

ARTICLE



Reproductive character displacement: insights from genital morphometrics in damselfly hybrid zones

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Reproductive Character Displacement (RCD) refers to the phenomenon of greater differences in reproductive characters between two species when they occur in sympatry compared to when they occur in allopatry to prevent maladaptive hybridization. We explored whether reinforcement of a mechanical barrier involved in the first contact point between male and female genital traits during copulation in the cross between *Ischnura graellsii* males and *Ischnura elegans* females has led to RCD, and whether it supports the lock-and-key hypothesis of genital evolution. We employed geometric morphometrics to analyze the shape and size of male and female genital traits, controlling for environmental and geographic factors. Consistent with an increase in mechanical isolation via reinforcement, we detected larger divergence in genital traits between the species in sympatry than in allopatry, and also stronger signal in females than in males. In the Northwest (NW) hybrid zone, we detected RCD in *I. graellsii* males and *I. elegans* females, while in the Northcentral (NC) hybrid zone we detected RCD only in *I. elegans* females and *I. elegans* males. The detection of RCD in both sexes of *I. elegans* was consistent with the lock-and-key hypothesis of genital evolution via female choice for conspecific males in this species. Our study highlights the importance of using geometric morphometrics to deal with the complexity of female reproductive structures while controlling for environmental and geographic factors to investigate RCD. This study contributes valuable insights into the dynamics of reproductive isolation mechanisms and genital coevolution.

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INTRODUCTION

Character displacement was defined by Brown and Wilson (1956) as a pattern in which two species with partially overlapping distributions present greater differences in certain ecological or reproductive characters where they occur in sympatry than in allopatry. When this pattern is favored by selection because it prevents maladaptive hybridization, it is called “Reproductive Character Displacement” (RCD) (Dobzhansky 1940; Servedio and Noor 2003; Coyne and Orr 2004). The decreased fitness of maladaptive hybrids leads to a natural selection process that decreases the prevalence of alleles associated with weakened ability to discriminate heterospecifics. This mechanism helps to avoid the costs of hybridization (West-Eberhard 1986), and consequently, it promotes the gradual enhancement of prezygotic isolation between species through RCD. Specifically, it promotes greater phenotypic divergence of reproductive traits when the species are in sympatry compared to an allopatric scenario (Howard and Harrison 1993). Reinforcement theory predicts increased levels of conspecific preference in females than in males, since hybridization costs are usually higher for females than for males (Coyne and Orr 2004).

Researchers who study reinforcement often view RCD as the pattern resulting from reinforcement (Dobzhansky 1936, 1940), as noted by Howard and Harrison (1993) and Servedio and Noor (2003). Pfennig and Pfennig (2009) defined RCD as the selective process where reproductive traits diverge to avoid costly

interactions with heterospecifics and included reinforcement as a form of character displacement. According to Pfennig and Pfennig (2009), this definition can help reduce confusion between RCD and reinforcement, addressing the difficulty in distinguishing pattern from process when discussing RCD. Available empirical evidence suggests that RCD plays an important role in the evolution of diversity (Pfennig and Pfennig 2005; Schluter 2000). This mechanism has been reported in diverse organisms, including insects (Brown and Wilson 1956; Walker 1974; Kawano 2002), fishes Schluter 2000; Crampton et al. 2011; Roth-Monzón et al. 2020), birds (Brown and Wilson 1956; Diamond et al. 1989; Seddon and Tobias 2010), reptiles (Melville 2002; Dayan and Simberloff 2005) and amphibians (Brown and Wilson 1956; Johanet et al. 2009).

RCD arising from reinforcement can lead to an escalation in trait divergence in either or both of the species involved (Cooley 2007). One limitation of the majority of existing studies of RCD is that they have focused on only one of the interacting species. This has left an important lack of empirical data about the patterns through which RCD arises, since increased differences in sympatry may be due to character displacement in both species (“bilateral”) or in only one of the species (“unilateral”). Unilateral displacement could result from unequal hybridization costs between the two species, biases in the likelihood of hybridization, asymmetrical effects of reproductive interference, evolutionary constraints, or historical accidents (see Cooley 2007).

