



Rapid evolution of prezygotic barriers in non-territorial damselflies

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A central question in evolutionary biology concerns the accumulation of reproductive barriers during speciation. However, separating the reproductive barriers that have led to speciation from those that have secondarily accumulated (i.e. after initial divergence) is a widely recognized problem. Ideal candidate species for overcoming this problem are young species, where time for additional barriers to accrue has been limited. In the present study, we add to previous studies investigating the strength of reproductive barriers between the parapatric damselflies *Ischnura elegans* and *Ischnura graellsii* by quantifying seven prezygotic barriers between the allopatric pairs of *I. elegans* and *Ischnura genei*, as well as *I. graellsii* and *I. genei*. Specifically, we measured four premating (temporal, sexual, mechanical I, and mechanical II) and three postmating (oviposition success, fecundity, and fertility) barriers using experimental approaches and, for first time, we investigated the mechanisms causing mechanical isolation, which is the strongest reproductive barrier in ischnurans. The findings of the present study support the notion that premating barriers are generally strong and contribute significantly to total reproductive isolation in young lineages (65–98%), although they never solely lead to complete isolation. Asymmetry was generally stronger in premating than in postmating barriers, and was driven mostly through asymmetry in mechanical isolation, which is caused by morphological divergence of secondary sexual appendages. We found that barriers act multiplicatively in all species combinations tested, with the exception of sexual isolation, which was not detected. Our results are consistent with a recent allopatric speciation scenario driven by differences in male anal appendages, either impeding copulation or affecting female preferences. Taken together, the results from this and previous studies in diverse odonate genera suggest that premating barriers have evolved rapidly in ischnuran damselflies and, although reproductive isolation in ischnurans is more commonly the result of several barriers acting together, morphological divergence of secondary sexual appendages appears to be a common factor facilitating premating isolation in this group. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: asymmetric reproductive isolation – odonata – prezygotic isolation – speciation.

INTRODUCTION

The accumulation of reproductive barriers during speciation has been a central question in evolutionary biology. Some reproductive barriers may evolve to

completion before others, such that a single reproductive barrier contributes disproportionately to the initial species' boundary (Mendelson, 2003; Coyne & Orr, 2004). More commonly, however, several reproductive barriers act together to prevent genetic exchange between lineages (Dopman, Robbins & Seaman, 2010; Sánchez-Guillén, Wellenreuther & Cordero-Rivera, 2012) and the relative importance of

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these barriers varies widely among groups. Knowledge about the order in which reproductive barriers accumulate allows the identification of which isolating mechanisms contribute disproportionately to the cessation of gene flow in a given lineage at the initial start of divergence. Identification of the first barriers that promote lineage divergence requires the study of young lineages to minimize the effect of additional barriers that evolve with increasing lineage divergence (Mendelson, Imhoff & Venditi, 2007).

Reproductive barriers are typically categorized depending on whether they take place before (pre mating) or after mating (post mating) and before (prezygotic) or after zygote formation (postzygotic) (Dobzhansky, 1937). Pre mating barriers take place before mating, whereas post mating prezygotic barriers act after mating or gametic contact and reduce the probability of successful fertilization. Postzygotic barriers act by reducing the fitness of hybrids and therefore the probability of genetic introgression (Dobzhansky, 1937). Frequently, the strength with which these reproductive barriers prevent hybrid formation differs between reciprocal crosses, a phenomenon termed asymmetric isolation. This asymmetry is common across a wide range of taxa (Coyne & Orr, 2004), including invertebrates (Coyne & Orr, 1989; Presgraves, 2002; Sánchez-Guillén *et al.*, 2012), vertebrates (Mendelson, 2003), and plants (Turelli & Moyle, 2007; Lowry *et al.*, 2008). Examples of animals showing asymmetry in prezygotic barriers include fruit flies (Kaneshiro, 1980), salamanders (Arnold, Verrel & Tilley, 1996), snakes (Shine *et al.*, 2002), and damselflies (Sánchez-Guillén *et al.*, 2012), although these studies have mainly focused on sexual isolation (Kaneshiro, 1980). By contrast, studies investigating asymmetry in postzygotic barriers have predominantly focused on reinforcement (Yukilevich, 2011), Darwin's corollary (Turelli & Moyle, 2007), and Haldane's rule (Haldane, 1922) in plants (Turelli & Moyle, 2007) and fishes (Bolnick *et al.*, 2008; Polačik & Reichard, 2011). Asymmetry in prezygotic isolation (sexual isolation) is predicted to be a transient stage characteristic of intermediate stages of divergence (Arnold *et al.*, 1996) or an outcome of reinforcement (Yukilevich, 2011), whereas asymmetry in post mating isolation requires genetic interactions involving genes to be asymmetrically transmitted (Turelli & Moyle, 2007).

The damselfly genus *Ischnura* is one of the most diverse genera of the family Coenagrionidae (Odonata), including approximately 70 species (Dumont, 2013). This genus consists of nonterritorial damselflies where males compete for mates in a scramble fashion (Cordero-Rivera, 2000). This genus represents an ideal model system to explore the accumulation of reproductive barriers during different

