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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.

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

71 and Nam, 2005; Nam et al. 2012). However, many candidate biocontrol agents remain

72 entirely unexplored, or underexploited in the context of container-style aquatic habitats where

73 vectorially-efficient mosquitoes can proliferate *en masse* (e.g. Townroe and Callaghan,

74 2014). Biological control of larval mosquito populations by deliberate application of

75 predatory copepod species has proven to be highly efficacious (reviewed by Marten and Reid,

76 2007). Presently, however, only those present within the cyclopoid order have been examined

77 and utilised for control. Yet, copepods represent a vastly extensive group of crustaceans,

78 comprising a broad range of orders adapted to both ephemeral and perennial hydrologic

79 ecosystems (Dussart and Defaye, 2001). Despite previous erroneous categorisation as

80 herbivorous, considered unable to prey upon mosquito larvae (Marten and Reid, 2007),

81 predatory calanoid copepods exist and can exert profound trophic impacts in aquatic

82 environments (Wasserman et al. 2016a; Dalu et al. 2016a; Cuthbert et al. 2018d). Moreover,

83 certain calanoid copepod species can be atypically large in size (e.g. Suárez-Morales et al.

84 2015), and can therefore handle larval mosquito stages throughout their ontogeny (Cuthbert

85 et al. 2018d). This contrasts to cyclopoid copepods which impart a size-refuge to larger prey

86 (Marten and Reid 2007). Therefore, examining the efficacy of calanoid copepods towards

87 container-breeding mosquitoes across ranging environmental contexts is of pertinence for the

88 applied biocontrol of mosquito-borne disease vectors.

89   Environmental context-dependencies can cause substantial variations in natural

90 enemy impacts on target populations (e.g. Cuthbert et al. 2018a), both as a result of biotic

91 (e.g. Alexander et al. 2013; Barrios-O'Neill et al. 2014; Wasserman et al. 2016c) and abiotic

92 (e.g. Wasserman et al. 2016b; Cuthbert et al. 2018a, b) factors, and is thus highly relevant to

93 biocontrol agent selection. However, the implications of these context-dependencies on the

94 efficacy of biocontrol agents often remain poorly understood. This, in turn, reduces the

95 capacity of practitioners to fully understand and quantify biocontrol agent impacts. As

96 vectorially-efficient mosquito species are adapted to breed in a highly variable range of

97 aquatic habitats (see Becker et al. 2010), understanding the implications of environmental

98 context is integral to robust quantifications of biocontrol agent impacts on target mosquito

99 species. Further, finding biocontrol agents that are also robust to environmental variability

100 would be desirable. In particular, water clarity is highly variable in hydrological

101 environments, and variations in water clarity can affect food webs though alterations of

102 predation efficacy by visual predators (e.g. van De Meutter et al. 2005; Lunt and Smee,

103 2015), manipulations of microhabitat structures and temperature regimes (e.g. Meysman et al.

104 2006; Paaijmans et al. 2017), and by directly impacting filter feeders (e.g. Rellstab and Spak,

105 2007), including larvae of many mosquito species. In addition, disease vector mosquitoes

106 have been shown be attracted to low-clarity habitats due to perceived higher nutritional loads

107 or greater depth (Ortiz-Perea and Callaghan, 2017; Cuthbert et al. 2018b), with concurrent

108 implications for mosquito abundances (e.g. Medlock and Vaux, 2014). Therefore, identifying

109 biocontrol agents to target disease vector mosquitoes which are not impacted by turbid

110 environments is crucial for successful field applications in diverse aquatic habitats (see

111 Cuthbert et al. 2018c).

112 Functional responses (FRs), i.e. the *per capita* consumption rates of consumers with

113 changes to resource densities (Solomon, 1949; Holling, 1959; Juliano, 2001), have been

114 applied extensively to quantify the resource regulation potential of consumers (e.g. Abrams,

115 1990; Dick et al. 2014), and can be applied to concurrently test environmental context-

116 dependencies of consumer impact (e.g. South et al. 2017; Cuthbert et al. 2018a, b). Given that

