Rapid reinforcement of reproductive isolation in a damselfly hybrid region

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Complete List of Authors:	Arce-Valdés, Luis ; INECOL, Evolutionary Biology Ballén-Guapacha, Andrea; INECOL, Evolutionary Biology Sánchez-Guillén, Rosa Ana; INECOL, Evolutionary Biology
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1 # Rapid reinforcement of reproductive isolation in a
2 # damselfly hybrid region
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4 **Luis Rodrigo Arce-Valdés[1]** https://orcid.org/0000-0001-6445-7534
5 **Andrea Viviana Ballén-Guapacha[1]** https://orcid.org/0000-0003-4299-8262
6 **Rosa Ana Sánchez-Guillén[1*]** https://orcid.org/0000-0001-6024-8321
7

8 [1]Instituto de Ecología A.C. (Red de Biología Evolutiva), Xalapa, Veracruz, México.
9 *Corresponding Address: Red de Biología Evolutiva, Instituto de Ecología A.C.
10 (INECOL). Xalapa, Veracruz, México, C.P. 91073: rosa.sanchez@inecol.mx

11

Abstract
12

13 Reinforcement is one of the most widely discussed mechanisms of speciation. When
14 reinforcement is operating, it produces a pattern of reproductive character displacement
15 in which stronger premating isolation develops in sympatric populations relative to
16 allopatric populations. This isolation is usually reinforced asymmetrically (between
17 reciprocal crosses), and this asymmetry is concordant between premating and postzygotic
18 barriers. Here, we investigated the potential role of reinforcement in the reproductive
19 isolation between two sympatric species of damselflies, *Ischnura elegans* and *I. graellsii*,
20 by quantifying five prezygotic (two mechanical and three gametic) reproductive barriers
21 in heterospecific crosses, and the same five barriers in backcrosses of *I. elegans* and *I.*
22 *graellsii* with F_1- and F_2-hybrids (postzygotic barriers). Then, we compared the strength
23 of these barriers with measurements from a previous experiment done approximately 40
24 generations ago (in 2000–2001). We detected greater premating isolation in the 2019–
25 2020 experiment compared to the 2000–2001 experiment. Moreover, premating barriers
26 were asymmetric, and this asymmetry was concordant in prezygotic and postzygotic
27 barriers, and more intense in *I. elegans* (the species with a smaller distribution) than in *I.*
28 *graellsii* females. Our results indicate that reinforcement plays a major role in the
29 evolution of mechanical isolation in *Ischnura*.
30

31 **Keywords:** concordant asymmetry, hybridisation, odonates, reproductive barriers,
   reproductive character displacement, speciation,

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Background

34   One central goal of modern evolutionary biology is to understand the processes that lead
35   to the origin and accumulation of reproductive barriers during speciation. Reinforcement
36   is one of the most widely discussed mechanisms of speciation. This phenomenon,
37   popularised by Dobzhansky [1,2], proposes one way in which natural selection can favour
38   a speciation event [1,3]. When formerly allopatric, closely related species come into
39   secondary contact, individuals with low heterospecific discrimination ability will
40   occasionally mate with heterospecifics and, in some cases, will form hybrids. If hybrids
41   are less viable than the parental species, natural selection will tend to eliminate them, thus
42   acting to avoid the excessive cost of hybridisation [4]. This gradually reduces and
43   eliminates alleles from the population due to individuals with low heterospecific
44   discrimination ability. Theory suggests that eventually, no hybridisation matings will be
45   present in sympatric populations, thus completing speciation [1]. Knowledge of
46   reinforcement has continuously been accumulated from both theoretical development
47   [1,3,5–9] and empirical evaluations [10–15].

48   When reinforcement operates, a pattern of reproductive character displacement (RCD)
49   arises, i.e., greater premating isolation in sympatric *versus* allopatric populations [9,13].
50   However, RCD in sympatry has alternative explanations in addition to reinforcement
51   [3,14]. For instance, the Templeton effect (differential fusion) explains the observed
52   pattern of higher isolation in sympatric populations than allopatric ones without appealing
53   to reinforcement; it proposes that sympatric populations persist only if interacting species
54   have already achieved strong prezygotic isolation in allopatry [16,17]. Moreover, RCD
55   patterns can also arise by species interference in sympatry, i.e., through selection for
56   divergence in mate recognition signals without hybridisation (noisy neighbours) or via
57   runaway sexual selection [3]. However, other specific predictions of RCD by
58   reinforcement can help distinguish between the aforementioned forces. Empirical
59   evidence has shown that under reinforcement, premating reproductive barriers will
60   increase in strength in the sympatric distribution in comparison to the same barriers
61   measured in the allopatric distribution [18–20]. Moreover, prezygotic and postzygotic
62   barriers, in sympatry, should evolve to be asymmetric (different intensity between
63   reciprocal crosses) in the same direction (concordant asymmetry), and more intense in
64   the reciprocal cross direction involving the female of the species with the smaller range
65   or population size (rarer-female effect) [14]. In recent decades, an increasing number of
66   empirical studies investigating reinforcement have tested concordant asymmetry in both
67   plants [21,22] and animals [14,23,24] as additional evidence of reinforcement
68   [14,21,22,24]. However, more studies on non-conventional taxa are still necessary.