stages of lineage divergence because of many of the species are very young (Sánchez-Guillén, Córdoba Aguilar, Cordero Rivera & Wellenreuther, 2014), can easily be observed in the field, and are suitable for laboratory experimental manipulation of reproductive behaviours. In previous studies, we have investigated the mechanisms leading to reproductive isolation between the sister parapatric species pair *Ischnura elegans* and *Ischnura graellsii* by measuring 19 pre- and postzygotic barriers (Monetti, Sánchez-Guillén & Cordero Rivera, 2002; Sánchez-Guillén, Van Gossum & Cordero-Rivera, 2005; Sánchez-Guillén *et al.*, 2012; Sánchez-Guillén, Córdoba-Aguilar & Cordero-Rivera, 2013a) and investigated whether reproductive isolation is correlated with genetic divergence in this and other damselfly genera (Sánchez-Guillén *et al.*, 2014). In these studies, we found that isolation is highly asymmetric and that mechanical isolation is the strongest component of isolation, and also detected a positive relationship between genetic divergence and the degree of reproductive isolation between taxa. In the present study, and unlike previous work, we investigated whether these previous findings hold when more species combinations are tested by quantifying the barriers causative of reproductive isolation between *I. elegans* and *Ischnura genei* and *I. graellsii* and *I. genei*. Furthermore, we are also investigating for the first time the mechanisms behind the strong mechanical isolation between ischnuran species by conducting a morphological analysis of the secondary genitalic structures and body size. We compare the strength of reproductive barriers in *I. elegans*, *I. genei*, and *I. graellsii*, which are all in the early stages of species divergence (the percentage of genetic divergence for cytochrome oxidase II is 0.31 between *I. elegans* and *I. genei*, and 0.09 between *I. genei* and *I. graellsii*) (Carchini, Cobolli, De Matthaëis & Utzeri, 1994; Sánchez-Guillén *et al.*, 2014), making them suitable candidates for the investigation of reproductive barriers causative in the cessation of gene flow. All three species occur in the Mediterranean basin, although their distributions are almost completely allopatric (Fig. 1). *Ischnura elegans* is the most widespread European damselfly, which extends from Ireland to Asia, whereas *I. graellsii* and *I. genei* are endemic species of Iberia and North Africa, and the Tyrrhenian Islands, respectively (Boudot *et al.*, 2009). In Spain, *I. elegans* and *I. graellsii* overlap at their distribution boundaries, forming a sympatric region where they hybridize and, consequently, genes of *I. graellsii* are introgressed into the *I. elegans* gene pool (Sánchez-Guillén *et al.*, 2011). *Ischnura genei* and *I. elegans* are allopatric but eventually come into contact and also show a pattern of introgressive hybridization of *I. genei* genes into the *I. elegans* gene pool (Sánchez-Guillén *et al.*, 2014). Finally, *I. graellsii*

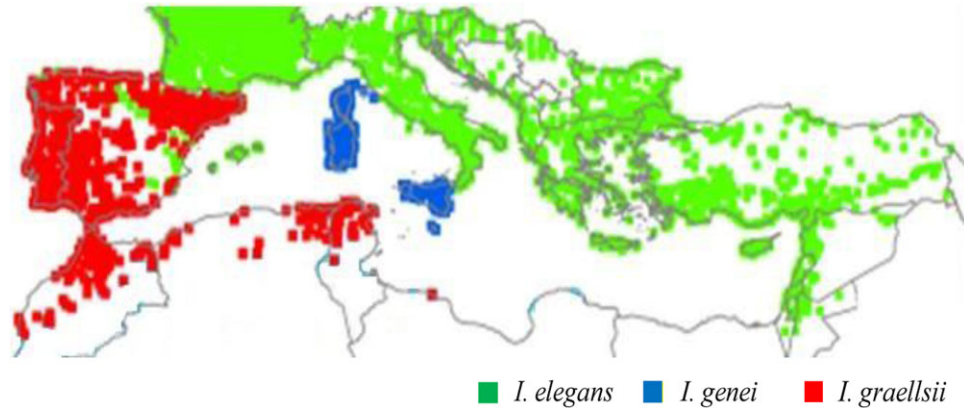


Figure 1. Mediterranean distribution of the three studied species: *Ischnura elegans*, *Ischnura genei*, and *Ischnura graellsii*.

and *I. genei* are completely allopatric. Moreover, all combinations of these three species can be induced to hybridize in the laboratory. Together, the aforementioned characteristics make these damselflies ideal model species to (1) identify initial reproductive barriers; (2) understand which barriers evolve most rapidly; and (3) identify possible asymmetry in barriers. We examined these aspects by quantifying the strength of seven prezygotic barriers (acting before fertilization) in the form of four premating (temporal, sexual, and two forms of mechanical) and three postmating (oviposition success, fecundity, and fertility) barriers, between *I. genei* and *I. elegans*, as well as between *I. genei* and *I. graellsii*, in both reciprocal cross directions using experimental approaches in the laboratory.

MATERIAL AND METHODS

LABORATORY REARING

Last-instar larvae of approximately 200 *I. elegans* (42°45'29.64"N, 9°5'39.84"W) and 200 *I. graellsii* (42°27'12.41"N, 8°50'54.88"W) individuals, both from allopatric Spanish populations, were sampled in August 2009. Additionally, eggs (approximately 1000) of 22 *I. genei* females (37°51'.54"N, 13°14'.38" and 37°01'.31"N, 14°20'.37"W) from allopatric Sicilian populations were collected in June 2009 by allowing females to oviposit on wet filter paper after capture. Resulting clutches were transported to the laboratory and maintained until larvae hatched (for details of the larval rearing methodology, see Sánchez-Guillén, Van Gossum & Cordero-Rivera, 2005; Van Gossum, Sánchez-Guillén & Cordero-Rivera, 2003), resulting in approximately 100 adults for each of the three species. Note that mortality at the last instar is almost zero, whereas mortality from after eggs hatching until the last-instar larvae (under laboratory conditions) is

almost 90% (Van Gossum *et al.*, 2003). Emerging individuals were immediately moved to small glass jars where they were kept for 1 day. Animals were then housed in insectaries (50 × 50 × 50 cm) separated by sex and species.