117 density- and context- dependencies of *per capita* impact may affect the viability of biocontrol

118 agents in regulating target organisms (O'Neil, 1990; Van Driesche and Bellows, 1996;

119 Cuthbert et al. 2018a), and the regulatory efficacy of many agents is yet to be explored, here,

120 we examine the predatory potential of *Lovenula raynerae* Suárez-Morales, Wasserman and

121 Dalu 2015, a recently described and remarkably large (4 – 5 mm) calanoid copepod species,

122 towards larvae of the disease vector complex *Culex pipiens* in container-style environments.

123 *Lovenula raynerae* is a predatory ephemeral pond specialist species which hatches from

124 dormant eggs within sediment during the early stages of hydroperiod (Suárez-Morales et al.

125 2015; Wasserman et al. 2016a). Such ephemeral aquatic systems are highly varied with

126 respect to their water clarity, particularly as a result of bioturbation which can heavily impact

127 ecosystem functioning (e.g. Waterkeyn et al. 2016). Although high predatory impacts of *L.*

128 *raynerae* have recently been described upon larval mosquitoes across their ontogeny

129 (Cuthbert et al. 2018d), further research is required to elucidate additional context-

130 dependencies of their impact, alongside assessments of their use in container-style habitats

131 which foster disease vector mosquitoes (Townroe and Callaghan 2014). Therefore, the

132 present study examines the FRs of *L. raynerae* towards larvae of the mosquito *C. pipiens*

133 across a water clarity gradient, and also assesses the predation potential of the copepod in

134 outdoor artificial container-style habitats under varying modes of predator and prey density.

135 **2. Materials and methods**

136 *2.1. Animal collection and rearing*

137 Adult male and female *L. raynerae* (4 – 5 mm) were collected from an ephemeral

138 pond in the Eastern Cape, South Africa (33° 10' 04.1" S 27° 16' 10.6" E) by towing a 64 μm

139 zooplankton net through the upper water column. Copepods were transported in source water

140 to a controlled environment (CE) room at Rhodes University, Grahamstown (25 °C ± 1 °C;

141 14:10 light:dark) and housed in 25 L aquaria containing strained (200 μm) water from the

142 collection site prior to the experiments. *Culex pipiens* complex larvae originated from egg

143 rafts collected from artificial container-style aquatic habitats on the Rhodes University

144 campus, and were reared to the desired size class on a diet of crushed rabbit food pellets

145 (Agricol, Port Eizabeth).

146 *2.2. Experimental protocols*

147   We conducted two experiments to discern the efficacy of the calanoid copepod *L.*

148 *raynerae* in mosquito control. In experiment 1, in the CE room, we quantified the effect of a

149 water clarity gradient on the predatory impact of *L. raynerae* towards larval mosquito prey.

150 Adult male *L. raynerae* were starved for 48 h prior to experimentation. Here, males were

151 selected for experimentation to provide standardisation of predator type, given the various

152 reproductive stages of female copepods that may influence predation rates. Functional

153 responses of copepods were constrained under three water clarity treatments, conducive with

154 the variability observed in ephemeral systems (Cuthbert, pers. obs.). Water clarity was

155 defined as 0%, 50% and 100% against a predefined scale using a water clarity tube

156 (GroundTruth, Leonard) by diluting turbid water to the prescribed clarity, with each treatment

157 continuously aerated and filtered (200 μm) prior to use. *Culex pipiens* (3.3 ± 0.2 mm) larvae

158 were established at five prey densities (2, 4, 8, 16, 32; $n = 4$ per density) in 80 mL arenas of

159 5.6 cm diameter containing the appropriate clarity treatment. Once predators were added,

160 they were allowed to feed undisturbed for 6 h, after which they were removed and remaining

161 prey counted to derive those killed. Controls consisted of three replicates at each density and

162 clarity treatment without predators.

