites tend to be limited to indirect mechanisms (Bordes & Morand, 2011), such as endoparasite-mediated energy loss reducing the grooming ability of willow ptarmigans (*Lagopus lagopus*), resulting in increased ectoparasite loads (Holmstad et al., 2008).

Water mites are aquatic parasites; thus, an intriguing possibility is that they could mediate an indirect relationship between the aquatic environment and the terrestrial gregarines. This could happen if, for example, aquatic environmental variables like pH affected mite parasitism (as in Gómez-Llano et al., 2023; Hasik & Siepielski, 2022; LoScerbo et al., 2020), and mite parasitism then affected gregarine parasitism. Such a relationship would then manifest as varying patterns of coinfection across environmental gradients. Therefore, we predict that aquatic variables will affect coinfection, and this will manifest as a mix of the direct relationship between the environment and mite infection and indirect, mite-mediated relationships between the environment and gregarine infection (e.g. Figure 1b). By simultaneously evaluating not only the relationships between the local environment and coinfection but also potential within-host interactions between ecto- and endoparasites, our study illuminates how coinfection emerges within complex ecological communities.

2 | MATERIALS AND METHODS

2.1 | Parasitism surveys, specimen processing and environmental sampling

All sampling was conducted with permission from the Arkansas Game & Fish Commission; no ethical approval was required. During the summers of 2017 and 2019, we sampled 13 lakes throughout Northwest AR (Table S1; Figure S1) to estimate parasite prevalence and intensity in damselfly hosts. Because diel activity varies among damselfly species, we sampled each lake at either one start time per sampling round once a week, alternating the start times each week (2019), or at three different start times per sampling round three times a week (2017). 2019 sampling occurred every week from early June to late July (39 sampling rounds), while 2017 sampling occurred every other week from late May through early August (141 sampling rounds), with each visit totalling six person-hours of sampling. This duration covered the majority of the flight season when damselflies emerge and are parasitized. Although mite parasitism can vary throughout the adult flight season (Nagel, Tonia, & Forbes, 2010), an analysis of temporal variation is beyond the scope of this study. We standardized spatial sampling effort by collecting damselflies in the same locations at each lake.

During each sampling event, we collected adult damselflies using aerial sweep-nets and stored them in 70% ethanol. We did not investigate sex differences in parasitism, as most captured hosts were male. Mites are known to not only reduce host survival but severe infections can also negatively impact flight ability (Åbro, 1982; Forbes & Baker, 1991). Furthermore, mite infection also reduces mating success (Gómez-Llano et al., 2020). Therefore, it is possible that our sampling was biased towards either less-infected individuals (due to mortality of heavily-infected individuals) or more-infected individuals (as we focused on single males, and those in copula with a female were less likely to be parasitized). Although we collected data on multiple odonate species, we excluded species with <10 individuals collected per lake, meaning that we were unable to compare coinfection patterns for every host species at every lake. We, therefore, focused our analyses on the five most common damselfly species: *Enallagma basidens*, *E. exsulans*, *E. signatum*, *E. traviatum* and *Ischnura posita*.

We quantified mite infection by examining damselflies for mites under a dissecting scope (Leica MZ7.5, Leica Microsystems). We also checked for 'scars'-melanized spots on the exoskeleton where mite-feeding tubes were located, which indicate successful mite parasitism (Smith et al., 2010). For each host species, we scored the prevalence of infection by dividing the number of infected individuals by the total number of individuals collected at a lake, and intensity of infection by calculating the median number of parasites per infected host.

To quantify infection by gregarines, we gently separated the abdomen from the thorax, after which we examined both the inside and outside of the gut under a dissecting scope (Leica MZ7.5, Leica Microsystems). We quantified infection prevalence and intensity in the same manner as the mites. For damselflies collected in 2019, we examined every individual for the presence of gregarines, while we examined 10 haphazardly chosen individual damselflies of every species collected from each sampling period in 2017.

Environmental sampling was completed during August of each year in the same area of each lake that we sampled damselflies. We focused on water pH and densities of fish, prey and macrophytes because previous studies have suggested that they can either directly or indirectly shape water mite and/or gregarine parasitism in this system (e.g. da Silva et al., 2021; Hasik & Siepielski, 2022; Hasik, Tye, et al., 2021; LoScerbo et al., 2020; McDevitt-Galles et al., 2018). Specifically, increasing fish density decreases damselfly activity and subsequent resource acquisition (Ousterhout et al., 2018; Siepielski et al., 2020; Strobbe et al., 2011), which may drive an increase in parasitism as immune defences are resource-limited in this system (Campero et al., 2008; De Block & Stoks, 2008; Hasik, Tye, et al., 2021). Increases in pH are positively correlated with *Arrenurus* infection in odonate hosts (Hasik & Siepielski, 2022; LoScerbo et al., 2020), likely due to the reduction in mite hatching success and viability at low pH (Edwards, 2004; Rousch et al., 1997). Mite infection is also positively correlated with macrophyte occurrence (da Silva et al., 2021), which is thought to be due to macrophyte occurrence constraining the occurrence of both odonates and their prey (Hykel et al., 2020) at sites where parasite transmission occurs (Hupało et al., 2014; Zawal & Buczyński, 2013). Fish density was measured as the mean of three standardized seine hauls through the littoral macrophyte bed; prey (e.g. ostracods, copepods and other invertebrates smaller than odonates) density as the mean of six replicate 6 L box samples; macrophyte density as the mean of 10 randomly placed 0.5 m × 0.5 m quadrats; and water pH as the mean of three readings from a YSI probe (YSI ProPlus, YSI Inc.). Full details of sampling procedures have been previously published (Siepielski et al., 2010) and can be found in the supplemental information.