After females (4–8 days old) and males (4–6 days old) reached sexual maturation, they were used for the different experiments. Each individual was used only once in a mating experiment to ensure that all experimental individuals were virgins, and to prevent mechanical damage, sperm depletion, and pseudoreplication (Sánchez-Guillén *et al.*, 2012). After matings, females were allowed to oviposit up to three clutches. Egg clutches were maintained in small Petri dishes until hatching (which took approximately 15 days). We obtained conspecific crosses of *I. genei* and crosses, in both reciprocal directions, between *I. genei* and *I. elegans*, and between *I. genei* and *I. graellsii*. Data on fecundity and fertility of conspecific crosses of *I. elegans* and *I. graellsii* (from the same populations and which were maintained and under the same conditions in the laboratory) were obtained from a previous study (Sánchez-Guillén *et al.*, 2012). Additionally, larvae and adults were reared and maintained under the same conditions in both studies.

Calculation details of the absolute contribution of each barrier, four prezygotic barriers (temporal, sexual, mechanical_I, and mechanical_{II}) and the three postmating prezygotic barriers (oviposition success, fecundity, and fertility) are summarized in Table 1. Results are provided as the mean ± SE unless stated otherwise.

MORPHOLOGICAL ANALYSIS: SECONDARY GENITALIC STRUCTURES AND BODY SIZE

The anatomical mis-match of the male's anal appendages and female pronotum causes mechanical isolation

Table 1. Formulae of the index of reproductive isolation (RI) of each isolation barrier: four pre mating prezygotic barriers (temporal, sexual, mechanical I, and mechanical II) and three post mating prezygotic barriers (oviposition success, fecundity and fertility)

Barrier	Isolation measure	Range	Formula
Premating	Temporal	0 to 1	$RI_{\text{temporal}} = 1 - (\text{conspecific matings of both species overlapping during the day})/(\text{total observed matings})$
	Sexual	-1 to 1	$RI_{\text{sexual}} = 1 - (\text{observed heterospecific tandem attempts})/(\text{heterospecific tandem attempts expected under random mating})$
	Mechanical I	0 to 1	$RI_{\text{mechanical I}} = 1 - (\text{tandems}/\text{attempts tandem})$
	Mechanical II	0 to 1	$RI_{\text{mechanical II}} = 1 - (\text{matings}/\text{tandems})$
Postmating, prezygotic	Oviposition	-1 to 1	$RI_{\text{oviposition}} = 1 - (\% \text{ females oviposited heterospecific matings}/\% \text{ females oviposited conspecific matings})$
	Fecundity	-1 to 1	$RI_{\text{fecundity}} = 1 - (\text{mean fecundity heterospecific matings}/\text{mean fecundity conspecific matings})$
	Fertility	-1 to 1	$RI_{\text{fertility}} = 1 - (\text{mean fertility heterospecific matings}/\text{mean fertility conspecific matings})$

in ischnurans damselflies (Sánchez-Guillén *et al.*, 2012) and in many related damselflies (Paulson, 1974; Robertson & Paterson, 1982; McPeck *et al.*, 2008; McPeck, Shen & Farid, 2009). This is because copulation is achieved when males grasp females by their pronotum (tandem position), using their anal appendages and, subsequently, females blend their abdomen to bring the genitalia into contact (wheel position). Differences in anal appendages have been reported between the three sister species. For this reason, the posterior male anal appendages (cerci and paraprocts) and the lateral female pronotum were described for the three species using a binocular microscope and digitized using a camera (Nikon Digital Sight DS-F11). Additionally, body lengths of males and females were measured using 278 samples (138 males and 140 females, from different allopatric populations) of the three species to test the prediction proposed by Grant & Grant (1997) that mechanical isolation can prevent hybridization between the male of the small species with the female of the bigger species. Multiple pairwise comparisons were performed using Dunn's procedure, with a Bonferroni corrected significance level of 0.0167.

PREMATING PREZYGOTIC BARRIERS

Experiment 1: temporal isolation

Ischnura elegans, *I. graellsii*, and *I. genei* have similar phenologies across both their sympatric (*I. elegans* and *I. graellsii*) and allopatric (*I. elegans*, *I. graellsii*, and *I. genei*) areas of distribution (Dijkstra & Lewington, 2006). However, because we investigated the strength of reproductive barriers between allopatric populations in the present study, it was not possible to estimate the seasonal components of temporal isolation in the wild. Instead, we measured the timing of

reproductive activity of all study species in the laboratory under a common setting. To estimate the amount of temporal isolation, we compared the time during the day at which conspecific copulations take place (only using males and females that were fully sexually mature, which occurred when adults were 4–6 or 4–8 days old, depending on the species). For this experiment, every day, we placed all mature males and all conspecific mature females in the focal insectary. Observations lasted from 07.00–19.00 h (clock time) and included conspecific copulations of *I. genei* ($N = 11$), which started at 07.47 h and finished at 14.02 h. We used data of conspecific *I. graellsii* ($N = 38$) copulations, which started at 11.03 h and finished at 17.15 h, and data of *I. elegans* ($N = 17$) copulations, which started at 13.31 h and finished at 17.37 h copulations from Sánchez-Guillén *et al.* (2012).

Experiment 2: sexual isolation

Sexual isolation was estimated as a male's attempt to mate (grasp females by their pronotum) with a conspecific versus heterospecific female. For this, we placed 10 males and five conspecific and five heterospecific females in the same insectary. The following species combinations were used: (1) *I. elegans* males, *I. elegans* females, and *I. genei* females; (2) *I. genei* males, *I. genei* females, and *I. elegans* females; (3) *I. graellsii* males, *I. graellsii* females, and *I. genei* females; and (4) *I. genei* males, *I. genei* females, and *I. graellsii* females. Observed and expected (under random mating) tandem attempts were compared using chi-squared tests (Table 2).

