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Muddy waters: efficacious predation of container-breeding mosquitoes by a newly-described calanoid copepod across differential water clarities

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Abstract

Mosquito-borne diseases induce unrivalled morbidity and mortality in human populations. In recent times, greater urbanisation has facilitated vector population expansion, particularly of those which proliferate in container-style habitats. To combat increased associated disease risk, we urgently require innovative and efficacious control mechanisms to be identified and implemented. Predatory biological control of vectorially-important mosquitoes can be effective. Despite their high prevalence in freshwater ecosystems, predatory calanoid copepods have yet to be examined comprehensively for mosquito control. Moreover, environmental context-dependencies can cause substantial variations in natural enemy impacts on target populations. Accordingly, improved understanding of the effects of context-dependencies upon predatory biocontrol is needed. Here, we use functional responses (FRs) to examine the predatory impact of a newly-described species of calanoid copepod, *Lovenula raynerae*, upon larval *Culex pipiens* prey across variations in prey supply and water clarity. Using outdoor field trials, we assess the viability of *L. raynerae* in reducing mosquito survival in container-style habitats. *Lovenula raynerae* displayed destabilising Type II FRs towards larval mosquito prey across all water clarities tested, with overall predation rates remaining largely unaffected across all clarity treatments. In the outdoor experiment, *L. raynerae* applications resulted in substantial reductions in larval *C. pipiens* populations, with close to total eradication achieved following the experimental period under higher predator densities. These results demonstrate that environmental context such as water clarity may have little effect on vector control by calanoid copepods, which suggests a predatory reliance on hydromechanical signalling. Further, for the first time, we demonstrate the applicability of calanoid copepods to artificial container-style habitats where mosquitoes proliferate. Therefore, our results indicate that further examination into the applicability of this species group to aid vector biocontrol strategies is warranted.

Keywords

biological control; predator-prey; functional response; turbidity; *Culex pipiens*; *Lovenula raynerae*

1. Introduction

The effective control of mosquito-borne diseases and their vectors is of substantial public health importance (Mehlhorn, 2012; Beneli and Mehlhorn, 2016; WHO, 2017). Currently, a variety of chemical, physical, genetic and biological approaches are used to control mosquitoes (see Becker et al. 2010). However, many population management approaches are associated with drawbacks which impede their sustainability (e.g. Baldacchino et al. 2015). For instance, commonly-used insecticidal chemicals have caused environmental pollution, and emergent effects of insecticide resistance have presented major challenges to mosquito control strategies (e.g. Scholte et al. 2004; Ranson et al. 2016; Main et al. 2018). Mosquitoes which exploit artificial container-style habitats are of particular public health importance due to an association with urban areas and thus high potential for contact with human populations, wherein urban ‘heat islands’ can result in higher disease vector mosquito abundances (Townroe and Callaghan, 2014). Indeed, exploitation of human environments has facilitated invasive mosquito species to radically extend their geographic range (e.g. Lambrechts et al. 2010).

Biological control (hereafter biocontrol) provides a relatively environmentally-friendly and economical option in vector control (Rodríguez-Pérez et al. 2012). Natural enemies can efficaciously suppress vectorially-important mosquito populations (Marten, 1990; Marten and Reid, 2007; Baldacchino et al. 2017; Cuthbert et al. 2018a, b; but see Thomas, 2018), and have successfully induced community-wide disease extirpations (Kay and Nam, 2005; Nam et al. 2012). However, many candidate biocontrol agents remain entirely unexplored, or underexploited in the context of container-style aquatic habitats where vectorially-efficient mosquitoes can proliferate *en masse* (e.g. Townroe and Callaghan, 2014). Biological control of larval mosquito populations by deliberate application of predatory copepod species has proven to be highly efficacious (reviewed by Marten and Reid, 2007). Presently, however, only those present within the cyclopoid order have been examined and utilised for control. Yet, copepods represent a vastly extensive group of crustaceans, comprising a broad range of orders adapted to both ephemeral and perennial hydrologic ecosystems (Dussart and Defaye, 2001). Despite previous erroneous categorisation as herbivorous, considered unable to prey upon mosquito larvae (Marten and Reid, 2007), predatory calanoid copepods exist and can exert profound trophic impacts in aquatic environments (Wasserman et al. 2016a; Dalu et al. 2016a; Cuthbert et al. 2018d). Moreover, certain calanoid copepod species can be atypically large in size (e.g. Suárez-Morales et al. 2015), and can therefore handle larval mosquito stages throughout their ontogeny (Cuthbert et al. 2018d). This contrasts to cyclopoid copepods which impart a size-refuge to larger prey (Marten and Reid 2007). Therefore, examining the efficacy of calanoid copepods towards container-breeding mosquitoes across ranging environmental contexts is of pertinence for the applied biocontrol of mosquito-borne disease vectors.

