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1 **Patterns of distribution, population genetics and ecological requirements of**
2 **field-occurring resistant and susceptible *Pseudosuccinea columella* snails to**
3 ***Fasciola hepatica* in Cuba**

4
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20 **Abstract**

21 *Pseudosuccinea columella* snails transmit the trematode *Fasciola hepatica*, but in Cuba, six
22 naturally occurring populations successfully resist parasite infection. Here, we present an
23 updated distribution of *P. columella* in Cuba; 68 positive sites with the earliest records more
24 abundant in west-central Cuba and with east-central populations generally corresponding to the
25 newest samples. No records were found farther east. The IPA site reported 10.5% prevalence of
26 *F. hepatica*-infected snails. Population genetics, studied through microsatellites, showed low
27 allelic and multilocus genotypic richness (MLGT), mainly in susceptible populations, strong
28 deviations from panmixia and high self-fertilization rates. Susceptible individuals were grouped
29 in one major cluster containing the majority of MLGT, and two independent clusters grouped
30 the MLGT of resistant individuals from western and central populations, respectively. From
31 these, we propose that several introductions of *P. columella* occurred in Cuba, primarily in the
32 west, with the early arrivals deriving on the resistant populations. A more recent introduction of
33 susceptible *P. columella* carrying MLGT *T* and *Y* may have occurred, where the latter spread
34 quickly through the island and possibly increase the risk of parasite transmission in Cuba since
35 all snails naturally infected with *F. hepatica* were carriers of the MLGT *Y*. Interestingly, even
36 though resistant populations are highly diverse and are likely the oldest within Cuba, they are
37 only found in six localities characterized by soft (total hardness, TH=6.3±1.03°d) and slightly
38 acidic (pH=6.2±0.12) waters with low richness in snail species (3.2±1.02). This tendency was also
39 observed in a two-year follow-up ecological study that was conducted on a farm where both
40 phenotypes occurred in sympatry; colonization events by resistant over susceptible snails
41 coincided with a reduction in the pH and TH of the water. A comparison of life traits in
42 susceptible and resistant isolates reared at two different pH/TH conditions (5.9/4°d or 7.8/14°d)
43 showed that low pH/TH negatively affects *P. columella*, irrespective of the phenotype. However,
44 evidence of higher tolerance (higher survival, life expectancy, egg viability) to such conditions
45 was observed in resistant isolates. Finally, we speculate that the limited distribution of resistant
46 populations might be related to a better exploitation of sites that are less suitable to snails
47 (thus, with lower competition), rather than to a differential ecological restriction to specific
48 environmental conditions from susceptible *P. columella*.

49 **Keywords:** ecology, population genetic, invasion, liver fluke, Lymnaeidae, microsatellites, life
50 traits, natural prevalence, cost of resistance

51 **1. Introduction**

52 Freshwater snails of the family Lymnaeidae (also known as pond snails) inhabit tropical,
53 temperate and cold regions, from sea level to high altitudes ¹. Many of the nearly 100 species
54 recently attributed to this family ² have become the target of taxonomical and parasitological
55 studies over the past few years, due to their important roles as intermediate hosts of several
56 medically relevant parasites, including the globally distributed liver fluke *Fasciola hepatica* (e.g.
57 ^{3,4}). Within Lymnaeidae, several species have been incidentally carried out of their native range
58 and introduced elsewhere (see the cases of *Galba cubensis* and *Austropeplea viridis* in Europe;
59 ^{5,6}). One of the most interesting examples is that of *Pseudosuccinea columella* (Say, 1817), a
60 globally introduced species with a considerable invasive ability ⁷. The transmission of *F. hepatica*
61 by *P. columella* is well-documented in its native range (North America; see ⁸) and in places
62 where it has been introduced such as Brazil ⁹, Argentina ¹⁰ and Australia ¹¹. This species is also
63 thought to enhance transmission in remote regions where it has invaded, like in Egypt ¹² and the
64 Pacific islands, including Hawaii and French Polynesia ^{13,14}.

65 In Cuba, the first report of *P. columella* dates back to 1858 in the western region and the latest
66 malacological surveys depict a western-central distribution ¹⁵. Although this species is thought
67 to play a secondary role in *F. hepatica* transmission in Cuba (with *Galba cubensis* being the main
68 host; see ^{16,17}), it is found naturally infected in the field ¹⁸. Important investigations have been
69 carried out on *P. columella* in recent decades due to the existence of a resistant phenotype
70 against *F. hepatica* infection in certain natural Cuban populations ^{19,20}. Experimental exposures
71 of these resistant snails to different sympatric and allopatric fluke isolates always result in the
72 failure of parasite larval stage development ¹⁹⁻²² which is associated with an effective
73 encapsulation of the parasite by snail immune cells ¹⁹.

74 Interestingly, thus far, all resistant *P. columella* can be easily differentiated from susceptible
75 individuals in the field though the use of a phenotypic marker consisting of a belt-like pattern of
76 small, sharp and whitish spots in the mid-region of the mantle with bigger spots scattered on
77 the upper and lower sides ¹⁹. Attempts to genetically differentiate susceptible from resistant
78 individuals have independently clustered both phenotypes by RAPD markers ²¹. More recently,

79 Cuban resistant populations were segregated from *P. columella* populations found elsewhere,
80 using mitochondrial haplotypes and microsatellites markers ⁷. It is important to stress that the
81 observed molecular and phenotypic differences are not a reliable mean of separating resistant
82 populations as a different species. Resistant snails displayed the same reliable features of the
83 shell and the same reproductive system anatomy defined for *P. columella* with only a slight
84 difference in two-nucleotide changes (0.17%) within the ITS1 and ITS2 when ribosomal genes
85 were sequenced and compared ²³. From an ecological point of view, previous comparative
86 studies on demographic dynamics in the laboratory between susceptible and resistant snails
87 have suggested the existence of a trade-offs against reproduction of the latter ^{24,25}.

88 The natural occurrence of populations that are susceptible and resistant to a parasite within the
89 same host species provides a unique model that can be addressed from several disciplines,
90 including evolutionary biology and host-pathogen interaction, environmental and health
91 sciences and snail control. Here, for the first time, we present a global overview of the ecology
92 of *P. columella* in Cuba, where both phenotypes are considered separately by the use of
93 different approaches. We present its updated distribution, study its infection status and its
94 genetic structure by microsatellites markers, and elucidate the ecological patterns associated
95 with the occurrence of resistant and susceptible populations through the analysis of field data
96 and experimental life history traits. From these, we gain insight into the history, colonization
97 and population structure of *P. columella* in Cuba, its relationship with *F. hepatica* and the
98 increasing risk of parasite transmission represented by certain widespread genotypes. In
99 addition, we provide further knowledge related to habitat preferences of *P. columella* and the
100 environmental conditions that contribute to its occurrence in the wild. All of these aspects are
101 essential to understand and to predict the distribution pattern and further spreading of *P.*
102 *columella* snails in Cuba. Findings from this study may play an important role in conservation
103 biology, parasitology and epidemiology and to devise intermediate host control strategies based
104 on the potential application of *P. columella* resistance.

105 **2. Material and Methods**

106 **2.1. Distribution of *P. columella* in Cuba**

107 The updated distribution of *P. columella* was obtained from the National Reference
108 Malacological Database for freshwater snails in Cuba, held by the Laboratory of Malacology of
109 the Institute of Tropical Medicine in Havana, through the revision of 496 sampled sites
110 (=populations) from 1982 to 2018. Each record includes the presence of a given freshwater
111 snail, geographic coordinates, habitat type, date and collector. Nationwide malacological
112 surveys are carried out by our laboratory on a 10-year sampling period basis complemented
113 with annual expeditions covering smaller areas. All sites positive for *P. columella* were plotted
114 on a map differentiating each record according to a 11-year time interval, to account for new
115 and historical data, using MapInfo v.15 (Pitney Bowes Software Inc., New York, USA, 2015). The
116 frequency of each habitat type where *P. columella* was observed was used as a measure of
117 habitat preference.

