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The effect of gregarine (Apicomplexa) colonisation on the functional response of the amphipod host

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ABSTRACT

Gregarines are a notably understudied but widespread group of protists that colonise aquatic and terrestrial invertebrates. This limited understanding of gregarines and their interactions with their hosts results partly from the absence of established culturing techniques and our understanding therefore has heavily relied on field collections. This study utilised for the first time cultured *Gammarus pulex* populations and comparative functional response models to explore the effects of gregarine colonisation on the host's consumption of Chironomid prey. This study shows that both positive and negative *G. pulex* displayed a Type II functional response. There were no statistical differences in the functional response parameters between the two groups. These results suggest that, under the study conditions, gregarines may function as commensal symbionts within their *G. pulex* host. This is consistent with growing evidence for gregarines acting across a range of symbiotic roles within their hosts. These findings provide insight into the role of gregarines in *G. pulex*, an invertebrate species frequently used for field- and lab-based experiments, contributing to the evidence of the complex and varied gregarine host-symbiont interactions.

1. Introduction

Species of the genus *Gammarus* (Amphipoda, Crustacea) can be hosts of many symbiotic/parasitic species, often as intermediate hosts (Bojko and Ovcharenko, 2019). The genus *Gammarus* is distributed throughout the Holarctic region and inhabits a range of aquatic habitats, including fresh, brackish, marine, and subterranean environments (Karaman and Pinkster, 1977; Barnard and Barnard, 1983). *Gammarus* spp. can exist in large quantities and can make up considerable portions of the biomass in a habitat (MacNeil et al., 1997). They are mainly detritivores, often acting as shredders but are omnivorous and can consume fresh plant matter, as well as animal prey (MacNeil et al., 1997). Furthermore, as a prey species for a variety of predators, this group of animals plays a prominent role in nutrient transfer within a food web (e.g. Costa et al., 2009). *Gammarus* species are also common model organisms used in aquatic experimental studies, both laboratory- and field-based. *Gammarus* spp. have been used extensively to assess biological differences such as abundance or feeding behaviour between native and analogous non-native amphipod species (e.g. Bollache et al., 2008; Dodd, et al., 2014; Pander et al., 2022) as well as for ecotoxicological studies (van den Heuvel-Greve et al., 2024; Porseryd et al., 2024; Rollin et al., 2023).

The parasite fauna of *Gammarus* spp. has been relatively well documented and includes representation from the acanthocephalans, microsporidians, trematodes, fungi, and protists (Thomas et al., 2000; Kelly et al., 2003; Helluy and Thomas, 2010; Gismondi et al., 2012; Fanton et al., 2020; Bącela-Spychalska et al., 2023; Prati et al., 2023, 2024). Among these, acanthocephalans, such as *Pomphorhynchus laevis* and *Polymorphus minutus*, are especially well-studied due to their ability to manipulate host behaviour (Gismondi et al., 2012; Fanton et al., 2020). Infected *Gammarus* spp. often exhibit altered phototactic or geotactic responses, which increase their susceptibility to predation by definitive hosts,

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typically birds or fish (Fanton et al., 2020). *Gammarus pulex* infected with *P. laevis* cover a greater distance and are less photophobic and more attracted to the water surface than uninfected amphipods (Fanton et al., 2020). This manipulation facilitates the parasite's transmission, making it a prime example of parasitic influence on *G. pulex* behaviour. Similarly, trematodes, which use *Gammarus* spp. as intermediate hosts, have been examined for their life cycles and their impacts on host physiology and behaviour (Thomas et al., 2000; Helluy and Thomas, 2010; Díaz-Morales et al., 2023). Microsporidians, including genera like *Nosema*, *Microsporidium*, *Dictyocoela* are a group of parasitic fungi frequently studied in *Gammarus* populations (Kelly et al., 2003; Bącela-Spychalska et al., 2023; Prati et al., 2023). These intracellular parasites can cause significant fitness reductions, influence host population dynamics and behaviour (Kelly et al., 2003; Prati et al., 2023).

Gregarines are smaller cryptic symbionts, a subgroup of the apicomplexans, a large and diverse phylum of parasitic protists. Some apicomplexans (e.g. *Plasmodium* spp., *Toxoplasma* sp.) have gained notoriety for their global disease impact (Striepen et al., 2007). Other groups are not yet well understood, particularly the gregarines, despite being a hugely diverse and evolutionarily specialised group (Leander, 2008). The impact on their hosts is under-explored and huge numbers of potential hosts are yet to be identified (Rueckert et al., 2019). Gregarines colonise invertebrates in nearly all groups across aquatic and terrestrial systems, residing in the intestine, gut, coelom, tissues and reproductive vesicles (Leander, 2008). Gregarine survival likely depends not only on intrinsic genetic and metabolic capacities, but also on the nutritional and microbial environment provided by the host environment. Gregarine trophozoites, which occupy extracellular niches in the host, are particularly dependent on their surrounding milieu. Surface pores in the gregarine pellicle, are believed to play roles in secretion and absorption, allowing the organism to exchange metabolites and nutrients essential for its survival (Warner, 1968). Gregarines have been observed to colonise the gut of *Gammarus* spp. including several eugregarine species such as *Cephaloidophora gammari*, *C. maculata*, *C. talitri*, *C. conus* and *Heliospora longissima* (Desportes and Schrével, 2013; McKinley et al., 2024).