Environmental context-dependencies can cause substantial variations in natural enemy impacts on target populations (e.g. Cuthbert et al. 2018a), both as a result of biotic (e.g. Alexander et al. 2013; Barrios-O’Neill et al. 2014; Wasserman et al. 2016c) and abiotic (e.g. Wasserman et al. 2016b; Cuthbert et al. 2018a, b) factors, and is thus highly relevant to biocontrol agent selection. However, the implications of these context-dependencies on the efficacy of biocontrol agents often remain poorly understood. This, in turn, reduces the capacity of practitioners to fully understand and quantify biocontrol agent impacts. As vectorially-efficient mosquito species are adapted to breed in a highly variable range of aquatic habitats (see Becker et al. 2010), understanding the implications of environmental context is integral to robust quantifications of biocontrol agent impacts on target mosquito species. Further, finding biocontrol agents that are also robust to environmental variability would be desirable. In particular, water clarity is highly variable in hydrological environments, and variations in water clarity can affect food webs though alterations of predation efficacy by visual predators (e.g. van De Meutter et al. 2005; Lunt and Smee, 2015), manipulations of microhabitat structures and temperature regimes (e.g. Meysman et al. 2006; Paaijmans et al. 2017), and by directly impacting filter feeders (e.g. Rellstab and Spak, 2007), including larvae of many mosquito species. In addition, disease vector mosquitoes have been shown be attracted to low-clarity habitats due to perceived higher nutritional loads or greater depth (Ortiz-Perea and Callaghan, 2017; Cuthbert et al. 2018b), with concurrent implications for mosquito abundances (e.g. Medlock and Vaux, 2014). Therefore, identifying biocontrol agents to target disease vector mosquitoes which are not impacted by turbid environments is crucial for successful field applications in diverse aquatic habitats (see Cuthbert et al. 2018c).

Functional responses (FRs), i.e. the *per capita* consumption rates of consumers with changes to resource densities (Solomon, 1949; Holling, 1959; Juliano, 2001), have been applied extensively to quantify the resource regulation potential of consumers (e.g. Abrams, 1990; Dick et al. 2014), and can be applied to concurrently test environmental context-dependencies of consumer impact (e.g. South et al. 2017; Cuthbert et al. 2018a, b). Given that density- and context- dependencies of *per capita* impact may affect the viability of biocontrol agents in regulating target organisms (O’Neil, 1990; Van Driesche and Bellows, 1996; Cuthbert et al. 2018a), and the regulatory efficacy of many agents is yet to be explored, here, we examine the predatory potential of *Lovenula raynerae* Suárez-Morales, Wasserman and

Dalu 2015, a recently described and remarkably large (4 – 5 mm) calanoid copepod species, towards larvae of the disease vector complex *Culex pipiens* in container-style environments. *Lovenula raynerae* is a predatory ephemeral pond specialist species which hatches from dormant eggs within sediment during the early stages of hydroperiod (Suárez-Morales et al. 2015; Wasserman et al. 2016a). Such ephemeral aquatic systems are highly varied with respect to their water clarity, particularly as a result of bioturbation which can heavily impact ecosystem functioning (e.g. Waterkeyn et al. 2016). Although high predatory impacts of *L. raynerae* have recently been described upon larval mosquitoes across their ontogeny (Cuthbert et al. 2018d), further research is required to elucidate additional context-dependencies of their impact, alongside assessments of their use in container-style habitats which foster disease vector mosquitoes (Townroe and Callaghan 2014). Therefore, the present study examines the FRs of *L. raynerae* towards larvae of the mosquito *C. pipiens* across a water clarity gradient, and also assesses the predation potential of the copepod in outdoor artificial container-style habitats under varying modes of predator and prey density.

