Importance of phenotypic plastic traits on invasion success: response of *Xenostrobus securis* to the predatory dogwhelk *Nucella lapillus*

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Running title: phenotypic plastic traits in response to a predator

28 **Abstract**

29 The ability of the invasive mussel *Xenostrobus securis* to activate defence mechanisms in response to the
30 novel predatory dogwhelk *Nucella lapillus* was explored using field and laboratory-based approaches. The
31 importance of the origin of mussels was investigated in relation to different environmental conditions and
32 levels of predation pressure (high and low). In the field, the responses of mussels were clearly asymmetrical,
33 as only individuals caged with dogwhelks at the site of high predation risk underwent phenotypical changes
34 (stronger attachment, thicker shells and heavier adductor muscle). By contrast, shell growth was faster in
35 mussels held in cages without dogwhelks at the high predation risk site, suggesting trade-off patterns
36 between growth and other energy-demanding actions. Nevertheless, *X. securis* activated inducible
37 morphological defences without any detrimental effect on soft tissue growth (i.e. condition index).
38 In the laboratory, the role of temperature on phenotypic responses of mussels exposed to dogwhelk was also
39 evaluated. Mussels originally from the site of low predation risk showed a weaker response to the predator *N.*
40 *lapillus* probably because of difficulties in correctly identifying predator cues. At higher temperatures,
41 mussels secreted stronger byssal threads regardless of their origin, while condition was poorer, shells thinner
42 and gametogenesis activated more rapidly, particularly in presence of dogwhelks. In summary, *X. securis*
43 appears to be highly capable of activating protective mechanisms in marine environments within its
44 geographical range of expansion through improved fitness.

45 **Keywords:** invasive mussel, plastic traits, anti-predatory actions, temperature

47 **1. Introduction**

48 Predator-prey interactions and the evolution of adaptive traits are major ecological factors controlling the
49 dynamics of populations, communities and ecosystems (Menge 1983, Freeman & Byers 2006). Such
50 interactions are also important in novel communities arising as a result of the establishment of non-
51 indigenous species (NIS) (Shinen et al. 2009, Hines et al. 2009). The success of NIS may primarily depend
52 on the responses and eco-physiological plasticity of organisms, which may in turn be mediated by factors
53 such as predator-prey density, distribution limits, spatial and temporal scales of interaction, environmental
54 conditions, feeding preferences and behaviour of predators and prey (Hines et al. 2009). Recent research

55 suggests that the predator avoidance behaviour of many invertebrates may depend on experience (Turner et
56 al. 2006). Indeed, unfed predators or predators feeding on prey that is unrelated to the target species usually
57 induce weak behavioural and morphological responses in prey, while predators feeding on conspecifics
58 induce strong responses (reviewed in Schoeppner & Relyea 2005). Studies with different taxa suggest that
59 prey may lose their antipredator behaviour in the absence of continued selection (Storfer & Sih 1998).

60 Predator-induced defence mechanisms are ecologically important forms of phenotypic plasticity whereby
61 prey show adaptive morphological, behavioural or physiological shifts that increase their resistance to
62 predation. The potential for inducible defence mechanisms to cause adaptive change over broad geographical
63 and temporal scales has been reported to be of comparable magnitude to any temperature-related latitudinal
64 effect (Trussell & Smith 2000). Common predator-induced responses include shell thickening in mussels and
65 production of defensive spines in bryozoans (Freeman 2007). Other common responses in some mussel
66 species include increased attachment strength and decreased clearance rates induced by risk cues (Naddafi &
67 Rudstam 2013). Moreover, predator-induced changes may lead to lower fecundity or reproduction rates in
68 individuals (Fässler & Kaiser 2008, Bourdeau 2010). These responses are often mediated by environmental
69 factors such as temperature, which regulates the evolution of life history traits via energetic costs (Lass &
70 Spaak 2003, Barbosa et al. 2014).

71 Chemical alarm responses (such as the release of infochemicals) represent a defensive strategy that is
72 triggered by an evolved signalling substance released from a conspecific victim of predation (Leonard et al.
73 1996). Defensive responses of prey may be directly induced by predators (enemy avoidance kairomones;
74 Kats & Dill 1998, Lowen et al. 2013) - even unfed predators (Trussell & Nicklin 2002, Freeman 2007) - as
75 well as by other prey at the moment of attack, e.g. by conspecifics or closely related species (heterospecifics,
76 as reported by Fässler & Kaiser 2008 for first time in mussels). These mechanisms “label” the predators so
77 that they can be recognised by the prey (alarm pheromones; Smith 1992, Trussell & Nicklin 2002, amongst
78 others). Both conspecific and heterospecific sources of chemosensory information are important for
79 assessing predation risk as an alarm response (Hagen et al. 2002). Prey must ensure that signals are reliable
80 (Harvell 1990) in order to prevent energetic losses or development of non-sense actions. There must
81 therefore be a balance between the enhancement of defence mechanisms and fitness strategies, e.g. growth or
82 reproduction, which may eventually lead to trade-offs (Hoverman & Relyea 2009). However, reduction in

83 the ability of the prey to respond appropriately to predation pressure may occasionally be an indirect
84 consequence of a reduction in feeding or somatic growth due to predator signals rather than trade-offs
85 (Bourdeau 2010).

86 Mussels are excellent target organisms for examining the mechanisms and costs of inducible defence
87 responses because they are prevalent on intertidal rocky shores and rely on morphological and chemical
88 defence actions to avoid predators (Leonard et al. 1999). The strong calcareous shell of mussels protects the
89 soft body of the organism, and shell size and thickness are therefore the main factors involved in anti-
90 predatory responses (Nagarajan et al. 2002). In the mussel *Mytilus edulis* Linnaeus, 1798, the presence of
91 water-borne effluents from crabs and starfish modifies the protective tissues and behaviour of individuals,
92 e.g. shell thickness and adductor muscle size and byssal tenacity (Reimer & Harms-Ringdahl 2001, Leonard
93 et al. 1999, Fässler & Kaiser 2008). Moreover, *M. edulis* is capable of distinguishing different predators and
94 expressing specific (inducible) defence mechanisms, although the eventual effectiveness of the mechanisms
95 is asymmetrical, and therefore the specific response to one cue does not deter the other (Freeman 2007).

