

Testing the predictions of reinforcement: long-term empirical data from a damselfly mosaic hybrid zone

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1 **Testing the predictions of reinforcement: long-term empirical data from**
2 **a damselfly mosaic hybrid zone**

3 **Abstract**

4 Theoretical work suggests that reinforcement can cause the strengthening of prezygotic
5 isolation in sympatry by mitigating the costs of maladaptive hybridization. However, only
6 a handful of studies have tested all predictions of this theory in natural populations. We
7 investigated reinforcement in a mosaic hybrid zone of the damselflies *Ischnura elegans*
8 and *I. graellsii*. Firstly, we tested if the conditions of reinforcement were fulfilled by
9 quantifying whether hybridization was costly, and prezygotic isolation was strengthening
10 in sympatry compared with in allopatry. Secondly, we investigated three specific
11 predictions of reinforcement: rarer female effect, presence of concordant prezygotic and
12 postzygotic isolation asymmetries in sympatry, and greater premating asymmetries
13 associated with weaker postzygotic isolation in sympatry. We found that reinforcement has
14 strengthened mechanical isolation in one cross direction between species in sympatry. Our
15 study details a case of reinforcement and heterospecific gene flow causing opposite effects
16 between reciprocal heterospecific crosses and describes a natural model in which
17 reproductive isolation is built by the simultaneous effects of reinforcement, the lock-and-
18 key model, Bateson-Dobzhansky-Müller incompatibilities and Haldane's rule.

19 **Keywords:** Hybridization, Odonates, Range expansion, Reproductive isolation, Speciation

20 Main Text

21 Introduction

22 One central goal of evolutionary biology is to understand the processes that lead to the
23 origin of reproductive isolation (RI) during speciation. Reinforcement is a process that can
24 strengthen reproductive barriers and is one of the most widely discussed mechanisms of
25 speciation (Coyne and Orr 2004; Lukhtanov 2011). This phenomenon, proposed and
26 popularized by Dobzhansky (1937, 1940), describes one way in which natural selection
27 can favor speciation (Noor 1999). Reinforcement acts on formerly allopatric, closely
28 related species that come into secondary contact in *de novo* created regions of sympatry. If
29 individuals show variation in their ability to distinguish conspecifics from heterospecifics,
30 some of them may occasionally try to mate with heterospecifics. Reduced fitness of
31 maladaptive hybrids will cause natural selection to reduce the frequency of alleles that are
32 linked with a diminished heterospecific discrimination ability, thus acting to reduce the
33 costs of hybridization (West-Eberhard 1986). This gradually enhances prezygotic isolation
34 between incipient species by Reproductive Character Displacement (RCD), i.e., it
35 enhances the development of greater phenotypic divergence of reproductive traits in
36 sympatry compared with an allopatry scenario (Howard 1993). Reinforcement acts usually
37 either on barriers acting before (prezygotic-premating barriers) or after mating, but before
38 zygote development (prezygotic-postmating barriers; Coyne 1974; Coyne and Orr 2004;
39 Matute 2010b). Theory suggests that this process is capable of gradually reducing the
40 extent of heterospecific matings in sympatric populations over time, and eventually, that
41 this can lead to the cessation of gene flow between sympatric populations, and ultimately
42 speciation (Dobzhansky 1937).

43 Historically, reinforcement theory has been viewed as a controversial idea (Coyne and Orr
44 2004), in the main because empirical evidence has been scarce. For instance, reinforcement
45 predicts stronger prezygotic isolation in heterospecific crosses in sympatry than in allopatry
46 (Coyne and Orr 1989; Howard 1993) and indeed, some evidence in support for this pattern
47 was found in nature (Ehrman 1965; Littlejohn 1965; Ratcliffe and Grant 1983; Noor
48 1995). However, just as quickly as evidence was documented in support for this prediction,
49 there was also a rise of alternative explanations for this enhanced isolation in sympatry.
50 For example, the Templeton effect, or differential fusion, posits that only species that have
51 already achieved strong isolation in allopatry will remain isolated in sympatry; others will
52 merge into a single taxon upon coming in contact (Paterson 1978; Templeton 1981). Thus,
53 higher prezygotic isolation can be observed in sympatry without invoking any selective
54 force. Additional alternative explanations include ecological character displacement (Otte
55 1989; Noor 1999; Coyne and Orr 2004) and RCD in response to runaway sexual selection
56 (Day 2000). Since Coyne and Orr's seminal work on *Drosophila* (Coyne and Orr 1989),
57 advocates of the reinforcement theory responded to some of these criticisms by proposing
58 other predictions that could distinguish reinforcement from alternative processes. Firstly,
59 since hybridization costs are usually higher for females than for males, reinforcement
60 theory predicts higher RCD in females than in males (Coyne and Orr 2004). Secondly,
61 since the rarer (the species with the smaller range) or smaller population size species is

62 more frequently involved in heterospecific matings owing to its low frequency in sympatry,
63 reinforcement theory predicts higher isolation in the reciprocal cross direction including
64 the female of the rarer species than in the one including the female of the common species
65 (rarer female effect; Yukilevich 2012). Thirdly, since hybrids produced from the two
66 reciprocal cross directions usually differ in fitness (Turelli and Moyle 2007), reinforcement
67 theory predicts a quicker strengthening of the premating isolation in the cross direction
68 producing hybrids with lower fitness (Yukilevich 2012). Fourthly, since asymmetrical
69 reinforcement increases premating asymmetries, and gene flow purges Bateson-
70 Dobzhansky-Müller (BDM) incompatibilities in sympatry, reinforcement theory predicts
71 both greater premating asymmetries and weaker postzygotic isolation in sympatry than in
72 allopatry (Turelli et al. 2014). Nowadays, reinforcement has been detected across
73 ubiquitous taxa. This indicates that speciation via reinforcement can be widespread in both
74 vertebrate (Hostert 1997; Vallin et al. 2012; Pfennig and Rice 2014; Baiz et al. 2019; St.
75 John and Fuller 2021) and invertebrate animals (Coyne and Orr 1989; Nosil et al. 2003;
76 Lessios 2007; Souza et al. 2008; Dillon et al. 2011; Porretta and Urbanelli 2012; Mérot et
77 al. 2017; Yukilevich 2021). While research on plants is being developed (Ramsey et al.
78 2003; Moyle et al. 2004; Silvertown et al. 2005; Hopkins 2013; Pellegrino 2016; Roda et
79 al. 2017), research on fungal species (Turner et al. 2010; Giraud and Gourbière 2012) is
80 lagging behind and not much is known so far. Despite the growing body of empirical
81 evidence in invertebrate and vertebrate species in support of reinforcement as an important
82 evolutionary process, not much is known about how consistently this kind of reinforcement
83 occurs in several contact regions of the same pair of species, or about the factors
84 influencing its evolution.

85 The damselfly species *Ischnura elegans* and *I. graellsii* (Odonata: Coenagrionidae), which
86 in the early 1900s came into secondary contact in Spain (Fig. 1), are a powerful model
87 system to study the evolution of RI. The expansion of *I. elegans* has resulted in a mottled
88 hybrid region, with two secondary contact zones (Sánchez-Guillén et al. 2011, 2023).
89 Mosaic and mottled hybrid zones, i.e., sympatric areas consisting of patches of alternating
90 populations of each parental species and admixed populations (Rand and Harrison 1989),
91 are ideal natural testbeds to study the evolution of reinforcement, for instance, its
92 repeatability across multiple contact areas within a hybrid zone (Cain et al. 1999; Hoskin
93 and Higgie 2013). This is the case of the north-west Spanish hybrid zone, which is
94 characterized by having introgressed populations of each parental species and hybrid
95 populations in which most individuals display different degrees of introgression, i.e., a
96 unimodal distribution (Sánchez-Guillén et al. 2023). Theory predicts that when sympatric
97 speciation occurs, disruptive selection (such as reinforcement) should convert a unimodal
98 distribution of genotypes to a bimodal one (Kondrashov et al. 1998; Jiggins and Mallet
99 2000). RI between *I. elegans* and *I. graellsii* in the north-west hybrid zone is incomplete
100 and asymmetric. While isolation is almost complete in crosses of *I. graellsii* males and *I.*
101 *elegans* females owing to mechanical incompatibilities, hybridization usually occurs in the
102 opposite direction (Monetti et al. 2002; Sánchez-Guillén et al. 2012). The incomplete RI,
103 the frequency distribution of the hybrid classes (Sánchez-Guillén et al. 2023), the

104 colonization and recolonization events, and the exceptional long-distribution data on this
105 system all indicate that this system is a good candidate example to evaluate reinforcement.

106 In this study, firstly, we evaluated reinforcement in the north-west hybrid zone, and
107 compared the strengths of five reproductive barriers (Fig. 2) in heterospecific crosses of *I.*
108 *elegans* and *I. graellsii* from the hybrid zone with the strengths of the same five
109 reproductive barriers in heterospecific crosses from allopatric populations. Secondly, we
110 measured the same reproductive barriers in hybrid crosses and backcrosses. We interpreted
111 these measurements as postzygotic barriers and, therefore, as hybridization costs.
112 Reinforcement theory is based on the principle that hybridization costs should be positively
113 correlated with selective pressures directing prezygotic isolation (Ortiz-Barrientos et al.
114 2009). Thirdly, since theoretical and empirical evidence suggests that the breakdown of
115 reproductive barriers is more likely than reinforcement (Abbott et al. 2013), we used a
116 dataset measuring the same reproductive barriers in other populations from this hybrid zone
117 (Sánchez-Guillén et al. 2012) as a replicate to evaluate the consistency of the hybridization
118 outcomes.

119 **Materials and Methods**

120 **Sympatry zone (north-west Spanish hybrid zone) description**

121 The north-west Spanish hybrid zone, henceforth called the sympatry zone, is found mainly
122 along the Galician coast (Fig. 1). This sympatry zone is a mosaic hybrid zone in which the
123 frequencies of *I. elegans* and *I. graellsii* vary between populations and thus in their degrees
124 of introgression (Sánchez-Guillén et al. 2023). First records of *I. elegans* in the sympatric
125 zone come from 1980 in Louro (both species), 1987 in Doniños (only *I. elegans*) and 1995
126 in Foz (both species and hybrids). After that, in between 2000 and 2001, we found *I.*
127 *elegans* with the occasional presence of *I. graellsii* in Laxe, Carnota and Louro, and
128 between 2001 and 2003 we found both species and hybrids in Cederia and the Corrubedo
129 complex (Table S1). All these populations were, previously to these dates, allopatric for *I.*
130 *graellsii* (details in Table S1). Currently, these populations mainly consist of introgressed
131 populations of *I. elegans* or introgressed *I. graellsii*, and only one of these populations,
132 Louro, from which *I. elegans* was removed because of salinization of the lagoon in 2010,
133 was after that recolonized in 2013 by both species, and displays different degrees of genetic
134 admixture (introgressed, hybrids, backcrosses, etc.; Sánchez-Guillén et al. 2023). Although
135 the sympatric region has only one hybrid population (Louro, since 2013), we expected to
136 find evidence of reinforcement: firstly, because included populations have recently
137 experienced hybridization, resulting in some cases with the local extinction of *I. graellsii*
138 (Laxe, Doniños, Louro, Foz; Table S1), and in other cases the local extinction of *I. elegans*
139 (e.g., Corrubedo complex; Table S1). The local extinction of one of the hybridizing species
140 has been found in several reinforcement models, when one species outnumbers the other
141 (Servedio and Noor 2003). Our second reason for this expectation was because we detected
142 a signature of RCD of the shape of the *I. elegans* and *I. graellsii* female's thorax involved
143 in the formation of the copula (Ballén-Guapacha *et al.*, *in press*).

144 **Field samplings**

145 We sampled five pure (allopatric) *I. elegans* populations [one in Sweden (Lund), one in
146 Belgium (De Maten), and three in France (Arles, Saint Cyprien and Marais D'Orx; Table
147 S1 and Fig. 1)], and four *I. graellsii* populations [one pure (allopatric) *I. graellsii*
148 population in Spain (Riomaior) and three *I. graellsii* populations in the Lanzada complex
149 (Lanzada, Montalvo and Cachadas) as localities with putative influence of *I. elegans* owing
150 to its geographic position between the sympatric localities of the Corrubedo complex in
151 the north and the *I. graellsii* allopatric localities in the south (Fig. 1)]. From the north-west
152 Spanish hybrid zone we sampled one *I. elegans* population from the sympatric region
153 (Laxe). Additionally, to evaluate the consistency of the hybridization outcomes, we
154 included (in our data-set) data from the north-west Spanish hybrid zone published in a
155 previous study (Table 1; Sánchez-Guillén et al. 2012), so that, we added to our data-set:
156 two pure (allopatric) *I. graellsii* populations from Spain (Alba, and Centeans), and two
157 populations from the north-west Spanish hybrid zone: one *I. graellsii* population from the
158 Corrubedo complex (Corrubedo, Xuño and Vilar), and two *I. elegans* populations (Laxe
159 and Louro; Table 1; Sánchez-Guillén et al. 2012). We categorized crosses involving these
160 localities as either allopatric or sympatric according to the population of origin of the *I.*
161 *elegans* individuals with which they were crossed. Finally, several crosses lacked
162 measurements of some of the reproductive barriers we measured (Table 1). These crosses
163 as well as those with a sample size of less than three during the mechanical barrier
164 estimation were excluded from the cumulative RI estimates (Table 1).

165 **Rearing in the laboratory and mating trials**

166 Last-instar larvae and teneral adults were maintained in the laboratory, until they reached sexual
167 maturity, with the conditions described by Van Gossum et al. (2003). Males and females
168 were kept separated in 50 x 50 x 50 cm wooden insectaries (Van Gossum et al. 2003).
169 During mating trials sexually mature males and females were placed in additional wooden
170 insectaries for observations. We repeated the methods implemented by Sánchez-Guillén et
171 al. (2012). In short, choice trials were made by placing multiple sexually mature male and
172 female damselflies of both species in contact during the hours in which they are most
173 reproductively active (i.e., from 9:00 to 12:00 for *I. elegans* and from 12:00 to 17:00 for *I.*
174 *graellsii*; thus, observations usually took place between 9:00 and 17:00). The numbers of
175 males and females per insectary were determined by the availability of sexually mature
176 individuals per day. We did not consider mate preference as a reproductive barrier because
177 of the high variability in the frequencies of the species during the experiments. Random
178 individuals per sex were placed in each insectary. All males and non-mated females were
179 placed in daily mating trials until females mated or they died. Couples which successfully
180 mated were isolated until sexual intercourse finished. Then, mated females were isolated
181 and we provided them with the conditions to oviposit (Van Gossum et al. 2003; Sánchez-
182 Guillén et al. 2012). Larvae were reared up to adulthood following standardized protocols
183 (Van Gossum et al. 2003; Sánchez-Guillén et al. 2012), and mating trials were repeated in
184 the following generations.

185 **Reproduction in *Ischnura* and reproductive barriers**

186 In damselflies the “tandem position” is achieved when the male successfully grasps the
187 female (by her prothorax) using his caudal appendages (Corbet 1999). Copulation begins
188 when the female bends her abdomen and mating organs (genitals) come in contact. This
189 position is usually referred to as “wheel position” (Cordero 1989). Once copulation is
190 achieved, the male first removes sperm from the female’s bursa and spermatheca from
191 previous matings and, after that, inseminates the female. After copulation the female lays
192 eggs until the sperm is finished or she mates again (Fig. 2).

193 We measured five sequential reproductive barriers: two premating barriers that prevent the
194 tandem (mechanical barrier) and wheel (mechanical-tactile barrier) positions and three
195 postmating barriers that prevent or reduce oviposition, fecundity, and fertility (Fig. 2; Table
196 2; Text S1). We used each male-female couple or mated female as units of observations
197 for premating and postmating barriers respectively (Table 1). To prevent pseudo-replicates,
198 we avoided the use of several observations from the same male-female pair (Text S1).

199 In allopatric crosses, all five reproductive barriers were measured across two generations.
200 F₀ consisted of conspecific crosses of *I. elegans*, conspecific crosses of *I. graellsii*, and
201 heterospecific crosses of *I. elegans* males and *I. graellsii* females, and *vice versa*; and F₁
202 consisted of backcrosses between both species’ males and females with F₁ hybrids from
203 the opposite sex and crosses between F₁-hybrids. In sympatric crosses, we were able to
204 additionally measure hybrid crosses and backcrosses in second generation hybrids (F₂);
205 however, to increase our sample sizes of postzygotic barriers we pooled data from the F₁
206 and F₂ generations. Each barrier was estimated using two values: i) An absolute value that
207 goes from 0 to 1, in which 0 means reproductive barrier absence (complete gene flow) and
208 1 means complete isolation (gene flow absence); and ii) a relative contribution factor to the
209 total cumulative isolation. See table 1 for the complete list of crosses categorized between
210 the allopatric and sympatric ecologies.

211 **Absolute and relative strength of the reproductive barriers**

212 Strength of the reproductive barriers in heterospecific and hybrid crosses is frequently
213 estimated using conspecific crosses of one or both parental species as controls (Sánchez-
214 Guillén et al. 2012; Barnard et al. 2017; St. John and Fuller 2021). These controls help
215 measure the mating preference between a conspecific and a heterospecific cross (Sobel and
216 Chen 2014) and are made employing indices such as the Stalker’s Index (Stalker 1942).
217 However, since our main interest was to compare the probability of gene flow between *I.*
218 *elegans* and *I. graellsii* from allopatry versus from the sympatry zone, we used the formula
219 proposed by Sobel and Chen (2014):

$$220 \quad RI = 1 - \frac{\text{observed hybridization}}{\text{expected hybridization}}$$

221 which represents the proportional decrease of hybridization relative to the null expectation
222 (random mating; Table 2). The advantage of this formula for our purpose, which is to
223 compare RI between allopatry and sympatry, is that it can be used to calculate average
224 values and variances when replicated measurements of RI are available. Thus, confidence
225 intervals can also be calculated, and used to calculate the potential range of average
226 reproductive isolation (see Sobel and Chen 2014 for further details). A detailed description
227 on our estimations of each of the five reproductive barriers can be found in the
228 Supplementary Text S1.

229 To estimate the contribution of each barrier to the total cumulative isolation in sequential
230 stages of reproduction, i.e., its relative contribution, we employed the multiplicative
231 function of individual components developed by Coyne and Orr (1989, 1997) and later
232 modified by Ramsey et al. (2003) to include any number of reproductive barriers (Sobel
233 and Chen 2014). We estimated the cumulative contribution (CC) of a component to the RI
234 at a stage n with the following formula:

$$235 \quad CC_n = RI_n(1 - \sum_{i=1}^{n-1} CC_i)$$

236 **GLM modeling**

237 To evaluate the effects of the different types of crosses and the “ecology” (sympatry vs
238 allopatry) on RI we modeled and compared generalized linear models (GLMs) for each
239 reproductive barrier. For prezygotic barriers (F_0 generation crosses) we measured the
240 influence of population origin by categorizing them between intrapopulation and
241 interpopulation crosses to create a new variable that we called “geography”. Then, we
242 modeled GLMs of each reproductive barrier isolation as a function of all possible
243 combinations of the types of crosses, the “ecology”, the “geography” and the interaction
244 between the types of crosses and the “ecology” variables. GLMs were modeled using the
245 *glm()* function in R 4.3.0 (R Core Team 2023) and compared using the AICc value with
246 the *dredge()* function of the MuMIN 1.47.5 library (Barton 2009). We modeled the
247 mechanical (successful tandem = 1 vs unsuccessful tandem = 0), mechanical-tactile
248 (successful mating = 1 vs unsuccessful mating = 0), oviposition (mated female that laid
249 eggs = 1 vs mated female that did not laid eggs = 0) and fertility (fertile egg = 1 vs infertile
250 egg = 0) barriers using the binomial distribution, and the fecundity barrier (eggs per clutch
251 index) using the Poisson distribution. We selected as the most probable model per
252 reproductive barrier the one with the lowest AICc score. Pairwise statistical comparisons
253 for the types of crosses and the interaction between the types of crosses and the “ecology”
254 variables were made through post hoc GLMs if these variables were included in the most
255 probable model. This procedure was also applied to postzygotic barriers (F_1 and F_2
256 generation crosses), with the single difference that we did not include the “geography”
257 variable. This variable was excluded because second and third generation crosses highly
258 increased the number of possible combinations of geographical origins of the ancestors of
259 the crossed samples (E. g. crosses between samples whose parents are from the same
260 population, vs crosses between samples product of intrapopulation crosses but whose
261 parents come from different populations, vs crosses between a sample from an

262 intrapopulation cross and a sample from an interpopulation cross, etc.). Statistical
263 significance tests were used to assess five theoretical predictions of reinforcement (Table
264 3). See the Supplementary Text S2 for details.

265 **Results**

266 **Rearing experiments**

267 Reproductive barriers were measured considering each male and female pair (pre mating)
268 and mated female (post mating) as units of observation respectively. While allopatric
269 reproductive barrier measurements were made with between 125 and 180 units of
270 observation per barrier, sympatric reproductive barriers estimations included between 191
271 and 327 units of observation per barrier (Table 1). While in allopatric crosses reproductive
272 barriers were measured in between one and four pairs of populations in sympatric crosses
273 reproductive barriers were measured in between two and five pairs of populations (Table
274 1).

275 **Conspecific crosses**

276 Conspecific crosses behaved similarly between allopatry and sympatry, although *I. elegans*
277 crosses were more successful (i.e., with lower isolation) between allopatric populations
278 than sympatric populations (Fig. 3). In all cases, reproductive success between conspecific
279 crosses was precluded by the cumulative action of all reproductive barriers (Fig. 4). In
280 conspecific *I. graellsii* crosses, reproductive success was largely precluded by low
281 fecundity and fertility, as pre mating barriers were mostly absent in both allopatric and
282 sympatric crosses (Fig. 4). Overall, reproductive success was similar or slightly higher in
283 conspecific crosses than in heterospecific and hybrid crosses (Fig. 3).

