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STRONG ASYMMETRY IN THE RELATIVE STRENGTHS OF PREZYGOTIC AND POSTZYGOTIC BARRIERS BETWEEN TWO DAMSELFLY SISTER SPECIES

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One of the longest debates in biology has been over the relative importance of different isolating barriers in speciation. However, for most species, there are few data evaluating their relative contributions and we can only speculate on the general roles of pre- and postzygotic isolation. Here, we quantify the absolute and cumulative contribution of 19 potential reproductive barriers between two sympatric damselfly sister species, *Ischnura elegans* and *I. graellsii*, including both premating (habitat, temporal, sexual and mechanical isolation) and postmating barriers (prezygotic: sperm insemination success and removal rate, oviposition success, fertility, fecundity; postzygotic: hybrid viability, hybrid sterility and hybrid breakdown). In sympatry, total reproductive isolation between *I. elegans* females and *I. graellsii* males was 95.2%, owing mostly to a premating mechanical incompatibility (93.4%), whereas other barriers were of little importance. Isolation between *I. graellsii* females and *I. elegans* males was also nearly complete (95.8%), which was caused by the cumulative action of multiple prezygotic ($n = 4$, 75.4%) and postzygotic postmating barriers ($n = 5$, 7.4%). Our results suggest that premating barriers are key factors in preventing gene flow between species, and that the relative strengths of premating barriers is highly asymmetrical between the reciprocal crosses.

KEY WORDS: **Asymmetric reproductive isolation, Darwin's corollary, Odonata, postzygotic isolation, prezygotic isolation, speciation.**

Knowledge about the importance of different isolating barriers during lineage divergence, and the intensity with which they prevent genetic exchange, is essential to understand the processes that result in the splitting of species. Reproductive isolation is seldom caused by a single strong isolating barrier, but is more commonly caused by multiple isolating mechanisms (Dobzhansky 1947, 1951; Coyne 1992; Schluter 2001; Price and Bouvier 2002; Coyne and Orr 2004). The comprehensive work by Coyne and Orr (1989, 1997, 2004) on reproductive isolation in the genus *Drosophila* pointed out the general leakiness of individual isolating barriers and inferred that the absence or low abundance of hybrids, despite some documented isolating barriers, implies that other barriers must exist. Isolating barriers have been intensively studied in plants (e.g., Lewis and Crowe 1958; Rick 1963; Ramsey et al. 2003; Kay 2006; Scopece et al. 2007; Widmer et al. 2009) and animal taxa [insects (Presgraves 1998, 2002; Slotman et al. 2005; Dopman et al. 2010), fishes (Mendelson 2003; Mendelson et al. 2004), amphibians (Sasa et al. 1998; Malone and Fontenot

2008), birds (Price and Bouvier 2002; Tubario and Litjamer 2002; Litjamer et al. 2003), and mammals (Fitzpatrick 2004; Bolnick and Near 2005; Fitzpatrick and Turelli 2006)]. These studies have provided important insights into the nature and evolution of different isolating barriers and their relative roles in reproductive isolation of animals, however, the vast majority of studies consider only a small subset of the potentially possible isolating barriers (Mayr 1963; Coyne and Orr 2004). The relative strength of isolating barriers depends on the direction of hybridization, that is, on the direction of gene exchange (Arnold et al. 1996; Tiffin et al. 2001; Takami et al. 2007) and in this context, asymmetries of premating barriers have been intensively discussed (e.g., Kaneshiro hypothesis). Several examples of asymmetric reproductive isolation have been observed in the genus *Drosophila* (Watanabe and Kawanishi 1979; Kaneshiro 1980) as well as in salamanders (Arnold et al. 1996), parasitic wasps (Bordenstein et al. 2000), and snakes (Shine et al. 2002). These studies have, among other things, shown that the contribution of postmating barriers to reproductive isolation is frequently asymmetric, a phenomenon that has been termed Darwin's corollary (Turelli and Moyle 2007). However, despite the interest in asymmetric isolating barriers, studies rarely investigate the contribution of multiple barriers between single pairs of animal species to reproduction. Therefore more studies both in animals and plants (Coyne and Orr 2004; Nosil et al. 2005; Lowry et al. 2008) are needed to quantify the relative contributions of reproductive barriers in closely related species. Studies on recently diverged sister taxa provide the most insights into the processes of speciation (Ramsey et al. 2003; Kay 2006) because the strength and the number of isolating barriers typically accumulates after speciation is complete. This is because the role and function of barriers to gene flow can change dynamically over time. For example, most extrinsic barriers are often ephemeral (Harrison 1998; McBride and Singer 2010), whereas many intrinsic barriers are essentially irreversible over the course of species divergence (e.g., Fuller 2008). Certain prezygotic barriers, such as habitat, temporal, or sexual isolation, for example, may fluctuate over time in relation to environmental conditions, such as range expansions, which can dramatically affect isolating barriers due to changes in species interactions and habitat (Wellenreuther et al. 2010a). Speciation can certainly occur by these aforementioned forces alone (e.g., by habitat isolation alone, see Munday et al. 2004), but it is conceivable that evolutionary trajectories could reticulate in the future. However, once two taxa achieve complete reproductive isolation by intrinsic postzygotic means, their permanence as independent lineages is essentially guaranteed (Sobel et al. 2009). These considerations are useful in comparing reproductively isolated taxa that differ markedly in their absolute levels of pre- and postmating isolation, despite equivalent levels of total isolation (Sobel et al. 2009).

Odonata (damselflies and dragonflies) are good model species to investigate the relative contributions of different isolating barriers to the total amount of reproductive isolation. This is because odonates can be easily observed in the wild, and many species can be reared and crossed successfully in captivity (Cordero 1990b; Andrés and Cordero 1999; Van Gossum et al. 2003; Sánchez-Guillén et al. 2005). Like in other animal taxa, reproductive barriers in odonates have been largely understudied and thus far only some components of premating isolation have been investigated, namely sexual isolation in the genus *Calopteryx* (Svensson et al. 2006, 2007; Tynkkynen et al. 2008a, 2008b; Wellenreuther et al. 2010a, b), and mechanical isolation in the genera *Nehalenia* (Van Gossum et al. 2007), *Enallagma* (McPeek et al. 2008; McPeek et al. 2011), and *Argia* (Paulson 1974). Even less studied than premating barriers are postmating mechanisms in odonates, which have so far been only examined in the genus *Ischnura* (Johnson 1975; Leong and Hafernik 1992).

In this study, we measure the relative contributions of different isolating components to the overall reproductive isolation between two recently diverged sister species of damselflies (Carchini et al. 1994), the blue tailed damselfly *Ischnura elegans* and the Iberian blue tail *I. graellsii*. The species are polyandrous (Robinson and Allgeyer 1996) and ecologically, morphologically (Monetti et al. 2002), and genetically similar (Nei's genetic distance is only 0.2%, Sánchez-Guillén et al. 2005), and co-occur over wide parts of their range (Askew 2004). *Ischnura elegans* is a common and widespread damselfly in most parts of Europe and has recently colonized several regions in Spain (Monetti et al. 2002). In contrast, *I. graellsii* is much more restricted in its range and is confined to southern Europe (Spain and Portugal) and northern Africa (Askew 1998; Dijkstra and Lewington 2006). Nonetheless, both species overlap on the Iberian Peninsula and frequently hybridize within the area of overlap (Monetti et al. 2002; Sánchez-Guillén et al. 2011).

The specific goals of our study were (1) to detect and quantify isolating barriers that contribute to premating (habitat, temporal, sexual, and mechanical isolation) and postmating isolation (prezygotic: sperm insemination success and removal rate, oviposition success, fertility, fecundity; postzygotic: hybrid viability, hybrid sterility and reduced hybrid vigor) and to detect if the strength of premating and postmating barriers to total reproductive isolation in the reciprocal crosses show asymmetry. We then (2) calculate the relative strength of each reproductive barrier to isolation and the overall total reproductive isolation, and lastly (3) we discuss the contribution of ecological divergence and the "joint action of multiple isolating barriers" to reproductive isolation in this species pair. To accomplish these aims, we integrated data from several sources, including data from previous studies on *I. elegans* and *I. graellsii*, field observations, laboratory mating and rearing experiments, and F_1- and F_2-hybrid offspring comparisons.

Methods

REPRODUCTION IN *ISCHNURA*

In damselflies, males grasp the female by the prothorax in the "tandem position" with or without courtship. The female can then accept the copulation by dorsally flexing her abdomen, which results in the formation of the "wheel position" and contact of the mating organs (Corbet 1999). Once copulation is achieved, the subsequent reproductive activity can be divided into three different behavioral phases, each of which corresponds to different measures of internal activity of the genitalia (Miller and Miller 1981). In the genus *Ischnura*, the first stage is characterized by sperm removal (Miller 1987a). *Ischnura graellsii* males have been shown to readily remove sperm from the bursa and spermatheca (Cordero and Miller 1992), whereas *I. elegans* males have only access to sperm in the bursa but not the spermatheca, the latter holding sperm for long-term usage (Miller 1987a, b). The second stage of copulation is characterized by the insemination of the female and the third stage is classified as "male mate guarding" and entails the end of insemination (Cordero-Rivera and Córdoba-Aguilar 2010). In *I. elegans* and *I. graellsii*, the whole process can take up to 5–7 h (Miller 1987a; Cordero 1990a) and favors last-male sperm precedence.