2. Materials and methods

2.1. Animal collection and rearing

Adult male and female *L. raynerae* (4 – 5 mm) were collected from an ephemeral pond in the Eastern Cape, South Africa (33° 10' 04.1" S 27° 16' 10.6" E) by towing a 64 µm zooplankton net through the upper water column. Copepods were transported in source water to a controlled environment (CE) room at Rhodes University, Grahamstown (25 °C ± 1 °C; 14:10 light:dark) and housed in 25 L aquaria containing strained (200 µm) water from the collection site prior to the experiments. *Culex pipiens* complex larvae originated from egg rafts collected from artificial container-style aquatic habitats on the Rhodes University campus, and were reared to the desired size class on a diet of crushed rabbit food pellets (Agricol, Port Eizabeth).

2.2. Experimental protocols

We conducted two experiments to discern the efficacy of the calanoid copepod *L. raynerae* in mosquito control. In experiment 1, in the CE room, we quantified the effect of a water clarity gradient on the predatory impact of *L. raynerae* towards larval mosquito prey. Adult male *L. raynerae* were starved for 48 h prior to experimentation. Here, males were selected for experimentation to provide standardisation of predator type, given the various reproductive stages of female copepods that may influence predation rates. Functional responses of copepods were constrained under three water clarity treatments, conducive with the variability observed in ephemeral systems (Cuthbert, pers. obs.). Water clarity was defined as 0%, 50% and 100% against a predefined scale using a water clarity tube (GroundTruth, Leonard) by diluting turbid water to the prescribed clarity, with each treatment continuously aerated and filtered (200 µm) prior to use. *Culex pipiens* (3.3 ± 0.2 mm) larvae were established at five prey densities (2, 4, 8, 16, 32; $n = 4$ per density) in 80 mL arenas of 5.6 cm diameter containing the appropriate clarity treatment. Once predators were added, they were allowed to feed undisturbed for 6 h, after which they were removed and remaining prey counted to derive those killed. Controls consisted of three replicates at each density and clarity treatment without predators.

In experiment 2, we ascertained the efficacy of *L. raynerae* in outdoor artificial container-style habitats at regulating *C. pipiens* populations. This was done in a partially shaded outdoor location within the Rhodes University campus, similar to the environments from which *C. pipiens* rafts were collected. *Culex pipiens* larvae (1.89 mm ± 0.08) were added at two densities (50, 100), to 2 L arenas of 13.5 cm diameter, each containing 1.5 L filtered (200 µm) aerated water from the copepod collection site and 0.3 g of crushed rabbit food pellets. Then, three predator densities were added (0, 4, 8) in a fully randomised array. We maintained copepod sex ratios of 3:1 male:female across predator treatments (i.e. 0:0, 3:1, 6:2) to minimise cannibalism (Lavens and Sorgeloos, 1996). After 72 h, the predators were removed and remaining live mosquito prey counted to derive the number eaten. We conducted at least three replicates per experimental group. Water temperatures within arenas were found to be within the 16 – 20 °C range across the duration of the experiment.

2.3. Statistical analyses

All statistical analyses were undertaken in R v3.4.2. (R Core Team, 2017). In experiment 1, generalised linear models (GLMs) assuming a Poisson error distribution were used to examine the effects of 'water clarity' and 'prey density' on raw prey consumption. All FR analyses were undertaken within the 'frair' package in R (Pritchard et al. 2017). Logistic regression considering the proportion of prey consumed as a function of the 'prey density' factor was used to infer FR types. Here, a Type II FR is determined categorically by a significantly negative first order term, and a Type III FR by a significantly positive first order term followed by a significantly negative second order term. We fit Rogers' random predator equation to account for non-replacement of prey during the experiment (Trexler et al. 1998; Juliano, 2001):

$$N_e = N_0(1 - \exp(a(N_e h - T)))$$

Eqn. 1.

where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack constant, h is the handling time and T is the total experimental period. We applied the Lambert W function to fit the random predator equation (Bolker, 2008). The difference (delta) method (see Juliano, 2001) was employed to compare FR attack rates and handling times between treatments with respect to the ‘water clarity’ factor. We applied Bonferroni corrections to account for multiplicity of comparisons (i.e. $\alpha = 0.017$). Furthermore, we employed a non-parametric bootstrapping procedure ($n = 2000$) to generate 95% confidence intervals around the FR curves (see Pritchard et al. 2017).