284 **Reproductive isolation asymmetry**

285 The hybridization direction, i.e., the cross in which hybridization occurs, remained
286 consistent within crosses of different populations within an ecology, but differed between
287 the sympatric and the allopatric ecologies (Fig. 3). In detail, in allopatry, hybridization
288 occurred through crosses between *I. graellsii* males and *I. elegans* females, but was
289 completely precluded in the opposite direction, owing to the cumulative effect of the five
290 measured reproductive barriers (Fig. 4). In contrast, in the sympatry zone, hybridization
291 occurred almost only via *I. elegans* male and *I. graellsii* female crosses. In fact, the
292 mechanical and mechanical-tactile barriers (Fig. 4; Table S2) precluded 94.7% and 100%
293 of the gene flow from the *I. graellsii* males' and *I. elegans* females' direction in the
294 sympatry zone in the population crosses of Cachadas and Laxe, and Lanzada and Louro
295 respectively. The exception to this pattern (in crosses between *I. graellsii* males and *I.*
296 *elegans* females) came from the cross involving Corrubedo and Louro in which total
297 cumulative RI reached only 73.4%. However, since only three females laid eggs in crosses
298 from these populations, low sample sizes precluded us from rearing hybrids from this cross.

299 Hybrid crosses also differed between allopatric and sympatric ecologies (Fig. 3). In
300 allopatry, matings occurred only via F₁-hybrid females and *I. elegans* or *I. graellsii* males,
301 i.e., no crosses involving F₁-hybrid males produced fertile eggs. Although allopatric F₂-
302 hybrid larvae were bred, the high cumulative RI and low sample sizes made it impossible
303 to obtain any adult F₂-hybrid. In sympatry, hybrids mated successfully in all cross
304 directions except with *I. graellsii* males. Additionally, RI was complete or almost complete
305 in crosses between hybrid males and *I. elegans* females in all three sympatric
306 interpopulation crosses (Fig. S1). In sympatry, F₂-hybrids were viable and fertile, and F₃-
307 hybrids were reared up to adulthood, although no reproductive fitness measurements were
308 made.

309 **GLM modeling**

310 Prezygotic-barrier GLM modeling and scoring using the AICc suggested that the
311 mechanical barrier was explained by crosses, ecology and the interaction between these
312 two variables (Fig. 5A; Table S3). *Post hoc* comparisons revealed that the heterospecific
313 cross between *I. graellsii* males and *I. elegans* females was significantly different from the
314 other three crosses ($p < 0.05/6$; Table S4). Additionally, significant differences were
315 detected in this cross between the allopatric and sympatric ecology ($p < 0.05/4$; Table S5).
316 In the mechanical-tactile barrier, the null model was selected as the most probable model
317 (Fig. S2A; Table S3). The oviposition barrier was explained by the crosses, ecology and
318 geography (Fig. S2B; Table S3). Finally, both fertility and fecundity barriers were
319 explained by the full model (Figs 5B and 5C; Table S3). All crosses' fecundities and
320 fertilities were statistically different between allopatry and sympatry ($p < 0.05/4$; Figs. 5B
321 and 5C; Table S5) except the fertility of *I. graellsii* males and *I. elegans* females crosses
322 ($p > 0.05/4$; Table S5).

323 Postzygotic-barrier GLM modeling described the mechanical, fecundity and fertility
324 barriers as explained by the crosses, the ecology and the interaction between them (Figs 6
325 and S3; Table S6). On the other hand, the mechanical-tactile and oviposition barriers were
326 explained only by the ecology (Fig. S3; Table S6). *Post hoc* analyses of the fecundity and
327 fertility barriers showed that each cross had significant differences between allopatry and
328 sympatry ($p < 0.05/5$; Figs. 6A and 6B; Tables S7 and S8).

329 **Testing reinforcement predictions**

330 *Sympatric strengthening of prezygotic barriers*

331 We detected the classical pattern expected under reinforcement, i.e., stronger prezygotic
332 isolation in sympatry than in allopatry (Table 3; Dobzhansky 1937, 1940), although
333 prezygotic barriers were asymmetric between heterospecific reciprocal crosses. Between *I.*
334 *graellsii* males and *I. elegans* females, total prezygotic isolation was stronger in sympatry
335 than in allopatry. The exception was the sympatric cross between Corrubedo and Louro, in
336 which prezygotic isolation was similar to allopatry. In the reciprocal cross direction,
337 between *I. elegans* males and *I. graellsii* females, cumulative prezygotic isolation was

338 similar between sympatry and allopatry, although in the latter all population crosses
339 reached complete isolation (Fig. 4).

340 We detected statistically significant differences in the strength of the mechanical barrier in
341 crosses between *I. graellsii* males and *I. elegans* females in comparison to the reciprocal
342 cross direction and the conspecific crosses of *I. elegans* and *I. graellsii* (Fig. 5A; Table S4).
343 *Post hoc* GLM modeling revealed that in this cross mechanical isolation was stronger in
344 sympatry than in allopatry (Fig. 5A; Table S5). Strong mechanical isolation in crosses
345 between *I. graellsii* males and *I. elegans* females was seen in two out of the three sympatric
346 interpopulation crosses (Fig. 5A). In contrast, in crosses between *I. elegans* males and *I.*
347 *graellsii* females mechanical isolation was similar to that in the conspecific crosses (Fig.
348 5A).

349 ***Rarer female effect***

350 Reinforcement theory predicts that selection will strengthen prezygotic barriers in the cross
351 direction which includes females of the less abundant species (Table 3; Yukilevich 2012).
352 We could not test this prediction as allopatric crosses showed strong asymmetry between
353 both crosses directions (Figs. 1 and 4). Since crosses between *I. elegans* males and *I.*
354 *graellsii* females were completely isolated in the allopatric condition reinforcement
355 pressures could have only operated in sympatry in the opposite direction (i. e. between *I.*
356 *graellsii* males and *I. elegans* females) independently of the relative abundance of both
357 species in the sympatry zone.

358 ***Concordant prezygotic and postzygotic isolation asymmetries***

359 Since costs of hybridization (postzygotic barriers) are usually asymmetric between
360 reciprocal crosses (termed Darwin's corollary; Darwin 1859; Turelli and Moyle 2007),
361 reinforcement is predicted to be more intense in the reciprocal cross direction which
362 produces more unfit hybrids (Table 3; Yukilevich 2012). Thus, concordant prezygotic and
363 postzygotic isolation asymmetries are expected between reciprocal crosses in sympatry but
364 not in allopatry. This prediction could not be tested in our data because RI was complete
365 in the cross direction between *I. elegans* males and *I. graellsii* females in allopatric crosses
366 (Fig. 3). Additionally, although the reciprocal cross direction, the one between *I. graellsii*
367 males and *I. elegans* females, was not completely precluded in either of the zones (allopatry
368 and sympatry), its high strength and the low sample size of the obtained larvae precluded
369 us to rear them until adulthood.

370 ***Greater premating asymmetries and weaker postzygotic isolation***

371 Turelli et al. (2014) proposed two additional predictions of the reinforcement theory, such
372 as a more definitive test of Yukilevich (2012) hypothesis about the role of intrinsic
373 postzygotic isolation in reinforcement (concordant isolation asymmetries). They proposed
374 that species pairs that have asymmetric postzygotic barriers in sympatry: i) should present
375 greater premating asymmetries in sympatry than in allopatry; and ii) since allopatrically

376 originated Bateson-Dobzhansky-Müller (BDM) incompatibility alleles are purged in
377 sympatry because of gene flow, species should present a reduction in the strength of
378 intrinsic postzygotic isolation in sympatry relative to allopatry (Table 3; Turelli et al. 2014).
379 Additionally, postzygotic isolation asymmetries should be reduced in sympatry by purging
380 of unidirectionally inherited BDM incompatibilities (Turelli and Moyle 2007). We could
381 not fully test Turelli's predictions since prezygotic RI was complete (or very high) in one
382 cross direction in both sympatry and allopatry, impeding our ability to test the asymmetry
383 of the postzygotic barriers between reciprocal crosses. However, we compared prezygotic
384 asymmetries between sympatry and allopatry, and estimated an overall measurement of
385 postzygotic isolation using each of the hybrids formed in allopatry and sympatry. We
386 detected greater prezygotic-premating asymmetries (Fig. S4, Table S9), weaker
387 prezygotic-postmating isolation (Fig. 5B; Table S5) and weaker postzygotic isolation in
388 sympatry than in allopatry (Fig. 6; Table S8); however, evidence from this prediction
389 should be taken carefully owing to the assumption we could not fulfill.

390 Firstly, consistent with the prediction of higher prezygotic-premating asymmetries in
391 sympatry than in allopatry, we detected significant asymmetries in the mechanical barrier
392 in sympatry using all the sympatric data (Fig. S4B; Table S9) and between the reciprocal
393 heterospecific crosses of Lanzada and Louro (Fig. S4E; Table S9), and Cachadas and Laxe
394 (Fig. S4F; Table S9). The exception was between the reciprocal heterospecific crosses of
395 Louro and Corrubedo (Fig. S4D; Table S9). In allopatry, neither by using all data (Fig.
396 S4A; Table S9) nor with the reciprocal heterospecific crosses of Cachadas and Belgium
397 (Fig. S4C; Table S9) were significant premating asymmetries detected. Additionally, all
398 prezygotic barriers in allopatry and prezygotic-postmating barriers in sympatry were
399 stronger in crosses between *I. elegans* males and *I. graellsii* females; however, in sympatry
400 the mechanical barrier was stronger in crosses between *I. graellsii* males and *I. elegans*
401 females (Fig. S4).

402 Secondly, despite the fact that we could not test the asymmetry of the postzygotic barriers
403 between reciprocal crosses, we detected overall weaker postmating isolation in hybrids
404 from sympatry than from allopatry. While in allopatry all crosses with hybrid males
405 produced a low number of infertile eggs and no F₂-hybrids could be reared up, in sympatry
406 only crosses between *I. graellsii* males and hybrids were completely isolated and adult F₂-
407 hybrids could not be bred, reared-up and reproduced (Figs 3 and S2). In all five
408 reproductive barriers the ecology was a significant factor influencing postzygotic isolation
409 (Figs 6 and S3; Table S6), although its effects differed between reproductive barriers.
410 While postzygotic-premating barriers were usually stronger in sympatry than in allopatry
411 (Figs S3A and S3B), in all three postzygotic-postmating barriers allopatric crosses
412 presented stronger isolation than sympatric crosses (Figs 6 and S3C). In fact, four out of
413 the five postzygotic types of crosses presented higher fecundities and fertilities values in
414 sympatric crosses than in allopatric crosses (Fig. 6). The exception was in crosses between
415 *I. graellsii* males and hybrid females that had very low sample sizes both for the allopatric
416 and sympatric ecology (Fig. 6; Table S2).

417 Interestingly, significant differences between the allopatric and sympatric ecology were
418 also detected in prezygotic-postmating barriers. In conspecific crosses data distribution
419 shows that in sympatry pure crosses produce a lower number of eggs than in allopatry (Fig.
420 5B), although no clear pattern could be inferred between allopatry and sympatry for fertility
421 values (Fig. 5C). However, heterospecific crosses between *I. elegans* males and *I. graellsii*
422 females presented an increment of both fecundity and fertility in sympatry than in allopatry
423 (Fig. 5). Since Turelli et al. (2014) prediction is based on evidencing gene flow in sympatric
424 heterospecific crosses, this pattern of increased fecundity and fertility in sympatric crosses
425 between *I. elegans* males and *I. graellsii* females could be evidence of the homogenizing
426 effects of historical gene flow in this direction. This is consistent with the fact that
427 sympatric hybridization occurs in this direction (Fig. 3). Recent genomic evidence has
428 shown reduced heterospecific differentiation and increased intraspecific genetic diversity
429 in both *I. elegans* and *I. graellsii* in sympatric samples in comparison to allopatric samples
430 (Sánchez-Guillén et al. 2023), which strengthens the evidence for heterospecific gene flow.

431 Finally, we not only detected statistically significant asymmetries in sympatry (but not in
432 allopatry) in the mechanical barrier, but also found that the strength of this barrier shifted
433 from being stronger in allopatry in crosses between *I. elegans* males and *I. graellsii* females
434 to being stronger in sympatry in crosses between *I. graellsii* males and *I. elegans* females
435 (Fig. S4). Additionally, sympatric backcrosses (but not allopatric) were successful in a
436 similar way, as were heterospecific crosses in the first generation. Specifically, while
437 hybridization occurred in sympatry in crosses between *I. elegans* males and *I. graellsii*
438 females, backcrosses were successful mostly with either *I. elegans* males or *I. graellsii*
439 females (Fig. 3). On the other hand, backcrosses with *I. graellsii* males or *I. elegans* females
440 were prevented by a strong mechanical barrier (Fig. S1). This pattern suggests that if
441 reinforcement has occurred in the mechanical isolation of *I. graellsii* males and *I. elegans*
442 females, then mechanical isolation could also have been strengthened in backcrosses
443 involving *I. graellsii* males and *I. elegans* females.

444 Discussion

445 Although our data were inconclusive in testing several reinforcement theoretical
446 predictions, our results suggest the presence of reinforcement (Table 3). This is consistent
447 with morphological evidence of RCD in sympatric *I. elegans* and *I. graellsii* females
448 (Ballén-Guapacha *et al.*, *in press*). We detected stronger prezygotic isolation in crosses
449 between *I. graellsii* males and *I. elegans* females in sympatry than in allopatry owing to
450 the strengthening of the mechanical barrier in these crosses. We also identified stronger
451 premating asymmetries in sympatry than in allopatry, an evidence of sympatric gene flow
452 in the form of reduced prezygotic-postmating barriers in sympatry than in allopatry, and
453 similar patterns of premating barriers in prezygotic and postzygotic barriers; i.e., the same
454 mating directions in heterospecific and backcrosses in sympatry but not in allopatry. Data
455 of two out of three population crosses in sympatry revealed a consistent pattern of
456 reinforcement.

457 Evolution of mechanical isolation in sympatry

458 The relative contributions of the five reproductive barriers to RI differed between allopatry
459 and sympatry and between reciprocal heterospecific crosses. In allopatry, premating
460 (mechanical and mechanical-tactile) barriers were moderate and similar between reciprocal
461 crosses, while postmating (oviposition, fecundity, and fertility) barriers were strong and
462 highly asymmetric between reciprocal crosses, preventing 100% of the hybrid formation
463 between *I. elegans* males and *I. graellsii* females. In two out of the three sympatric crosses
464 between *I. graellsii* males and *I. elegans* females, premating barriers were stronger than
465 postmating barriers, and most of the isolation was due to the action of the mechanical
466 barrier preventing the tandem formation. The low mechanical isolation detected in the
467 heterospecific crosses involving *I. graellsii* males from Corrubedo (Fig. 4Q) could be due
468 to a misclassification of hybrids as *I. graellsii*, because of the high prevalence of hybrids
469 in this population during the sampling year (Table S1). In the cross direction between *I.*
470 *elegans* males and *I. graellsii* females, gene flow was prevented by the joint action of both
471 premating and postmating barriers.

472 Mechanical and mechanical-tactile barriers preventing the formation of successful tandem
473 or copula formation are (with a few exceptions; Nava-Bolaños et al. 2017) important
474 reproductive barriers across a variety of non-territorial odonate species, such as the
475 *Enallagma* and *Ischnura* damselflies, which lack visual recognition and precopulatory
476 courtship behaviors (Robertson and Paterson 1982; Barnard et al. 2017; Solano et al. 2018).
477 The role of mechanical barriers in RI has been used as evidence for the lock-and-key model
478 (Paulson 1974; Eberhard 1985; Masly 2012), which suggests that the morphology of sexual
479 structures is under rapid male-female coevolution via reinforcement to enhance RI
480 (Eberhard 1985; Masly 2012), and explains the wide diversity and taxonomic importance
481 of sexual structures (Monetti et al. 2002; Barnard et al. 2017; Solano et al. 2018). Thus, the
482 lock-and-key theory predicts enhanced mechanical isolation in sympatry compared with
483 allopatry, and a correlation with low hybrid fitness (Eberhard 1985; Shapiro and Porter
484 1989; Brennan and Prum 2015). Our results are consistent with both predictions and
485 suggest that sexual structures involved in the tandem formation could be evolving because
486 of reproductive character displacement (RCD) in *I. elegans* and *I. graellsii*. This is
487 consistent with recent morphological evidence showing RCD in the pronotum of females
488 in sympatry (Ballén-Guapacha *et al.*, *in press*). RCD in these structures could also explain
489 why premating barriers in sympatry behaved similarly in backcrosses and in heterospecific
490 crosses, i.e., reducing gene flow in backcrosses with *I. graellsii* males or *I. elegans* females.
491 If tandem related structures have mainly been reinforced in *I. graellsii* males and *I. elegans*
492 females, then these structures could also be mechanically incompatible with hybrids with
493 intermediate morphology. This provides an explanation to why sympatric backcrossing
494 occurred mainly with *I. elegans* males or *I. graellsii* females.

495 **Testing specific predictions of reinforcement**

496 Refinements of the reinforcement theory during the 1990s concluded that reinforcement
497 could occur under a broad range of conditions (Coyne and Orr 2004), although several
498 factors need to be fulfilled. For example, the outcomes of hybridization would range from
499 species fusion and extinction to speciation via reinforcement as a function of hybridization

500 costs and initial differences in reproductive characteristics between species (Liou and Price
501 1994). The higher the hybridization costs (lower fitness of hybridizing individuals), and
502 the higher the initial variance in reproductive characteristics, the higher the probability of
503 speciation via reinforcement (Liou and Price 1994). In allopatry, the cross between *I.*
504 *elegans* males and *I. graellsii* females is completely isolated by prezygotic barriers.
505 Unsuccessful mating attempts (complete prezygotic isolation) can still act as a selective
506 pressure that strengthens earlier-acting barriers (e.g., premating barriers) to avoid
507 unnecessary wastage of gametes, time, energy (Hoskin and Higgie 2013), or other
508 reproductive costs. However, reinforcement pressures increase as further reproductive
509 barriers act on hybridization. Intrinsic postzygotic isolation is usually more costly (at least
510 to females) than prezygotic isolation, as energy has been invested in maladaptive hybrid
511 formation (Ortiz-Barrientos et al. 2009). Data from allopatric populations showed that
512 crosses between *I. graellsii* males and *I. elegans* females are more prone to be reinforced
513 than the opposite direction, based on the formation of costly F₁-hybrids which are highly
514 unfit owing to their high infertility, and because both species are morphologically well
515 differentiated by reproductive characters related to the tandem position, i.e., male caudal
516 appendages and female pronotum (Monetti et al. 2002). Importantly, the fact that hybrids
517 from the allopatric crosses between *I. graellsii* males and *I. elegans* females are highly, but
518 not completely, unfit (not achieving complete isolation in the F₁ generation) suggests that
519 some gene flow is possible, and that these species are not yet “good” species *sensu* Butlin
520 (1987). This distinction is important, as several authors (Butlin 1987; Coyne and Orr 2004)
521 argue that sympatric strengthening of prezygotic isolation in cases in which taxa already
522 produce completely unfit hybrids (no gene flow) in allopatry should not be considered as
523 reinforcement, since such enhancement of prezygotic isolation would have then happened
524 *after* allopatric speciation. Consistently, our sympatric experiments crosses showed
525 evidence that reinforcement has strongly enhanced the prezygotic RI between *I. graellsii*
526 males and *I. elegans* females. This is evident by a stronger prezygotic isolation between *I.*
527 *graellsii* males and *I. elegans* females in sympatry than in allopatry due to the strengthening
528 of the mechanical barrier.

529 We could not test neither the rarer female effect nor the “concordant isolation asymmetries”
530 predictions (Yukilevich 2012), and we could only test partially the greater premating
531 asymmetries and weaker postzygotic isolation in sympatry than in allopatry pattern (Turelli
532 et al. 2014) because F₁-hybrids from one cross direction, in both allopatry and sympatry,
533 were not obtained due to the completeness of the prezygotic isolation. However, the shift
534 in hybridization directions between allopatry and sympatry, the higher mechanical
535 isolation in the latter than the former, and recent evidence of higher RCD in *I. elegans*
536 females than in *I. graellsii* males (Ballén-Guapacha *et al.*, *in press*) is all consistent with
537 the reinforcement of reproductive isolation theory. Future studies should increase the
538 sample size of experimental crosses in an attempt to obtain F₁ hybrids from both reciprocal
539 cross directions. This will open the possibility to test the predictions that we could not.

540 Our results show that reinforcement can act rapidly, since differences in prezygotic
541 isolation have been formed at most during the last 100–120 years since the presence of *I.*
542 *elegans* was detected in Spain (Sánchez-Guillén et al. 2011, 2023; Wellenreuther et al.

2018). Our results are consistent with *Drosophila* experiments showing that reinforcement can act rapidly in just a few generations (Matute 2010a). Additionally, our data show that reinforcement can quickly shift hybridization directions, i.e., from hybridization occurring between *I. graellsii* males and *I. elegans* females in allopatry to between *I. elegans* males and *I. graellsii* females in sympatry. This could be, to our knowledge, the first report of such natural shifting in hybridization directions in a time scale of between tens to some hundreds of generations due to reinforcement. We hypothesize that during the initial secondary contact between *I. elegans* and *I. graellsii*, hybridization should have occurred in the allopatric direction, i.e., between *I. graellsii* males and *I. elegans* females. High hybridization costs of this cross direction (infertile hybrid males) could have induced reinforcement to displace tandem-related reproductive characters in *I. elegans* females (Ballén-Guapacha *et al.*, *in press*), reducing the mechanical compatibility between *I. graellsii* males and *I. elegans* females. However, as introgression occurred between the species (Sánchez-Guillén *et al.* 2023), purging of BDM incompatibilities reduced postzygotic isolation in sympatry, and reduced heterospecific genetic differentiation could have reduced prezygotic-postmating isolation by increasing heterospecific fecundity and fertility. Since reinforcement could have been occurring mostly between *I. graellsii* males and *I. elegans* females, both the reduction of prezygotic-postmating and postzygotic isolation could have allowed sympatric hybridization to occur in crosses between *I. elegans* males and *I. graellsii* females. Once sympatric hybridization was possible between *I. elegans* males and *I. graellsii* females, reinforcement in this cross direction could occur, albeit slower than in *I. elegans* females because hybridization costs (postzygotic isolation) have been reduced. Whether introgression will increase by hybridization between *I. elegans* males and *I. graellsii* females, or reinforcement will increase prezygotic isolation also in this direction, is an interesting question to evaluate in the future.

While asymmetrically reinforcement has been documented before (Jaenike *et al.* 2006; Turner *et al.* 2010; Yukilevich 2012; Zhou and Fuller 2014; Ostevik *et al.* 2021; St. John and Fuller 2021), to our knowledge this could be the first study suggesting reinforcement and gene flow causing opposite consequences between reciprocal crosses, i.e., reinforcement increasing prezygotic isolation in one direction and gene flow reducing in the other. Future studies should evaluate the asymmetrical effects of reinforcement and gene flow between reciprocal crosses in species pairs in which asymmetrical reinforcement has been documented.