Gregarine colonisations in other invertebrates have previously been demonstrated to affect host feeding behaviour. For example, colonisation by the gregarine *Gregarina cochlearium* reduces consumption index in male host beetles while infected individuals also display slower growth rate (Barber et al., 2025). In a social wasp (*Polybia occidentalis*) gregarine infection reduced the time spent foraging for nectar and prey (Bouwma et al., 2005). Therefore, investigating the potential effects that gregarines have on their gammarid hosts is important to better understand and interpret outcomes of experimental studies (e.g. ecotoxicological studies) utilizing *Gammarus* spp., as often studies only check for visible parasite presence and not for cryptic species (Agatz and Brown, 2014; Dodd et al., 2014) which limits the groups of parasites that have been screened.

Whilst apicomplexans have previously been viewed as exclusively parasitic, there is evidence of variation in relationships between gregarines and their hosts. They appear to exist across the symbiotic spectrum, with mutualistic, commensalistic and parasitic interactions having been observed across several different host species (Rueckert et al., 2019). Due to the high level of host specificity of gregarines, the position of a gregarine species on the symbiotic spectrum is highly varied and species or even circumstance-specific (Rueckert et al., 2019).

There are different ways to study the potential influence of a gregarine colonisation, such as the functional response which describes the relationship between food or resource availability and the consumption rate of that resource (Juliano, 2001), measured by calculating the number of prey consumed as a function of the density of prey supplied (Holling, 1966). The relationship between prey availability and prey consumption plays a crucial role in understanding predator–prey interactions. It can provide a measure of a species' ability to utilise resources, allowing the ecological impact of stressors on a predator or the feeding efficiency of different predators to be compared (Jeschke et al., 2002; Dick et al., 2013). Functional response curves provide a phenomenological estimation of the attack rate, handling time of prey and the maximum feeding capacity (Juliano, 2001).

Functional responses are typically categorised into Type I, Type II and Type III. Type I responses are characterised by a linearly increasing prey consumption rate (Pritchard et al., 2017). Type II responses show a decrease in consumption as prey density increases (Juliano, 2001). Type III responses exhibit an initial increase in consumption with increased density, followed by a reduction in the attack rate due to predator interference, prey switching, or through refugia provided by the environment (Barrios-O'Neill et al., 2015). These response types are not always fixed and are sensitive to changes in the environment. It is also important to note that functional responses provide phenomenological-based estimates only and caution should be taken when drawing conclusions. Despite this, they can provide useful estimates for comparing groups or populations (Jeschke and Hohberg, 2008). For example, functional response studies have compared invasive and native species to elucidate differences between species (e.g. Dodd et al. 2014, Alexander et al., 2014) and between different environmental contexts (e.g. Paterson et al., 2015; Barrios-O'Neill et al., 2015). However, very few applications of these models have been used to investigate the effects of parasitism. Parasite infections have been demonstrated to affect functional response curves; for example, a reduced attack rate and maximum consumption were observed in the crab species (*Eurypanopeus depressus*) (Toscano et al., 2014). In addition, functional response curves have been used as a tool to investigate the effect of acanthocephalan parasite in *G. pulex*, revealing increases in attack rates and maximum feeding rates (Dick et al., 2010).

This study aims to investigate the impact of gregarine colonisation by comparing functional response models of gregarine-positive and gregarine-negative *G. pulex*. It is hypothesised that there will be differences in the functional response parameters linked with the colonisation status. Gregarine presence was determined pre- and post-experiment and applied to models to investigate effects of parasite/symbiont presence on predatory response, which has not been frequently explored (Dick et al., 2010; Haddaway et al., 2012; Toscano et al., 2014). This provides another facet to our insight to potential consequences of gregarine infections with its potential to alter prey consumption.

2. Materials and methods

2.1. Sample collection

This study utilised *Gammarus pulex* specimens collected from the Water of Leith river in Scotland (55.896010, −3.307785) in January 2022, employing a standard kick sampling technique (Cheshmedjiev et al., 2011). Sample site collection was based on personal experience recently published in McKinley et al. (2024) who identified three gregarine species (*Cephaloidophora gammari,* C. *conus* and *Heliospora longissima*) in the gut of *G. pulex* from the Water of Leith (Fig. 1B–D).