Reproductive barriers between the two possible crosses of *I. elegans* and *I. graellsii* were investigated using data from the literature, field surveys, behavioral experiments, and breeding experiments in the laboratory. The details of the procedures are outlined in the relevant sections below, and formula and calculations are given in Table 1.

PREMATING MECHANISMS: HABITAT, TEMPORAL, SEXUAL, AND MECHANICAL ISOLATION

Even when species coexist in the same general geographic area, they might interact little because of differences in fine-scale habitat associations (Ramsey et al. 2003; Stoks and McPeek 2006; Wellenreuther et al. 2007, 2008; Wellenreuther and Clements 2007; Hilton et al. 2008). The degree of habitat isolation (Table 1) as a reproductive barrier was calculated using population frequency counts of both species in two sympatric areas (details in Table S1). Populations were classified as sympatric if both species co-existed in space and time, that is, if both species were found together. Allopatric populations were those where only one species was found, or where the two species coexisted spatially, but not temporally.

To estimate the amount of temporal isolation, we compared the time during the day at which copulations take place (Table 1). Data were taken from a previous study by Monetti et al. (2002) and consisted of conspecific copulations of *I. graellsii* ($n = 21$) that occurred from 1110 to 1955 h and *I. elegans* ($n = 23$) ranging from 0855 to 1640 h, and heterospecific copulations between *I. graellsii* females and *I. elegans* males and ($n = 13$) which occurred from 0853 h to1705 h.

It was not possible to delineate sexual versus mechanical isolation in the field, because all natural copulations are the direct result of the amount of attraction and mechanical compatibility between individuals. Therefore, we refer to our measure as sexual-mechanical-isolation (Table 1). The proportion of conspecific and heterospecific copulations was quantified in the sympatric population of Xuño in northwest Spain (42°37′40.23″N and 9°02′23.71″O) over a period of five days in 2003. The observed proportions were later compared with the expected proportions using a chi-square test.

Mechanical isolation was estimated in more detail by measuring the incompatibility between the male cerci and female prothorax (when the male is unable to grasp the female), and the incompatibility between the genitalia (Table 1). We measured both types of incompatibilities in crosses between *I. graellsii* females and *I. elegans* males and between *I. elegans* females and *I. graellsii* males.

LABORATORY REARING

Laboratory experiments were conducted from 2000 to 2002 during which three damselfly generations were reared. The first generation was started in June 2000 and included the last-instar larval stage of *I. elegans* (collected from Louro 42°69′08.8″N, –8°66′03″5O), and of *I. graellsii* (collected from Corrubedo 42°34′35.29″N, 9°4′30.52″O; Lanzada 42°25′44.46″N, 8°52′20.20″O and Alba 42°26′29.39″N, 8°38′40.99″O). The second generation consisted of eight crosses of *I. elegans*, four crosses of *I. graellsii*, three crosses of *I. elegans* females and *I. graellsii* males (to measure oviposition success, fertility and fecundity), and 10 crosses of *I. graellsii* females and *I. elegans* males (to measure oviposition success, fertility and fecundity, and to raise F_1-hybrids). The third generation was composed of four crosses of *I. elegans*, four crosses of *I. graellsii*, six crosses of F_1-females and *I. elegans* males and two crosses of *I. graellsii* females and F_1-males (F_2-hybrids). F_2-hybrids were reared until adulthood. Larvae were reared to adulthood and matings were obtained following the methodology developed by Sánchez-Guillén et al. (2005). Each individual was only used once in a mating experiment to ensure that only virgins were used, and to prevent mechanical damage, sperm depletion, and pseudoreplication.

POSTMATING MECHANISMS: PREZYGOTIC AND POSTZYGOTIC ISOLATION

Prezygotic mechanisms: sperm insemination success and removal rate, oviposition success, fecundity, and fertility

Sperm insemination and removal were measured in both species by conducting mating experiments using individuals reared in

Table 1. Calculation details for the relative strength of the reproductive isolation index "S" for each isolating barrier.

Barrier/formula	Isolation measure/ range	Estimate
Premating	Habitat	Population overlap
$RI_{habitat}=1-$(sympatric populations/total populations)	0 to 1	Isolation due to micro-allopatry
Premating	Temporal	Timing of copulations
$RI_{temporal}=1-$(matings occurring at the same time/total matings)	0 to 1	Isolation due different reproductive times
Premating	Mechanical-sexual	Matings in the field
$RI_{sexual\text{-}mechanical}=1-$(observed heterospecific matings/expected heterospecific matings under random mating)	−1 to 1	Preference for conspecifics
Premating	$Mechanical_I$	Incompatibility between genitalia
$RI_{mechanicalII}=1-$(tandem attempts/tandems)	0 to 1	Mechanical incompatibility
Premating	$Mechanical_{II}$	Incompatibility between genitalia
$RI_{mechanicalII}=1-$(tandems/matings)	0 to 1	Mechanical incompatibility
Postmating: prezygotic	Failed insemination	Failed sperm insemination
$RI_{failed\_insemination}=1-$(% females with sperm heterospecific matings/% females with sperm conspecific matings)	−1 to 1	Isolation oviposition failure
Postmating: prezygotic	Insemination success	Reduction in sperm insemination success
$RI_{insemination\_success}=1-$(sperm volume one heterospecific mating/sperm volume one conspecific matings)	−1 to 1	Lower sperm insemination in heterospecifics
Postmating: prezygotic	Sperm removal	Sperm removal from previous matings
$RI_{sperm\_removal}=1-$(sperm volume two matings (conspecific and heterospecific)/sperm volume two conspecific matings)	−1 to 1	Lower sperm removal in heterospecifics
Postmating: prezygotic	Oviposition	Percentage females that oviposited
$RI_{oviposition}=1-$(% females oviposited heterospecific matings/% females oviposited conspecific matings)	−1 to 1	Lower fitness in heterospecifics
Postmating: prezygotic	Fecundity	Number of eggs laid in the first three clutches
$RI_{fecundity}=1-$(mean fecundity heterospecific matings/mean fecundity conspecific matings)	−1 to 1	Lower fitness in heterospecifics
Postmating: prezygotic	Fertility	Number of fertile eggs in each mating treatment
$RI_{fertility}=1-$(mean fertility heterospecific matings/mean fertility conspecific matings).	−1 to 1	Lower fitness in heterospecifics
Postmating: postzygotic	F_1-survivorship (viability)	Survivorship of F_1-hybrids
$RI_{F1\text{-}survivorship}=1-$(fitness hybrid/fitness parent species)	−1 to 1	Isolation due to mortality
Postmating: postzygotic	F_1-sex-ratio (viability)	Sex-ratio of F_1-hybrids
$RI_{F1\text{-}sex\text{-}ratio}=1-$(fitness hybrid/fitness parent species)	−1 to 1	Isolation due to skewed sex-ratio
Postmating: postzygotic	F_2-oviposition (sterility)	Proportion of F_1-females that laid eggs
$RI_{F2\text{-}oviposition}=1-$(fitness hybrid/fitness parent species)	−1 to 1	Isolation oviposition failure
Postmating: postzygotic	F_2-failed insemination (sterility)	Failed sperm insemination of F_1-hybrids
$RI_{F2\text{-}failed\_insemination}=1-$(% females with sperm heterospecific matings/% females with sperm conspecific matings)	−1 to 1	Isolation insemination failure
Postmating: postzygotic	F_2-fecundity (sterility)	Eggs in the first three clutches of F_1-females
$RI_{F2\text{-}fecundity}=1-$(fitness hybrid/fitness parent species)	−1 to 1	Isolation due to lack of fecundity

Continued.

Table 1. Continued.

Barrier/formula	Isolation measure/ range	Estimate
Postmating: postzygotic	F_2-fertility (sterility)	Proportion of hatched eggs of F_1-females
$RI_{F2\text{-fertility}}=1-$(fitness hybrid/fitness parent species)	-1 to 1	Isolation due to lack of fertility
Postmating: postzygotic	F_2-survivorship (vigor)	Survivorship of F_2-hybrids
$RI_{F2\text{-survivorship}}=1-$(fitness hybrid/fitness parent species)	-1 to 1	Isolation due to mortality of F_2-hybrids
Postmating: postzygotic	F_2-sex-ratio (vigor)	Sex-ratio of F_2-hybrids
$RI_{F2\text{-sex-ratio}}=1-$(fitness hybrid/fitness parent species)	-1 to 1	Isolation due to skewed sex-ration in F_2-hybrids

the laboratory. For conspecific matings we used (1) females of both species with conspecific males, (2) females of both species with two conspecific males (one day between matings), and (3) females of both species with two conspecific males, where the second mating was interrupted after 5, 10, 20, 40, 60, 90 minutes in *I. graellsii* and up to a maximum time of 120 minutes in *I. elegans*. For the heterospecific mating treatments, *I. graellsii* females were mated with a (1) heterospecific, (2) conspecific and the day after with a heterospecific, and (3) heterospecific followed by a conspecific. No matings between *I. elegans* females and *I. graellsii* males were achieved because of the high mechanical isolation. Following the mating experiments, the bursa and spermatheca were dissected under a binocular microscope and compressed to a uniform thickness under a supported coverslip on a slide following Miller (1987a). The area of the storage organs was measured twice at a magnification of 40× with an image analyzer (UTHSCSA ImageTool Version 3.0). The volume of the storage organs was estimated as mean area multiplied by thickness. Mean sperm volumes were normally distributed and therefore an ANOVA was used to compare single- and double-mated females from the conspecific treatments, and double mated *I. graellsii* females mated with two con- or heterospecifics. The relationship between sperm volume and the time at which the second mating was interrupted was analyzed using a linear regression, with sperm volume as the response variable and time as the predictor. The degree of reproductive isolation was evaluated using three metrics (Table 1): failed sperm insemination, reduction in sperm insemination success following mating, and the degree of sperm removal of previous matings.

