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1 Variation in the immune state of *Gammarus pulex*
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15

16

17 **Abstract**

18 Temperature is known to impact host-parasite interactions in various ways. Such effects are
19 often regarded as the consequence of the increased metabolism of parasites with increasing
20 temperature. However, the effect of temperature on hosts' immune system could also be a
21 determinant. Here we assessed the influence of temperature on the immunocompetence of
22 the crustacean amphipod *Gammarus pulex*. Amphipods play a key ecological role in
23 freshwater ecosystems that can be altered by several parasites. We investigated the
24 consequences of three weeks of acclimatization at four temperatures (from 9°C to 17°C) on
25 different immunological parameters. Temperature influenced both hemocyte concentration
26 and active phenoloxidase enzymatic activity, with lower values at intermediate
27 temperatures, while total phenoloxidase activity was not affected. In addition, the ability of
28 gammarids to clear a bacterial infection was at the highest at intermediate temperatures.
29 These results suggest a dysregulation of the immune system of gammarids in response to
30 stress induced by extreme temperature.

31

32 **Keywords**

33 Gammarid, Immunocompetence, Environmental conditions, Phenoloxidase, Hemocyte,
34 Bacterial resistance

35

1. Introduction

37 Understanding the consequences of human-mediated climate changes on organism physiology or
38 ecology must take into account the effects that such changes may have on complex biotic
39 interactions (Gilman et al., 2010), including in particular host-parasite interactions (Morley and
40 Lewis, 2014). Indeed, temperature is known to be one of the most important factors affecting host-
41 parasite relationships (Murdock et al., 2012a).

42 Temperature may first affect the biology and physiology of parasites (Barber et al., 2016;
43 Gillooly et al., 2001; Harvell et al., 2002), influencing, for example, development time (Olson and
44 Pratt, 1971; Tokeson and Holmes, 1982) and infection success in helminths (Mouritsen and Jensen,
45 1997; Okaka, 1989; Studer and Poulin, 2013), or replication rate in micro-organisms (e.g. Dunn et al.,
46 2006). However, temperature can also affect hosts. In particular, the immunocompetence of hosts
47 might be of crucial importance for the successful establishment, growth, and survival of parasites
48 within their hosts (Marcogliese, 2001). This is especially true of ectotherm hosts, where a range of
49 behavioral and physiological processes is temperature-dependent. In insects, several studies
50 highlighted that, besides increasing metabolic rate, an increase in temperature has a positive effect
51 on the activity of several immune parameters, such as phenoloxidase enzymes or antibacterial
52 responses (Adamo and Lovett, 2011; Catalán et al., 2012). However, other studies have revealed that
53 such an enhanced immune activity is not the rule. Increased temperature can affect immune
54 parameters in a more complex way, depending on which immune pathway is considered (Murdock et
55 al., 2012b; Silva and Elliot, 2016), with immune responses being dependent on complex interactions
56 between temperature and time (Murdock et al., 2012b).

57 Crustacean amphipods are widespread and abundant in many freshwater and marine
58 ecosystems worldwide. They occupy a central place within food webs, where they constitute both an
59 important prey for many species (Degani et al., 1987; Friberg et al., 1994) and a predator modulating
60 other macroinvertebrate populations (Kelly et al., 2006; MacNeil et al., 1997). In particular,
61 freshwater amphipods feed on leaf litter, participating in their decaying process (Foucreau et al.,
62 2013; Piscart et al., 2009), and, thus, in the maintenance of water quality (Maltby et al., 2002).
63 Amphipods can be infected by various parasitic and pathogenic species, ranging from bacteria to
64 macro-parasites such as cestodes, trematodes or acanthocephalans (Thomas et al., 2002; Van Maren,
65 1979). Infection of amphipods can ultimately affect their functional role as predator or shredder
66 species (Fielding et al., 2003; Labaude et al., 2017; Médoc et al., 2011). Understanding how variation
67 in abiotic environment modulates the capacity of gammarids to cope with infections is therefore
68 important to assess the effect of global change on this keystone species group (Labaude et al., 2017).

69 Amphipod immunocompetence varies among natural populations, and causes of this
70 variation are poorly understood. Levels of circulating immune effectors in amphipods have been
71 found to be negatively related to parasite prevalence (Cornet et al., 2009a), and environmental
72 conditions, in particular food resources, have been shown to modulate the immune system of
73 gammarids (Babin et al., 2015, 2010). However, the effect of other environmental factors on the
74 immune system of amphipods remain undocumented.

75 Temperature is known to affect several components of the physiology of amphipods.
76 Common species in European rivers, such as *Gammarus fossarum*, *G. pulex* or *G. roeseli*, have a high
77 thermal plasticity. For example, the thermal optimum range for adult survival is comprised between
78 3°C and 17°C (Issartel et al., 2005; Maazouzi et al., 2011), but varies between populations (Foucreau
79 et al., 2014). On the other hand, Pöckl & Humpesch (1990) found that the optimal temperature for
80 reproductive success and egg survival is around 12°C for *G. fossarum* and 15°C in *G. roeseli*. Several
81 physiological parameters (e.g. oxygen consumption or glycogen content) are impacted by
82 temperature in amphipods, with temperature above 15°C negatively impacting the physiology of
83 Northern French populations of *G. pulex* (Foucreau et al., 2014). As for insects, temperature is known
84 to influence the immune system of crustaceans (reviewed by Le Moullac & Haffner, 2000), although,
85 so far, most studies have focused on crustacean species of economic interest, such as those raised
86 for farming, and mostly tropical ones (e.g. Cheng & Chen, 2000; Cheng et al., 2003, 2005). Overall,
87 these studies suggest that increasing temperature until some value tends to have a positive effect on
88 various immunological parameters, after which a decrease is observed, although this pattern varies
89 between studies and/or crustacean species. To what extent this may apply to crustacean amphipods
90 is unknown.

