

1 **Phenotypic responses of invasive species to removals affect ecosystem functioning**
2 **and restoration: implications for invasion management**

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5 Running title: Invaders response to removal impacts ecosystem

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25

26 ***Abstract***

27 Reducing the abundances of invasive species by removals aims to minimize their
28 ecological impacts and enable ecosystem recovery. Removal methods are usually
29 selective, modifying phenotypic traits in the managed populations. However, there is
30 little empirical evidence of how removal driven changes in multiple phenotypic traits
31 of surviving individuals of invasive species can affect ecosystem functioning and
32 recovery. Overcoming this knowledge gap is highly relevant because individuals are
33 the elemental units of ecological processes and so integrating individual-level responses
34 into the management of biological invasions could improve their efficiency. Here, we
35 provide novel demonstration that removals by trapping, angling and biocontrol from
36 multiple lakes of the globally invasive crayfish *Procambarus clarkii* induced
37 substantial changes in multiple phenotypic traits. A mesocosm experiment then
38 revealed that these changes in phenotypic traits constrain recovery of basic ecosystem
39 functions (decomposition of organic matter, benthic primary production) by acting in
40 the opposite direction than the effects of reduced invader abundance. However, only
41 minor ecological impacts of invader abundance and phenotypic traits variation
42 prevailed a year after its complete eradication. Our study provides quantitative evidence
43 to an original idea that removal driven trait changes can dampen recovery of invaded
44 ecosystems even when the abundance of invasive species is substantially reduced. We
45 suggest that the phenotypic responses of invaders to the removal program thus have
46 strong effects on ecosystem recovery and should be considered within the management
47 of biological invasions, particularly when complete eradication is not achievable.

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51 ***Introduction***

52 The common goal of invasive species management is to restore the ecosystem
53 properties and functions to their pre-invaded state, including in native biodiversity and
54 ecosystem services (Bellard *et al.*, 2016; Kopf *et al.*, 2017). Considerable resources are
55 deployed globally to manage invasive species, yet the outcomes of this management
56 remain equivocal, with numerous failures to permanently reduce or eradicate invaders
57 and, ultimately, achieve biodiversity and ecosystem recovery (Pluess *et al.*, 2012; Kopf
58 *et al.*, 2017). A central tenet of invasive species management is that removal programs
59 reduce the ecological impacts of invaders through decreasing their abundance (Hulme
60 2006). However, this approach does not consider phenotypic responses to the removals
61 in the surviving individuals.

62 While the ecological impacts of biological invasions are determined by the
63 number of individuals in the invasive populations, it is also affected by the *per capita*
64 ecological effects of individuals (Parker *et al.*, 1999; Dick *et al.*, 2017). Removal efforts
65 generally involve selective methods, including harvesting (*e.g.* fishing), and
66 applications of biocides and biological agents (Myers *et al.*, 2000; Britton *et al.*, 2011).
67 This selective removal of individuals from populations can become a principal driver
68 of rapid trait change (*e.g.* in behavior, morphology and life-history traits) as driven by
69 phenotypic plasticity and selection (*i.e.* contemporary evolution Mimura *et al.*, 2017;
70 Fugère & Hendry 2018). This is important, because intraspecific phenotypic trait
71 variability can have strong effects on ecosystem functioning (Des Roches *et al.*, 2018;
72 Palkovacs *et al.*, 2018; Raffard *et al.*, 2019), and the distribution of phenotypic traits
73 across invasive populations influences the rate, extent and impacts of their invasion
74 (Britton *et al.*, 2011; Evangelista *et al.*, 2019). For example, a recent study revealed that
75 harvest induced reduction in activity of gray snapper (*Lutjanus griseus*) decreased

76 nutrient supply to the water column in a coastal ecosystem (Allgeier et al. 2020). Thus,
77 it can be predicted that invaded ecosystems will suffer additional ecological impacts if
78 removals induce strong trait changes in the surviving individuals (Závorka *et al.*,
79 2018a). However, there remains a considerable knowledge gap in how ecological
80 impacts manifest from reduced invader abundances and any consequent removal-
81 induced trait changes.

82 Evidence also suggests that historic variation in invader abundance can affect
83 the dynamics of the ecosystem following eradication of invader, thus altering the long-
84 term trajectory of ecosystem recovery (Marchante *et al.*, 2009; Reynolds *et al.*, 2017).
85 Therefore, it can also be expected that, should the removal-induced trait changes of an
86 invader occur before its complete eradication from an ecosystem, these trait changes
87 will affect the long-term trajectory of ecosystem recovery. However, there is scant
88 knowledge on how historic intraspecific variation in phenotype and abundance within
89 managed populations of invasive species affects the long-term trajectory of ecosystem
90 recovery after eradication, despite this information being of high importance to
91 managers whose aim is to reduce invasion impacts using removal methods.

92 The aim of this study was first to quantify the effects of removal programs on
93 the phenotypic traits of the invasive red swamp crayfish (*Procambarus clarkii*), a high
94 impacting global invader (Souty-Grosset *et al.*, 2016) that has been subjected to
95 numerous control attempts (*e.g.* Aquiloni *et al.*, 2010). We compared a suite of
96 ecologically important traits among invasive populations from lakes with and without
97 removal programs, where removals are through trapping, angling and biocontrol. We
98 then used experimental mesocosms to decouple the effects of reduced crayfish
99 abundance from removal-induced phenotypic changes on macroinvertebrate
100 community and ecosystem functioning (benthic and pelagic primary production, litter

101 decomposition, ecosystem metabolism and nutrient cycling). The use of an
102 experimental approach is important for teasing apart of the two effects, given that
103 removal programs typically reduce abundance whilst simultaneously inducing pressure
104 that can drive trait changes (caused by phenotypic plasticity and selection) in the target
105 invasive species. Finally, we removed all crayfish from the mesocosms to simulate a
106 successful and complete eradication and re-evaluated the macroinvertebrate community
107 and ecosystem functioning a year later to determine the long-term trajectory of
108 ecosystem recovery. The three approaches enabled testing of the following hypotheses:
109 *i)* removal programs induce changes in ecologically significant phenotypic traits of the
110 invasive species, *ii)* the direction of the ecological effects induced by invader trait
111 changes and abundance reduction are opposite and can reduce the efficiency of removal
112 programs, and *iii)* historic variation in invader phenotype and abundance alters the long-
113 term trajectory of ecosystem recovery.