2.2. Aquarium set-up

The collected specimens were transported back to the laboratory at Edinburgh Napier University, where they were transferred

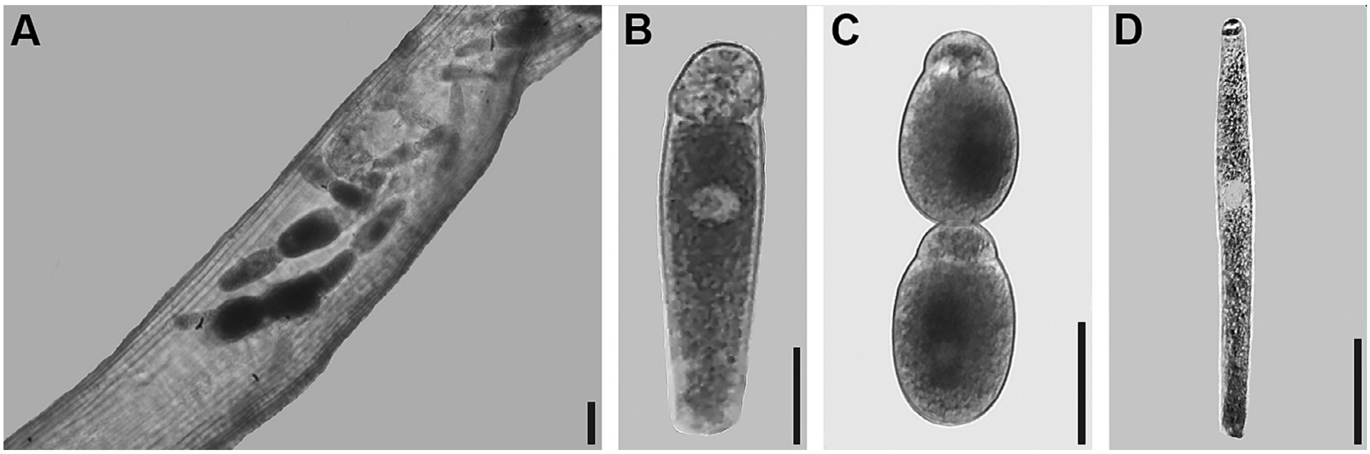

Fig. 1. Light micrographs of gregarine trophozoites colonising *G. pulex*. (A) Gregarine trophozoites visible in extracted *G. pulex* gut. (B) *Cephaloidophora conus* (C) An association of two *Cephaloidophora gammari* trophozoites paired up in caudo-frontal syzygy. (D) *Heliospora longissima* trophozoite. Background was cleaned in Adobe Photoshop for better visualization. Scale bars (A) 30 µm, (B) 20 µm, (C), (D) 25 µm.

to aquaria. These specimens formed the gregarine-positive population used in this study. The gregarine-negative population was created using the protocol to establish gregarine-negative *Gammarus pulex* lab cultures (see Supplementary Material). Aquaria (5L) were filled with synthetic freshwater (2.4 g of magnesium sulphate, 3.84 g of sodium bicarbonate, 0.16 g of potassium chloride, and 2.4 g of calcium sulphate dihydrate dissolved in 20L of purified water; process adapted from EPA (2002), hereafter fresh water). Aquaria were maintained in a temperature-controlled room at 18̊C and fresh water oxygen saturation maintained at high levels through aeration. PVCU pipes (5 cm in length, ¾") were provided as refugia to enrich the environment. Thirty individual wild-caught *G. pulex* were housed in 5L of fresh water. *Gammarus pulex* were fed a diet consisting of organic lettuce leaves washed with fresh water.

2.3. Pre-experiment determination of colonisation status

Individual *G. pulex* (male, 10–15 mm length, rostrum to final urosome segment) were placed in 250 mL glass beakers filled with continually aerated fresh water (200 mL), leaf material (food) and a 5 cm piece of plastic pipe (refugia). The protocol, as described above, was employed, using visual checks for cysts to identify colonisation status. Faecal matter was collected and checked for gregarine cysts every 48–72 h over a 14-day period, to pre-determine the colonisation status of each *G. pulex*. After screening, gregarine-negative and gregarine-positive *G. pulex* were housed in separate 5L-aquaria.