118 Differentiation of resistant and susceptible *P. columella* populations to *F. hepatica* infection
119 were primarily based on the presence or absence of a characteristic band of small sharp spots in
120 the mid-region of the mantle, as its occurrence is associated with the resistant phenotype¹⁹.
121 Additionally, both phenotypes, and particularly that of the resistant populations, were double-
122 checked in the laboratory by experimental infection using different panels of potentially
123 sympatric (retrieved from infected cattle reared at the site or in nearby areas) and allopatric *F.*
124 *hepatica* isolates (e.g.²⁰⁻²²). The latter confirmed the reliability of the mantle pigmentation
125 pattern as a distinctive phenotypic marker for *F. hepatica* resistance in *P. columella*.
126 Experimental infections were carried out following the methodology previously described²⁰,
127 exposing 30 snails per population to a dose of five *F. hepatica* miracidia.

128 **2.2. Population genetics of *P. columella* from Cuba**

129 *2.2.1 Snail sampling, DNA extraction, amplification and genotyping of microsatellite loci*

130 We sampled 20 populations of *P. columella* (17 susceptible and 3 resistant, *i.e.* displaying the
131 reliable phenotypic mantle marker described by Gutiérrez et al.¹⁹). When possible, up to 30
132 individuals were collected from each population, up to a total of 329 snails. The snails were
133 carefully dissected in the laboratory for screening of possible parasite infection. A piece of tissue

134 from the foot was used for DNA extraction to avoid foreign genetic contamination (*e.g.* sperm
135 from cross-fertilization). The extraction of DNA was performed using Chelex methodology
136 following²⁶ and slightly modified for 96-well plates. In brief, a small portion of tissue was added
137 to a mixture of 100 μ L of 5% Chelex[®]100 (Bio-Rad) and 5 μ L of proteinase K (50 mg/mL;
138 Promega) in each well. The plate was mixed in vortex, incubated overnight at 56°C and placed
139 for 10 min at 95°C. The mixture was centrifuged at 6000 x *g* for 6 min and the supernatant
140 containing the DNA was collected and stored at -20°C until use.

141 PCR amplifications were performed in a 96-well MJ-Research PTC 100 for all samples. Six
142 microsatellite loci, previously described for *P. columella* species by Nicot et al.²⁷, were amplified
143 (GenBank Access number): PCO01 (EU04295), PCO02 (EU049296), PCO07 (EU049299), PCO12
144 (EU049303), PCO13 (EU049304) and PCO20 (EU049309). Each locus was amplified through PCR
145 using 1 μ L of extracted DNA in 10 μ L of reaction volume containing 2 μ L buffer 5X (Promega), 1
146 μ L of 25 mmol/L MgCl₂, 0.5 μ L of 2mmol/L dNTP (Invitro-gen/Life Technology), 0.2 μ L of each
147 primer (10 pmol) and 0.2 μ L of *Taq* DNA polymerase (Promega). Thermocycling consisted of 10
148 min of initial denaturalization at 94°C, 30 cycles at 94°C for 30 s, annealing at 55°C (for each
149 locus) for 30 s, 60 s at 72°C and a final elongation step at 60°C for 30 min.

150 Primers were fluorescently labelled to be used in an ABI automated sequencer (ABI Prism 310
151 Genetic Analyser, Applied Biosystem, Perkin-Elmer, USA). Amplified products for each individual
152 were used for sequencing where 1 μ L of diluted amplicon (1/100) was added to a mix containing
153 15 μ L of Hi-Di Formamide and 0.27 μ L of internal size standards (GENESCAN 500 LIZ, Applied).
154 Each allele's length was read using GeneMapper[®] v.4.0 software (Applied Biosystems[™]).

155 2.2.2 Population genetics analysis

156 Current parameters of population genetics like the mean number of alleles (*a*), the observed
157 (*H_o*) and expected (*H_s*) heterozygosity and *F_{IS}* were estimated for each locus. Pairwise
158 differentiation between populations (*F_{ST}*) was also tested. Calculations were computed using
159 FSTAT v2.9.3.2²⁸ and Bonferroni corrections were applied for multiple tests²⁹. The selfing rate
160 (*s*) for each population was calculated using the equation $s = 2F_{IS}/(1+F_{IS})$ ³⁰. Identical multilocus

161 genotypes (MLGT) were identified based only on individuals with all microsatellite loci amplified
162 and coded using a combination of letters (one letter or letters' combination = one MLGT =
163 unique combination of alleles across all analysed loci). Nei's ^{31,32} genetic distances between
164 populations were estimated using Genetix ³³. The obtained distance matrix was used to build a
165 neighbour-joining network of all MLGT in SplitsTree4 ³⁴. The genotypic diversity was calculated
166 in each population with an adaptation of the Simpson index using GENCLONE 2.0 ³⁵.

167 **2.3. Ecological patterns of resistant and susceptible *P. columella***

168 The ecological patterns of the two phenotypes of *P. columella* were explored using three
169 approaches: laboratory field records, field study and experimental life tables.

170 *2.3.1 Revisiting laboratory field records of resistant and susceptible *P. columella* populations*

171 A thorough literature review which included published papers and 37 years of unpublished data
172 from our laboratory's field notes, was conducted. We gathered data for species composition of
173 the mollusc assemblage and field abiotic factors (phosphates, nitrates, dissolved oxygen, pH and
174 total hardness) measured for resistant and susceptible populations investigated within this
175 study.

176 *2.3.2 Ecological follow up of resistant and susceptible populations of *P. columella**

177 We performed a two-year follow-up ecological study on the La Coca (= locality La Coca) cattle
178 farm located in south-western Havana, as it harbours resistant and susceptible populations of *P.*
179 *columella* (see Figure 1A). The region is characterized by a lowland meadow that is commonly
180 flooded during the rainy season, which extends for nearly 10 km². The area includes several
181 channels used for irrigation and livestock watering, usually no more than 60 centimetres in
182 depth. Gramineous vegetation is abundant but aquatic plants in the channels are scarce. These
183 features contribute to the presence of several water bodies (sites) scattered throughout the
184 locality where freshwater snail populations thrive. The *P. columella* species is present at five
185 sites from the La Coca locality, four of which harbour susceptible populations. The fifth site is

186 known as Segundo Potrero and is characterized by the occurrence of a resistant *P. columella*
187 population named La Coca after the locality.

188 Thus, based on these characteristics, we selected two sites from the locality La Coca for the
189 ecological follow-up: (i) Segundo Potrero, due to the presence of the resistant *P. columella*
190 population (La Coca population) and (ii) a nearby site called Negrines, 500 m away and
191 connected by one of the channels, where a population of susceptible individuals (Negrines
192 population) occurs. Each site was sampled at monthly intervals from January 2011 to December
193 2012 always by the same person during the early hours of the morning, using soft forceps and
194 sieves (1 mm mesh). We used the capture per unit of effort method over a 15 minute interval ³⁶
195 and relative abundance was measured as individuals/15' for each species. Aquatic and shore
196 vegetation were swept for every sample. Resistant snails were easily identified in the field by
197 the phenotypic marker of mantle pigmentation ¹⁹. Abiotic factors including pH, temperature,
198 total hardness (TH), carbonate hardness (CH), dissolved oxygen, phosphates and nitrites were
199 measured using a field kit (Merck). Species diversity was calculated for each site using the
200 Simpson index ³⁷ and a canonical correspondence analysis ³⁸ was done to detect unimodal
201 response of mollusc species abundances to ecological factors.