114

115 ***Materials and methods***

116 *Study area*

117 The study was conducted from May 2017 to August 2018. We used a well-studied
118 model system of invasive populations of red swamp crayfish *Procambarus clarkii* that
119 have invaded gravel-pit lakes along the flood plain of the Garonne River in
120 southwestern France (Alp *et al.*, 2016; Zhao *et al.*, 2016; Jackson *et al.*, 2017; Raffard
121 *et al.*, 2017; Evangelista *et al.*, 2019). Invasive red swamp crayfish was introduced into
122 the study area in the mid-1990's and virtually all lakes are now colonized by the species.
123 Red swamp crayfish occur primarily in the littoral habitats of these lakes (Jackson *et*
124 *al.*, 2017). The present study was performed using invasive crayfish collected in six
125 gravel pit lakes (mean \pm SD water surface: 11 \pm 7 ha and water depth: 2.8 \pm 1.1 m) that

126 were generally similar in their biotic and abiotic conditions, but differed in the presence
127 / absence of a program dedicated to remove invasive red swamp crayfish (*i.e.* removal
128 programs, Supplement SI 1). Three lakes (BID, BVI, LIN) have invasive crayfish being
129 removed by a combination of angling, trapping and the introduction of predatory fish
130 (see Supplement SI 1), while the three other lakes (CEA, SAB, SOA) have never been
131 subjected to any removal programs. Angling by hoop nets and introductions of
132 predatory fish in the three lakes with removals programs have been on-going for more
133 than 20 years prior the experiment, while trapping commenced 10 years and 1 year prior
134 the experiment in BID and LIN respectively. All other biotic and abiotic environmental
135 factors related to lake hydro-morphology, water quality, crayfish density did not
136 significantly differ between the two groups of lakes (Supplement SI 1), indicating that
137 the main difference between these groups was the presence/absence of crayfish removal
138 programs. In addition, crayfish populations from lakes with and without removal
139 programs displayed very similar genetic characteristics in term of expected
140 heterozygosities, allelic richness, private allelic richness and within-population genetic
141 uniqueness value. Invasive crayfish populations in the area were highly structured
142 genetically, indicating that gene flow between lakes is extremely limited and that each
143 lake represent a genetically distinct population, except for lakes BVI and LIN which
144 belong to the same genetic cluster (Paz-Vinas *et al.*, *unpublished data.*). Consequently,
145 phenotypic differences between populations were assumed to be the direct outcomes of
146 phenotypic trait changes induced by the removal program applied in the lakes.

147

148 *Crayfish phenotypes scoring*

149 Red swamp crayfish were collected between May 29th and June 2nd 2017 using pairs of
150 baited Promar mesh 503 and 501 traps set overnight (Alp *et al.*, 2016). Trapping can be

151 a selective method of crayfish sampling, reducing the variation of phenotypes among
152 captured individuals compared to the variation in the whole population (Biro &
153 Dingemanse, 2009). This results from issues such as trap selectivity arising from the
154 mesh and entrance sizes used (Green *et al.*, 2018), and their deployment in specific
155 habitats and/ or their use in the presence/ absence of predators. However, the sampling
156 method used here has recently been shown to be highly efficient (De Palma-Dow *et al.*,
157 2020), and we used the same method and effort to collect crayfish in all lakes. For this
158 reason, the chance of sampling bias across the lakes was minimised and was considered
159 unlikely to increase phenotypic differences among the populations with and without
160 removal programs. A total of 238 individual crayfish were collected (BID: 40, BVI: 42,
161 LIN: 40, CEA: 40, SAB: 40, and SOA: 36, respectively). These were then transported
162 to the experimental facility and kept in aerated holding tanks (one population per tank,
163 cattle tank: 550 L) containing shelters and covered by a mesh net.

164 On June 3rd 2017, each individual crayfish was measured (carapace length to
165 0.01 mm, body mass to 0.01 g). Mean (\pm SD) of carapace length and body mass was
166 46.98 ± 4.75 mm and 24.75 ± 8.98 g, respectively. Then, chelae strength was quantified
167 with individual pinching a sensor (Magtrol SA, Switzerland), which recorded the
168 maximum applied force (nearest 0.001 N). To induce the crayfish to pinch, individuals
169 were held by the carapace and the sensor was placed between dactylopodite and the
170 propodite of the left chelae (Malavé & Styga, 2018). We took a single measurement of
171 pinch force of each individual, but each individual was given sufficient time to produce
172 maximum pinching force. This enabled a relatively robust record of maximum pinch
173 force to be measured across individuals, while limiting the potential negative effects of
174 handling on individuals subsequently used in the mesocosm experiment. Finally,
175 crayfish were individually tagged with a passive integrated transponder (PIT) tag (8 x

176 1.4 mm and 12 x 2.15 mm, FDX-B tags, Oregon RFID, USA), inserted at the base of
177 the fifth pereopod pair through an incision made with a sterile scalpel (Bubb *et al.*,
178 2002). Individuals were then returned to their holding tank for recovery. From all
179 tagged crayfish (n = 238), 144 individuals were subsequently used (n = 12 males and
180 12 females from the six populations, selected randomly) for further phenotypic scoring
181 and in the mesocosm experiment. All individuals were sexually mature adults and the
182 experiment was performed before the spawning season.

183 Three behavioural traits (namely boldness, activity, and voracity) were
184 quantified before the experiment commenced. Scoring was conducted from 08.00 until
185 17.00 under the natural light conditions and stable temperature (water ~ 20 °C, air ~ 25
186 °C). Crayfish were fasted in acclimation tanks for 24 h prior to scoring to standardize
187 their hunger levels. Movement of crayfish was quantified in contexts of terrestrial and
188 aquatic environment. Movement of individuals was measured using open field test
189 conducted in barren white translucent rectangular plastic tanks (65 × 36.5 × 15 cm) with
190 no refugia that were positioned underneath a camera (HD Webcam C525, Logitech,
191 USA). The whole experimental setup was placed under a translucent tent. When
192 subjected to the trial, individuals were gently netted from the acclimation tank and
193 placed into trial tanks (one per tank). Terrestrial movement was recorded in an empty
194 tank for 10 minutes after 10 minutes of acclimation. Immediately after the terrestrial
195 movement scoring, tanks were filled with 50 mm of tap water and aquatic movement
196 was recorded for 10 minutes following acclimation for 10 minutes. Tanks were emptied
197 and cleaned between each trial. Crayfish movements (measured as distance moved
198 during the trial) were analyzed using video tracking software (LoliTrack 4.0, Loligo
199 Systems ApS, Denmark). Terrestrial movement was assumed to correspond to
200 boldness, given that red swamp crayfish moves overland rarely and only under certain

201 climatic conditions (*e.g.* rain) and it has demonstrated that crayfish are at high predation
202 and desiccation risk during movements in terrestrial environments (Ramaldo &
203 Anastácio 2015). Aquatic movement was assumed to correspond to activity of
204 individuals in a familiar environment (*i.e.* individuals had time to habituate to the
205 environment of the tank during the scoring of terrestrial movement). Therefore, this
206 represented relatively low-stress conditions, as suggested for measuring activity (Réale
207 *et al.*, 2007).

208 Following the open field test, voracity (*i.e.* individual foraging linked to its
209 behavior and metabolism; Pintor *et al.*, 2008) was quantified by placing each individual
210 into a white translucent circular tank (18 cm deep, 21.5 cm diameter, covered by lid)
211 with 15 live red maggots (Diptera), with the number consumed in 15 minutes
212 determined. The measurement was repeated in three consecutive trials that followed
213 immediately after one another. Maggot consumption rate ($\text{ind}\cdot\text{min}^{-1}$) was decreasing
214 over the three consecutive trials ($F_{2;401} = 33.77$; $p < 0.001$), but individual differences
215 were significantly repeatable across the three trials ($R_{\text{adj}} = 0.389$, 95% CI [0.280,
216 0.493]). Therefore, we used the mean of the three trials as a measurement of voracity
217 rate.