96 The black pygmy mussel *Xenostrobus securis* Lamarck, 1819, which like *M. edulis* belongs to the family
97 Mytilidae, is a NIS native to Australia and New Zealand that has successfully invaded the Mediterranean
98 Sea (Streftaris & Zenetos 2006) and the Atlantic coast of the Iberian Peninsula (Garci et al. 2007, Pascual et
99 al. 2010, Adarraga & Martínez 2012). It was first reported in the Ría de Vigo (NW Spain) near the mouth of
100 the Verdugo river in 2002 (Garci et al. 2007). Since then it has gradually spread towards the middle part of
101 the ría and into the nearby Ría de Pontevedra (Gestoso et al. 2012). It forms monospecific and mixed
102 aggregations with the commercially-important mussel *Mytilus galloprovincialis* Lamarck, 1819. Its success
103 as an invasive species in the invaded area can be attributed to its ability to tolerate a wide range of
104 environmental conditions, e.g. salinity fluctuations, and to a reduced biotic resistance by native communities,
105 especially in the innermost areas of the rías (Babarro & Lassudrie 2011, Gestoso et al. 2012). However, in
106 the outermost areas of rías, predation may be an important factor controlling the abundance of the invader
107 (Gestoso et al. 2014).

108 We carried out field and laboratory-based experiments to investigate how novel predators and
109 environmental conditions affect the life history traits of the invasive *X. securis*. Specifically, we tested the
110 effect of the predatory dogwhelk *Nucella lapillus* Linnaeus, 1758, which is one the most abundant benthic

111 predators on rocky shores of the inner areas of Galician rías (Gestoso et al. 2014), although it is absent in
112 areas characterised by low salinity. We carried out a transplant experiment between two locations that
113 differed in predation pressure (absence versus presence of dogwhelks) and environmental conditions. We
114 then carried out a laboratory experiment to evaluate how mussels completing their life cycle under different
115 environmental conditions and predation pressure respond to water-borne cues from the dogwhelk *N. lapillus*.
116 As temperature is known to influence the balance of energy expenditure by organisms and thus life history
117 responses (Broomhall 2004; Barbosa et al. 2014), we also investigated the role of temperature in shaping the
118 responses of mussels.

119

120 **2. Material and Methods**

121 *2.1. Study area and field experiment*

122 The field experiment was conducted at two different locations in the Ría de Vigo (NW Spain), between
123 the end of April and the end of July 2014: the inner location of Pontesampaio, at the mouth of the Verdugo
124 river, and the outermost Cesantes, under a stronger oceanic influence (Figure 1). The two locations differ in
125 environmental conditions, predation pressure and abundance of the invader (Gestoso et al. 2014). At
126 Pontesampaio, where the invader *X. securis* is most abundant (origin of the invasion process), the average
127 salinity (19.05 ± 9.49 , range 0 - 32.53, data reported as mean $\pm$ S.D.) and water temperature (mean 15.95°C
128 ± 3.90 , range 8.36 - 23.73) are both lower than at the outer location (salinity: 29.4 ± 2.73 , range 6.9 - 33.27;
129 temperature $16.48^{\circ}\text{C} \pm 2.76$, range 11 - 22.34) (*in situ* one-year data obtained with Star Oddi mini DST
130 CTDs), although the values vary widely due to the river influence and tidal cycles. Flow regimes also vary
131 from $8\text{--}123.1\text{ cm s}^{-1}$ at Pontesampaio and from $0\text{--}2.3\text{ cm s}^{-1}$ at Cesantes (Babarro & Lassudrie 2011).
132 Pontesampaio is also characterized by lower total and organic particulate matter contents (TPM: $1.98\text{ mg/L} \pm$
133 0.52 ; POM: $0.78\text{ mg/L} \pm 0.12$) and a lower chlorophyll *a* content ($2.39\text{ }\mu\text{g/L} \pm 0.61$) than at the outer
134 location (TPM: $2.20\text{ mg/L} \pm 0.72$; POM: $0.87\text{ mg/L} \pm 0.24$; chlorophyll *a*: $3.24\text{ }\mu\text{g/L} \pm 1.28$), where *X.*
135 *securis* is less abundant. Pontesampaio is characterized as an environment with a low predation risk
136 (hereafter LP), whereas Cesantes, where benthic predators are abundant, is characterized as an environment
137 with a high predation risk (hereafter HP). Although only few shell-drilling muricids and some fish have been

138 reported as predators of *Xenostrobus* species in its native range (Morton & Leung 2015), diverse benthic
139 species might prey upon it in the invaded area. The potential predators include the muricids *N. lapillus* and
140 *Ocenebra erinaceus* Linnaeus, 1758, the crustacean *Carcinus maenas* Linnaeus, 1758, and fish of the
141 families Gobiidae and Labridae (Filgueira & Castro 2011, Veiga et al. 2011, Gestoso et al. 2014).

142 We carried out a reciprocal transplant experiment to assess the effects of the physical environment and
143 predation pressure on the physiological responses of the invader. A previous study reported that the
144 physiological responses of *X. securis* were not affected by handling, caging or the biodegradable mesh used
145 (Gestoso et al. 2014). Although the experimental design included Predation (presence (+) and absence (-) of
146 dogwhelks) and Origin (LP and HP) as fixed factors, it was not fully orthogonal, because dogwhelks do not
147 occur naturally at the LP site.