2.2 | Does coinfection vary predictably with the local environment?

We conducted all of the following analyses in R v4.1.2 (R Core Team, 2021). We constructed individual, species-specific logistic regressions between the proportion of hosts infected with either water mites, gregarines or coinfected as response variables and environmental predictors, in addition to including sampling year as the probability of coinfection varied among years within species (interaction between species and year: $X^2_4 = 13.63$, $p = 0.01$, Figure S2a). Comparing the relationships between environmental variables, single infections by both parasites and coinfection by both parasites will provide information on whether the relationship between the local environment and coinfection (if present) is simply due to the relationship between the prevalence of either water mites or gregarines (or both) varying across a gradient of that environmental variable (as in Figure 1a). All environmental predictors were centred to have a mean of 0 and SD of 1. To ensure that the difference between years was not due to a difference in the sampling methods (i.e. all hosts checked for both parasites in 2019 vs. a subset of $n = 10$ hosts from each species and population sampling event in 2017 checked for all parasites), we created $n = 1000$ data sets by randomly sampling $n = 10$ individuals without replacement from each species and population sampling event for the full 2019 data to create a sampling distribution. This procedure mimics an expectation of what our estimates of infection would be on average had we only sampled 10 individuals (as we did with the 2017 data). We then used this distribution to quantify the probability of coinfection, finding that it once again varied among years within species (interaction between species and year: $X^2_4 = 10.25$, $p < 0.04$, Figure S2b). Because there were no qualitative differences between the full 2019 data set and the estimate derived from the sampling distribution, we used the full 2019 data set for analyses.

We also conducted a co-occurrence analysis to test whether water mites and gregarines tend to co-occur within hosts more or less than expected by chance. For our test statistic we utilized the Checkerboard score (C-score), a measure of the 'checkerboard units' that can be found for each parasite species pair (Stone & Roberts, 1990). In short, the C-score is a measure of the degree of segregation of species across sites (or parasite taxa across hosts), ranging from 0 (no segregation, always co-occurring) to 1 (complete segregation, never co-occurring) (Gotelli & Rohde, 2002). We first calculated the observed C-score for each host species across lakes, after which we compared each species-specific observed value to 1000 randomly assembled null assemblages (as in Gotelli & Rohde, 2002). Importantly, the row sums of each species infection matrix was preserved (Connor & Simberloff, 1979), meaning that the observed frequency of infection by each parasite type was consistent across all randomly assembled matrices. We then calculated the observed C-score at the population for each species for those populations where we collected a minimum of $n = 30$ individual hosts to avoid the bias associated with smaller samples (Jiménez-Valverde, 2020). We again compared these observed values to 1000 randomly assembled null assemblages (as above). Statistical significance of comparisons between observed and simulated C-scores was based on the lack of overlap between observed C-score and 2 SD's of the simulated C-scores (as in Gotelli & Rohde, 2002).

2.3 | Is there evidence for direct or indirect (primary parasite-mediated) relationships between the environment and secondary parasites?

Gregarines are terrestrial parasites, and as such they should have no direct relationship with aquatic environmental variables (though the aquatic environment may affect the dipteran prey species which act as vectors, a point we return to in Section 4). We therefore asked if water mites (primary parasite) mediate the relationship between the aquatic environment and gregarines (secondary parasite, i.e. Figure 1b). Alternatively, aspects of the local environment may decrease either the prevalence or intensity of infection by gregarines via mechanisms other than mediation by water mites, which would result in declining coinfection across an increasing gradient of that environmental variable (i.e. Figure 1a). Such an apparent 'direct effect' of the environment on gregarines may instead indicate that the local environment affects the host, and not the gregarines, directly. Here, we focused our analyses on water mite and gregarine intensity, as we were interested in uncovering within-host interactions. Intensity is a measure of the average number of parasites per infected host *individual*, while prevalence is a measure of how many hosts in the *population* are infected (Bush et al., 1997; Reiczigel et al., 2019). Intensity therefore provides a more meaningful representation of how any environmental effects on a primary parasite subsequently affect secondary host infection.