218 There was no difference in activity ($F_{1;121} = 0.708$; $p = 0.402$), voracity ($F_{1;104} =$
219 1.913 ; $p = 0.170$), and growth rate ($F_{1;112} = 0.008$; $p = 0.928$) between males and
220 females. However, males were bolder than females ($F_{1;128} = 6.460$; $p = 0.012$). Body
221 mass of individuals was negatively correlated to growth rate ($F_{1;128} = 50.80$; $p < 0.001$),
222 but activity ($F_{1;121} = 0.016$; $p = 0.901$), boldness ($F_{1;128} = 1.732$; $p = 0.190$), and voracity
223 ($F_{1;104} = 2.085$; $p = 0.152$) were not significantly correlated to body mass. The effect
224 of body mass on phenotypic traits was controlled in the models testing the phenotypic

225 divergence between the populations with and without removal programs by adding
226 body mass as co-variable (*see* details in the Statistical analyses section).

227 At the end of the mesocosm experiment (see details below), all crayfish were
228 collected from the mesocosms using traps and a small hand net on August 3rd 2017,
229 euthanized, and body mass, carapace length (nearest 0.01 mm) measured with a caliper
230 and dorsal pictures of body and right chela were taken. Specific growth rate (SGR) was
231 then calculated as:

$$232 \quad SGR = \frac{\ln M_f - \ln M_i}{T} \cdot 100$$

233 where M_f and M_i were the final and initial body mass, respectively and T the time
234 interval between two measurements, expressed in days (*i.e.* 62).

235 Morphological analysis quantifying body and chela shape was performed using
236 geometric morphometric analysis performed using the R package ‘GEOMORPH’
237 (Adams & Otárola-Castillo *et al.*, 2013). Body and chela shape analyses were based on
238 17 and 7 homologous landmarks respectively (Supplement SI 2). Partial warps, which
239 represent the non-uniform components of the body and chela shape variation, were
240 constructed and further examined by principal component analysis (PCA).
241 Consequently, each component of the PCA corresponded to a component of the shape
242 represented by partial warps (Adams & Otárola-Castillo *et al.*, 2013). The first partial
243 warp of body shape and the first partial warp of chelae shape explained substantial
244 morphological variance (body shape PC1 = 20.5 %, chela shape PC1 = 27.7 %;
245 Supplement SI 2) and were used for the analyses of morphological variation.

246

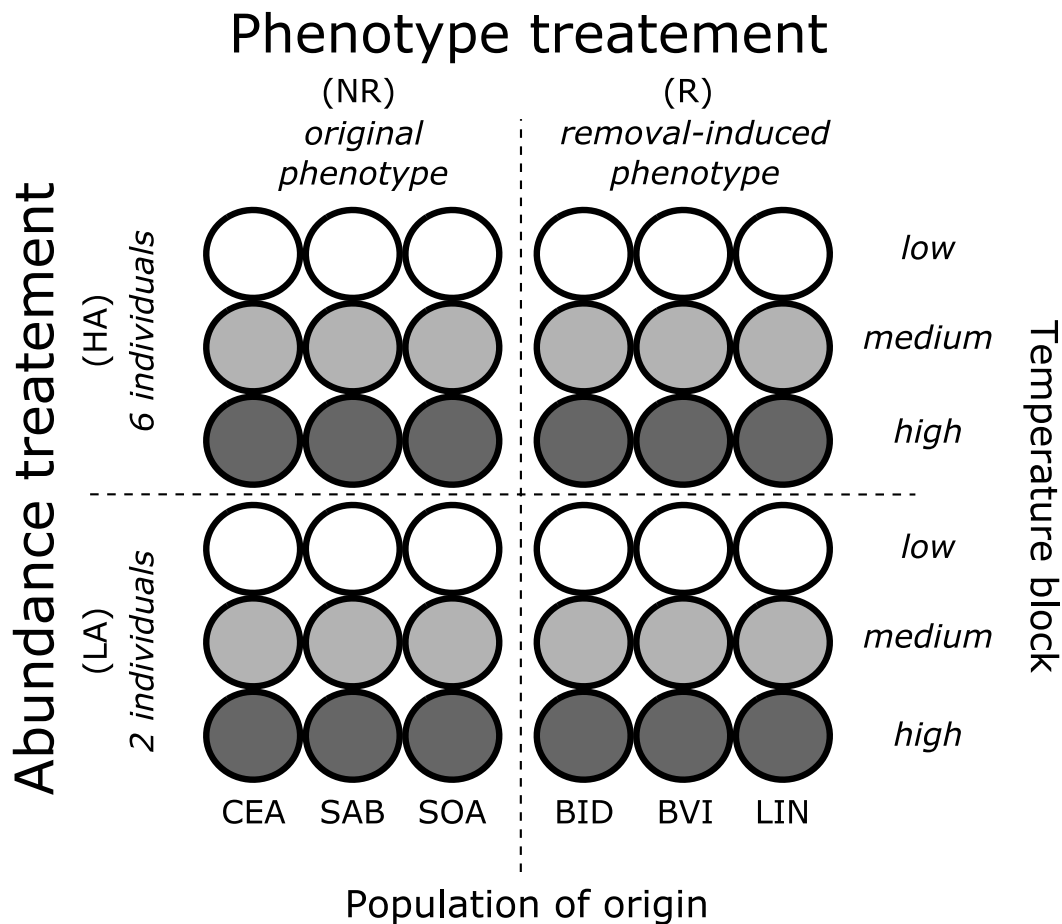
247 *Mesocosm experiment*

248 The main mesocosm experiment lasted 7 weeks from June 8th 2017 (*i.e.* introduction of
249 crayfish into the mesocosms) until July 31st 2017 (*i.e.* final measurement and sampling

250 of the community and ecosystem metrics) using 36 outdoor mesocosms (circular tanks,
251 550 L, 0.63 m deep, 1.28 m diameter). On May 3rd to 5th 2017, each mesocosm was
252 provided with 5 cm of gravel substrate (to mimic the substrate in the lakes), 400 L of
253 dechlorinated tap water, 30 ml of liquid fertilizer (N 3% and K 5%) and 20 L of
254 unfiltered water from a gravel pit lake containing an inoculum of autotrophic and
255 heterotrophic microorganisms. The mesocosms were also inoculated with periphyton
256 and zooplankton collected from a nearby gravel pit lake. On May 9th 2017, benthic
257 macroinvertebrates were introduced to each mesocosm from mesh bags containing 5 g
258 of a leaf litter mixture that have been placed in a gravel pit lake for 20 days. In addition,
259 7 freshwater snails (*Physa* sp.) collected from local ponds were added to each
260 mesocosm. On May 16th 2017, in each mesocosm, 7 pieces of drainpipe (3 pieces 10 ×
261 20 cm and 4 pieces 4 x 20 cm) and a half of an alveolar construction brick (50 × 15 ×
262 15 cm) were added to provide crayfish shelters. On May 19th 2017, 20 g (wet mass) of
263 macrophytes (*Ceratophyllum* sp.), collected from local ponds, were added to each
264 mesocosm.