After copulation, fertilization (i.e., zygote formation) can be impeded by five mean causes: (1) poor transfer or storage sperm; (2) inability of gametes in foreign reproductive tract; (3) inability of gametes to affect fertilization due to poor movement or cross-attraction; (4) failure of fertilization when gametes contact each other; and (5) foreign ejaculate fails to stimulate oviposition or reduces rate of oviposition (following Coyne and Orr 2004). Consequences of these barriers can be measured as oviposition success, fecundity, and fertility [the mean number of fertile eggs (eggs that hatched or showed a developing embryo) in each mating treatment]. Oviposition success, fecundity, and fertility were estimated using mating experiments with individuals raised in the laboratory. The following crosses were obtained: *I. graellsii* and *I. elegans* females both mated with one conspecific male ($n = 8$ and $n = 18$, respectively); *I. elegans* and *I. graellsii* females mated with heterospecific males ($n = 3$ and $n = 20$, respectively), and *I. graellsii* mated with two conspecific males ($n = 6$) and with a con- and heterospecific male ($n = 9$): three with a conspecific and then with a heterospecific male, and six with a heterospecific followed by a conspecific male. Oviposition success was estimated by comparing the percentage of *I. elegans* and *I. graellsii* females that oviposited after conspecific matings with the percentage of females that oviposited after heterospecific matings. Fecundity was estimated as the total number of eggs laid in the first three clutches, and was analyzed with a generalized linear model (GLM) using a Poisson distribution and a logit link function. The total number of eggs was the response variable and the number of clutches was the predictor. Fertilization was assessed by a change in the egg color; no fertile eggs remain white whereas fertilized eggs are transparent and the zygote is clearly visible. Fertility was quantified as the mean number of fertile eggs in each mating treatment, and only eggs that hatched or showed a developing embryo were considered fertile. The fertility was analyzed using a GLM with a binomial distribution and a logit-link function, with the number of hatched eggs as the response variable, clutch size as binomial totals, and treatment as predictors. Details of the formulae for the isolation index for oviposition, fecundity, and fertility can be found in Table 1.

Postzygotic mechanisms: hybrid viability, hybrid sterility, and hybrid breakdown

Postzygotic isolation can be divided into hybrid viability, hybrid sterility, and hybrid breakdown. To determine the extent of

postzygotic isolation in these three categories, we mated laboratory-reared individuals and raised the F_1 and F_2 offspring resulting from matings between *I. graellsii* females and *I. elegans* males. Hybrid viability was measured in terms of sex-ratio and the survivorship of F_1-hybrids. Furthermore, we also crossed F_1-hybrid females and *I. elegans* males, *I. graellsii* females, and F_1-hybrid males, and measured hybrid sterility as oviposition success (proportion of females that laid eggs), fecundity (number of eggs in the first three clutches), and fertility (proportion of hatched eggs). Finally, to estimate reduction in hybrid vigor, we estimated the sex-ratio and survivorship of F_2-hybrids obtained from those crosses. Detailed information about the isolation indices and the formulae are listed in Table 1.

TOTAL ISOLATION: PREMATING AND POSTMATING MECHANISMS

To estimate the cumulative reproductive isolation between *I. graellsii* and *I. elegans*, we used the multiplicative function of individual components of isolation in sequential stages of mating following the methods published in Coyne and Orr (1989, 1997) and Ramsey et al. (2003). This method allows the quantification of the contribution of each individual barrier to the total reproductive isolation. The absolute contribution (AC) of a component to reproductive isolation (RI) at stage n was calculated in the following manner:

$$\mathrm{AC}_n = \mathrm{RI}_n \left(1 - \sum_{i=1}^{n-1} \mathrm{AC}_i\right).$$

Asymmetry of reproductive isolating barriers between the two possible reciprocal crosses was calculated for each individual barrier (pre- and postmating), by calculating the absolute value of the difference between barriers.

Results

PREMATING MECHANISMS

Habitat isolation

The relative proportions of sympatric and allopatric populations in the two sympatric Spanish regions La Rioja and Valencia (Table S1) were 13 of 52 and six of 106, respectively, and the resulting respective habitat isolation index between the two species was $\mathrm{RI}_{\mathrm{habitat}} = 0.88$ (Table 2). This high value indicates that fine-scale habitat divergence between the two species is large, despite occurring in the same general geographic area.

Temporal isolation

Temporal isolation between the species was, in contrast, low ($\mathrm{RI}_{\mathrm{temporal}} = 0.05$) and contributed much less to the total RI between species (Table 2), suggesting that the daily time of reproductive activity largely overlaps between species.

Sexual-mechanical isolation

Sexual-mechanical isolation was observed in the field and a total of 102 copulations were documented, of which 80 were conspecific copulations (*I. elegans* $n = 5$; *I. graellsii* $n = 75$) and 22 heterospecific copulations between *I. graellsii* females and *I. elegans* males. The observed (22) and expected (16) frequencies of the heterospecific copulations between the latter cross were similar ($P = 0.224$, Table 3), whereas none of the expected (8.76) copulations between *I. elegans* females and *I. graellsii* males were observed in the field ($P = 0.009$, Table 3). The index of sexual-mechanical isolation between *I. graellsii* females and *I. elegans* males crosses was $\mathrm{RI}_{\mathrm{sexual-mechanical}} = 0.00$ (Table 2), which suggests that there was no sexual-mechanical isolation. In contrast, sexual-mechanical isolation between *I. elegans* females and *I. graellsii* males in the field was complete; $\mathrm{RI}_{\mathrm{sexual-mechanical}} = 1.00$ (Table 2).

Mechanical isolation

Mechanical isolation was subsequently estimated in more detail in the laboratory by observing heterospecific mating attempts in insectaries (Table 4). Twenty of 23 attempts to form a tandem were successful between *I. graellsii* females and *I. elegans* males and all tandems were transformed into copulations. On the other hand, only five of 44 attempts to form a tandem between *I. elegans* females and *I. graellsii* males were successful, and three of those ended in copulations. The index of mechanical isolation$_{\mathrm{I}}$ (unsuccessful tandems) between *I. graellsii* females and *I. elegans* males was $\mathrm{RI}_{\mathrm{mechanicalI}} = 0.00$, whereas the index between *I. elegans* females and *I. graellsii* males was $\mathrm{RI}_{\mathrm{mechanicalI}} = 0.89$ (Table 2), suggesting a strong mechanical barrier in the latter cross. The index of mechanical isolation $_{\mathrm{II}}$ (unsuccessful copulations) between *I. graellsii* females and *I. elegans* males was $\mathrm{RI}_{\mathrm{mechanicalII}} = 0.13$, whereas the index between *I. elegans* females and *I. graellsii* males was $\mathrm{RI}_{\mathrm{mechanicalII}} = 0.40$ (Table 2), again indicating a much stronger mechanical barrier between *I. elegans* females and *I. graellsii* males than in the other reciprocal cross.

POSTMATING MECHANISMS: PREZYGOTIC AND POSTZYGOTIC ISOLATION

Sperm insemination success and removal rate

The ability of males to remove sperm from previous matings was estimated using sperm volume measurements of the females' bursa and spermatheca after one or two matings with either con- or heterospecifics. Figure 1 shows the results of the sperm removal analyses. Two *I. graellsii* females (total $n = 12$) were found to have empty sperm storage organs following conspecific copulations (both bursa and spermatheca), indicating unsuccessful insemination by the male, and these two females were thus excluded from the analyses (see Fig. S1). For conspecific trials, the

Table 2. Contributions to total reproductive isolation calculated for sequential reproductive barriers, with the sum of contributions equaling total isolation. S denotes the relative strength and AC the absolute contribution of different pre- and postzygotic isolating barriers in reciprocal crosses between *I. elegans* and *I. graellsii* (EG: *I. elegans* female and *I. graellsii* male, GE: *I. graellsii* female and *I. elegans* male), calculated as shown in the main text, accounting for either barriers acting in sympatry, that is when there is no isolation due to temporal or spatial factors, which was estimated (1) taking into account sexual/mechanical isolation in the field and (2) taking into account mechanical isolation in the laboratory, and in sympatry and allopatry, when the species experience all reproductive isolation barriers. "na" denotes cases were the isolation index could not be estimated, and empty cell denotes the isolation index that were not took into account. Total reproductive isolation for a set of components is the sum of all the AC.