To address this question, we first constructed structural equation models for each species (SEMs, Shipley, 2009). The supplemental information contains a graphical representation of the initial SEM we used for each species to test our hypothesis of water mite-mediated indirect relationships between the local aquatic environment and gregarine intensity. We also included year as a covariate in each SEM to control for the correlated structure of the data set, but do not explicitly consider temporal variation in the analyses as 2 years is not sufficient replication. For these analyses, we focused on hosts that were coinfected by both parasites; thus, our sample sizes were reduced compared to our analysis of the proportion of individuals coinfected, which included data from all individuals collected. For all models, we compared goodness of fit using the comparative fit index (CFI, a measure of the amount of variance accounted for in the covariance matrix, Fan et al., 2016) and root mean square error of approximation (a measure of how far a hypothesized model is from a 'perfect' fit, Xia & Yang, 2018). Models with CFI >0.95 and RMSE <0.06 are considered to fit the data well and were compared to one another using AIC values (as in Hasik & Siepielski, 2022).

Our initial SEM tested the hypothesis that water mites mediate the relationship between the local aquatic environment and terrestrial parasitism by gregarines. However, it is possible that the local environment affects gregarine parasitism via other mechanisms (i.e. affecting the host directly). To test for the possibility of other mechanisms affecting gregarine parasitism, we identified additional terms to fit to each model by analysing modification indices using the 'modindices' argument in the lavaan package (Rosseel, 2012). Modification indices with $\chi^2 > 3.84$ identify what terms, if added to a candidate model, improve model fit (Whittaker, 2012). This method allowed us to build a statistically informed network of relationships between the local environment and gregarine parasitism. Because we were only interested in the network of direct and indirect relationships between gregarine intensity and the local environment, we iteratively added direct environmental predictors of gregarine intensity with $\chi^2 > 3.84$ until no further modification indices were identified and the model had a CFI >0.95 and RMSE <0.06. For the models analysing *E. basidens* and *E. exsulans*, fish density was highly correlated with at least one other variable ($r > 0.80$), so we removed fish density from the initial SEMs for these species. We *ln*-transformed both water mite and gregarine intensity to meet model assumptions for all SEMs (as in Hasik & Siepielski, 2022), and centred all environmental variables to have a mean of 0 and SD of 1.

3 | RESULTS

3.1 | Coinfection varies predictably with the local environment

The occurrence, magnitude and direction of the relationships between environmental factors and the probability of coinfection varied among host species (Table 1). Summary information on parasitism can be found in Table S2. However, one consistent pattern emerged: The predicted probability of coinfection strongly declined as pH increased for all five host species (Table 1; Figure 3). While there was no consistent relationship between pH and mite prevalence (Figure 3; Table S3), the pattern of coinfection likely arose due to the negative relationship between pH and gregarine prevalence for all five host species (Figure 3; Table S4), absent the influence of water-mite mediation. For both *E. basidens* and *E. signatum*, the strength of the association between the probability of coinfection and the environmental variables varied among years, with the probability of coinfection for both species being slightly greater in 2019 than 2017 (Table 1; Figure 3; Figures S3–S5). Of the five host species, *E. signatum* was most likely to be coinfected across environmental gradients, while *I. posita* showed similar patterns of coinfection (Figure 3). Of particular note is how, with few exceptions, the predicted probability of coinfection for both *E. exsulans* and *E. traviatum* was very low (i.e. Pr(Coinfection) <0.03 in almost all cases).

3.2 | Co-occurrence within species and across environments

Our analysis of C-scores at the species level revealed that water mites and gregarines co-occur more often than would be expected by chance for *I. posita* (Figure 4a), as the observed C-scores were significantly lower than the mean of the randomly assembled data, but this was not true for the other four host species. When comparing C-scores at the population level for each species, we found that the majority of observed values were also not significantly different

TABLE 1 Results from the logistic regression model of coinfection among species.

Species	Factor	Estimate	SE	z	p
E. basidens	Intercept	−3.57	0.69	−5.13	2.96e-07
	Fish density m^{-2}	0.55	0.44	1.24	0.22
	Prey density L^{-1}	−0.71	0.56	−1.30	0.21
	pH	**−1.42**	**0.45**	**−3.15**	**0.002**
	Macrophyte density m^{-2}	**0.72**	**0.23**	**3.14**	**0.002**
	Year	**1.25**	**0.62**	**2.01**	**0.045**
E. exsulans	Intercept	−6.29	0.60	−10.53	<2e-16
	Fish density m^{-2}	−0.14	0.31	−0.43	0.67
	Prey density L^{-1}	0.79	0.56	1.40	0.16
	pH	**−1.20**	**0.35**	**−3.43**	**0.001**
	Macrophyte density m^{-2}	0.38	0.87	0.43	0.66
	Year	1.40	0.84	1.67	0.10
E. signatum	Intercept	−2.71	0.19	−14.63	<2e-16
	Fish density m^{-2}	−0.01	0.15	−0.07	0.94
	Prey density L^{-1}	**0.44**	**0.15**	**2.89**	**0.004**
	pH	**−0.32**	**0.15**	**−2.10**	**0.04**
	Macrophyte density m^{-2}	−0.04	0.28	−0.16	0.88
	Year	**0.66**	**0.31**	**2.17**	**0.03**
E. traviatum	Intercept	−6.96	1.18	−5.92	3.33e-09
	Fish density m^{-2}	−1.19	0.80	−1.50	0.14
	Prey density L^{-1}	−0.66	0.58	−1.13	0.26
	pH	**−2.004**	**0.50**	**−4.02**	**5.76e-05**
	Macrophyte density m^{-2}	0.31	0.49	0.64	0.53
	Year	1.50	1.12	1.33	0.18
I. posita	Intercept	−2.49	0.19	−13.11	<2e-16
	Fish density m^{-2}	−0.19	0.11	−1.73	0.08
	Prey density L^{-1}	0.22	0.11	1.87	0.06
	pH	**−0.46**	**0.09**	**−4.91**	**9.18e07**
	Macrophyte density m^{-2}	0.09	0.13	0.73	0.47
	Year	−0.06	0.22	−0.26	0.79