265 The experiment was based on a factorial design with two main treatments:
266 crayfish phenotype (two levels, *i.e.* crayfish with and without removal-induced
267 phenotypic changes) and crayfish abundance (two levels, *i.e.* low abundance – 2
268 individuals per mesocosm – and high abundance – 6 individuals; Fig. 1). The treatment
269 combinations were: 2 individuals with removal-induced phenotypic changes, mean ±
270 SD crayfish biomass: 56.3 ± 14.0 g (low abundance and removal program), 6
271 individuals with removal-induced phenotypic changes, mean ± SD biomass: 153.0 ±
272 41.1 g (high abundance and removal program), 2 individuals without removal-induced
273 phenotypic changes, mean ± SD biomass: 43.2 ± 13.6 g (low abundance and no removal
274 program), and 6 individuals without removal-induced phenotypic changes, mean ± SD

275 biomass: 123.6 ± 15.3 g (high abundance and no removal program). Density of crayfish
276 was chosen to simulate the range of typical densities that are apparent in invaded lakes
277 (Jackson *et al.*, 2017; Evangelista *et al.*, 2019). Size of crayfish corresponded to an
278 average size of adult individuals in the invaded lakes. Crayfish were always stocked to
279 the mesocosms with individuals from the same population and each treatment
280 combination was replicated nine times, totalizing 36 mesocosms (Fig. 1). Sex ratio was
281 1M:1F in all mesocosms to control for the potential effect of sex ratio on ecosystem
282 dynamics (Fryxell *et al.*, 2015). Temperature loggers (HOBO Temperature/Light Data
283 Logger UA-002-64; Onset Computer Corporation, USA) were placed in each
284 mesocosm and temperature differences among mesocosms caused by the spatial
285 structure of the mesocosms platform was measured during their set-up in May.
286 Consequently, mesocosms were divided into three temperature blocks prior to crayfish
287 introduction to account for temperature variability (Fig. 1, Supplement SI 3). Overall,
288 there was no difference throughout the experiment in mean water temperature (21.2 °C
289 ± 2.5 SD) between the mesocosms with different crayfish abundances ($F_{1,321} = 0.02$; p
290 $= 0.90$) and with different crayfish phenotypes ($F_{1,321} = 0.02$; $p = 0.89$). Dechlorinated
291 tap water was added to all mesocosms to balance the effect of evaporation on July 3rd
292 2017 (15, 30, 45 L in the low, medium, and high temperature block, respectively).



293

294 **Fig. 1.** Design of mesocosm experiment. Diagram of treatments distribution between
 295 the mesocosms.

296

297 At the end of the experiment (August 1st 2017), macroinvertebrates were
 298 sampled in each mesocosm using a hand-net pulled around the edge of the tank for two
 299 turns (Evangelista *et al.*, 2019). Prior to sampling, macroinvertebrates were dislodged
 300 by disturbing bottom sediments and stirring round the water of the mesocosms. Samples
 301 were stored in 90% ethanol and subsequently identified to the lowest taxonomic level
 302 (mainly Family). In addition, individual snails (*Physa sp.*) attached to the wall of the
 303 mesocosm were counted at 5 cm above and 5 cm below the water surface around the
 304 mesocosm perimeter (*i.e.* the count was done without removing individuals from the
 305 mesocosms). A total of 10 macroinvertebrate taxa (*Physa*, Chironomidae, other

306 Diptera, Corixidae, Ephemeroptera, Odonata, Oligochaeta, Coleoptera, Notonecta, and
307 Hydra) were identified and counted (Supplement SI 6).

308 On July 31st 2017, we also quantified a total of nine response metrics related to
309 ecosystem functioning. Gross and net primary productivity (GPP and NPP) and
310 respiration (R) were estimated using diurnal changes in dissolved oxygen (DO)
311 concentrations (mg.L⁻¹) (Harmon *et al.*, 2009). These measurements were conducted
312 using a DO probe (ProDSS Multiparameter Water Quality Meter, YSI, USA) at dusk
313 and dawn (July 31st/ August 1st 2017). Benthic algae production was measured as
314 chlorophyll-a concentration (µg chlo-a.cm⁻²) on ceramic tiles (10 x 10 cm) placed in
315 the mesocosms on June 6th 2017 using a portable fluorometer (BenthoTorch, BBE
316 moldaenke GmbH, Germany) (Kahlert & McKie 2014). Production of pelagic algae
317 was assessed by measuring total chlorophyll-a concentration in the water column (µg
318 chlo-a.L⁻¹) using a portable fluorometer (AlgaeTorch, BBE moldaenke GmbH,
319 Germany). The decomposition rate of leaf litter was quantified by measuring
320 breakdown of 3 g bouquet of leaves of black poplar *Populus nigra* placed into the
321 mesocosms on June 13th and retrieved on July 31st 2017. Decomposition rate (K, day⁻¹)
322 was calculated following (Lecerf *et al.*, 2005):

$$323 \quad K = \frac{-\ln \frac{M_f}{M_i}}{T}$$

324 where M_f is final and M_i is initial oven-dried mass of leaf litter, T the duration of leaf
325 exposure in mesocosms (48 days). Soluble reactive phosphorous (PO₄³⁻), ammonium
326 (NH₄⁺) and dissolved organic carbon (DOC) were quantified from a filtered water
327 samples (50 mL) collected with a syringe with filter (Whatman GF/C, pore size 1.2
328 µm). Concentration of PO₄³⁻ and NH₄⁺ was quantified using the molybdenum blue and
329 phenol-hypochlorite methods respectively, performed by an automated continuous-

330 flow colorimetric analyzer (ALPKEM Corporation, Clackamas, OR, U.S.A). DOC
331 concentration was quantified by samples pacification using HCl and analyses using a
332 TOC analyzer (TOC-L, Shimadzu, Japan).

333 Before crayfish introduction to the mesocosms, there were no significant
334 differences in the nine metrics of ecosystem functioning between the mesocosms
335 stocked with different crayfish abundance and phenotype (*i.e.* measurements at the
336 beginning of the experiment, Supplement SI 4). At the end of the first part of the
337 experiment (August 3rd 2017), all crayfish were removed from the mesocosms to
338 simulate the successful eradication of an invasive species. Nearly one year after this
339 crayfish eradication (June 27th 2018), we assessed the effects of the historic treatments
340 (*i.e.* abundance and phenotype of crayfish) on ecosystem response. This sampling was
341 conducted following the same procedure as described above (for details see Supplement
342 SI 5). This aimed to determine how the ecological effects caused by abundance
343 reduction and removal-induced phenotypic changes affected the trajectory of
344 ecosystem response if complete eradication of invader is achieved.