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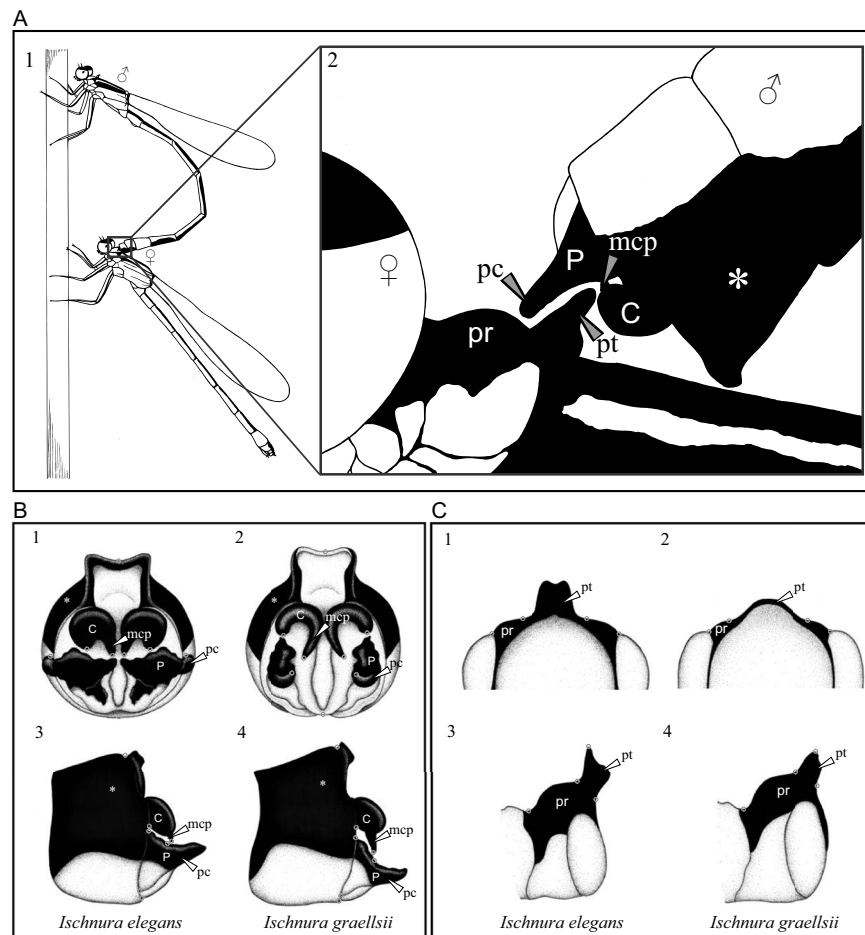


Fig. 1 Graphical representation of the tandem position and reproductive structures in *Ischnura* species. **A** The male grasps the female by her thorax with his caudal appendages (1); close-up of the point of contact: the caudal appendages, located on the last segment of the male's abdomen, and the pronotum, located on the female's prothorax (2). **B** Graphical representation and the landmark positions on the male caudal appendages of *Ischnura elegans* (1, posterior view and B3, left lateral view) and of *I. graellsii* (2, posterior view and 4, lateral view). **C** Graphical representation and the landmark positions on the female prothorax of *Ischnura elegans* (1, posterior view, and 3, left lateral view) and of *I. graellsii* (2, posterior view, and 4, left lateral view). C cerci, mcp medio-ventrally directed cercal process, P paraproct, pc paraproctal claspers, *tenth abdominal segment, Pr prothorax, pt pronotum. The red circles represent the combination of landmarks that were assigned.