91 The aim of this study was therefore to measure the immunocompetence baseline of *G. pulex*
92 amphipods exposed to different temperatures within their natural range, in order to understand if
93 temperature variation may account for the variation in immunity among populations. Both the
94 inactive ProPO enzyme, representing the maintenance of the ProPO system, and its active form,
95 indicative of its activity, were measured. The number of hemocytes was counted, as well as the
96 ability of gammarids to clear a bacterial infection. Because immunocompetence may interact with
97 infection by some helminth parasites (Cornet et al., 2009b), this study was conducted on uninfected
98 gammarids, and the clearance of bacterial infection was investigated using a bacteria that did not, *a*
99 *priori*, co-evolve with amphipods. Finally, we relied on field data, collected in various rivers in winter
100 and spring, to compare if variation in phenoloxidase activity observed in our experiments were
101 reflecting those observed in the natural environment of gammarids.

103 **2. Material and methods**

104 *2.1. Sampling and acclimatization*

105 *G. pulex* (Linnaeus 1758) individuals were collected in April 2014 from a small tributary of the Suzon
106 River (Burgundy, eastern France, 47°24'12.6"N 4°52'58.2"E), using the kick sampling method with a
107 hand net. Gammarids were first maintained during one week under standard laboratory conditions
108 at 10°C, corresponding to the temperature of the river measured at the time of collection. Only
109 males showing no sign of infection with helminth parasites were kept for the experiments to avoid
110 any confounding effect of sex or parasitic infection.

111 Gammarids were randomly assigned to one of four temperature regimes: 9, 11, 14 or 17°C
112 for three weeks. This range of temperatures corresponds to naturally fluctuating temperatures
113 experienced by gammarids in their habitat (Pöckl et al., 2003) and current temperatures in Burgundy
114 (Gunn and Crumley, 1991; Rowell, 2005). Gammarids were individually maintained in plastic tubes
115 (1.5 x 4 cm) closed at both ends with fine mesh, allowing water exchanges. All tubes were then
116 placed in a tank, with one different tank being used for each temperature. Tanks were filled with a
117 mix of water from the river and dechlorinated, UV-treated tap water. Water from each tank was
118 constantly pumped toward a device (TANK TK-1000 Chiller, Teco®, Ravenna, Italy) controlling its
119 temperature. The tanks were replicated at 9°C but not at other temperatures due to a logistical
120 problem. However, all precautions were taken to avoid a tank effect. All tanks were strictly identical,
121 located next to each other in the same room, and maintenance and measures were made
122 concomitantly for each temperature. The temperature was measured every five minutes during the
123 whole experiment, using automatic thermometer recorders. As the actual temperatures that were
124 measured slightly diverged from the intended ones, we took into account the mean of actual
125 temperatures measured over the total duration of the experiment (respectively 8.8°C, 11.1°C, 14.2°C
126 and 17.0°C). Water temperature oscillated around these average values with a variation of $\pm 2^\circ\text{C}$.
127 Gammarids were fed once a week with elm leaves conditioned at 14°C, thus ensuring that food did
128 not differ between treatments, although the biofilm on leaves surface could develop at different
129 rates after leaves were provided to gammarids. Individuals were maintained under a 12:12 light:dark
130 cycle regime.

131

132 *2.2. Measures of immune system*

133 Crustaceans have an innate immune system that relies on several components (Söderhäll and
134 Cerenius, 1992; Vazquez et al., 2009). The prophenoloxidase (ProPO) cascade, in particular, is
135 involved in the response toward non-self (Söderhäll and Cerenius, 1998). The active catalyzing

136 phenoloxidase (PO) enzyme has the capacity to adhere to foreign organisms, ranging from micro-
137 organisms to macro-parasites, and induces the deposition of melanin on their surface (Cerenius and
138 Söderhäll, 2004). The inactive form of the enzyme is stored in the hemolymph and in hemocytes, and
139 is rapidly activated upon infection. Hemocytes are circulating cells that are also involved in several
140 defense mechanisms, such as phagocytosis, encapsulation, or coagulation (Johansson et al., 2000;
141 Vazquez et al., 2009).

142

143 2.2.1. Resistance to bacterial challenge

144 To assess the ability of the immune system of gammarids to clear bacterial infection, resistance to a
145 bacterial challenge was tested using the protocol described in Cornet et al. (2009). The day before
146 the experiment, tetracycline-resistant *Escherichia coli* bacteria (strain CIP 103410, Pasteur Institute,
147 Paris, France) were grown in 10 ml of broth medium (10 g of bactotryptone, 5 g of yeast extract and
148 10 g of NaCl per liter of distilled water, pH 7.0) containing 10 µl of tetracycline (2.5 mg/ml), at 30°C in
149 a shaking incubator. The solution of bacteria was then centrifuged (4°C, 15000 rpm, 30 min), and the
150 bacteria were rinsed twice using PBS buffer before being re-suspended in PBS buffer at a
151 concentration of 1×10^5 bacteria per microliter, using a counting chamber (Neubauer Improved)
152 under a compound microscope (magnification x 400).