Note: Factors with significant *p*-values ($p < 0.05$) are bolded.

from the mean of the randomly assembled data (Figure 4b). The only exception was, again, the C-score for *I. posita* at Lake Sequoyah, where the observed C-score was significantly lower (Figure 4b).

3.3 | Little evidence for within-host interactions between water mites and gregarines

We found minimal evidence for indirect relationships between the aquatic environment and terrestrial parasitism. For four of the five species we analysed, the SEM that best fit the data included only the direct influence of the aquatic environment on both gregarine and water mite intensity. For *E. basidens*, gregarine intensity decreased strongly with pH, while water mite intensity strongly decreased with pH and slightly with prey density (Figure 5a). For *E. exsulans*, gregarine intensity slightly decreased with pH and increased with prey density, while water mite intensity slightly decreased with pH and prey density (Figure 5b). For *E. signatum*, gregarine intensity slightly decreased and increased with pH and prey density, respectively, while water mite intensity moderately and strongly increased with fish density and pH, respectively (Figure 5c). For *E. traviatum*, gregarine intensity strongly and slightly decreased with pH and prey density, respectively, while water mite intensity greatly increased with macrophyte density (Figure 5d). For *I. posita*, gregarine intensity strongly decreased with pH and slightly decreased with fish density and macrophyte density, while water mite intensity slightly

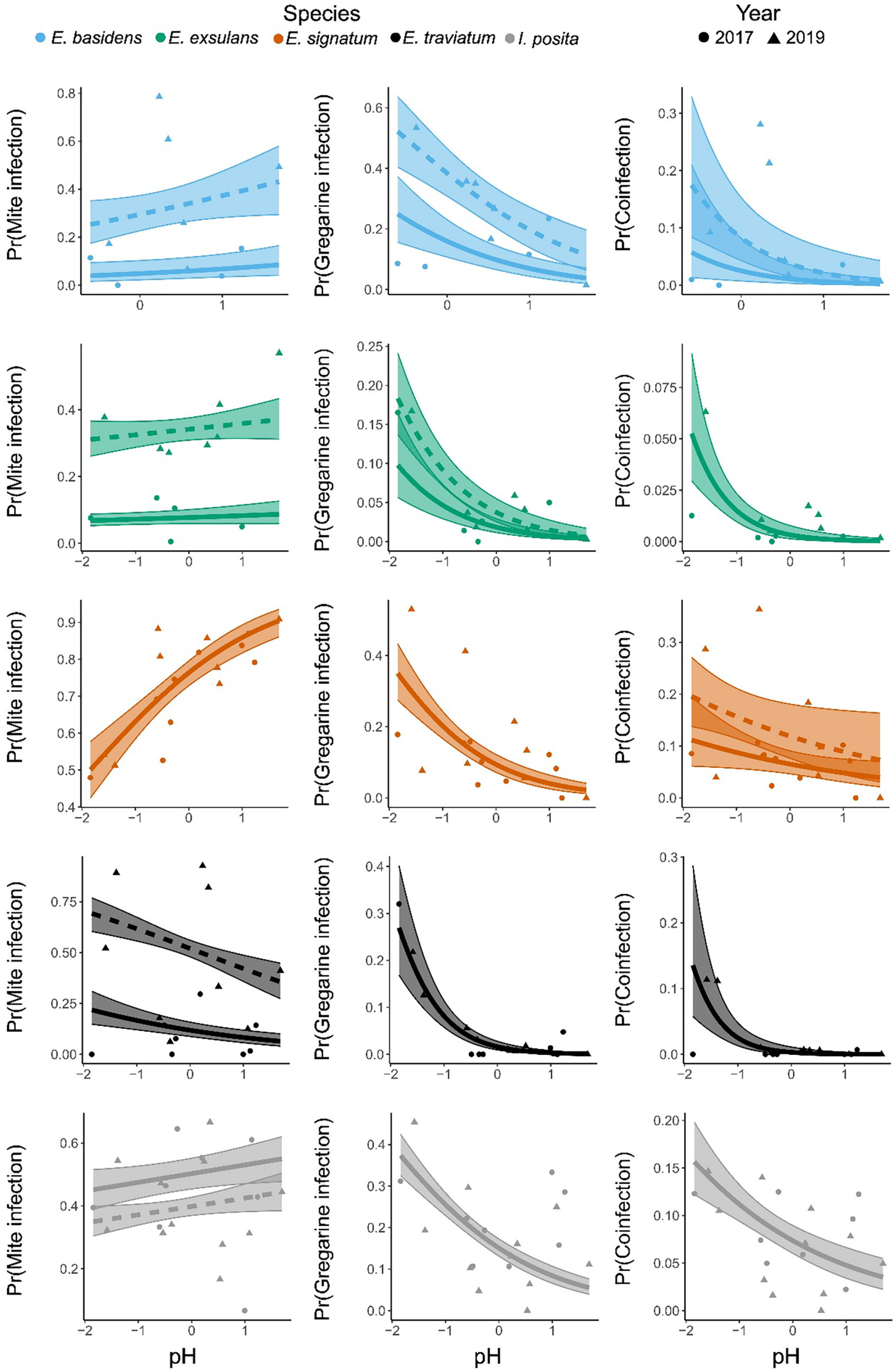
Species
● E. basidens ● E. exsulans ● E. signatum ● E. traviatum ● I. posita
Year
● 2017 ▲ 2019
Pr(Mite infection)
Pr(Gregarine infection)
Pr(Coinfection)
pH
pH
pH
−2
−1
0
1