Experiment 3: mechanical isolation

Mechanical isolation was estimated by measuring incompatibility between the male cerci and female

Table 2. Estimates of sexual isolation in insectaries in laboratory

Species combination	Males		Females conspecific		Females heterospecific		Observed attempts to mating		Expected attempts to mating		P
			conspecific	heterospecific	conspecific	heterospecific	Conspecific	Heterospecific	Conspecific	Heterospecific	
♀ <i>elegans</i> -♀ <i>genei</i> -♂ <i>elegans</i>	10	5	5	5	6	5	5.5	5.5	5.5	5	0.763
♀ <i>genei</i> -♀ <i>elegans</i> -♂ <i>genei</i>	10	5	5	5	15	9	12	12	12	6	0.221
♀ <i>graellsii</i> -♀ <i>genei</i> -♂ <i>graellsii</i>	10	5	5	5	4	8	6	6	6	5	0.248
♀ <i>genei</i> -♀ <i>graellsii</i> -♂ <i>genei</i>	10	5	5	5	6	4	5	5	5	5	0.527

The number of conspecific and heterospecific attempts to tandem observed in insectaries and expected under random mating is shown. The columns of males and conspecific and heterospecific females indicate the number of individuals used in each presentation. The last two columns represent the number of conspecific and heterospecific attempts to mating observed and expected under random mating, and the value of P, obtained from a χ^2 contingency table.

prothorax (mechanical isolation I, males attempt to grasp females but fail to engage them) and the incompatibility between male–female genitalia (mechanical isolation II, both genitalia come into contact but fail to engage) in heterospecific versus conspecific crosses (Table 3). For this experiment, we placed males of *I. elegans* with females of *I. genei*, males of *I. genei* with females of *I. elegans*, males of *I. graellsii* with females of *I. genei*, males of *I. genei* with females of *I. graellsii*, and males of *I. genei* with females of *I. genei* in the same insectary and, for each species and sex combination, we estimated the proportion of males that failed when attempting to form the tandem, as well as the proportion of males that failed when attempting copulation (genitalic contact).

POSTMATING PREZYGOTIC BARRIERS

During copulation, females receive stimulus-based information of aberrant morphology and inappropriate movements that affects the reproductive output of females even after successful copulation. This can occur, for example, when females prematurely interrupt copulation, expel sperm (Coyne & Orr, 2004) or avoid oviposition (Sánchez-Guillén *et al.*, 2012).

Experiment 4: gametic isolation

Oviposition success, fecundity, and fertility were measured in all heterospecific crosses between *I. elegans* × *I. genei* and *I. graellsii* × *I. genei* and conspecific crosses of *I. genei* that achieved copulation (for samples sizes, see Table 3). Oviposition, fecundity and fertility data for conspecific crosses between *I. elegans* and *I. graellsii* were obtained from a previous study (Sánchez-Guillén *et al.*, 2012). Reduction in oviposition success was estimated by comparing the percentage of females mated by a conspecific male that oviposited versus the percentage of females mated by a heterospecific male that oviposited. Isolation by a reduction in the fecundity of females (measured as the number of eggs in the first three clutches) and by a reduction of fertility (proportion of fertile eggs) were compared between conspecific and heterospecific pairs. We considered all eggs as fertile if they hatched or showed a developing embryo, aiming to minimize biases as a result of laboratory conditions (Sánchez-Guillén *et al.*, 2012).

TOTAL REPRODUCTIVE ISOLATION

Cumulative reproductive isolation for each species combination was estimated using Eqns 1–6 in Ramsey, Bradshaw & Schemske (2003). The effect of reproductive barriers that act later in the mating cycle is reduced by the strength of earlier barriers. In our case, the chronological order is: temporal, sexual,

Table 3. Estimates of pre- and postmating prezygotic isolation in insectaries in the laboratory: mechanical isolation, oviposition, fecundity, and fertility

Species combination	N	Tandem-attempt			Mating	Oviposition			Fecundity			Fertility		
		Tandem	Mating	Mean		N	Mean ± SE	N	Mean ± SE	N	Mean ± SE			
												N	Mean	Mean ± SE
1. ♀ <i>elegans</i> -♂ <i>elegans</i>	NA	NA	NA	NA	8	1.00	8	496.00 ± 129.30	8	0.83 ± 0.04				
2. ♀ <i>graellsii</i> -♂ <i>graellsii</i>	NA	NA	NA	NA	20	1.00	17	603.53 ± 62.16	18	0.97 ± 0.01				
3. ♀ <i>genei</i> -♂ <i>genei</i>	15♂, 15♀	14	13	13	11	1.00	11	493.64 ± 59.08	11	0.87 ± 0.04				
4. ♀ <i>genei</i> -♂ <i>elegans</i>	15♂, 15♀	13	13	10	11	0.91	10	310 ± 45.66	10	0.57 ± 0.07				
5. ♀ <i>elegans</i> -♂ <i>genei</i>	15♂, 15♀	19	1	1	1	0	0		0					
6. ♀ <i>genei</i> -♂ <i>graellsii</i>	15♂, 10♀	16	11	11	10	0.70	7	252.14 ± 72.51	10	0.80 ± 0.08				
7. ♀ <i>graellsii</i> -♂ <i>genei</i>	20♂, 15♀	55	22	13	11	0.91	10	303.27 ± 59.31	10	0.79 ± 0.09				

Columns of attempts to tandem, tandem, and matings represent the absolute number of the observed sexual activity. Sample size (*N*) indicates the number of females. NA, cases where the isolation index could not be estimated. Data of (oviposition, fecundity, and fertility) of conspecific crosses of *Ischnura elegans* and *Ischnura graellsii* are from a previous study (Sánchez-Guillén *et al.*, 2012).

mechanical isolation, oviposition success, fecundity, and fertility. The relative contribution of each reproductive barrier is estimated as the ratio of its absolute contribution to the total strength of reproductive isolation. Total reproductive isolation was calculated as the sum of all relative contributions. Calculation details of the absolute contribution of each barrier of reproductive isolation are summarized in Table 1.