153 Gammarids were briefly anesthetized on ice and gently immobilized on sticky gum under a
154 dissecting microscope. Their second or third coxal plate was then laterally perforated with a fine
155 sterile needle. Using a Hamilton syringe with a fine needle, 0.5 µl of the bacterial solution was
156 injected through the hole. Injected gammarids were kept individually in hermetic glass vials, to avoid
157 water contamination, and placed back at their acclimatization temperature for seven hours and
158 fifteen minutes. After this time, about half of bacteria are expected to have been cleared, and
159 variation between individuals is likely to be visible (Cornet et al., 2009a). A small hole was then
160 pierced again in the cuticle of the individual, and 2 µl of hemolymph were extracted using a sterile
161 glass capillary, and immediately diluted in 198 µl of PBS buffer. After homogenization, this mixture
162 was divided into two replicates of 100 µl that were spread on Petri dishes containing 5 µg/ml of
163 tetracycline. Petri dishes were incubated at 30°C until bacterial colonies were clearly visible, and
164 CFUs (colony-forming units) were counted using an automatic colony counter (Scan® 500 version
165 6.1.2.0, Interscience, Saint Nom, France). For each gammarid, the number of colonies was adjusted
166 to 1 µl of pure hemolymph and the mean of the two replicates was used. In four cases, no colony
167 developed on the Petri dishes. As we could not exclude that this was due to an experimental error,
168 these samples (n = 4) were removed from the data.

169 The optimal temperature for the growth of *E. coli* is around 37°C. Therefore, the
170 temperatures used in our experiments might not only have an effect on the immune system of
171 gammarids, but also on the survival of the bacteria. To provide information about this effect, a side
172 experiment was done using a similar protocol as described above, but with gammarids being
173 replaced by plastic tubes containing 50 µl of PBS buffer. After the addition of 1 µl of the bacterial
174 solution (1 x 10⁵ bacteria/µl), five tubes were kept at each temperature during 6.5 hours. Two
175 microliters of the solution were then diluted in 198 µl of PBS buffer and bacteria were spread on
176 Petri dishes and counted as described above. This additional experiment should not be seen as a
177 proper control for the bacterial replication in absence of an immune system, both because a culture
178 medium was not provided and because of low temperatures investigated. It should rather be seen as
179 a test of how *E. coli* is maintaining itself in a non-favorable habitat at different low temperatures.

180

181 2.2.2. Hemolymph extraction, hemocyte concentration and enzyme activity of the ProPO 182 system

183 Hemolymph extractions to measure immune parameters were performed on different gammarids
184 than those used for the bacterial challenge experiment. Following a brief anesthetization on ice,
185 individuals were immobilized on sticky gum under a dissecting microscope and a hole was dorsally
186 made on their cuticle using a fine sterile needle. Hemolymph was extracted using sterile pre-chilled
187 graduated glass capillaries, and the total volume that was extracted was recorded. Hemolymph was
188 immediately mixed with 20 µl of cold PBS buffer. Ten microliters of the mix were immediately used
189 to assess hemocyte concentration under a phase-contrast microscope (magnification x 400), using a
190 Neubauer counting chamber. Three types of hemocytes can be differentiated in crustaceans
191 (Johansson et al., 2000), including in amphipods (Dezfuli et al., 2008). However, because of their
192 large overlapping functions (Johansson et al., 2000), we felt conservatory to count all hemocyte
193 types. The rest of the mix was frozen in liquid nitrogen and stored at -80°C for later enzymatic activity
194 assays.

195 For all individuals, the activity of naturally activated phenoloxidase enzymes (hereafter
196 referred as “active phenoloxidase activity”) and the combined activity of PO and its ProPO
197 (prophenoloxidase) proenzyme (hereafter referred as “total phenoloxidase activity”) were measured
198 using a spectrometric assay (Cornet et al., 2009b). Hemolymph samples were thawed on ice and 5 µl
199 were added to microplate wells containing 20 µl of PBS buffer and either 140 µl of distilled water to
200 measure active phenoloxidase activity or 140 µl of chymotrypsin solution (0.07 mg per ml of distilled
201 water), used to activate the ProPO into PO, to measure total phenoloxidase activity. The enzymatic

202 reaction started with the addition of 20 μ l of cold L-Dopa solution (4 mg per ml of distilled water) in
203 each well. The reaction was allowed to proceed for 40 min at 30°C, and change in optical density was
204 followed using a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.
205 Enzymatic activity was analyzed using the SOFT-MaxPro 4.0 software (Molecular Devices, Sunnyvale,
206 CA, USA), and measured as the maximum slope (V_{max} value in OD min^{-1}) of the linear phase of the
207 reaction curve.

208 There was a strong linear relationship between gammarid size and hemolymph sampling
209 ($F_{1,216} = 97.18$, $P < 0.0001$) but the size of gammarids did not vary among temperatures ($F_{3,214} = 0.94$, P
210 $= 0.42$). Therefore, measures of hemocyte concentration and enzymatic activities were both adjusted
211 for 1 μ l of pure hemolymph for all individuals to avoid any effect of hemolymph sampling volume.

212

213 2.3. Measurements and genetic analyses

214 All the gammarids used in the experiment were measured (from the height of the fourth coxal plate,
215 see Bollache et al., 2000), using a microscope and Lucia G 4.81 software. Although the cryptic species
216 *G. fossarum* can also occur in the river, *G. pulex* is known to be largely dominant (Lagrue et al., 2014).
217 Genetic analyses were therefore conducted on 20% of tested individuals ($n = 88$), that confirmed the
218 large majority of *G. pulex* (95%). Thus, apart from these *G. fossarum* individuals that were removed,
219 all other individuals were considered as belonging to *G. pulex*.