202 Additionally, all collected lymnaeid snails were brought to the laboratory and dissected to
203 account for *F. hepatica* larval stage infection.

204 2.3.3. Laboratory life tables of resistant and susceptible *P. columella*

205 Four natural populations of *P. columella*; two composed of susceptible (Negrines, 22.9577°N; -
206 82.4649°W and Aurora, 23.0789°N; -81.9176°W) and two of resistant (La Coca, 22.9554°N; -
207 82.4598°W and La Palma, 22.7695°N; -83.5443°W) individuals (see Figure 1B), were sampled
208 and reared under laboratory conditions for one generation. Given the results from field data
209 concerning the ecological patterns related to the occurrence of both phenotypes, two different
210 conditions of pH/total hardness (TH) were set up: common (7.6/14°d; according to Perera ³⁶,
211 those conditions are the most commonly found for Cuban freshwater habitats) and lower
212 (5.9/14°d) conditions. Snails were then reared in the two conditions using separate 25 L
213 aquariums. Five-day-old snails from each isolate (n=30 per population at each condition) were

214 placed in the aquariums and this moment of transfer was considered as time zero for the
215 experiment. All aquariums were provided with 50 mg each of CaCO₃ and MgCO₃ to contribute to
216 shell formation. Water acidification down to pH 5.9 (low pH condition) was achieved through an
217 automatic CO₂ pump connected to a setter pH meter. Total hardness conditions were adjusted
218 to the required range for each aquarium by differentially mixing aged tap water with distilled
219 water. Both pH and TH were systematically checked along with other abiotic parameters (Cl⁻,
220 NO₃⁻, NO₂⁻, CH) with a commercial aquarium kit to guarantee optimal and reproducible
221 conditions during the experiment. Temperature was always maintained at 25-26°C and the
222 snails were fed three times per week with a mixture of algae cultured for this purpose following
223 Sánchez et al.³⁹. All populations were followed until the death of all individuals. Live and dead
224 snails, the number of eggs, egg masses and newly hatched snails (NHS) were counted at weekly
225 intervals. At the end of the experiment, the following life table parameters were determined
226 according to Margalef⁴⁰: proportion of living individuals (lx), probability of survival (px), life
227 expectancy (ex), fecundity rate (mx), net reproduction rate (Ro), and intrinsic (r) and finite (λ)
228 rates of natural increase.

229 Snail survival curves were constructed based on the percentage of live snails at each time point
230 and pairwise log-rank tests of Kaplan-Meier curves were performed to assess the statistical
231 differences on survivorship data. The factorial ANOVAs followed by the *post hoc* multiple
232 comparison Tukey test were carried out to assess statistical significance between the mean
233 values of eggs, egg masses and egg/egg masses per population. The overall percentage of viable
234 eggs (*i.e.* viable eggs = NHS/number of eggs) per strain at each condition was compared through
235 a Fisher's Exact Test. Similarly, the percentage of NHS from the first, second and third week
236 after egg laying was determined for each strain and compared between experimental settings
237 to assess possible delays. Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA 2014) software was used
238 for calculations and differences were considered significant when $P < 0.05$.

239 **2.4. Ethics approval**

240 French laboratory holds permit #A66040 for experiments on animals, which was obtained from
241 the French Ministry of Agriculture and Fisheries and the French Ministry of National Education,

242 Research and Technology. The housing, breeding and care of the utilized animals followed the
243 ethical requirements of France. The experimenter possesses an official certificate for animal
244 experimentation from both of the above-listed French ministries (Decree #87–848, October 19,
245 1987). The various protocols used in this study have been approved by the French Veterinary
246 Agency of the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de
247 l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083). The Cuban laboratory
248 of Malacology holds permit for experiments on animals obtained from the Cuban government.
249 The housing, breeding and care of the utilized animals followed the ethical requirements of
250 Cuba. The experimenter possesses an official certificate for animal experimentation from the
251 Cuban government.

252 **3. Results**

253 **3.1. Distribution of *P. columella* in Cuba**

254 Knowing the distribution of a snail species is particularly important to assess its epidemiological
255 significance and to attempt its effective control. The National Reference Database contains
256 nearly 500 records in Cuba (Figure 2A), from which 68 verified sites harbour *P. columella* species
257 (Figure 2B). The distribution of *P. columella* ranges from the westernmost region (Pinar del Río
258 province) to the east-central (Camagüey province) region of Cuba (Figure 2B). No records exist
259 for the easternmost provinces. Notably, 59% of sampled populations were documented in the
260 last 15 years. Overall, earliest records are more abundant in the west-central region while
261 populations from east-central Cuba correspond mostly to newer samplings. From the 68 verified
262 *P. columella* populations, only six are acknowledged as resistant to *F. hepatica* infection (*i.e.*
263 snails displaying the characteristic mantle pigmentation pattern described by Gutiérrez et al.¹⁹
264 and showing no record of parasite development following exposure to different *F. hepatica*
265 isolates). Populations of resistant individuals are mostly recorded in the west (Pinar del Río –
266 Havana provinces) with the exception of Babiney, in Cienfuegos (central Cuba; Figure 2B).

267 Concerning habitat type preferences, permanent ponds, rivers and dams harbour 62.5% of all *P.*
268 *columella* records, with up to six different habitats concentrating the remaining populations
269 (Figure 2C). We did not find a clear pattern of habitat types for resistant populations which were

270 observed in permanent ponds (2), flooded lands (1), springs (1), ditches (1) and canals (1).
271 Globally, the number of populations recorded in open or connected water systems (*e.g.* rivers,
272 streams, canals) is fewer than those of closed habitats (*e.g.* dams, ponds, flooded lands; Figure
273 2C).

274 **3.2. Population genetic structure of *P. columella* in Cuba**

275 Population genetic studies on *P. columella* could give insights into important questions for
276 biological and health sciences; its evolutionary history in Cuba, its pattern of colonization, the
277 genetic structure of its populations and its role in *F. hepatica* transmission. Thus, from the
278 distribution of *P. columella* presented above, we selected 20 populations (see Figure 3 for
279 details) to analyse the genetic structure of this species in Cuba. Records of *P. columella* infected
280 with different trematode species in Cuba exist in our laboratory but, in the present study,
281 dissection of 329 snails only evidenced infection of *F. hepatica* in two individuals from IPA
282 population (10.5% prevalence, IPA site). No other infections, by *F. hepatica* or other trematode
283 species, were recorded at any other site.

284 The six microsatellite loci explored were polymorphic in *P. columella*, especially PCO12 which
285 displays seven different alleles in Cuba. Population genetic parameters are presented in Table 1.
286 Allelic richness is very low in the 20 studied populations (mean 2.034 ± 0.646 SD) and five
287 populations (Pilón, El Antojo, Puesto de Mando, Guillén and Arroyo) are completely
288 monomorphic at the six loci. The highest allelic richness was observed in the two resistant
289 populations of La Coca and Babiney (2.17 ± 0.75 SD). However, the analysis of the third resistant
290 population (El Azufre) showed a lower allelic richness (1.17 ± 0.41 SD). Expected heterozygosis
291 (mean 0.1 ± 0.09 SD) and observed heterozygosis (0.03 ± 0.056 SD) were very low. Most
292 populations had high and significant values of F_{IS} after Bonferroni correction (mean $0.667 \pm$
293 0.313 SD) for which estimated selfing rates were also high (> 64%). Although an overall value of
294 F_{ST} of 0.339 ($P > 0.05$) was observed, significant differentiation was only recorded in resistant
295 populations (La Coca, Babiney and El Azufre) that not only differed from susceptible populations
296 but also from each other (Supplementary Table S1).