RESULTS

MORPHOLOGICAL ANALYSIS

Males and females of different species differed in body size (males: Kruskal–Wallis test = 73.49, $P < 0.0001$; females: Kruskal–Wallis test = 74.034, $P < 0.0001$). The body size of *I. elegans* males (30.39 ± 0.23 mm) was the largest of all males. Similarly, females *I. elegans* were larger (31.06 ± 0.22 mm) than females of *I. graellsii* and *I. genei* (Fig. 2). However, body size distributions of *I. graellsii* and *I. genei* males and females were similar (males ranged from 26.80 to 27.01 mm and females from 27.01 to 27.34 mm, respectively) (Fig. 2). Visual microscope investigations of female pronotum and male secondary genitalia (i.e. male anal appendages; cerci and paraprocts) revealed that *I. elegans* can also be separated from the other two species through the upright projection of the pronotum hind border, and through the overall larger size of the pronotum, compared to the other species (Fig. 2), and further in males because of the parallel anal cerci and long paraprocts (Fig. 2). Anal cerci are morphologically different between the species; namely, in *I. graellsii*, the cerci are parallel but, in *I. genei*, they cross over. In both species, paraprocts are shorter compared to *I. elegans*. In addition, in these two species, the pronotum is also shorter than in *I. elegans* (Fig. 2).

PREMATING PREZYGOTIC BARRIERS

In both species, combinations of *I. genei* × *I. elegans* and *I. genei* × *I. graellsii*, we detected significant temporal and mechanical isolation, whereas sexual isolation was not detected (i.e. matings were random with respect to species status).

In the first species combination, between *I. genei* and *I. elegans*, temporal isolation impeded 50% of the heterospecific interactions (i.e. only 25 out of the 50 observed conspecific matings of *I. elegans* and of *I. genei* overlapped temporally). Between *I. genei* females and *I. elegans* males, we detected random mating (i.e. the observed and expected attempts to tandem were similar) (Table 2). Furthermore, although mechanical isolation did not impede tandem formation, it did impede 23.07% of copulations (Table 3). Matings between *I. elegans* females and

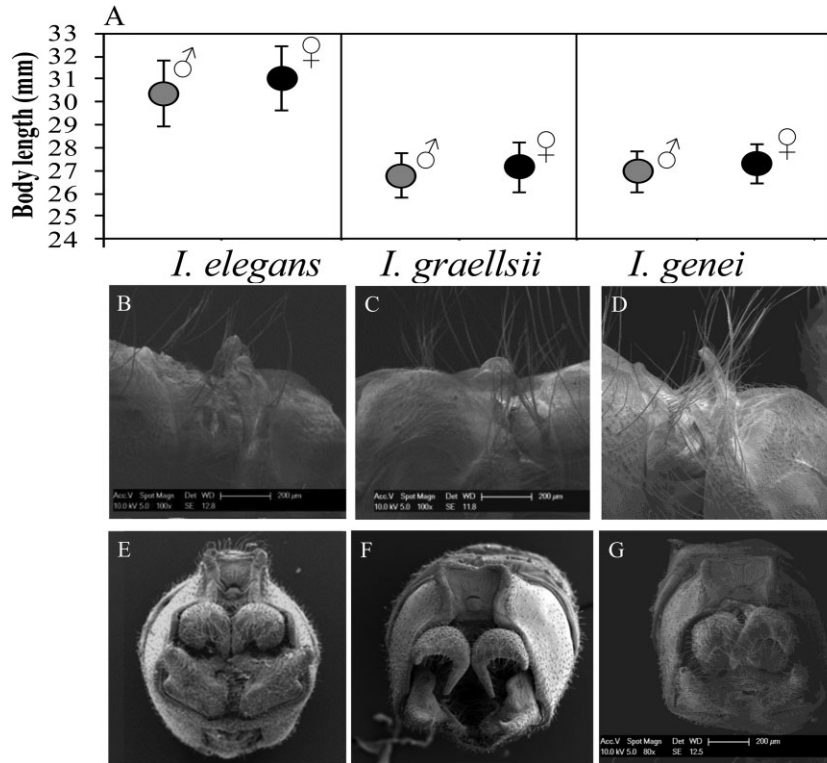


Figure 2. Body length (A). Anterior view of the pronotum of the female (B–D) and male abdominal appendages in anal view (E, F, G) for the three sister species (*Ischnura elegans*, *Ischnura graellsii*, and *Ischnura genei*), respectively.

I. genei males were also random (Table 2). However, although mechanical isolation impeded in 95% of tandems in this cross direction, some resulted in copulation (one copulation of a total of 19 tandem attempts) (Table 3).

In the second species combination, between *I. genei* and *I. graellsii*, temporal isolation prevented a high proportion of putative heterospecific matings between *I. genei* and *I. graellsii* because only 30% (eight of the 27) of conspecific matings overlapped temporally. Between *I. genei* females and *I. graellsii* males, there was no sexual isolation (Table 2) and, although mechanical isolation impeded 31% of tandems, it had no effect on the copulation probability (Table 3). Between *I. graellsii* females and *I. genei* males, matings were also random (Table 2) and mechanical isolation prevented 60% of tandems and 41% of copulations (Table 3).