69   Different approaches have been used to empirically evaluate the presence of
70   reinforcement. The traditional approach is *via* RCD through the comparison of phenotype
71   distribution of sexually related traits, by quantifying chemical, mechanical, visual,
72   auditory, or courtship-related behaviours between heterospecific organisms living in
73   sympatry *versus* in allopatry [11,25,26] (but see [18] for a case of reinforcement without
74   clear RCD). This method, however, lacks the ability to recognise reinforcement from the

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Templeton effect on its own. Motivated by this, other strategies have been implemented, such as the direct measurement of the strength of the reproductive isolation (RI) in heterospecific crosses between individuals living in sympatry and those living in the ancestral allopatric distributions [12,18,27]. No differences should be found if high RI had already developed in ancestral allopatric areas. Finally, other consequences of reinforcement, such as reductions in hybrid individual frequencies in sympatric populations [28,29], the presence of introgressed diagnostic alleles in sympatric zones where complete RI has been achieved [30], higher frequency of recently diverged sister taxa presenting RCD in sympatry than in allopatry [31], or concordant asymmetry [14], can be measured. Using these methods, reinforcement has been detected across several taxonomic groups, suggesting speciation via reinforcement to be widespread in both vertebrate [11,25,32–34] and invertebrate animals [10,13,35–40]. Continuous work has been developed on testing reinforcement in plant [18,41–43] and fungi species [44]. Because biological evolution is characterised by the change in inherited traits over successive generations, a fourth approach arises. If sufficient generations have passed and reinforcement has been operating, RI should increase between two sampling periods of sympatric heterospecific crosses.

Climate-induced range expansions can be seen as 'natural laboratories' to investigate reinforcement of RI between closely related species that have recently come into secondary contact after allopatric speciation. This is the case of damselflies *Ischnura elegans* and *I. graellsii*, which have recently formed a hybrid region in Spain (first records of *I. elegans* in Spain come from early 1900). The expansion of *I. elegans* in Spain has resulted in several secondary contact zones with varying proportions of both parental species and hybrids [45]. In the north-western Spanish hybrid zone, *I. elegans* was found for the first time in the early 1980s [46]. This hybrid zone is characterised by having introgressed populations of each parental species and hybrid populations in which most individuals display different degrees of introgression (unimodal distribution) (Sánchez-Guillén, under review). Theory predicts that when sympatric speciation occurs, disruptive selection (such as reinforcement) converts a unimodal distribution of genotypes to a bimodal one [47,48]. The frequency of genotypes in the north-western Spanish hybrid zone and the time since *I. elegans* colonisation suggests that this area could be in an early stage of the speciation process. This makes the north-western Spanish hybrid zone a candidate zone to investigate the presence of RI reinforcement using a long-term temporal approach.

Our specific goal was to investigate reinforcement of RI in a north-western Spanish hybrid zone by using a long-term temporal approach. To this end, we measured (experiment 2019–2020) the strength of five prezygotic reproductive barriers (two premating and three postmating) between *I. elegans* and *I. graellsii*, and five postzygotic barriers in two generations of backcrosses and hybrid crosses. By reanalysing previous data from a similar study that measured 19 pre- and postzygotic reproductive barriers between *I. elegans* and *I. graellsii* from the north-western Spanish hybrid zone done 20 years ago (experiment 2000–2001) [27,49], we statistically tested whether: i) the total

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117   strength of the five prezygotic reproductive barriers was higher in the 2019–2020
118   experiment compared to the 2000–2001 experiment (about 40 generations later); and ii)
119   which barriers were reinforced in this time frame. Finally, we evaluated the presence of
120   concordant isolation asymmetries between premating and postzygotic barriers as
121   additional evidence for reinforcement. This work enhances the evaluation of
122   reinforcement within Odonata, where to our knowledge, only a few previous studies have
123   been done [26,50].

Methods

124

Laboratory rearing experiments

125

126   During the 2000–2001 experiment, a total of 94 interactions (conspecific, heterospecific,
127   hybrid, and backcrosses) were done in the laboratory: first generation included 28
128   interactions and were done with last-instar larvae or teneral-adults of *I. elegans* from
129   Louro and *I. graellsii* from Corrubedo and Lanzada-complex (Lanzada; Fig. 1); the
130   second generation included 33 interactions; and the third generation included 33
131   interactions (details in Supplementary Table S1). During the 2019–2020 experiment, a
132   total of 202 interactions (conspecific, heterospecific, hybrid, and backcrosses) were
133   breed; the first generation included 114 interactions done with tenerals of *I. elegans* from
134   Laxe (Louro population became extinct in 2012 due to saltwater entry into the lagoon due
135   to a spring tide) and *I. graellsii* from Lanzada-complex (Cachadas and Montalvo; Fig. 1).
136   the second generation included 55 interactions, and the third generation included 33
137   interactions. Based on the introgression presented by *I. elegans* and *I. graellsii* from
138   northwest Spain, the reproductive success of allopatric *I. elegans* and *I. graellsii* was also
139   estimated. A total of 31 conspecific interactions of *I. elegans* from France, and of *I.
140   graellsii* from Alba (Fig. 1) were performed in the laboratory. Following a standardised
141   rearing protocol [51,52], larvae from both experiments (2000–2001 and 2019–2020) and
142   allopatry were reared to adulthood. When maturity was reached, mating experiments were
143   done following the protocol used by Sánchez-Guillén et al. [27]. Unfortunately, two
144   factors precluded the full comparison between rearing experiments: i) in 2000–2001
145   conspecifics premating barriers were not estimated based on the assumption that no
146   reproductive isolation was expected in conspecific matings; and ii) in 2019–2020, fungi
147   contamination of the eggs' substrate (filter paper) precluded the estimation of the
148   proportion of fertile eggs in all crosses from the second generation (F_1).

