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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7   *Authors*

8   Libor Závorka[1†*], Rémy Lassus[1], J. Robert Britton[2] & Julien Cucherousset[1]

9

10   [1] Laboratoire Évolution & Diversité Biologique (EDB UMR 5174), CNRS, Université

11   de Toulouse, 31062 Toulouse, France

12   [2] Department of Life and Environmental Sciences, Faculty of Science and Technology,

13   Bournemouth University, Poole, BH12 5BB, United Kingdom

14   [†] Present address: WasserCluster Lunz–Inter-university Centre for Aquatic

15   Ecosystem Research, A-3293, Lunz am See, Austria

16

17   [*]*Corresponding author*: Libor Závorka, E-mail: liborzavorka@email.cz

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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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Introduction

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

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

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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

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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±SD water surface: 11±7 ha and water depth: 2.8±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 heterozygozities, 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.

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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

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

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

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

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

There was no difference in activity ($F_{1;121} = 0.708$; $p = 0.402$), voracity ($F_{1;104} = 1.913$; $p = 0.170$), and growth rate ($F_{1;112} = 0.008$; $p = 0.928$) between males and females. However, males were bolder than females ($F_{1;128} = 6.460$; $p = 0.012$). Body mass of individuals was negatively correlated to growth rate ($F_{1;128} = 50.80$; $p < 0.001$), but activity ($F_{1;121} = 0.016$; $p = 0.901$), boldness ($F_{1;128} = 1.732$; $p = 0.190$), and voracity ($F_{1;104} = 2.085$; $p = 0.152$) were not significantly correlated to body mass. The effect 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    $$SGR = \frac{\ln M_f - \ln M_i}{T} \cdot 100$$

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).

Phenotype treatement
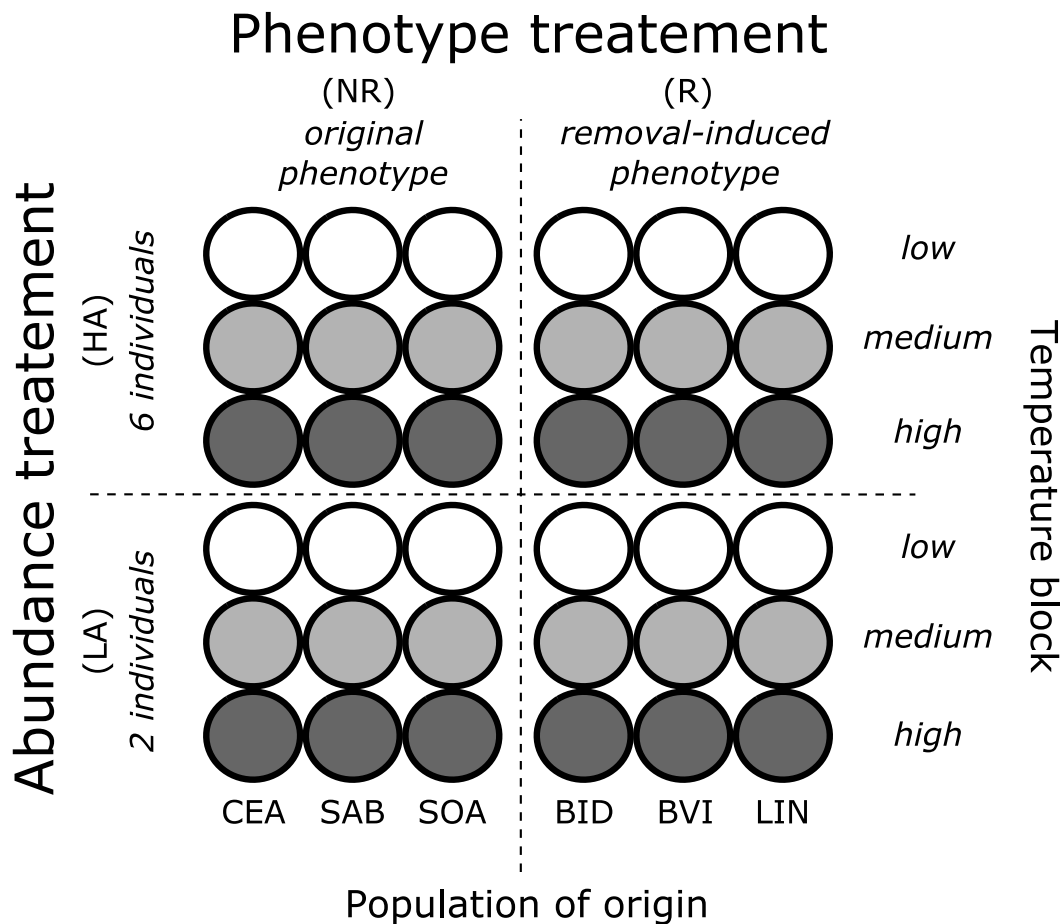
Fig. 1. *Design of mesocosm experiment. Diagram of treatments distribution between the mesocosms.*