		Sympatry								Sympatry and Allopatry			
		G♀E♂		E♀G♂		G♀E♂		E♀G♂		G♀E♂		E♀G♂	
Barrier	Type	(S)	(AC)	(S)	(AC)	(S)	(AC)	(S)	(AC)	(S)	(AC)	(S)	(AC)
Premating	Habitat									0.880	0.880	0.880	0.880
Premating	Temporal									0.050	0.006	0.050	0.006
Premating	Mechanical-sexual	0.000	0.000	1.00	1.000								
Premating	$\text{Mechanical}_{\text{I}}$					0.000	0.000	0.890	0.890	0.000	0.000	0.890	0.101
Premating	$\text{Mechanical}_{\text{II}}$					0.130	0.130	0.400	0.044	0.130	0.015	0.400	0.005
Postmating, prezygotic	$\text{Failed insemination}_{\text{I}}$	0.580	0.580			0.580	0.505	0.000	0.000	0.580	0.058	0.000	0.000
Postmating, prezygotic	$\text{Insemination success}_{\text{II}}$	0.000	0.000	na	na	0.000	0.000	na	na	0.000	0.000	na	na
Postmating, prezygotic	Sperm removal	0.130	0.055	na	na	0.130	0.048	na	na	0.130	0.005	na	na
Postmating, prezygotic	Oviposition	0.000	0.000			0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Postmating, prezygotic	Fecundity	0.370	0.135			0.370	0.118	0.190	0.013	0.370	0.013	0.190	0.001
Postmating, prezygotic	Fertility	0.420	0.097			0.420	0.084	0.100	0.005	0.420	0.010	0.100	0.001
Postmating, postzygotic	F_1-survivorship (viability)	0.000	0.000	na	na	0.000	0.000	na	na	0.000	0.000	na	na
Postmating, postzygotic	F_1-sex-ratio (viability)	0.000	0.000	na	na	0.000	0.000	na	na	0.000	0.000	na	na
Postmating, prezygotic	F_2-failed insemination (sterility)	0.375	0.050	na	na	0.375	0.044	na	na	0.375	0.005	na	na
Postmating, postzygotic	F_2-oviposition (sterility)	0.000	0.000	na	na	0.000	0.000	na	na	0.000	0.000	na	na
Postmating, postzygotic	F_2-fecundity (sterility)	0.200	0.017	na	na	0.20	0.015	na	na	0.200	0.002	na	na
Postmating, postzygotic	F_2-fertility (sterility)	0.280	0.019	na	na	0.280	0.016	na	na	0.280	0.002	na	na
Postmating, postzygotic	F_2-survivorship (vigour)	0.020	0.001	na	na	0.020	0.001	na	na	0.020	0.000	na	na
Postmating, postzygotic	F_2-sex-ratio (vigour)	−0.020	−0.001	na	na	−0.020	−0.001	na	na	−0.020	0.000	na	na
Premating	Total		0.000		1.000		0.130		0.934		0.901		0.992
Postmating (prezygotic)	Total		0.866				0.754		0.018		0.086		0.002
Postmating (postzygotic)	Total		0.085				0.074		na		0.008		na

Table 3. **Estimates of sexual-mechanical isolation in the field. Table shows the number of conspecific and hybrid matings observed in north-western Spain at the population Xuño. The columns of solitary males and females indicate the number of individuals found alone of each species: G (*I. graellsii*) and E (*I. elegans*). The number of matings found for each combination is indicated as follows: GG (*I. graellsii* female and *I. graellsii* male), EE (*I. elegans* female and *I. elegans* male), GE (*I. graellsii* female and *I. elegans* male), and EG (*I. elegans* female and *I. graellsii* male). The last columns represent the number of heterospecific matings expected under random mating, and the value of *P*, obtained for χ^2 contingency. P_1 value for conspecific (GG and EE) and heterospecific (GE) crosses and P_2 value for conspecific and heterospecific (EG) crosses.**

	Solitary males		Solitary females		Observed matings (con- and heterospecific)				Expected matings (con- and heterospecific)					
Date	G♂	E♂	G♀	E♀	G♀G♂	E♀E♂	G♀E♂	E♀G♂	G♀G♂	E♀E♂	G♀E♂	E♀G♂	P_1	P_2
05/06	155	8	41	3	15	0	2	0	13.22	0.07	0.78	1.10		
27/06	119	8	34	1	13	0	0	0	11.15	0.04	0.80	0.35		
07/08	108	10	67	1	24	0	3	0	24.73	0.00	2.25	0.36		
11/09	99	77	54	13	10	1	2	0	5.49	1.28	4.58	1.42		
17/09	na	na	na	na	13	4	15	0	13.52	3.16	11.28	3.49		
All data	481	103	196	18	75	5	22	0	68.11	4.55	16.00	8.76	0.224	0.009

***Expected heterospecific matings on 17 September were estimated using the population frequencies of both species collected at 11 SeptemberhaS NOT BEEN LINKED TO TABLE BODY.**

total sperm volume (bursa and spermatheca combined) of double-mated *I. graellsii* females was not significantly different from single mated females ($F_{1,13} = 2.25$, $P = 0.157$), and the same result was obtained when comparing bursal and spermathecal volume separately ($P > 0.050$). *I. graellsii* females whose second copulation was interrupted showed a decrease in sperm volume (regression coefficient = –0.00029, SE = 0.000001, $P = 0.008$), which was due to a clear diminution in the bursa ($r^2 = 0.33$, $P < 0.008$) and a similar trend in the spermatheca ($r^2 = 0.18$, $P < 0.060$; Fig. S1).

Insemination success of conspecific *I. elegans* crosses was 100% (total $n = 12$), and evidence for this came from two independent trials namely the oviposition trials ($n = 8$, see additional results below), and sperm volume measurements after copulation ($n = 4$). Figure 1 shows the results of the insemination analyses. For the conspecific trials, the sperm volume of single- and double-mated females was similar (total volume: $F_{1,8} = 0.27$, $P = 0.620$), although there was a tendency for the bursal sperm to increase after two matings ($F_{1,8} = 5.11$, $P = 0.054$), though the same was not found for the spermatheca ($F_{1,8} = 0.05$, $P = 0.822$). Sperm volume decreased with time of interruption of the second mating from 1 to 60 min, but increased again thereafter (Fig. S1), resulting in no significant slope (bursa: $r = 0.01$, $P = 0.730$; spermatheca: $r = 0.03$, $P = 0.582$).

Heterospecific matings between *I. graellsii* females with *I. elegans* males were less successful, and seven of the 12 females had a completely empty bursa and spermatheca after the end of copulation, again suggesting unsuccessful insemination by the male. The sperm volume received by the remaining five females (0.0051 ± 0.0012) was, nevertheless, similar to that of females mated twice with conspecifics (sequence *I. elegans–I. graellsii*; 0.0067 ± 0.0001; *I. graellsii–I. elegans*: 0.0065 ± 0.0009; $F_{2,11} = 0.84$, $P = 0.459$, Fig. 1). This suggests that once copulation is achieved, sperm transfer is not impaired in either con- or heterospecific trials. Total sperm volume of *I. graellsii* females

Table 4. **Estimates of the combined effects of mechanical isolation$_{I}$ and $_{II}$ in the laboratory. The table shows the number of conspecific and hybrid matings observed in north-western Spain at the population Xuño. First and second columns explain the generation and the type of crossing: GE (*I. graellsii* female and *I. elegans* male), EG (*I. elegans* female and *I. graellsii* male), GH (*I. graellsii* female and Hybrid male), EH (*I. elegans* female and Hybrid male), HH (Hybrid female and Hybrid male), HG (Hybrid female and *I. graellsii* male), and HE (Hybrid female and *I. graellsii* male). Columns of attempts to tandem, tandem and matings represent the absolute number of the observed sexual activity. RI represents the absolute value of the index of isolation for each fitness component. "na" denotes cases were the isolation index could not be estimated.**

Generation	Cross	Attempt to tandem	Tandem	Mating	RI
First: Parental	G♀G♂	na	na	na	0.00
species	E♀E♂	na	na	na	0.00
Second:	G♀E♂	23	23	20	0.13
F_1-hybrids	E♀G♂	44	5	3	0.93
Third:	G♀H♂	12	8	4	0.67
F_2-hybrids	E♀H♂	11	0	0	1.00
	H♀H♂	37	3	1	0.97
	H♀G♂	4	1	0	1.00
	H♀E♂	8	8	8	0.00