297 Multilocus genotyping at the six microsatellite loci showed the existence of 36 MLGT. Observed
298 mean number of MLGT in susceptible populations was 2.764 (\pm 1.562 SD) while a higher average
299 of 6.333 (\pm 3.785 SD) was attained in resistant populations. In particular, differences in MLGT
300 between each group of populations were statistically significant (t value (df: 18) = 3.58; P =
301 0.002). Mean genotypic diversity was 0.397 (\pm 0.357) with the highest values in the resistant
302 populations of La Coca (0.893) and Babiney (0.836). In the case of resistant individuals, 18 MLGT
303 were observed for which only MLGT *E* is shared in two populations (El Azufre and La Coca).
304 Figure 3A shows the distribution of the 36 MLGT in Cuba with the MLGT *Y* widespread in most
305 sampled sites and the only one observed in susceptible monomorphic populations. This MLGT *Y*
306 is absent in resistant populations (Figure 3A). Interestingly, the two infected individuals found in
307 the IPA population and the population from Pilón previously reported as infected (see Gutiérrez
308 et al. ¹⁸), shared the same MLGT *Y*. A different MLGT (*T*) was observed only in the easternmost
309 populations (Río Manaquita, Río Central and Matadero Aves) within several individuals. The rest
310 of the MLGT are considered rare and differ from one another by a single or few alleles. The
311 obtained MLGT network from Nei's genetic distance displays three majoritarian clusters (Figure
312 3B). Susceptible MLGT gathered within one major cluster. Two different major clusters and one
313 small cluster contain most of the MLGT from resistant individuals (Figure 3B).

314 **3.3. Ecological patterns associated with the occurrence of resistant and susceptible *P.*** 315 ***columella* in Cuba: field and laboratory observations**

316 The higher allelic and MLGT richness of resistant populations presented herein contrasts with
317 their restricted distribution, as they have been identified in only six of 68 sites harbouring *P.*
318 *columella*. In this sense, studies depicting more general ecological interactions of both
319 susceptible and resistant *P. columella* in the mollusc assemblage and their interaction with the
320 abiotic factors, which could explain the differential distribution observed, are lacking.
321 Considering that such resistance could hold promise as a control strategy to disengage *F.*
322 *hepatica* transmission in the field, it is important to account for its ecological or demographical
323 patterns. Thus, we analysed the occurrence of each phenotype in the wild in association with
324 different environmental factors and we carried out experimental life tables to address this topic.

325 3.3.1 *Reviewing data from natural populations*

326 Since no apparent pattern concerning the type of habitat was found in either resistant or
327 susceptible *P. columella* (see section 3.1), we aimed to identify specific ecological features
328 associated with the occurrence of each phenotype. For this, we used data from 14 sites
329 harbouring *P. columella* mostly concerning different abiotic factors (see Table 2). It can be
330 noted, that both susceptible and resistant populations seem to prefer water with low nitrite and
331 ammonium concentrations. Interestingly, the recorded ecological data showed a tendency for
332 resistant populations to settle in waterbodies with lower pH (6-6.5), TH (4-9°d) and mollusc
333 richness (see Table 2).

334 3.3.2 *Ecological factors affecting the abundance of resistant and susceptible P. columella in the*
335 *La Coca locality.*

336 We found the two distinct phenotypes (resistant and susceptible) of *P. columella* occurring in
337 the La Coca locality, but with a marked degree of ecological segregation depending on the
338 particular sampled site, with Segundo Potrero only harbouring resistant snails (La Coca resistant
339 population). *Galba cubensis* (Lymnaeidae), *Physa acuta* (Physidae), *Drepanotrema anatinum* and
340 *Drepanotrema lucidum* (Planorbidae) were recorded in Negrines. In this site, *P. acuta* and *D.*
341 *anatinum* showed similar variations while *G. cubensis* performed two ephemeral colonisations
342 with rapid extinctions (Supplementary Figure 1). *Physa acuta* was dominant in Negrines but it
343 did not seem to affect the population dynamic of *P. columella* (Supplementary Figure 1). In
344 contrast, the scenario at Segundo Potrero was completely different with a very low snail species
345 richness marked by low abundances of *D. anatinum* and *Gundlachia radiata*, and a considerable
346 dominance by resistant *P. columella* snails (Supplementary Figure 1).

347 The ecological follow-up was only carried out in two sites, which limits replication. However, the
348 closeness and constant water flow interchange between both sites and the ecological variations
349 recorded on some variables in the Negrines site allowed us to observe an interesting pattern.
350 Concerning the different phenotypes of *P. columella*, we should note that, while in Segundo
351 Potrero only resistant individuals are found (*i.e.* La Coca population), in Negrines susceptible

352 individuals occurred in sympatry with resistant snails at certain times of the study (Figure 4B).
353 The canonical correspondence analysis performed with all the ecological data recorded at both
354 sites showed a particular relationship between the abundances of resistant and susceptible
355 individuals with variations in pH and both TH and CH (Figure 4A). We found a strong negative
356 association between resistant snails and pH demonstrating a certain tolerance for slightly acidic
357 waters. The remaining factors had a minor effect on species abundances. In this sense, while in
358 Negrines the mean values of pH and TH were around 7.5 and 14°d respectively, Segundo
359 Potrero showed lower values (pH: 5.5-6; TH: 2°d-4°d) with an annual stability and a high
360 abundance of resistant *P. columella*. However, in Negrines, the population of *P. columella*
361 remained stable at low effective size but with peaks of resistant individuals when susceptible
362 snails decreased or disappeared. Interestingly, such colonization events by the resistant snails in
363 Negrines matched a drop in pH and TH values highlighting their particular association with
364 water acidity and TH observed in the canonical analysis (Figure 4) and with the overall pattern
365 recorded from the previous analysis (section 3.3.1). Globally, susceptible snails were more
366 abundant when pH ranged between 7 and 8.

367 Notably, we failed to detect *F. hepatica* infection from *P. columella* during the two-year
368 ecological follow-up study in La Coca. However, while susceptible snails from Negrines were
369 experimentally infected, it was not possible to infect resistant snails with different *F. hepatica*
370 isolates.

371 3.3.3 Influence of pH and total hardness on experimental life history traits of resistant and 372 susceptible *P. columella*

373 Given that the evidences gathered from the historical field notes and from the population
374 dynamics in the La Coca locality suggested a differential relation between the occurrence of
375 susceptible and resistant individuals concerning the pH/TH (*i.e.* positive association of resistant
376 populations with acidic and soft waters with low species richness), we performed an
377 experimental life table study to assess the effect of these abiotic factors on several life-history
378 traits of *P. columella*. We measured survival and reproductive traits as shell size showed no
379 significant differences between *P. columella* phenotypes in a previous study²⁴. Age-

380 independent parameters, and life expectancy and fecundity rates for weeks 0, 5, 9 and 14;
381 considering the start of the experiment, sexual maturity, middle and late snail developmental
382 stages respectively, are shown in Table 3. To facilitate comprehension and to elucidate patterns,
383 we will address the results from two different levels of analysis: a) the overall effects on the
384 species and b) the differential effects on the resistant/susceptible phenotypes.