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

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

323

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 (PO$_4^{3-}$), ammonium

326　(NH$_4^+$) 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 PO$_4^{3-}$ and NH$_4^+$ was quantified using the molybdenum blue and

329　phenol-hypochlorite methods respectively, performed by an automated continuous-

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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

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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

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

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

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

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

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

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

Results

Removal-induced phenotypic changes in lakes

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

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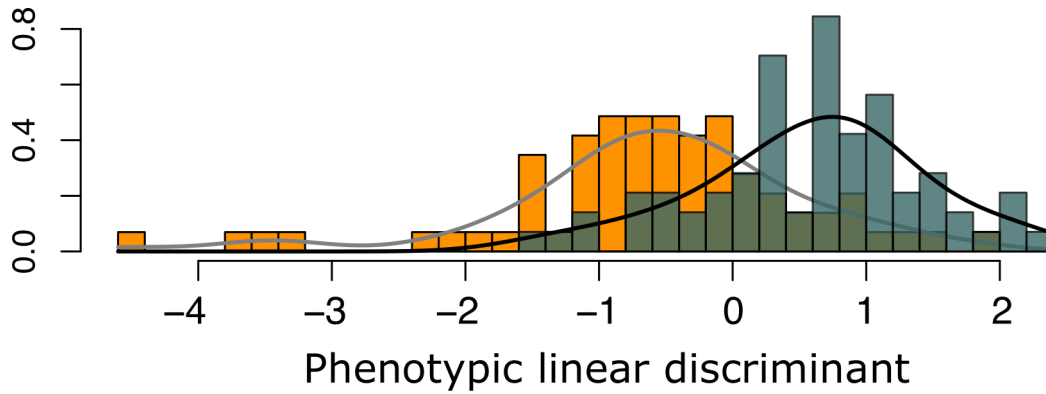
432

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 (± 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 (± 247.86)	381.392 (± 151.159)	0.007 (± 0.004)	0.001 (± 0.010)	0.005 (± 0.024)	6.484 (± 6.071)	0.065 (± 0.180)	27.358 (± 9.724)
NR	562.429 (± 195.683)	329.668 (± 137.154)	0.004 (± 0.002)	-0.001 (± 0.011)	-0.005 (± 0.023)	7.097 (±5 .742)	0.166 (±0 .356)	22.029 (± 7.038)
Difference between groups	NS	**R>NR**	**R>NR**	NS	NS	NS	NS	**R>NR**