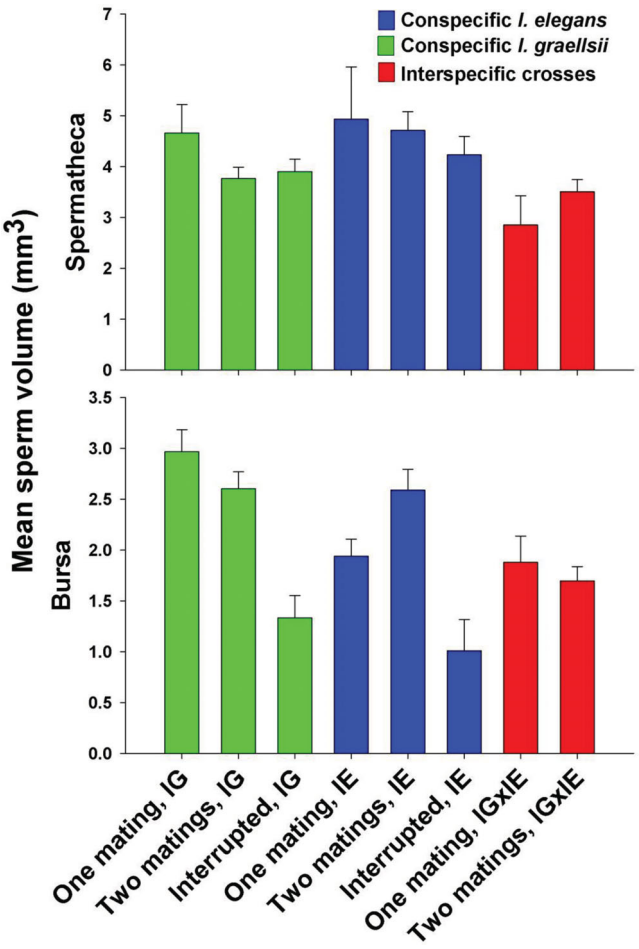

Figure 1. **The volume of sperm in females of *I. elegans* (IE) and *I. graellsii* (IG) after one or two conspecific and heterospecific matings. Interrupted matings represent females whose second mating was interrupted after 10–60 min of stage I of the second copulation, when the male was removing sperm. Heterospecific matings involved only *I. graellsii* females with one *I. elegans* male (one mating), or two *I. elegans* and *I. graellsii* males in both types of sequences (two matings). Unsuccessful transfers from conspecific and heterospecific crosses have been excluded from this Figure.**

mated with a conspecific and heterospecific male was similar to the volume after two conspecific matings, irrespective of the mating order (ANOVA, $F_{3,21} = 0.44$, $P = 0.728$), and the same results were obtained when the bursa and spermatheca were analyzed separately ($P > 0.050$, Fig. 1).

All *I. elegans* females ($n = 3$) mated with *I. graellsii* males got inseminated, as determined from the successful development of eggs (see below) following heterospecific copulations (note that the low sample size was due to the high mechanical isolation for this cross). Based on the data above, insemination failure for crosses of *I. graellsii* females and *I. elegans* males was $RI_{failed\_insemination} = 0.58$ and for *I. elegans* females and *I. graellsii* males $RI_{failed\_insemination} = 0.00$, respectively (Table 2), indicating a reduction in sperm insemination between *I. graellsii* females and *I. elegans* males. The postmating RI index for insemination success and sperm removal was only measured in the cross between *I. graellsii* females and *I. elegans* males because all *I. elegans* females refused to remate with a heterospecific male after mating with a conspecific male. Insemination success in this cross was not impaired ($RI_{insemination\_success} = 0.00$, Table 2), whereas reduced sperm removal was indicated ($RI_{sperm\_removal} = 0.13$, Table 2), suggesting that heterospecific males show a reduction in the ability to remove sperm from previous matings.

Oviposition success

All eight *I. elegans* and all 18 *I. graellsii* females mated to conspecific males laid eggs. Moreover, all three *I. elegans* females mated with *I. graellsii* males laid eggs. In contrast, only 13 of 20 *I. graellsii* females mated to *I. elegans* males oviposited. Upon dissection, all seven females that failed to lay eggs were found to have empty genitalia, indicating insemination failure and not a failure to oviposit, and these females were therefore removed from further analyses. As a result, the oviposition isolation index for both crosses was $RI_{oviposition} = 0.00$ (Table 2), showing no isolation by reduction in oviposition success.

Fecundity

Fecundity estimates after conspecific matings showed that *I. graellsii* females laid on average more eggs (642.2 ± 50.82, $n = 17$, one female that laid less than 50 eggs was excluded) than *I. elegans* females (496.0 ± 129.3, $n = 8$), but this was because *I. graellsii* females laid more clutches, so that the average number of eggs per clutch was similar (225.3 ± 17.8 for *I. graellsii* and 238.7 ± 37.3 for *I. elegans*). The number of eggs laid after heterospecific matings for *I. graellsii* females mated with *I. elegans* males was 366.9 ± 68.3 ($n = 11$, two females that laid less than 50 eggs were excluded) and for *I. elegans* females mated with *I. graellsii* males was 402.0 ± 4.5 ($n = 3$). Despite the average number of eggs being smaller after heterospecific matings, again, number of clutches explained most of the variation, and the number of eggs per clutch was similar between treatments (GLM with Poisson errors and log link, corrected for overdispersion, deviance ratio = 1.69, $P = 0.134$). Between *I. graellsii* females and *I. elegans* males, the isolation index for the reduction of fecundity was $RI_{fecundity} = 0.37$ and between *I. elegans* females and *I. graellsii* males $RI_{fecundity} = 0.19$ (Table 2), pointing towards a higher isolation for the cross between *I. graellsii* females and *I. elegans* males.

Fertility

Mean fertility was also examined in the mating treatments by comparing the mean number of fertile eggs. Figure 2 shows the results of the fertility analyses, which are summarized in Table 5. Female

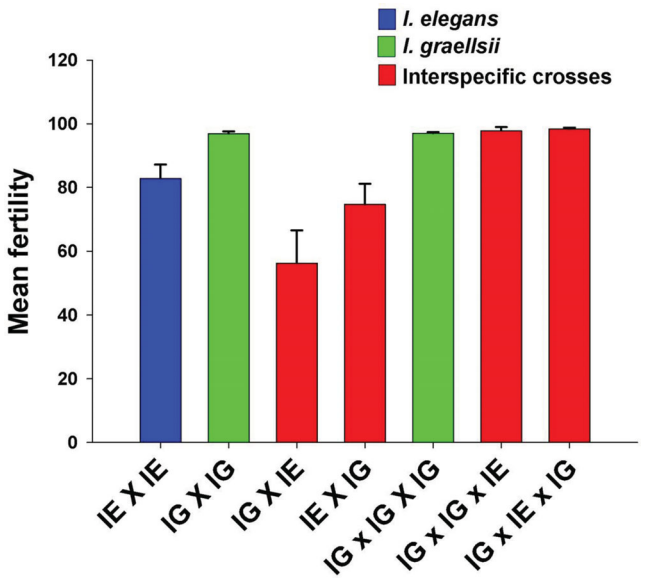

Figure 2. Mean fertility after conspecific and heterospecific matings of *I. elegans* (IE) and *I. graellsii* (IG) with one and two males. The female species is denoted first.

I. graellsii mated with one conspecific showed a fertility of 97% but when was mated with a heterospecific males had a fertility rate of 56%. In *I. elegans,* the results showed a similar decrease in fertility after heterospecific matings, with 82.8% fertility in conspecific matings and 74.7% in heterospecific matings (Table 5). The fertility derived from matings between *I. elegans* females and *I. graellsii* males was lower compared to conspecific *I. elegans* matings, but this difference was only close to significance, ($t_{51} = 1.92, P = 0.060$). However, the values obtained for the remaining groups were significantly lower from the value obtained for conspecific *I. elegans* matings (GLM with binomial errors corrected for overdispersion, *P*-values ranged between 0.030 and <0.001). Between *I. graellsii* females and *I. elegans* males, the isolation index for the reduction of fertility was $RI_{fertility} = 0.42$ and between *I. elegans* females and *I. graellsii* males $RI_{fertility} = 0.10$ (Table 2), indicating again a higher isolation between *I. graellsii* females and *I. elegans* males.

Hybrid viability

Hybrid viability was assessed in terms of F_1-sex-ratio and survivorship until adulthood from heterospecific crosses between *I. elegans* males and *I. graellsii* females (Table 5). The F_1-sex-ratio was similar among all crosses ranging from 1:0.33 to 1:1.27 (χ^2test, *P* ranged from 1 to 0.225). Furthermore, the same proportion of F_1-hybrid larvae (64%) and *I. graellsii* larvae (62%) reached adulthood (GLM with binomial errors, deviance ratio = 0.58, $P = 0.574$). The isolation index based on deviation in sex-ratio was $RI_{F1\_sexratio} = 0.00$ (Table 5), and the isolation index based on survivorship until adulthood was $RI_{F1\_survivorship} = 0.00$ (Table 5).

Hybrid sterility

Hybrid sterility was examined by conducting hybrid mating experiments between F_1-offspring derived from crosses between *I. graellsii* females and *I. elegans* males. These showed that *I. graellsii* females were only mechanically compatible with F_1-hybrid males and F_1-hybrid females with *I. elegans* males (Table 2). Crosses in these two possible directions were conducted and then the oviposition success, fecundity, and fertility quantified. Only two of four *I. graellsii* females mated with F_1-hybrid males (50%) and six of eight F_1-hybrid females (75%) mated with *I. elegans* males laid eggs. Upon dissection, all females that failed to lay eggs were found to have empty genitalia, indicating insemination failure and not a failure to oviposit, and these females were therefore removed from further analyses. The isolation index based on the failure to oviposit for *I. graellsii* females mated with F_1-hybrid males was thus $RI_{F2\_insemination\_success} = 0.50$, and for F_1-hybrid females mated with *I. elegans* males $RI_{F2\_insemination\_success} = 0.25$, and the combined isolation index was $RI_{F2\_insemination\_success} = 0.375$. The isolation index based on the oviposition success for *I. graellsii* females mated with F_1-hybrid males was thus $RI_{F2\_oviposition} = 0.00$, and for F_1-hybrid females mated with *I. elegans* males $RI_{F2\_oviposition} = 0.00$, and the combined isolation index was $RI_{F2\_oviposition} = 0.00$ (Table 5).