Weakening of intrinsic postzygotic isolation

In addition to the evidence of reinforcement of mechanical isolation, we detected weaker postzygotic-postmating isolation, and a lower number of hybrid crosses completely isolated by postmating barriers in sympatry than in allopatry. Hybrid fecundity and fertility fitness relative to those of pure species are mixed, and highly dependent on the genetic divergence between the parental species (Burke and Arnold 2001; Orr and Turelli 2001). They range from: i) reductions in both F₁ and F₂ hybrids fecundity or fertility (Naisbit *et al.* 2002); ii) no differences in fecundity and fertility between the parental species and hybrids (Van Der Sluijs *et al.* 2008); to iii) equal or higher F₁-hybrid reproductive success than conspecific

585 crosses but lower in F₂ or later generation hybrids (hybrid breakdown; Vetukhiv 1956;
586 Edmands 1999; Dunham and Argue 2000). Reductions in hybrid fecundity or fertility are
587 best explained by the Bateson-Dobzhansky-Müller (BDM) incompatibilities model
588 (Dobzhansky 1934; Orr 1996). That model describes how reductions in hybrid fitness occur
589 in response to negative interactions between introgressed alleles from different populations
590 and the genomic background of hybrids. Hybrid breakdown due to BDM incompatibilities
591 is more prone to occur as species diverge, accumulate mutations and increase in genetic
592 distance (Orr and Turelli 2001). Despite conflicting evidence as to whether BDM
593 incompatibilities accumulate linearly (Leppälä et al. 2013) or faster (i.e. the snowball
594 effect; Orr 1995; Presgraves 2010) over time, empirical research in both plants (Moyle and
595 Nakazato 2010; Leppälä et al. 2013) and animals (Matute et al. 2010) converges to a
596 continuous accumulation of BDM incompatibilities as taxa diverge. This BDM
597 incompatibilities property, i.e., higher frequency at increased genetic divergence, is
598 consistent with our observations. Overall genetic distance between *I. elegans* and *I.*
599 *graellsii* in the north-west hybrid zone ($F_{ST}=0.625$) is lower than in allopatry ($F_{ST}=0.725$)
600 (Sánchez-Guillén et al. 2023). However, future studies should attempt to rear up hybrids
601 from *I. elegans* and *I. graellsii* in both cross directions in both ecologies to help distinguish
602 whether the sympatric reduction of postzygotic isolation in sympatry is due to purging via
603 gene flow (Turelli et al. 2014), because species inherit BDM incompatibilities
604 asymmetrically (Turelli and Moyle 2007), or a combination of both of these factors.

605 Future studies should also formally evaluate the genetic bases of these apparent BDM
606 incompatibilities. Since in allopatric heterospecific crosses, male hybrids were infertile,
607 and since males are the hemizygous sex in these species, some of these BDM
608 incompatibilities may be related to the X chromosome (Haldane's rule). These results are
609 consistent with recent evidence suggesting a role of the X chromosome in the reproductive
610 isolation of these species (Swegers et al. 2022). Evidence gathered since the origin of the
611 Haldane's rule in 1922 (Haldane 1922) has established this phenomenon as one of the most
612 robust generalizations in evolution (Delph and Demuth 2016), i.e., that hybrids from the
613 heterogametic (or hemizygous; Koevoets and Beukeboom 2009) sex are the ones with
614 reduced fitness. Not only are there plenty of cases reported in vertebrates, invertebrates and
615 plants (reviewed in Schilthuizen et al. 2011; Delph and Demuth 2016), but also recent
616 evidence has shown that there are a high number of independent evolutionary origins of
617 the Haldane's rule (Delph and Demuth 2016).

618 **Conclusions**

619 Our results provide not only new empirical evidence of reinforcement of RI in Odonata,
620 but also contribute to a better understanding of the mechanisms leading to speciation, by
621 describing a natural model in which several mechanisms such as reinforcement, Bateson-
622 Dobzhansky-Müller incompatibilities and the Haldane's rule are driving RI
623 simultaneously. Our work describes a case where reinforcement increases prezygotic
624 isolation in one cross direction, while simultaneously, gene flow weakens postzygotic
625 isolation in the opposite cross direction. Since the study of the asymmetrical effects of
626 reinforcement between reciprocal crosses (Jaenike et al. 2006; Turner et al. 2010;

627 Yukilevich 2012; Zhou and Fuller 2014; Ostevik et al. 2021; St. John and Fuller 2021) is
628 an important growing field in evolutionary biology, our study opens the possibility of
629 testing the interaction between these processes in other taxa.

630 **Data availability**

631 All datasets and scripts used in this manuscript were uploaded to OSF at:
632 https://osf.io/k6jyg/?view_only=c68a5102dea44045ab9dd922c425e7f3

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- 874

875 **Tables**

876 **Table 1.** Sample sizes per reproductive barrier measured for each population cross pair. Although all data was used for the absolute
 877 isolation estimates and GLM modeling only crosses with a sample size equal or higher than 3 during the mechanical barrier were used
 878 for cumulative isolation (CI) estimates (*). The last column shows data reanalyzed from Sánchez-Guillén et al. (2012).

Ecology	Type	Cross	Populations crossed	Mec [†]	Mec-Tac	Ovi	Fec	Fer	CI	Sánchez-Guillén et al. 2012
Allopatry	Conspecifics	E♂E♀	Arl×Arl	3	3	19	16	16	*	
		E♂E♀	Bel×Bel	8	8	8	8	8	*	
		E♂E♀	Bel×Swe	5	5	5	5	5	*	
		E♂E♀	Swe×Swe	4	3	3	2	2	*	
		G♂G♀	Alb×Alb	0	0	14	13	13		
		G♂G♀	Cac×Cac	11	10	10	10	10	*	
		G♂G♀	Rio×Rio	14	14	0	0	0	**	
	Heterospecifics	E♂G♀	Arl×Cac	7	7	10	9	9	*	
		E♂G♀	Bel×Cac	42	34	11	8	8	*	
		E♂G♀	Lai×Cac	6	4	3	1	1	*	
		E♂G♀	Swe×Cac	15	13	6	1	1	*	
		G♂E♀	Cac×Bel	11	9	5	5	5	*	
		G♂E♀	Cac×Mar	1	1	1	1	1		
	Postzygotics	E♂H♀	AEle×(H:Bel×Cac)	7	7	11	10	10	*	
		G♂H♀	AGra×(H:Bel×Cac)	2	2	1	1	1	**	
		H♂E♀	(H:Bel×Cac)×AEle	12	9	8	5	5	*	
		H♂G♀	(H:Bel×Cac)×AGra	14	12	8	6	6	*	
		H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	18	18	29	24	24	*	
Sympatry		Conspecifics	E♂E♀	Lou×Lou	5	3	10	8	8	*
	E♂E♀		Lax×Lax	41	34	28	25	25	*	
	G♂G♀		Lan×Lan	2	2	4	4	4		*
	G♂G♀		Mon×Mon	10	10	12	12	12	*	
	Heterospecifics	E♂G♀	Lou×Cen	0	0	3	2	2		*
		E♂G♀	Lou×Cor	1	1	2	2	2		*
		E♂G♀	Lou×Lan	7	7	11	8	8	*	*
		E♂G♀	Lax×Cac	63	50	53	50	50	*	
		E♂G♀	Lax×Mon	34	25	13	13	13	*	
		G♂E♀	Cor×Lou	4	3	3	3	3	*	*

Ecology	Type	Cross	Populations crossed	Mec [†]	Mec-Tac	Ovi	Fec	Fer	CI	Sánchez-Guillén et al. 2012
		G♂E♀	Lan×Lou	24	1	0	0	0	*	*
		G♂E♀	Cac×Lax	19	2	0	0	0	*	*
	Postzygotics	E♂H♀	SEle×(H:Lou×Cen)	0	0	1	1	1		*
		E♂H♀	SEle×(H:Lou×Lan)	8	8	12	11	11	*	*
		E♂H♀	SEle×(H:Lax×Cac)	4	4	4	4	4	*	*
		E♂H♀	SEle×(H:Lax×Mon)	15	11	11	9	9	*	*
		G♂H♀	SGra×(H:Lou×Lan)	6	1	0	0	0	*	*
		G♂H♀	SGra×(H:Lax×Mon)	1	1	1	1	1		
		H♂E♀	(H:Lou×Cen)×SEle	5	0	0	0	0	*	*
		H♂E♀	(H:Lou×Lan)×SEle	10	5	3	3	3	*	*
		H♂E♀	(H:Lax×Mon)×SEle	8	3	2	2	2	*	*
		H♂G♀	(H:Lou×Cen)×SGra	8	8	2	2	2	*	*
		H♂G♀	(H:Lou×Lan)×SGra	2	0	1	1	1		*
		H♂G♀	(H:Lax×Mon)×SGra	5	5	4	4	4	*	*
		H♂H♀	(H:Lou×Cen)×(H:Lou×Cen)	9	0	0	0	0	*	*
		H♂H♀	(H:Lou×Cen)×(H:Lou×Lan)	1	0	0	0	0		*
		H♂H♀	(H:Lou×Lan)×(H:Lou×Cen)	1	1	0	0	0		*
		H♂H♀	(H:Lou×Lan)×(H:Lou×Lan)	16	4	9	9	9	*	*
		H♂H♀	(H:Lou×Mon)×(H:Lou×Mon)	18	16	20	17	17	*	*

879 [†]Mec: Mechanical barrier; Mec-Tac: Mechanical-Tactile barrier; Ovi: Oviposition; Fec: Fecundity; Fer: Fertility; CI: Cumulative Isolation. Population labels are
880 explained in Table S1 (E = *I. elegans*; G = *I. graellsii*; H = hybrids; AEle = Pooled pure allopatric *I. elegans* samples; AGra = Pooled pure allopatric *I. graellsii*
881 samples; SEle = Pooled pure sympatric *I. elegans* samples; SGra = Pooled pure sympatric *I. graellsii* samples). [‡]Cumulative isolation was not estimated with this
882 cross due to the lack of postmating barriers measurements. [§]We included this cross in cumulative isolation measurements despite its low samples sizes since it was
883 the only allopatric cross between *I. graellsii* males and female hybrids.

884 **Table 2.** Summary of absolute reproductive isolation formulas per barrier (fitness component). We used the formula proposed by Sobel
 885 and Chen (2014): $RI = 1 - \frac{\text{observed hybridization}}{\text{expected hybridization}}$ which represents the proportional decrease of hybridization relative to the null
 886 expectation.

Fitness component	Formula	Isolation range	Estimate
Premating: estimated using as replicates male-female interacting couples			
I. Mechanical	$RI = 1 - \frac{\text{observed hybridization (number of succesful tandems)}}{\text{expected hybridization (number of tandem attempts)}}$	0–1	Incompatibility between secondary genitalia to form the tandem position
II. Mechanical-Tactile	$RI = 1 - \frac{\text{observed hybridization (number of succesful copulations)}}{\text{expected hybridization (number of succesful tandems)}}$	0–1	Male fails to stimulate the female to form the wheel position or primary genitalia are incompatible
Postmating: estimated using as replicates isolated mated females, i.e., which successfully formed copulation positions with a single male			
I. Oviposition	$RI = 1 - \frac{\text{observed hybridization (number of mated females that laid eggs)}}{\text{expected hybridizaton (number of total mated females)}}$	0–1	Sperm fails to stimulate females' oviposition
II. Fecundity	$RI = 1 - \frac{\text{observed hybridization } (2 * \frac{\sum_{i=1}^n \text{eggs per clutch index}}{n})}{\text{expected hybridization (maximum eggs per clutch Sp1 + maximum eggs per clutch Sp2)}}$	0–1	Sperm reduces rate of females' oviposition (fecundity)
III. Fertility	$RI = 1 - \frac{\sum_{i=1}^n \text{observed hybridization (number of fertile eggs)}}{\text{expected hybridization (total laid eggs)}} \frac{1}{n}$	0–1	Poor transfer or sperm storage, inability of gametes in foreign reproductive tract, poor movement or cross-attraction, or failure of fertilization when gametes contact each other.

887

888 **Table 3.** Summary of reinforcement theoretical predictions tested in *Ischnura elegans* and *I. graellsii*.

Predictions	Expected patterns	Observed patterns [†]			Result
		Sympatry		Allopatry	
Strengthening of prezygotic barriers in sympatry (Dobzhansky 1937, 1940)	Stronger prezygotic isolation in sympatry than in allopatry	Mechanical RI G♂E♀ (Cac×Lax)	>	Mechanical RI G♂E♀ (Cac×Bel & Cac×Mar)	✓
		Mechanical RI G♂E♀ (Cor×Lou)	≈		X
		Mechanical RI G♂E♀ (Lan×Lou)	>		✓
Rarer female effect (Yukilevich 2012)	Stronger prezygotic isolation in sympatry, but not in allopatry, in the cross-involving females of the rarer species [‡]	This prediction could not be tested as allopatric samples presented strong reproductive isolation asymmetries, thus, confounding the effects population frequencies could have had on reinforcement selective pressures.			NA
Concordant prezygotic and postzygotic isolation asymmetries (Yukilevich 2012)	The asymmetry, in the strength of RI between reciprocal crosses, has the same direction in prezygotic and postzygotic barriers in sympatry (but not in allopatry).	This prediction could not be tested in our data because RI was complete or almost complete (100–94.7%) in one reciprocal cross direction in both allopatry (between <i>I. elegans</i> males and <i>I. graellsii</i> females) and sympatry (between <i>I. graellsii</i> males and <i>I. elegans</i> females). This made impossible the comparison between reciprocal crosses either in allopatry or in sympatry.			NA
Greater premating asymmetries and weaker postzygotic isolation in sympatry than in allopatry (Turelli et al. 2014)	Species pairs with asymmetric postzygotic isolation have: i) higher premating asymmetries and ii) weaker postzygotic isolation in sympatry than in allopatry.	i) Mechanical asymmetry (All sympatric data)	>	Mechanical asymmetry (All allopatric data)	✓
		i) Mechanical asymmetry (CorvsLou)	≈	Mechanical asymmetry (BelvsCac)	X
		i) Mechanical asymmetry (LanvsLou)	>		✓
		i) Mechanical asymmetry (CacvsLax)	>		✓
		ii) Postzygotic RI E♂H♀	<	Postzygotic RI E♂H♀	? [‡]
		ii) Postzygotic RI G♂H♀	>	Postzygotic RI G♂H♀	? [‡]
		ii) Postzygotic RI H♂H♀	<	Postzygotic RI H♂H♀	? [‡]
		ii) Postzygotic RI H♂E♀	<	Postzygotic RI H♂E♀	? [‡]
		ii) Postzygotic RI H♂G♀	<	Postzygotic RI H♂G♀	? [‡]

889 [†]E: *I. elegans*; G: *I. graellsii*; H: Hybrid. [‡]Inconclusive, since we could not rear up hybrids from both directions either from allopatry or from sympatry; thus we
890 cannot distinguish if the weaker postzygotic isolation in sympatry was due to purging of BDM incompatibilities via gene flow as predicted by reinforcement, or
891 because species inherit BDM incompatibilities asymmetrically.

892 **Figure legends**

893 **Figure 1.** *Ischnura elegans* (blue) and *I. graellsii* (orange) field observations across the
894 north-west Spanish hybrid zone (left) and continental Europe (down-right) from 1758 to
895 2022 shared by Adolfo Cordero Rivera (*Personal communication*). Crosses show sampled
896 localities. In the top right *I. graellsii* and *I. elegans* males.

897 **Figure 2.** Schematic representation of damselfly reproduction and reproductive barriers
898 measured. A) *Ischnura* damselflies achieving the tandem position (mechanical barrier). B)
899 The female bends her abdomen and primary genitalia come into contact, achieving the
900 mating position (mechanical-tactile barrier). C) Sperm transfer induces the female to
901 oviposit (oviposition barrier). Left-up close-up: sperm is deposited in the female bursa and
902 spermatheca. Left-down close-up: Empty female reproductive tract. D) Female laying
903 eggs. We counted the numbers of eggs in the first three clutches (females were allowed to
904 laid one egg clutch per day, starting from the second day of mating) and averaged them
905 (eggs per clutch index; fecundity barrier). We also measured the ratio of fertile eggs (with
906 visible larvae eyes or opened due to hatching) to the total number of eggs (fertility barrier).

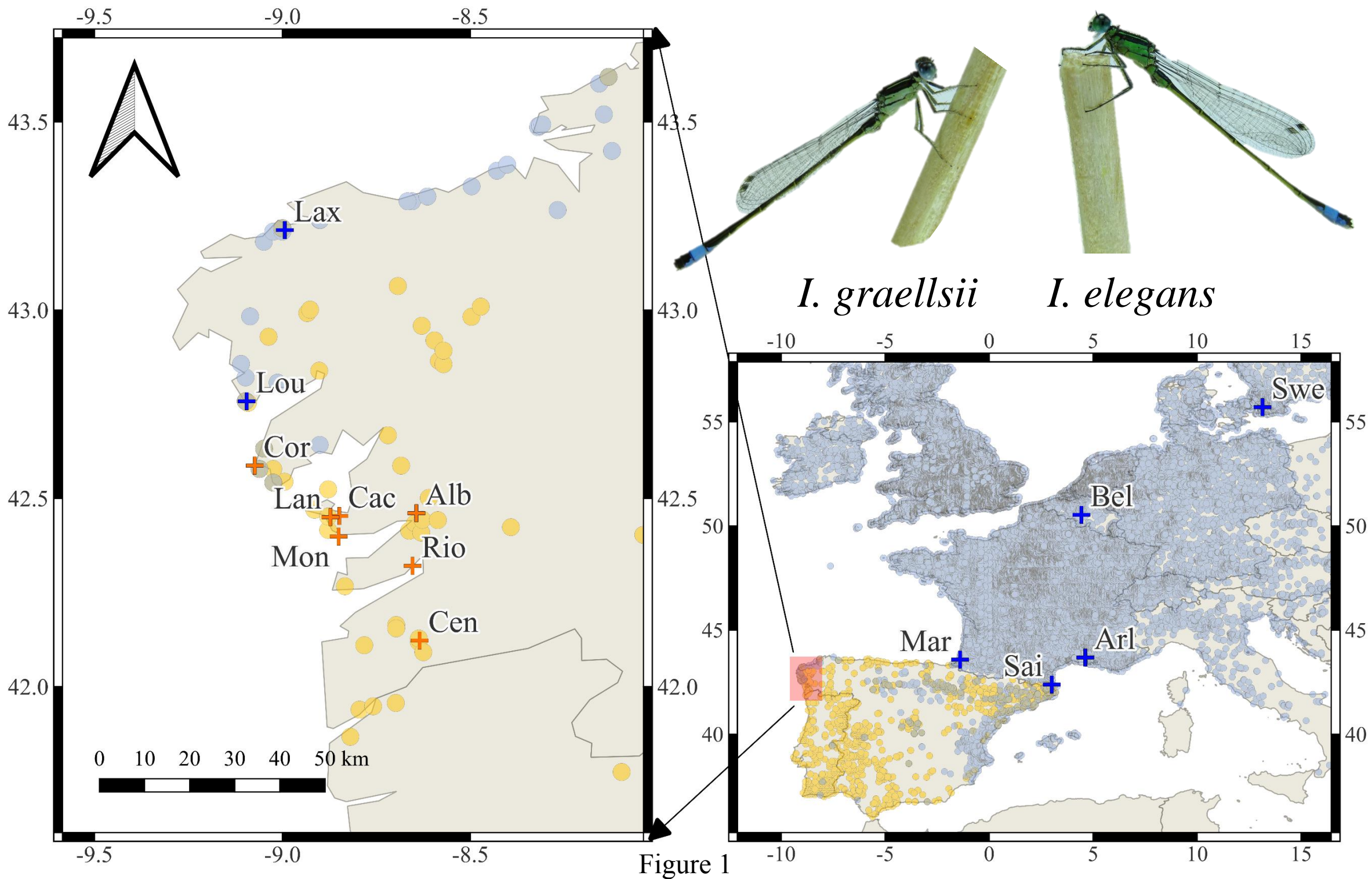
907 **Figure 3.** Schematic representation of the hybridization directions between *Ischnura*
908 *elegans* and *I. graellsii*, comparing crosses between allopatry and sympatry. Solid arrows
909 show gene flow direction and dashed arrows pointing to “CI” letters mark complete
910 isolation. Additionally, we include the total cumulative RI for crosses not in complete
911 isolation. Population labels are explained in Table S1. In allopatry, hybrids were bred only
912 from crosses between *I. graellsii* males and *I. elegans* females, and we could not rear adult
913 F₂-hybrids. RI was high but not complete in crosses with pure-species males and hybrid
914 females, which leaves the possibility of breeding F₂-hybrids from these backcrosses. In
915 sympatry, hybrids were bred from crosses between *I. elegans* males and *I. graellsii* females,
916 and most later-generation hybrids were bred from the hybrid crosses and from backcrosses
917 involving *I. elegans* males or *I. graellsii* females †: Crosses between *I. graellsii* males from
918 Corrubedo and *I. elegans* females from Louro did not achieve high total cumulative
919 isolation; however, owing to small sample sizes we could not rear up adult hybrids from
920 this cross. ‡: F₂-hybrid adults from allopatry were not reared because of the low numbers
921 of obtained larvae.

922 **Figure 4.** Cumulative RI of five prezygotic barriers in conspecific and heterospecific
923 *Ischnura* crosses from allopatry and sympatry. Only crosses with a sample size equal or
924 higher than 3 during the mechanical barrier were used for cumulative isolation estimates.
925 Color lines within each subplot show data for a population cross pair: A) Arles×Arles; B)
926 Belgium×Belgium; C) Belgium×Sweden; D) Sweden×Sweden; E) Cachadas×Cachadas;
927 F) Arles×Cachadas; G) Belgium×Cachadas; H) SaintCyprien×Cachadas; I)
928 Sweden×Cachadas; J) Cachadas×Belgium; K) Louro×Louro; L) Laxe×Laxe; M)

929 Montalvo×Montalvo; N) Louro×Lanzada; O) Laxe×Cachadas; P) Laxe×Montalvo; Q)
930 Corrubedo×Louro; R) Lanzada×Louro; S) Cachadas×Laxe.