The occurrence of RCD between closely related species in the genital morphology of males and females has been used as support for the lock-and-key hypothesis of genital evolution (e.g., in the Odonoptera ground beetles; Nishimura et al. 2022). Lock-and-key genital evolution can drive nishimura coevolution in a way that maintains the integrity of species boundaries and prevents hybridization (see Sloan and Simmons 2019). In insects, the genital lock-and-key has been proposed as a potent selector against hybridization (Sota and Kubota 1998). However, despite the increasing number of studies testing RCD in genital divergence in insects (e.g., Augustijn et al. 2022; Kawano 2002, 2003; Usami et al. 2006) and arachnids (Kuntner et al. 2009; Costa-Schmidt and De Araújo 2010; Muster and Michalik 2020), the majority of these studies have focused only on male divergence, which has prevented a comprehensive investigation of the lock-and-key hypothesis of genital evolution.

In damselflies, copulation involves two contact points. First, the male grasps the female prothorax using his caudal appendages, thus achieving the first contact point known as the "tandem position" (see Fig. 1A). Even though they are not intromissive, contact and grasping structures that are involved in copulation have been included in the category of genital traits (Brennan 2016). There is wide variation in male caudal

appendages among odonates (e.g., McPeck et al. 2011) and mechanical isolation due to incompatibility in forming the tandem position is the strongest component of prezygotic isolation in damselflies (Sánchez-Guillén et al. 2014; Wellenreuther and Sánchez-Guillén 2016; Nava-Bolaños et al. 2017; Barnard et al. 2017). McPeck et al. (2008, 2009) pioneered three-dimensional morphometric studies in *Enallagma* damselflies, which have provided empirical support for "lock-and-key" coevolutionary divergence in damselflies. In their research, McPeck et al. (2008, 2009) identified correlated evolution between male and female genital traits, specifically, male cerci and female mesostigmal plates (see Fig. 1A). They quantified both tempo and mode of male and female genital evolution, and they found a similar pattern of punctuated evolution in both structures, proposing that this pattern might be indicative of a lock-and-key mechanism in the tandem position in damselflies (see also Masly 2012; Paulson 1974).

Ischnura elegans and *I. graellsii* are non-territorial damselflies that perform few visual displays, but rather use mechanical-tactile stimuli for species recognition (e.g., Robertson and Paterson 1982). The expansion of *I. elegans* has led to the formation of two mottled hybrid zones between *I. elegans* and *I. graellsii* in Spain, characterized by two secondary contact zones (Sánchez-Guillén et

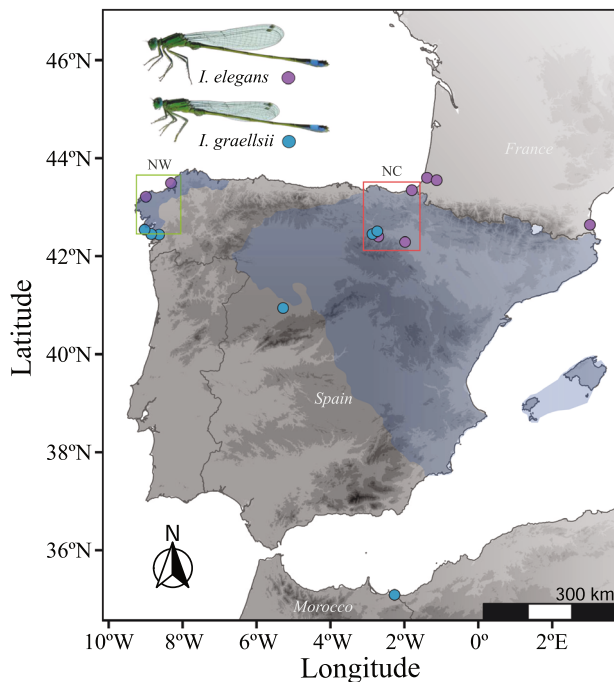


Fig. 2 Map of study localities in allopatry and hybrid zones of *Ischnura elegans* and *I. graellsii*. Green dots show sampled localities in the northwest hybrid zone (NW), orange dots show sampled localities in the north central hybrid zone (NC), light blue dots show allopatric *I. graellsii* localities, and purple dots show allopatric *I. elegans* localities. The dark blue area represents the area in which the two species coexist.

