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Fear alone reduces energy processing by resident 'keystone' prey threatened by an invader; a non-consumptive effect of 'killer shrimp' invasion of freshwater ecosystems is revealed

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22 **Abstract**

23 Non-consumptive effects (NCEs) of predators – so called ‘fear’ responses – encompass costly
24 antipredator behaviours, such as reduced feeding efficiency. NCEs can influence prey
25 population dynamics and community structure, if prey are ‘keystone’ species such as
26 *Gammarus* spp. amphipod ‘shrimps’. These freshwater macroinvertebrates have the
27 ecosystem functional role of shredding fallen leaf litter, making it accessible to other taxa.
28 Across Europe, the invasive predatory ‘killer shrimp’ *Dikerogammarus villosus* is replacing
29 resident *Gammarus* spp., potentially threatening this vital ecosystem function. While
30 predation (consumptive effects (CEs)) of this invader has been well studied, for the first time
31 we test whether NCEs can be evident in prey only exposed to *D. villosus* presence and
32 whether this could potentially impact on the prey’s functional role. In mesocosms, exposure
33 to constrained *D. villosus* did not result in mortalities of any of three *Gammarus* prey species
34 but the leaf shredding efficiencies of all prey were significantly reduced compared to a
35 control treatment. This clear NCE has the potential to propagate through the ecological
36 community via decreased energy processing. This study demonstrates the potential for fear of
37 invasive predator presence alone to impact on ecosystem function.

38

39 **Key words:** Consumptive effect (CE), *Dikerogammarus*, killer shrimp, non-consumptive
40 effect (NCE), shredders

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45 **Introduction**

46 The ‘naive prey’ hypothesis posits that resident prey lacking shared evolutionary history with
47 invaders can be heavily predated because of ineffective anti-predator responses [1]. While
48 effective anti-predator responses reduce predation rates (i.e. consumptive effects, CEs),
49 tactics such as predator avoidance by prey can be costly resulting in non-consumptive, or
50 ‘trait mediated’ effects (NCEs) such as reduced foraging efficiency [2]. Described as ‘fear’
51 responses, these tactics used by individuals can influence population densities and dynamics
52 through impacts on survival and reproduction [2]. If prey species perform key ecosystem
53 functions such as energy processing, NCEs may propagate further through ecological
54 communities. Thus, there may be cumulative effects of NCEs alongside CEs that impact the
55 structure of resident communities subject to invasion by predatory species [3].

56 Studies on the impacts of biological invasion have traditionally focussed on
57 competition, predation and biodiversity loss [4]. Within three decades, the Ponto-Caspian
58 amphipod *Dikerogammarus villosus* has invaded the freshwaters of nearly twenty European
59 countries [5]. *D. villosus* is a voracious predator consuming a vast range of macroinvertebrate
60 taxa across different trophic groups, it can be super-abundant within invaded sites, dominating
61 assemblages and its invasion is linked to local extinctions [5]. In the Netherlands, declines of
62 two resident amphipods, the native *Gammarus duebeni* and a previous invader *G. tigrinus*,
63 shortly after the arrival of *D. villosus*, have been attributed to predation by the invader [6] and
64 another amphipod, *G. pulex*, may also be under threat of species replacement by *D. villosus* in
65 areas of Central Europe and the U.K. [5]. Nevertheless, *D. villosus* and its resident prey can
66 also co-exist, both during and following range expansion of the invader [5-6].

67 Freshwater *Gammarus* amphipods are archetypal leaf shredders, having the functional
68 role in river and lake ecosystems of processing major allochthonous energy inputs of fallen leaf
69 litter from the riparian zone [7]. *Gammarus* spp. can form 85% (numerically and biomass) of
70 taxa in riverine macroinvertebrate assemblages [8-9] and are ‘keystone species’ capable of
71 influencing the population dynamics of other trophic levels [7]. They can be ‘key’ shredders,
72 for instance individual *Gammarus* species can account for up to 16% of litter breakdown [7]
73 and *Gammarus* spp. collectively up to 75% [8]. Litter processing via direct consumption,
74 particle fragmentation and faeces production by shredders facilitates energy transfer between
75 trophic levels, making these allochthonous energy inputs accessible to many other
76 macroinvertebrate taxa [9]. In contrast, *D. villosus* is described as a ‘predatory omnivore’ and
77 the feeding behaviour of this opportunistic invader is very flexible and its trophic function,
78 seems to vary between ecosystems [10]. In some invaded systems it may be a relatively
79 efficient and significant shredder / processor of leaf material [11-13], while in others it is a very
80 poor leaf shredder [14]. Despite this variability, the continuing range expansion and associated
81 predatory impact of *D. villosus* on resident shredder prey is an exemplar of a biological invasion
82 that could dramatically affect ecosystem function in some invaded systems.