931 **Figure 5.** Fitness-component measurements and summary of GLM modeling results for
932 the A) mechanical (green = successful tandem and orange = unsuccessful formation of a
933 tandem), B) fecundity, and C) fertility prezygotic reproductive barriers in *Ischnura*. The
934 equation in the left-bottom corner of each subplot shows the model with the lowest AICc
935 value. Values between parentheses on each population cross show the sample size.
936 Population labels are explained in Table S1. Letters superscripts of crosses boxes at the top
937 of each subplot show different groups inferred with *post hoc* GLM analyses for crosses; e.g.,
938 in A) crosses between *I. graellsii* males and *I. elegans* females (B) differed significantly in
939 pairwise comparisons from the other three types of crosses (A; $p < 0.05/6$). * = *Post hoc*
940 statistically significant differences between the sympatric and allopatric ecology within
941 each cross; **Bold** = Intrapopulation crosses.

942 **Figure 6.** Fitness-component measurements and summary of GLM modeling results for
943 the A) fecundity and B) fertility postzygotic reproductive barriers in *Ischnura*. The
944 equation in the left-bottom corner of each subplot shows the model with the lowest AICc
945 value. Values between parentheses on each population cross show the sample size.
946 Population labels are explained in Table S1 (AEle = Pooled pure allopatric *I. elegans*
947 samples; Agra = Pooled pure allopatric *I. graellsii* samples; SEle = Pooled pure sympatric
948 *I. elegans* samples; SGra = Pooled pure sympatric *I. graellsii* samples). Letters superscripts
949 of crosses boxes at the top of each subplot show different groups inferred with *post hoc*
950 GLM analyses for crosses; e.g., in B) crosses between hybrids with *I. graellsii* males (B)
951 and *I. elegans* females (C) differed significantly in pairwise comparisons from the other
952 three types of crosses (A), and between them ($p < 0.05/10$). Purple and triangles = allopatric
953 crosses; Pink and circles = sympatric crosses; * = *Post hoc* statistically significant
954 differences between the sympatric and allopatric ecology within each cross.



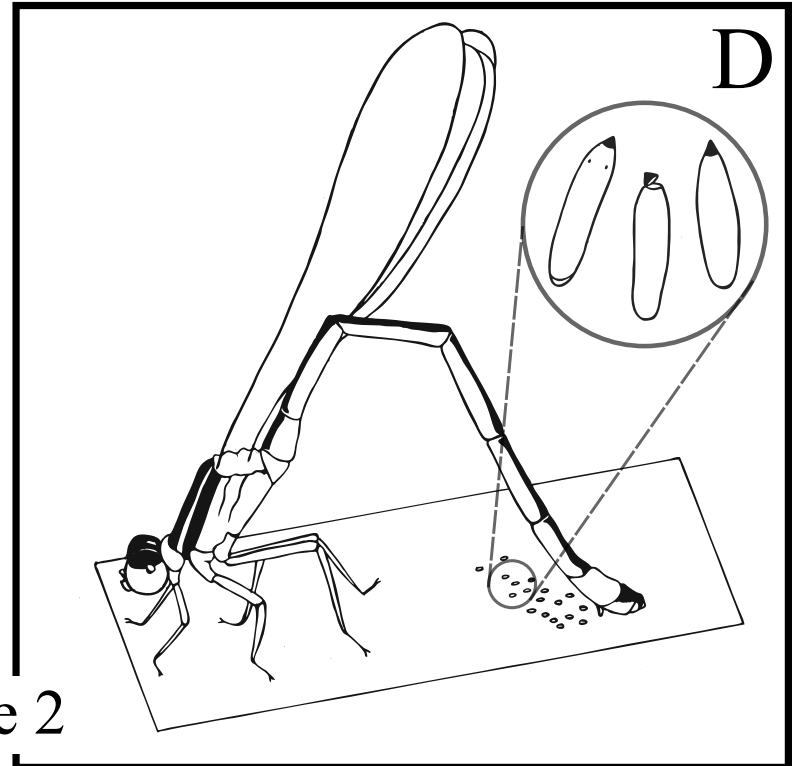
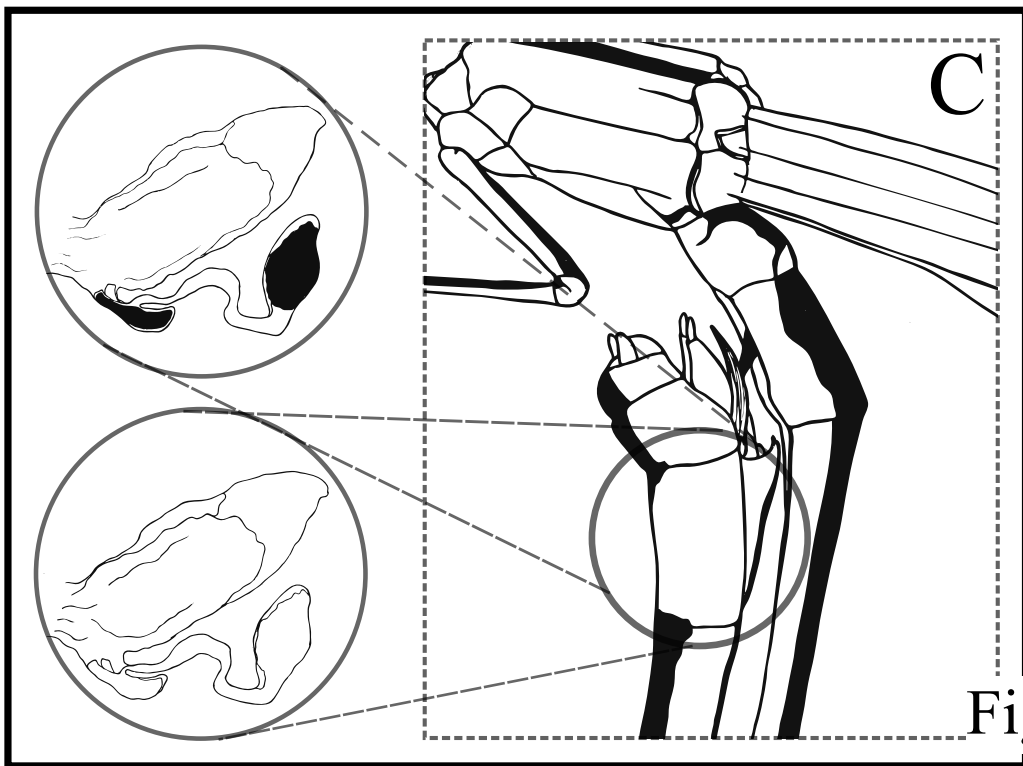
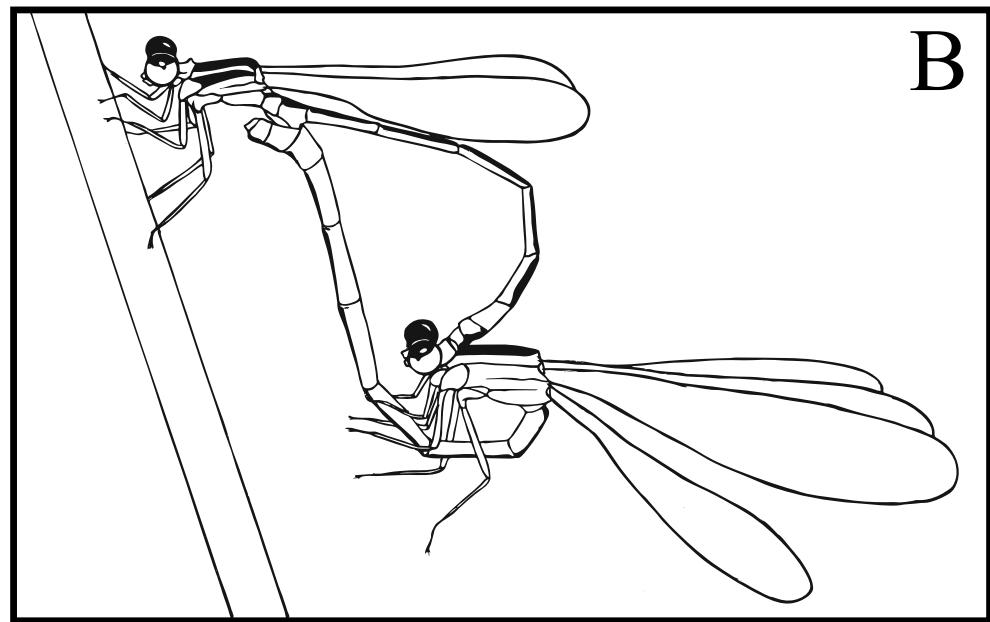
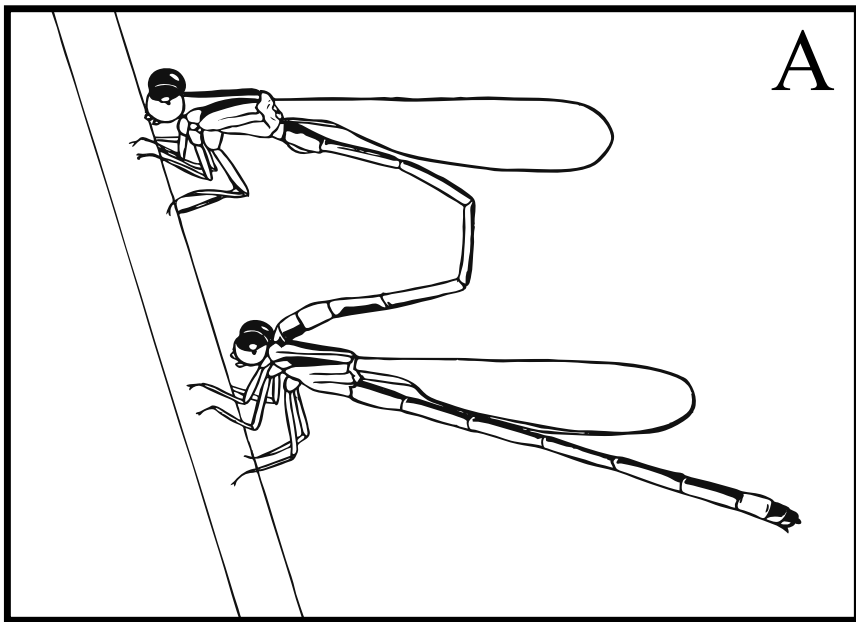


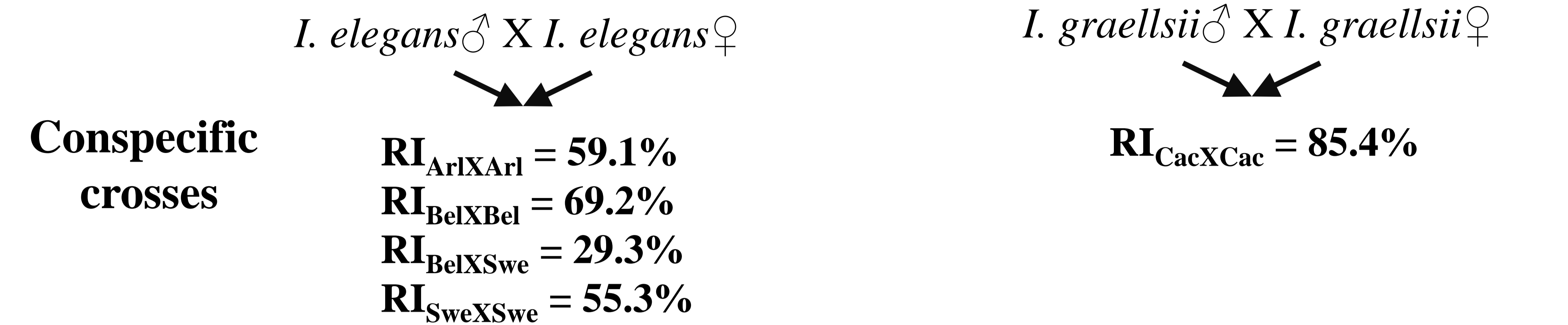
Figure 2

Figure 3

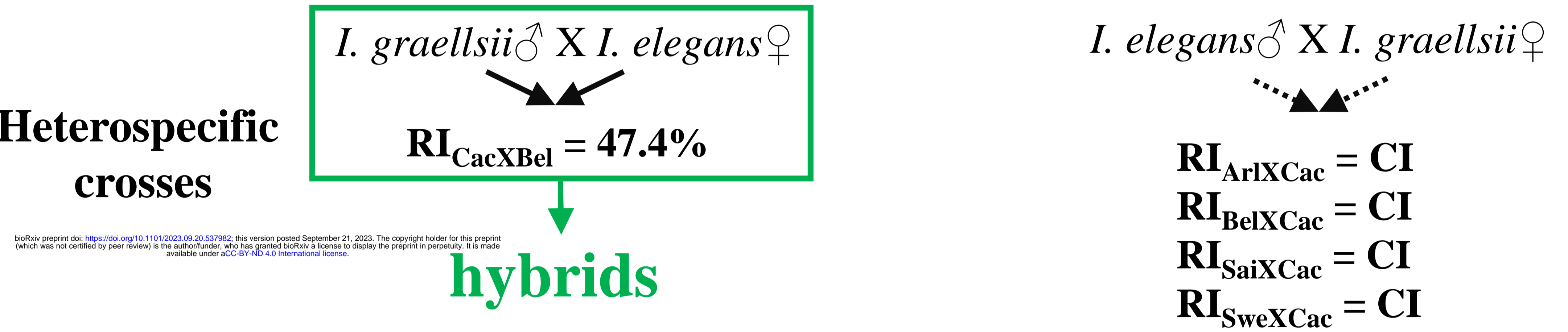
Allopatry

Sympatry

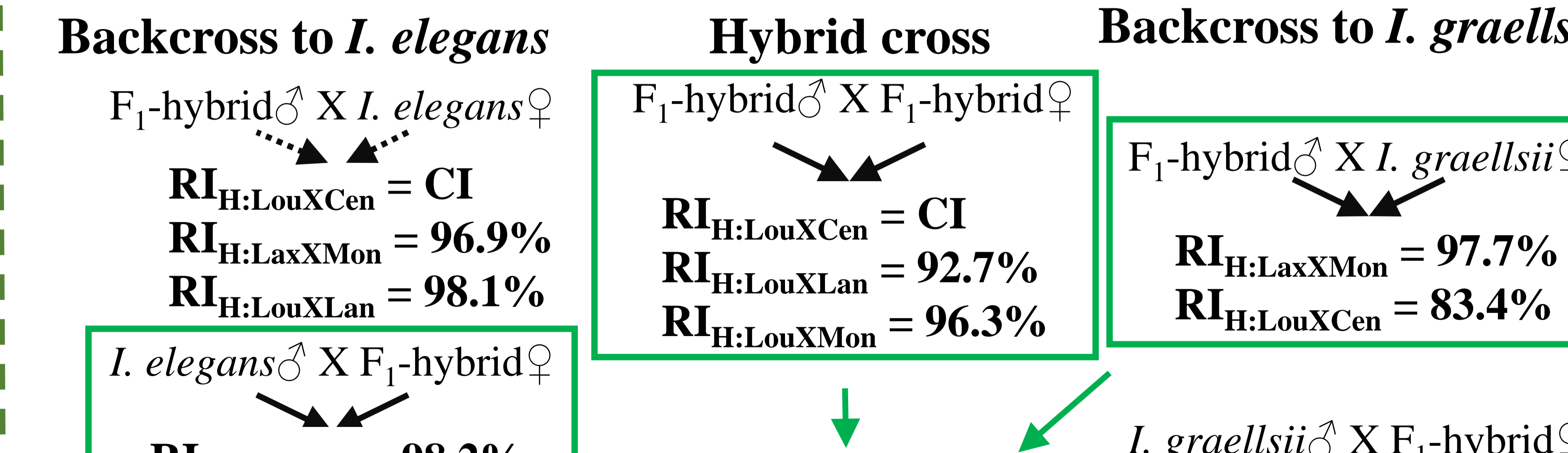
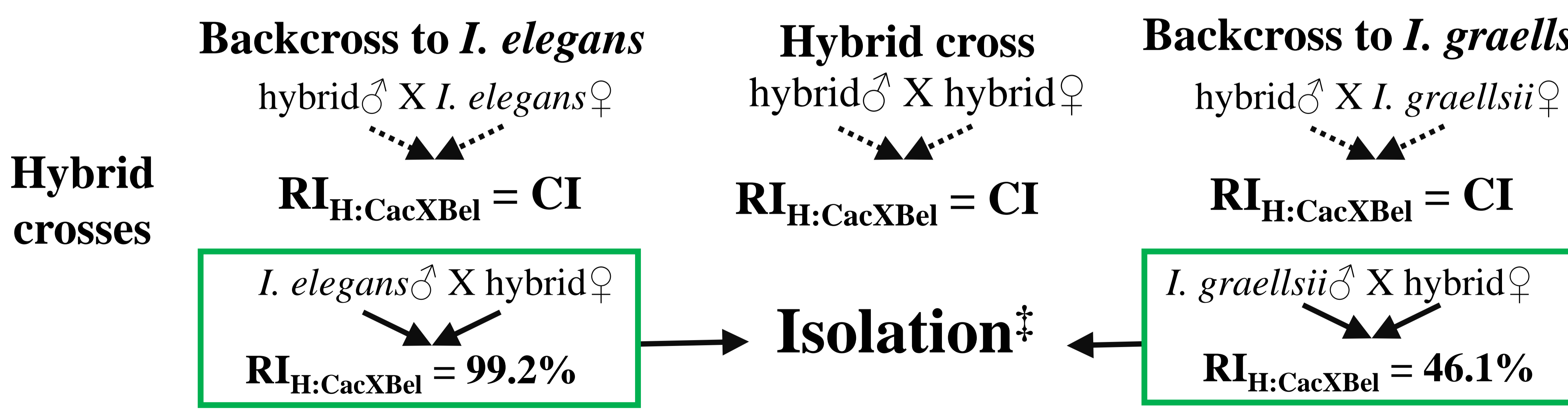
Barriers



- Prezygotic**
- Premating**
1. Mechanical
 2. Mechanical-Tactile
- Postmating**
3. Oviposition
 4. Fecundity
 5. Fertility



- Prezygotic**
- Premating**
1. Mechanical
 2. Mechanical-Tactile
- Postmating**
3. Oviposition
 4. Fecundity
 5. Fertility

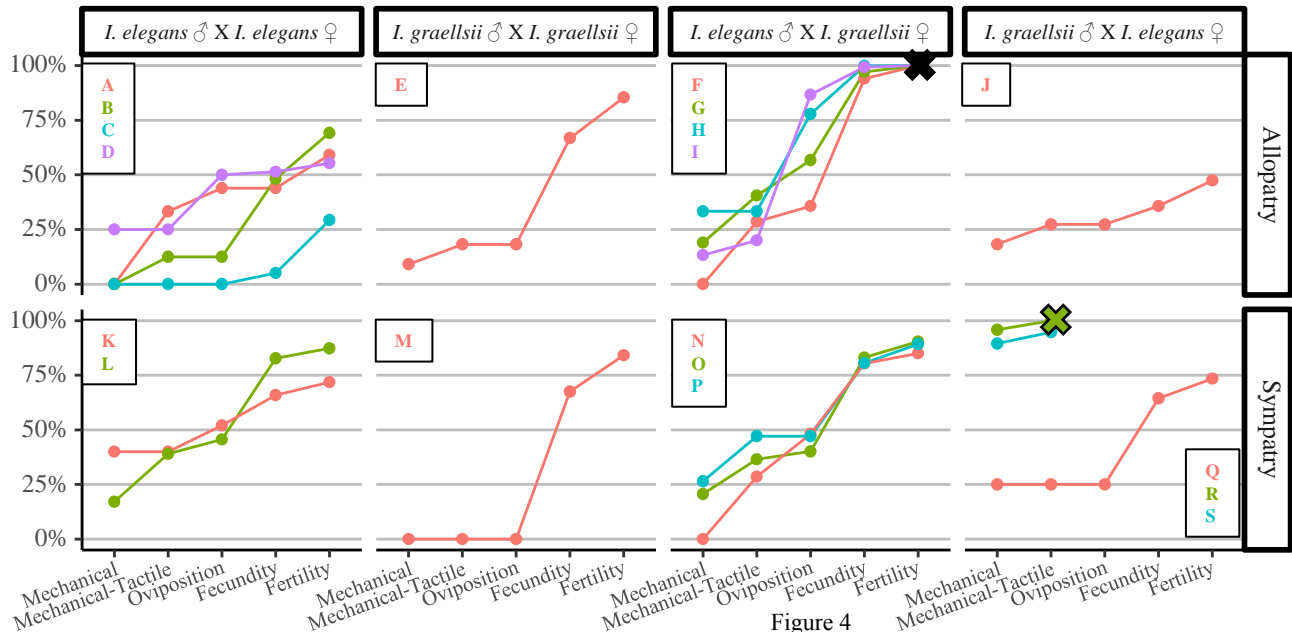


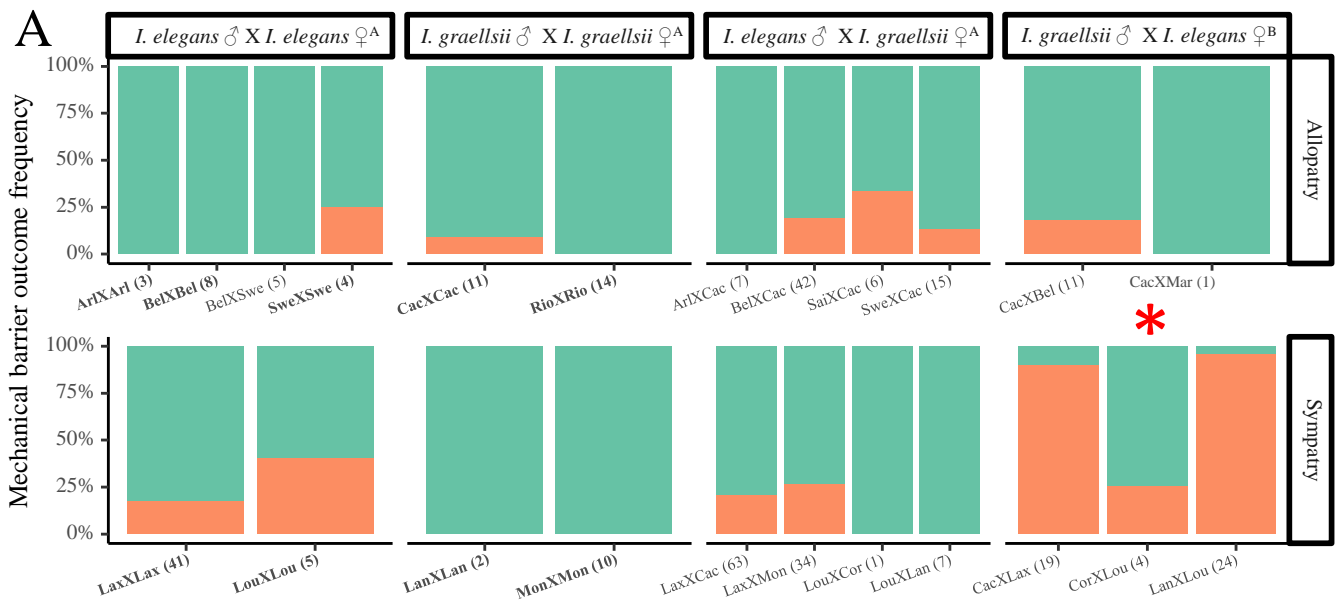
- Postzygotic**
- Premating**
1. Mechanical
 2. Mechanical-Tactile
- Postmating**
3. Oviposition
 4. Fecundity
 5. Fertility