The number of eggs laid (fecundity) after conspecific *I. graellsii* matings (642.2 ± 50.8) was similar ($U = 20, P = 0.853$, Mann–Whitney *U*-test) to the number of eggs laid by *I. graellsii* females mated with a F_1-hybrid male (533.3 ± 379.0). In addition, the number of eggs laid after conspecific *I. elegans* matings (496.0 ± 129.3) was similar ($U = 26, P = 0.852$, Mann–Whitney *U*-test) to the number of eggs laid by F_1-hybrid females mated with *I. elegans* males (424.0 ± 123.9). Moreover, the number of eggs laid by *I. graellsii* females mated with a F_1-hybrid male was similar ($U = 6, P = 0.857$, Mann–Whitney *U*-test) to the number of eggs laid by F_1-hybrid females mated with *I. elegans* males. The isolation indices for *I. graellsii* females mated with F_1-hybrid males were $RI_{F2\_fecundity} = 0.11$, and $RI_{F2\_fecundity} = 0.29$ for the F_1-hybrid females mated with *I. elegans* males, and the combined index was $RI_{F2\_fecundity} = 0.20$.

Fertility measurements showed that 97% of the eggs hatched when *I. graellsii* females were mated with conspecifics, whereas 65.7% hatched when females were mated with F_1-hybrids (GLM with binomial errors, deviance = 59.30, $P < 0.001$). The same was found for *I. elegans*, where 82.8% of the eggs hatched after conspecific matings but only 59.5% after F_1-hybrid matings (GLM, deviance = 10.73, $P = 0.007$). The latter fertility measure was similar to that obtained for matings between *I. graellsii* females and F_1-hybrid males (65.7%, GLM, deviance ratio = 0.68, $P = 0.442$). The combined fertility isolation index for both types of crosses was $RI_{F2\_fertility} = 0.30$, for *I. graellsii*

Table 5. Estimates of postmating isolation mechanisms; prezygotic: oviposition success, fertility, and fecundity; postzygotic: hybrid viability, hybrid sterility, and hybrid breakdown for hybrid matings between *I. graellsii* and *I. elegans*. Second and third columns represent the type of crossing with G denoting *I. graellsii*, E *I. elegans*, and H hybrids. Sample size (*N*) indicates the number of females, SE denotes standard error. RI represents the value of the index of isolation for each fitness component. "na" denotes cases were the isolation index could not be estimated

Fitness component	Generation	Cross	*N*	Mean	SE	RI
Oviposition rate	First: Parental species	E♀E♂	8	1.00		
		G♀G♂	18	1.00		
	Second: F_1-hybrids	G♀E♂	13	1.00		0.00
		G E♀♂	3	1.00		0.00
	Third: F_2 –hybrids	G♀H♂	2	1.00		0.00
		H♀E♂	6	1.00		0.00
Fecundity	First: Parental species	E♀E♂	8	496.00	129.30	
		G♀G♂	17	642.20	50.82	
	Second: F_1-hybrids	G♀E♂	11	366.90	68.30	0.37
		E♀G♂	3	402.00	4.50	0.19
	Third: F_2 –hybrids	G♀H♂	2	533.31	379.00	0.11
		H♀E♂	6	424.01	123.92	0.29
Fertility	First: Parental species	E♀E♂	8	0.83	0.04	
		G♀G♂	18	0.97	0.01	
	Second: F_1-hybrids	G♀E♂	13	0.56	0.10	0.42
		E♀G♂	3	0.75	0.06	0.10
	Third: F_2 –hybrids	G♀H♂	2	0.66	0.09	0.32
		H♀E♂	6	0.59	0.08	0.28
Sex-ratio	First: Parental species	E♀E♂	10	0.52	0.02	
		G♀G♂	3	0.48	0.04	
	Second: F_1-hybrids	G♀E♂	8	0.52	0.02	0.00
		E♀G♂	na	na	na	na
	Third: F_2 –hybrids	G♀H♂	2	0.46	0.05	0.08
		H♀E♂	6	0.56	0.03	−0.13
Viability	First: Parental species	E♀E♂	2	0.48	8.90	
		G♀G♂	3	0.59	3.81	
	Second: F_1-hybrids	G♀E♂	8	0.36	7.54	0.00
		E♀G♂	na	na	na	na
	Third: F_2 –hybrids	G♀H♂	2	0.58	12.99	−0.55
		H♀E♂	4	0.41	9.97	0.36

with F_1-hybrid males $RI_{F2\_fertility} = 0.32$; and for F_1-hybrid females with *I. elegans* males $RI_{F2\_fertility} = 0.28$.

Hybrid breakdown

Hybrid breakdown was estimated based on the F_2-sex-ratio and survivorship until adulthood of the descendents (F_2) in two types of crosses; in *I. graellsii* females with F_1-males ($n = 2$) and in F_1-females mated with *I. elegans* males ($n = 6$). The male: female sex-ratio ranged from 1:0.47 to 1:1.28, respectively, for both crosses, and significant differences in the expected sex-ratios (1:1) were detected (χ^2, P ranged from 0.048:1). F_2-larval survivorship until adulthood was 58% for the F_2-hybrids resulting from *I. graellsii* females and F_1-males, and 41% for the F_2-hybrids resulting from F_1-females and *I. elegans* males. In addition, these two types of crosses also showed a lower survivorship than conspecific crosses, where survivorship was 59% for *I. graellsii* and 48% for *I. elegans*. However, there were no significant differences in viability depending on the type of cross (GLM with binomial errors, deviance ratio = 0.76, $P = 0.460$). The isolation index based on the deviation in sex-ratio was $RI_{F2\_sex\_ratio} = 0.08$ for *I. graellsii* females and F_1-males and $RI_{F2\_sex\_ratio} = -0.13$ for F_1-females and *I. elegans* males, and the combined index was $RI_{F2\_sexratio} = -0.02$. The isolation index based on survivorship until adulthood was $RI_{F2\_survivorship} = -0.16$ for *I. graellsii* females with F_1-males and $RI_{F2\_survivorship} = 0.20$ for F_1-females and *I. elegans* males, and the combined index was $RI_{F2\_survivorship} = 0.02$.

TOTAL CUMULATIVE RI

When accounting for sympatric barriers only, total premating isolation between *I. elegans* females and *I. graellsii* males was high

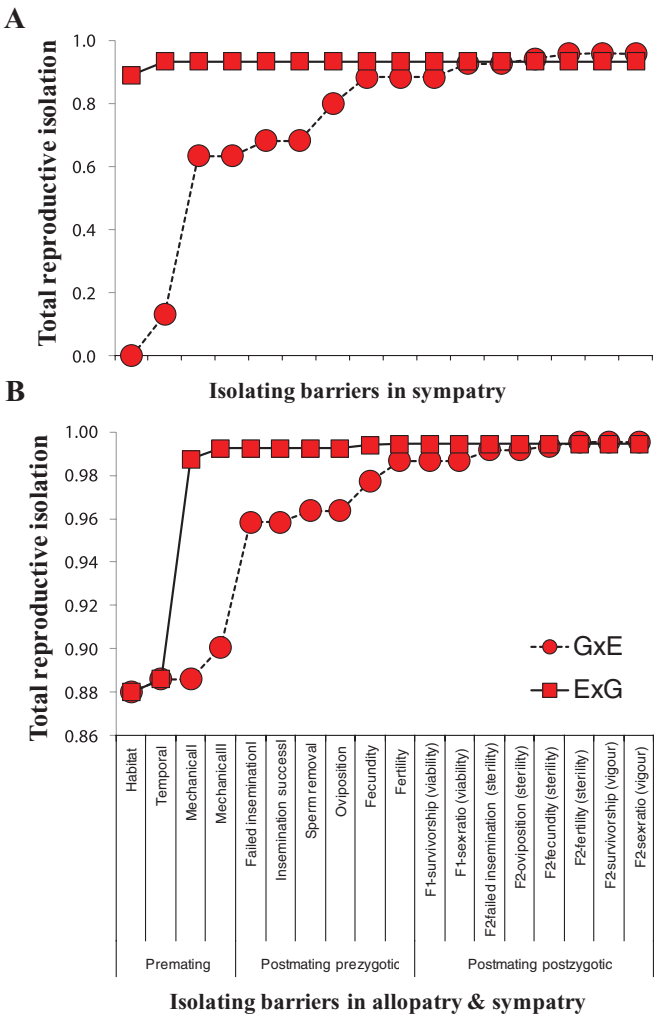

Figure 3. Relative contributions to total isolation (see Table 4) in reciprocal crosses (G × E: *I. graellsii* females and *I. elegans* males, E × G: *I. elegans* females and *I. graellsii* males following the method described by Ramsey et al. (2003). Graph (A) includes all sympatric barriers, that is, excluding habitat and temporal isolation while graph (B) includes all sympatric and allopatric barriers. * Different scales on *y*-axis were used to present the relative contributions to total isolation: (0.0–1.0) in graph A) and (0.86–1.0) in graph B).