83 While consumptive effects of *D. villosus* eliminating shredder prey species would
84 obviously affect energy processing and ecosystem function, MacNeil *et al.* [13] showed that
85 NCEs might occur alongside CEs, as while many shredders were simply predated, the leaf
86 shredding efficiency of survivors of the ongoing predation also decreased. The contribution of
87 NCEs to biological invasion and its wider ecosystem level impacts could thus be even greater
88 than previously appreciated [1,3]. We test whether exposure to just presence alone (with no
89 possibility of physical interaction) of a predatory invader, could generate NCEs on naive prey
90 behaviour. Specifically we assess if a NCE of *D. villosus* presence only (using caged
91 confinement of the predator to isolate this) is evident on the shredding behaviour of three

92 different *Gammarus* prey species and which, alongside more obvious CEs such as injury by a
93 predator and predation itself, could undermine the prey species' functional role [7,10].

94

95 **Materials and methods**

96 We used a laboratory mesocosm approach to expose naïve prey to an invasive predator, which
97 allowed us to investigate NCEs without the possibility of accidental release of the invader.
98 During October 2003, *G. duebeni*, *G. pulex* and *G. tigrinus* were collected from three separate
99 locations in the Netherlands where *D. villosus* had not yet invaded (see Appendix 1 for details),
100 allowing simulation of initial interspecific contact and invasion [14]. Species were maintained
101 separately in holding tanks in the laboratory before being introduced to mesocosms [14]. Five
102 pre-weighed adults of either *G. duebeni*, *G. pulex*, *G. tigrinus* or *D. villosus* (mean wet weights
103 of each species \pm SE = 44.2 \pm 3.9 mg, 40.3 \pm 3.7 mg, 24.2 \pm 2.8 mg and 68.0 \pm 4.1 mg
104 respectively) were introduced into mesocosms and these were able to swim freely and feed on
105 30 pre-weighed discs of 6mm diameter stream conditioned *Acer pseudoplatanus* leaf [14]. In
106 additional amphipod-free mesocosms submerged control leaf discs did not lose mass over the
107 experimental period (see Appendix 1). In half of the mesocosms a small plastic mesh cage
108 containing a single male *D. villosus* (standardised wet weight range 70-78 mg) was present
109 (caged *D. villosus* were not fed during the 4 days of the actual mesocosm experiment). This
110 placed the shredders in the chemical and visual presence of *D. villosus*, while preventing the
111 *D. villosus* from directly interacting with them. The remaining mesocosms were identical
112 except that *D. villosus* and cages were absent. We acknowledge inclusion of empty cages in *D.*
113 *villosus* 'free' mesocosms would have removed any potential 'cage effect' on amphipod
114 shredding activity and if resources had allowed we would have done this, but we do assume

115 any impact of a small empty plastic cage on the leaf shredding activity of five amphipods over
116 a 4 day period, in a much larger mesocosm to be marginal at best.

117 Mesocosms were examined daily for 4 days and numbers of disks (to the nearest quarter
118 of a disk) consumed each day recorded. When the number of remaining disks fell to 10 in any
119 replicate, 10 further pre-weighed disks were added, ensuring material was always available in
120 excess. Leaf shredding efficiency as leaf consumption per unit mass of shredder (mg wet
121 weight leaf consumed per mg wet weight animal) in each separate 24hr period was estimated
122 for each mesocosm [14]. There were $n = 6$ mesocosms for each shredder species and *D. villosus*
123 treatment, such that $n = 48$ replicate mesocosms, with 192 observations of shredding efficiency
124 across the 4 days.