163   In experiment 2, we ascertained the efficacy of *L. raynerae* in outdoor artificial

164 container-style habitats at regulating *C. pipiens* populations. This was done in a partially

165 shaded outdoor location within the Rhodes University campus, similar to the environments

166 from which *C. pipiens* rafts were collected. *Culex pipiens* larvae (1.89 mm $\pm$ 0.08) were

167 added at two densities (50, 100), to 2 L arenas of 13.5 cm diameter, each containing 1.5 L

168   filtered (200 μm) aerated water from the copepod collection site and 0.3 g of crushed rabbit

169   food pellets. Then, three predator densities were added (0, 4, 8) in a fully randomised array.

170   We maintained copepod sex ratios of 3:1 male:female across predator treatments (i.e. 0:0,

171   3:1, 6:2) to minimise cannibalism (Lavens and Sorgeloos, 1996). After 72 h, the predators

172   were removed and remaining live mosquito prey counted to derive the number eaten. We

173   conducted at least three replicates per experimental group. Water temperatures within arenas

174   were found to be within the 16 – 20 °C range across the duration of the experiment.

175   *2.3. Statistical analyses*

176   All statistical analyses were undertaken in R v3.4.2. (R Core Team, 2017). In

177   experiment 1, generalised linear models (GLMs) assuming a Poisson error distribution were

178   used to examine the effects of 'water clarity' and 'prey density' on raw prey consumption.

179   All FR analyses were undertaken within the 'frair' package in R (Pritchard et al. 2017).

180   Logistic regression considering the proportion of prey consumed as a function of the 'prey

181   density' factor was used to infer FR types. Here, a Type II FR is determined categorically by

182   a significantly negative first order term, and a Type III FR by a significantly positive first

183   order term followed by a significantly negative second order term. We fit Rogers' random

184   predator equation to account for non-replacement of prey during the experiment (Trexler et

185   al. 1998; Juliano, 2001):

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

187   Eqn. 1.

188

189   where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack constant,

190   h is the handling time and T is the total experimental period. We applied the Lambert W

191   function to fit the random predator equation (Bolker, 2008). The difference (delta) method

192   (see Juliano, 2001) was employed to compare FR attack rates and handling times between

193   treatments with respect to the 'water clarity' factor. We applied Bonferroni corrections to

194   account for multiplicity of comparisons (i.e. $\alpha = 0.017$). Furthermore, we employed a non-

195   parametric bootstrapping procedure ($n = 2000$) to generate 95% confidence intervals around

196   the FR curves (see Pritchard et al. 2017).

197   In experiment 2, GLMs assuming a quasibinomial error distribution, as residuals were

198   found to be over-dispersed relative to degrees of freedom, were used to model mortality rates

199   with respect to the 'predator density' and 'prey density' factors. Here, we used Tukey's

200   comparisons *via* the 'multcomp' package in R (Hothorn et al. 2008). In all cases, non-

201   significant terms and interactions were removed stepwise to obtain models with maximal

202   parsimony (as per Crawley, 2007).

203   **3. Results**

204   In experiment 1, survival in control groups was 100% and so experimental deaths of

205   larval mosquitoes were attributed to predation by copepods, which were also observed eating

206   the larvae. Overall consumption was not significantly affected by water clarity ($\chi^2 = 1.76$, *df*

207    $= 2$, $p = 0.42$) but increased significantly with higher prey densities ($\chi^2 = 80.45$, *df* $= 4$, $p <$

208   0.001). The consumptive effect of 'prey density' was not dependent on the water clarity as

209   the 'water clarity × prey density' effect was not significant ($\chi^2 = 7.46$, *df* $= 8$, $p = 0.49$). Type

210   II FRs were detected in all water clarity treatments (Table 1; Figure 1). Functional response

211   parameters (attack rate, *a*; handling time, *h*) did not differ significantly between any water

212   clarity treatment pairs (Table 1; Figure 1; *a*, low – medium, $z = 0.64$, $p = 0.52$; *a*, medium –

213   high, $z = 0.25$, $p = 0.80$; *a*, low – high, $z = 0.87$, $p = 0.39$; *h*, low – medium, $z = 0.55$, $p =$

214   0.58; *h*, medium – high, $z = 0.79$, $p = 0.43$; *h*, low – high, $z = 0.32$, $p = 0.75$).