385 *Overall life-history traits for Pseudosuccinea columella – common pH/TH conditions seem better*
386 *suited to P. columella species:* Survival of *P. columella* was highest during the first eight weeks
387 with a lifespan ranging from 15 to 23 weeks (3 to 5 months). Sexual maturity was reached
388 between the third and fifth weeks, depending on the population and experimental setting.
389 Reproductive traits showed a steady increase since first egg laying until reaching a peak
390 between week 13 and week 16 (see Table 3 and Figure 5 for details).

391 Overall, *P. columella* performed better at common pH/TH conditions irrespective of the
392 phenotype (see Table 3; Figure 5). Low pH/TH values had a negative impact on snail survival
393 with the lowest life expectancies compared to common conditions (Table 3). In particular, with
394 the exception of La Palma, the other populations considerably decreased their life expectancy at
395 birth (La Coca: 1.7-fold, Aurora: 1.5-fold, Negrines: 2.9-fold). Regarding mortality, this trait
396 peaked at week 1 for Negrines and around week 7 for the remaining populations (*i.e.* all
397 populations but La Palma were reduced by more than half; Figure 5A, Table 3). Furthermore,
398 fertility traits were also negatively affected under these conditions. A significant delay in snail
399 hatching within the first week following egg laying was recorded at low pH/TH for all
400 populations but La Palma (NHS within the first week in Table 3). Additionally, several egg masses
401 laid and incubated at 5.9/4°d needed up to three weeks to hatch. The combination of these
402 later effects also affected population increases (r and λ) which were lower at low pH/TH. Net
403 reproductive rate was also lower at 5.9/4°d except for La Coca which presented a two-fold
404 higher R_0 (Table 3). All these impairments on survival and reproductive parameters of *P.*
405 *columella* reared at low pH/TH strongly suggests that common conditions of 7.6/14°d are better
406 suited to this species, irrespective of the phenotype.

407 Interestingly, overall higher fecundity rate was observed at 5.9/4°d (Table 3) probably to
408 compensate for the reduction in self-maintenance and reproduction recorded on *P. columella*
409 under these conditions. However, a significant increase of the mean number of eggs, egg
410 masses and egg per egg masses was only attained by La Coca and Aurora snails at low pH/TH
411 (Figure 5B; Tukey's Test post hoc, $P < 0.05$).

412 *Differential effects of pH and TH at the resistant/susceptible phenotype level – higher tolerance*
413 *to low pH/TH conditions is possibly associated with the resistant phenotype:* Despite the overall
414 decrease in self-maintenance and reproduction of *P. columella* at 5.9/4°d presented above,
415 differences can be seen between phenotypes that points at a higher tolerance for low pH/TH of
416 resistant populations. In general, higher survival (Log rank Tests, $P < 0.05$; Figure 5A) and life
417 expectancy at birth (Table 3) were observed in resistant snails at low pH/TH conditions. In
418 addition, we recorded a higher percentage of viable eggs in resistant compared to susceptible
419 populations at low pH/TH (Table 3; Fisher's Exact Test, $P < 0.05$). No significant variation in this
420 trait in resistant *P. columella* at common versus low pH/TH (Table 3; Fisher's Exact Tests, $P >$
421 0.05) was recorded. Conversely, an overall significant decrease in the proportion of viable eggs
422 was observed on susceptible populations at 5.9/4°d compared to 7.6/14°d (Table 3; Fisher's
423 Exact Test, $P < 0.05$).

424 *Differences in life traits between populations (irrespective of the experimental setting) with focus*
425 *on reproductive outputs:* It is worth mentioning that different patterns regarding reproductive
426 traits were recorded for each population independent of the pH/TH conditions. In susceptible *P.*
427 *columella*, the Aurora isolate was characterized by high values of R_o , r and λ (Figure 5; Table 3)
428 while a more discrete output of laid eggs per snail was recorded in Negrines for both conditions
429 (Figure 5B for details). Interestingly, the resistant La Coca population presented an overall lower
430 fecundity rate than both susceptible populations (see Table 3). In addition, egg masses/snail
431 (attempts to reproduce) in this population always peaked the latest (weeks 16-17 at low pH/TH
432 and 19-20 at common pH/TH) with the exception of Negrines at low pH/TH that did not show a
433 marked peak but a relatively steady production since early stages. In the case of resistant La
434 Palma we observed that, regardless of the experimental condition, around 30% of their eggs

435 needed more than one week to hatch (see NHS in Table 3). Even at 7.6/14°d, where the
436 reproductive output of this population was higher (high values of R_0 , r and λ), this delay in egg
437 hatching remained unaltered (Fisher's Exact Test, $P > 0.05$; Table 3). Additionally, even after the
438 increase of R_0 , r and λ observed at 7.6/14°d (see Table 3), the age-independent reproductive
439 traits of La Palma, although outperforming Negrines, were lower to the susceptible population
440 of Aurora.

441 **4. Discussion**

442 **4.1. Distribution and population genetics of *P. columella* in Cuba: insights into its** 443 **history and colonization and its relationship with *Fasciola hepatica* transmission**

444 *Pseudosuccinea columella* is native to North America but has been widely introduced outside of
445 its native range due to natural (e.g. aquatic birds, flooding) or human-mediated invasion ⁷. The
446 date of introduction of *P. columella* in Cuba is unknown, but it was first reported in 1858 by
447 Poey under the name of *Lymnaea francisca*. It is thus plausible that a first introduction in the
448 beginning of the 19th century allowed *P. columella* to settle in the western part of Cuba, the
449 region where the oldest and abundant known documentations have been described. The close
450 proximity of Cuba to the North American continent provides an easy opportunity for natural
451 introductions, particularly in the westernmost region. Our results reveal several facts suggesting
452 that successive introductions of *P. columella* occurred successfully in Cuba, mainly in the west
453 but also in the central region. A sluggish invasion of this species to the eastern region of the
454 island, mostly by more recently introduced invasive genotypes (e.g. MLGT Y), is presumed to be
455 ongoing. Perhaps the most obvious evidences to support this assumption are: (1) the increased
456 number of records in recent years from the east-central region and its complete absence in the
457 easternmost region; (2) the higher allelic and multilocus genotypic richness in the west
458 (particularly in resistant populations); (3) the existence of three major clusters of MLGTs and the
459 presence of some MLGTs only found in the central region of Cuba, which contrasts with (4) the
460 similar number of MLGTs shared by most susceptible *P. columella* populations (with five that
461 were completely monomorphic) and the widespread distribution of MLGT (Y) in Cuba. The
462 overall low genetic structuration and diversity found in *P. columella* can be explained by recent

463 genetic bottlenecks that usually occur after introduction events, followed by the expansion of
464 certain invasive genotypes such as MLGT Y (see Lounnas et al. ⁷).

465 In particular, previous studies showed that resistant *P. columella* populations differ from those
466 which are susceptible. Gutiérrez et al. ²³ detected different RAPD profiles using 17 different
467 primers. Another study by Calienes et al. ²¹ was the first to attempt to detect clear segregation
468 when comparing polymorphic DNA of three resistant populations (La Palma, El Azufre, Babiney)
469 and nine susceptible populations from Cuba using RAPD profiles. In the present study, we found
470 that the genetic structure of the three resistant populations analysed (El Azufre, La Coca and
471 Babiney) is significantly different in terms of presence and amount of MLGTs from susceptible
472 populations, with higher genotypic diversity. The clear segregation into different susceptible
473 and resistant MLGT associated clusters observed here, support the idea of an early, different
474 and detached history from susceptible *P. columella* populations. In contrast to highly susceptible
475 populations, resistant MLGTs from western and central populations cluster separately from
476 each other, and it is thus likely that they originated after different introduction events.
477 However, we should note that we have only tested three out of the six known resistant
478 populations, and thus the three others remain unexplored.