In experiment 2, GLMs assuming a quasibinomial error distribution, as residuals were found to be over-dispersed relative to degrees of freedom, were used to model mortality rates with respect to the ‘predator density’ and ‘prey density’ factors. Here, we used Tukey’s comparisons *via* the ‘multcomp’ package in R (Hothorn et al. 2008). In all cases, non-significant terms and interactions were removed stepwise to obtain models with maximal parsimony (as per Crawley, 2007).

3. Results

In experiment 1, survival in control groups was 100% and so experimental deaths of larval mosquitoes were attributed to predation by copepods, which were also observed eating the larvae. Overall consumption was not significantly affected by water clarity ($\chi^2 = 1.76$, $df = 2$, $p = 0.42$) but increased significantly with higher prey densities ($\chi^2 = 80.45$, $df = 4$, $p < 0.001$). The consumptive effect of ‘prey density’ was not dependent on the water clarity as the ‘water clarity × prey density’ effect was not significant ($\chi^2 = 7.46$, $df = 8$, $p = 0.49$). Type II FRs were detected in all water clarity treatments (Table 1; Figure 1). Functional response parameters (attack rate, a; handling time, h) did not differ significantly between any water clarity treatment pairs (Table 1; Figure 1; a, low – medium, $z = 0.64$, $p = 0.52$; a, medium – high, $z = 0.25$, $p = 0.80$; a, low – high, $z = 0.87$, $p = 0.39$; h, low – medium, $z = 0.55$, $p = 0.58$; h, medium – high, $z = 0.79$, $p = 0.43$; h, low – high, $z = 0.32$, $p = 0.75$).

In experiment 2, overall, the presence of *L. raynerae* resulted in significant larval mosquito reductions given that the ‘predator density’ factor significantly affected mortality rates ($F_{2, 17} = 72.59$, $p < 0.001$; Figure 1). Greater mortality rates were found between all incremental predator density increases (0 – 4, $z = 6.69$, $p < 0.001$; 4 – 8: $z = 4.35$, $p < 0.001$; 0 – 8, $z = 9.90$, $p < 0.001$). Significantly greater mortality rates of larval mosquito prey were demonstrated under the lower prey density treatments overall ($F_{1, 16} = 10.23$, $p = 0.006$; Figure 2). There was no ‘predator density × prey density’ interaction ($F_{2, 14} = 1.37$, $p = 0.29$), and so the efficacy of *L. raynerae* at different densities was robust to treatment variations associated with prey density.

4. Discussion

Here, for the first time, we demonstrate high *per capita* predation potential of an ephemeral pond specialist calanoid copepod, *L. raynerae*, towards vector mosquito prey irrespective of water clarity regime. Equally, in outdoor trials, we show that this species can substantially reduce larval mosquito abundances in container-style habitats which frequently foster vectorially-efficient mosquito species (Townroe and Callaghan, 2014). Copepods are highly efficacious predatory biocontrol agents for disease vectoring mosquitoes (Marten, 1984; Marten and Reid, 2007; Cuthbert et al 2018a, b). Although biocontrol examinations have hitherto focused on cyclopoid copepods, other groups of copepods are also predatory and thus may be of value in biocontrol strategies (Wasserman et al. 2016a; Cuthbert et al. 2018d).