215   In experiment 2, overall, the presence of *L. raynerae* resulted in significant larval

216   mosquito reductions given that the 'predator density' factor significantly affected mortality

217   rates ($F_{2,\,17} = 72.59$, $p < 0.001$; Figure 1). Greater mortality rates were found between all

218   incremental predator density increases (0 – 4, $z = 6.69$, $p < 0.001$; 4 – 8: $z = 4.35$, $p < 0.001$; 0

219   – 8, $z = 9.90$, $p < 0.001$). Significantly greater mortality rates of larval mosquito prey were

220   demonstrated under the lower prey density treatments overall ($F_{1,\,16} = 10.23$, $p = 0.006$;

221   Figure 2). There was no 'predator density × prey density' interaction ($F_{2,\,14} = 1.37$, $p = 0.29$),

222   and so the efficacy of *L. raynerae* at different densities was robust to treatment variations

223   associated with prey density.

4. Discussion

225   Here, for the first time, we demonstrate high *per capita* predation potential of an

226   ephemeral pond specialist calanoid copepod, *L. raynerae*, towards vector mosquito prey

227   irrespective of water clarity regime. Equally, in outdoor trials, we show that this species can

228   substantially reduce larval mosquito abundances in container-style habitats which frequently

229   foster vectorially-efficient mosquito species (Townroe and Callaghan, 2014). Copepods are

230   highly efficacious predatory biocontrol agents for disease vectoring mosquitoes (Marten,

231   1984; Marten and Reid, 2007; Cuthbert et al 2018a, b). Although biocontrol examinations

232   have hitherto focused on cyclopoid copepods, other groups of copepods are also predatory

233   and thus may be of value in biocontrol strategies (Wasserman et al. 2016a; Cuthbert et al.

234   2018d).

235   Functional responses of the calanoid copepod *L. raynerae* were not significantly

236   affected by variations in water clarity, either in terms of form or magnitude. In a predation

237   context, both FR form and magnitude are powerful predictors of the interaction strengths

238   between predators and prey (Dick et al. 2014), and may be combined with predator

239   population responses to holistically assess ecological impact (Dick et al. 2017; Cuthbert et al.

240   2018a, b). Three broad forms of FR have been defined (Hassell, 1978): the linear Type I,

241   hyperbolic Type II and sigmoidal Type III. Whilst Type I FRs are mechanistically restricted

242   to filter feeders (Jeschke et al. 2004), Type II FRs are conducive to high ecological impact as

243   a result of high predation pressures at low prey densities (Dick et al. 2014). Accordingly,

244   Type II FRs are particularly desirable in biocontrol contexts as they trend towards target prey

245   eradications (Cuthbert et al. 2018a). However, Type III FRs are regarded as more stabilising,

246   wherein there is a provisioning of low-density refugia for prey, driven, empirically, by

247   processes such as prey switching (Hassell, 1978; Cuthbert et al. 2018e).

248   The present study observed Type II FRs regardless of water clarity regime. Therefore,

249   *L. raynerae* is effectively able to locate, capture and handle prey at low densities, even in

250   highly turbid conditions. These results are pertinent as ephemeral aquatic ecosystems, both

251   natural and artificial, are highly varied with respect to their water clarity regime, for instance

252   due to detritus inputs or bioturbation (Cuthbert, pers. obs.). Indeed, bioturbation associated

253   with biocontrol agents can also affect the viability of multiple management interventions in

254   aquatic habitats which target larval mosquitoes (e.g. Fry-O'Brien and Mulla, 1996).