Gene Flow

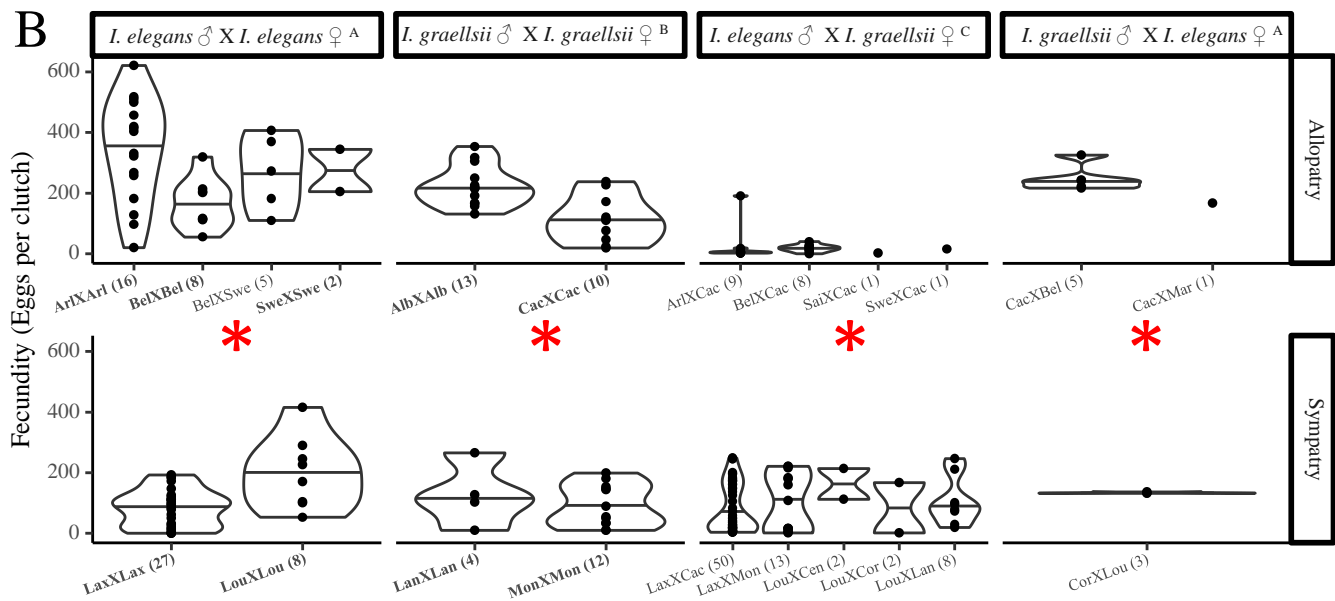
bioRxiv preprint doi: <https://doi.org/10.1101/2023.09.20.557982>; this version posted September 21, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Cumulative Reproductive Isolation

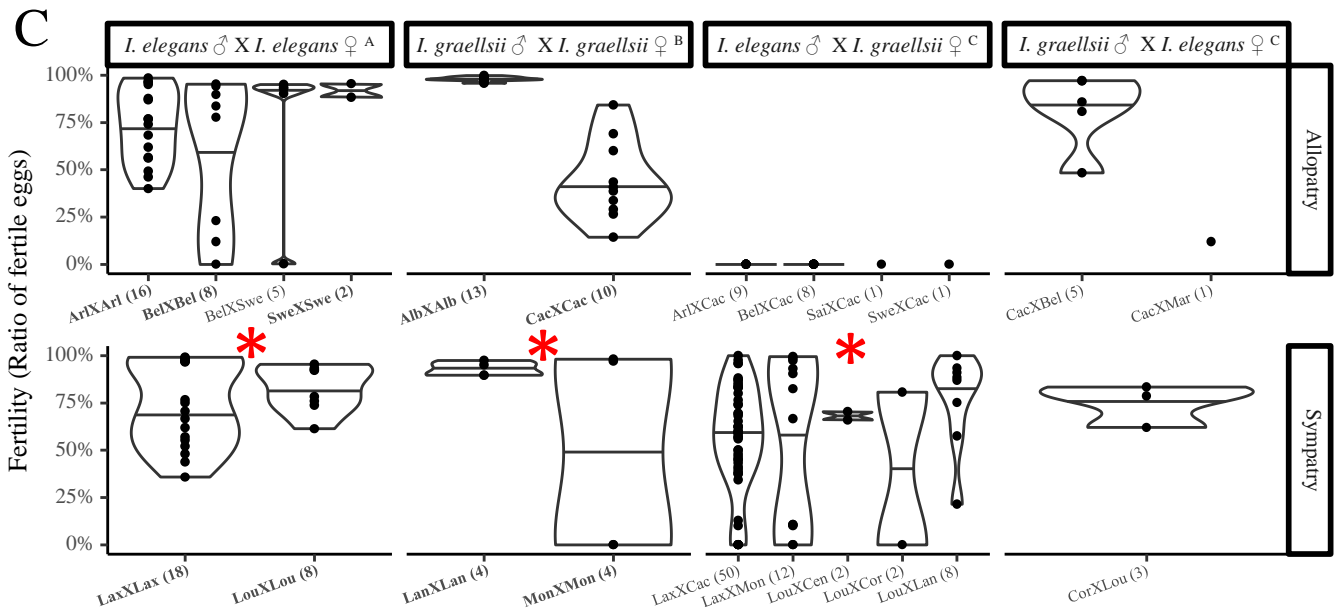




Mechanical Isolation ~ Cross + Ecology + (Cross:Ecology)

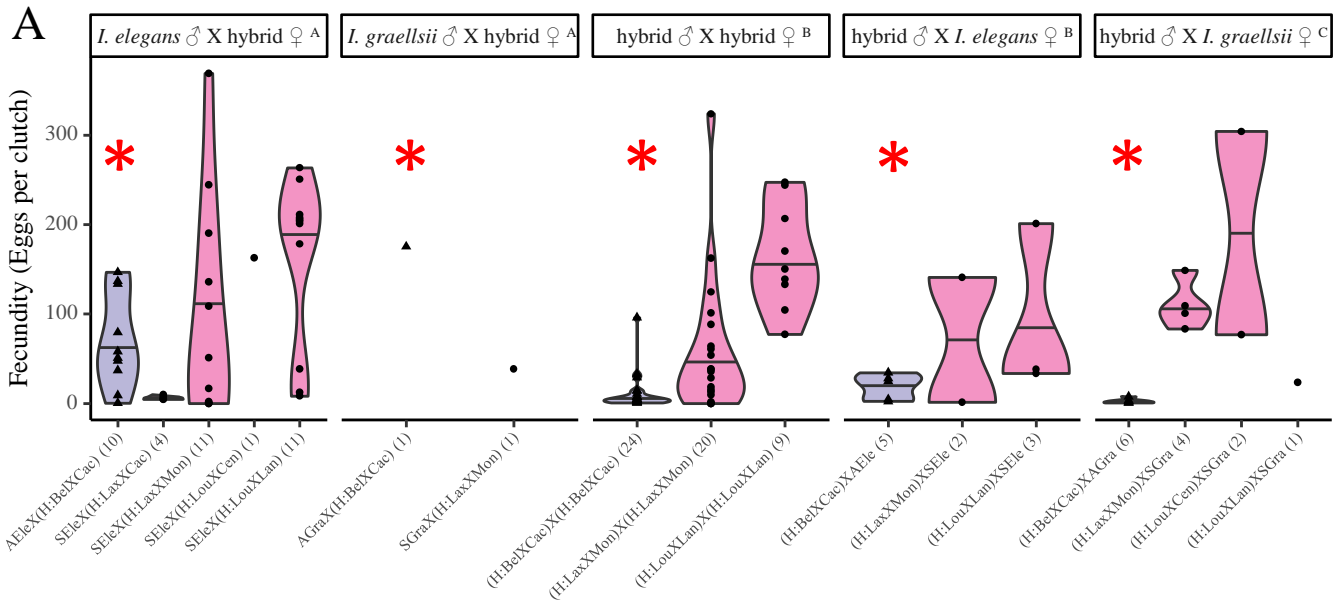


Fecundity ~ Cross + Ecology + Geography + (Cross:Ecology)

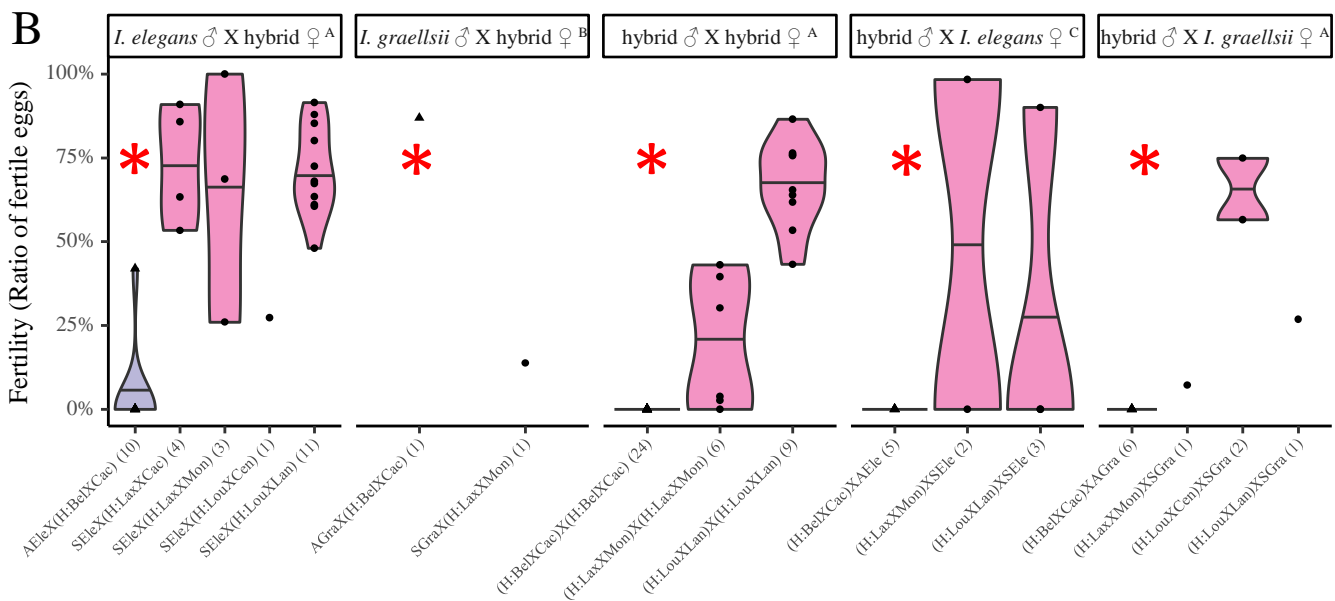


Fertility ~ Cross + Ecology + Geography + (Cross:Ecology)

Figure 5



Fecundity ~ Cross + Ecology + (Cross:Ecology)



Fertility ~ Cross + Ecology + (Cross:Ecology)

Figure 6

Supplementary Information for

Testing the predictions of reinforcement: long-term empirical data from a damselfly mosaic hybrid zone

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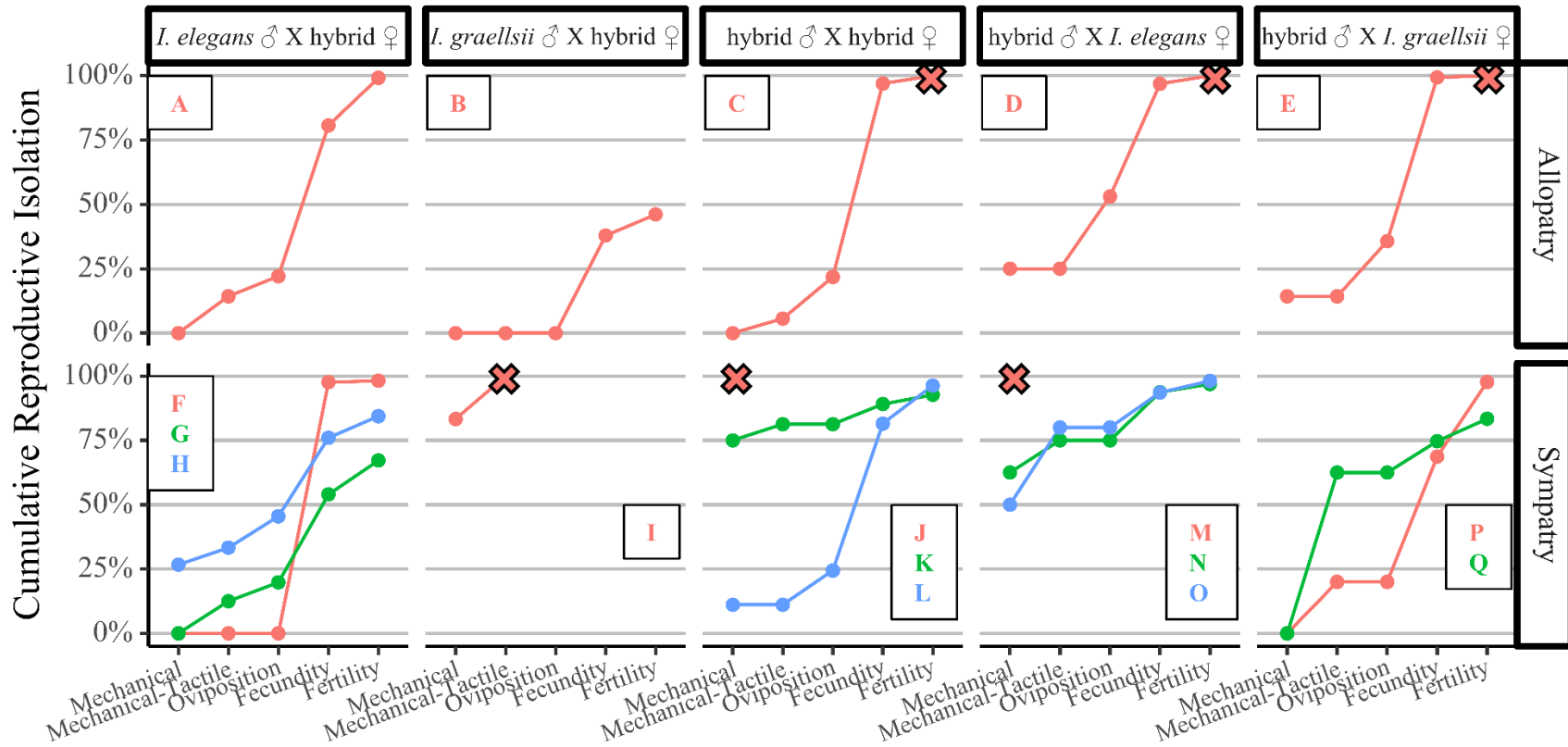


Fig. S1. Cumulative RI of five postzygotic barriers in conspecific and heterospecific crosses from allopatry and sympatry. Color lines within each subplot show data for a population cross pair: A) AElle×(H:Bel×Cac); B) AGra×(H:Bel×Cac); C) (H:Bel×Cac)×(H:Bel×Cac); D) (H:Bel×Cac)×AElle; E) (H:Bel×Cac)×AGra; F) SEle×(H:Lax×Cac); G) SEle×(H:Lou×Lan); H) SEle×(H:Lax×Mon); I) SGra×(H:Lou×Lan); J) (H:Lou×Cen)×(H:Lou×Cen); K) (H:Lou×Lan)×(H:Lou×Lan); L) (H:Lou×Mon)×(H:Lou×Mon); M) (H:Lou×Cen)×SEle; N) (H:Lax×Mon)×SEle; O) (H:Lou×Lan)×SEle; P) (H:Lax×Mon)×SGra; Q) (H:Lax×Cen)×SGra.. Population labels are explained in the Table S1 (AElle = Pooled pure allopatric *I. elegans* samples; AGra = Pooled pure allopatric *I. graellsii* samples; SEle = Pooled pure sympatric *I. elegans* samples; SGra = Pooled pure sympatric *I. graellsii* samples).

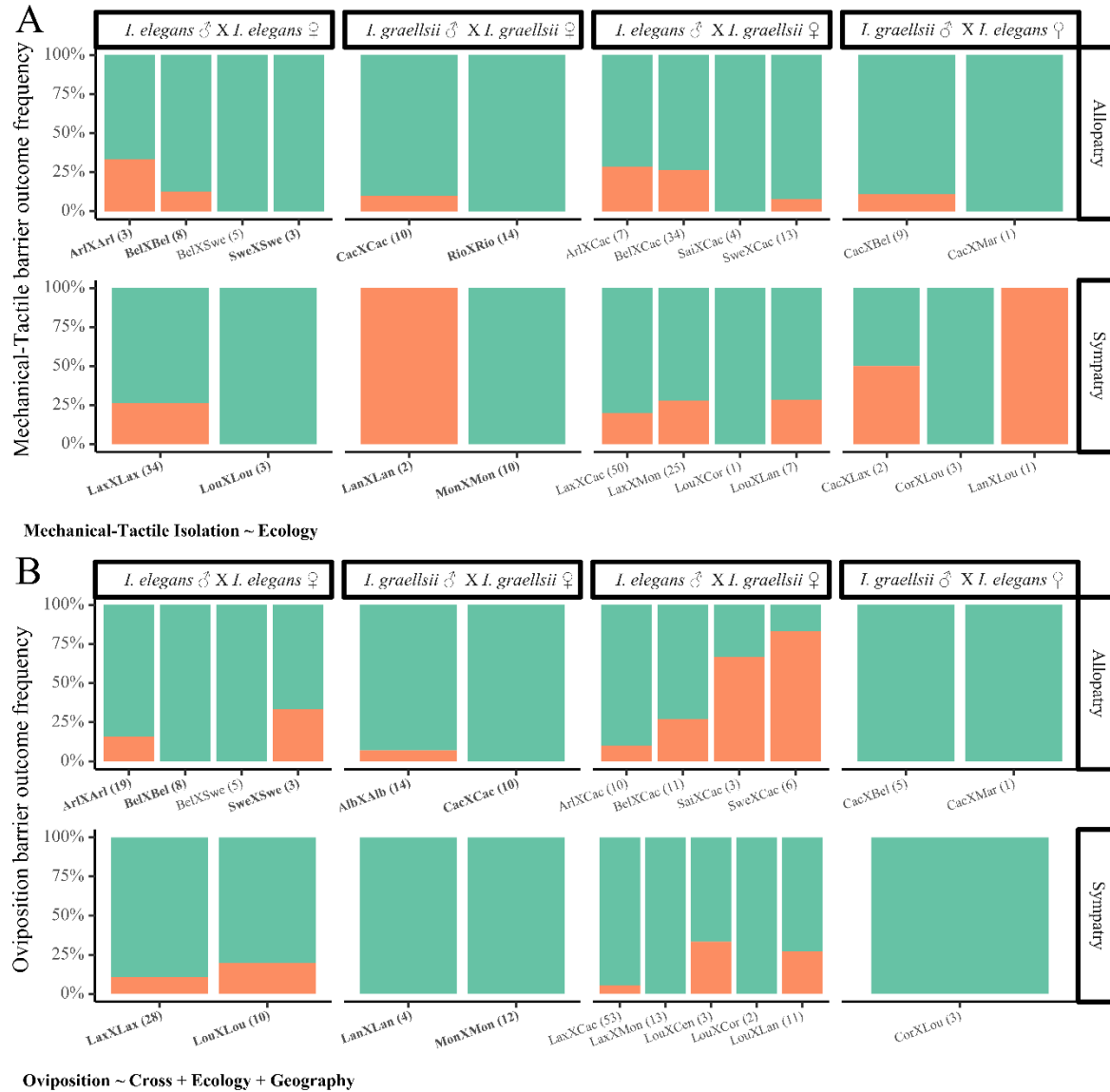


Fig. S2. Fitness components measurements for *Ischnura* and summary of GLM modeling results for the A) mechanical-tactile (green = successful mating and orange = unsuccessful mating) and B) oviposition prezygotic reproductive barriers (green = successful oviposition and orange = unsuccessful oviposition). The equation in the left-bottom corner of each subplot shows the model with the lowest AICc value. Values between parentheses on each population cross show the sample size. Population labels are explained in the Table S1. No statistically significant differences between crosses were detected with *post hoc* GLM ($p > 0.05/6$). **Bold** = Intrapopulation crosses.