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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).
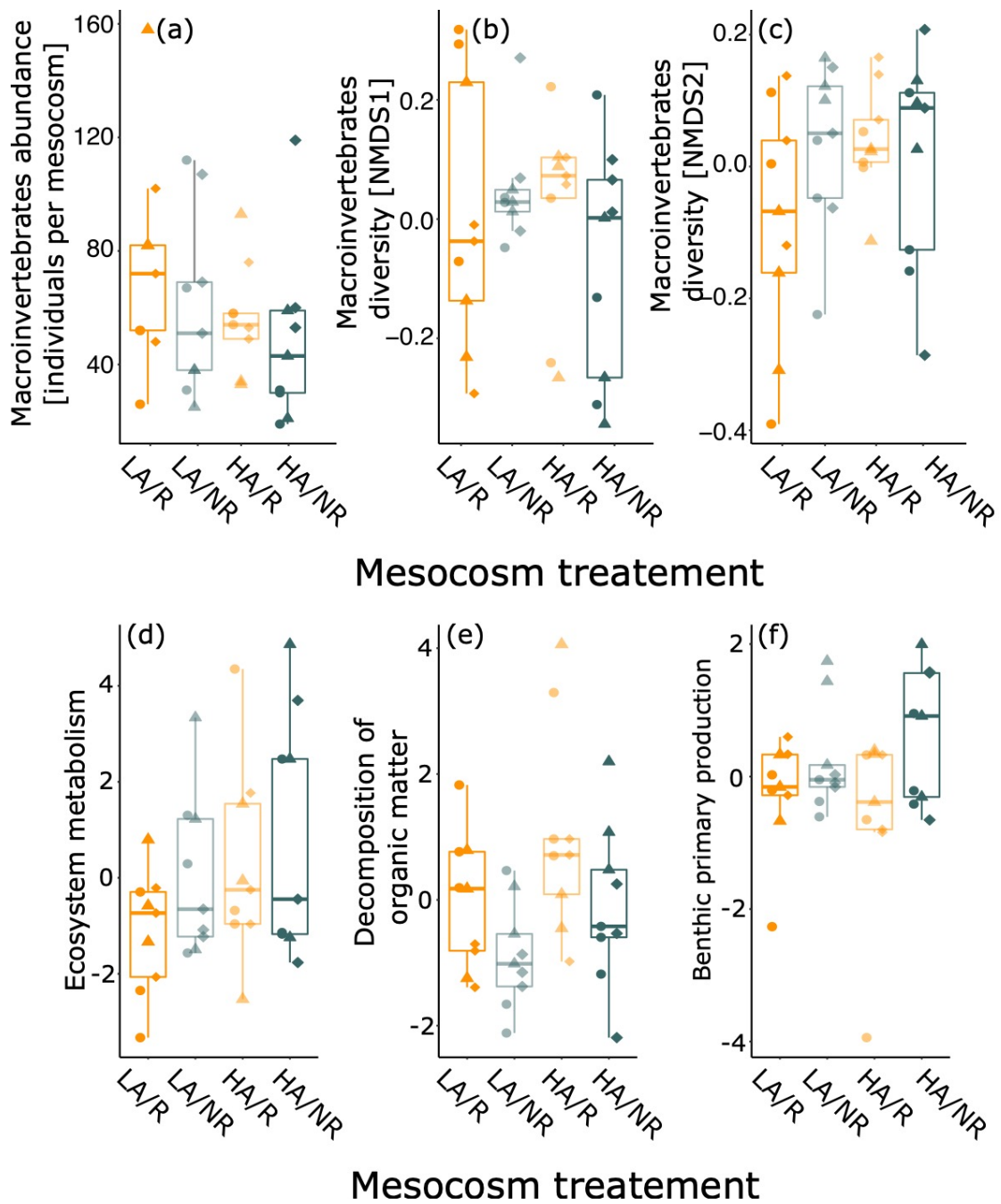
Fig. 3. *Ecological effects in the experimental mesocosms. Effects of crayfish phenotype and abundance on community of macroinvertebrates (a-c) and on ecosystem multifunctional components (d-f) in the experimental mesocosms. Boxplots show effects of treatment combinations (LA/R - 2 crayfish with removal-induced phenotypic changes, LA/NR - 2 crayfish with original phenotype, HA/R - 6 crayfish with removal-induced phenotypic changes, HA/NR - 6 crayfish with original phenotype). Orange and 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
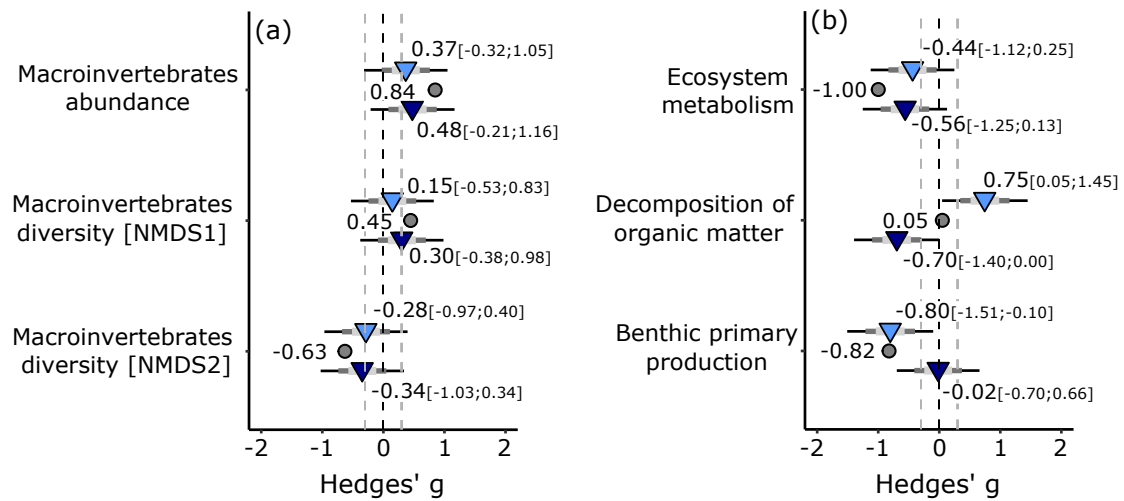
481

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 cycles*

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

22

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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