255   Mosquitoes often exhibit predator avoidance behaviours when ovipositing (see Vonesh and

256   Blaustein, 2010), and water clarity can offset this avoidance behaviour under certain

257   conditions (Cuthbert et al. 2018b). For this reason, identifying predatory agents which are

258   unaffected by water clarity variations is imperative for effective biocontrol applications. Our

259   results suggest a reliance on hydromechanical cues by this predatory calanoid copepod when

260   detecting and capturing mosquito prey, as opposed to visual cues, which may account for the

261   lack of overall consumptive variation between water clarity treatments. Indeed, these results

262   corroborate with those demonstrating a lack of reliance on visual signals in cyclopoid

263   copepods when detecting prey across diurnal and water clarity regime shifts (e.g. Hwang and

264   Strickler, 2001; Cuthbert et al. 2018c).

265   Attack rates of *L. raynerae* were relatively unaffected by variations in water clarity,

266   and even trended towards being higher under lower water clarities. Attack rates correspond to

267   the initial slope steepness in FR curves, and thus high attack rates can be particularly

268   destabilising to prey populations at low prey densities. On the other hand, handling times

269   reflect the asymptote in FR curves, and can be reciprocated to infer maximum feeding rates

270   of predators (Dick et al. 2014; Cuthbert et al. 2018a). Here, although larval mosquitoes can

271   exhibit high responsiveness to predatory cues (e.g. Zuharah and Lester 2011), it is feasible

272   that low water clarity increases vulnerability to predation in larval mosquito prey, in turn

273   enhancing the capture efficiency by predators at low prey densities. Furthermore, handling

274   times were not significantly different across the water clarity gradient in the present study,

275   and so maximum feeding rates were similar between treatments. Yet, handling times trended

276   towards being lowest, and thus maximum feeding rates highest, at intermediate water

277   clarities. Hence, we present strong and sustained destabilising predatory impacts of the

278   calanoid copepod *L. raynerae* towards varying prey supplies of larval *C. pipiens* irrespective

279   of this environmental context. Importantly, although FR examinations here only considered

280   males, female *L. raynerae* are also voracious consumers of larval mosquitoes, with the

281   potential to kill over 5 first instar larvae per hour and the ability to handle late instar prey

282   (Cuthbert et al. 2018d). This intake rate is considerably higher than cyclopoid copepods

283   which are often used in biological control, and which also impart a size refuge to late instar

284   mosquito prey (Marten and Reid, 2007).

285   The study also highlights that the *L. raynerae* predation efficiency observed under

286   controlled laboratory conditions also persists in outdoor environments exposed to natural

287   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

313 adapted to occupy relatively high trophic levels (Dalu et al. 2016a). Further, their especially

314 large size may make *L. raynerae* less vulnerable to higher-order predation as compared to

315 physically smaller copepod species. Generally, biotic interactions within ephemeral aquatic

316 ecosystems are often poorly studied due to spatial and temporal heterogeneity (Dalu et al.

317 2016b), and thus these systems hold much potential for biocontrol agent exploration, as

318 demonstrated in the present study. Our results show strong and destabilising predatory

319 impacts of *L. raynerae* towards larvae of *C. pipiens* across a water clarity regime, which may

320 enable consistently high impacts upon target populations under differing environmental

321 conditions. Furthermore, we show efficacious predatory potential of this species in artificial

322 container-style habitats in outdoor environments. Future research should test additional

323 environmental contexts as to their effects on biotic interaction strengths between biocontrol

324 agents and target organisms, and further explore the sustained potential of calanoid copepods

325 over longer hydroperiods within container-style habitats which harbour disease vector

326 mosquitoes.

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499 **Acknowledgements**

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513   **Tables and Figure Captions**

514   Table 1. First order terms generated from logistic regression of proportional prey

515   consumption as a function of prey density alongside parameter outputs from Rogers' random

516   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

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528   Figure 1. Functional responses of *Lovenula raynerae* towards larval *Culex pipiens* prey

529   across a water clarity gradient (low, 0%; medium, 50%; high, 100%). Shaded areas represent

530   bootstrapped ($n = 2000$) confidence intervals.

531   Figure 2. Mortality rate (±SE) of larval *Culex pipiens* at different densities in outdoor trial

532   resulting from the presence of predatory copepod *Lovenula raynerae* at three densities.

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