Functional responses of the calanoid copepod *L. raynerae* were not significantly affected by variations in water clarity, either in terms of form or magnitude. In a predation context, both FR form and magnitude are powerful predictors of the interaction strengths between predators and prey (Dick et al. 2014), and may be combined with predator population responses to holistically assess ecological impact (Dick et al. 2017; Cuthbert et al. 2018a, b). Three broad forms of FR have been defined (Hassell, 1978): the linear Type I, hyperbolic Type II and sigmoidal Type III. Whilst Type I FRs are mechanistically restricted to filter feeders (Jeschke et al. 2004), Type II FRs are conducive to high ecological impact as a result of high predation pressures at low prey densities (Dick et al. 2014). Accordingly, Type II FRs are particularly desirable in biocontrol contexts as they trend towards target prey eradications (Cuthbert et al. 2018a). However, Type III FRs are regarded as more stabilising, wherein there is a provisioning of low-density refugia for prey, driven, empirically, by processes such as prey switching (Hassell, 1978; Cuthbert et al. 2018e).

The present study observed Type II FRs regardless of water clarity regime. Therefore, *L. raynerae* is effectively able to locate, capture and handle prey at low densities, even in highly turbid conditions. These results are pertinent as ephemeral aquatic ecosystems, both natural and artificial, are highly varied with respect to their water clarity regime, for instance due to detritus inputs or bioturbation (Cuthbert, pers. obs.). Indeed, bioturbation associated with biocontrol agents can also affect the viability of multiple management interventions in aquatic habitats which target larval mosquitoes (e.g. Fry-O'Brien and Mulla, 1996). Mosquitoes often exhibit predator avoidance behaviours when ovipositing (see Vonesh and Blaustein, 2010), and water clarity can offset this avoidance behaviour under certain conditions (Cuthbert et al. 2018b). For this reason, identifying predatory agents which are unaffected by water clarity variations is imperative for effective biocontrol applications. Our results suggest a reliance on hydromechanical cues by this predatory calanoid copepod when detecting and capturing mosquito prey, as opposed to visual cues, which may account for the lack of overall consumptive variation between water clarity treatments. Indeed, these results corroborate with those demonstrating a lack of reliance on visual signals in cyclopoid copepods when detecting prey across diurnal and water clarity regime shifts (e.g. Hwang and Strickler, 2001; Cuthbert et al. 2018c).

Attack rates of *L. raynerae* were relatively unaffected by variations in water clarity, and even trended towards being higher under lower water clarities. Attack rates correspond to the initial slope steepness in FR curves, and thus high attack rates can be particularly destabilising to prey populations at low prey densities. On the other hand, handling times reflect the asymptote in FR curves, and can be reciprocated to infer maximum feeding rates of predators (Dick et al. 2014; Cuthbert et al. 2018a). Here, although larval mosquitoes can exhibit high responsiveness to predatory cues (e.g. Zuharah and Lester 2011), it is feasible that low water clarity increases vulnerability to predation in larval mosquito prey, in turn enhancing the capture efficiency by predators at low prey densities. Furthermore, handling times were not significantly different across the water clarity gradient in the present study, and so maximum feeding rates were similar between treatments. Yet, handling times trended towards being lowest, and thus maximum feeding rates highest, at intermediate water clarities. Hence, we present strong and sustained destabilising predatory impacts of the calanoid copepod *L. raynerae* towards varying prey supplies of larval *C. pipiens* irrespective of this environmental context. Importantly, although FR examinations here only considered males, female *L. raynerae* are also voracious consumers of larval mosquitoes, with the potential to kill over 5 first instar larvae per hour and the ability to handle late instar prey (Cuthbert et al. 2018d). This intake rate is considerably higher than cyclopoid copepods which are often used in biological control, and which also impart a size refuge to late instar mosquito prey (Marten and Reid, 2007).