POSTMATING PREZYGOTIC BARRIERS

In heterospecific crosses of *I. genei* × *I. elegans* and *I. genei* × *I. graellsii*, we were able to quantify partial isolation in all three postmating barriers. Ninety-one percent ($N = 11$ females) of *I. genei* females that mated with *I. elegans* males oviposited eggs but females were less fecund (310 ± 45.66 ; $U = 23.0$, $P = 0.024$, Mann–

Whitney U -test) and eggs less fertile [57%; generalized linear model (GLM) with binomial errors and logit link, deviance ratio = 21.32, $P < 0.001$] compared to conspecific combinations (fecundity: 493.6 ± 59.08 ; fertility: 87%) (Table 3). In the only mating obtained between *I. elegans* females × *I. genei* males, the female did not oviposit (Table 3).

Seventy percent ($N = 10$ females) of *I. genei* females that mated with *I. graellsii* males oviposited but females were again less fecund (252 ± 72.5 ; $U = 14.0$, $P = 0.027$, Mann–Whitney U -test), although they had similar fertility (80%; GLM with binomial errors and logit link, deviance ratio = 0.03, $P = 0.866$) relative to conspecific combinations (fecundity: 493 ± 59.08 ; fertility: 87%) (Table 3). When *I. graellsii* females mated with *I. genei* males, 91% ($N = 11$ females) of the females oviposited. These females again were less fecund (303 ± 59.31 ; $U = 31.0$, $P = 0.002$, Mann–Whitney U -test) and less fertile (79%; GLM with binomial errors and logit link, deviance ratio = 16.49, $P < 0.001$) relative to conspecific crosses (fecundity: 603.05 ± 62.16 ; fertility: 97%) (Table 3).

TOTAL REPRODUCTIVE ISOLATION

Temporal isolation between crosses was important and prevented between 50–70% of possible interactions

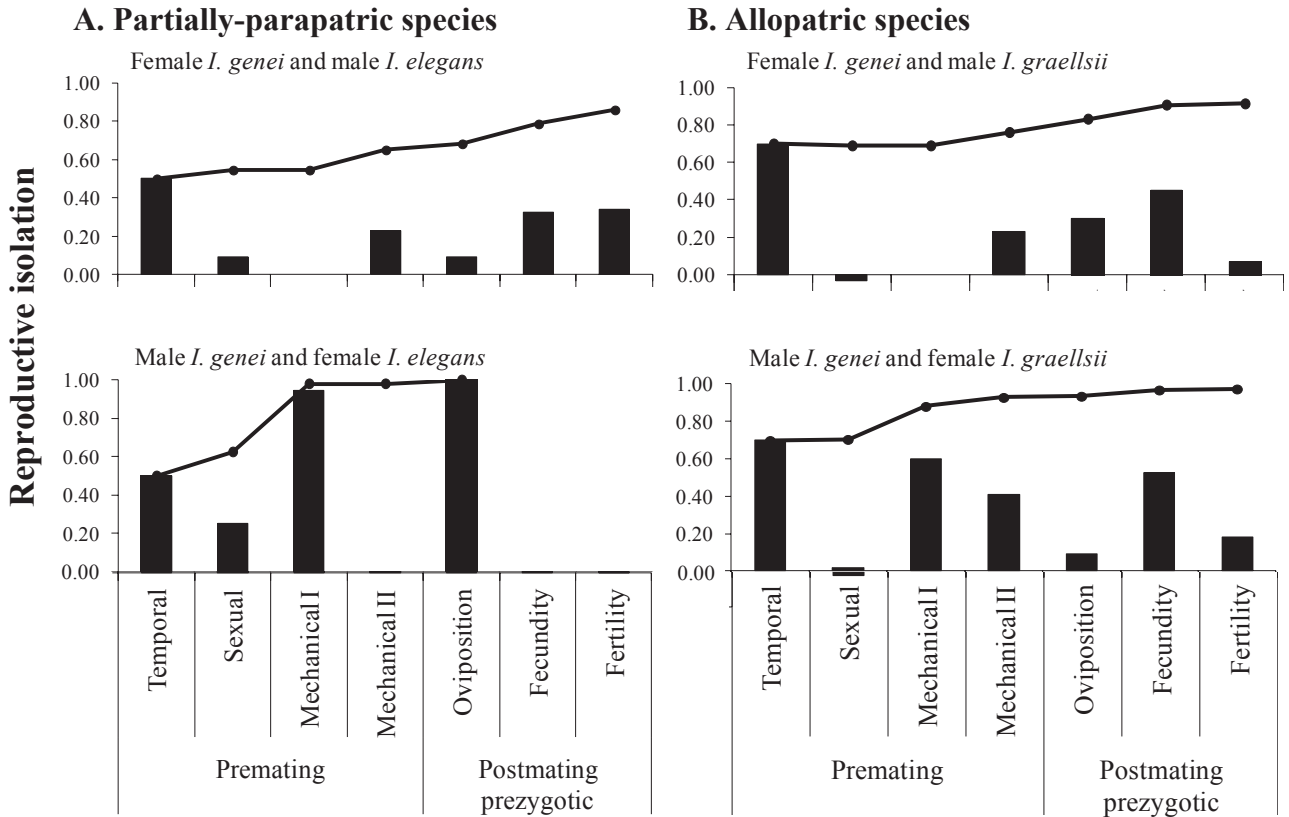


Figure 3. Total reproductive isolation. Absolute contribution (represented by bars) and relative accumulated contribution (represented by lines) of each reproductive barrier to total reproductive isolation between *Ischnura genei* × *Ischnura elegans* (A) and *Ischnura genei* × *Ischnura graellsii* (B).

between pairs. Likewise, mechanical isolation was also a strong barrier between crosses and acted highly asymmetrically between crosses: *I. elegans* females × *I. genei* males (98%) (Fig. 3A), *I. genei* females × *I. elegans* males (65%) (Fig. 3A); *I. graellsii* females × *I. genei* males (76%) (Fig. 3B), and *I. genei* females × *I. graellsii* males (31%) (Fig. 3B). No evidence was found for sexual isolation.