220

221 2.4. Variation of active phenoloxidase activity in the field

222 To investigate whether temperature-dependent variation in immune parameters could explain
223 natural variations observed between populations in the field, eleven gammarid populations were
224 investigated twice, in winter 2006-2007 (November-February, sampling dates different according to
225 sites) and spring 2007 (early June for all sites). Gammarids were sampled at some sites described in
226 Cornet et al. (2007) in rivers surrounding Dijon: Corberon (river Meuzin downstream), Dijon (river
227 Ouche intermediate location), Grancey le Chateau (river Ruisseau des Tilles), Lusigny sur Ouche (river
228 Ouche upstream), Les Maillys (river Tille downstream), Noiron sur Bèze (river Bèze), Saulon la
229 Chapelle (River Sansfond), Talmay (river Vingeanne), Tarsul (river Tille upstream), Trouhans (river
230 Ouche downstream), and Villy le Brulé (river Meuzin upstream). At each site and on each sampling
231 occasion, temperature was recorded to the nearest 0.1°C. Gammarids were captured with a hand net
232 and immediately brought to the laboratory. For each site and for each season (winter or spring), 28

233 to 48 individuals were measured, and their hemolymph was extracted to measure the active
234 phenoloxidase activity, as described above.

235

236 *2.5. Statistical analyses*

237 Data from resistance to bacterial challenge were natural-log transformed to meet homoscedasticity
238 conditions and analyzed using a linear model with gammarid size and temperature as covariates. A
239 quadratic effect of temperature was also considered, after visual inspection of the results. Similarly,
240 bacterial dynamics in tubes under different temperatures were natural-log transformed and analyzed
241 separately using a linear model, with temperature and its quadratic effect as factors.

242 Data on hemocyte concentration (number of hemocytes per microliter of pure hemolymph)
243 were square-root transformed. Active and total phenoloxidase enzymatic activities were transformed
244 using Box-Cox procedures. As the three parameters were measured on the same individuals, a
245 multivariate analysis of variance (MANOVA, Pillai's trace) was conducted, testing for the influence of
246 gammarid size, temperature, temperature's quadratic effect, and their interactions. Linear models
247 were then used for each parameter, with the same factors. Difference between temperatures can be
248 assessed by the degree of overlap of the 95 % confidence intervals (CI), following the
249 recommendations of Cummings and Finch (2005). The difference can be considered significant when
250 the 95% CI did not overlap on more than half of their length (see Fig. 1 and 2 in supplementary
251 materials).

252 For field data, the active phenoloxidase activity was log-transformed, and a nested ANOVA
253 was conducted testing for the influence of gammarid size, season (winter vs spring) nested within
254 sampling site, and sampling site as sources of variation.

255 Non-significant interactions were removed for all models. Data were analyzed using JMP
256 version 10.0.0 software (SAS Institute, Cary, NC, U.S.A.).

257

258 **3. Results**

259 Statistical analyses showed that both hemocyte concentration and total phenoloxidase activity
260 slightly differed between the two replicates at the lowest temperature, while other parameters did
261 not (table 1 in supplementary materials). However, using one replicate or the other did not affect the
262 results of our analyses on the effect of temperature (tables 2 and 3 in supplementary materials).
263 Therefore, data from the two replicates were pooled in the following analyses.

264

265 *3.1. Bacterial survival*

266 The number of bacterial colonies was negatively affected by gammarid size, and showed a convex
267 relationship with temperature (Table 1, Fig. 1A). Examination of 95% CI suggests that clearance at
268 14.2°C was more efficient than at 8.8°C and 17°C (Fig. 1 in supplementary materials). The number of
269 *in vitro* bacterial colonies was significantly affected by temperature, with a linear increase of
270 surviving bacteria with increased temperature (Table 1, Fig. 1B).

271

272 *3.2. Immunological parameters*

273 All three immunological parameters were significantly affected by gammarid size, temperature and
274 its quadratic effect (MANOVA, Table 2). The quadratic effect of temperature on the combination of
275 the three immunological parameters (as estimated by the coordinates on the first canonical axis,
276 which were generated using the eigenvectors used to construct multivariate test statistics) is
277 illustrated in Fig. 2A. As suggested by 95% CI, the global immunocompetence of gammarids was
278 higher at low and high temperatures, and lower at intermediate ones (Fig. 2 in supplementary
279 materials). When analyzing these immunological parameters separately with linear models,
280 gammarid size was found to have a significant effect only on active phenoloxidase activity, with
281 larger animals showing a lower active phenoloxidase activity for 1 µl of hemolymph (Table 2). Active
282 phenoloxidase activity was also close to be significantly affected by the quadratic effect of
283 temperature, with a decrease of activity between 8.8 and 11.1°C, followed by an increase (Table 2,
284 Fig. 2C). The effect of temperature on hemocyte concentration was also marginally non-significant
285 (Table 2, Fig. 2B). For these two parameters, in accordance with the analyses, 95% CI were not able
286 to firmly discriminate between temperatures (Fig. 2 in supplementary materials). No factor was
287 found to significantly influence the total phenoloxidase activity (Table 2, Fig. 2D).

288

289 *3.3. Variation of active phenoloxidase activity in the field*

290 In winter, the average temperature among the 11 sites was 8.4°C ± 0.41 (s.e.m.; min: 6.1°C at
291 Corberon, max: 9.4°C at Lusigny and Noiron). In spring, the average highest temperature was 13.7°C
292 ± 0.49 (min: 11.4°C at Lusigny, max: 15.8°C at Trouhans). These temperatures therefore fall within
293 the range of our lowest and medium range experimental temperatures, respectively.

294 The nested ANOVA (Global model: $F_{22, 784} = 4.02$, $P < 0.0001$; $R^2 = 0.10$) showed that, despite
295 variation among populations ($F_{10, 784} = 5.71$, $P < 0.0001$, Fig. 3A) and a negative effect of gammarid
296 size on the level of active phenoloxidase activity ($F_{1, 784} = 8.05$, $P = 0.005$), the active phenoloxidase
297 activity, nested within population, was higher at winter temperature than at spring temperature ($F_{11,$
298 $784} = 2.28$, $P = 0.01$, Fig. 3B).