Absolute and relative strength of five sequential reproductive isolation barriers

149

150   During damselfly copulation, the "tandem position" is achieved when males successfully
151   grasp females by the prothorax using their caudal appendages [53]. Copulation begins
152   when the female accepts the male by bending her abdomen and placing the genitals in
153   contact. This position is usually referred to as the "wheel position" [54]. Once copulation
154   is achieved, males inseminate females, and their sperm is allocated to the female's bursa
155   and spermatheca. Immediately after mating, females lay eggs until the sperm is used or

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156   they mate again with a new male. We measured five sequential reproductive barriers in
157   both rearing experiments (2000–2001 and 2019–2020): two premating mechanical
158   barriers that prevented tandem and wheel positions and three post-mating barriers,
159   oviposition success, fecundity, and fertility. These barriers were measured across three
160   generations (F_0: heterospecific crosses between *I. graellsii* females and *I. elegans* males,
161   and vice versa; F_1: backcrosses between both species males and females with F_1 hybrids
162   from the opposite sex and crosses between F_1-hybrids; and F_2: backcrosses between both
163   species males and females with F_2-hybrids from the opposite sex and crosses between F_2-
164   hybrids; details in Table S1). Due to small sample sizes of hybrid families in the third
165   generation, we merged all individuals whose genetic composition was not pure into the
166   "F_2 hybrid" category (i.e., offspring from backcrosses and hybrid crosses of the previous
167   generation).

168   The strength of the reproductive barriers in heterospecific and hybrid crosses are
169   frequently estimated using conspecific crosses of one or both parental species as controls
170   [27,34,55]. These controls help measure the mating preference between a conspecific and
171   a heterospecific cross [56]. Corrections are made employing indices, such as the Stalker's
172   Index [57], which values range from –1 to 1, where –1 describes the full preference for
173   the heterospecific mating; 0 describes no differences in gene flow between conspecific
174   *versus* heterospecific crosses; and 1 describes the complete preference for conspecific
175   matings. A review and unification of the methods used to estimate reproductive isolation
176   (RI) was made by Sobel and Chen [56]. Since we do not have data on the strength of the
177   mechanical barriers in sympatric conspecific crosses of *I. elegans* and *I. graellsii* during
178   the 2000–2001 experiment (Table S1), and because our main interest was to evaluate the
179   reinforcement of the reproductive barriers between the 2000–2001 and 2019–2020
180   experiments, we did not use any conspecific correction (except for a fecundity barrier).
181   The way in which we measured RI barriers allowed us to do so, since most of them
182   evaluated gene flow as a parameter with a binomial distribution (details in Table 1). The
183   first mechanical barrier (Mechanical I "unsuccessful tandems") was estimated as:

184
$$RI_{Mechanical\ I} = 1 - \frac{successful\ tandems}{tandem\ attempts}$$
(1)

185   The second mechanical barrier (Mechanical II "unsuccessful copulations") was estimated
186   as:

187
$$RI_{Mechanical\ II} = 1 - \frac{succesful\ wheel\ positions\ (copulations)}{succesful\ tandems}$$
(2)

188   These mechanical barriers measure the incompatibility between the males' caudal
189   appendages and the females' prothorax and the incompatibility between the males' and
190   females' genital structures. Three gametic barriers were measured during the first three
191   clutches. The first gametic barrier (Gametic I "oviposition") was estimated as:

192
$$RI_{Gametic\ I} = 1 - \frac{number\ of\ females\ that\ succesfully\ laid\ eggs}{total\ mated\ females\ that\ were\ allowed\ to\ lay\ eggs}$$
(3)

5

193   Since the second gametic barrier (Gametic II "fecundity") was measured as the mean
194   number of eggs laid per clutch, a mathematical correction was needed to estimate the RI
195   strength on the same scale as the other barriers (0 to 1). Thus, we used the following
196   index, employing the mean allopatric conspecific fecundity of both parental species:

197    $$RI_{Gametic\,II} = 1 - \frac{2 * mean\,heterospecific\,fecundity}{mean\,allopatric\,fecundity\,Sp1 + mean\,allopatric\,fecundity\,Sp2}$$ (4)

198   For sympatric conspecific crosses, fecundity RI was measured for both species as:

199    $$RI_{Gametic\,II} = 1 - \frac{mean\,sympatric\,fecundity}{mean\,allopatric\,fecundity}$$ (5)

200   The two previous gametic barriers measure how the heterospecific ejaculate fails to
201   stimulate oviposition and how the heterospecific ejaculate reduces the rate of oviposition
202   [58]. Finally, the third gametic barrier (Gametic III "fertility") was measured as:

203    $$RI_{Gametic\,III} = 1 - \frac{fertile\,eggs}{total\,laid\,eggs}$$ (6)

204   Fertile eggs showed evidence of hatching or the presence of an embryo. This gametic
205   barrier measures several processes: poor transfer or storage sperm, unviability of gametes
206   in the foreign reproductive tract, poor movement or cross-attraction, or failure of
207   fertilisation when gametes contact each other [58]. All calculations and statistical
208   analyses were done using R statistical software [59]. To discard inbreeding effects, the
209   strength of the five reproductive barriers were statistically compared between second-
210   generation conspecific crosses (F_1) made up of siblings and non-related parents. Since no
211   statistically significant differences in the reproductive isolation barriers of conspecific
212   crosses were found between mated siblings and unrelated samples (see Supplementary
213   Fig. S1), we proceeded with the analyses, merging data from both types of families.
214   Finally, to estimate the relative contribution of each barrier to the total isolation in
215   sequential stages of reproduction, we employed the multiplicative function of individual
216   components developed by Coyne and Orr [13,60] and later modified by Ramsey et al.
217   [41] to include any number of barriers. The accumulative contribution (AC) of a
218   component to the reproductive isolation (RI) at stage *n* was estimated with the following
219   formula:

220    $$AC_n = RI_n(1 - \sum_{i=1}^{n-1} AC_i)$$ (7)

221   **Premating and postzygotic isolation asymmetries**

222   Reinforcement theory predicts that the premating isolation in sympatry should evolve to
223   be asymmetric in the same direction as postzygotic isolation [14]. Following Yukilevich
224   [14], we calculated premating (mechanical I and mechanical II) isolation asymmetry as:

225    $$Asymmetry = Premating\,AC_{\,I.\,graellsii\,males\,with\,I.\,elegans\,females} -$$
226    $$Premating\,AC_{\,I.\,elegans\,males\,with\,I.\,graellsii\,females}$$ (8)

227 Postzygotic isolation asymmetry was estimated in the F_1 and F_2 generations for each
228 backcross as:

$$Asymmetry = PrematingAC_{Backcross} - PrematingAC_{Reciprocal\ cross} \qquad (9)$$

Results

Absolute strength of reproductive barriers

Mechanical I (unsuccessful tandems)

233 In the 2000–2001 experiment, mechanical I isolation (unsuccessful tandems) barrier was
234 absent in crosses of *I. elegans* males with *I. graellsii* females (0.00), F_1-hybrid females
235 (0.00), and F_2-hybrid females (0.00). However, this barrier was high and similar in crosses
236 of *I. graellsii* males with *I. elegans* females (0.89) and F_1-hybrid females (0.90) and in
237 crosses of F_1-hybrid males with *I. elegans* females (0.86) and F_1-hybrid females (0.85)
238 but not with *I. graellsii* females (0.38) (Table S1 and Fig. S2A). In the 2019–2020
239 experiment, this barrier was present in all crosses of *I. elegans* males with *I. graellsii*
240 females (0.41), F_1-hybrid females (0.55), and F_2-hybrid females (0.25). Additionally, this
241 barrier was high in crosses of *I. graellsii* males with *I. elegans* females (0.95) and with
242 F_2-hybrid females (0.67). Surprisingly, this barrier was almost absent in crosses of F_1-
243 hybrid males with *I. elegans* females (0.00) and F_1-hybrid females (0.12) and between F_2-
244 hybrid males and females (0.18). When comparing the strength of this barrier between
245 experiments (2000–2001 *vs*. 2019–2020), we detected statistically significant differences
246 between: 1) *I. elegans* males mated with *I. graellsii* females ($p < 0.001$); and 2) with F_1-
247 hybrid females ($p < 0.05$; Table S2 and Fig. S2A) who increased their isolation; and 3)
248 between F_1-hybrid males mated with F_1-hybrid females who reduced their isolation ($p <
249 0.001$; Table S2 and Fig. S2A).

Mechanical II (unsuccessful copulations)

251 During the 2000–2001 experiment, mechanical II (unsuccessful copulations) barrier
252 caused complete reproductive isolation (RI) in the cross between *I. graellsii* males and
253 F_1-hybrid females. A cross that had already reduced gene flow from the previous barrier.
254 Additionally, intermediate levels of RI were recorded in both directions during the F_0
255 generation (*I. graellsii* males with *I. elegans* females = 0.40; *I. elegans* males with *I.
256 graellsii* females = 0.13) and low RI across all crosses of the F_1 and F_2 generations (Table
257 S1 and Fig. S2B). During the 2019–2020 experiment, a similar trend was observed. This
258 barrier caused complete RI in two crosses, with reduced gene flow from the mechanical
259 I barrier. Both crosses involved pure *I. elegans* females with 1) pure *I. graellsii* males and
260 2) F_1-hybrid males. Interestingly, this barrier was absent in the cross between *I. elegans*
261 females and F_2-hybrid males (0.00). Overall, as in the 2000–2001 experiment,
262 intermediate levels of RI were also registered during the first generation with decreasing
263 isolating strengths during the second and third generation (Table S1 and Fig. S2B). When

264   comparing the strength of this barrier between experiments (2000–2001 *vs*. 2019–2020),
265   we did not find statistically significant differences in heterospecific backcrosses to *I.*
266   *elegans* and *I. graellsii* or in hybrid crosses (Table S2, Fig. S2B).

Gametic I (oviposition)

268   Gametic I (oviposition) barrier was absent in most cross combinations (Table S1; Fig.
269   S2C). In the 2000–2001 experiment, this barrier was present in crosses of *I. elegans* males
270   with *I. graellsii* females (0.33) and in crosses of F_1-hybrid males with *I. elegans* females
271   (1.00) and F_1-hybrid females (0.67) (Table S1 and Fig. S2C). In the 2019–2020
272   experiment, this barrier was detected but in lower levels in crosses of *I. elegans* males
273   with *I. graellsii* females (0.04) and between both generation hybrid crosses (F_1-hybrids =
274   0.08; F_2-hybrids = 0.25). When comparing the strength of this barrier between
275   experiments (2000–2001 *vs*. 2019–2020), we found a statistically significant reduction of
276   this barrier between *I. elegans* males mated with *I. graellsii* females ($p < 0.05$; Table S2
277   and Fig. S2C).

Gametic II (fecundity)

279   In the 2000–2001 experiment, gametic II (fecundity) barrier was present in all cross
280   combinations except in the cross between F_1-hybrid males and *I. graellsii* females (0.01).
281   Consistently, this barrier was present in all cross combinations in the 2019–2020
282   experiment (Table S1; Fig. S2D). In fact, when comparing the strength of the fecundity
283   barrier between experiments (2000–2001 *vs*. 2019–2020), statistically significant
284   differences were detected only in two cross combinations, both involving F_2-hybrid
285   females. When they mated with *I. elegans* males ($p < 0.05$; Table S2 and Figs. S2D and
286   S3) and with F_2-hybrid males ($p < 0.001$; Table S1 and Figs. S2D and S3).