Postmating prezygotic barriers were generally less strong and asymmetric (Fig. 3). Between *I. genei* females and *I. elegans* males, postmating isolation reduced 21% of zygote formations but, between *I. elegans* females and *I. genei* males, the reduction was only 2% (Fig. 3A). Between *I. genei* females and *I. graellsii* males, the reduction in zygote formation was 12%, whereas zygotes between *I. graellsii* females and *I. genei* males were prevented in 3% of cases (Fig. 3B).

Total prezygotic (premating and postmating) isolation between *I. genei* females and *I. elegans* males was 86% (Fig. 3A) and much lower than between *I. elegans* females and *I. genei* males, which was complete (Fig. 3A). Between *I. genei* females and *I. graellsii* males, total prezygotic was also high (93%) (Fig. 3B) and similar to the total prezygotic isolation index

obtained for *I. graellsii* females and *I. genei* males (99%) (Fig. 3B).

DISCUSSION

The present study investigated reproductive barriers in two *Ischnura* species combinations (*I. genei* × *I. elegans* and *I. genei* × *I. graellsii*) that are sister species, by quantifying the strength of isolating barriers using experimental approaches in the laboratory. *Ischnura genei* and *I. elegans* are mainly allopatric, although they occur sympatrically over a small part of their geographical distribution and, where this occurs, the species show extensive introgression (Sánchez-Guillén *et al.*, 2014). *Ischnura genei* and *I. graellsii* are allopatric across their range. In the laboratory, both species combinations can easily hybridize, suggesting that geographical isolation is fundamental to species persistence in the latter pair. Overall, the present study shows that premating barriers in the studied species are generally strong and contribute disproportionately to reproductive isolation, which is consistent with previous findings on barriers between *I. elegans* and

I. graellsii. We also found that multiple barriers are at work in all species combinations, and that some barriers are highly asymmetric. Below, we discuss premating and postmating barriers in turn and assess their importance in species maintenance, and, lastly, speculate what the strength of barriers can tell us about possible speciation routes in this group.

PREMATING PREZYGOTIC BARRIERS

We did not detect signs of sexual barriers between the study species, which is consistent with previous studies investigating *I. elegans* and *I. graellsii* (Sánchez-Guillén *et al.*, 2012), as well as several nonterritorial damselfly genera without courtship, such as *Lestes* and *Enallagma* (Turgeon *et al.*, 2005). Future work should aim to increase the sample sizes of experiments investigating sexual premating barriers in odonates and test whether sexual isolation is indeed absent in this group. Ischnuran species show no pre-copulatory courtship, which raises the question of how males recognize females. Males and at least one of the several female morphs have colourful (predominantly, green and blue patterns) body patches, and stripes, whereas there is consistently one pale-like female morph (Van Gossum *et al.*, 2008). We are unaware of any work in our study species in relation to the use of such body colour variation in mate discrimination. However, a recent study in *Ischnura heterosticta* indicates that males are able to find and discriminate female morphs on the basis of female body colour (Huang *et al.*, 2013). This result may apply to other ischnuran species (including those we used in the present study) given their relatively uniform body colour pattern and sexual biology. Males of *I. elegans* show an innate preference for the male-mimicking androchrome female colour morph (for colour morph descriptions, see Sánchez-Guillén *et al.*, 2005) but undergo ontogenetic changes in male mate preferences towards a preference for gynochrome, likely as a result of learned mate recognition (Sánchez-Guillén *et al.*, 2013b). This is in contrast to studies performed in other groups where sexual isolation can be fundamental in starting divergence between lineages (Coyne & Orr, 2004) and the rapid diversification of species groups (Price, 1998; Gray & Cade, 2000; Boake, 2005; Mendelson & Shaw, 2005), especially in damselfly species with strong sexual selection (for theoretical treatment, see McPeck & Gavrillets, 2006; Wellenreuther, Vercken & Svensson, 2010). Our study indicates that sexual isolation is less important in odonate species lacking courtship and elaborated secondary sexual traits (Cordero, 1989), which contrasts strongly with work on territorial damselflies with courtship, such as species in the genus *Calopteryx* (Córdoba-Aguilar & Cordero Rivera, 2005).

By contrast to sexual isolation, we found evidence that temporal isolation does minimize the window of reproductive overlap between species (reproductive activity overlapped 30–50%), which is consistent with previous studies in *I. elegans* and *I. graellsii* (Sánchez-Guillén *et al.*, 2012) and many studies showing that this barrier is indeed an important isolating mechanism in insects (Hölldobler, 1976). However, there is little evidence of significant temporal isolation in other taxa, except for corals and some algal species (Coyne & Orr, 2004). Analyses of mechanical isolation showed that this barrier is pervasive in all species combinations and, overall, the strongest barrier preventing reproduction. Premating structural isolation, presumably caused by a mis-match between the male anal appendages and mesostigmal plates of the female pronotum (mechanical isolation _I), was important in males with short anal appendages and females with large pronotum (60–95%). This situation occurs, for example, when *I. genei* males mate with *I. elegans* and *I. graellsii* females. Our results are consistent with previous studies in *I. elegans* and *I. graellsii* (Sánchez-Guillén *et al.*, 2012), as well as *Enallagma* and *Argia* (Paulson, 1974; Robertson & Paterson, 1982), in which matings were also impeded between males with short anal appendages and females with a large pronotum. However, by contrast to the prediction of Grant & Grant (1997) (working with birds) suggesting that mechanical isolation can prevent hybridization between the male of the small species with females of the bigger species, differences in body size alone could not explain mechanical isolation. Although there was no mechanical isolation between the largest male (*I. elegans*) and the shortest females (*I. graellsii* and *I. genei*), mechanical isolation was almost complete between short males (*I. graellsii* and *I. genei*) and large females (*I. elegans*). The asymmetry in mechanical isolation in reciprocal crosses of *I. graellsii* and *I. genei* could not be explained by differences in body size because both species have similar body sizes (Fig. 2a). In damselflies that lack pre-copulatory courtship, body size and intromittent genital size does not scale allometrically (Nava-Bolaños *et al.*, 2014). Consistent with this, we detected that incompatibility between primary male and female genitalia (mechanical isolation _{II}) impeded 0% to 40% of copulations, independently of the body size. Taken together, these mechanical premating barriers decrease copulation success in the different species combinations to a significant degree (range: 23–95%).