148 Artificial mussel aggregations each comprising eight individuals were constructed on previously sanded
149 PVC plates (14 x 14 x 0.5 cm). Similarly sized individuals (25.61 ± 3.09 mm of shell length) were collected
150 from each location (LP and HP) and transported to the laboratory. The mussels were cleaned by removing
151 biofouling and remains of byssal threads from the ventral margin and were labelled individually for later
152 identification (by supergluing a piece of paper printed with a number to the shell). The mussels were placed
153 on the PVC plates, which were held in the laboratory for 2-3 days, to enable primary attachment, before
154 being situated in the field. A biodegradable mesh was used to facilitate attachment of mussels to the plates.
155 Plastic cages (14 x 12 x 8 cm) were used to exclude any other predators from the PVC plates to which the
156 experimental animals were attached; the cages were divided into two equal compartments by a double layer
157 of plastic mesh (1 x 1 cm) to prevent direct contact between the prey (mussels) and predators (dogwhelks).
158 Eight mussels and two dogwhelks (29.04 ± 1.71 mm; apex-base length) were placed in separate
159 compartments in each cage (Figure 2A). The densities (number of individuals per m⁻²) were chosen on the
160 basis of the natural densities of both species at Cesantes (*X. securis*: 8.47 ± 0.46 , n= 60; *N. lapillus*: $1.67 \pm$
161 0.18 , n= 60; results from 2011-2012 pooled data; unpublished results). Once assembled, the experimental
162 cage units (n=4) were transported to the field and screwed to rocky platforms with stainless steel screws. The
163 cages were randomly placed at the same height on the shore and separated by a minimum distance of ~1 m
164 (Figure 2B). Fouling was removed from cages every week, and the dogwhelks were replaced every two
165 weeks by others maintained in a reservoir tank in the laboratory and fed on *X. securis*.

166 The design included the following treatments: (1) local mussels from HP without dogwhelks (HP>HP-);
167 (2) local mussels from HP with dogwhelks (HP>HP+); (3) mussels transferred from LP to HP without
168 dogwhelks (LP>HP-); (4) mussels transferred from LP to HP with dogwhelks (LP>HP+); (5) local mussels
169 from LP without dogwhelks (LP>LP-), and (6) mussels transferred from HP to LP without dogwhelks
170 (HP>LP-).

171 Shell thickness index (STI), specific growth rate of shell (SGR) mussel (byssal) tenacity in an aggregation
172 (TEN), weight of the posterior adductor muscle (PAM), condition index (CI) and gonadal developmental
173 stage (GS) were measured to test the effect of experimental treatments on mussel performance. Prior to the
174 experiment, PAM (n= 20), CI (n= 12) and GS (n=10) were also measured in some individuals in each
175 population to evaluate the initial physiological status of mussels.

176 To evaluate growth, STI and SGR were measured in randomly selected and individually marked mussels
177 of each experimental treatment at the beginning of the experiment (n= 32). STI was calculated as follows:

178
$$STI = 1000 * \text{dry shell wt} / [L * (H^2 + W^2)^{0.5} * \pi / 2]$$

179 where L, H and W are respectively length, height and width of the shell (Freeman et al. 2009) measured
180 with a digital vernier caliper (± 0.1 mm). The immersed mass of each mussel was also obtained and
181 converted to dry shell weight by using individual destructive regressions for each of the two mussel
182 populations, i.e. twenty mussels/location (Palmer 1982). To estimate shell weight, mussels were sacrificed,
183 the tissue was dissected out and the shells were patted dry with paper towels and weighed on a Sartorius
184 precision digital balance (± 0.01 mg). After removing residuals of organic material, the shells were dried in a
185 muffle furnace at 100°C for two hours to remove moisture.

186 SGR was calculated as follows:

187
$$SGR = \ln(\text{final length} / \text{initial length}) * t^{-1}$$

188 where final length and initial length are the shell lengths at the end and beginning of the experiment,
189 respectively and t is the duration of the experimental period (90-d) in months (Christensen et al. 2015).

190 The strength of byssal attachment (referred to as tenacity) of mussels in each aggregation was measured
191 by connecting a single mussel to a spring scale (Digital Force Gauge DN431, 0.01 N resolution) with the aid
192 of custom-made forceps (see Babarro & Comeau 2014 for details of procedure). Care was taken to avoid
193 disturbing neighboring mussels when dislodging one individual. Individuals that were immediately adjacent
194 to those selected for dislodgement were not considered for trials if they had interconnected byssus threads.

195 This restriction explains why sample sizes were variable and lower than the total number of individuals.
196 Dislodgement measurements were made with wet mussels to prevent modification of the mechanical
197 properties of the byssus. Attachment force (F) was normalized by mussel size in order to calculate tenacity
198 (TEN, N m⁻²), as follows:

$$199 \quad \text{TEN} = F / AP$$

200 where *AP* is the projected area of the individuals pulled for dislodgement, approximately an ellipse
201 obtained by the product of width and height values of shells (n=13-15).

202 To determine PAM weight, the whole posterior adductor muscle of four mussels was removed with a
203 knife and pooled into a single replicate (n=4) to yield a sufficient amount of sample, which was then dried at
204 60 °C for 48 h and weighed. PAM values were standardized to mussel shell area (mg cm⁻²) and obtained
205 from shell length, height and width values (see STI for shell area formula).

206 The CI was calculated as follows:

$$207 \quad \text{CI} = (\text{DW}_{\text{tissue}} / \text{DW}_{\text{shell}}) \times 100$$

208 where *DW_{tissue}* is the dry weight of the soft-tissue and *DW_{shell}* is the dry shell weight (Freeman 1974).