345

346 *Statistical analyses*

347 The effect of removal program on eight phenotypic traits (*i.e.* activity, boldness,
348 voracity, body and chelae shape, pinch force, specific growth rate, and body mass) was
349 tested using Multivariate analysis of variance (MANOVA) with the presence or absence
350 of removal program in the lake of origin as a response variable. The divergence of
351 phenotypes was further tested by Linear Discriminant Analysis (LDA), which
352 evaluated the probability of correct assignment of individuals to the two classes (*i.e.*
353 lakes with and without removal program) based on linear combination of the eight
354 phenotypic traits. The missing data in the matrix of phenotypic traits were imputed

355 using the regularized iterative PCA algorithm (Josse & Husson 2012). The divergence
356 between the groups in single phenotypic traits was tested by linear models with the
357 removal program as a response variable and body mass as covariate (note that the model
358 for body mass did not include body mass as covariate). The divergence in body and
359 chelae shape was tested with Procrustes ANOVA with 9999-round randomized residual
360 permutation procedures and controlled for the centroid size. P-values of the models for
361 single phenotypic traits we adjusted by the false discovery rate method. Generalized
362 linear models (GLM), with initial body mass and population of origin as co-variables,
363 were used to test the effect of sex and tag size on behaviour and growth rate of crayfish.
364 Repeatability of maggot consumption rate across the three trials adjusted for body mass
365 was quantified using the intra-class correlation coefficient (ICC) extracted from linear
366 mixed models (LMM) with individual identity as a random factor (Nakagawa *et al.*,
367 2010).

368 The effects of the treatments on the macroinvertebrate community in the
369 mesocosm experiment was assessed using non-metric multidimensional scaling
370 (NMDS) ordinations based on Euclidean distance, calculated from untransformed
371 abundances of each taxa in each mesocosm that resulted in two dominant axes, NMDS
372 1 and NMDS 2 (Supplement SI 6). We then used a multifunctional approach to quantify
373 ecosystem response to treatments (Antiqueira *et al.*, 2018) and quantified the
374 distribution of ecosystem metrics (n = 9) between the mesocosms using PCA. This
375 method allows quantification of dominant axes of multifunctionality, synergies and
376 trade-offs among functions. Therefore, this approach provides a novel integrative
377 perspective on how global change drivers, such as biological invasions, will impact the
378 simultaneous provisioning of multiple ecosystem functions (Giling *et al.*, 2019). All
379 ecosystem metrics were centered, scaled, and transformed if needed to approach normal

380 distribution. This procedure resulted in three PC axes (eigenvalue > 1) that represented
 381 the majority of variation in the original nine metrics (74.4% in total, PC 1: 42.0 %, PC
 382 2: 19.6 %, and PC 3: 12.8 %). We interpreted these three independent dimensions as
 383 ecosystem multifunctional components, related to and summarizing different and
 384 important ecosystem properties (Antiqueira *et al.*, 2018; Supplement SI 7). Ecosystem
 385 metabolism was the first ecosystem multifunctional component and was positively
 386 related to GPP ($r = 0.95$), NPP ($r = 0.94$), R ($r = 0.94$) and pelagic algae production (r
 387 $= 0.59$). Decomposition of organic matter was the second ecosystem multifunctional
 388 component positively related to decomposition rate of leaf litter ($r = 0.72$) and
 389 concentration of dissolved organic carbon ($r = 0.70$) and reactive phosphorous ($r =$
 390 0.51). Finally, benthic primary production was the third ecosystem multifunctional
 391 component positively related to the production of benthic algae ($r = 0.68$) and
 392 negatively to the concentration of ammonium in the water ($r = -0.59$).

393 Hedges' g effect sizes compared the effects of crayfish phenotypic change (*i.e.*
 394 effect of phenotypes from lakes with and without removal program) and abundance on
 395 macroinvertebrates community and ecosystem multifunctional components (Des
 396 Roches *et al.*, 2018). They were calculated using the following formula:

$$397 \quad Hedges' g = \frac{m_{imp} - m_{ctrl}}{\sqrt{\frac{(n_{imp} - 1)SD_{imp}^2 + (n_{ctrl} - 1)SD_{ctrl}^2}{n_{imp} + n_{ctrl} - 2}}}$$

398 where m is the group mean and SD is the group standard deviation of a response variable
 399 determined as control *ctrl* ($n = 18$) and impact *imp* ($n = 18$). When calculating the effects
 400 size of crayfish phenotype, variables measured in the mesocosms stocked with crayfish
 401 from lakes without a removal program (*i.e.* crayfish with assumed original phenotypes)
 402 were used as control and variables measured in mesocosms stocked with crayfish from
 403 the lakes with a removal program (*i.e.* crayfish with removal-induced phenotypic

404 changes) were used as impact. When calculating the effect size of crayfish abundance,
405 variables measured in mesocosms with six individuals were used as a control (*i.e.* high
406 abundance before removal) and variables measured in mesocosms with two individuals
407 (*i.e.* low abundance after removal) were used as impact. Following Des Roches *et al.*,
408 (2018), values of Hedges' g were interpreted as negligible if $|g| < 0.20$, small if $|g| <$
409 0.30 , medium if $|g| < 0.80$, and large if $|g| \geq 0.80$.

410 We used GLMs to test the effects of crayfish phenotype, abundance, their
411 interaction term, and temperature block as co-variables on macroinvertebrate
412 communities and ecosystem multifunctional components. The interaction term between
413 crayfish phenotype and abundance was not significant in any tested model and was thus
414 removed from all final models. The difference between the absolute effect sizes (*i.e.*
415 magnitude) of crayfish ecological impacts measured before (August 2017) and one year
416 after (June 2018) crayfish removal was tested by a paired t-test. All analyses were
417 conducted in R v. 3.4.1 (R Core Development Team).

418

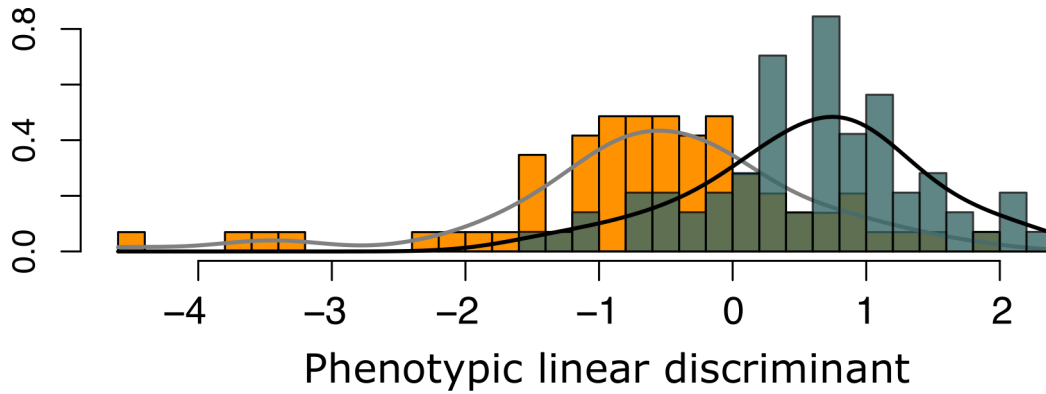
419 **Results**

420 *Removal-induced phenotypic changes in lakes*

421 Ecologically significant phenotypic traits of invasive crayfish differed between
422 populations from lakes with and without removal programs (MANOVA $F_{8,134} = 5.934$,
423 $p > 0.001$). Linear discriminant analysis indicated that individuals from lakes with and
424 without removal programs could be correctly identified with a mean probability of 76.9
425 % based on the 8 recorded phenotypic traits (*i.e.* activity, boldness, voracity, body and
426 chelae shape, pinch force, specific growth rate, and body mass; Fig. 2). Supplement SI
427 2 has further details on variation of phenotypic traits between individual lakes. At the
428 single trait level, removal-induced phenotypic changes were most distinctively

429 demonstrated in higher body mass and a mass-independent increase of boldness and
 430 voracity in crayfish from lakes with removal programs (Table 1).