Current theory predicts that, in species with strong sexual selection, sexual conflict may act on male traits and female preferences to drive the evolution of pre- and postmating prezygotic barriers through mechanical isolation (both structural and tactile)

(Coyne & Orr, 2004). For example, in Diplopoda, divergence in genital shape appears to contribute to reproductive isolation among isolated populations (Wojcieszek & Simmons, 2013). As a result of mechanical incompatibilities, gene flow between species can either be impeded before mating, by the incompatibility between morphological features, or after mating because of aberrant morphology or inappropriate movements, prompting the female to prematurely interrupt copulation or to expel sperm (Coyne & Orr, 2004). Studies in beetles (Eberhard, 2002), butterflies (Lorkovic, 1958), damselflies (Paulson, 1974; Robertson & Paterson, 1982), fruit flies (Coyne & Orr, 2004), and plants (Niklas, 1982) support the central role of mechanical isolation in reproductive isolation. In damselflies, for example, the rapid development of mechanical isolation through the divergence of anal appendages is common in genera such as *Ischnura*, *Enallagma*, and *Argia* (Paulson, 1974; Robertson & Paterson, 1982; McPeck *et al.*, 2008; Sánchez-Guillén *et al.*, 2012) and it has been suggested that the driving process of this divergence are antagonistic mating interactions and sexual conflict (Cordero-Rivera & Córdoba-Aguilar, 2010), as well as divergent ecological selection (McPeck *et al.*, 2008).

POSTMATING PREZYGOTIC BARRIERS

Postmating isolation, likely caused by species-specific differences in mating interactions and cues (e.g. tactile interactions during copulation), reduced the reproductive output of females between 9% and 100%, which is similar to heterospecific crosses between *I. graellsii* and *I. elegans* (Sánchez-Guillén *et al.*, 2012). A proportion of 9% and 30% of *I. genei* females mated with *I. elegans* and *I. graellsii* males, respectively, did not lay eggs. Additionally, 9% of *I. graellsii* females and 100% (only one female) of *I. elegans* females did not lay eggs when mated with *I. genei* males. Given the scramble competition nature of these species, as well as fitness costs associated with excessive matings in damselflies (Cordero, Santolamazza Carbone & Utzeri, 1998), females may show a general tendency to avoid matings (Gosden & Svensson, 2009). Females can avoid producing hybrids, first by not bending their abdomen to form wheel position (copulation) or by avoiding egg laying after mating. The different body lengths (and thus body weights) of the three species may exert an influence on hybrid production. In particular, when the heterospecific male is smaller in size than the conspecific male, females commonly resist copulations (e.g. by not bending her abdomen), presumably because smaller males would lack the strength to maintain the mating position for sufficient time.

However, when the heterospecific male is larger in size than conspecific males, females do not resist copulations, and the relatively large and heavy males can thus form and maintain mating wheels for long times. In the latter case, females commonly avoid subsequent egg laying. Such a situation might occur in *I. genei* females that are mated by *I. graellsii* males (which are both of similar size). Here, 23% of females avoided copulations and 30% of females did not lay eggs, whereas, in *I. graellsii* females mating with *I. genei* males (where the male is of a smaller size), 41% of females avoided copulations but only 9% avoided oviposition.

We measured two other postmating prezygotic barriers, namely reduced fecundity and reduced fertility, and detected that 10–52% of zygotes failed (compared to conspecific crosses). It has been documented that heterospecific sperm can have high fertilization success, even higher than conspecific sperm (Rugman-Jones & Eady, 2007). We did not find evidence for this and this is corroborated by heterospecific sperm precedence appearing to be absent in female *I. graellsii* that had mated with *I. elegans* males (Sánchez-Guillén *et al.*, 2013a). Finally, studies comparing the overall asymmetry of pre- versus postmating barriers have suggested that the overall asymmetry is much stronger between postmating barriers. Lowry *et al.* (2008), for example, estimated that they can be up to three times higher than premating barriers in a study on flowering plants. The present study, and a previous study on *I. elegans* and *I. graellsii* (Sánchez-Guillén *et al.*, 2012), did not support this finding and, if anything, we found that the opposite was true and that the overall asymmetry was more pronounced between premating barriers (Fig. 2).

CONCLUSIONS

The finding that premating barriers prevented most gene flow between the genetically close species *I. elegans* and *I. genei*, *I. genei* and *I. graellsii* (present study), and *I. elegans* and *I. graellsii* (Sánchez-Guillén *et al.*, 2012), suggests that premating barriers have evolved rapidly in this group, and that the morphological divergence of anal appendages and pronotum is a core factor facilitating premating isolation. Our results strongly indicate that premating prezygotic barriers are more asymmetric than postmating prezygotic barriers for preventing hybrid formation, which is consistent with previous studies showing that reproductive isolation is more commonly caused by several barriers acting together. Sexual isolation was not detected, in contrast to other odonate species where sexual selection has been implicated as a key driver of diversification

(McPeck & Gavrilets, 2006; Svensson, Karlsson & Eroukhmanoff, 2007), indicating that environmental driven range expansions and the subsequent geographical overlap of these species could lead to the species breakdown (Sánchez-Guillén *et al.*, 2011, 2013c).

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