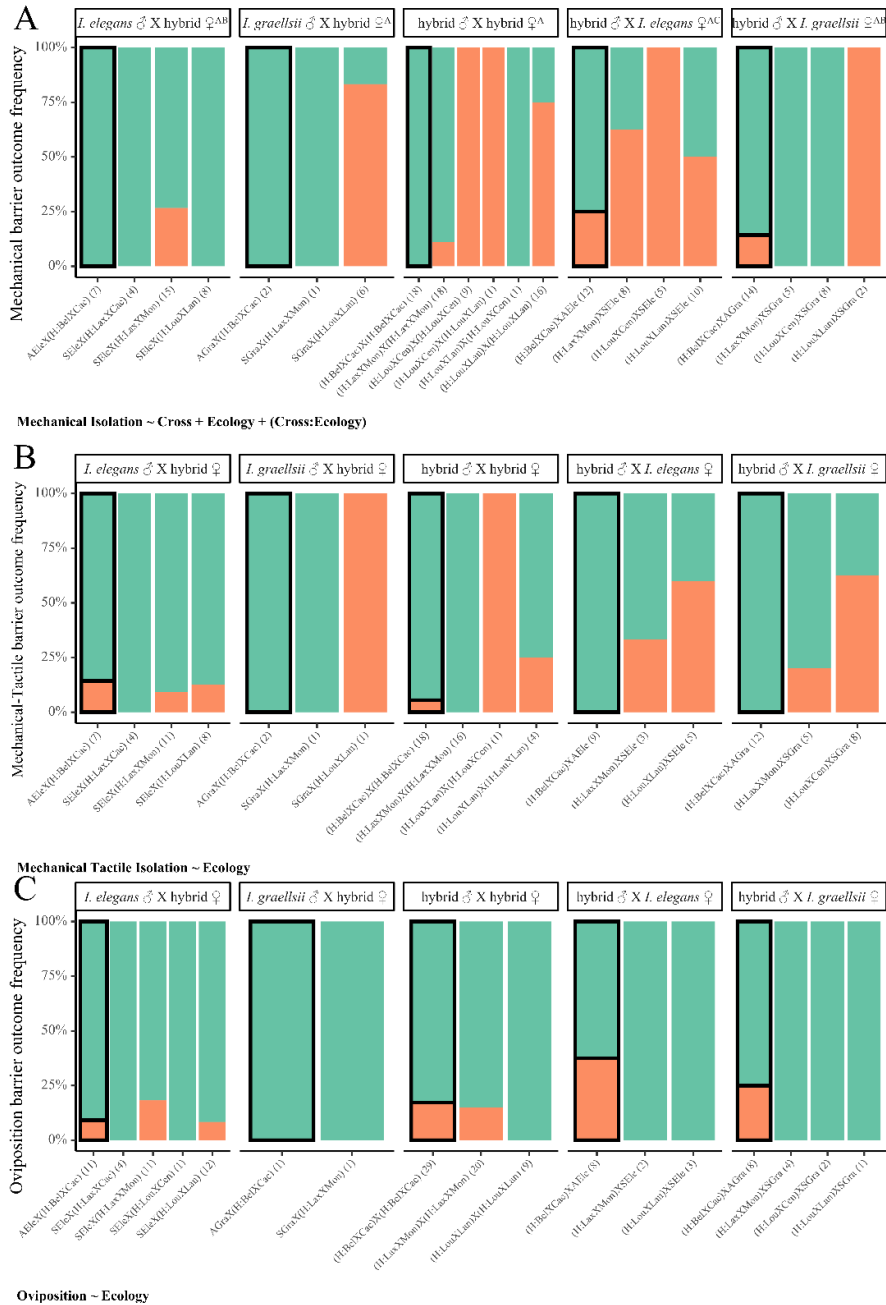


Fig. S3. Fitness components measurements for *Ischnura* and summary of GLM modeling results for the A) mechanical (green = successful tandem and orange = unsuccessful tandem), B) mechanical-tactile (green = successful mating and orange = unsuccessful mating) and C) oviposition (green = successful oviposition and orange = unsuccessful oviposition) postzygotic reproductive barriers. The equation in the left-bottom corner of each subplot shows the model with the lowest AICc value. Values between parentheses on each population cross show the sample size. Population labels are explained in the Table S1 (AEle = Pooled pure allopatric *I. elegans* samples; Agra = Pooled pure allopatric *I. graellsii* samples; SEle = Pooled pure sympatric *I. elegans* samples; SGra = Pooled pure sympatric *I. graellsii* samples). Letters superscripts of crosses boxes at the top of each subplot show different groups inferred with *post hoc* GLM analyses for crosses. In A) crosses between hybrids and *I. elegans* females (AC) differed significantly in pairwise comparisons from their reciprocal cross and from crosses between hybrids and *I. graellsii* females (AB; $p < 0.05/10$).

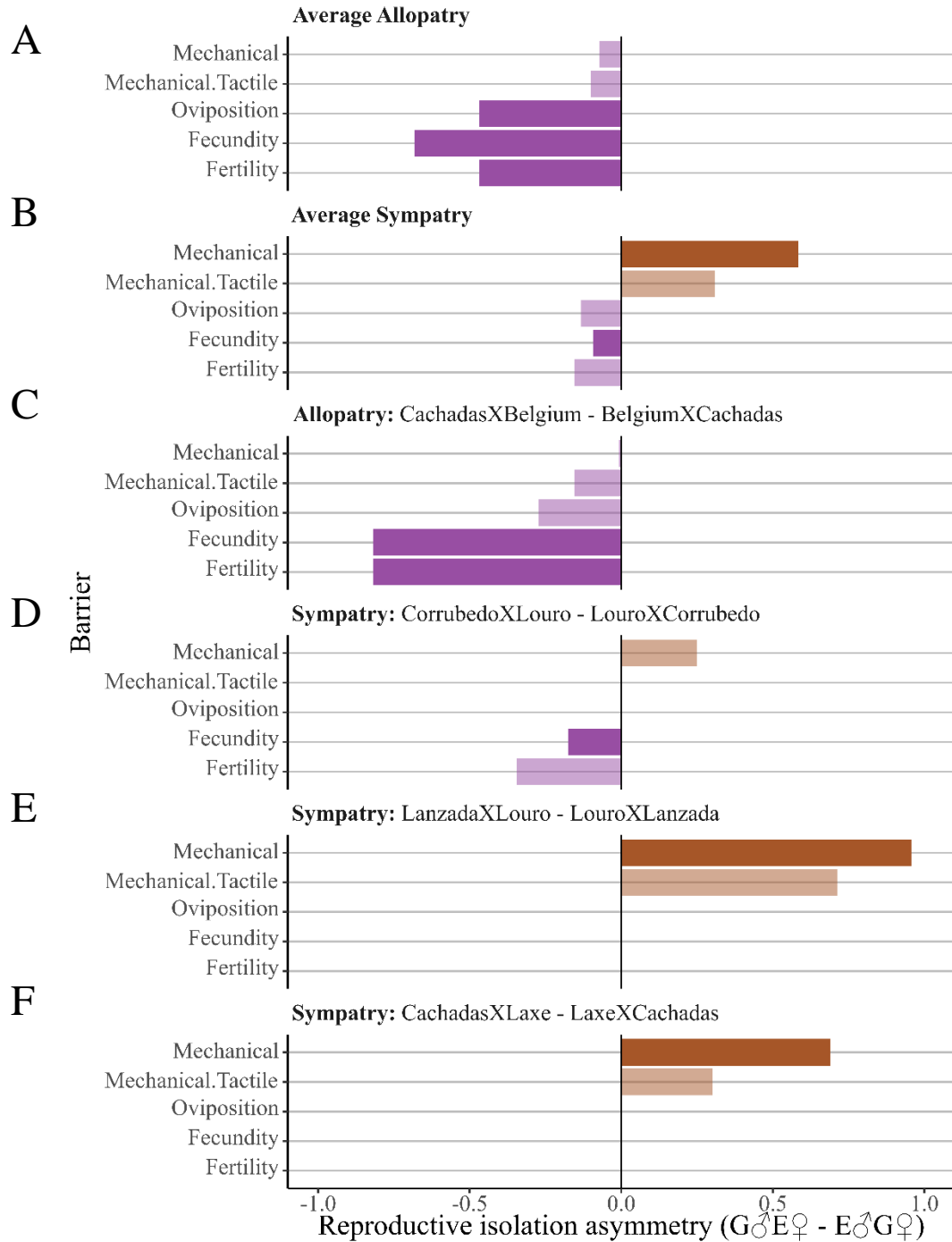


Fig. S4. Prezygotic reproductive asymmetries in A) allopatry (averaged between all populations), B) sympatry (averaged between all populations) and populations in which both reciprocal crosses were sampled both in allopatry and sympatry (E to F). Asymmetries were measured as the absolute RI from crosses between *Ichnura graellsii* males with *I. elegans* females minus the RI in the reciprocal direction and are categorized between stronger isolation in the former (brown) or stronger isolation in the latter (purple). Solid bars represent barriers in which GLM models including the cross variable presented lower AICc values than models excluding it. GLM modeling for allopatry and sympatry were done using all data; the plot shows the difference between population crosses averages. E: *I. elegans*; G: *I. graellsii*.

Table S1. Historical and molecular data from the sampled localities of *Ischnura* damselflies.

Locality	Distribution	Historic data [†]	Sampled-years for RI measurements	Molecular data [‡]	Observations
Cedeira, northwest Spain	Sympatric	2001: both species and hybrids 2003: both species 2007: only <i>I. elegans</i> . Occasionally <i>I. graellsii</i>			
Foz, northwest Spain	Sympatric	1990: <i>I. elegans</i> and hybrids 2001–2004: both species and hybrids 2007: <i>I. elegans</i> 2010: <i>I. elegans</i>			
Doniños, northwest Spain	Sympatric	1987: only <i>I. elegans</i> . Occasionally <i>I. graellsii</i> 2001–2021: <i>I. elegans</i> . Occasionally <i>I. graellsii</i>		2007: introgressed <i>I. elegans</i> and hybrids (SSR) 2014: introgressed <i>I. elegans</i> (SNPs)	
Laxe, northwest Spain (Lax)	Sympatric	2000: only <i>I. elegans</i> 2001: dried locality, <i>I. elegans</i> removed 2001–2021: <i>I. elegans</i> . Occasionally <i>I. graellsii</i>	2019–2020: <i>I. elegans</i> . Occasionally <i>I. graellsii</i>	2007: introgressed <i>I. elegans</i> and hybrids (SSR) 2014: introgressed <i>I. elegans</i> (SNPs)	
Louro, northwest Spain (Lou)	Sympatric	1980: both species and hybrids 1995: both species and hybrids 1998–2001: <i>I. elegans</i> . Occasionally <i>I. graellsii</i>	2000–2001: <i>I. elegans</i> . Occasionally <i>I. graellsii</i> (Sánchez-Guillén et al. 2012).	2007: introgressed <i>I. elegans</i> and hybrids (SSR) 2013: introgressed <i>I. graellsii</i> ; F ₁ –F ₂ hybrids, backcrosses to <i>I.</i>	Postmating barriers in crosses between <i>I. elegans</i> from Louro and <i>I. graellsii</i> from Centeans were not measured.

Locality	Distribution	Historic data [†]	Sampled-years for RI measurements	Molecular data [‡]	Observations
		2010: both species were removed (brackish water in the lagoon). 2013: <i>I. graellsii</i> . Occasionally <i>I. elegans</i>		<i>elegans</i> and to <i>I. graellsii</i>	
Carnota	Sympatric	2000–2001: <i>I. elegans</i>			
Corrubedo complex, northwest Spain [Xuño, Vilar and Corrubedo (Cor)]	Sympatric	1988: only <i>I. graellsii</i> 1988–2002: only <i>I. graellsii</i> 2003–2006: both species at similar proportions 2007–2014: only <i>I. graellsii</i>	2000–2001: only <i>I. graellsii</i> (Sánchez-Guillén et al. 2012).	2014: introgressed <i>I. graellsii</i>	
Lanzada complex, north-west Spain [Lanzada (Lan), Montalvo (Mon), Cachadas (Cac)]	Zone of putative influence of <i>I. elegans</i>	1999: only <i>I. graellsii</i> 2000–2015: only <i>I. graellsii</i>	2000–2001: only <i>I. graellsii</i> (Sánchez-Guillén et al. 2012). 2015: only <i>I. graellsii</i>	2015: pure <i>I. graellsii</i>	
Alba, campus, northwest Spain (Alb)	Allopatric	2001: only <i>I. graellsii</i> 2002–2005: only <i>I. graellsii</i>	2000–2001: only <i>I. graellsii</i> (Sánchez-Guillén et al. 2012).	2005: pure <i>I. graellsii</i>	Premating barriers in <i>I. graellsii</i> conspecific crosses were not measured.
Riomaior, northwest Spain (Rio)	Allopatric	2001: only <i>I. graellsii</i> 2002–2005: only <i>I. graellsii</i>	2023: only <i>I. graellsii</i>		Postmating barriers in <i>I. graellsii</i> conspecific crosses were not measured.
Centears, northwest	Allopatric	1995: only <i>I. graellsii</i>	2000–2001: only <i>I.</i>		Postmating barriers in

Locality	Distribution	Historic data [†]	Sampled-years for RI measurements	Molecular data [‡]	Observations
Spain (Cen)			<i>graellsii</i> (Sánchez-Guillén et al. 2012).		crosses between <i>I. elegans</i> from Louro and <i>I. graellsii</i> from Centeans were not measured.
Lund, Sweden (Swe)	Allopatric		2015: only <i>I. elegans</i>	2015: pure <i>I. elegans</i>	
De Maten, Belgium (Bel)	Allopatric		2015: only <i>I. elegans</i>	2015: pure <i>I. elegans</i>	
Arles, France (Arl)	Allopatric		2015: only <i>I. elegans</i>	2015: pure <i>I. elegans</i>	
Saint Cyprien, France (Sai)	Allopatric		2015: only <i>I. elegans</i>	2015: pure <i>I. elegans</i>	
Marais D'Orx, France (Mar)	Allopatric		2015: only <i>I. elegans</i>	2015: pure <i>I. elegans</i>	

[†]Data from (Sánchez-Guillén et al. 2005, 2011, 2012, 2023).

[‡]Genetic evidence from microsatellites (Sánchez-Guillén et al. 2011) and RADseq genome-wide SNPs (Sánchez-Guillén et al. 2023) from the studied localities distributed across this hybrid zone (Laxe, Louro, Corrubedo and Cachadas) identified introgression and hybridization.

Table S2. Sample size (a single interaction was recorded for each male and female *Ischnura* couple for pre mating barriers, while sample sizes refer to number of females for postmating barriers), success events and absolute reproductive isolation per cross between populations. G = *Ischnura graellsii*; E = *I. elegans*. Population labels are explained in the Table S1 (AEle = Pooled pure allopatric *I. elegans* samples; Agra = Pooled pure allopatric *I. graellsii* samples; SEle = Pooled pure sympatric *I. elegans* samples; SGra = Pooled pure sympatric *I. graellsii* samples).

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
<i>Premating I – Mechanical barrier</i>									
Conspecific crosses	E♂E♀	Arl×Arl	3	3	0.000	Lou×Lou	5	3	0.400
	E♂E♀	Bel×Bel	8	8	0.000	Lax×Lax	41	34	0.171
	E♂E♀	Bel×Swe	5	5	0.000				
	E♂E♀	Swe×Swe	4	3	0.250				
	G♂G♀	Alb×Alb	0	NA	NA	Lan×Lan	2	2	0.000
	G♂G♀	Cac×Cac	11	10	0.091	Mon×Mon	10	10	0.000
	G♂G♀	Rio×Rio	14	14	0.000				
Heterospecific crosses	E♂G♀	Arl×Cac	7	7	0.000	Lou×Cen	0	NA	NA
	E♂G♀	Bel×Cac	42	34	0.190	Lou×Cor	1	1	0.000
	E♂G♀	Sai×Cac	6	4	0.333	Lou×Lan	7	7	0.000
	E♂G♀	Swe×Cac	15	13	0.133	Lax×Cac	63	50	0.206
	E♂G♀					Lax×Mon	34	25	0.265
	G♂E♀	Cac×Bel	11	9	0.182	Cor×Lou	4	3	0.250
	G♂E♀	Cac×Mar	1	1	0.000	Lan×Lou	24	1	0.958
	G♂E♀					Cac×Lax	19	2	0.895
Postzygotic crosses	E♂H♀	AEle×(H:Bel×Cac)	7	7	0.000	SEle×(H:Lou×Cen)	0	0	NA
	E♂H♀					SEle×(H:Lou×Lan)	8	8	0.000
	E♂H♀					SEle×(H:Lax×Cac)	4	4	0.000
	E♂H♀					SEle×(H:Lax×Mon)	15	11	0.267
	G♂H♀	AGra×(H:Bel×Cac)	2	2	0.000	SGra×(H:Lou×Lan)	6	1	0.833
	G♂H♀					SGra×(H:Lax×Mon)	1	1	0.000

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
	H♂E♀	(H:Bel×Cac)×AEle	12	9	0.250	(H:LouxCen)×SEle	5	0	1.000
	H♂E♀					(H:LouXLan)×SEle	10	5	0.500
	H♂E♀					(H:Lax×Mon)×SEle	8	3	0.625
	H♂G♀	(H:Bel×Cac)×AGra	14	12	0.143	(H:Lou×Cen)×SGra	8	8	0.000
	H♂G♀					(H:Lou×Lan)×SGra	2	0	1.000
	H♂G♀					(H:Lax×Mon)×SGra	5	5	0.000
	H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	18	18	0.000	(H:Lou×Cen)×(H:Lou×Cen)	9	0	1.000
	H♂H♀					(H:Lou×Cen)×(H:Lou×Lan)	1	0	1.000
	H♂H♀					(H:Lou×Lan)×(H:Lou×Cen)	1	1	0.000
	H♂H♀					(H:Lou×Lan)×(H:Lou×Lan)	16	4	0.750
	H♂H♀					(H:Lou×Mon)×(H:Lou×Mon)	18	16	0.111
		Total	180			Total	327		
Premating II – Mechanical-tactile barrier									
Conspecific crosses	E♂E♀	Arl×Arl	3	2	0.333	Lou×Lou	3	3	0.000
	E♂E♀	Bel×Bel	8	7	0.125	Lax×Lax	34	25	0.265
	E♂E♀	Bel×Swe	5	5	0.000				
	E♂E♀	Swe×Swe	3	3	0.000				
	G♂G♀	Alb×Alb	0	NA	NA	Lan×Lan	2	0	1.000
	G♂G♀	Cac×Cac	10	9	0.100	Mon×Mon	10	10	0.000
	G♂G♀	Rio×Rio	14	14	0.000				
Heterospecific crosses	E♂G♀	Arl×Cac	7	5	0.286	Lou×Cen	0	NA	NA
	E♂G♀	Bel×Cac	34	25	0.265	Lou×Cor	1	1	0.000
	E♂G♀	Sai×Cac	4	4	0.000	Lou×Lan	7	5	0.286
	E♂G♀	Swe×Cac	13	12	0.077	Lax×Cac	50	40	0.200
	E♂G♀					Lax×Mon	25	18	0.280
	G♂E♀	Cac×Bel	9	8	0.111	Cor×Lou	3	3	0.000

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
	G♂E♀	Cac×Mar	1	1	0.000	Lan×Lou	1	0	1.000
	G♂E♀					Cac×Lax	2	1	0.500
Postzygotic crosses	E♂H♀	AEle×(H:Bel×Cac)	7	6	0.143	SEle×(H:Lou×Cen)	0	NA	NA
	E♂H♀					SEle×(H:Lou×Lan)	8	7	0.125
	E♂H♀					SEle×(H:Lax×Cac)	4	4	0.000
	E♂H♀					SEle×(H:Lax×Mon)	11	10	0.091
	G♂H♀	AGra×(H:Bel×Cac)	2	2	0.000	SGra×(H:Lou×Lan)	1	0	1.000
	G♂H♀					SGra×(H:Lax×Mon)	1	1	0.000
	H♂E♀	(H:Bel×Cac)×AEle	9	9	0.000	(H:LouxCen)×SEle	0	NA	NA
	H♂E♀					(H:LouxLan)×SEle	5	2	0.600
	H♂E♀					(H:Lax×Mon)×SEle	3	2	0.333
	H♂G♀	(H:Bel×Cac)×AGra	12	12	0.000	(H:Lou×Cen)×SGra	8	3	0.625
	H♂G♀					(H:LouxLan)×SGra	0	NA	NA
	H♂G♀					(H:Lax×Mon)×SGra	5	4	0.200
	H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	18	17	0.056	(H:Lou×Cen)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Cen)×(H:Lou×Lan)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Cen)	1	0	1.000
	H♂H♀					(H:Lou×Lan)×(H:Lou×Lan)	4	3	0.250
H♂H♀					(H:Lou×Mon)×(H:Lou×Mon)	16	16	0.000	
		Total	159			Total	205		
Postmating I – Oviposition									
Conspecific crosses	E♂E♀	Arl×Arl	19	16	0.158	Lou×Lou	10	8	0.200
	E♂E♀	Bel×Bel	8	8	0.000	Lax×Lax	28	25	0.107
	E♂E♀	Bel×Swe	5	5	0.000				
	E♂E♀	Swe×Swe	3	2	0.333				

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
	G♂G♀	Alb×Alb	14	13	0.071	Lan×Lan	4	4	0.000
	G♂G♀	Cac×Cac	10	10	0.000	Mon×Mon	12	12	0.000
	G♂G♀	Rio×Rio	0	NA	NA				
Heterospecific crosses	E♂G♀	Arl×Cac	10	9	0.100	Lou×Cen	3	2	0.333
	E♂G♀	Bel×Cac	11	8	0.273	Lou×Cor	2	2	0.000
	E♂G♀	Sai×Cac	3	1	0.667	Lou×Lan	11	8	0.273
	E♂G♀	Swe×Cac	6	1	0.833	Lax×Cac	53	50	0.057
	E♂G♀					Lax×Mon	13	13	0.000
	G♂E♀	Cac×Bel	5	5	0.000	Cor×Lou	3	3	0.000
	G♂E♀	Cac×Mar	1	1	0.000	Lan×Lou	0	NA	NA
	G♂E♀				Cac×Lax	0	NA	NA	
Postzygotic crosses	E♂H♀	AEle×(H:Bel×Cac)	11	10	0.091	SEle×(H:Lou×Cen)	1	1	0.000
	E♂H♀				SEle×(H:Lou×Lan)	12	11	0.083	
	E♂H♀				SEle×(H:Lax×Cac)	4	4	0.000	
	E♂H♀				SEle×(H:Lax×Mon)	11	9	0.182	
	G♂H♀	AGra×(H:Bel×Cac)	1	1	0.000	SGra×(H:Lou×Lan)	0	NA	NA
	G♂H♀				SGra×(H:Lax×Mon)	1	1	0.000	
	H♂E♀	(H:Bel×Cac)×AEle	8	5	0.375	(H:LouxCen)×SEle	0	NA	NA
	H♂E♀				(H:LouxLan)×SEle	3	3	0.000	
	H♂E♀				(H:Lax×Mon)×SEle	2	2	0.000	
	H♂G♀	(H:Bel×Cac)×AGra	8	6	0.250	(H:Lou×Cen)×SGra	2	2	0.000
	H♂G♀				(H:LouxLan)×SGra	1	1	0.000	
	H♂G♀				(H:Lax×Mon)×SGra	4	4	0.000	
	H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	29	24	0.172	(H:Lou×Cen)×(H:Lou×Cen)	0	NA	NA
H♂H♀				(H:Lou×Cen)×(H:Lou×Lan)	0	NA	NA		

