1 **Fear alone reduces energy processing by resident 'keystone' prey threatened**

2 **by an invader; a non-consumptive effect of 'killer shrimp' invasion of**

3 **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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Introduction

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

Studies on the impacts of biological invasion have traditionally focussed on competition, predation and biodiversity loss [4]. Within three decades, the Ponto-Caspian amphipod *Dikerogammarus villosus* has invaded the freshwaters of nearly twenty European countries [5]. *D. villosus* is a voracious predator consuming a vast range of macroinvertebrate taxa across different trophic groups, it can be super-abundant within invaded sites, dominating assemblages and its invasion is linked to local extinctions [5]. In the Netherlands, declines of two resident amphipods, the native *Gammarus duebeni* and a previous invader *G. tigrinus*, shortly after the arrival of *D. villosus*, have been attributed to predation by the invader [6] and another amphipod, *G. pulex*, may also be under threat of species replacement by *D. villosus* in areas of Central Europe and the U.K. [5]. Nevertheless, *D. villosus* and its resident prey can 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 allochthonus 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 ± SE = 44.2 ± 3.9 mg, 40.3 ± 3.7 mg, 24.2 ± 2.8 mg and 68.0 ± 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

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

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.

8

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.

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251

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
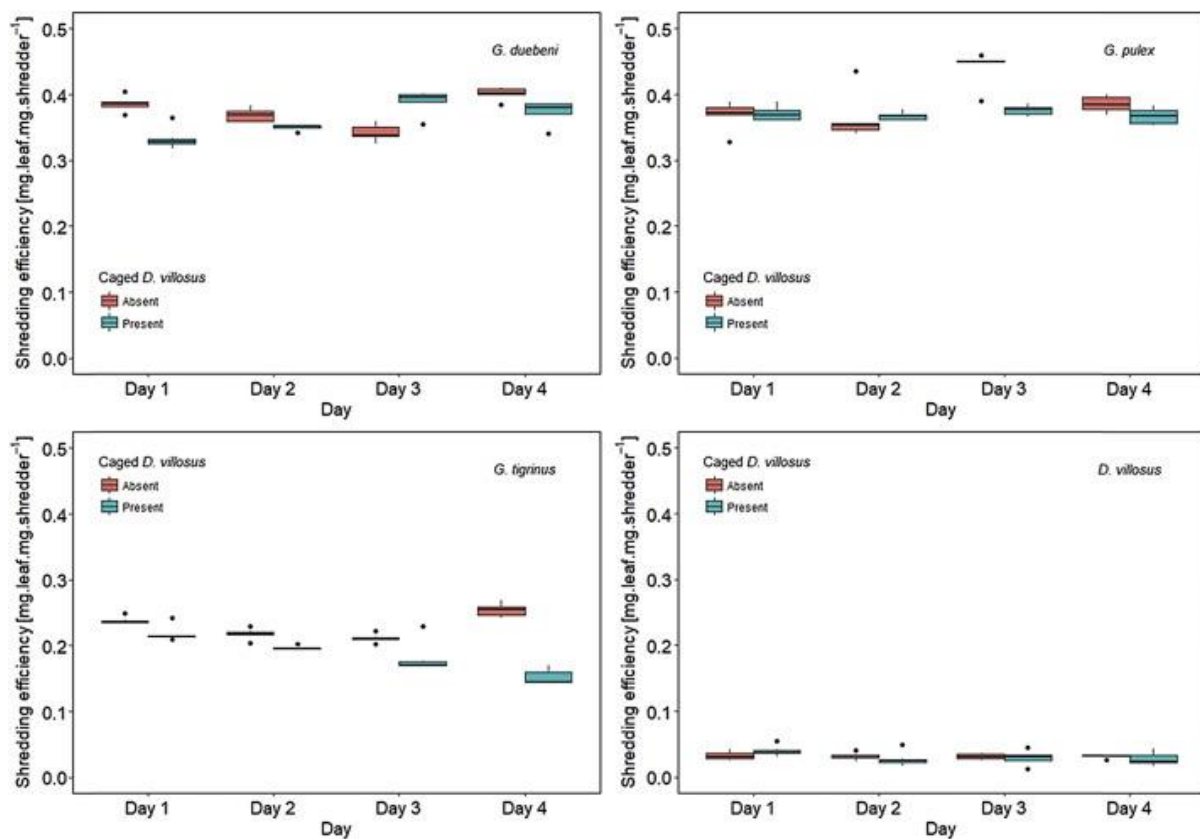
Figure 1: The three way interaction between caged *D. villosus* presence, free swimming

shredder species and day, on shredding efficiency. Data for each species is shown on

different panels. Lower and upper hinges show first and third quartiles respectively and

whiskers represent the first and third quartiles – and + 1.5 interquartile ranges. Data points

outside of these bounds are shown by dots. Untransformed raw data shown for clarity.

Appendix 1

METHODOLOGICAL DETAILS

Animal collection, holding tanks and mesocosms

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

Mesocosms consisted of plastic aquaria of 20 x 20 x 8 cm (length x width x height) were supplied with aerated, filtered mixed source water (17°C; mean ± SE dissolved oxygen 9.3 ± 0.24 mgl-1). Five clear glass pebbles (each 2 cm diameter) were placed in the aquaria, permitting animals to retreat into crevices, while still allowing observation.

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

13

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 ± 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 log.10 (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
```