2.4. Experimental set-up

Individuals of *G. pulex* (10 gregarine-negative and 10 gregarine-positive) were selected randomly from the two experimental populations and placed separately in an experimental arena (a 200 mL glass beaker filled with 100 mL of fresh water and a small plastic tube). Individuals were starved for 24 h to standardise hunger levels and check their health status. Ten densities of live chironomids were selected (1, 2, 4, 6, 8, 10, 16, 20, 30, and 40) based on the results of a range-finding trial to determine prey consumption levels, ensuring that functional response curves could reach asymptote. Chironomids at each density were added to the experimental arenas with a *G. pulex* present. Chironomids were sourced from a cultured stock from Aquatic Live Fish Foods (UK supplier). This was repeated for both populations until a gregarine-negative and a gregarine-positive *G. pulex* were presented with each of the ten densities. Experiments ran for 24 h, starting at 14:00 and stopping at 14:00 on the following day, with the removal of *G. pulex*. Each *G. pulex* specimen was used once and provided with only one prey density, with a new individual used in each replicate. The experiment was repeated three times, and each replicate included a control group of 10 chironomids with no *G. pulex* present. Following the removal of *G. pulex*, the number of chironomids consumed was calculated as the number that had been completely consumed or were dead with evidence of attack damage. Each *G. pulex* removed from the arena was placed into an individual 1.5 mL microfuge tube containing 80 % ethanol and labelled. The length of each *G. pulex* was measured, and the mass was recorded in milligrams (mg) of dry mass (air drying following submersion in ethanol).

2.5. Post-experiment determination of colonisation status

Post-experimentation colonisation status was confirmed through dissection. The intestinal tract was removed and macerated. Macerated material was then processed for parasites under an inverted microscope. The ultimate colonisation status of *G. pulex* was assigned based on this assessment. If dissection revealed a different colonisation status from that determined by faecal inspection, the result was removed from the dataset. In this case, the experiment was repeated for the affected combination of chironomid density and gregarine colonisation status, to ensure a complete data set.

2.6. Statistical analysis

To investigate prey consumption differences between *G. pulex* with two colonisation statuses, linear and non-linear approaches were undertaken. Linear relationships between the number of chironomids consumed (as a proportion of those supplied) and the colonisation status (factor, 2 levels), supplied prey density (factor, 10 levels), and individual size (length, continuous) were analysed using a general linear model with binomial error distribution. The maximal model included a three-way interaction between colonisation status, supplied prey density and length. Interactions provide insight to non-additive relationships in prey consumption between the two colonisation status groups and the size of the individuals. Model selection was undertaken through the backward removal of non-significant terms. Difference in model significance was undertaken by comparing AIC values of candidate model pairs; models within 2 AIC units were considered statistically indistinguishable. Non-linear relationships were investigated via functional response analysis using the *frair* package (Pritchard et al., 2017). The Type (I, II, III) of functional response was determined for the different colonisation status' using a logistic regression. As the prey were not replaced during the feeding challenges, functional responses were modelled using the Rogers II ('random predator') equation (Pritchard, 2014). Functional response curves allow phenomenological attack rate, handling time and maximum consumption rate parameters to be estimated using maximum likelihood estimation (MLE) via the *bbmle* package (Bolker, 2008), which implements the model using the Lambert W function (Pritchard et al., 2017). To investigate the reliability of identifying colonisation status of live *G. pulex* by examining faecal material, a two-by-two contingency table analysis (Fisher's exact test) was undertaken. All statistical analyses were undertaken in R (R Core Team, 2024).

3. Results

3.1. Prey consumption experiment

In total, 63 *G. pulex* were processed throughout the experiment. Experimental *G. pulex* had a mean length of 13.46 mm (±1.175SD; negative = 13.71 mm ± 1.115SD, positive = 13.17 mm ± 1.197SD) and a mean mass of 0.039 mg (±0.0062SD; negative = 0.040 m g ± 0.00629SD, positive = 0.038 mg ± 0.00613SD). There was no significant statistical difference between the individuals in each colonisation status group for length ($F_{(1,61)}$ = 3.36, p = 0.072; Supplementary Fig. S1a) or mass ($F_{(1,61)}$ = 1.47, p = 0.229; Supplementary Fig. S1b).

The minimum length recorded was 11 mm and the maximum was 16 mm, the minimum mass was 0.023 mg and the maximum was 0.05 mg. An ANOVA of mass regressed on length with an interaction term for colonisation status revealed no statistical difference in the length-to-mass relationship for the gregarine-negative and gregarine-positive groups, either as a difference in the relationship (interaction p = 0.354) or as an additive effect (p = 0.726).

A comparison of the initial assignment of colonisation status (based on faecal matter examination) with ultimate colonisation status (intestinal dissection) revealed a high success rate (86.2 % successful assignment for negative and 96.6 % success for positive; Supplementary Fig. S2). There was no statistical difference (Fisher's exact test, p = 0.363) in the likelihood of incorrect identification of colonisation status, meaning there was no bias in miss-identifying an individual as gregarine-negative or gregarine-positive.