Gametic III (fertility)

288   Gametic III (fertility)was present in all cross combinations from both experiments (2000–
289   2001 and 2018–2019), ranging from 0.15 to 0.93, and was larger in crosses involving F_2
290   hybrids from both sexes in both experiments (Table S1 and Fig. S2E). When comparing
291   experiments (2000–2001 *versus* 2019–2020), only a statistically significant increase in
292   the strength of this barrier was detected in crosses between F_2 hybrids ($p < 0.01$; Table
293   S2 and Figs. S2E and S4).

Total cumulative reproductive isolation

295   The total cumulative reproductive isolation of the five reproductive barriers in
296   heterospecific backcrosses to *I. elegans* and to *I. graellsii* and hybrid crosses is given in
297   Figure 2. In the 2000–2001 experiment, total isolation (TI) between *I. elegans* males and
298   *I. graellsii* females was high (77%), owing mostly to gametic isolation (no oviposition
299   and reduced fecundity; Table S1, Fig. 2A). In contrast, TI between *I. graellsii* males and
300   *I. elegans* females was much higher (96%), mainly due to mechanical isolation

301   (unsuccessful tandems Table S1, Fig. 2A). In the 2019–2020 experiment, TI between *I.*
302   *elegans* males and *I. graellsii* females was high (84%), and between *I. graellsii* males and
303   *I. elegans* females, it was complete (100%), owing, in both cross directions, to high
304   mechanical isolation (unsuccessful tandems), which accounted for 41% and 95% of the
305   total reproductive isolation, respectively (see Table S1, Fig. 2B).

306   TI in backcrosses of *I. elegans* males with F_1-hybrid females (2000–2001: 57% and 2019–
307   2020: 59%; Figs 2D-G) and F_2-hybrid females in the 2000–2001 experiment (50%; Fig.
308   2J) was moderate, while it was high (97%; Fig. 2M) in 2019–2020 experiment when *I.*
309   *elegans* mated with F2-hybrid females.

310   In backcrosses of *I. graellsii* males mated with F_1- and F_2-hybrid females, the component
311   that contributed most to TI was mechanical I isolation (unsuccessful tandems). The latter
312   prevented 90% and 67% of the TI (100% and 99%) observed in experiment 2000–2001
313   (matings with F_1-hybrids; Fig. 2C) and 2019–2020 (matings with F_2 hybrids; Fig. 2L)
314   respectively.

315   In hybrid crosses between F_1- and F_2-hybrid males mated with *I. elegans* females, almost
316   complete reproductive isolation was observed in both generations and years (100% in
317   both F_1 crosses and 98% in the F_2 cross during 2019–2020; Figs 2D, 2G and 2M).
318   Mechanical barriers (unsuccessful tandems and copulations) contributed the most to these
319   crosses. When F_1- and F_2-hybrid males mated with *I. graellsii* females, moderate-to-high
320   levels (but no complete isolation) of TI were achieved. Higher values of TI were seen in
321   the F_2 generation (2000–2001: 98%; 2019–2020: 98%; Figs. 2I and 2L) than in the F_1
322   generation (2000–2001: 76%; 2019–2020: 53%; Figs. 2C and 2F). TI for these crosses
323   was achieved with the gradual contributions of the five reproductive barriers. The gametic
324   barriers gained importance in comparison with the crosses with *I. elegans* females.

325   Hybrid crosses between F_1-hybrids showed a reduction in TI, from 99% in 2000–2001
326   experiment to 67% in 2018–2019 experiment, mainly due to the reduction of the relative
327   importance of the mechanical barrier (unsuccessful tandems; Figs. 2E and 2H). In
328   contrast, TI in hybrid crosses between F_2 hybrids presented an important increment, from
329   55% in 2000–2001 experiment to 97% in 2018–2019 experiment, due to the increase of
330   strength in the mechanical I and the three gametic barriers (Figs. 2K and 2N).

331   **Premating and postzygotic isolation asymmetries**

332   Consistent with accumulative isolation patterns, 2000–2001 and 2019–2020 experiments
333   showed higher premating RI in crosses with *I. graellsii* males or *I. elegans* females than
334   in the reciprocal cross direction, both for prezygotic and postzygotic isolation (Table 2).
335   Moreover, all backcrossing experiments in the two experimental years presented positive
336   values of asymmetry, as in the prezygotic, premating barriers. This shows a consistent
337   preference for the gene flow direction of prezygotic, premating barriers. Less premating
338   asymmetry was calculated during 2019–2020 than in 2000–2001 due to an increment in
339   RI in the cross between *I. elegans* males and *I. graellsii* females.

Discussion

340

341　Reinforcement has been a controversial mechanism of speciation; however, since Coyne
342　and Orr's work [13,60], it has increasingly been recognised as playing an important role
343　in speciation and evolution. We evaluated whether a hybrid zone with at least 40 years
344　has increased its strength of reproductive isolation in the last 40 generations (approx. 2
345　generations per year). We also evaluated which barriers had been enhanced, and whether
346　the strengthening of the reproductive isolation was consistent with reinforcement
347　predictions about asymmetry and concordance. Our results indicate that reinforcement
348　plays a major role in the evolution of mechanical isolation in *Ischnura* damselflies. We
349　detected stronger premating isolation between *I. elegans* and *I. graellsii* in the 2019–2020
350　experiment than in the 2000–2001 experiment. Moreover, premating and postzygotic
351　barriers were concordantly asymmetric between reciprocal crosses, and more intense for
352　*I. elegans* than for *I. graellsii* females, which belongs to the species with the smaller
353　distribution in Spain.