FIGURE 3 Predicted probability of infection by water mites, gregarines and coinfection among damselfly hosts in relation to pH. Shown are model-derived logistic regressions (bands denote 95% CI) between the predicted probability of either infection by water mites, gregarines or coinfection and pH. Note the different *y*-axes among panels. Points denote the proportion of individuals infected with a given parasite or coinfected within individual populations and years. Colour denotes species and shape denotes year. For models where year was a significant predictor, the solid line denotes the predicted probability for a given relationship for 2017, while the dashed line denotes the same for 2019.

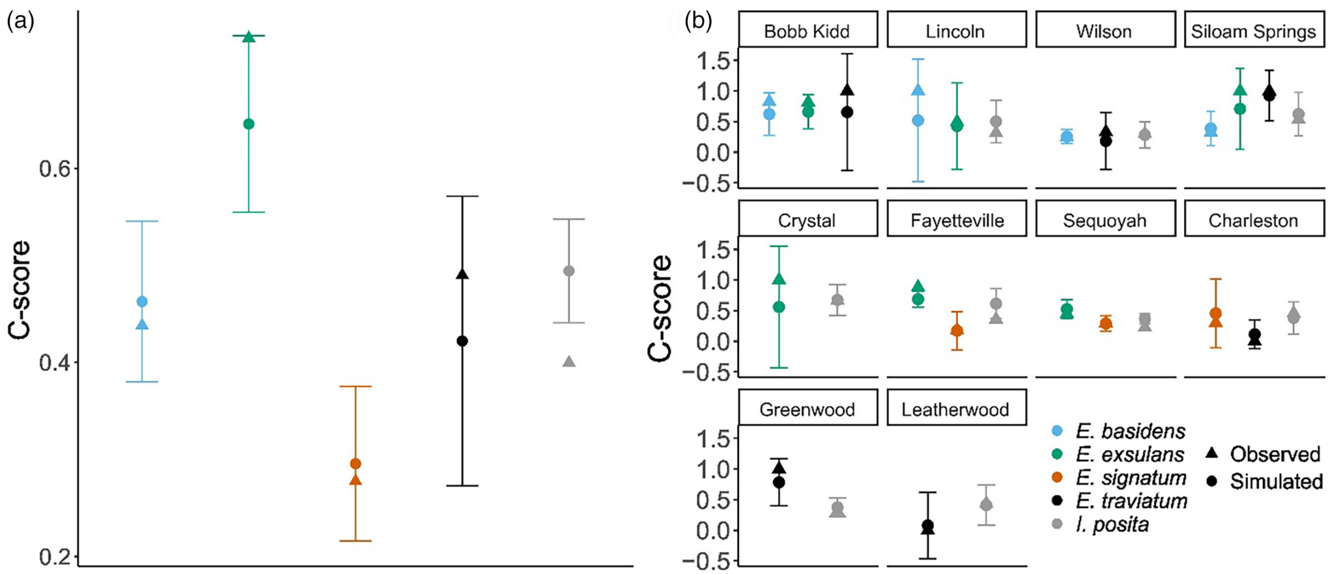

FIGURE 4 Plots of the C-scores from the randomly assembled data sets for each species at the species (a) and population (b) levels. A C-score of 1.0 means the two parasites never co-occurred, a C-score of 0 means they always co-occurred. Circles represent the mean of the simulated data, error bars represent ±2 SD and the triangles represent the observed C-score for each species.

increased with prey density (Figure 5e). *I. posita* was the only host species for which we found evidence for a water mite-mediated indirect relationship between the aquatic environment and gregarine intensity, as the SEM revealed a significant, though weak positive relationship between water mite intensity and gregarine intensity (Figure 5e).