The study also highlights that the *L. raynerae* predation efficiency observed under controlled laboratory conditions also persists in outdoor environments exposed to natural conditions, where vector mosquitoes proliferate. In outdoor experiments within container-style habitats, *L. raynerae* induced substantial mortality rates in larval mosquito populations. As vectorially-important mosquitoes increasingly proliferate in such artificial, container-style habitats (Townroe and Callaghan, 2014), exploring the suitability for biocontrol agents over longer-term experiments in these environments is critical for empirical derivations of their efficacy. Indeed, these aquatic habitats can often be minute and ephemeral in nature, negating the use of larger larval mosquito antagonists, such as fish (see Azevedo-Santos et al. 2016). Our results demonstrate that, over the experimental period, higher densities of *L. raynerae* exhibited higher predation capacities relative to lower densities towards all prey supplies. Thus, multiple *L. raynerae* conspecifics may be additive in their consumption of *C. pipiens* larvae. Moreover, as *L. raynerae* is an ephemeral pond specialist and is capable of producing dormant, drought-resistant eggs, applications of this species to ephemeral aquatic habitats which foster mosquitoes may enable predator hatching *in situ* prior to, or simultaneous with, colonisation by mosquitoes. Although our results are theoretically promising in this respect, further research is required to test the efficacy of single applications of dormant eggs of *L. raynerae* over recurrent hydroperiods, and thus over longer experimental times overall. Furthermore, examinations of prey preferences and cannibalism in *L. raynerae* towards juveniles would be of value in further discerning factors that may impede their applied efficacy in biocontrol. However, it has been proposed that the most efficacious copepod species in biocontrol are able to curtail overpopulation and growth stunting *via* cannibalism of juveniles when the population becomes too high relative to the food supply (Marten and Reid, 2007).

In conclusion, our results suggest that calanoids and other copepod groups warrant further consideration as biocontrol agents of disease vector mosquitoes. In particular, ephemeral pond specialist species, such as *L. raynerae*, may be particularly promising candidates, as they are often particularly large, develop rapidly, exhibit dormancy, and have adapted to occupy relatively high trophic levels (Dalu et al. 2016a). Further, their especially large size may make *L. raynerae* less vulnerable to higher-order predation as compared to physically smaller copepod species. Generally, biotic interactions within ephemeral aquatic ecosystems are often poorly studied due to spatial and temporal heterogeneity (Dalu et al. 2016b), and thus these systems hold much potential for biocontrol agent exploration, as demonstrated in the present study. Our results show strong and destabilising predatory impacts of *L. raynerae* towards larvae of *C. pipiens* across a water clarity regime, which may enable consistently high impacts upon target populations under differing environmental conditions. Furthermore, we show efficacious predatory potential of this species in artificial container-style habitats in outdoor environments. Future research should test additional environmental contexts as to their effects on biotic interaction strengths between biocontrol agents and target organisms, and further explore the sustained potential of calanoid copepods over longer hydroperiods within container-style habitats which harbour disease vector mosquitoes.

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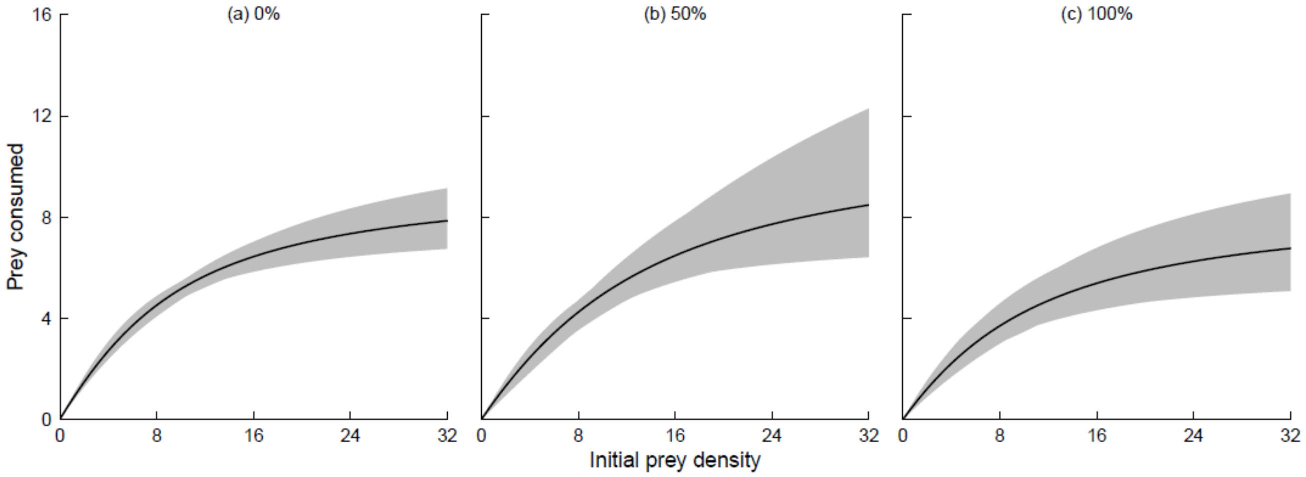
Tables and Figure Captions