209 To determine GS, a piece of gonad from each individual and for each experimental condition (n=10)
210 was dissected and routinely processed for histology, i.e. fixed in Davidson formaldehyde for 24 h,
211 dehydrated in an ethanol series, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and
212 eosin. Gonadal development stage was scored following a modified version of the scale proposed by
213 Martínez-Castro & Vázquez (2012): resting, gametogenesis, maturity, spawning, post-spawning and
214 exhaustion. When more than one developmental stage was evident within a single individual, the
215 reproductive stage was assigned according to the stage observed in most follicles.

216

217 *2.2. Laboratory experiment*

218 A mesocosm experiment was carried out between the end of January and the end of April 2014 to
219 evaluate the effects of predation and mussel origin on physiological performance of individuals and how
220 these responses are shaped by temperature. The experimental design included Origin (LP and HP),
221 Temperature (13 and 18 °C) and Predation (presence (+) and absence (-) of dogwhelks) as orthogonal fixed
222 factors.

223 Similar sized individuals to those used in the field experiment (see section 2.1.) were collected from the
224 same two locations and transported to the laboratory, where they were cleaned and marked following the
225 same protocol described above. Eight individuals were placed in glass Petri dishes and allowed to establish
226 primary attachment on a biodegradable mesh, which facilitated the final attachment. In this case, 3-L plastic
227 containers (19 cm diameter x 13 cm depth) were used as experimental units (n=4). The containers were
228 divided into two compartments using a double layer of plastic mesh (1 x 1 cm), and the mussels in the glass
229 Petri dish and two dogwhelks (when required) were placed in separate compartments (Figure 2C).
230 Experimental units were placed in 350-L PVC tanks inside an isothermal room and received light from
231 above, with a 12/12 light/dark photoperiod. The water temperature in the tanks was controlled using titanium
232 heaters and two levels were chosen to reflect mean temperature in the study area during late winter and early
233 summer, respectively.

234 Prior to the experiment, mussels were acclimated at 13°C for 7 days and the temperature was then either
235 maintained or increased gradually (1°C per day) until reaching 18°C. Salinity of the seawater in the
236 experimental units was maintained at 28 ± 0.2 and the seawater was renewed every two days. Dogwhelks,
237 which were replaced in experimental units every two weeks, were maintained in reservoir tanks under the
238 same conditions as in the experimental treatments and were fed *ad libitum* with *X. securis*. Mussels in the
239 experimental cage units were fed on a mixed diet composed of *Isochrysis galbana* clone T-ISO (40%),
240 *Chaetoceros gracilis* (25%), *Phaeodactylum tricornutum* (25%) and *Rhodomonas lens* (10%); a ration of 3%
241 of total tissue dry-weight was supplied in two doses every two days coinciding with seawater renewal.

242 The STI, TEN, CI and GS were measured to evaluate the effects of the experimental treatments on
243 performance of mussels (see for detailed description of procedure section 2.1). Prior to the experiment, TEN
244 (n= 25), CI (n= 12) and GS (n= 20) were measured in some (n) individuals in each population to evaluate the
245 initial physiological status of mussels. As in the field experiment, initial STI values were measured in
246 randomly selected and marked individuals of each experimental treatment (n= 32).

247

248 2.3. Statistical analysis

249 Initial differences (i.e. status of mussels before field and laboratory experiments) in STI, TEN, PAM
250 weight and CI from both locations were evaluated by one-way ANOVAs (two-tailed tests) with Origin as a
251 fixed factor.

252 In the field experiment, the response variables TEN, PAM and CI were analysed using distinct two-way
253 ANOVAs. Two-way ANOVA was first applied with Origin (LP and HP) and Exposure site (LP and HP) as
254 fixed factors, excluding the effect of the dogwhelk presence. A second two-way ANOVA was then applied
255 with Origin (LP and HP) and Predation (presence and absence of dogwhelks) as fixed factors only for site
256 HP where dogwhelks occur naturally.

257 In the laboratory experiment, the response variables TEN and CI were analysed by three-way ANOVAs
258 with Origin (LP and HP), Predation (presence and absence of dogwhelks) and Temperature (13 and 18°C) as
259 fully orthogonal fixed factors.

260 For the specific case of STI and SGR, changes in these variables over time were evaluated by ANCOVAs
261 with the same design as for the ANOVAs, but with initial STI and initial shell length values, respectively as
262 covariates. The interaction terms for each factor and the covariate were included in the design to test whether
263 slopes of the regression lines were significantly different. No significant interactions, i.e. p values > 0.05 ,
264 with the covariates indicated homogeneity of slopes and the analysis was re-run without considering
265 interactions (McDonald 2009).

266 Normality and homogeneity of variances were examined respectively by the Shapiro-Wilk W-test and
267 Levene's test. Data were transformed when necessary, and if heterogeneity persisted, rank transformation
268 was used (Conover 2012). Significant differences between experimental groups were tested using *a*
269 *posteriori* Tukey tests.

270 Gonadal stage was analysed by multinomial logistic regression. As the field experimental design was not
271 fully orthogonal, the data were split into two datasets and two separate analyses were carried out. The first
272 analysis tested the effect of Origin on mussels at LP, and the second analysis tested the effect of Predation
273 and Origin on mussels at HP. For the laboratory experiment, multinomial logistic regression was used to test
274 the effect of the three factors: Origin, Predation and Temperature.

275 All analyses were performed using the STATISTICA 7.0 software (Tulsa, OK, USA), except for
276 multinomial logistic regressions, which were performed with the multinom function from the multcomp
277 package for R 2.12.1 (R Development Core Team 2010). All data are reported as means $\pm$ SD.