4 | DISCUSSION

Previous work on coinfection has focused on how infection with one parasite facilitates or inhibits infection with a second (Gipson et al., 2019; Hood, 2003; Marchetto & Power, 2018), with limited work investigating how these patterns change across environmental gradients (but see Bolnick et al., 2020). These studies have provided an important foundation for understanding the mechanisms and outcomes of coinfection. Yet a critical step is to investigate how within-host interactions manifest within complex ecological communities. In this study, we developed and implemented a framework for investigating within-host interactions across environmental gradients, in addition to comparing direct and indirect relationships between the local environment and potentially coinfecting parasites. We found that the probability of coinfection varied across environmental gradients, with pH emerging as a consistent predictor. Furthermore, with one exception, we found no support for our hypothesis of indirect, primary parasite-mediated relationships between the local environment and infection by a secondary parasite, as there was little evidence for within-host interactions. Our results suggest that either the direct effect of pH on gregarines or a latent environmental effect on the host itself drives coinfection by water mites and gregarines. The proposed framework is useful for examining within-host interactions across environmental gradients in other host–parasite systems.

Among factors considered here, we found that the local environment is the best predictor of coinfection in this system. Although there was variation in which environmental factors related to which parasite in which host species, one consistent pattern emerged: Coinfection declined as pH increased. Given the observational nature of our study, we do note that there could be other environmental factors correlated with pH that we did not measure, but are actually the key factor(s) shaping parasitism. Surprisingly, our SEMs revealed that this relationship did not manifest through an indirect relationship between pH and gregarine intensity via mediation by water mites. That is, the effect of pH on gregarine infection did not arise because of how pH seemed to affect water mite infection first. Instead, pH had a direct effect on gregarine intensity in the SEMs for all five species. This apparent relationship between the aquatic environment and gregarines was contrary to our predictions because gregarine infection occurs terrestrially. We do not know of a mechanism by which pH could directly affect gregarine intensity; however,