354　***Premating and postmating reproductive barriers***

355　In both experiments (2000–2001 and 2019–2020), the contribution of the five
356　reproductive barriers in heterospecific crosses of *I. elegans* and *I. graellsii* was
357　asymmetric, i.e., markedly different between reciprocal crosses. In the heterospecific
358　crosses between *I. graellsii* males and *I. elegans* females, the premating barriers were
359　stronger than the post-mating barriers, and most of the isolation was due to the action of
360　the mechanical barrier preventing the tandem formation. However, in the heterospecific
361　crosses between *I. elegans* males and *I. graellsii* females, gene flow was prevented by the
362　joint action of both premating and post-mating barriers [27]. Mechanical and tactile
363　barriers preventing the formation of successful tandem or copula are (with few exceptions
364　[61]) important reproductive barriers across a variety of non-territorial odonate species,
365　such as the *Enallagma* and *Ischnura* genera, which lack visual recognition and
366　precopulatory courtship behaviours [55,62,63]. The role of mechanical barriers in RI has
367　been used as evidence for the lock-and-key mechanism [64,65], which suggests that the
368　morphology of sexual structures must be under rapid evolution [65], explaining the wide
369　diversity and taxonomic importance of sexual structures [49,55,63], and thus, these
370　structures could be under RCD in *Ischnura* damselflies. However, the first efforts
371　investigating RCD in female's prothoracic sensilla in *Enallagma anna* and *E.*
372　*carunculatum* damselflies, which hybridise in nature, failed to detect RCD despite its role
373　in species recognition [26]. Since the work of Waage [50,66], RCD in the visual
374　recognition system of damselflies *Calopteryx aequabilis* and *C. maculata* has been
375　regarded as a classic reinforcement example outside *Drosophila* [58,67]. However, a
376　molecular study with *C. aequabilis* and *C. maculata* showed that speciation between them
377　precedes the evolution of wing colour used by them as an interspecific recognition system
378　[68]. This rejects the RCD by reinforcement in these species and proposes the "noisy
379　neighbour" theory as the most plausible alternative hypothesis [68]. Since our results
380　suggest that reinforcement operates between *I. elegans* and *I. graellsii*, RCD should be

morphologically tested in the structures involved in the tandem (i.e., male caudal appendages and the female prothorax).

In addition to the increase of premating RI, the strengthening of RI was also detected in the gametic barriers reducing fecundity and fertility in F_2-hybrid crosses, and fecundity in backcrosses to *I. elegans* (F_2-hybrid males mated with *I. elegans* females). Hybrid fecundity and fertility fitness relative to pure species are mixed and highly dependent on the genetic distance between the parental species [69,70], ranging from reductions in both F_1- and F_2-hybrids fecundity or fertility [71], to no differences in fecundity and fertility between the parental species and hybrids [72], or equal or higher F_1-hybrid reproductive success than conspecific crosses but lower in F_2 or later generation hybrids (hybrid breakdown [73–75]) explained by the Bateson–Dobzhansky–Muller (BDM) incompatibilities model [76,77]. This model describes reductions in hybrid fitness as the recombination of alleles from different populations in hybrid genomes. Hybrid breakdown due to BDM incompatibilities may be more easily observed after the F_1 generation, when recessive incompatible factors are increasingly presented [78]. Consistently, post-mating reductions in reproductive success (increased RI) between 2000–2001 and 2019–2020 experiments were detected in crosses involving F_2 hybrids. The genetic bases of BDM incompatibilities have not yet been formally evaluated in *Ischnura* damselflies.

Reinforcement of reproductive isolation

The mechanical barrier preventing tandem formation, but not the mechanical barrier preventing copula or gametic barriers, was significantly stronger in 2019–2020 experiment compared to the 2000–2001 experiment, showing a significant strengthening in approximately 40 generations. The strengthening of this mechanical barrier is consistent with the main prediction of the reinforcement theory about selection, acting preferably in premating barriers [1,3] (however see [79] for an example of reinforcement acting in post-mating barriers), especially those related to partner selection and even those that are under sexual selection [80–82]. Consequently, a change was detected in the relative contribution of the reproductive barriers, becoming premating mechanical barriers, the main barriers of RI between *I. elegans* and *I. graellsii* in both reciprocal crosses.

During tandem, mechanical and tactile incompatibilities have been described as important sources of RI in two damselfly families (Lestidae and Coenagrionidae) [20,27,55,62,64,83,84]. While mechanical recognition involves structural compatibility between male caudal appendages and female prothorax, tactile recognition involves the ability of the male to correctly stimulate the female for the latter to accept copulation by blending her abdomen. In *Enallagma* damselflies, both kinds of incompatibilities are important components of RI [55], however a clear RCD has not been found, at least, in the number, density, or location of sensilla in the females' mesostigmal plates [26]. Our results support the idea that mechanical, instead of tactile, incompatibilities are the main source of RI in *Ischnura* [20,84], leaving the latter a secondary contributor to RI. If reinforcement has been acting on mechanical isolation between *I. elegans* and *I. graellsii*,

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423   as our results suggest, the reduced number of successful tandem formations should leave
424   "small room" for selection to operate in tactile incompatibilities, explaining the
425   strengthening of RI we detected in the former but not in the latter species.