3.2. Linear patterns of prey consumption

The proportion of prey consumed showed a weak relationship with the colonisation status of the individual. The final model (Table 1) included an interaction between colonisation status and prey supplied, and length which has a residual deviance of $81.937_{(42)}$ which provided a statistically significant description of the relationships in the data (null deviance = $206.742_{(62)}$; χ^2 test on deviance difference $124.81_{(20)}$, $p < 0.001$; AIC_{NULL} = 338.22, AIC_{final} = 253.42; Table 1). Specifically, the interaction between colonisation status and supplied prey density was weak (p = 0.0015) and post-hoc testing showed that this interaction was driven by statistical differences between colonisation status and supplied prey density at eight prey and 16 prey (Fig. 2). Gregarine-negative individuals were consuming a greater proportion of prey when prey density was 8 and gregarine-positive individuals were consuming a greater proportion of prey when the supplied prey density was 16. The inclusion of the length of the individual improved model fit but did not show statistical significance (p = 0.103).

3.3. Non-linear patterns of prey consumption – functional response

A significant negative regression coefficient in the relationship between the proportion of chironomids consumed and the density of chironomids supplied for both gregarine-negative (coefficient(SE) = −0.04 (±0.01); z-value = −5.047, $p < 0.001$) and gregarine-positive (coefficient(SE) = −0.04 (±0.01); z-value = −4.473, $p < 0.001$) *G. pulex* indicated the presence of a Type II functional response in both cases. There was no statistical difference in the number of chironomids consumed between gregarine-positive and gregarine-negative *G. pulex* (Mann-Witney U; W = 467.5, p = 0.729). Bootstrapped estimates (95 % CI) of attack rate (negative: 0.443–3.938; positive: 0.525–1.676) and handling time (negative: 0.035–0.211; positive: 0.059–0.142) showed a high degree of overlap in both the estimated coefficients, indicating little statistical difference (Fig. 3). There was a greater variability in the response of gregarine-negative *G. pulex* highlighted by larger ranges of both attack rate and handling time.

4. Discussion

We were able to investigate the potential onward impacts of cryptic parasitism on a commonly used experimental approach: comparative functional responses. The utilisation of comparative functional response models to determine effect of symbiotic relationships on the host is not yet a widespread method (Dick et al., 2010). However, these models can act as valuable tools for determining relative resource acquisition ability and consumption. They have notable importance for revealing individual survival and estimating wider ecological effects, as functional response types are major determinants of predator–prey dynamics and population stability (Toscano and Griffen, 2014).

The gregarine-positive and gregarine-negative *G. pulex* groups both exhibited Type II functional responses, the most observed type of response of non-filter feeders (Jeschke et al., 2021). This is consistent with other studies of *G. pulex* (Dick et al., 2010; Dodd et al., 2014; Médoc et al., 2015). Type II responses are characterised by a rising monotonic curve that gradually flattens as prey density increases, as the host spends an increasing amount of time capturing, handling and consuming prey, without changing foraging tactics (Jeschke et al., 2021). However, as this was an in vitro study, several variable factors were removed, which may influence the functional response type, such as additional stress imposed by competition and habitat complexity (Toscano and Griffen, 2013), along with different prey species (Haddaway et al., 2012; Dodd et al., 2014) or large prey species (Kalinkat et al., 2013; Kreuzinger-Janik et al., 2019). The *G. pulex* were also maintained under controlled conditions with plentiful food material for several weeks prior to the commencement of the feeding trials, which may have enhanced their health and longevity beyond that of wild populations. Despite this, the presence of a gregarine symbiont colonisation did not cause the functional response to be altered, thus it can be inferred that they are unlikely to be putting significant physiological stress on the *G. pulex* host.

Comparison of attack rate, handling time and maximum feeding rate using functional response parameters revealed no differences in the mean value between gregarine-positive and gregarine-negative *G. pulex*. Notably, these findings contrast with the effects caused by presence of other parasites of *G. pulex*. For example, Dianne et al. (2014) found that G. *pulex* infected with the

Table 1
Summary of deviance explained by the maximal and final models (null deviance for both models is 206.742). Interaction terms are denoted with a ":".

Model	Variable	Deviance	df, residual df	*p*-value
MAXIMAL MODEL: Proportion of Prey Consumed ~ Colonisation Status * Prey Supplied * Length				
	Model AIC = 263.6			
	Colonisation Status	0.368	1, 61	0.544
	Prey Supplied	95.033	9, 52	<0.0001
	Length	2.658	1, 51	0.10301
	Colonisation Status: Prey Supplied	26.746	9, 42	0.00154
	Colonisation Status: Length	0.320	1, 41	0.57131
	Prey Supplied: Length	11.199	9, 32	0.26232
	Colonisation Status: Prey Supplied: Length	14.343	8, 24	0.07325
FINAL MODEL: Proportion of Prey Consumed ~ Colonisation Status * Prey Supplied + Length				
	Model AIC = 253.4			
	Colonisation Status	0.368	1, 61	0.544
	Prey Supplied	95.033	9, 52	<0.0001
	Length	2.658	1, 51	0.10301
	Colonisation Status: Prey Supplied	26.746	9, 42	0.00154