125

126 *Statistical methods*

127 To determine the effects of predator treatment (*D. villosus*, absent or caged), shredder species
128 (*G. duebeni*, *G. pulex*, *G. tigrinus* or *D. villosus*), day (1-4) and their interactions on shredding
129 efficiency we used a linear mixed effects model. Due to the repeated measures nature of the
130 data, we allowed random intercepts for replicates (mesocosms) to account for variation
131 between them in shredding efficiency on day 1 and we also allowed random slopes to account
132 for differences between them in changes in shredding efficiency across days 1-4. The analysis
133 was implemented using the lme4 [15] and lmerTest [16] packages running under R version
134 3.4.1 [17], which in combination allow traditional F and P values to be calculated for fixed
135 effects (see Appendix 2). Prior to analysis, data were $\log_{10}(x+1)$ transformed (as shredding
136 efficiency could be <1).

137

138

139 **Results**

140 Not a single individual died during the 4 days. Shredding efficiency was, however, influenced
141 by a 3-way interaction between presence of caged *D. villosus*, free swimming shredder species
142 and day ($F_{6,176.09} = 11.74, P < 0.0001$) (Table 1; Figure 1). *G. duebeni* and *G. pulex* shredded
143 more efficiently than *G. tigrinus*. Except for day 2 in the case of *G. pulex* and day 3 in the case
144 of *G. duebeni*, all *Gammarus* spp. showed reduced shredding efficiency in the presence of caged
145 *D. villosus*. In contrast, free swimming *D. villosus* showed minimal amounts of shredding
146 relative to the *Gammarus* spp. and this was unaffected by the presence of a caged conspecific.
147 After 4 days, each *Gammarus* species showed lower shredding efficiency in the presence of
148 caged *D. villosus* compared to the treatments where *D. villosus* was absent; *G. tigrinus*, the
149 least efficient and smallest shredder, showing the greatest sensitivity to the presence of *D.*
150 *villosus* (Figure 1).

151

152 **Discussion**

153 This study demonstrates an unappreciated and indirect impact of a biological invasion by a
154 voracious predator; that the mere presence of an invader can influence resident prey behaviour,
155 in this case the feeding efficiency of naïve residents. MacNeil *et al.*, [14] demonstrated strong
156 CEs of uncaged *D. villosus* and indicated potential NCEs in surviving prey, with reduced
157 shredding potentially reflecting injuries and loss of feeding time due to avoidance responses to
158 predation-event cues (e.g. conspecific alarm cues or damaged conspecifics) and predator cues
159 [9]. In contrast, the current analysis reveals that NCEs are not dependent on the occurrence of
160 injuries or predation event cues. Rather, they resulted from the presence of caged *D. villosus*
161 (unable to physically interact with prey), clearly demonstrating that prey responses to predator

162 cues caused by predator presence alone can lead to reduced shredding activity of all prey
163 species.

164 The *Gammarus* spp. in our experiment had no prior exposure to *D. villosus* and would
165 not have been under selection to respond to specific alarm cues from this predator [1]. Thus,
166 this reduced shredding efficiency may reflect a generalized response to the presence of novel
167 heterospecifics [18-19]. Here, the caged predator could have been detected via visual or semio-
168 chemical cues [14, 19] but further studies would be needed to elucidate the exact mechanism
169 driving this NCE. In addition, investigation of consumption of conspecifics in cages could
170 produce larger behavioural responses from the prey [9]. Our data indicate that the elimination
171 of resident prey may be driven by processes additional to direct predation, as at least to some
172 degree, any reduced foraging efficiency may contribute to reduced survival and fecundity [1,7].
173 Our results also indicate that NCEs on functionally important prey species (key shredders [8-
174 9] and keystone species [7]) may have repercussions at the ecosystem level. For example,
175 recovery of stream communities following perturbation is dependent upon shredder
176 facilitation, whereby recovery of other taxa and ecosystem processes is dependent on the
177 recovery of shredders due to their contribution to energy flow [20].

178 The impact of NCEs on shredding activity seems unlikely to be uniform across
179 locations or different naive prey species. The reduction in shredding efficiency was greatest in
180 the least efficient shredder, *G. tigrinus*. This is unsurprising, as although *Gammarus* spp. are
181 regarded as archetypal leaf shredders, all three species used here, are flexible omnivores and
182 *G. tigrinus* is arguably the most predatory of the three [7]. Indeed, the ability of *Gammarus* to
183 switch feeding strategy may generate a greater reduction in shredding efficiency, than would
184 be the case for obligate herbivore-shredders, with a consequent greater reduction in energy
185 processing.