299

300 4. Discussion

301 Our results show that the maintenance of *G. pulex* individuals at different temperatures (around 9,
302 11, 14 and 17°C) during three weeks led to differences in several basal components of their immune
303 system. Overall, immunological parameters were found to vary according to a quadratic effect of
304 temperature. Combination of hemocyte concentration, active phenoloxidase activity and total
305 (ProPO + PO) enzymatic activity was lower at intermediate temperatures, but the ability to clear
306 bacterial infection was higher at these intermediate temperatures.

307 Variation in temperature affected the ability of gammarids to clear a bacterial infection.
308 Indeed, although bacterial survival *in vitro* presented a positive linear relationship with increasing
309 temperature, the CFU concentration in the hemolymph of gammarids decreased between 9°C and
310 14°C, before increasing again. Thus, the ability of gammarids to deal with bacteria increased with
311 temperature, but only to a certain extent, in accordance with other results obtained in crustaceans
312 (Cheng et al., 2003) or in insects (Catalán et al., 2012). In the crab *Carcinus maenas*, for example,
313 Chrisholm & Smith (1994) evidenced a seasonal change in the antibacterial activity by hemocytes
314 according to temperature. The antibacterial activity was the lowest at both the lowest and highest
315 water temperatures of the year. Although this result seems contradictory with the other immune
316 parameters measured in our study (see discussion below), defense against *E. coli* was already found
317 to show no correlation with basal PO activity in *G. pulex* (Cornet et al., 2009a), suggesting that
318 resistance against bacteria might go through other immune pathways. Some authors even suggested
319 the existence of a trade-off between antibacterial activity and PO activity (Moret and Schmid-
320 Hempel, 2009; Siva-Jothy et al., 2005).

321 The overall lower level of other components of immune activity at intermediate
322 temperatures seemed to be mostly due to the hemocyte concentration and active phenoloxidase
323 activity. Indeed, the two parameters were at their lowest value at intermediate temperatures
324 (respectively 14°C and 9°C), and increased at lower and higher temperatures. This similarity is
325 consistent with the positive correlation already reported between these two parameters in other
326 crustaceans (Cheng et al., 2005). However, the range of temperatures chosen in our study did not
327 induce any effect on the total phenoloxidase activity of gammarids. Active phenoloxidase activity

328 represents the amount of naturally active enzymes, while total phenoloxidase activity also takes into
329 account the inactive ProPO enzymes present in the hemolymph, thus rather measuring the
330 maintenance of the ProPO system (Söderhäll and Cerenius, 1992). It seems therefore that the
331 acclimatization temperature had an effect on the use of the ProPO system, without influencing its
332 maintenance. Consistent with this result, the total phenoloxidase enzymatic activity was found to
333 show low variations between years in several populations of *G. pulex*, while their PO activity was
334 highly variable (Cornet et al., 2009a), suggesting that investment in the maintenance of the ProPO
335 system resulted from local adaptation, whereas its activation depended on proximate environmental
336 conditions (Cornet et al., 2009a). Our results therefore suggest that one relevant environmental
337 condition could be temperature, and the present field data confirmed a part of the experimental
338 results. Because the tanks were not replicated in our experiment, part of the variation observed
339 could be due to a tank effect. Such pseudo-replication is a recurrent problem in thermal biology
340 literature, and future studies should seek to overcome it when possible. In our study, all possible
341 precautions were taken to avoid a tank effect. In addition, field data confirmed the impact of
342 temperature on gammarids immune system. At each of the eleven sampling sites, lowest
343 temperatures taken in winter and highest taken in spring fell within the frame of our lowest and
344 middle-range experimental temperatures, respectively. The active phenoloxidase activity measured
345 on the animals captured at these sites varied between sites. Even after taking this variation into
346 account, individuals sampled during winter had a higher active phenoloxidase activity than those
347 sampled during spring. These data are therefore compatible with our experimental ones, where
348 gammarids exposed to low temperatures (around 9°C) had higher active phenoloxidase activity than
349 those exposed to medium-range temperatures (around 14°C). Our data nevertheless did not include
350 temperatures higher than 16°C, such that we are not able to extrapolate the effect of higher
351 temperatures at these sites. A long term study with monthly records of rivers temperature and
352 measures of gammarids immune system would bring a better understanding of variations linked to
353 temperature on a finer scale.

354 While it is widely recognized that temperature can alter the immune system of crustaceans,
355 there is no consensus about the direction of these alterations, as many studies have found
356 contradictory results (Le Moullac and Haffner, 2000). However, it is generally accepted that increased
357 temperatures tend to have a positive effect on crustacean immunocompetence, at least to a certain
358 extent (see Le Moullac & Haffner, 2000; Matozzo et al., 2011 and references therein). Here, highest
359 levels of immune defense were found at both the highest and lowest temperatures tested. Contrary
360 to many studies testing the effects of extreme temperatures (e.g. Gomez-Jimenez et al., 2000;
361 Pascual et al., 2003; Brockton & Smith, 2008), the temperature conditions chosen here were within
362 the optimal thermal range of gammarids (Issartel et al., 2005; Pöckl and Humpesch, 1990), and are

363 consistent with the temperatures currently recorded in Burgundy (Gunn and Crumley, 1991; Rowell,
364 2005), thus providing information about changes most likely experienced by gammarids following
365 fluctuations of temperatures in their natural habitat (Cornet et al., 2009a; Pöckl et al., 2003). The
366 effects of temperature on different components of the immune system of ectotherms have been
367 found to be dependent on the time of exposure, with rapid modifications occurring early after
368 temperature modification, followed by a stabilization of the different parameters over time (Pan et
369 al., 2008; Raffel et al., 2006). In bivalves transferred from 17°C to 11°C, 23°C or 28°C, hemocyte
370 density reached its highest value at 23°C and its lowest value at 28°C within one hour. However, after
371 three days of acclimatization, the shape of the effect of temperature was similar to the one found in
372 our study, with the highest value being at the lowest temperature (Chen et al., 2007). Thus, the
373 relatively long acclimatization time chosen in our study allowed us to observe temperature effects
374 due to individual plasticity.