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
	H♂H♀					(H:Lou×Lan)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Lan)	9	9	0.000
	H♂H♀					(H:Lou×Mon)×(H:Lou×Mon)	20	17	0.150
		Total	152			Total	209		
Postmating II – Fecundity									
Conspecific crosses	E♂E♀	Arl×Arl	16	340.6 [†]	0.000	Lou×Lou	8	200.6	0.290
	E♂E♀	Bel×Bel	8	167.3	0.408	Lax×Lax	25	89.5	0.683
	E♂E♀	Bel×Swe	5	268.1	0.051				
	E♂E♀	Swe×Swe	2	274.8	0.027				
	G♂G♀	Alb×Alb	13	224.2 [†]	0.206	Lan×Lan	4	126.3	0.553
	G♂G♀	Cac×Cac	10	114.6	0.594	Mon×Mon	12	91.9	0.675
	G♂G♀	Rio×Rio	0	NA	NA				
Heterospecific crosses	E♂G♀	Arl×Cac	9	26.4	0.906	Lou×Cen	2	163.0	0.423
	E♂G♀	Bel×Cac	8	18.7	0.934	Lou×Cor	2	84.3	0.701
	E♂G♀	Sai×Cac	1	2.0	0.993	Lou×Lan	8	106.5	0.623
	E♂G♀	Swe×Cac	1	15.5	0.945	Lax×Cac	50	79.9	0.717
	E♂G♀					Lax×Mon	13	103.6	0.633
	G♂E♀	Cac×Bel	5	249.8	0.115	Cor×Lou	3	134.0	0.526
	G♂E♀	Cac×Mar	1	167.0	0.409	Lan×Lou	0	NA	NA
	G♂E♀					Cac×Lax	0	NA	NA
Postzygotic crosses	E♂H♀	AEle×(H:Bel×Cac)	10	69.9	0.752	SEle×(H:Lou×Cen)	1	162.7	0.424
	E♂H♀					SEle×(H:Lou×Lan)	11	161.9	0.427
	E♂H♀					SEle×(H:Lax×Cac)	4	6.8	0.976
	E♂H♀					SEle×(H:Lax×Mon)	9	124.3	0.560
	G♂H♀	AGra×(H:Bel×Cac)	1	175.3	0.379	SGra×(H:Lou×Lan)	0	NA	NA

Type of cross	Allopatry					Sympatry			
	Cross	Populations	N	Success	RI	Populations	N	Success	Isolation
	G♂H♀					SGra×(H:Lax×Mon)	1	38.7	0.863
	H♂E♀	(H:Bel×Cac)×AEle	5	18.8	0.934	(H:LouxCen)×SEle	0	NA	NA
	H♂E♀					(H:LouxLan)×SEle	3	90.9	0.678
	H♂E♀					(H:Lax×Mon)×SEle	2	71.2	0.748
	H♂G♀	(H:Bel×Cac)×AGra	6	2.7	0.990	(H:Lou×Cen)×SGra	2	191	0.325
	H♂G♀					(H:LouxLan)×SGra	1	24	0.916
	H♂G♀					(H:Lax×Mon)×SGra	4	110	0.609
	H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	24	10.9	0.961	(H:Lou×Cen)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Cen)×(H:Lou×Lan)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Lan)	9	164	0.421
	H♂H♀					(H:Lou×Mon)×(H:Lou×Mon)	17	69	0.755
		Total	125			Total	191		
Postmating III – Fertility									
Conspecific crosses	E♂E♀	Arl×Arl	16	0.729	0.271	Lou×Lou	8	0.828	0.172
	E♂E♀	Bel×Bel	8	0.594	0.406	Lax×Lax	25	0.737	0.263
	E♂E♀	Bel×Swe	5	0.744	0.256				
	E♂E♀	Swe×Swe	2	0.919	0.081				
	G♂G♀	Alb×Alb	13	0.980	0.020	Lan×Lan	4	0.930	0.071
	G♂G♀	Cac×Cac	10	0.440	0.560	Mon×Mon	12	0.488	0.512
	G♂G♀	Rio×Rio	0	NA	NA				
Heterospecific crosses	E♂G♀	Arl×Cac	9	0.000	1.000	Lou×Cen	2	0.682	0.319
	E♂G♀	Bel×Cac	8	0.000	1.000	Lou×Cor	2	0.404	0.597
	E♂G♀	Sai×Cac	1	0.000	1.000	Lou×Lan	8	0.767	0.233
	E♂G♀	Swe×Cac	1	0.000	1.000	Lax×Cac	50	0.565	0.435

Type of cross	Cross	Allopatry				Sympatry			
		Populations	N	Success	RI	Populations	N	Success	Isolation
	E♂G♀					Lax×Mon	13	0.549	0.451
	G♂E♀	Cac×Bel	5	0.818	0.182	Cor×Lou	3	0.747	0.253
	G♂E♀	Cac×Mar	1	0.120	0.880	Lan×Lou	0	NA	NA
	G♂E♀					Cac×Lax	0	NA	NA
Postzygotic crosses	E♂H♀	AEle×(H:Bel×Cac)	10	0.042	0.958	SEle×(H:Lou×Cen)	1	0.273	0.727
	E♂H♀					SEle×(H:Lou×Lan)	11	0.714	0.286
	E♂H♀					SEle×(H:Lax×Cac)	4	0.733	0.267
	E♂H♀					SEle×(H:Lax×Mon)	9	0.649	0.351
	G♂H♀	AGra×(H:Bel×Cac)	1	0.869	0.131	SGra×(H:Lou×Lan)	0	NA	NA
	G♂H♀					SGra×(H:Lax×Mon)	1	0.138	0.862
	H♂E♀	(H:Bel×Cac)×AEle	5	0.000	1.000	(H:Lou×Cen)×SEle	0	NA	NA
	H♂E♀					(H:Lou×Lan)×SEle	3	0.300	0.700
	H♂E♀					(H:Lax×Mon)×SEle	2	0.492	0.508
	H♂G♀	(H:Bel×Cac)×AGra	6	0.000	1.000	(H:Lou×Cen)×SGra	2	0.657	0.343
	H♂G♀					(H:Lou×Lan)×SGra	1	0.268	0.732
	H♂G♀					(H:Lax×Mon)×SGra	4	0.072	0.928
	H♂H♀	(H:Bel×Cac)×(H:Bel×Cac)	24	0.000	1.000	(H:Lou×Cen)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Cen)×(H:Lou×Lan)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Cen)	0	NA	NA
	H♂H♀					(H:Lou×Lan)×(H:Lou×Lan)	9	0.669	0.331
H♂H♀					(H:Lou×Mon)×(H:Lou×Mon)	17	0.199	0.801	
		Total	125			Total	191		

†Maximum average fecundity values for conspecific allopatric crosses were used as conspecific correction for the estimation of the fecundity barrier reproductive isolation.

Table S3. GLM model comparisons per prezygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Models are sorted by increasing values of the AICc. “+” signs on each parameter shows the inclusion of each parameter in each model. Cross = Types of crosses; Ecology = Sympatry vs Allopatry; Geography = Intrapopulation vs Interpopulation crosses; Cross:Ecology = Interaction between crosses and ecology; df = degrees freedom; logLik = log-likelihood. The model with the lowest scoring AICc per barrier was selected as the best model.

Model	Intercept	Cross	Ecology	Geography	Cross:Ecology	df	logLik	AICc	delta	weight
<i>Premating I – Mechanical barrier (Binomial distribution)</i>										
12	2.944	+	+		+	8	-140.233	296.9	0	0.676
16	17.57	+	+	+	+	9	-139.937	298.4	1.52	0.316
4	2.52	+	+			5	-148.278	306.7	9.83	0.005
8	16.57	+	+	+		6	-147.876	308	11.1	0.003
2	1.723	+				4	-153.317	314.8	17.85	0
6	15.57	+		+		5	-152.459	315.1	18.19	0
7	1.645		+	+		3	-174.481	355	58.13	0
3	1.937		+			2	-183.104	370.2	73.34	0
5	0.7438			+		2	-184.627	373.3	76.38	0
1	1.04					1	-193.517	389	92.14	0
<i>Premating II – Mechanical-tactile barrier (Binomial distribution)</i>										
3	1.781		+			2	-120.511	245.1	0	0.299
1	1.432					1	-122.064	246.1	1.07	0.175
7	1.675		+	+		3	-120.038	246.2	1.1	0.172
5	1.326			+		2	-121.618	247.3	2.21	0.099
6	16.57	+		+		5	-118.907	248.1	2.99	0.067
4	1.754	+	+			5	-118.987	248.2	3.15	0.062
2	1.409	+				4	-120.059	248.3	3.21	0.06
8	16.57	+	+	+		6	-118.136	248.6	3.55	0.051
12	2.14	+	+		+	8	-117.774	252.1	7.08	0.009
16	16.57	+	+	+	+	9	-117.123	253	7.93	0.006

Model	Intercept	Cross	Ecology	Geography	Cross:Ecology	df	logLik	AICc	delta	weight
<i>Postmating I – Oviposition (Binomial distribution)</i>										
8	17.57	+	+	+		6	-76.568	165.5	0	0.32
4	1.483	+	+			5	-77.661	165.6	0.08	0.308
12	2.048	+	+		+	8	-75.024	166.7	1.18	0.177
16	18.57	+	+	+	+	9	-74.366	167.5	2.03	0.116
2	1.962	+				4	-81.312	170.8	5.29	0.023
6	17.57	+		+		5	-80.629	171.5	6.01	0.016
7	1.257		+	+		3	-82.708	171.5	6.01	0.016
3	1.597		+			2	-83.932	171.9	6.41	0.013
1	1.996					1	-85.701	173.4	7.91	0.006
5	1.792			+		2	-84.992	174	8.53	0.005
<i>Postmating II – Fecundity (Poisson distribution)</i>										
16	5.592	+	+	+	+	9	-7780.176	15579.3	0	0.586
12	5.635	+	+		+	8	-7781.616	15580	0.7	0.414
8	5.592	+	+	+		6	-9300.452	18613.3	3034.06	0
4	5.456	+	+			5	-9314.166	18638.6	3059.37	0
6	5.592	+		+		5	-9831.48	19673.3	4094	0
2	5.246	+				4	-9910.267	19828.7	4249.47	0
7	4.912		+	+		3	-10138.447	20283	4703.75	0
3	5.217		+			2	-10782.699	21569.5	5990.2	0
5	4.553			+		2	-11074.958	22154	6574.72	0
1	4.87					1	-12160.64	24323.3	8744.04	0
<i>Postmating III – Fertility (Binomial distribution)</i>										
16	1.785	+	+	+	+	9	-10261.8	20542.6	0	1
12	1.422	+	+		+	8	-10308.74	20634.3	91.68	0
8	1.785	+	+	+		6	-10910.35	21833.2	1290.57	0

Model	Intercept	Cross	Ecology	Geography	Cross:Ecology	df	logLik	AICc	delta	weight
4	1.288	+	+			5	-10997.73	22005.8	1463.2	0
6	1.785	+		+		5	-11169.1	22348.5	1805.94	0
2	1.466	+				4	-11201.49	22411.2	1868.6	0
7	0.9834		+	+		3	-11342.68	22691.5	2148.9	0
5	1.092			+		2	-11369.85	22743.8	2201.17	0
1	1.308					1	-11594.82	23191.7	2649.07	0
3	1.321		+			2	-11593.89	23191.8	2649.25	0

Table S4. *Post hoc* GLM modeling for reproductive isolation as a function of types of crosses (RI ~ Cross) per prezygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Mechanical-tactile barrier was excluded as crosses were not a significant parameter in its GLM modeling (Table S3). GLMs were modeled using each cross direction as model intercept to allow pairwise comparisons between types of crosses. S.E. = Standard error; * = Significant p value for differences between a cross and the model intercept ($p < 0.05/6$).

Barrier	Cross	Estimate	S.E.	z value	p	p<0.05/6
Mechanical	Intercept: elegans♂×graellsii♀					
	Intercept	1.4224	0.1911	7.45E+00	9.71E-14	NA
	elegans♂×elegans♀	0.3004	0.3929	0.765	0.4446	
	graellsii♂×graellsii♀	2.1611	1.0316	2.095	0.0362	
	graellsii♂×elegans♀	-2.411	0.3497	-6.90E+00	5.37E-12	*
	Intercept: graellsii♂×graellsii♀					
	Intercept	3.584	1.014	3.535	0.000408	NA
	elegans♂×graellsii♀	-2.161	1.032	-2.095	0.036184	
	elegans♂×elegans♀	-1.861	1.07	-1.738	0.082128	
	graellsii♂×elegans♀	-4.572	1.055	-4.33E+00	1.47E-05	*
	Intercept: elegans♂×elegans♀					
	Intercept	1.7228	0.3433	5.02E+00	5.22E-07	NA
	graellsii♂×graellsii♀	1.8608	1.0703	1.738	0.0821	
	elegans♂×graellsii♀	-0.3004	0.3929	-0.765	0.4446	
	graellsii♂×elegans♀	-2.7114	0.4512	-6.01E+00	1.87E-09	*
	Intercept: graellsii♂×elegans♀					
	Intercept	-0.9886	0.2928	-3.376	0.000736	NA
	elegans♂×elegans♀	2.7114	0.4512	6.01E+00	1.87E-09	*
	graellsii♂×graellsii♀	4.5721	1.0552	4.33E+00	1.47E-05	*
	elegans♂×graellsii♀	2.411	0.3497	6.90E+00	5.37E-12	*
Oviposition	Intercept: elegans♂×graellsii♀					
	Intercept	1.6529	0.2573	6.425	1.32E-10	NA
	elegans♂×elegans♀	0.3087	0.4392	0.703	0.4821	
	graellsii♂×graellsii♀	2.0106	1.0449	1.924	0.0543	
	graellsii♂×elegans♀	15.9131	1318.7268	0.012	0.9904	
	Intercept: graellsii♂×graellsii♀					
	Intercept	3.664	1.013	3.617	0.000297	NA
	elegans♂×graellsii♀	-2.011	1.045	-1.924	0.054327	
	elegans♂×elegans♀	-1.702	1.073	-1.585	0.112877	
	graellsii♂×elegans♀	13.903	1318.727	0.011	0.991589	
	Intercept: elegans♂×elegans♀					
	Intercept	1.9617	0.356	5.51	3.58E-08	NA
	graellsii♂×graellsii♀	1.7019	1.0735	1.585	0.113	
	elegans♂×graellsii♀	-0.3087	0.4392	-0.703	0.482	
graellsii♂×elegans♀	15.6044	1318.7268	0.012	0.991		

Barrier	Cross	Estimate	S.E.	z value	p	p<0.05/6
	Intercept: graellsii♂×elegans♀					
	Intercept	17.57	1318.73	0.013	0.989	
	elegans♂×elegans♀	-15.6	1318.73	-0.012	0.991	
	graellsii♂×graellsii♀	-13.9	1318.73	-0.011	0.992	
	elegans♂×graellsii♀	-15.91	1318.73	-0.012	0.99	
Fecundity	Intercept: elegans♂×graellsii♀					
	Intercept	4.32343	0.01187	364.1	<2E-16	NA
	elegans♂×elegans♀	0.92248	0.01486	62.08	<2E-16	*
	graellsii♂×graellsii♀	0.65578	0.01782	36.81	<2E-16	*
	graellsii♂×elegans♀	0.98539	0.02628	37.49	<2E-16	*
	Intercept: graellsii♂×graellsii♀					
	Intercept	4.97921	0.01328	374.9	<2E-16	NA
	elegans♂×graellsii♀	-0.65578	0.01782	-36.81	<2E-16	*
	elegans♂×elegans♀	0.2667	0.01601	16.66	<2E-16	*
	graellsii♂×elegans♀	0.32961	0.02695	12.23	<2E-16	*
	Intercept: elegans♂×elegans♀					
	Intercept	5.245907	0.008935	587.12	<2E-16	NA
	graellsii♂×graellsii♀	-0.266701	0.016007	-16.661	<2E-16	*
	elegans♂×graellsii♀	-0.922479	0.01486	-62.076	<2E-16	*
	graellsii♂×elegans♀	0.062911	0.025092	2.507	0.0122	
	Intercept: graellsii♂×elegans♀					
	Intercept	5.30882	0.02345	226.42	<2E-16	NA
	elegans♂×elegans♀	-0.06291	0.02509	-2.507	0.0122	
	graellsii♂×graellsii♀	-0.32961	0.02695	-12.232	<2E-16	*
	elegans♂×graellsii♀	-0.98539	0.02628	-37.493	<2E-16	*
Fertility	Intercept: elegans♂×graellsii♀					
	Intercept	0.98859	0.01645	60.082	<2E-16	NA
	elegans♂×elegans♀	0.47708	0.02348	20.316	<2E-16	*
	graellsii♂×graellsii♀	0.67899	0.02833	23.967	<2E-16	*
	graellsii♂×elegans♀	0.04076	0.03572	1.141	0.254	
	Intercept: graellsii♂×graellsii♀					
	Intercept	1.66758	0.02306	72.309	<2E-16	NA
	elegans♂×graellsii♀	-0.67899	0.02833	-23.967	<2E-16	*
	elegans♂×elegans♀	-0.20191	0.02851	-7.083	1.41E-12	*
	graellsii♂×elegans♀	-0.63823	0.03921	-16.278	<2E-16	*
	Intercept: elegans♂×elegans♀					
	Intercept	1.46567	0.01675	87.479	<2E-16	NA
	graellsii♂×graellsii♀	0.20191	0.02851	7.083	1.41E-12	*
	elegans♂×graellsii♀	-0.47708	0.02348	-20.316	<2E-16	*
graellsii♂×elegans♀	-0.43632	0.03586	-12.167	<2E-16	*	

Barrier	Cross	Estimate	S.E.	z value	p	p<0.05/6
	Intercept: graellsii♂×elegans♀					
	Intercept	1.02935	0.03171	32.464	<2E-16	NA
	elegans♂×elegans♀	0.43632	0.03586	12.167	<2E-16	*
	graellsii♂×graellsii♀	0.63823	0.03921	16.278	<2E-16	*
	elegans♂×graellsii♀	-0.04076	0.03572	-1.141	0.254	

Table S5. *Post hoc* GLM modeling for reproductive isolation as a function of the interaction ecology and types of crosses (RI ~ Ecology:Cross) per prezygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Mechanical-tactile and oviposition barriers were excluded as ecology and cross interaction were not significant parameters in its GLM modeling (Table S3). Although each cross in each ecology was compared with each other combination, here we report only results for differences between ecologies within each type of cross. * = Significant p value for differences between the allopatric and sympatric ecology (p<0.05/4).

Barrier	Cross	Estimate	Std. Error	z value	p	p<0.05/4
Mechanical	elegans♂×graellsii♀	0.2477	0.3976	0.623	0.5332	
	graellsii♂×graellsii♀	-13.39	692.69	-0.019	0.985	
	elegans♂×elegans♀	1.5308	1.0912	1.403	0.160683	
	graellsii♂×elegans♀	3.5313	0.8894	3.97	7.18E-05	*
Fecundity	elegans♂×graellsii♀	-1.42837	0.05111	-27.945	<2.00E-16	*
	graellsii♂×graellsii♀	0.5644	0.02947	19.152	<2.00E-16	*
	elegans♂×elegans♀	0.93497	0.01937	48.271	<2.00E-16	*
	graellsii♂×elegans♀	-0.5667	0.05651	-10.028	<2.00E-16	*
Fertility	elegans♂×graellsii♀	-6.18228	0.50183	-12.319	<2.00E-16	*
	graellsii♂×graellsii♀	-0.78251	0.08106	-9.654	<2.00E-16	*
	elegans♂×elegans♀	-0.12752	0.03562	-3.58	0.000343	*
	graellsii♂×elegans♀	0.07011	0.07545	0.929	0.35275	

Table S6. GLM models comparison per postzygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Models are sorted by increasing values of the AICc. “+” signs on each parameter shows the inclusion of each parameter in each model. Cross = Types of crosses; Ecology = Sympatry vs Allopatry; Cross:Ecology = Interaction between crosses and ecology; df = degrees freedom; logLik = log-likelihood. The model with the lowest scoring AICc per barrier was selected as the best model.

Model	Intercept	Cross	Ecology	Cross:Ecology	df	logLik	AICc	delta	weight
<i>Premating I – Mechanical barrier (Binomial distribution)</i>									
8	18.57	+	+	+	10	-79.845	181.1	0	0.566
4	3.991	+	+		6	-84.545	181.6	0.53	0.434
3	2.262		+		2	-96.419	196.9	15.84	0
2	2.015	+			5	-96.243	202.9	21.78	0
1	0.7376				1	-107.015	216.1	34.98	0
<i>Premating II – Mechanical-tactile barrier (Binomial distribution)</i>									
3	3.135		+		2	-43.943	92	0	0.514
4	4.161	+	+		6	-40.18	93.1	1.14	0.29
8	1.792	+	+	+	10	-36.036	94.2	2.19	0.171
1	1.752				1	-48.176	98.4	6.39	0.021
2	2.197	+			5	-45.63	101.8	9.82	0.004
<i>Postmating I – Oviposition (Binomial distribution)</i>									
3	1.431		+		2	-48.435	101	0	0.61
1	1.867				1	-49.994	102	1.05	0.36
4	1.605	+	+		6	-47.796	108.3	7.32	0.016
2	2.169	+			5	-49.078	108.7	7.69	0.013
8	2.303	+	+	+	10	-45.653	113.2	12.23	0.001
<i>Postmating II – Fecundity (Poisson distribution)</i>									
8	4.25	+	+	+	10	-3892.949	7808	0	1
4	3.589	+	+		6	-4453.062	8918.9	1110.89	0
3	3.3		+		2	-4656.369	9316.8	1508.83	0

Model	Intercept	Cross	Ecology	Cross:Ecology	df	logLik	AICc	delta	weight
2	4.63	+			5	-5508.288	11027.1	3219.11	0
1	4.278				1	-5888.144	11778.3	3970.31	0
<i>Postmating III – Fertility (Binomial distribution)</i>									
8	-2.372	+	+	+	10	-2140.936	4304.7	0	1
4	-2.211	+	+		6	-2582.392	5177.8	873.14	0
3	-1.501		+		2	-3113.13	6230.4	1925.74	0
2	0.1178	+			5	-4321.67	8654.1	4349.4	0
1	0.2091				1	-4392.622	8787.3	4482.63	0

Table S7. *Post hoc* GLM modeling for reproductive isolation as a function of types of crosses (RI ~ Cross) per postzygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Mechanical-tactile and oviposition barriers were excluded as crosses were not significant parameters in their GLM modeling (Table S6). GLMs were modeled using each cross direction as model intercept to allow pairwise comparisons between types of crosses. S.E. = Standard error; * = Significant p value for differences between a cross and the model intercept ($p < 0.05/10$).