431



432

433 **Fig. 2.** Frequency histogram and kernel density distribution of the phenotypic linear
 434 discriminant of individuals from the lakes with (orange bars and grey curve) and
 435 without (green bars and black curve) removal program.

436

437 **Table 1.** Differences in single phenotypic traits. Phenotypic traits mean (\pm SD) of
 438 individuals from lakes with (R) and without (NR) removal program. For units and
 439 scoring methods see the method section. Difference between the groups is based on
 440 models controlled for body size. Significant differences (adjusted $p < 0.05$) are
 441 displayed in bold.

442

	Activity	Boldness	Voracity	Body shape	Chelae shape	Pinch force	SGR mass	Body mass
R	549.57 (\pm 247.86)	381.392 (\pm 151.159)	0.007 (\pm 0.004)	0.001 (\pm 0.010)	0.005 (\pm 0.024)	6.484 (\pm 6.071)	0.065 (\pm 0.180)	27.358 (\pm 9.724)
NR	562.429 (\pm 195.683)	329.668 (\pm 137.154)	0.004 (\pm 0.002)	-0.001 (\pm 0.011)	-0.005 (\pm 0.023)	7.097 (\pm 5.742)	0.166 (\pm 0.356)	22.029 (\pm 7.038)
Difference between groups	NS	R>NR	R>NR	NS	NS	NS	NS	R>NR

443

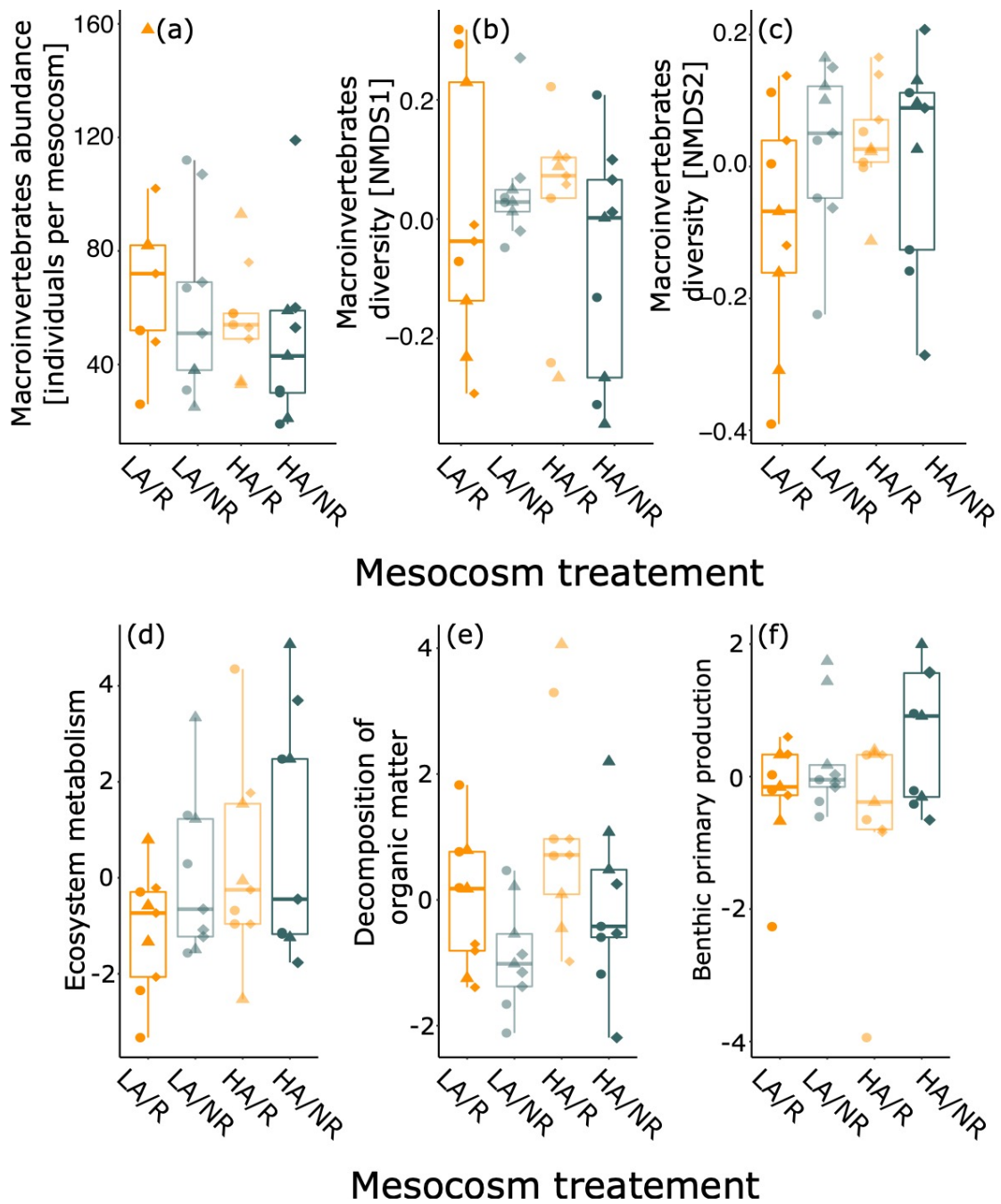
444

445

446 *Ecological consequences of removal programs*

447 There were no significant effects of removal-induced phenotypic changes and
448 abundance reduction of crayfish on either the abundance of macroinvertebrates
449 (crayfish phenotype: $F_{1,31} = 1.35$, $p = 0.25$; crayfish abundance: $F_{1,31} = 2.24$, $p = 0.14$;
450 Fig. 3a, 4a) or the diversity of the macroinvertebrate community (NMDS1 - crayfish
451 phenotype: $F_{1,31} = 0.20$, $p = 0.66$; crayfish abundance: $F_{1,31} = 0.83$, $p = 0.37$; Fig. 3b and
452 NMDS2 - crayfish phenotype: $F_{1,31} = 0.72$, $p = 0.40$; crayfish abundance: $F_{1,31} = 1.04$,
453 $p = 0.32$; Fig. 3c,4a; Supplement SI 6).