375 The highest levels of immune defense associated to low and high temperatures observed in
376 our experiments may *a priori* suggest that extreme temperatures enhance the immune protection of
377 gammarids. However, this is not consistent with previous findings in *Gammarus* (e.g. Pöckl &
378 Humpesch, 1990), according to which intermediate temperature values were those for which
379 physiological functions (other than immunity) are the most efficient. This raises the question about
380 how to interpret changes in circulating immune effectors with respect to actual host
381 immunocompetence. In other words, should we consider higher levels of circulating immune defense
382 to be a good estimation of host immunocompetence or, alternatively, to be indicative of a
383 dysregulation of the immune system in response to stress? On the one hand, our results on baseline
384 immune measurements suggest that while highest temperatures reduced gammarids ability to deal
385 with microbial infections, they may, in contrast, improve their resistance to macro-parasites through
386 higher defense potentialities (higher active phenoloxidase activity or hemocyte counts). However,
387 both the prevalence of infection by macro-parasites and parasite growth have been found to be
388 increased with increasing temperatures in amphipods (Dunn et al., 2006; Mouritsen and Jensen,
389 1997; Tokeson and Holmes, 1982). This can be then hardly explained by a positive effect of high
390 temperature on the basal immunocompetence of gammarids. Higher infection parameters at high
391 temperatures could then derive from other effects of temperature on gammarids. First, high
392 temperature, beside any direct effect on immunity, might constitute a general physiological stress.
393 For instance, the effects of ocean acidification on a lobster were more pronounced at high
394 temperatures, while temperature alone did not influence its immune system (Hernroth et al., 2012).
395 Moreover, high temperatures can be linked to other stressors, such as lower water oxygenation.
396 Second, interactions between parasites and their hosts are not restricted to the hosts' immune
397 system, and changes in other traits might have an impact on parasite infection success. For instance,

398 temperature has been shown to increase food consumption in gammarids (Foucreau et al., 2016;
399 Labaude et al., 2017; Pellan et al., 2015), thus increasing their probability of consuming parasite eggs,
400 and, hence, becoming infected. Moreover, temperature is also known to have direct effects on
401 parasites themselves, affecting several traits linked to infection (Barber et al., 2016; Gillooly et al.,
402 2001; Harvell et al., 2002).

403 On the other hand, it is often suggested that environmental variation leads to stress-induced
404 immunosuppression in crustaceans (Le Moullac and Haffner, 2000). However, crabs transferred from
405 17°C to 4°C or 30°C for seven days displayed higher PO activity, as well as increased hemocyte
406 proliferation, compared with those which were maintained at intermediate temperature, which is
407 optimal for their physiology (Matozzo et al., 2011). Similar results were found in a mollusk exposed
408 for one day to temperatures ranging from 20 to 32°C, with hemocyte counts being minimal for
409 intermediate temperature values (Cheng et al., 2004). Therefore, one may consider that the optimal
410 level of hemocyte counts or active phenoloxidase activity (in the absence of parasite infection, i.e.
411 without any immune response) may not correspond to highest values, but rather the lowest ones.
412 Since extreme temperatures induce tissue damages in some crustaceans (Madeira et al., 2015), an
413 interpretation of the higher immune parameters at extreme temperatures could be that the immune
414 system is dealing with tissue damages imposed by these temperatures. Whatever the underlying
415 cause, this would mean that extreme temperatures would induce an over-functioning of some
416 immune defenses, with the risk of increasing immune pathologies. Indeed, the release of cytotoxic
417 compounds, resulting from enhanced immune activity, can lead to self-damages on host tissues and
418 organs (Sadd and Siva-Jothy, 2006). In gammarids, between-population variation in the level of PO or
419 ProPO enzymes positively correlates with the level of circulating carotenoids, suggesting that these
420 anti-oxidant molecules are important to limit the negative effects of high levels of toxic compounds
421 delivered through this immune pathway (Cornet et al., 2007). Experimental results verified this
422 hypothesis (Babin et al., 2015, 2010), suggesting that high levels of immunocompetence can be
423 detrimental if not compensated by high levels of carotenoid storage. Therefore one possibility is that
424 extreme temperatures induce higher circulating immune effectors because of the immune system
425 response to stress.

426 In the context of global change, temperature is predicted to increase in several parts of the
427 world, including temperate areas. Our results suggest that a moderate raise in temperature, within
428 the range studied here, could affect amphipod populations. First, amphipod could show an increased
429 sensitivity to bacterial infection, particularly in the context of an up surging of wildlife pathogens
430 (Johnson and Paull, 2011). Second, dysregulation in the level of basal immunocompetence due to
431 temperature-related stress could further decrease the ability of amphipods to face increased levels
432 of infection. As a secondary consequence, an increase temperature, through its effect on the general

433 condition of amphipods, may alter their role in ecosystems, for example by limiting their shredding
434 ability. However, given the complex effects of multiple stressors on interspecific interactions,
435 communities and ecosystems (e.g. Woodward et al., 2010; Labaude et al., 2015), further
436 investigations are necessary to assess the precise effect of an increase in temperature on the
437 functional role of amphipods within natural ecosystems. Moreover, the consequences of a raise of
438 temperature beyond the range studied here can hardly be extrapolated from our results.