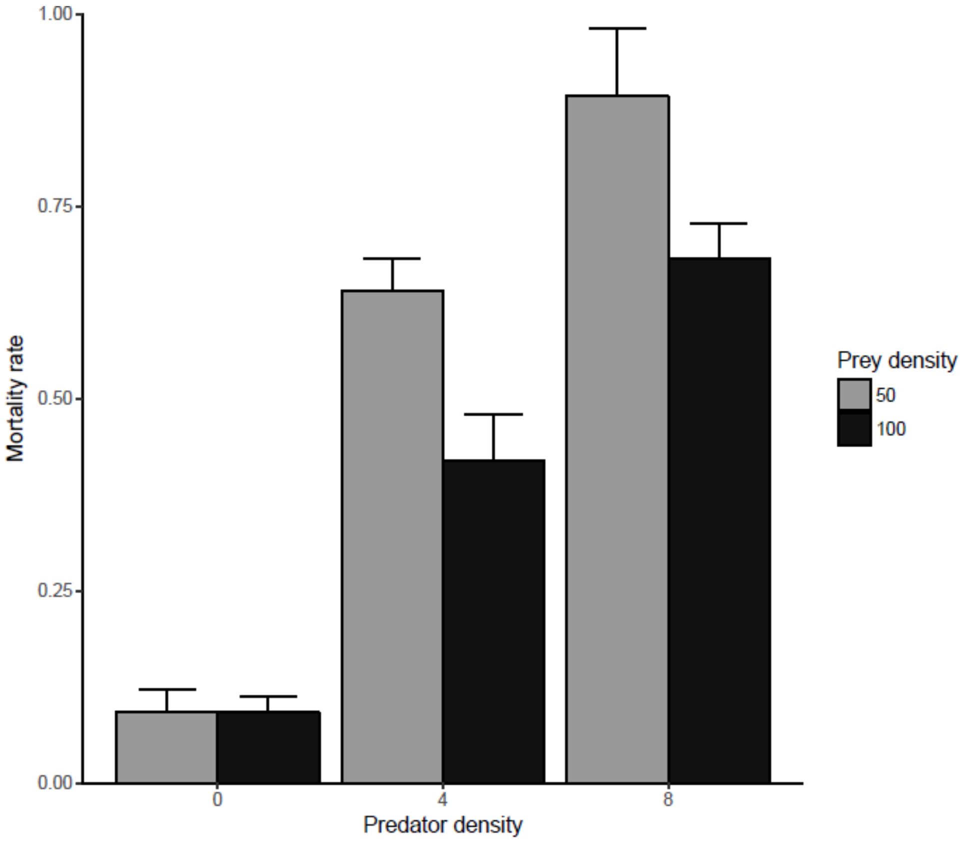
Table 1. First order terms generated from logistic regression of proportional prey consumption as a function of prey density alongside parameter outputs from Rogers' random predator equation across water clarity regimes.

Water clarity	First order term, p	a, p	h, p
0%	-0.058620, < 0.001	1.572822 , < 0.001	0.104547, < 0.001
50%	-0.044384, < 0.001	1.201939¸ < 0.001	0.087762, < 0.001
100%	-0.049398, < 0.001	1.082802, < 0.001	0.115452, < 0.001

Figure 1. Functional responses of *Lovenula raynerae* towards larval *Culex pipiens* prey across a water clarity gradient (low, 0%; medium, 50%; high, 100%). Shaded areas represent bootstrapped (*n* = 2000) confidence intervals.

Figure 2. Mortality rate (±SE) of larval *Culex pipiens* at different densities in outdoor trial resulting from the presence of predatory copepod *Lovenula raynerae* at three densities.


(a) 0%
(b) 50%
(c) 100%
Prey consumed
0
4
8
12
16
0
8
16
24
32
Initial prey density


1.00
0.75
0.50
0.25
0.00
Mortality rate
0
4
8
Predator density
Prey density
50
100

563