(93.4%), owing mostly to a strong mechanical isolation (Table 5, Fig. 3 and 4). In contrast, premating isolation between *I. graellsii* females and *I. elegans* males was much lower (13%), due to a less strong mechanical barrier in this cross. However, in the field, premating barriers were completely (100%) between *I. elegans* females and *I. graellsii* males, whereas were not detected between *I. graellsii* females and *I. elegans* males. Postmating prezygotic isolation was, however, much stronger for this cross, accounting for 75.4% ($n = 4$, see Table 5) of total RI. The prezygotic component that contributed most to the isolation between *I. graellsii* females and *I. elegans* males was the failure to inseminate, which accounted for 50.5%, followed by a reduction in fecundity, which accounted for 11.8%. In addition, postzygotic barriers ($n = 5$, see Table 5) increased the total RI value by an additional 7.4% to an overall value of 95.8%. Prezygotic barriers were much weaker between *I. elegans* females and *I. graellsii* males (1.8%), however, mechanical isolation isolated this cross to a high degree, resulting in an overall isolation index of 95.2% (Fig. 3). Postzygotic isolation in hybrids from crosses between *I. elegans* females and *I. graellsii* males could not be tested due to the high prezygotic isolation (Fig. 3). Nevertheless, the component that contributed most to postzygotic isolation in F_1- and F_2-hybrids derived from crosses between *I. graellsii* females and *I. elegans* males was the failure to inseminate (4.4%). When habitat and temporal isolation were also accounted for (sympatric and allopatric barriers), then total RI was almost complete in both possible directions, with crosses between *I. elegans* females and *I. graellsii* males being isolated by 99.4% and *I. graellsii* females and *I. elegans* males by 99.5%.

The pre-and postmating barriers that were most asymmetric between the two reciprocal crosses (see Table 2) were (1) premating; $\text{mechanical}_{\text{I,}}$ and $\text{mechanical}_{\text{II,}}$ which had a degree of asymmetry of 0.89 and 0.27; respectively and (2) postmating; failed insemination and fertility, which had a degree of asymmetry of 0.58 and 0.32; respectively, whereas fecundity was almost symmetric (accounting for less than 0.20).

Discussion

Our study showed that RI between the sympatric sister species *I. graellsii* and *I. elegans* is high but incomplete. We quantified 19 potential isolating barriers and showed that the contributions of different barriers to total RI differed markedly between the two reciprocal crosses. Heterospecific crosses between *I. elegans* females and *I. graellsii* males were prevented in the field by strong premating sexual-mechanical barriers (mechanical barriers in the laboratory accounted for 93.4% of total isolation). On the other hand, not a single isolating barrier had a large effect on the hybridization rate between *I. graellsii* females and *I. elegans* males, but we found that the joint action of multiple barriers prevented a significant and large proportion of gene flow in this cross. Premating sexual-mechanical barriers were not present in the crosses between *I. graellsii* females and *I. elegans* males in the field although they were present in the laboratory (mechanical barriers accounted for 13.0% of total isolation), whereas postmating prezygotic barriers had a large effect (75.4%) in the reduction of gene flow between the species.

PREMATING ISOLATION: THE ROLE OF PREZYGOTIC BARRIERS

Although it has been widely recognized that even partial habitat isolation can play a key role in speciation (Ramsey et al. 2003;

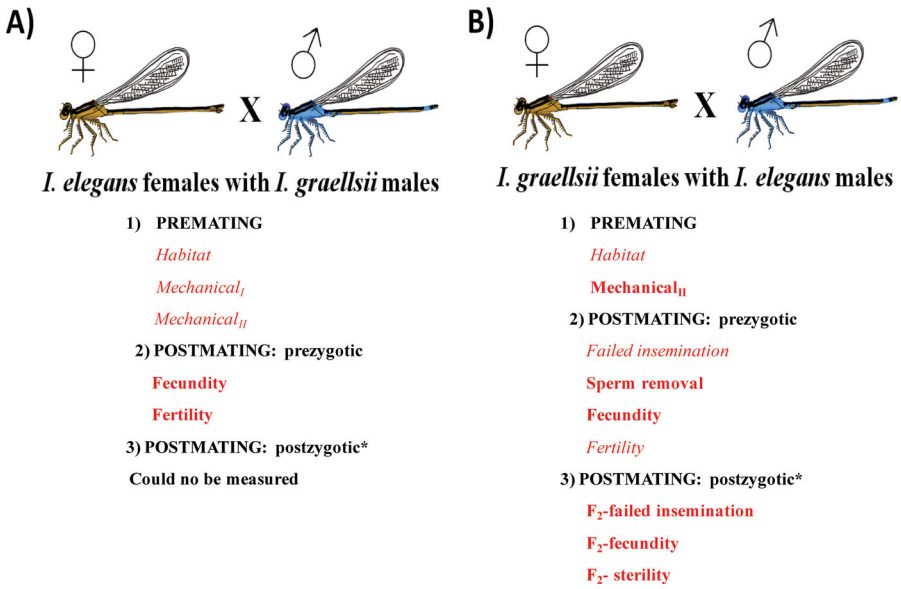

Figure 4. Schematic diagram of the most important isolating barriers in the two crosses (A) *I. elegans* females with *I. graellsii* males and (B) *I. graellsii* females and *I. elegans* males. All barriers having a strength of more than 0.1 are shown (see Table 4) and the strongest three barriers are italicized.

Gavrilets 2004; Munday et al. 2004; Wellenreuther et al. 2007; Wellenreuther and Clements 2008), many studies still ignore this barrier (Kay 2006). The results of our study show that habitat isolation between *I. elegans* and *I. graellsii* is very important in preventing heterospecific matings. Although both species co-occur in three sympatric regions on the Iberian Peninsula (two were investigated in this study), fine-scale habitat associations prevent a large amount of overlap. Both species have quite similar habitat preferences, but some differences do occur. In northwestern Spain one of the clearest differences is that *I. elegans* prefers coastal habitats, whereas *I. graellsii* is often found further inland. Nevertheless, this is not the case in many other regions, particularly the Mediterranean coast of Spain and in Italy, where *I. elegans* occupies the same habitats that *I. graellsii* uses in Spain. Despite these coarse comparisons of species-specific distribution patterns, little work has been done to try to quantify habitat preferences of both species. Recently, however, we have compiled an updated distribution map of the two species in Spain, and from these maps it can be seen that both species, and in particular *I. elegans*, have expanded their geographic range in southern Europe, leading to a greater degree of overall sympatry (Sánchez-Guillén et al. 2011). This pattern could suggest that the species are still expanding from their glacial refugia; however, the refugia would be expected to be somewhat similar for the two species because they share a similar lifestyle (Askew 2004). An alternative is that this pattern has emerged as a response to the warming climate. It has been shown for many insect species (Parmesan 1996; Parmesan et al. 1999), and for odonates in particular (Hickling et al. 2005; Wellenreuther et al. 2010a), that the recent increase in temperatures over the last few decades has led to a rapid increase in geographic range size, which can result in newly formed sympatric areas. In addition, recent molecular studies on *I. elegans* have corroborated the general view that this species is a good disperser and shows high gene flow between populations (Wellenreuther et al. 2011), further supporting the view that the distribution of this species could quickly change in response to climatic variations, It therefore appears most likely that the differences in geographical distributions reflect mostly differences in ecological niches. This highlights that species-specific spatial preferences need to be considered more explicitly in future studies to understand the environmental factors behind the spatial segregation.

Temporal isolation, on the other hand, although considered an important isolating mechanism in insects in general (Hölldobler 1976) and in *I. graellsii* and *I. elegans* in particular (Cordero 1989; Cordero-Rivera and Egido 1998; Monetti et al. 2002), did not function as an efficient barrier against hybridization in this case (isolation index 0.05). Given that odonates, like all insects, are ectotherms and hence are intimately bound to ambient temperatures (e.g., Deutsch et al. 2008), it is not surprising that reproductive activities are governed by almost identical temperature optima, leading to similar daily activity cycles, although *I. elegans* tends to mate somewhat earlier in the day. In addition, temporal isolation may be due to differences in phenology of the parental species. However, *I. elegans* and *I. graellsii* have been documented to share similar phenologies in the sympatric areas that they occupy on the Iberian Peninsula (see Dijkstra and Lewington 2006).

Mechanical isolation was strongly asymmetric between the crosses. This mechanical isolation is presumably caused by a mismatch in the anatomy of anal appendages. In odonates, reproduction proceeds by the male first grasping the head or prothorax of the female with his anal appendages, and the female then applies her genitalia to the secondary genitalia of the male, which lie on the ventral side of the second abdominal segment, and it is thus possible to envisage the development of two sets of "lock-and-key" isolating mechanisms (Watson 1966). The first type is a mismatch between the anal appendages of the male and the mesogtigmal plates of the female, and the other at the link between the genitalia. Differences in the anatomy of the anal appendages in odonates have been described for many groups or related species (Paulson 1974; McPeek et al. 2008; McPeek et al. 2009), and it seems plausible that such anatomical differences are impairing copulations between *I. elegans* females and *I. graellsii* males. In addition to mechanical differences, many female odonates have been shown to possess mechanoreceptors in the mesostigmal plates, which are possibly stimulated by the male during tandem formation (Robertson and Patterson 1982). These mechanoreceptors may also be involved in the mechanical isolation between this cross, as inferred from the high percentage of females in heterospecific tandems that did not accept copulations (R. A. Sánchez-Guillén, pers. obs.).