279 **3. Results**280 *3.1. Field experiment*

281 At the beginning of the experiment, the shells of the mussels from site HP were 16% thicker ($F_{1,190}=$
 282 43.19; $P < 0.001$) than those of the mussels from site LP (Figure 3A). The differences related to initial STI
 283 values lasted for the whole experimental period as the shell thickness index (STI) only differed significantly
 284 in relation to the origin of mussels (Table 1; Figures 3A) when the effects of origin and exposure site were
 285 tested simultaneously. Both origin and predation had significant, non-interactive effects on the STI index of
 286 mussels at HP (Table 1). Mussels originally from HP had thicker shells (0.70 ± 0.10) than those transferred
 287 from LP (0.57 ± 0.06) at the end of the experiment (Figure 3A). At the end of the experimental period,
 288 mussels also had thicker shells (6-7%) when exposed to predators and regardless of their origin (Figure 3A).

289 The interaction between origin and site of exposure of mussels had a significant effect on SGR (Table 1;
 290 Figure 3B). Mussels originally from LP grew faster ($0.042 \pm 0.012 \text{ mo}^{-1}$) than mussels from HP when
 291 transferred to HP ($0.032 \pm 0.002 \text{ mo}^{-1}$). Similarly, both origin and predation had a significant interactive
 292 effect on SGR of mussels at HP (Table 1). The SGR only increased significantly in mussels originally from
 293 LP and transferred to HP without dogwhelks (0.050 mo^{-1} ; Figure 3B). Although mussels grew, no significant
 294 variation in SGR was detected when mussels were transplanted from HP to LP (Figure 3B).

295 Byssal tenacity (TEN) did not vary significantly with origin of mussels or exposure site (two-way
 296 ANOVA, Table 2) with values ranging from 6.38 to 7.99 ($\times 10^{-4}$) N. m^{-2} (Figure 3C). By contrast, in mussels
 297 at site HP, TEN was significantly affected by origin of mussels, but depended on the presence of predators
 298 (i.e. significant interaction Origin x Predation; Table 2; Figure 3C). Byssus was stronger in mussels
 299 originally from LP transplanted to HP with dogwhelks (up to 11.32×10^{-4} N. m^{-2}).

300 The PAM weight did not differ significantly between mussels from different locations at the beginning of
 301 the experiment (range of 0.64-0.67 mg cm^{-2} ; Figure 4A). After 3 months in the field, differences in PAM
 302 weight were due to the exposure site condition (Table 2; Figure 4A), and mussels from site HP had heavier
 303 adductor muscles ($0.75 \text{ mg cm}^{-2} \pm 0.08$) than those from site LP ($0.66 \text{ mg cm}^{-2} \pm 0.04$). Although the origin

304 of mussels did not significantly affect PAM weight at site HP, the presence of predators did have an effect
305 (Table 2; Figure 4A), with increments of 6% and 12% respectively for mussels originally from HP and LP.

306 The CI differed significantly between mussels from the two locations at the beginning of the experiment
307 ($F_{1,8} = 6.28$, $P < 0.05$), with higher values in mussels at site LP ($11.3\% \pm 1.10$) than in mussels at site HP
308 ($9.4\% \pm 0.76$; Figures 4B). After three months, CI values differed significantly depending on the origin of
309 mussels and exposure site, but with no significant interaction (Table 2). Although the condition of all
310 mussels originally from LP ($16.24\% \pm 3.10$) was better than that of mussels from HP ($11.96\% \pm 2.35$), at the
311 end of experiment CI was only higher in mussels from LP transferred to HP (Figures 4B). The interaction
312 between the origin of mussels and predation affected the condition of mussels at site HP (Table 2; Figure
313 4B). The condition of mussels originally from LP transferred to HP increased, especially in the absence of
314 dogwhelks (about 19.9%; Figure 4B).

315 At the beginning of the experiment, mussels from both locations were at an advanced stage of
316 maturation (Figure 5) as most had all follicles filled with ripe gametes. The gametogenetic stage of mussels
317 at LP did not differ significantly in relation to origin of mussels ($\chi^2 = 1.98$, $df = 2$, $P = 0.371$), although the
318 percentage of individuals originally from LP that spawned was slightly lower than that of individuals
319 transferred from site HP (50% and 70%, respectively). By contrast, the gametogenetic stage of mussels at
320 site HP differed depending on the origin of mussels ($\chi^2 = 7.84$, $df = 3$, $P = 0.049$; Figure 5). The mussels
321 originally from HP were at a more advanced stage of gametogenesis than the mussels transferred from LP.
322 This trend was more evident in the presence of dogwhelks, although the differences were not significant
323 (Figure 5).

324 3.2. Laboratory trial

325 Shells of mussels originally from HP were thicker (about 16%) at the beginning of the experiment ($F_{1,238} =$
326 24.38 ; $P < 0.001$; Figure 6A). ANCOVA applied to the STI values at the end of the experiment highlighted
327 the significant impact of origin and temperature as well as the interaction between the initial STI and both
328 factors (Table 3, Figure 6A). The shells were thicker at low temperature (0.53 ± 0.02) than at high
329 temperature (0.50 ± 0.02). The shells of mussels from HP were thicker (values ranged between 0.51 and 0.57)
330 than those of mussels from LP (values ranged between 0.46 and 0.50).

331 At the beginning of the experiment (i.e. one week under experimental conditions), the byssal attachment
332 strength was only significantly affected by temperature, with an increase in TEN at high temperature ($F_{1,43}=$
333 9.25 , $P < 0.01$ and $F_{1,49}= 7.91$, $P < 0.01$ for respectively LP and HP; Figure 6B). At the end of the experiment,
334 TEN varied significantly with temperature (Table 3), but also with origin of mussels, although depending on
335 the presence of predators (i.e. significant interaction Origin x Predation (Table 3, Figure 6B). Byssal tenacity
336 increased by up to 21% in mussels exposed to high temperature. Moreover, the strength of attachment in
337 mussels originally from HP increased, but only in the presence of dogwhelks (TEN up to 65% higher).