439 Finally, this study investigated the effects of temperature on a short term, at the ecological
440 scale. At longer, evolutionary, time-scales, gammarids might adapt to changes in temperature, and
441 eventually evolve differences in their immune system. It would thus be valuable to assess if such an
442 adaptation does select for higher immunocompetence, in response to the possible increase in
443 infection probability, or rather to a lower level of circulating immune effectors, thus reducing the
444 cost of immunity.

445

446

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449

450 **Competing interests**

451 The authors declare no competing interests.

452

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638

639 Table 1.

640 Linear model (ANOVA) analyzing the number of bacteria remaining in 1 μ l of gammarid hemolymph
641 (“bacteria in gammarids”), or remaining in PBS (“bacteria *in vitro*”), as a function of gammarids size,
642 temperature and its quadratic effect (temperature²).

Models	Source of variation	<i>num d.f.</i> ^a , <i>den d.f.</i> ^b	F	P
Bacteria in gammarids	Global Model	3, 167	4.90	0.027
	Size	1, 167	7.56	0.0067
	Temperature	1, 167	2.24	0.14
	Temperature ²	1, 167	6.94	0.0093
Bacteria <i>in vitro</i>	Global Model (temperature)	1, 23	5.01	0.036

643 ^a Degrees of freedom of the numerator

644 ^b Degrees of freedom of the denominator

645 Non-significant interactions were removed from the model. Significant values are presented in bold.

646 Table 2.

647 Multivariate (MANOVA, Pillai's trace) and univariate (ANOVA) analyses of variance for the three
648 immunological parameters (hemocyte concentration, active and total phenoloxidase enzymatic
649 activity) as a function of gammarids size, temperature and its quadratic effect (temperature²).

Models	Source of variation	<i>num d.f.^a, den d.f.^b</i>	F	P
MANOVA	Global Model	9, 633	4.83	< 0.0001
	Size	3, 209	9.28	< 0.0001
	Temperature	3, 209	3.21	0.024
	Temperature ²	3, 209	4.69	0.0034
ANOVA hemocyte concentration	Global Model	3, 211	2.22	0.086
	Size	1, 211	2.32	0.13
	Temperature	1, 211	3.24	0.073
	Temperature ²	1, 211	2.02	0.16
ANOVA active PO	Global Model	3, 211	7.36	0.0001
	Size	1, 211	15.49	0.0001
	Temperature	1, 211	3.14	0.078
	Temperature ²	1, 211	3.61	0.059
ANOVA total PO	Global Model	3, 211	0.25	0.86
	Size	1, 211	0.29	0.59
	Temperature	1, 211	0.05	0.82
	Temperature ²	1, 211	0.19	0.66

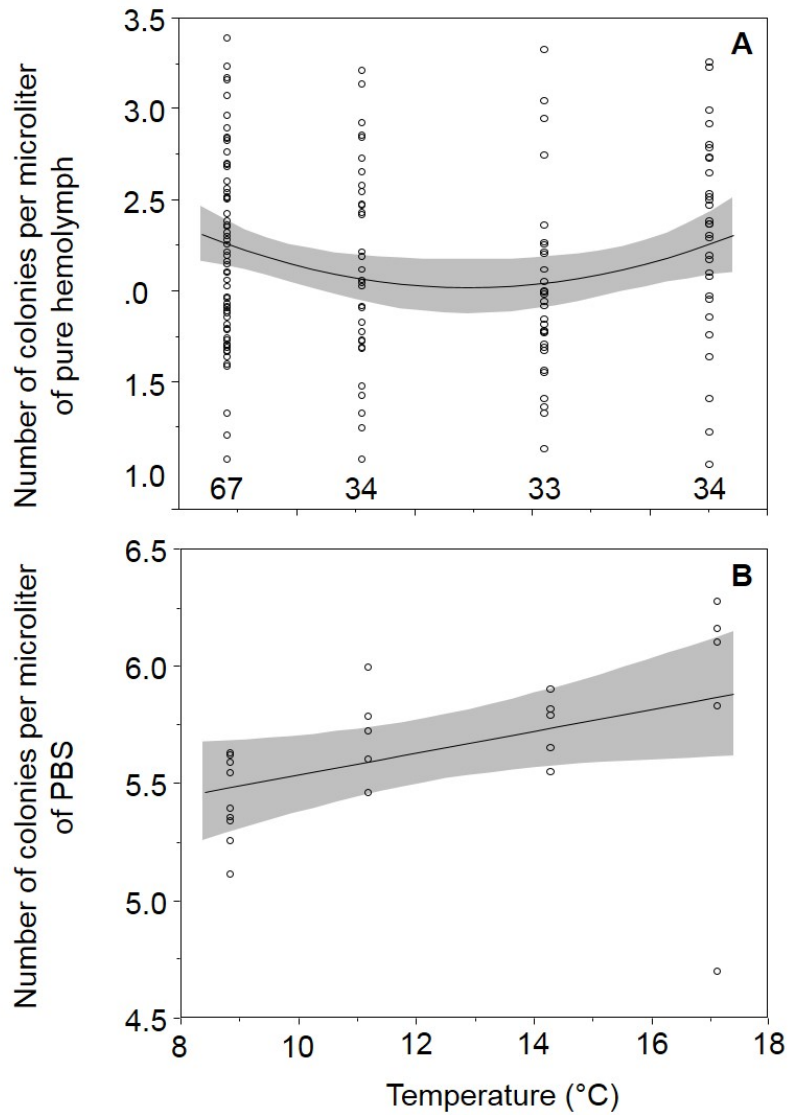
650 ^a Degrees of freedom of the numerator

651 ^b Degrees of freedom of the denominator

652 Non-significant interactions were removed from the model. Significant values are presented in bold.

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656 Fig. 1.