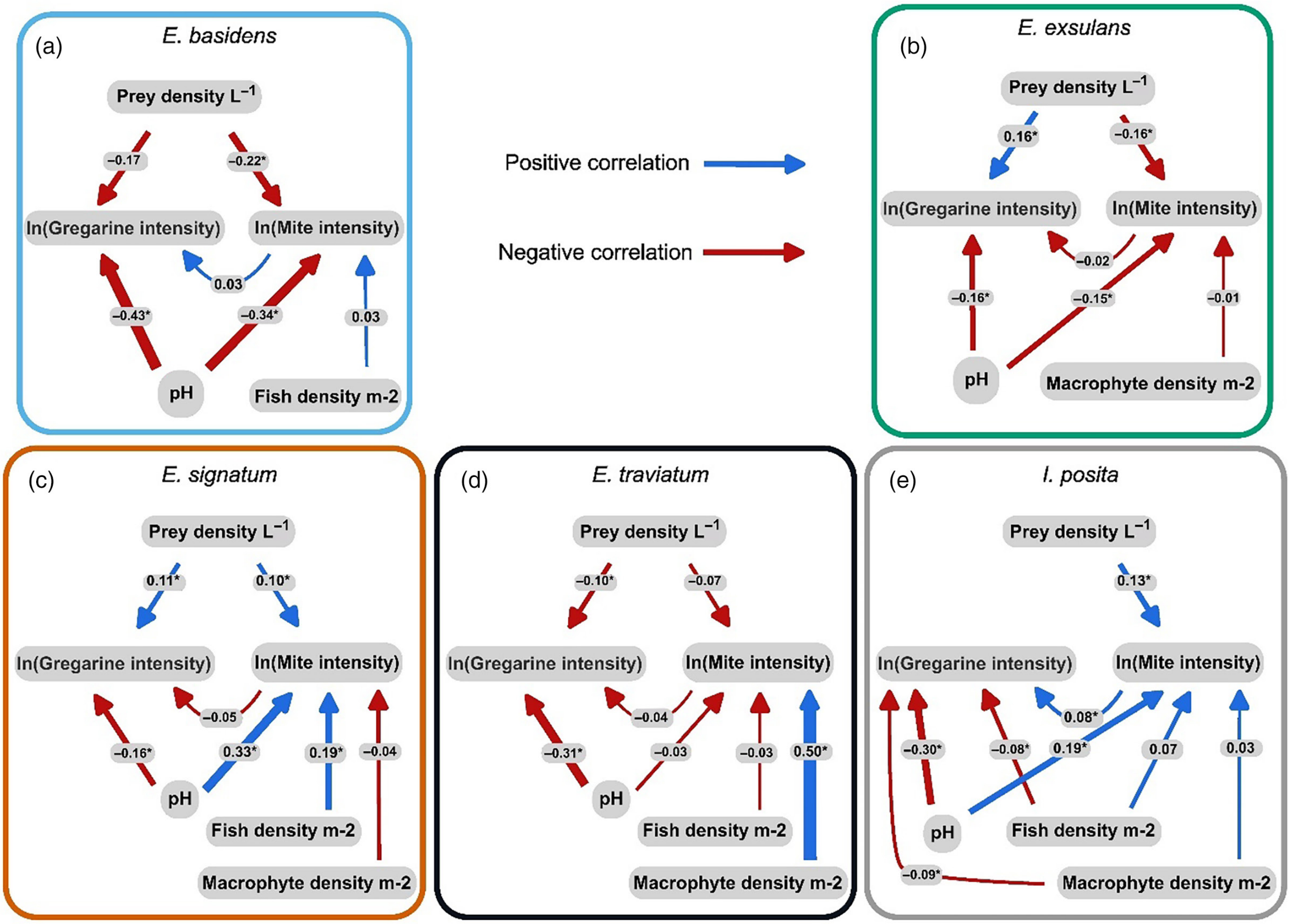

FIGURE 5 SEM plots representing the relationships between environmental factors, mite intensity and gregarine intensity in five damselfly host species. Solid lines represent standardized path coefficients of each predictor on mite intensity or gregarine intensity, which can be interpreted as effect sizes. Blue lines denote positive relationships, while red denotes negative relationships. Significant effect sizes ($p < 0.05$) are denoted with (*), and the thickness of the lines is proportional to the strength of the effects.

there are several indirect pathways that may explain our results. Gregarine oocysts are ingested by odonates when they consume prey items, mainly dipterans that also have an aquatic larval stage (Åbro, 1976; Hasik, Ilvonen, et al., 2023; Locklin & Vodopich, 2010). Thus, one possibility is that the development of these prey items may be impaired in more alkaline water, which would reduce their population size and reduce gregarine infection intensity via an effect on vector abundance. Alternatively, pH may directly affect the host's ability to defend itself from gregarine parasites. This would happen if, for instance, odonate host condition was negatively affected in more acidic water, making them more suitable targets for eventual gregarine infection, though odonates can tolerate low pH (Hudson & Berrill, 1986). Although little is known about the relationships between environmental variables and infection with gregarines at this point, our study has identified a key line of investigation for future research to better understand the ecology of gregarine infection. Gregarines are one of the most common parasites of invertebrates, infecting terrestrial, freshwater and marine hosts (Bollati & Ceballos, 2014; Criado-Fornelio et al., 2016). Given that they not only occur across wildly varying habitats but can also sometimes have beneficial effects on their hosts (Arcila & Meunier, 2020), identifying the environmental factors influencing infection by gregarines and developing experimental tests of their roles will provide needed insight into these ubiquitous parasites.

Local environmental conditions can also affect the transmission of parasites (Lafferty & Kuris, 2005; Morley, 2007; Wolinska & King, 2009). It may be that pH affects either the distribution or viability of gregarine oocysts as suggested by Hupało et al. (2014), which would then manifest as a direct effect of pH on gregarine intensity. pH is already known as a key predictor for infection dynamics in water mites infecting odonate hosts, as both water mite prevalence and intensity increase with pH (Gómez-Llano et al., 2023; Hasik & Siepielski, 2022; LoScerbo et al., 2020), with the proposed mechanism being that low pH environments reduce mite hatching success and survival (Edwards, 2004; Rousch et al., 1997). It is possible that low pH environments increase the transmission efficiency/infectivity of gregarine infective stages or the viability of gregarine oocysts. Indeed, in a study of root-knot nematodes (*Meloidogyne incognita*) and their fungal parasites (*Pochonia chlamydosporia*), Luambano et al. (2014) found that fungal prevalence was highest in low-pH soil and decreased in more alkaline soil. While it was not tested in that study, higher prevalence in low-pH soil may have been due to the low-pH-mediated increase in infectivity seen in other nematophagous fungi (Jaffee & Zasoski, 2007). These findings from prior studies, combined with the results from our study, suggest that abiotic factors are key mediators of infection dynamics for many host–parasite systems, highlighting the need to consider factors beyond the focal host–parasite relationship.

That we found little evidence for within-host interactions between water mites and gregarines is surprising, as within-host interactions between coinfecting parasites explain infection dynamics in many hosts, including rabbits (Lello et al., 2004), salmon (Byrne et al., 2003), field voles (Telfer et al., 2010), African buffalo (Ezenwa et al., 2010) and ants (Hughes & Boomsma, 2004). Identifying what hosts and what parasites are likely to display the patterns of within-host coinfection remains an important issue. Indeed, parasites in general are overdispersed, with most of the parasites being harboured by only a few individuals in a population (Hoverman et al., 2013; Johnson et al., 2015; Locklin & Vodopich, 2010; Rynkiewicz et al., 2019; Woolhouse et al., 1997). However, the ecology and life histories of the two parasites in this study would seemingly promote priority effects. Infection with water mites has been shown to reduce flight ability (Nagel, Zanuttig, & Forbes, 2010), and any such reduction in flight ability may limit the ability of odonate hosts to acquire prey (and infective gregarine oocysts). Alternatively, if the host invests in immune defences to fight off mite infection, it could leave them in a compromised position to defend themselves from gregarines (see Rodgers & Bolnick, 2023 for an example in sticklebacks). This would open the door for prey density to potentially affect gregarine parasitism, as immune defences in this system are food limited (Campero et al., 2008; Hasik, Tye, et al., 2021), providing another avenue by which the local environment can impact coinfection. However, while we did not measure immune defences, our SEMs revealed both positive and negative correlations between prey density and gregarine intensity, so the role of immune defences in gregarine infection in this system is still unclear. Importantly, despite evidence for within-host interactions contributing to coinfection from multiple host–parasite systems, our findings are consistent with other studies finding no support for within-host interactions (with the exception of *I. posita*). In their study of the helminth communities of perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*), Poulin and Valtonen (2002) found little evidence for within-host interactions between coinfecting parasites. Likewise, Nilssen et al. (1998) found no evidence for within-host interactions in the macroparasite community of reindeer (*Rangifer tarandus*). In this system, it may very well be that within-host interactions rarely occur (as in Rynkiewicz et al., 2019), as we only found weak evidence in *I. posita*. It is unclear why *I. posita* exhibits possible within-host infection dynamics, but *Enallagma* does not. *Ischnura* is the sister taxa to *Enallagma*, and so it may be that within host infection dynamics arise more across genera, rather than within genera. Indeed, key ecological differences in resource use and other aspects of the ecology that could mediate infection differ markedly between the genera, not among species (McPeek, 1990a, 1998; Siepielski et al., 2010). Determining under what context within-host interactions among parasites lead to coinfection remains an important goal.