186 Studies of invasion impacts have traditionally focussed on predation and biodiversity
187 loss [2]. Here we demonstrate the potential for ecosystem level changes, simply due to the
188 behavioural NCEs of mere exposure to a novel predator. A better understanding of the role of
189 NCEs during biological invasions could enhance our ability to predict their progress and, in
190 some cases, their wider ecosystem level ramifications.

191

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195

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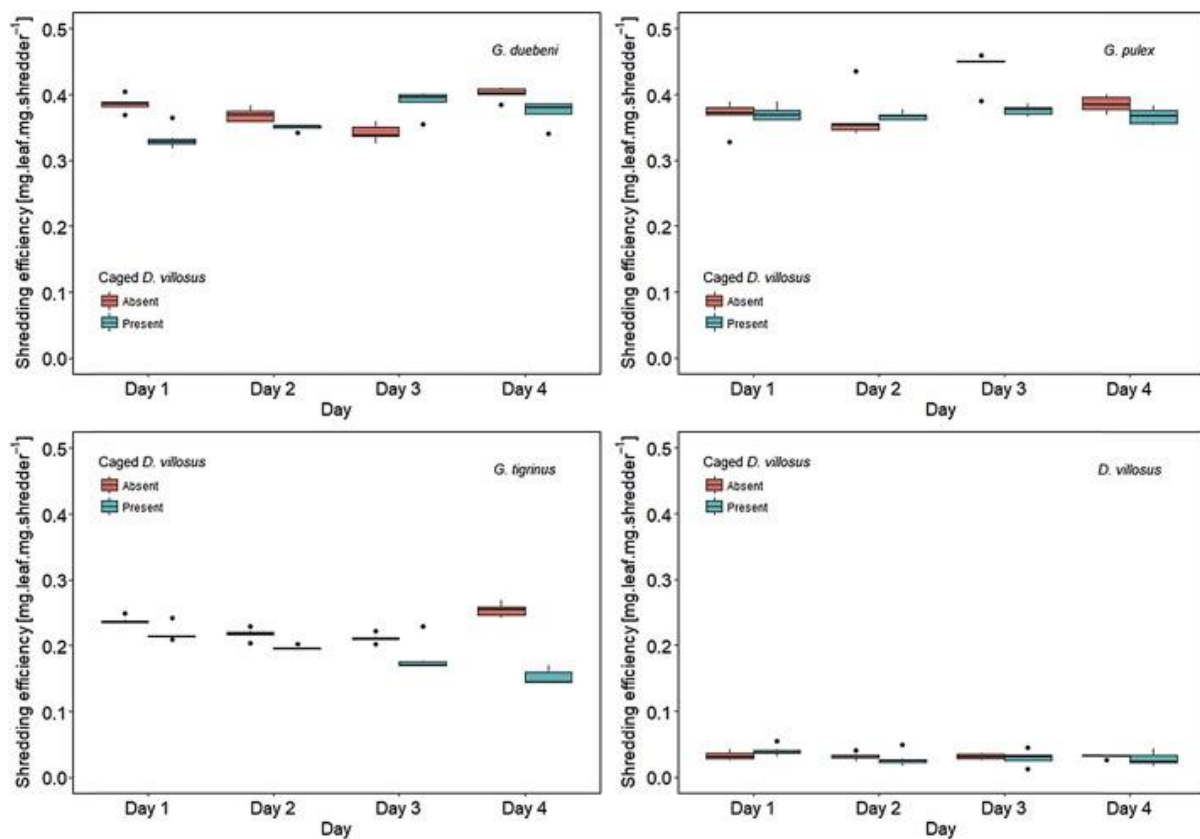
251

252 **Table 1:** Significance tests for fixed effects in the linear mixed model.