479 Concerning parasite transmission, the observed distribution pattern (range and number of
480 populations) in *P. columella* compared to the other lymnaeid *G. cubensis* (over 100 recorded
481 populations all over the Cuban archipelago; see ¹⁵) supports the presumed secondary role for
482 this species as an intermediate host of *F. hepatica* in Cuba. A recent global-scale genetic study
483 on *P. columella* considered one of our recorded MLGT (*T*) as highly invasive after it was detected
484 in Colombia, Peru, Venezuela, South Africa and the Indian Ocean ⁷. Surprisingly we only found
485 this MLGT in two eastern populations in Cuba (Río Central and Matadero Aves) suggesting that
486 it may be a recent introduction and may pose a risk of further spread in Cuba. Additionally,
487 MLGT Y which is only found in monomorphic populations, is most likely the result of a recent
488 introduction and may in fact present itself as ecologically adaptable (found in 15 out of 20
489 analysed populations) and highly compatible with *F. hepatica*. For instance, the five individuals
490 found naturally-infected with *F. hepatica* in Cuba correspond to this specific MLGT (two

491 individuals from IPA and three from Pilón). From this we could expect that if populations with a
492 tendency to become infected matched those that present a high ecological plasticity, a highly
493 favourable scenario for *F. hepatica* transmission in Cuba would result, since it would increase
494 the probability for the parasite to find a suitable host. However, this hypothesis would benefit
495 from the identification of more infected individuals that share the same GTML. In any case, the
496 lack of genetic diversity has already been recognised as an advantage for parasites to proliferate
497 in a given host population ⁴¹, and overall, susceptible populations of *P. columella* showed no
498 significant differentiation (low F_{ST} , $P > 0.05$).

499 In the present study, the low allelic richness per population as well as the strong deviations from
500 panmixia suggest a high self-fertilization rate in this species, as also demonstrated by Lounnas et
501 al. ⁷. Other lymnaeid snails such as *G. truncatula* ⁴² and *Omphiscola glabra* ⁴³ also prefer self-
502 fertilization as a reproductive mode. Similarly, other studies observed the same low allelic
503 richness pattern in *P. columella* that we found ²⁷. However, here, three populations of *P.*
504 *columella* (IPA, Río Central and Río Arimao) showed lower self-fertilization rates than those
505 previously reported. Several studies indicated that cross-fertilization may represent a selective
506 advantage when populations are under severe parasitic pressures ^{44,45}. Conversely, self-
507 fertilization can be selected in stochastic environments ⁴⁶ securing reproduction even if only one
508 individual survives the harsh conditions. Thus, we should expect lower self-fertilization rates in
509 stable habitats or endemic areas for parasite transmission. The IPA site matches both criteria
510 since it was previously suggested as a fasciolosis transmission site by Gutiérrez et al. ⁴⁷, and
511 consists of a permanent pond used to stock water. In fact, the two infected individuals belonged
512 to this population. The other two sites (Rio Central and Rio Arimao) are recognised as fasciolosis
513 areas as high bovine prevalence are reported ⁴⁸.

514 While the establishment of resistant populations decreases the chances of transmission of the
515 liver fluke, the observed distribution pattern of such phenotype even with a high genetic
516 diversity and the older presence of the resistant population in Cuba suggests that there is an
517 ecological cost for resistance. The latter was previously proposed by Gutiérrez et al. after
518 performing experimental history traits following *F. hepatica* infection ²⁴ and under competition
519 ²⁵. Thus, a deeper look into the ecological patterns associated with the occurrence of this

520 phenotype in nature is necessary in the pursuit of using this phenomenon to develop novel
521 control strategies for *F. hepatica* transmission.

522 **4.2. Ecological patterns associated with resistant and susceptible *P. columella* from**
523 ***Cuba: insights into the cost of resistance***

524 Lymnaeid snails are known to occur worldwide in an extensive range of habitat types¹. In Cuba,
525 it has been previously shown that *P. columella* does not exhibit particular preferences regarding
526 natural or transformed habitats and contrasts with its relative *G. cubensis*, commonly
527 established in anthropic sites¹⁵. *Pseudosuccinea columella* is highly aquatic and consequently
528 could be more severely affected by the physical and chemical factors of the water, a factor that
529 could be linked to its overall preference for closed freshwater systems and, in particular, to
530 habitats like permanent ponds as recorded herein. A previous study by Gutiérrez⁴⁹ in Pinar del
531 Río, Cuba, showed a positive correlation between the abundances of *P. columella*, the pH and
532 the concentrations of nitrites. Here, we reveal a different effect when the overall pattern of
533 occurrence of resistant and susceptible individuals and the ecological dynamics of those living in
534 sympatry, are considered separately. As presented here, the conditions for occurrence of
535 susceptible individuals in terms of pH, TH and the concentration of nitrites and phosphates are
536 similar to those reported by Perera³⁶ elsewhere in Cuba. However, resistant snails present a
537 marked association to lower values of water pH and TH and a tendency to colonize sites with
538 low species richness. This result could be explained by two hypotheses:

539 (1) One hypothesis could be that these conditions are essential for the survival of resistant snails
540 in the field (*i.e.* resistant snails could not tolerate other conditions), suggesting that they have a
541 different ecology than susceptible snails. Such marked ecological segregation might lead us to
542 think in a strong differentiation between phenotypes that, over time, would result in speciation.
543 However, we have successfully kept resistant *P. columella* in our laboratory for more than 20
544 years at pH/TH conditions ranging between 7-8/12-18°d. In addition, invasive *F. hepatica*-
545 susceptible *P. columella* populations have been reported in France at sites characterized by
546 acidic soils and soft waters^{50,51}. Both facts suggest that, as a single species, both *P. columella*
547 phenotypes can tolerate pH and TH variations rather than be differentially-restricted to specific

548 values of these parameters. Our experimental life-history traits also support that since *P.*
549 *columella*, regardless of the phenotype, can survive within the same range of pH/TH conditions
550 (5.9-7.6/4-12°d). However, while the species seems to be better suited to 7.6/12°d conditions,
551 an overall higher performance (*i.e.* higher tolerance) at low pH/TH conditions, was observed for
552 resistant individuals compared to susceptible populations, in terms of self-maintenance (survival
553 and life expectancy) and reproductive (egg viability) traits.

554 (2) Thus, a second hypothesis could be that low pH and TH are much less suitable for other
555 species (with narrower tolerance levels) and that these low values directly reduce competition.
556 In this sense, our results demonstrate that such conditions negatively affect some demographic
557 traits of *P. columella* in favour of resistant phenotypes. It is therefore difficult to provide an
558 explanation for how resistant populations are maintained in 'neutral' (where common pH/TH
559 levels are present) sites unless colonisation events by competitor snails are rare and that
560 resistant individuals were the only ones to settle. This scenario was observed within our field
561 results since the colonization of Negrines by resistant snails coincided with a decrease of pH and
562 TH, and lower abundances of susceptible individuals (Figure 4). This hypothesis can also be
563 supported by the observed pattern of low richness of snail species in slightly acidic/soft water-
564 sites where resistant populations are found. In a colonisation event of suitable sites by
565 susceptible *P. columella* a replacement of the resistant snails might be expected. In fact, under
566 experimental conditions, Gutiérrez et al.²⁵ reported that resistant *P. columella* are less
567 competitive than susceptible snails in terms of reproductive traits (reduced R_0 when raised in
568 the presence of susceptible snails). The latter contrasted with an increase in shell growth and R_0
569 on susceptible *P. columella* when reared in competition with resistant individuals²⁵. To
570 summarise, the restricted distribution and the overall negative correlation of resistant *P.*
571 *columella* and species diversity may be interpreted as an ecological cost of resistance, explained
572 by a less competitive potential of resistant snails.