Our study contributes a framework for examining within-host interactions across environmental gradients, but it is important to mention several caveats. First, we were not able to identify either the water mites or gregarines to the species level. While our results do not support the hypothesis of water-mite mediation on gregarine infection intensity, it could be that infection with water mites increases the species richness of gregarines. Indeed, Rodgers and Bolnick (2023) showed that stickleback hosts (*Gasterosteus aculeatus*) infected with helminths (*Schistocephalus solidus*) had a richer community of coinfecting parasites than those not infected by the helminths. Another possibility is that water mites do indeed mediate the relationship between the local environment and gregarines, but on a species-specific basis. We currently lack information on the community structure of both water mites and gregarines infecting odonates, but further work in this area may provide insight into the potential for within-host interactions between these parasites. The dimensionality of our SEMs was limited by the number of host populations included, especially given the various environmental predictors we fit to our models. Our analyses were also limited to comparing coinfection among individuals of five species, and patterns of water-mite mediated coinfection may only emerge at levels beyond the individual host, for example, populations or species (Ilvonen et al., 2018). Such scale-dependent effects are not uncommon (Bolnick et al., 2020). Another limitation of our approach, like many such studies, is the correlational nature of our study. While SEMs are useful for identifying and testing the strength and direction of the various direct and indirect relationships between the local environment and infection by water mites and gregarines, we cannot infer causality.

In conclusion, our study suggests that abiotic and biotic factors, not within-host interactions among coinfecting parasites, can be the key factors determining the patterns of coinfection. Furthermore, our results indicate how important it is to consider the various possibilities presented by such a complex network. Had we not considered within-host interactions and instead simply considered how the probability of coinfection varied across environmental gradients and uncovered the relationship between coinfection and pH we may have erroneously concluded that within-host inhibition/limitation of gregarines by water mites was the likely culprit, due to the known increases in mite parasitism with increases in pH. By utilizing methods that allowed us not only to relate coinfection to the local environment but also to relate infection by one parasite to another, and then connect that relationship to the local environment, we were able to tease apart the intermingled components of this intricate host–parasite network. Host–parasite interactions occur within complex ecological communities, making it crucial to not only account for environmental variation but also investigate the multifarious effects of abiotic and biotic factors. Our proposed framework makes it possible to identify among a set of candidate environmental factors which ones predict coinfection patterns, while also disentangling the direct and indirect pathways between the local environment and within-host interactions between coinfecting parasites. Future research including more host–parasites systems should allow for a more complete understanding of the dynamic and multifaceted nature of host–parasite interactions in nature.

AUTHOR CONTRIBUTIONS

Adam Z. Hasik, Jason T. Bried and Adam M. Siepielski designed the study and collected data. Adam Z. Hasik, Daniel I. Bolnick and Adam M. Siepielski developed the analytical framework. Adam Z. Hasik analysed the data and wrote the initial draft. All authors contributed substantially to revisions.

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CONFLICT OF INTEREST STATEMENT

We declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.4tmpg4fht (Hasik et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1: Map of the study area in NW Arkansas, USA.

Figure S2: Coinfection patterns for the five most common damselfly host species.

Figure S3: Predicted probability of infection by water mites, gregarines, and coinfection among damselfly hosts in relation to fish density.

Figure S4: Predicted probability of infection by water mites, gregarines, and coinfection among damselfly hosts in relation to prey density.

Figure S5: Predicted probability of infection by water mites, gregarines, and coinfection among damselfly hosts in relation to macrophyte density.

Table S1: Summary information from the 2017 and 2019 parasitism surveys for all of the damselfly species at the 13 lakes in northwest Arkansas, USA.

Table S2: Summary information on species-level mean parasite prevalence and median parasite infection intensity.

Table S3: Results from the logistic regression model of mite infection among species.

Table S4: Results from the logistic regression model of gregarine infection among species.

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