426   The concordance in the asymmetry in the strength of the RI between reciprocal crosses is
427   an additional pattern expected to be caused by reinforcement [14]. We observed that the
428   asymmetry in the strength of the RI between reciprocal crosses was concordant between
429   premating and postzygotic barriers. In both experiments (2000–2001 and 2019–2010), we
430   detected higher premating isolation in the crosses between *I. graellsii* males and *I. elegans*
431   females than in the reciprocal cross direction. This asymmetry was concordant with the
432   asymmetry observed in the strength of the postzygotic, premating barriers in both
433   experiments (2000–2001 and 2019–2010), in which mechanical isolation was stronger in
434   crosses involving *I. graellsii* males (with F_1- and F_2-hybrids) than *I. elegans* males (with
435   F_1- and F_2-hybrids). Moreover, we detected higher isolation in crosses involving *I.*
436   *elegans* females. *Ischnura elegans* is the species that more recently expanded its
437   distribution into the Iberian Peninsula [46] and thus the species with the smaller
438   distribution and population sizes in Spain [45,52,85], which is consistent with the rarer-
439   female effect [14], another pattern expected to be caused by reinforcement.

440   Reinforcement theory is based on the principle that hybridisation costs should be
441   positively correlated with selective pressures directing premating isolation [86].
442   Additionally, hybridisation costs might differ between reciprocal crosses, which is
443   evidenced by the frequent observation that reciprocal crosses produce hybrids with
444   different fitness (Darwin's corollary) [87]. Typically, postzygotic isolation asymmetries
445   are calculated in survival or reproductive differences between hybrids formed in the two
446   reciprocal cross directions [14,21,22,24]. However, because prezygotic isolation is almost
447   complete in crosses between *I. graellsii* males and *I. elegans* females, we did not get any
448   hybrid larvae (from this cross direction) in the 2019–2020 experiment. In contrast, in the
449   2000–2001 experiment, prezygotic isolation in crosses between *I. graellsii* males and *I.*
450   *elegans* was not complete, but hatched eggs were not reared. Because of this, all measured
451   adult F_1-hybrids were produced by the *I. elegans* males and *I. graellsii* females cross. The
452   difficulty in growing up adult hybrids from the *I. graellsii* males with *I. elegans* females
453   direction suggests that hybridisation cost should be higher in this cross than the reciprocal
454   one. Within the context of reinforcement, this explains why there were higher premating
455   barriers measured in this direction than the opposite one. The lack of F_1 adult hybrids
456   from this cross, however, limited us to contrasting postzygotic barriers in the more
457   "traditional way." However, if asymmetric reinforcement is acting between *I. elegans* and
458   *I. graellsii*, we expected that these asymmetries should also be observed in backcrosses.
459   Higher postzygotic premating isolation was expected in crosses involving *I. elegans*
460   females and *I. graellsii* males than the opposite, with F_1-hybrids, organisms that should
461   reduce RCD by homogenising the morphological differences between the two species.
462   This was the case not only with F_1-hybrids but also with F_2-hybrids, which were samples
463   that differed in their *I. elegans* and *I. graellsii* genetic background proportions. This
464   suggests the presence of concordant isolation asymmetries because of reinforcement in
465   this damselfly hybrid zone. However, as suggested by Turelli *et al*. [88] stronger evidence

466 for reinforcement is found when concordant asymmetries are tested between sympatric,
467 heterospecific crosses and allopatric, heterospecific ones. Theory predicts concordant
468 asymmetries in the former, but not in the latter. This is a design yet to be tested in *I.*
469 *elegans* and *I. graellsii.*

Conclusions

471 Premating and post-mating barriers contributed differently to the isolation of *I. elegans*
472 and *I. graellsii* in the north-western Spanish hybrid zone; while premating mechanical
473 barriers were strong and asymmetric in the heterospecific crosses, post-mating barriers
474 were more important in later generations (back- and hybrid-crosses). This is consistent
475 with the key-and-lock model and the hybrid breakdown due to BDM incompatibilities.
476 The combined effect of both processes could accelerate speciation and complete RI in the
477 north-western Spanish hybrid zone by removing individuals with low heterospecific
478 discrimination abilities, increasing the difference of reproductive structures between the
479 species (RCD), and reducing hybrid post-mating fitness. Our results indicate that
480 reinforcement plays a major role in the evolution of mechanical isolation in *Ischnura*.
481 Future studies should investigate the pattern of character displacement in the structural
482 and morphological bases of premating reinforcement between these species.
483 Additionally, research about reinforcement between *I. elegans* and *I. graellsii* should also
484 be directed at comparing RCD between allopatric and sympatric populations and testing
485 for reduced gene flow between them.

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731

732 **Tables**

733 **Table 1.** Reproductive isolation formulas and statistical tests.