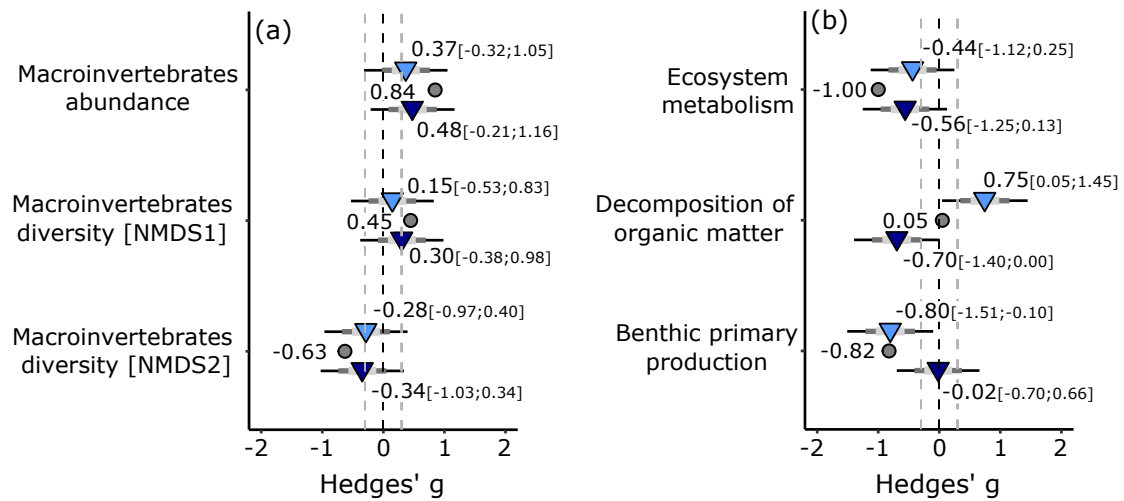
454 All ecosystem metrics were summarized by the three ecosystem multifunctional
455 components that represented the synergies and trade-offs among the important
456 ecosystem functions of ecosystem metabolism, decomposition rate of organic matter
457 and benthic primary production (Supplement SI 7). The cumulation of negative effects
458 of removal-induced phenotypic changes ($F_{1,31} = 3.88$, $p = 0.06$; Fig 3d) and abundance
459 reduction ($F_{1,31} = 6.20$, $p = 0.02$; Fig 3d) of crayfish on ecosystem metabolism resulted
460 in a strong decrease in ecosystem metabolism (Hedges' $g = -1.00$; Fig. 4b). The
461 significantly increased decomposition rate due to removal-induced phenotypic changes
462 ($F_{1,31} = 5.65$, $p = 0.02$; Fig 3e) contrasted with the significant decrease of decomposition
463 rate caused by crayfish abundance reduction ($F_{2,31} = 5.01$, $p = 0.03$; Fig 3e), which
464 resulted in a negligible overall effect of removal program on decomposition (Hedges'
465 $g = 0.05$; Fig. 4b). Removal-induced phenotypic changes of crayfish ($F_{1,31} = 5.97$, $p =$
466 0.02 ; Fig 3f), but not reduction of crayfish abundance ($F_{1,31} = 0.00$, $p = 0.95$; Fig 3f),
467 led to a decrease in benthic primary production (Hedges' $g = -0.82$; Fig. 4b).



468

469 **Fig. 3.** Ecological effects in the experimental mesocosms. Effects of crayfish phenotype
 470 and abundance on community of macroinvertebrates (a-c) and on ecosystem
 471 multifunctional components (d-f) in the experimental mesocosms. Boxplots show effects
 472 of treatment combinations (LA/R - 2 crayfish with removal-induced phenotypic
 473 changes, LA/NR - 2 crayfish with original phenotype, HA/R - 6 crayfish with removal-
 474 induced phenotypic changes, HA/NR - 6 crayfish with original phenotype). Orange and
 475 green boxplots represent mesocosms containing crayfish with and without removal-

476 induced phenotypic changes respectively. Shape and color of the points in the box plot
 477 also correspond to the specific lake of crayfish origin (orange circle - BVI (R), orange
 478 triangle - BID (R), orange diamond - LIN (R), green circle - SAB (NR), green triangle
 479 - CEA (NR), and green diamond - SOA (NR)).
 480



481
 482 **Fig. 4.** Effect size of ecological impacts of phenotypic changes and abundance
 483 reduction. Overall effect size (Hedges' g) and CI (95 % black line, 75 % dark grey line,
 484 50 % light grey line) of crayfish removal-induced phenotypic changes (light blue) and
 485 abundance reduction (dark blue) on ecological metrics of (a) community of
 486 macroinvertebrates and (b) ecosystem multifunctional components. Light grey vertical
 487 lines represent a range of small effect size i.e. Hedges' d (-0.3, 0.3). Empty circles
 488 illustrate the cumulative effect size of phenotypic change and abundance effects.

489

490

491 Ecological consequences of historic variation in invader phenotype and abundance

492 The magnitude of the ecological effects of crayfish invasion on macroinvertebrate
 493 community and ecosystem functioning was significantly lower one year after crayfish
 494 eradication ($|t| = 3.27, p < 0.01$; Supplement SI 5). The decrease in average magnitude

495 of the ecological effects was similar for crayfish phenotype (Δ |Hedges' g | = -0.14) and
496 abundance (Δ |Hedges' g | = -0.23). Overall, the eradication of crayfish resulted in a
497 relatively small mean magnitude of the ecological effects of historical variation in
498 invasive crayfish phenotype: |Hedges' g | = 0.30 and abundance: |Hedges' g | = 0.21 on
499 macroinvertebrate community and ecosystem functioning.

500

501 **Discussion**

502 Our novel findings reveal that reductions in the population abundances of invasive
503 species do not necessarily minimise their invasion impacts and facilitate restoration of
504 the ecosystem back to its pre-invaded state, thus challenging a central tenet of invasive
505 species management. Instead, these highly original results indicate that ecosystems
506 actually suffer additional impacts if removals of invasive species induce strong
507 phenotypic responses in the surviving individuals. We confirmed our first hypothesis
508 that the removal program induced changes in ecologically significant phenotypic traits
509 of the invasive crayfish. Our findings were also consistent with our second hypothesis,
510 as we demonstrated that the direction of the ecological effects of removal-induced
511 phenotypic changes and abundance reduction can be opposite and result in the lower
512 efficacy of removal programs that aim to limit invader impacts. This result was
513 strongest in the ecosystem multifunctional components that are susceptible to the
514 consumptive effects of invasive crayfish *i.e.* the decomposition of organic matter and
515 benthic primary production (Twardochleb *et al.*, 2013). Conversely, the effects of
516 phenotypic changes and abundance reduction acted in the same direction on the
517 ecosystem multifunctional component susceptible to non-consumptive effects, *i.e.*
518 ecosystem metabolism. Finally, we found that, a year after crayfish eradication, the
519 effects of historic variation in crayfish phenotype and abundance on ecosystem

520 functioning were reduced and generally minor. This was inconsistent with our third
521 hypothesis that historic variation in invader phenotype and abundance alters the long-
522 term trajectory of ecosystem recovery. It is an encouraging result, as it indicates that
523 ecological impacts of changes in invaders phenotype and abundance caused by
524 previously unsuccessful eradication programs (Pluess *et al.*, 2012; Zavaleta *et al.*, 2001)
525 might not constrain future ecosystem recovery providing that eradication is achieved,
526 although this remains to be tested and quantified in more natural and complex settings.