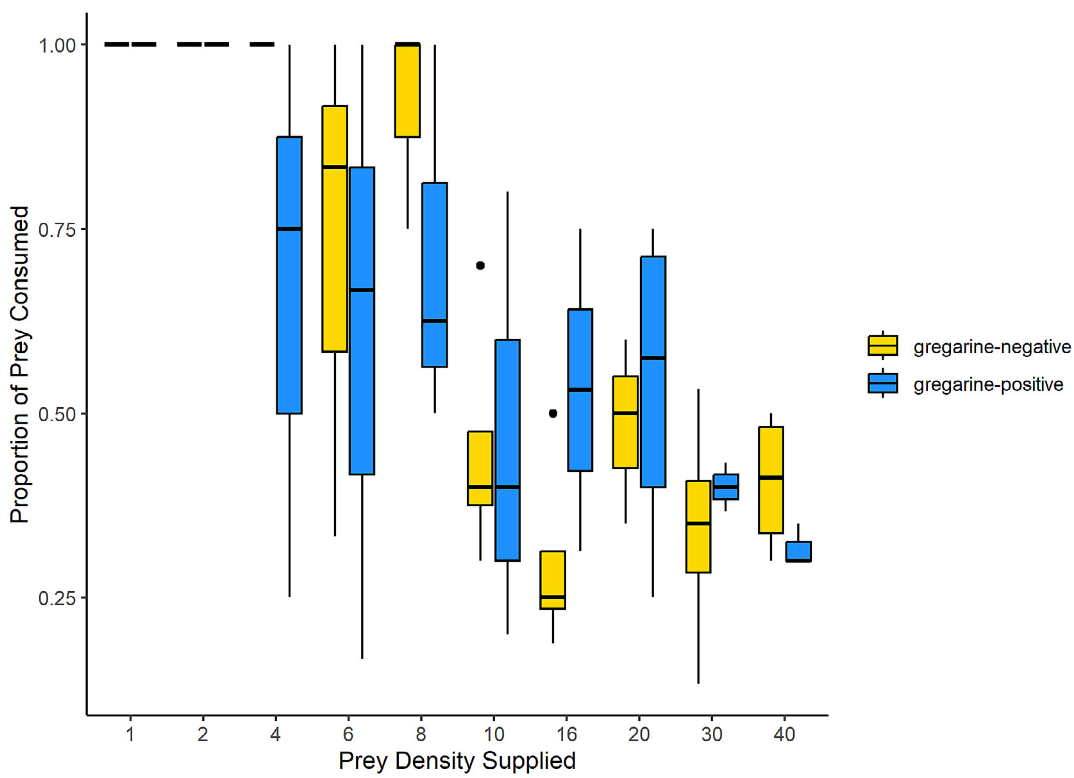

Fig. 2. Relationship between proportion of prey consumed and prey density supplied and colonisation status.

acanthocephalan parasite (*Pomphorhynchus laevis*) negatively affected foraging and food intake at low densities of food. Using comparative functional responses, Dick et al. (2010) found that the acanthocephalan parasite, *Echinorhynchus truttae*, increased attack rate and maximum feeding rate in *G. pulex*. This confirms that analysis of feeding rates and the use of functional response models can detect differences in parasitised and unparasitised hosts, however, in this study none were detected.

Functional response models have not previously been applied to detect gregarine impact on hosts; however, gregarine colonisations have been found to have a range of impacts on their host's feeding capacity. Johny et al. (2000) identified reduced food consumption rates in gregarine-positive tobacco grasshoppers (*Atractomorpha crenulata*) in comparison to the gregarine-negative group. On the other hand, a study of a millipede species (*Rossiulus kessleri*) found no significant impact of a gregarine colonisation on food consumption (Brygadyrenko and Svyrydchenko, 2015). It appears that the effect gregarines have on food consumption of the host, alongside body size traits, is highly dependent on several factors, which may include gregarine species, host species and food type or availability.

The application of functional response models for investigating the effects of parasite infection is useful as a phenomenological approach to provide comparable estimates of the parameters across distinct groups. It has the potential to be used more widely as a comparative tool due to its relative simplicity and accessibility. Some caution should be taken in interpretation, however. There may be variation in the response types and feeding parameters depending on the prey type provided (Dodd et al., 2014). Furthermore, as highlighted by Toscano et al. (2014), maximum feeding rate is directly related to handling time and assumes continuous foraging. Foraging or activity levels were not observed