338 At the beginning of the experiment, CI differed significantly between mussels from both locations ($F_{1,6}$
339 $=25.99$, $P < 0.01$; Figure 6C), and the condition of mussels from site LP was better ($12.36\% \pm 0.70$) than that
340 of mussels from site HP ($9.27\% \pm 0.81$). The differences due to origin of mussels were maintained after the
341 experimental period (Table 3; Figure 6C), with values ranging from 10 - 12% and from 7.4 - 8.9% for
342 mussels from respectively LP and HP. The mussel condition index was higher at low temperature ($10.40\% \pm$
343 1.70) than at high temperature ($8.60\% \pm 1.40$) (Table 3; Figure 6C).

344 Mussels collected from both locations were at the end of the gametogenetic cycle as almost all of them
345 were already spent (Figure 7), and a new reproductive cycle started during the experimental period. The
346 presence of dogwhelks affected maturation of the mussels, although the effect varied with temperature (i.e.
347 Temperature x Predation interaction, $\chi^2= 12.46$, $df 5$, $P= 0.028$; Figure 7). Gametogenesis occurred faster at
348 high temperature, particularly in the presence of dogwhelks (i.e. more mussels were spawning or already
349 spent) than at lower temperature. These patterns were not affected by the origin of mussels.

350

351 4. Discussion

352 Although we are aware that the results of this study would be more robust if we had replicated
353 populations, it was not possible in practice within Ría de Vigo, especially at the mouth of the river, because
354 both environmental and biotic conditions varied at a very small spatial scale. Furthermore, there is no other
355 river mouth in the ría with similar density of *X. securis*. The best alternative as a complement to the field
356 survey was to carry out a mesocosm experiment in which individuals from the two distinct populations were
357 maintained under controlled conditions and exposed to different temperature and predation pressure during a
358 period of 3 months. Results of the mesocosm experiment pointed in the same direction than those of the field

359 survey and suggested that the origin of mussels is an important factor influencing phenotypic responses. To
360 mitigate potential pseudo-replication problems and draw more accurate conclusions, future studies including
361 mussel populations from other areas outside the Ría de Vigo, which experience similar abiotic and biotic
362 conditions and present similar pattern of invasion by *X. securis*, e.g. Ría de Pontevedra, would be necessary.

363 Results indicated that the non-indigenous mussels were clearly capable of activating phenotypic
364 responses to predation risk after 3 months of exposure, although they had not been exposed to the predator
365 signals in their original habitat (i.e. population at LP). The field transplant experiment revealed asymmetry in
366 relation to the impact of transplant direction as the most significant changes occurred in mussels transferred
367 to site HP, where the oceanic influence was stronger. The presence of dogwhelks induced development of
368 protective tissues in mussels, e.g. greater byssal tenacity, and to a lesser extent thicker shells and heavier
369 adductor muscle, but with different effects depending on the origin of mussels. Similar predator-induced
370 phenotypic responses (i.e. enhanced attachment strength, shell thickening and heavier adductor muscle) have
371 previously been reported for other mussel species (Leonard et al. 1999, Lowen et al. 2013). In the laboratory,
372 exposure of mussels to higher temperature caused an increase in attachment strength (TEN), but STI and CI
373 were significantly lower than in mussels maintained in colder water. Gametogenesis occurred faster at higher
374 temperature in the presence of dogwhelks.

375 Of all response variables considered here, mussel tenacity most clearly illustrates the plasticity linked to
376 the origin of mussels in both field and laboratory experiments. Byssus secretion represents a relatively short-
377 term response of individuals and can be activated by different abiotic and biotic factors in 6-50 hours (Côté
378 1995, Cheung et al. 2004, Shin et al. 2008). Mussels originally from site HP may have reacted to dogwhelks
379 as an important predator in their original habitat based on recognition experiences, as confirmed in the
380 laboratory experiment. By contrast, other responses such as weight of the adductor muscle and shell
381 thickness (involving calcium carbonate deposition and energy uptake allocated towards soft tissues) may
382 take longer to be modified significantly than byssus secretion. This may partly explain the smaller magnitude
383 of differences in adductor muscle and shell thickness between mussels in the presence and absence of
384 dogwhelks (6-17%) in both experiments. Mussels are able to recognize and differentiate between predator
385 species and to apply different types of phenotypic plasticity (Reimer & Harms-Ringdahl 2001). In the
386 present study, the increase in weight of the adductor muscle may represent a minor response that counteracts
387 the most common type of attack used by dogwhelks (see also Freeman 2007), i.e. drilling holes, although it

could confer mussels with a general strategy to respond to predation risk in a novel marine environment because dogwhelks can also feed on mussels through the gap between valves (Ebling et al. 1964). In the case of shell thickness, the initial differences between mussels from both populations, which lasted until the end of the experiment, may have minimized or masked any potential response to dogwhelk presence. The abundance of native predators in the wild (see Caro & Castilla 2004, Babarro & Abad 2013) and environmental factors such as wave exposure, temperature and salinity may have accounted for the initial variation in shell properties across locations (Dickinson et al. 2012).