The lack of species recognition between *I. graellsii* females and *I. elegans* stands in contrast to other damselfly species, where strong species recognition and species-specific sexual selection has been documented (Svensson et al. 2006, 2007; Wellenreuther et al. 2010a). This may be due to the fact that sexual isolation appears to be more important in damselfly species with exaggerated secondary sexual traits, such as the colored wings in *Calopteryx* spp. (Svensson et al. 2006, 2007; Tynkkynen et al. 2008a, b). In the genus *Ischnura*, elaborated secondary sexual traits are not well developed and courtship is largely lacking (Cordero 1989). This combination of factors is probably causative in the relative high number of interfamily matings between *Ischnura* spp. and other damselfly species that can be commonly observed in the wild (e.g., *I. elegans* male with *P. nymphula* female Monetti et al. 2002).

POSTMATING: PREZYGOTIC ISOLATION

Between 35% and 58% of *I. graellsii* females mated to *I. elegans* males did not receive any sperm, while insemination was never impaired between *I. elegans* females and *I. graellsii* males. This is a first barrier for hybridization, which might be caused by males refusing to inseminate females, by females impeding insemination, or by an incompatibility between genitalia. Alternatively, rather than a failure to inseminate, lack of sperm in the genitalic structures may also be caused by females expelling sperm following insemination, a process that has been documented in several insect species, including odonates (Cordero and Miller 1992; Cordero et al. 1995; Córdoba-Aguilar et al. 2003). When heterospecific insemination occurred, then the volume of sperm transferred was similar to the volume during conspecific matings, but the fertility rate was clearly reduced (Table 4). These results point toward noncompetitive gametic isolation between the crosses, due to sperm from heterospecific males being less successful (Matute 2010), and could either be caused by a lower viability of heterospecific sperm in the female reproductive tract, or by lower heterospecific sperm mobility, as found in crickets (Gregory and Howard 1994). Sometimes, heterospecific sperm is unable to fertilize the egg (Vacquier 1998), a mechanism that seems particularly common in animals with external fertilization (Palumbi and Metz 1991), or sperm fails to elicit oviposition, a mechanism identified in *Drosophila* (Fuyama 1983; Price et al. 2001) and crickets (Gregory and Howard 1993).

Many males in the order Odonata are able to remove sperm (Córdoba-Aguilar and Cordero-Rivera 2009), and *I. graellsii* and *I. elegans* can access and remove stored sperm from the bursa (Miller 1987a, b; Cordero and Miller 1992), and in *I. graellsii* also from the spermatheca (Cordero and Miller 1992). We were able to corroborate these results for the bursa, but the findings were less clear for the spermatheca (Fig. S1). Unfortunately, we were unable to directly study sperm removal in heterospecific matings, because *I. graellsii* females refused to remate if a heterospecific male was involved. Nevertheless, it seems that sperm removal occurs when *I. graellsii* females mate with *I. elegans* males, because sperm volume measurements were similar when the female was mated with males of both species or with two conspecific males (Fig. S1). These results show that postcopulatory prezygotic events can be an important factor in the prevention of gene flow between closely related species, and in particular, between *I. graellsii* females and *I. elegans* males.

POSTMATING: POSTZYGOTIC ISOLATION

Postmating postzygotic barriers could only be measured between *I. graellsii* females and *I. elegans* males because it was not possible to obtain a sufficient number of F_1-hybrids for *I. elegans* females and *I. graellsii* males. Therefore, the results cannot be used to make relative statements about the contributions of different isolating barriers for each type of cross.

In comparison to the premating and postmating prezygotic isolating barriers, postzygotic isolation contributed much less to the total isolation between *I. graellsii* females and *I. elegans* males. Overall, early postzygotic fitness effects were small and showed no evidence of Haldane's Rule (Haldane 1922), because both males and females showed similar levels of hybrid sterility and viability. However, although the F_1-hybrid fitness was generally high and we did not detect problems in the F_1-hybrid development in the laboratory, we found a significant reduction

in F_1-hybrid fertility. In addition, we detected evidence for reproduction isolation by partial F_2-hybrid sterility, caused by a failure to inseminate or to stimulate females to oviposit and a reduction in female fertility. There was also a slight indication for a reduction in F_2-hybrid vigor, due to decreased offspring viability between F_2-hybrid males with *I. elegans* females.

TOTAL RI

Habitat isolation was identified as a key reproductive barrier between species and when this barrier was taken into account, total RI was close to complete (over 99%). However, even when the species co-occurred in sympatry, several isolation barriers reduced the realized amount of gene flow, and total RI was still around 95%. The finding that RI between the species is high but not complete is supported by both field observations (Monetti et al. 2002) and molecular studies (Sánchez-Guillén et al. 2011). Hybridization between the two species occurs frequently in the wild and is highly asymmetric, with the vast majority of crosses being between *I. graellsii* females and *I. elegans* males (Sánchez-Guillén et al. 2005; Sánchez-Guillén et al. 2011). This highly unidirectional hybridization indicates that the costs of interspecific interactions are higher in *I. graellsii*, explaining the recent displacement of *I. graellsii* in some Spanish regions (Monetti et al. 2002).

We have found a particularly high degree of asymmetry in premating barriers between the reciprocal crosses, although significant levels of asymmetry were also found among postmating barriers. Asymmetry in premating isolation is common across a wide range of taxa (Coyne and Orr 2004, pp. 226–227). Nevertheless, in a recent comprehensive analysis involving 19 pairs of plant taxa, Lowry et al. (2008) assessed the strength and patterns of asymmetry of multiple prezygotic and postzygotic reproductive isolating barriers and found that postmating barriers are approximately three times more asymmetrical in their action than premating barriers. This finding is consistent with earlier studies (Tiffin et al. 2001; Turelli and Moyle 2007), and could indicate that the relative asymmetries of reproductive barriers between plant and animal taxa follows different rules. The mechanisms underlying these asymmetries can be manifold and often vary among taxa (Turelli and Moyle 2007). In our species pair, one major selection pressure seems to be direct—on the number of hybrid offspring produced—rather than indirect—on the fitness of hybrid offspring. In this case, the simplest mechanism appears to be Dobzhansky–Muller incompatibilities.

The data on asymmetric prezygotic isolation in our species pair can also be interpreted in light of Kaneshiro's (1980) hypothesis, which seeks to explain asymmetry in behavioral isolation between species. Kaneshiro (1980) proposed that when one species is derived from another species via a founder event, the females from the ancestral species are more likely to reject males from the derived species would have changed to a greater extent (especially due to loss of behavioral [mating] traits) than males from the ancestral species. The underlying rationale of the hypothesis is that, via genetic drift, males from the derived species would have changed to a greater extent than males from the ancestral species. In our case, *I. graellsii* has a more restricted range than *I. elegans* (see Introduction), and would hence be equivalent to the "derived species," according to Kaneshiro's hypothesis. Therefore, the finding of the strong premating (mechanical) isolation in the case of *I. graellsii* males and *I. elegans* females is consistent with a modified version of Kaneshiro's hypothesis.

EVOLUTION OF THE ISOLATION MECHANISMS

Speciation often involves the evolution of numerous prezygotic and postzygotic isolating barriers between divergent populations, and we have measured 19 prezygotic and postzygotic barriers to reproduction between our study species. Detailed knowledge of the strength and nature of those barriers provides insight into ecological and genetic factors that directly or indirectly influenced their origin, and may help predict whether they will be maintained in the face of sympatric hybridization and introgression and is central to establishing general principles of species formation. Previous studies uncovered over 10 distinct reproductive barriers that act over the entire life cycle (Nosil et al. 2005; Matute 2010), ranging from habitat isolation to hybrid dysfunction, although no single barrier typically impedes all gene flow. The formation and evolution of early-acting isolating barriers has been suggested to be governed by reinforcement (Dobzhansky 1940), a process by which natural selection increases RI between populations (Matute 2010). Under such a scenario, prezygotic isolating mechanisms are expected to evolve more quickly between sympatric than allopatric species pairs (Coyne and Orr 1989), and this has been observed in several taxa such as *Drosophila* (Coyne and Orr 1989, 1997), and other animal species (e.g., Noor 1995; Coyne and Orr 2004).

In summary, the importance of multiple isolating barriers in preventing hybridization and introgression has been reported in other insects, and often involves multiple premating barriers (Mendelson and Shaw 2002; Bailey et al. 2004). Our results together with other studies in plants (Ramsey et al. 2003; Kay 2006) and fishes (Mendelson 2003; Mendelson et al. 2004, 2007) reinforce the hypothesis that the relative contribution of prezygotic isolation to total isolation is high. Moreover, our study highlights the general need to quantify the relative contributions of potential barriers to gene flow in other species pairs if we want to gain a better understanding how RI evolves and whether there are general patterns in the accumulation of RI between different taxa. The lack of thorough studies and the overwhelming concentration on a few or single isolating barriers suggests that many other genetic barriers have been overlooked in the past.

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Supporting Information

The following supporting information is available for this article:

Table S1. Population frequency counts of *Ischnura elegans* and *I. graellsii* in two sympatric Spanish regions; La Rioja (T. Latasa, unpubl. data) and the community of Valencia (Baixeras et al., 2006).

Figure S1. The relationship between the time until interruption of copulation and sperm volume in the bursa and spermatheca of *I. elegans* and *I. graellsii* females mated to conspecific males.

Supporting Information may be found in the online version of this article.

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