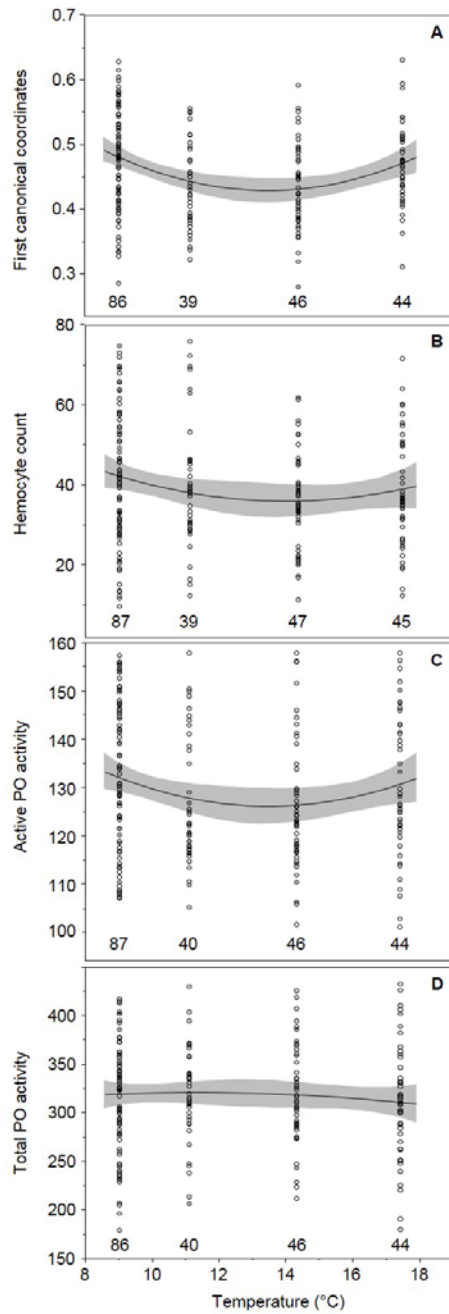
657 Effect of temperature on the number of bacteria. (A) Number of bacteria (colony-forming units)

658 remaining in 1 μ l of gammarid hemolymph; (B) Number of bacteria remaining in 1 μ l of PBS buffer.

659 Dots are individual data (the transformed data are presented here), the line is the predicted fit and

660 the grey area stands for its 95% confidence interval.

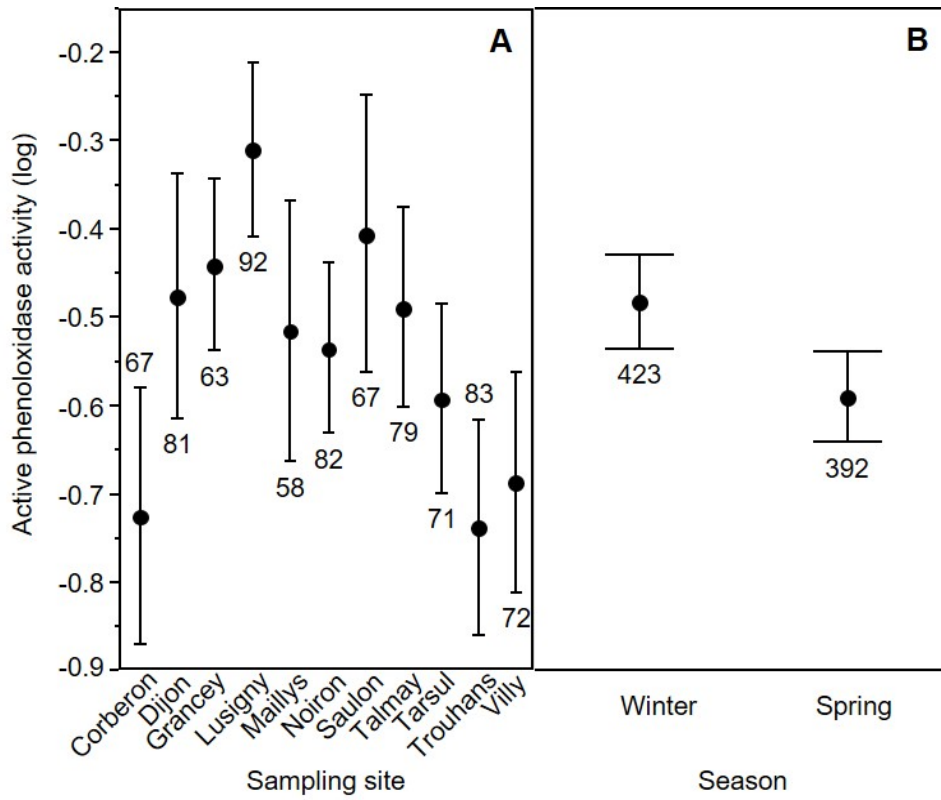
661



662

663 Fig. 2.

664 Effect of acclimatization temperature on the different immunological parameters. (A) Coordinates of
 665 the first canonical axis generated by the MANOVA (combination of the three immunological
 666 parameters), (B) hemocyte concentration, (C) active phenoloxidase activity and (D) total
 667 phenoloxidase activity. The transformed data are presented here. Dots are individual data (the
 668 transformed data are presented here), the line is the predicted fit and the grey area stands for its
 669 95% confidence interval.



670

671 Fig. 3.

672 Effects of sampling site (A) and season (B) on the active phenoloxidase activity of gammarids
 673 captured in the field. The transformed data are presented here. Dots represent mean values, error
 674 bars stand for 95% confidence intervals and sample sizes are indicated.

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