As with any other trait, there is a cost associated with activation of inducible defences (Harvell 1990, Trussell & Smith 2000). Induced-predatory responses (especially byssal tenacity) in mussels transferred from the LP site were made at the expense of growth of shells and soft tissues (i.e. condition index), which only increased significantly in the absence of dogwhelks at the HP site. Thus, our findings support the notion of a trade-off between energy allocated to byssus production and growth, as previously found for other mussel species (Garner & Litvaitis 2013). Indeed, byssus production constitutes a substantial cost for some mussel species and may require up to 44% of total carbon and 21% of total nitrogen produced (Hawkins & Bayne 1985). Moreover, mussels from HP had thicker shells together with a poorer condition (i.e. CI) at the beginning of the experiments. The reduction in somatic growth may be the result of a direct trade-off between tissue growth and shell thickness in response to higher predation pressure at HP site. Nevertheless, reduced somatic growth in response to predation risk may also be a consequence of reduced or even suppressed feeding, rather than a direct trade-off associated with production of thicker shells (Smee & Weissburg 2006, Bourdeau 2010). Our results indicated a direct (active) physiological response of *X. securis* to predation risk as the most likely underlying mechanism, for the following reasons: (1) mussels originally from LP transferred to HP did not fully exploit their growth potential in the presence of dogwhelks; (2) in the presence of dogwhelks, the byssal tenacity, posterior adductor muscle weight and shell thickness increased in the transplanted animals, as did soft tissue weight i.e. condition (Figure 4B), which would be only plausible under optimal feeding or physiological rates (see Paige 1992); and (3) there was no relationship between the increase in linear shell growth and shell thickening, which suggests no direct constraints on energy investment in both shell characteristics.

The fact that the origin of mussels was an important factor explaining phenotypical responses, especially in the laboratory experiment, is consistent with previous findings (Trussell & Nicklin 2002; Turner et al.

2006). In this study, mussels originally from site LP did not respond significantly to the presence of kairomones, i.e. signals emanating from the predator itself. In contrast to other mussel species like *M. edulis*, which shows poor phenotypic integration with distinct predation cues (Freeman et al. 2009), the invader showed inducible changes specifically activated in the presence of dogwhelks and not disrupted by cues from other potential predators in the surroundings (Filgueira & Castro 2011, Gestoso et al. 2014). The ability of *X. securis* to respond to (new) predation risk would be extremely important for warning other conspecifics, with an eventual impact on other species of the community and their interactions. In the study area, dogwhelks seem to prefer to prey on *M. galloprovincialis* rather than on the invader (Gestoso et al. 2014) and, consequently, alarm cues may not only have emanated from conspecifics but eventually also from heterospecifics, with the magnitude depending on feeding preferences (Fässler & Kaiser 2008). Although the responses of *X. securis* reported here may have depended on experience (i.e. results of laboratory tests), other factors such as responses to conspecific and heterospecific cues may have made some contribution, according to field results. Further research with replicated populations from very distinct environments previously exposed or not exposed to dogwhelks would be necessary in order to draw more accurate conclusions.

Temperature, as a key parameter that regulates physiological and behavioural responses of organisms (Barbosa et al. 2014), had a significant impact on byssal tenacity, shell thickness and soft tissues of mussels, independently of the predator presence. The increase in byssal tenacity with increasing temperature was accompanied by a faster gametogenetic cycle, which may support the hypothesis that tenacity increases after spawning events in mussel displaying low reproductive activity (Carrington 2002). By contrast, STI and CI values were higher at low temperature. These results are surprising as thinner shells are commonly secreted at lower temperature because calcium carbonate saturation decreases and dissolution rates increase with decreasing temperature (Trussel & Smith 2000). Clearly, the lower condition index reported at high temperature can be explained by the fact that more than 80% of the population was spawning or already spent. As mussels used in the laboratory experiment were collected in winter, we can also hypothesize that the sudden increase in temperature up to 18 °C may have caused metabolic adjustments to optimized fitness (Barbosa et al. 2014). In addition, exposure to higher temperature may have increased the energy demands in mussels (see Mackenzie et al. 2014), while food availability was maintained constant in both temperature treatments. In contrast to the other physiological responses, gametogenesis was interactively affected by

446 temperature and predation. Indeed, thermal conditions can affect reproductive traits of individuals in
447 response to predation risk (Barbosa et al. 2014). The fact that most of response variables were not
448 interactively affected by temperature and predation can have different explanations. At 18 °C, dogwhelks
449 were observed laying egg capsules on the walls of containers throughout most of the experimental period.
450 Reproduction may have negatively affected the production of kairomones because of the associated cost, e.g.
451 dogwhelks begin to forage optimally only after 2-3 weeks of reproduction (Gosselin & Bourget 1989), with
452 significant consequences on perception of the predator. Alternatively, as kairomones released from predators
453 may decompose over time, it is possible that degradation process was faster at the higher temperature, i.e. 18
454 °C (see Lass & Spaak 2003).

455 In conclusion, the cost of constitutive defences and the variability in predation pressure in estuarine areas
456 favour the development of inducible defence mechanisms in *X. securis*. The study findings also demonstrate
457 that the environment with the strongest marine influence colonised by the invader offers natural resources
458 that allow the individuals to activate inducible defences without compromising growth (e.g. of soft tissues).
459 However, the activation of protective responses of mussels to the presence of predators came at a cost, as
460 indicated by the observed trade-off between shell growth and byssus tenacity. The fact that the origin of
461 mussels helped to explain individual's responses indicates that the invader is able to adapt and respond to
462 new environments. The ubiquity and magnitude of predator-induced changes suggest that phenotypic
463 plasticity plays an important role in determining the invasiveness of a NIS in new invaded habitats and thus
464 in shaping marine communities. Further studies integrating the topics of biological invasions and phenotypic
465 plasticity are urgently needed for accurate assessment of the invasion risk associated with other species.

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470

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599

Figure Legends

Figure 1. Map of the Ría de Vigo (NW Spain) showing the experimental sites where the mussels were collected (Sampaio [LP] and Cesantes [HP]).

Figure 2. Details of (A) experimental units, (B) arrangement of experimental units in the field experiment, and (C) experimental units used in the laboratory experiment. In all experimental units, mussels and dogwhelks were held in different compartments separated by a double layer of plastic mesh.

Figure 3. Mean values ($\pm$ S.D.) of (A) shell thickness index, (B) specific (shell) growth rate, and (C) mussel tenacity at the beginning ($t=0$) and at the end (i.e. 3 months after starting) of the field experiment according to exposure site (HP and LP) and predator presence (+) or absence (-). See Material and Methods, and Results sections for information on the transplant direction and animals used in each experimental comparison.