527 Trapping, angling and stocking of fish predators used in the lakes of invasive
528 crayfish origin have yet to result in significantly decreased invasive crayfish abundance
529 (*i.e.* based on data from 2016 survey; Supplement SI 1). Nonetheless, we have already
530 observed that crayfish from lakes with removal programs were bolder, more voracious
531 and had larger body mass. Previous studies suggest that large crayfish displaying bold
532 behaviors are less likely to be consumed by fish predators (Stein & Magnuson 1976;
533 Roth & Kitchell 2005). In contrast, large size, bold behaviours, and voracity are the
534 traits most likely to increase the probability of individuals being harvested by angling
535 or trapping (Biro & Sampson 2015; Green *et al.*, 2018; Koeck *et al.*, 2019). However,
536 because harvest and stocking of predators were applied simultaneously in our study
537 systems, it is not possible to decouple the relative effects of each removal method on
538 invader phenotype. The selection pressure induced by harvesting may leave surviving
539 individuals more susceptible to predation and *vice versa* (*e.g.* Olsen & Moland 2011).
540 As little is known about the effects of harvesting in truly natural contexts, future work
541 should aim to identify the mechanisms driving phenotypic responses of invaders in
542 removal programs and the relative contributions of different control methods to
543 observed phenotypic changes when multiple removal methods are used. Interestingly,
544 while there were some differences among the lakes in the way the removals were

545 performed (duration and effort) due to individual differences between managers in their
546 methods, we found similar phenotypic trait changes across all lakes with removal
547 programs (Supplement SI 2). Individual differences in boldness and voracity have been
548 shown to be highly consistent overtime and influence the trophic ecology of invasive
549 crayfish (Raffard *et al.*, 2017). Therefore, the removal-induced phenotypic changes of
550 the invasive crayfish could magnify their consumptive effects, which are a dominant
551 driver of their ecological impacts (Twardochleb *et al.*, 2013).

552 Invasive crayfish are known to accelerate the decomposition rate of organic
553 matter (Alp *et al.*, 2016), and reduce macrophyte production (Nyström & Strand 1996)
554 and standing algae (Rudnick & Resh 2005) through consumption. We revealed that the
555 removal-induced phenotypic changes caused accelerated decomposition of organic
556 matter and reduced benthic primary production, highlighting that, even if invader
557 abundance was lowered, these important ecosystem functions do not recover. This
558 could possibly be the consequence of increased consumption of benthic algae and leaf
559 litter by the individual crayfish from the lakes that had a removal program. Our results
560 thus directly corroborate previous findings indicating that boldness and foraging rates
561 (which were found to be higher in crayfish from lakes with removal programs) are often
562 associated within a functional syndrome that has direct impacts on consumptive effects
563 of crayfish invasion (Pintor *et al.* 2008; Raffard *et al.*, 2017). We also found that
564 removal-induced phenotypic changes of crayfish reduced ecosystem metabolism,
565 despite their consumption not directly impacting the pelagic ecosystem where most of
566 the oxygen production and respiration occurs (Harmon *et al.*, 2009). Previous work
567 showed that crayfish can impact the pelagic components of ecosystems indirectly
568 through nutrient recycling (Vanni 2002) and bioturbation (Angeler *et al.*, 2001), which
569 are the processes that could have been affected by removal-induced phenotypic changes

570 in the surviving populations of invasive crayfish (Raffard *et al.*, 2017; Evangelista *et*
571 *al.*, 2019). Our findings showed that the non-consumptive effects of phenotypic
572 changes can combine with the effects of reduced crayfish abundance and lead to
573 reduced ecosystem metabolism. This suggests that removal programs could facilitate
574 recovery of ecosystem metabolism. We observed only limited effects of crayfish
575 abundance and phenotype on the macroinvertebrate communities, although the
576 abundance of macroinvertebrates tended to increase in response to the effect of
577 removal-induced phenotypic changes and crayfish abundance reduction. The limited
578 response of macroinvertebrate communities indicates that effects of invasive crayfish
579 on ecosystem functioning were unlikely to have been mediated by a trophic cascade
580 (Twardochleb *et al.*, 2013; Souty-Grosset *et al.*, 2016). The lack of community response
581 to crayfish phenotype and abundance could be partially due to relatively low taxonomic
582 diversity of macroinvertebrate community in mesocosms (Supplement SI6).

583 Our study provides the first direct quantitative evidence supporting the idea that
584 removal methods causing complex changes in phenotype of invasive species can alter
585 the ecological impacts of invasion even when the abundance of invasive species is
586 substantially reduced (Palkovacs *et al.*, 2018; Závorka *et al.*, 2018a). While this finding
587 is based on a single species, the novel concept we describe requires further attention,
588 given that single species studies have been shown to provide key insights into dynamics
589 of biological invasions (Pyšek *et al.*, 2008). The density of invasive crayfish in the
590 mesocosms was within the range occurring in invaded lakes (Jackson *et al.*, 2017;
591 Evangelista *et al.*, 2019), but the scale of mesocosm studies can limit the complexity of
592 ecological interactions therein (*e.g.* intimidation effect by predators; Stein & Magnuson
593 1976; Aquiloni *et al.*, 2010). The scale of the mesocosms can also affect ecosystem
594 processes, but strong effects of phenotypic variability on ecosystem functioning have

595 previously been shown in both mesocosms and larger, natural experiments (Des Roches
596 *et al.*, 2018; Raffard *et al.*, 2019). We found that ecosystems can be highly resilient, as
597 relatively small effects of historical variation in invasive crayfish phenotype and
598 abundance were observed a year after complete eradication of crayfish from the
599 mesocosms. However, caution is needed in the interpretation of these results, as
600 ecosystem resilience depends on intensity of ecological impacts of invasions and occur
601 at time scales that are dependent upon the ecological context. For example, a previous
602 study found that the negative ecological impacts of an invasive species decreased over
603 time, probably due to the rapid response of native organisms *e.g.* their adaptation, but
604 also local extinction (Závorka *et al.* 2018b). These responses of native organisms could
605 reduce the capacity of ecosystem to return to its pre-invaded state even after invasive
606 species eradication. The removal-induced phenotypic changes of invasive species
607 should thus be accounted for in ecosystem management planning, especially in cases
608 where eradication of an invader is not possible and population containment via
609 removals is the only practicable option. Previous studies have shown that rapid
610 evolutionary responses depend not only on selection pressures, but also on genetic
611 architecture and phenotypic plasticity of the target population (Kokko *et al.*, 2017),
612 which could limit the heritability of phenotypic changes induced by selective removal
613 methods. However, removal programs are often relatively short term (Britton *et al.*,
614 2011), and thus are likely to primarily impact invader phenotype through phenotypic
615 plasticity. Therefore, studies testing how phenotypic plasticity and genetic divergence
616 of ecologically significant traits can affect ecosystem functioning requires further
617 attention in context of invasion biology (Lundsgaard-Hansen *et al.*, 2013).

618 In conclusion, our field and experimental approaches provided novel results that
619 revealed the phenotypic responses of invasive species can have fundamental

620 implications for how recipient ecosystems respond to invader removals and eradication.
621 The successful testing of our first two hypotheses demonstrated that invaded
622 ecosystems can suffer additional ecological impacts via strong responses of phenotypic
623 traits in the surviving invasive individuals and, whilst these phenotypic responses
624 facilitate recovery of some ecosystem functions, they simultaneously constrain the
625 recovery of others. Considering this trade-off should thus become an integral part of
626 risk-benefit assessment of invasive species control efficiency in order to avoid negative
627 consequences on recipient ecosystems and native biota.

628

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