573 Assessing the competitive potential of resistant isolates in relation to susceptible *P. columella*
574 and other freshwater snail species could be experimentally challenging and a demonstration of
575 a definitive cost of resistance will require rearing in competition with one another, both *P.*

576 *columella* phenotypes in the presence and absence of the parasite. However, the present study
577 takes an important step forward in advancing our understanding of the suggested cost of
578 resistance of *P. columella* from previous laboratory studies ^{24,25}, by recording a natural pattern
579 and integrating field data and new experimental results into a plausible hypothesis. In this
580 sense, while we cannot assure that susceptible populations outperform resistant isolates,
581 evidence of possible trade-offs against reproduction in resistant populations were also found
582 here (La Coca: lower fecundity rates compared to both susceptible isolates, late reproductive
583 peaks); La Palma: delay of egg hatching regardless of the pH/TH condition, lower R_0 compared
584 to Aurora snails). Trade-offs are expected to occur from selecting advantageous features (*e.g.*
585 resistance to parasite, ecological tolerance) and thus contribute to their low occurrence in the
586 wild. In this sense, a previous study similarly observed reproductive constraints for La Palma
587 (resistant) snails compared to other susceptible *P. columella* populations, particularly in terms
588 of lower R_0 ²⁴. Even considering the discrete performance of Negrines, the limited reproductive
589 output whether in quantity (low fecundity rates) or in time (late peak of egg production or delay
590 of egg hatching) observed here in resistant isolates, may negatively affect their success in
591 colonizing new and highly suitable habitats. While several factors could account for the preclude
592 of a natural dispersion of this phenotype in Cuba where the most common conditions of Cuban
593 freshwater bodies include pH and TH ranging from 7-8 and 12-18°d ³⁶, identifying potential
594 trade-offs associated with this phenotype might also bring forward some understanding of all
595 possible outcomes of the suggested cost of resistance.

596 ***Concluding remarks***

597 The introductions of vectors and intermediate hosts are significant issues because of the
598 epidemiological risks they pose. In fact, recently introduced populations suffer strong genetic
599 bottlenecks that drag off former genetic diversity preventing them from acquiring resistance to
600 local parasites ⁴¹. Genetic flow could act as a force that increases this diversity but, in contrast to
601 what may be occurring in the liver fluke populations from Cuba (high cross-fertilization and bad
602 management of cattle that mixes the strains; ⁵²), it is unlikely to take place at effective rates in
603 *P. columella* snails. However, as discussed above, populations carrying some highly invasive

604 MLGTs are colonizing a number of available sites in Cuba, one of which (MLGT Y) was also
605 directly related to *F. hepatica* transmission in the present study. Thus, it is plausible that the
606 ability of *P. columella* individuals with this MLGT to invade represents a true danger regarding
607 transmission of the liver fluke. In any case, it is of major importance to maintain an active
608 surveillance of parasite prevalence in *P. columella* and of potentially new introductions of this
609 species in different and distant regions of Cuba and the world.

610 On the other hand, while the spread of resistant *P. columella* could definitively aid in the
611 effective control of *F. hepatica* transmission, our results suggest that a resistance-related cost
612 exists, as previously discussed by Gutiérrez et al.^{24,25}. It is, thus, highly improbable that
613 genotypes with invading abilities also correspond to those resistant to *F. hepatica*. The present
614 work is a step forward towards the rational application of *P. columella* resistance as a potential
615 variant to tackle parasite transmission. Our results provide insights into the ecological cost of
616 resistance and the patterns associated with its occurrence in nature that could be used for
617 planning a human-mediated introduction of resistant snails in particular high-risk transmission
618 foci. Other strategies to incorporate resistant features into susceptible populations by out-
619 crossing could be challenging if the high self-fertilization rates of *P. columella* showed herein
620 and elsewhere⁷ are considered. On the other hand, other approaches like genetic manipulation,
621 the controlled selection of resistant features or induction of resistance-mediated mechanisms in
622 wild vector populations might turn out to be feasible. However, for any of these approaches to
623 be applied, a deeper and wider comprehension of the molecular and immunological bases that
624 mediate the natural resistance of *P. columella* to *F. hepatica* is required.

625

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772 **Author contribution statement**

773 AA designed, performed and analysed the experiments and drafted the manuscript. AV, JS, ML,
774 JPP, SH and BG participated in design of the experiments, analysis and the reviewing process. JS,
775 ML participated in the experiments. All authors read and approved the final manuscript.

776

777 **Additional information**

778 **Competing Interests statement**

779 The authors declare that no financial and/or non-financial competing interest exists.

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782 **Legends to Figures:**

783 **Figure 1.** A) Overview of the locality La Coca showing the two sites, *i.e.* Segundo Potrero and
784 Negrines, sampled during the two-year follow-up ecological study where the resistant (R) La
785 Coca population and the susceptible (S) Negrines population of *Pseudosuccinea columella*
786 occurred. B) Localities from western Cuba where the four populations of resistant (1: La Palma,
787 2: La Coca) and susceptible (2: Negrines within La Coca locality, 3: Aurora) *P. columella* used in
788 the life table experiments were collected from.

789
790 **Figure 2.** A) Distribution of all registered records in the National Malacology Reference Database
791 accounting for freshwater mollusc species from 1982-2018. B) Updated distribution of
792 *Pseudosuccinea columella* from Cuba. Positive sites were plotted with different points
793 representing 11-year time intervals to account for historical and newly recorded data. Resistant
794 populations are named in the map, consistently with previous published works. The year of first
795 record of resistant populations is also indicated. C) Number of records of *P. columella* per type
796 of habitat. (Laboratory of Malacology, IPK).

797
798 **Figure 3.** A) Map showing the distribution of the different multilocus genotypes found (MLGT;
799 one letter or letters' combination = one MLGT = unique combination of alleles across all
800 analysed loci) on sampled *Pseudosuccinea columella* populations genotyped through 6
801 microsatellite loci (3, 7, 10: *resistant *P. columella* populations). B) MLGT of *P. columella*
802 clustered following a joining-neighbour network using Nei's genetic distance from each MLGT
803 (different filling pattern correspond to different clusters). 1: Tio Pancho, 2: SJM, 3: El Azufre*, 4:
804 Río Hondo, 5: IPA, 6: Pilón, 7: La Coca*, 8: Parque Lenin, 9: V7, 10: Babiney*, 11: Vega Grande,
805 12: El Antojo, 13: El Cacao, 14: Río Manaquita, 15: Río Yayabo, 16: Puesto de Mando, 17:
806 Guillén, 18: Río Central, 19: Matadero Aves, 20: Arroyo.

807

808 **Figure 4.** A) Canonical correspondence analysis of the two-year follow up study in the La Coca
809 locality (sampled sites: Negrines and Segundo Potrero). B) Monthly relative abundances of
810 *Pseudosuccinea columella* and dynamics of pH and water hardness in the two sampled sites
811 within the La Coca locality. AV: aquatic vegetation, CH: Carbonate hardness, D: Simpson's
812 Diversity index, S: susceptible *P. columella*, R: resistant *P. columella*, T: temperature, TH: Total
813 hardness.