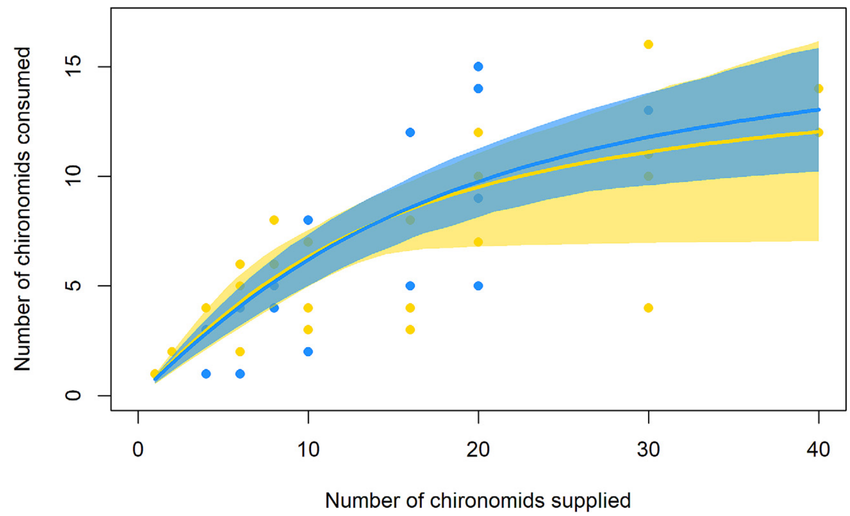

Fig. 3. Type II Functional Response curves for gregarine-negative (yellow) and gregarine-positive (blue) *G. pulex*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

directly in this study, so variation in some behaviours remain unknown. However, as no differences were detected in any of the parameters (attack rate, handling time or maximum feeding rate), it is unlikely that the consequences of these were notably different. It may be beneficial for future work to observe handling time directly between groups, allowing mechanistic conclusions to be drawn. This also allows for the identification of any differences in activity levels, as behavioural traits, such as distance travelled by the host and social interactions, are not included in functional response analysis. For example, Bojko et al. (2019) found no significant differences in activity levels (travelling around the experimental arena) in gregarine-positive and gregarine-negative gammarids (*Dikerogammarus haemobaphes*), but they were not tested in isolation from other gammarid parasite infections. However, Dick et al. (2010) noted acanthocephalan parasite infected *G. pulex* displayed increased refuge use compared to uninfected *G. pulex*. This is important as these differences may have further impacts on survival rates or reproductive success.

As no detectable mean effects were found in this study, it could be suggested that gregarines do not have an impact on *G. pulex* in the context of this study and may, in fact, be existing as commensal symbionts. This is surprising as gregarines can exist in sizes and quantities that appear to fill or block the midgut of the host (Fig. 1A), which would suggest altered feeding or food processing ability (Klingenberg et al., 1997). Combining the evidence for no difference in feeding parameters and no difference in the host body size traits measured in this study, it appears gregarines are not causing the typical symptoms observed in hosts infected by parasitic species. These include reduced predation due to the energy cost of parasite presence (Dick et al., 2010) or increased feeding due to increased energy demands (Fielding et al., 2003), which have both been identified to occur in *G. pulex* infected with an acanthocephalan parasite. However, while our study distinguished between *G. pulex* individuals colonized or not colonized by gregarines, we did not quantify gregarine load or identify the specific gregarine species within individuals following the functional response assays. In a recent study by the authors, McKinley et al. (2024), determined that *H. longissima* was the most prevalent (~40 %) gregarine species infecting *G. pulex* in the Water of Leith, Scotland in January 2022. *Cephaloidophora gammari* and *C. conus* both reached around 20 %. We expect that we encountered similar proportions of infection in the current study, as samples were taken at the same time. Variation in symbiont abundance could influence host feeding rates, potentially contributing to the greater variability observed among colonized amphipods. Likewise, the three gregarine species detected in the guts of *G. pulex* may differ in their interactions with the host, ranging from commensal to more parasitic, and such species-specific effects could also underlie the observed variation in feeding responses. Future work incorporating quantitative estimates of gregarine load and species-level identifications within functional response experiments would be valuable to refine our understanding of how gregarines affect the host.

Commensal gregarine-host relationships have been implied in several studies. Research by Wróblewski et al. (2020) identified gregarine species in *G. pulex* consistent with those identified in this study (*Heliospora longissima* and *Cephaloidophora gammari*). The authors determined that gregarine infections may cause decreased *G. pulex* survival in autumn but reproduction rates in spring and summer potentially negate these effects. It has been suggested that gregarines may normally be non-detrimental in most hosts due to their notably widespread existence (Yaman and Baki, 2010). This is supported by a high prevalence of gregarine colonisation within *G. pulex* populations. McKinley et al. (2024) showed that gregarine prevalences were highest in autumn and spring, reaching almost 50 % in *G. pulex* in the Water of Leith in Scotland. The present study identified a 1:2 colonisation rate and this is comparable with a study of gregarine presence in *G. pulex* from the Baltic region, although this has been found to vary seasonally (Wróblewski et al., 2020). Studies of other gammarid species such as *Gammarus fasciatus* have demonstrated rates of gregarine colonisation of up to 78 % of the hosts examined (Grunberg and Sukhdeo, 2017). With the prevalence of gammarids within many habitats and these proven high rates of gregarine colonisation, it would be unlikely that gregarines cause serious harm.