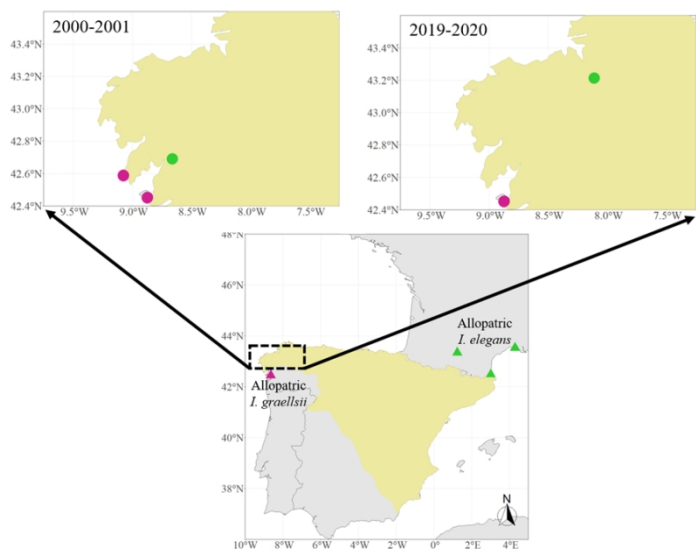| BARRIER/FORMULA	ISOLATION / RANGE	ESTIMATE	Statistical Tests
Premating: F_0, F_1 and F_2	Mechanical		
RI mechanical I = 1- (Tandem/Tandem attempts)	0—1	Incompatibility between secondary genitalia to form **tandem position**	Fisher's exact test
RI mechanical II = 1- (Copula/Tandems)	0—1	Incompatibility between primary genitalia to form **wheel position**	Fisher's exact test
Postmating: F_0, F_1 and F_2	Gametic		
RI oviposition = 1- (mated females that oviposited/total mated females)	0—1	Sperm fails to stimulate females' **oviposition**	Fisher's exact test
RI fecundity =1 - (2 * mean heterospecific fecundity/ (mean allopatric fecundity Sp. 1 + mean allopatric fecundity Sp. 2)	-1—1	Sperm reduces rate of females' oviposition (**fecundity**)	Wilcoxon rank sum test
RI fertility = 1- (total fertile eggs/total laid eggs).	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.	Wilcoxon rank sum test

734

21

735   **Table 2**. Isolation asymmetry calculated as the difference in the strength of accumulative
736   premating isolation between reciprocal crosses of both, prezygotic and postzygotic
737   barriers.

Generation	Reciprocal crosses	RI Strength	RI Strength	Asymmetry
2000−2001 experiment				
F_0: Prezygotic	G♂E♀ - E♂G♀	0.93	0.13	0.93-0.13 = 0.80
F_1: Postzygotic	G♂H♀ - H♂G♀	1	0.63	1-0.63 = 0.37
F_1: Postzygotic	H♂E♀ - E♂H♀	0.86	0	0.86-0 = 0.86
F_2: Postzygotic	G♂H♀ - H♂G♀	NA	0.38	NA
F_2: Postzygotic	H♂E♀ - E♂H♀	NA	0.14	NA
2019−2020 experiment				
F_0: Prezygotic	G♂E♀ - E♂G♀	1	0.61	1-0.61 = 0.39
F_1: Postzygotic	G♂H♀ - H♂G♀	NA	0.25	NA
F_1: Postzygotic	H♂E♀ - E♂H♀	1	0.55	1-0.55 = 0.45
F_2: Postzygotic	G♂H♀ - H♂G♀	0.67	0.50	0.67-0.50 = 0.17
F_2: Postzygotic	H♂E♀ - E♂H♀	0.91	0.38	0.91-0.38 = 0.53

738

739   **Figure Captions**

740   **Figure 1.** Sampled populations from the hybrid region (north-west Spain) and
741   surrounding allopatric populations of *I. elegans* (south France) and *I. graellsii* (north-west
742   Spain) in both experiments, 2000–2001 and 2019–2020. Colours indicate species
743   localities: green, *I. elegans* and purple *I. graellsii.* Gray area represents the allopatric
744   distribution of both species and yellow area represents the sympatric distribution.

745   **Figure 2**. Accumulative reproductive isolation across the five reproductive barriers in
746   heterospecific crosses, backcrosses and F_1- and F_2-hybrid crosses in two experimental
747   years (2000–2001 and 2019–2020). G, indicates *I. graellsii*; E, indicates *I. elegans*; and
748   H, indicates F_1- and F_2-hybrids.

749

Sampled populations from the hybrid region (north-west Spain) and surrounding allopatric populations of I. elegans (south France) and I. graellsii (north-west Spain) in both experiments, 2000–2001 and 2019–2020. Colours indicate species localities: green, I. elegans and purple I. graellsii. Gray area represents the allopatric distribution of both species and yellow area represents the sympatric distribution.

855x481mm (38 x 38 DPI)
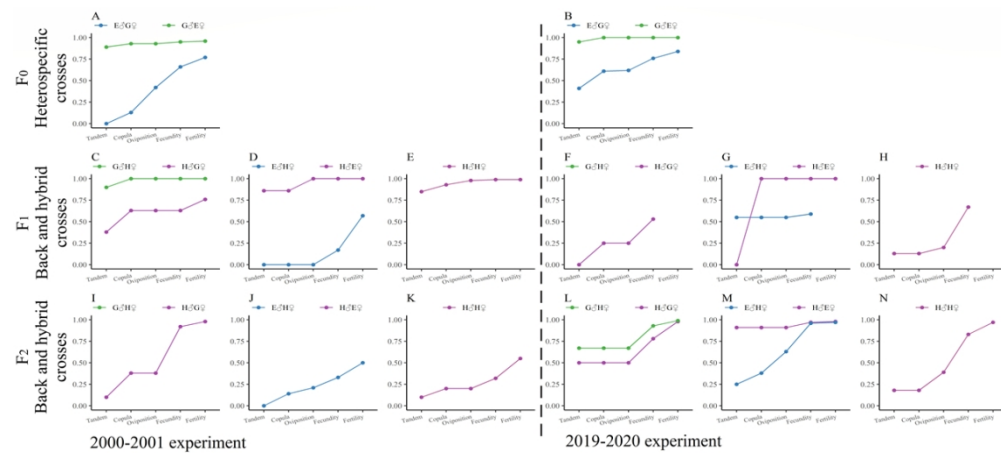
Accumulative reproductive isolation across the five reproductive barriers in heterospecific crosses, backcrosses and F1- and F2-hybrid crosses in two experimental years (2000–2001 and 2019–2020). G, indicates I. graellsii; E, indicates I. elegans; and H, indicates F1- and F2-hybrids.

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