Effect	df	F	P
Predator treatment	1, 176.09	0.01	0.94
Shredder species	3, 176.09	570.51	<0.0001
Day	3, 176.09	0.31	0.58
Predator treatment x Shredder species	2, 176.09	5.12	0.002
Predator treatment x Day	3, 176.09	8.98	<0.005
Shredder species x Day	6, 176.09	10.56	<0.0001
Predator treatment x Shredder species x Day	6, 176.09	11.74	<0.0001

253

254



255

256 **Figure 1:** The three way interaction between caged *D. villosus* presence, free swimming
 257 shredder species and day, on shredding efficiency. Data for each species is shown on
 258 different panels. Lower and upper hinges show first and third quartiles respectively and
 259 whiskers represent the first and third quartiles – and + 1.5 interquartile ranges. Data points
 260 outside of these bounds are shown by dots. Untransformed raw data shown for clarity.

261 **Appendix 1**

262 **METHODOLOGICAL DETAILS**

263

264 Animal collection, holding tanks and mesocosms

265 *D. villosus* was collected from the Gouwzee lake (52°30'N 05°05'E), part of the Markermeer/IJsselmeer
266 complex near Hoorn in the Netherlands (see MacNeil et al., 2008 for site details). *G. duebeni* was
267 collected from a stream at Amstelmeer (52°53'N 4°53'E), *G. pulex* from a stream near Staverden (the
268 Hierdense Beek; 52°17'N 05°44'E) and *G. tigrinus* from a lake at Oosterpoel (52°30'N 05°05'E) near
269 Monnickendam. Each species was maintained separately in the laboratory in aerated tanks (60 x 40
270 x 10 cm deep) and acclimated in an equal mix of the 4 species' source waters (mean conductivity
271 850 μScm^{-1} for mixed water, range 707 – 890 μScm^{-1} for individual source waters) with substrate
272 such as cobbles and pebbles, macrophytes, non-amphipod fauna such as mayflies, chironomids and
273 snails and leaves from collection sites. All of the test animals can be maintained for several months
274 under these conditions (C. MacNeil, personal observation). Animals were acclimatized for seven
275 days in a light:dark cycle of 10:14 hours and a water temperature 17°C, both these being appropriate
276 for the time of year for the majority of streams sampled

277 Mesocosms consisted of plastic aquaria of 20 x 20 x 8 cm (length x width x height)
278 were supplied with aerated, filtered mixed source water (17°C; mean \pm SE dissolved oxygen
279 $9.3 \pm 0.24 \text{ mg l}^{-1}$). Five clear glass pebbles (each 2 cm diameter) were placed in the aquaria,
280 permitting animals to retreat into crevices, while still allowing observation.

281 Cages used to isolate *D. villosus* in mesocosms were plastic mesh containers (8 x 7.5 x
282 4.5 cm) and were preconstructed aquarium shop products, used to isolate individual adult fish
283 in tanks, either for treatment or to prevent adults eating eggs / fry. The mesh size was 1.8 mm.
284 The cages were housed for 12 hours in a tank of the source water 'mix' used to fill mesocosms,
285 before being used in mesocosm experiments.

286 *Acer pseudoplatanus* is an indigenous tree common at the collection sites and previous studies
287 have shown its leaves to be highly palatable to amphipods. The 6-mm diameter disks were cut using a
288 cork borer, avoiding the midribs (mean \pm SE disk wet weight of 3.9 ± 0.5 mg, n = 200). Leaf disks in
289 additional control aquaria (no animals present) showed negligible weight loss of <3% (range 0.9-2.8%
290 of weight of initial disks added) over an 8 day observation period.

291

292 **Appendix 2**

293 **STATISTICAL DETAILS**

294

295 Model assumptions (normality of residuals and homogeneity of variance) were assessed via
296 inspection of quantile and residual plots respectively. To improve the normality of residuals, data
297 were log₁₀(x+1) transformed. To allow for significance testing via F-tests, the degrees of freedom
298 were estimated using the Satterthwaite approximation method. The analysis was coded as follows:

299

300

301

302 `#requires packages:`

303 `#lme4`

304 `#lmerTest`

305

306 `data <- read.csv`

307 `ID <- data$ID`

308 `shred <- data$Shredder`

309 `dv <- data$Predator`

310 `day <- data$Day`

311 `eff <- data$efficiency`

312

313 `log.eff <- log10(eff+1)` `#log x+1 transform`

314 `m1<-lmer(log.eff ~ shred*dv*day + (day|ID))` `#LMM`

315 anova(m1)

#Significance testing

316

317