Figure 4. Mean values ($\pm$ S.D.) of (A) weight of posterior adductor muscle and (B) condition index of mussels at the beginning ($t=0$) and at the end (i.e. 3 months after starting) of the field experiment according to exposure site (HP or LP) and predator presence (+) or absence (-). See Material and Methods, and Results sections for information on the transplant direction and animals used in each experimental comparison.

Figure 5. Gonadal stage of mussels ($n=10$) in the field experiment at the beginning of the experiment ($t=0$) and in relation to exposure site (HP or LP), and predator presence (+) or absence (-).

Figure 6. Mean values ($\pm$ S.D.) of (A) shell thickness index, (B) mussel tenacity, and (C) condition index of mussels from sites HP and LP at the beginning ($t=0$) and at the end (i.e. 3 months after starting) of the laboratory exposure to different temperatures (13 and 18°C), and predator presence (+) or absence (-). See Material and Methods, and Results sections for information on the animals used in each experimental comparison.

Figure 7. Gonadal stage of mussels ($n=10$) in the laboratory experiment at the beginning of the experiment ($t=0$) and in relation to temperature (13 and 18 °C), and predator presence (+) or absence (-). The data from HP and LP were pooled as neither the origin nor any interaction with this factor was significant.

Figure 1

Figure 2

A. Field

B. Field.

C. Laboratory.

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1. Field experiment. Results of ANCOVAs to determine the effect of the initial STI and shell length at t=0 as covariates as well as Origin and Exposure site (A) and Origin and Predation (B) on shell thickness index (STI) and shell length growth (SGR) values, respectively. See main text (Materials and Methods) for specific details of each factor.

STI				SGR		
A. ANCOVAs						
Factor	df	F	P	df	F	P
initial t_0	1	88.56	2.11×10^{-15}	1	147.45	$< 1.00 \times 10^{-18}$
Origin (OR)	1	18.58	3.80×10^{-5}	1	0.92	0.340
Exposure site (ES)	1	0.37	0.547	1	0.18	0.675
OR x ES	1	0.36	0.548	1	13.44	3.86×10^{-4}
Error	99			106		
B. ANCOVAs						
Factor	df	F	P	df	F	P
initial t_0	1	162.74	$< 1.00 \times 10^{-18}$	1	184.07	$< 1.00 \times 10^{-18}$
Origin (OR)	1	55.89	2.41×10^{-11}	1	0.68	0.412
Predation (PR)	1	9.16	3.11×10^{-3}	1	2.34	0.129
OR x PR	1	1.55	0.215	1	5.39	0.022
Error	105			109		

Table 2. Field experiment. Results of two-way ANOVAs to determine the effect of Origin and Exposure site (A) and also Origin and Predation (B) on byssal tenacity (TEN), weight of the posterior adductor muscle (PAM) and condition index (CI) of the mussels. See Material and Methods for specific details about each factor. All analyses were subjected to log transformation prior to the analysis.

A. Two-way ANOVAs									
Factor	TEN			PAM			CI		
	df	F	P	df	F	P	df	F	P
Origin (OR)	1	3.95	0.053	1	2.94	0.112	1	15.70	1.88 x 10⁻³
Exposure site (ES)	1	1.10	0.318	1	7.90	0.015	1	17.74	1.21 x 10⁻³
OR x ES	1	1.97	0.212	1	0.15	0.708	1	3.36	0.091
Error	54			12			12		

B. Two-way ANOVAs									
Factor	TEN			PAM			CI		
	df	F	P	df	F	P	df	F	P
Origin (OR)	1	11.33	1.44 x 10⁻³	1	0.81	0.385	1	13.53	3.16 x 10⁻³
Predation (PR)	1	5.90	0.018	1	5.20	0.040	1	1.37	0.265
OR x PR	1	4.39	0.041	1	0.99	0.340	1	6.66	0.024
Error	52			12			12		

Table 3. Laboratory. Results of ANCOVA (A) to determine the effect of the initial STI ($t=0$) as covariate as well as Origin, Temperature and Predation on shell thickness index (STI) and three-way ANOVA (B) for tenacity (TEN) and condition index (CI) of the mussels with the same factors. See main text (Materials and Methods) for specific details of each factor. STI was Rank transformed prior to the analysis. TEN and CI were log transformed.

STI				
A. ANCOVA				
Factor	df	F	P	
initial t ₀	1	78.05	4.41 x 10⁻¹⁶	
Origin (OR)	1	4.57	0.033	
Temperature (TEMP)	1	15.06	1.40 x 10⁻⁴	
Predation (PR)	1	0.30	0.584	
OR x TEMP	1	0.01	0.793	
OR x PR	1	0.08	0.933	
TEMP x PR	1	2.52	0.116	
OR x TEMP x PR	1	0.94	0.334	
OR x STI _{t=0}	1	5.47	0.020	
TEMP x STI _{t=0}	1	10.67	1.27 x 10⁻³	
Error	206			

TEN				CI		
B. Three-way ANOVAs						
Factor	df	F	P	df	F	P
Origin (OR)	1	2.27	0.135	1	48.06	3.61 x 10⁻⁷
Temperature (TEMP)	1	4.98	0.028	1	20.22	1.49 x 10⁻⁴
Predation (PR)	1	18.31	4.30 x 10⁻⁵	1	0.04	0.864
OR x TEMP	1	1.20	0.275	1	1x10 ⁻⁴	0.994
OR x PR	1	5.14	0.025	1	0.16	0.688
TEMP x PR	1	1.15	0.287	1	0.10	0.755
OR x TEMP x PR	1	0.04	0.843	1	0.03	0.869
Error	99			24		