Some studies have only identified detrimental effects of gregarines appearing under stressors such as food limitation. For example, gregarine-positive field crickets (*Gryllus pennsylvanicus*) only displayed increased weight loss and reduced survival when kept on sub-optimal diets (Zuk, 1987). In some cases, stressors triggered a potential form of mutualism between gregarines and their host. For instance, high gregarine (*Gregarina ovata*) presence had an apparent positive effect on European earwig survival during food shortages (Arcila and Meunier, 2020). Similarly, in a species of damselfly (*Enallagma boreale*), a high gregarine load enhanced survival under food stress (Hecker et al., 2002). This was suggested to be due to nutritional benefits or improved immune systems. For this reason, further work investigating gregarines in *G. pulex* applying functional response methods may benefit from manipulating starvation periods or reducing densities of prey to increase food stress, or exposure to other additional stressors such as increasing temperature or salinity (common under global change developments).

It should also be noted that only males were used in this study, to remove sex-specific bias such as increased energy output of reproductive traits. This may, however, impact conclusions as gregarine infections have been demonstrated to have differential effects on the sexes. It has been found that under food stress, female field crickets (*Teleogryllus oceanicus*) had less immune response to a gregarine infection than males, presumably due to energy resource allocation (Zuk et al., 2004). Comparing the functional responses of male and female *G. pulex* colonised by gregarines may be beneficial to highlight any sex-specific effects.

With such varied findings across different invertebrate hosts, this family of symbionts exists on a spectrum of symbiotic roles (Rueckert et al., 2019). The relationships are complex, however, and in the case of commensal symbiosis, it is challenging to differentiate between total absence of any impacts and some form of resistance to the effects from the host. Thus, further work should investigate different stressors on the host, such as food limitation, as well as female *G. pulex* to identify sex-specific effects in order to identify the origins of the parasitic, commensal and mutualistic behaviours exhibited.

The utilisation of any organism under experimentation, where the goal is to describe potential patterns in the real world or to test e.g. the effects of pollutants, depends on our ability to account for as much random variation as possible through good experimental design. Understanding where sources of random variation may arise, is therefore, a key component of the experimental process. Grabner and Sures (2019) and Grabner et al. (2023) have shown, for example, that parasites can change the response of typical environmental toxicological test organisms, influencing the test results, but did not consider any protists. Similarly, Nahar et al. (2025) focused on the effects of acanthocephalans and microsporidia on the stress response of *Gammarus fossarum* when exposed to herbicides. Herein, we have established a screening protocol for cryptic parasites in experimental animals, as well as a protocol to establish positive and negative laboratory host cultures for one of these groups, the gregarine apicomplexans.

Gregarine colonisations may present significant implications for the health and ecology of aquatic organisms (Zakariah et al., 2019; Rueckert et al., 2019). Some gregarines have been shown to impact their hosts' nutrition, behaviour, and fecundity (Arcila and Meunier, 2020). Our established protocols for *G. pulex* could be used (potentially also with other amphipod species) to determine, under controlled conditions, if gregarine colonisations can influence not only *G. pulex* predation, behaviour, and competition dynamics, but also stress responses when exposed to multiple stressors, and whether these could potentially further imply impacts on community and ecosystem level. Understanding the dynamics of gregarine colonisations in these hosts requires robust culturing and identification techniques. These techniques are pivotal not only for advancing scientific knowledge, but also for managing ecological impacts and developing potential biocontrol measures.

5. Conclusion

This study provides insight into the role gregarine symbionts play within their host by applying comparative functional response models to compare gregarine-positive and gregarine-negative *G. pulex*. No significant differences in body size traits (body length and dry weight) were detected, and no significant differences across the feeding parameters highlighted by functional response analysis (attack rate, handling time or maximum feeding rate). This is suggestive of a commensal relationship between the gregarine parasite and the *G. pulex* host under the conditions of this study. While no differences were observed between the two experimental groups, there was a much greater (almost double) variation in response parameters described for the gregarine-negative group, highlighting that while no statistical differences were identified in the mean values, variation in response was different. This is an important step in understanding the relationship between gregarines and their *G. pulex* host. Furthermore, it adds to the growing evidence of varied and complex interactions that gregarines appear to display.

CRediT authorship contribution statement

Heather J. Knight: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Kevin O. McKinley:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Anastasios D. Tsaousis:** Writing – review & editing, Supervision, Funding acquisition. **Jennifer A. Dodd:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Sonja Rückert:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.10.002.

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