Barrier	Cross	Estimate	S.E.	z value	p	$p < 0.05/10$
Mechanical	Intercept: hybrid ♂ × hybrid ♀					
	Intercept	0.4855	0.2594	1.871	0.0613	NA
	elegans ♂ × hybrid ♀	1.5294	0.5921	2.583	0.0098	
	graellsii ♂ × hybrid ♀	-0.7087	0.7192	-0.985	0.3245	
	hybrid ♂ × elegans ♀	-0.5427	0.4262	-1.273	0.203	
	hybrid ♂ × graellsii ♀	1.3471	0.5978	2.254	0.0242	
	Intercept: elegans ♂ × hybrid ♀					
	Intercept	2.0149	0.5323	3.785	0.000153	NA
	hybrid ♂ × hybrid ♀	-1.5294	0.5921	-2.583	0.0098	
	graellsii ♂ × hybrid ♀	-2.238	0.8563	-2.613	0.008962	
	hybrid ♂ × elegans ♀	-2.0721	0.6306	-3.286	0.001017	*
	hybrid ♂ × graellsii ♀	-0.1823	0.7572	-0.241	0.809719	
	Intercept: hybrid ♂ × graellsii ♀					
	Intercept	1.8326	0.5385	3.403	0.000666	NA
	elegans ♂ × hybrid ♀	0.1823	0.7572	0.241	0.809719	
	hybrid ♂ × hybrid ♀	-1.3471	0.5978	-2.254	0.024223	
	graellsii ♂ × hybrid ♀	-2.0557	0.8602	-2.39	0.016861	
	hybrid ♂ × elegans ♀	-1.8897	0.6359	-2.972	0.002961	*
	Intercept: hybrid ♂ × elegans ♀					
	Intercept	-0.05716	0.3382	-0.169	0.86579	NA
	hybrid ♂ × graellsii ♀	1.88974	0.63591	2.972	0.00296	*
	elegans ♂ × hybrid ♀	2.07206	0.63064	3.286	0.00102	*
	hybrid ♂ × hybrid ♀	0.54267	0.42625	1.273	0.20297	
	graellsii ♂ × hybrid ♀	-0.16599	0.75125	-0.221	0.82514	
	Intercept: graellsii ♂ × hybrid ♀					
	Intercept	-0.2231	0.6708	-0.333	0.7394	NA
	hybrid ♂ × elegans ♀	0.166	0.7513	0.221	0.82514	
	hybrid ♂ × graellsii ♀	2.0557	0.8602	2.39	0.01686	
	elegans ♂ × hybrid ♀	2.238	0.8563	2.613	0.00896	
	hybrid ♂ × hybrid ♀	0.7087	0.7192	0.985	0.32449	
Fecundity	Intercept: elegans ♂ × hybrid ♀					
	Intercept	4.63026	0.01623	285.203	<2E-16	NA
	graellsii ♂ × hybrid ♀	0.04257	0.07026	0.606	0.545	
	hybrid ♂ × hybrid ♀	-0.62293	0.02463	-25.292	<2E-16	*
	hybrid ♂ × elegans ♀	-0.70236	0.04724	-14.866	<2E-16	*

Barrier	Cross	Estimate	S.E.	z value	p	p<0.05/10	
	hybrid♂×graellsii♀	-0.43363	0.0377	-11.503	<2E-16	*	
	Intercept: hybrid♂×hybrid♀						
	Intercept	4.00733	0.01852	216.359	<2E-16	NA	
	elegans♂×hybrid♀	0.62293	0.02463	25.292	<2E-16	*	
	graellsii♂×hybrid♀	0.6655	0.07082	9.397	<2E-16	*	
	hybrid♂×elegans♀	-0.07944	0.04808	-1.652	0.0985	.	
	hybrid♂×graellsii♀	0.18929	0.03874	4.887	1.03E-06	*	
	Intercept: hybrid♂×graellsii♀						
	Intercept	4.19662	0.03402	123.355	<2E-16	NA	
	hybrid♂×hybrid♀	-0.18929	0.03874	-4.887	1.03E-06	*	
	elegans♂×hybrid♀	0.43363	0.0377	11.503	<2E-16	*	
	graellsii♂×hybrid♀	0.47621	0.07636	6.237	4.47E-10	*	
	hybrid♂×elegans♀	-0.26873	0.05591	-4.806	1.54E-06	*	
	Intercept: hybrid♂×elegans♀						
	Intercept	3.9279	0.04437	88.53	<2E-16	NA	
	hybrid♂×graellsii♀	0.26873	0.05591	4.806	1.54E-06	*	
	hybrid♂×hybrid♀	0.07944	0.04808	1.652	0.0985		
	elegans♂×hybrid♀	0.70236	0.04724	14.866	<2E-16	*	
	graellsii♂×hybrid♀	0.74493	0.08149	9.141	<2E-16	*	
	Intercept: graellsii♂×hybrid♀						
	Intercept	4.67283	0.06836	68.358	<2E-16	NA	
	hybrid♂×elegans♀	-0.74493	0.08149	-9.141	<2E-16	*	
	hybrid♂×graellsii♀	-0.47621	0.07636	-6.237	4.47E-10	*	
	hybrid♂×hybrid♀	-0.6655	0.07082	-9.397	<2E-16	*	
	elegans♂×hybrid♀	-0.04257	0.07026	-0.606	0.545		
	Fertility	Intercept: hybrid♂×hybrid♀					
		Intercept	0.16714	0.0267	6.259	3.87E-10	NA
		elegans♂×hybrid♀	-0.04939	0.03491	-1.415	0.157	
		graellsii♂×hybrid♀	0.86205	0.09351	9.219	<2E-16	*
		hybrid♂×elegans♀	0.32044	0.05865	5.464	4.66E-08	*
hybrid♂×graellsii♀		0.08488	0.05952	1.426	0.154		
Intercept: elegans♂×hybrid♀							
Intercept		0.11775	0.02248	5.238	1.63E-07	NA	
hybrid♂×hybrid♀		0.04939	0.03491	1.415	0.1571		
graellsii♂×hybrid♀		0.91144	0.09239	9.865	<2E-16	*	
hybrid♂×elegans♀		0.36983	0.05685	6.505	7.75E-11	*	
hybrid♂×graellsii♀		0.13427	0.05775	2.325	0.0201		
Intercept: hybrid♂×elegans♀							
Intercept		0.48758	0.05222	9.338	<2E-16	NA	
elegans♂×hybrid♀		-0.36983	0.05685	-6.505	7.75E-11	*	

Barrier	Cross	Estimate	S.E.	z value	p	p<0.05/10	
	hybrid♂×hybrid♀	-0.32044	0.05865	-5.464	4.66E-08	*	
	graellsii♂×hybrid♀	0.54161	0.10372	5.222	1.77E-07	*	
	hybrid♂×graellsii♀	-0.23556	0.07454	-3.16	0.00158	*	
	Intercept: graellsii♂×hybrid♀						
	Intercept	1.0292	0.08962	11.484	<2E-16	NA	
	hybrid♂×elegans♀	-0.54161	0.10372	-5.222	1.77E-07	*	
	elegans♂×hybrid♀	-0.91144	0.09239	-9.865	<2E-16	*	
	hybrid♂×hybrid♀	-0.86205	0.09351	-9.219	<2E-16	*	
	hybrid♂×graellsii♀	-0.77717	0.10422	-7.457	8.84E-14	*	
	Intercept: hybrid♂×graellsii♀						
	Intercept	0.25202	0.0532	4.737	2.16E-06	NA	
	graellsii♂×hybrid♀	0.77717	0.10422	7.457	8.84E-14	*	
	hybrid♂×elegans♀	0.23556	0.07454	3.16	0.00158	*	
	elegans♂×hybrid♀	-0.13427	0.05775	-2.325	0.02008		
	hybrid♂×hybrid♀	-0.08488	0.05952	-1.426	0.15388		

Table S8. *Post hoc* GLM modeling for reproductive isolation as a function of the interaction ecology and types of crosses (RI ~ Ecology:Cross) per postzygotic reproductive barrier in *Ischnura graellsii* and *I. elegans*. Mechanical-tactile and oviposition barriers were excluded as ecology and cross interaction were not significant parameters in its GLM modeling (Table S6). Although each cross in each ecology was compared with each other combination, here we report only results for differences between ecologies within each type of cross. * = Significant p value for differences between the allopatric and sympatric ecology (p<0.05/5).

Barrier	Cross	Estimate	Std. Error	z value	p	p<0.05/5
Mechanical	hybrid♂×hybrid♀	18.6996	1537.4007	0.012	0.9903	
	elegans♂×hybrid♀	16.81687	2465.32572	0.007	0.994557	
	hybrid♂×graellsii♀	-0.08004	1.07715	-0.074	0.94076	
	hybrid♂×elegans♀	1.7272	0.7976	2.166	0.030341	
	graellsii♂×hybrid♀	19.4824	4612.2021	0.004	0.99663	
Fecundity	elegans♂×hybrid♀	-0.49114	0.04183	-11.741	<2e-16	*
	hybrid♂×hybrid♀	-2.13006	0.06487	-32.835	<2e-16	*
	hybrid♂×graellsii♀	-2.40932	0.0708	-34.032	<2e-16	*
	hybrid♂×elegans♀	-1.49568	0.11473	-13.037	<2e-16	*
	graellsii♂×hybrid♀	1.5012	0.1771	8.478	<2e-16	*
Fertility	hybrid♂×hybrid♀	-5.67083	0.57957	-9.785	<2e-16	*
	elegans♂×hybrid♀	-3.16696	0.0831	-38.109	<2e-16	*
	hybrid♂×elegans♀	-5.62044	0.5841	-9.622	<2e-16	*
	graellsii♂×hybrid♀	3.7232	0.2986	12.467	<2e-16	*
	hybrid♂×graellsii♀	-4.2015	1.01183	-4.152	3.29E-05	*

Table S9. GLM models comparison per prezygotic reproductive barrier comparing the two reciprocal heterospecific crosses (crosses between *Ischnura elegans* males and *I. graellsii* females vs crosses between *I. graellsii* males and *I. elegans* females). Models are sorted per reproductive barrier by increasing values of the AICc. “+” sign on the cross parameter show the inclusion of heterospecific crosses as a parameter explaining RI. df = degrees freedom; logLik = log-likelihood. If the model including the cross parameter had the lowest AICc value, we concluded significant prezygotic asymmetries were present on that barrier.

Barrier	Model	Intercept	Cross	df	logLik	AICc	delta	weight
All allopatric data								
Mechanical	1	1.58		1	-37.478	77	0	0.741
	2	1.576	+	2	-37.477	79.1	2.1	0.259
Mechanical-Tactile	1	1.442		1	-33.179	68.4	0	0.669
	2	1.344	+	2	-32.82	69.8	1.41	0.331
Oviposition	2	0.5465	+	2	-19.715	43.8	0	0.789
	1	0.821		1	-22.158	46.4	2.64	0.211
Fecundity	2	3.062	+	2	-462.131	928.8	0	1
	1	4.289		1	-1629.01	3260.2	2331.39	0
Fertility	2	-5.061	+	2	-875.601	1755.7	0	1
	1	0.5421		1	-1575.386	3152.9	1397.2	0
All sympatric data								
Mechanical	2	1.328	+	2	-71.849	147.8	0	1
	1	0.3455		1	-103.124	208.3	60.5	0
Mechanical-Tactile	1	1.175		1	-48.627	99.3	0	0.709
	2	1.214	+	2	-48.47	101.1	1.78	0.291
Oviposition	1	2.411		1	-24.181	50.4	0	0.687
	2	2.372	+	2	-23.918	52	1.57	0.313
Fecundity	2	4.49	+	2	-3243.226	6490.6	0	1
	1	4.509		1	-3271.21	6544.5	53.86	0
Fertility	1	1.119		1	-2830.357	5662.8	0	0.711
	2	1.121	+	2	-2830.204	5664.6	1.8	0.289
Allopatry: Cachadas×Belgium vs Belgium×Cachadas								
Mechanical	1	1.459		1	-25.668	53.4	0	0.746
	2	1.447	+	2	-25.666	55.6	2.16	0.254
Mechanical-Tactile	1	1.194		1	-23.321	48.7	0	0.639
	2	1.022	+	2	-22.789	49.9	1.14	0.361
Oviposition	1	1.466		1	-7.721	17.7	0	0.511
	2	0.9808	+	2	-6.445	17.8	0.09	0.489
Fecundity	2	2.931	+	2	-82.398	170	0	1
	1	4.679		1	-872.914	1748.2	1578.2	0
Fertility	2	-5.056	+	2	-374.343	753.9	0	1

Barrier	Model	Intercept	Cross	df	logLik	AICc	delta	weight
	1	1.122		1	-851.08	1704.5	950.64	0
Sympatry: Corrubedo×Louro vs Louro×Corrubedo								
Mechanical	1	1.386		1	-2.502	8.3	0	0.956
	2	18.57	+	2	-2.249	14.5	6.16	0.044
Mechanical-Tactile	1	23.57		1	0	4	0	0.998
	2	23.57	+	2	0	16	12	0.002
Oviposition	1	24.57		1	0	3.3	0	0.966
	2	24.57	+	2	0	10	6.67	0.034
Fecundity	2	4.431	+	2	-124.964	259.9	0	1
	1	4.736		1	-138.639	280.6	20.68	0
Fertility	1	1.168		1	-47.679	98.7	0	0.615
	2	1.389	+	2	-44.814	99.6	0.94	0.385
Sympatry: Lanzada×Louro vs Louro×Lanzada								
Mechanical	2	19.57	+	2	-4.157	12.7	0	1
	1	-1.056		1	-17.702	37.5	24.8	0
Mechanical-Tactile	1	0.5108		1	-5.293	13.3	0	0.682
	2	0.9163	+	2	-4.188	14.8	1.52	0.318
Sympatry: Cachadas×Laxe vs Laxe×Cachadas								
Mechanical	2	1.347	+	2	-38.465	81.1	0	1
	1	0.55		1	-53.85	109.8	28.67	0
Mechanical-Tactile	1	1.316		1	-26.831	55.7	0	0.659
	2	1.386	+	2	-26.406	57.1	1.31	0.341

Text S1. Supplementary Methods: Estimation of the absolute strength of the reproductive barriers between *Ischnura graellsii* and *I. elegans*

Mechanical and mechanical-tactile barriers measure the incompatibility between the males' caudal appendages and the females' prothorax, the failure in the stimulation by the male to the female in the tandem position, and the incompatibility between the males' and females' genital structures.

We estimated the first premating barrier (Premating I – mechanical barrier) as:

$$RI_{mechanical} = 1 - \frac{\text{number of successful tandems}}{\text{number of tandem attempts}} \quad (1)$$

in which we defined a tandem attempt when a male flew towards a female and curled his abdomen to try to grab her with his caudal appendages. If a male tried several times to grab a specific female (either on the same day or on multiple experimental days), we only counted this interaction as a single tandem attempt. By doing this, the sample size shows the number of male-female pairs in which at least one tandem was attempted. If, in at least one of these tandem attempts, the male correctly grabbed the female and the couple remained together in tandem position (Fig. 2A), a successful tandem was recorded; i.e., multiple tandems made by the same male-female pair were recorded as a single successful event.

We estimated the second premating barrier (prematuring II – mechanical-tactile barrier) as:

$$RI_{mechanical-tactile} = 1 - \frac{\text{number of successful copulations}}{\text{number of successful tandems}} \quad (2)$$

in which a successful copulation was recorded if a female in the tandem position bent her abdomen and placed it in contact with male genitalia (Fig. 2B). Male-female pairs that formed the mating position, reverted to a tandem (or free themselves completely) and then formed a second mating position were considered as a single successful copulation event; i.e., the number of successful copulations shows the number of male-female pairs that achieved at least one successful mating position. Pairs that achieved this position were carefully observed and isolated on individual jars. To avoid additional copulations of females, we considered as “mated” each female that achieved this position without regarding the length pairs remained in copula or the number of copulas they formed.

The first two postmating barriers measure: 1) postmating I – oviposition, how the heterospecific ejaculate fails to stimulate female oviposition (number of females that laid eggs; Fig. 2C); and 2) postmating II – fecundity, how the heterospecific ejaculate reduces the frequency of oviposition [number of laid eggs by female (Coyne and Orr 2004); Fig. 2D]. The third postmating barrier (postmating III – fertility) measures several processes: poor transfer or storage sperm, unviability of gametes in the foreign reproductive tract, poor movement or cross-attraction, or failure of fertilization when gametes contact each other [(Coyne and Orr 2004); Fig. 2D]. We measured postmating barriers II and III using the first three clutches.

We estimated the first postmating barrier (postmating I – oviposition) as:

$$RI_{oviposition} = 1 - \frac{\text{number of mated females that laid eggs}}{\text{number of total mated females}} \quad (3)$$

For the second postmating barrier (postmating II – fecundity), first for each mated female we measured the mean number of eggs they laid per clutch in the first three clutches. We excluded females that did not lay eggs (oviposition barrier) and, if females survived less than the first three oviposition days, we averaged the number of eggs they laid on the days they lived (i.e. in one or two clutches). We refer to this value as the eggs per clutch index. Then, we averaged this number for all females of the same type of cross per population (i.e. the population column in Table S2), and used a mathematical correction to estimate a RI strength value in a range from 0 to 1, as we had with the other barriers:

$$RI_{fecundity} = 1 - \frac{2 * \frac{\sum_{i=1}^n \text{eggs per clutch index}}{n}}{340.6 + 224.2} \quad (4)$$

in which n refers to the number of laying females for each type of cross per cross of populations. The 340.6 and 224.4 values on the denominator of equation 4 refers to the maximum average eggs per clutch index seen in allopatric conspecific crosses. While the former refers to the average fecundity of *I. elegans* allopatric crosses in Arles, the latter refers to the average fecundity of *I. graellsii* allopatric crosses in Alba (Table S2). By using the same conspecific values in all fecundity RI estimations, our results reflected only the changes in heterospecific eggs per clutch indices. When the average eggs per clutch index of a population cross was higher than the average of the conspecific corrections, and thus a negative value of RI was estimated, we rounded up the RI value to zero.

Finally, we estimated the third postmating barrier (postmating III – fertility) as:

$$RI_{fertility} = 1 - \frac{\sum_{i=1}^n \frac{\text{number of fertile eggs}}{\text{total laid eggs}}}{n} \quad (5)$$

in which we identified fertile eggs as those having evidence of hatching or of a developing embryo, and n refers to the number of laying females per type of cross per population.

Text S2. Supplementary Methods: Testing reinforcement predictions

Strengthening of prezygotic barriers

Since Dobzhansky's earliest work in reinforcement (Dobzhansky 1937, 1940; Dobzhansky and Koller 1938), the classical test of reinforcement is done by contrasting the strength of prezygotic isolation in sympatry *versus* in allopatry (Coyne and Orr 1989, 1997, 2004). We expected stronger total prezygotic isolation in sympatry than in allopatry in *Ischnura graellsii* and *I. elegans*, and stronger absolute isolation in sympatry than in allopatry in the reproductive barrier under reinforcement.

Rarer female effect

Since usually females pay higher fitness costs of hybridization than males (Coyne and Orr 2004), and females of the rarer species have a higher chance of being involved in an heterospecific mating than females of the more common species, reinforcement is expected to strengthen prezygotic isolation faster in the cross direction involving females of the rarer species (Yukilevich 2012). Since *I. elegans* is the invader species in Spain, this species is less frequent in the sympatry zone than *I. graellsii* (Sánchez-Guillén et al. 2011). Thus, for this prediction we expected stronger prezygotic isolation in crosses between *I. graellsii* males and *I. elegans* females than the reciprocal cross in sympatry but not in allopatry. Additionally, since in a local-scale species frequencies vary between localities (Sánchez-Guillén et al. 2023), we expected stronger prezygotic isolation in sympatric crosses between Corrubedo (*I. graellsii* males) and Louro (*I. elegans* females), and Lanzada (*I. graellsii* males) and Louro (*I. elegans* females) than in crosses between Cachadas (*I. graellsii* males) and Laxe (*I. elegans* females). The reason for this is that while historically Louro has been an *I. graellsii*-dominant locality, Laxe has been an *I. elegans*-dominant locality (Table S1).

Concordant prezygotic and postzygotic isolation asymmetries

Unidirectionally inherited Bateson-Dobzhansky-Müller (BDM) incompatibilities associated with sex or cytoplasmic chromosomes cause postzygotic isolation to be asymmetric between reciprocal crosses (Turelli and Moyle 2007). Since under reinforcement, hybridization costs (postzygotic barriers) and prezygotic isolation are expected to be positively correlated (Ortiz-Barrientos et al. 2009), concordant prezygotic and postzygotic isolation asymmetries between reciprocal crosses are expected in sympatry but not in allopatry (Yukilevich 2012). For this prediction we expected that cross directions with stronger postzygotic isolation (highest hybridization costs) have also stronger total prezygotic isolation in sympatry but not in allopatry.

Greater premating asymmetries and weaker postzygotic isolation in sympatry than in allopatry

Species pairs with asymmetric postzygotic isolation in sympatry are expected to have higher premating asymmetries in sympatry than in allopatry under reinforcement (Turelli et al. 2014). Additionally, since gene flow operates only in sympatry, crosses from sympatry should also have weaker postzygotic isolation owing to the suppression of BDM incompatibilities (Turelli et al. 2014). For this prediction we expected statistically significant differences in premating isolation (mechanical or mechanical-tactile barriers) between reciprocal heterospecific crosses in sympatry but not in allopatry, and weaker postzygotic isolation in sympatry than in allopatry. To detect significant asymmetries in prezygotic isolation we compared the null model GLM to one that included RI as a function of the two heterospecific crosses per prezygotic barrier. If the model including the crosses variable scored a lower AICc value, then we considered this statistical support for difference between heterospecific crosses. These tests were made: i) pooling all sympatric and allopatric data, and ii) in specific population crosses in which we measured the two reciprocal directions.

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