814

815 **Figure 5.** A) Survival curves of resistant (R) and susceptible (S) *Pseudosuccinea columella*
816 populations in which pairwise comparisons of snail survival between population at each
817 experimental setting, performed by log-rank Tests, are also shown. B) Egg production data of
818 experimentally reared *P. columella* snails at pH and total hardness (pH/TH) of 5.9/5°d or
819 7.6/14°d. Arrows within the egg production data indicate the week at which 100% mortality was
820 observed for the given population at each condition. Results of Factorial ANOVAs were $P < 0.05$.

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822

823 **Table 1.** Mean allelic number (a), observed (H_o) and expected (H_e) heterozygosity, F_{IS} (level of
 824 significance after Bonferroni correction) and estimated auto-fecundity rates (s) for resistant (R)
 825 and susceptible (S) populations of *Pseudosuccinea columella* (n: number of analysed individuals).

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Population	N	a ($\pm DS$)	H_o ($\pm DS$)	H_e ($\pm DS$)	F_{IS} (P)	S
R La Coca	37	2.17 \pm 0.75	0.056 \pm 0.074	0.304 \pm 0.227	0.816 (<0.01)	0.9
R Babiney	26	2.17 \pm 0.75	0.068 \pm 0.059	0.249 \pm 0.197	0.726 (<0.01)	0.84
S IPA	19	2 \pm 1.1	0.068 \pm 0.121	0.127 \pm 0.164	0.469 (<0.01)	0.64
S Parque Lenin	26	1.67 \pm 0.82	0.019 \pm 0.045	0.087 \pm 0.104	0.786 (<0.01)	0.88
S Río Arimao	13	1.83 \pm 0.75	0.249 \pm 0.259	0.239 \pm 0.267	0.038 (NS)	0.08
S Río Hondo	15	1.5 \pm 0.84	0.025 \pm 0.039	0.092 \pm 0.191	0.733 (<0.01)	0.85
S Río Manaquita	15	1.5 \pm 0.84	0.028 \pm 0.044	0.149 \pm 0.25	0.814 (<0.01)	0.9
S Río Yayabo	20	1.33 \pm 0.52	0.01 \pm 0.025	0.174 \pm 0.27	0.94 (<0.01)	0.97
S Tío Pancho	22	1.17 \pm 0.41	0.008 \pm 0.019	0.008 \pm 0.02	0 (NS)	1
S V7	13	1.33 \pm 0.52	0.031 \pm 0.049	0.101 \pm 0.212	0.689 (<0.05)	0.82
S Vegas Grandes	10	1.33 \pm 0.52	0.05 \pm 0.083	0.117 \pm 0.24	0.571 (NS)	0.73
S Arroyo	16	1 \pm 0	0	0	-	1
S Puesto de Mando	6	1 \pm 0	0	0	-	1
S El Antojo	6	1 \pm 0	0	0	-	1
R El Azufre	19	1.17 \pm 0.41	0	0.076 \pm 0.186	1 (<0.01)	1
S Guillén	15	1 \pm 0	0	0	-	1
S Río Central	7	1.5 \pm 0.84	0.048 \pm 0.073	0.091 \pm 0.163	0.478 (NS)	0.65
S Matadero de Aves	24	1.5 \pm 0.84	0.007 \pm 0.017	0.17 \pm 0.263	0.959 (<0.01)	0.98
S Pilón	6	1 \pm 0	0	0	-	1
S SJM	14	1.17 \pm 0.41	0	0.024 \pm 0.058	1 (NS)	1

827

828

829 **Table 2.** Mollusc species richness (S), dissolved ions (PO_4^{3-} , NO_3^- , NO_2^- , NH_4^-), O_2 and pH and total
 830 hardness (TH) from sampled sites harbouring resistant (^R) and susceptible (^S) *Pseudosuccinea*
 831 *columella*. Data from unpublished field notes archived in the Laboratory of Malacology, IPK,
 832 Cuba are referred to in this table as ‘this study’. SE: standard error.

833

Population	S	PO_4^{3-}	NO_3^-	NO_2^-	NH_4^-	O_2	pH	TH (°d)	Reference
^R La Palma	3	-	50	0.1	0.2	-	6.5	6	This study
^R El Azufre	7	0.5	10	0.05	0.2	-	6	9	⁴⁹
^R La Coca	3	0.25	-	0.05	0.2	15	6	4	This study
^R La Playita	2	-	-	-	-	-	6.5	-	This study
^R Candelaria	1	-	-	-	-	-	6	6	This study
^S Negrines	5	0.5	-	0.05	0	20	7.5	20	This study
^S Batabanó	11	-	-	-	-	-	8	20	⁵³
^S Botánico	7	-	0	0.1	0.4	-	8	11	³⁶
^S El Rubio	7	-	25	0.05	0.2	64	8	22	This study
^S Parque Lenin	15	0.25	5	0.1	0	68	8	18	This study
^S Bodegón	4	-	25	0	0	-	7.5	22	³⁶
^S San Juan Martinez	7	0.5	10	0.05	0.2	-	7.5	8	This study
^S Camagüey	8	-	-	-	-	-	7	18	This study
^S Hanabanilla	12	-	21	-	0.03	-	7.2	4	⁵⁴
^R Mean ± SE	3.2 ± 1.02	0.38 ± 0.13	30 ± 20.00	0.067 ± 0.01	0.2 ± 0.00	-	6.2 ± 0.12	6.3 ± 1.03	-
^S Mean ± SE	8.4 ± 1.18	0.41 ± 0.08	14.33 ± 4.4	0.058 ± 0.01	0.12 ± 0.06	50.7± 15.37	7.6 ± 0.13	16.2 ± 2.19	-

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835

836 **Table 3.** Experimental life-history traits of resistant (R: La Palma and La Coca) and susceptible (S:
837 Aurora and Negrines) *Pseudosuccinea columella* at different pH and total hardness (TH)
838 conditions (e_x : life expectancy, m_x : fecundity rate, NHS within one week: snails that hatched
839 within one week after egg laying, R_o : net reproductive rate, FR: week at first reproduction, r :
840 intrinsic rate of natural increase, λ : finite rate of natural increase. Data corresponds to weeks
841 (W) 0, 5, 9 and 14. The (*) represents significant differences ($P < 0.05$) for each population when
842 comparing the given trait between experimental settings by Fisher's Exact Tests.

Condition (pH/TH)	Strain	Age-dependent traits								Age-independent traits				NHS	Viable
		W0		W5		W9		W14		FR	Ro	r	Λ	within one	eggs
		e_x	e_x	m_x	e_x	m_x	e_x	m_x	e_x	m_x				week (%)	(%)
5.9/4°d	La Palma ^R	10.9	5.9	0.7	3.7	12.7	2.3	115.5	4	37.4	0.72	2.06	72.9	99.37	
	La Coca ^R	10.3	5.3	0.6	5.5	27.8	6.1	96.7	5	116.8	0.79	2.20	68.4*	96.46	
	Aurora ^S	7.9	2.9	1.5	5.0	76.5	1.5	147.0	4	37.8	0.73	2.06	53.9*	95.4*	
	Negrines ^S	4.4	4.4	25.0	3.3	28.0	-	-	4	19.2	0.59	1.80	64.8*	67.5*	
7.6/14°d	La Palma ^R	8.6	6.7	1.1	4.0	5.3	2.2	56	3	45.1	0.95	2.59	71.5	98.3	
	La Coca ^R	17.4	13.8	0.2	9.8	0.7	5.0	10.6	4	56.9	0.81	2.24	97.4*	98.7	
	Aurora ^S	12.1	7.6	0.8	5.0	2.6	2.7	33.4	3	56.4	1.00	2.74	92.5*	96.6*	
	Negrines ^S	12.7	8.9	0.3	5.2	0.3	2.5	20.4	4	21.8	0.62	1.85	87.6*	99.2*	

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