al. 2011, 2023) (see Fig. 2). Previous genomic studies have detected ongoing hybridization in both zones: there was bilateral introgression in the NW hybrid zone, while in the NC zone introgression was unilateral, occurring only in *I. graellsii*, and hybrids were less frequent than in the NW zone (Sánchez-Guillén et al. 2011, 2023; Wellenreuther et al. 2018). Recently, in the NW hybrid zone, Arce-Valdés et al. (2023) detected asymmetric reinforcement of the mechanical barrier to tandem formation only in the cross between *I. graellsii* males and *I. elegans* females. In this cross, the strength of the mechanical barrier was stronger in the hybrid zone than it was in allopatry, and hybrids had lower survivorship and reduced fertility and fecundity than the parental species. However, in the other reciprocal cross (*I. elegans* males with *I. graellsii* females) the strength of this mechanical barrier was similar to that in allopatry. Although reinforcement has not yet been investigated in the NC hybrid zone, Sánchez-Guillén et al. (2023) found that the strength of the mechanical barrier to form the tandem was comparable to that of the barrier in the NW hybrid zone. Additionally, hybrids were observed, albeit at a lower frequency (Sánchez-Guillén et al. 2023) suggesting potential reinforcement of this mechanical barrier in the NC hybrid zone.

In this study, we investigated whether reinforcement of the mechanical barrier that prevents the tandem formation detected in the NW hybrid zone (Arce-Valdés et al. 2023) and putative reinforcement in the NC hybrid zone) has led to RCD in the traits crucial for tandem formation (male caudal appendages and female prothorax) of *I. graellsii* and *I. elegans* from both hybrid zones, using geometric morphometrics analyses of both shape and Centroid Size (CS) to deal with the complexity of reproductive structures. Since phenotypic characters could vary along environmental gradients (Goldberg Emma and Lande 2006) we controlled for environmental and geographical factors (c.f., Johanet et al. 2009). Genetic differentiation between populations was not

included because it is correlated with the geographical distance between populations (Wellenreuther et al. 2011). In addition, we investigated the effect of overall body size on RCD, since previous studies have detected a correlation between body size and genital traits (Polihronakis 2006; Eberhard 2009, 2010; Nava-Bolaños et al. 2012, 2014). We have three specific predictions: first, since some studies explicitly link asymmetrical gene flow to asymmetrical RCD (see Cooley 2007), we expected to detect a pattern of RCD in *I. graellsii* males and *I. elegans* females since they form the heterospecific cross in which reinforcement has been detected (Arce-Valdés et al. 2023). Second, we predicted RCD in the *I. elegans* males, since lock-and-key genital evolution is expected to drive genital coevolution to prevent hybridization (Sloan and Simmons 2019). Third, we expected the signal of RCD to be stronger in the *I. elegans* females than *I. graellsii* males, since hybridization costs are usually higher for females than for males (Coyne and Orr 2004).

METHODOLOGY

Population sampling

During summers from 2003 to 2021, adult males and females were collected from 15 localities: six localities in which the two species are allopatric, four localities within the NW hybrid zone, and five localities within the NC hybrid zone (Table 1; Fig. 2). From each locality, we sampled 14–31 adult males and 9–28 adult females, with larger sample sizes for the dominant species in the localities where both species occur (details in Table 1). Populations from both hybrid zones were selected for this study based on our knowledge of reproductive isolation and hybridization patterns from previous studies (Sánchez-Guillén et al. 2011, 2012; Arce-Valdés et al. 2023; Sánchez-Guillén et al. 2023). Morphological identification of *I. elegans* and *I. graellsii* was based on male caudal appendage, thorax color (only in young females), and prothoracic tubercle (in both males and females) (see Monetti et al. 2002). Despite the less reliable species assignment in females compared to males our team has extensive expertise in the morphology of these species. Individuals were morphologically identified as either *I. elegans* or *I. graellsii*, with no assignment to a hybrid status, because of hybrids resemble the maternal phenotype (normally *I. graellsii*) and are intermediate to both parental species (Monetti et al. 2002). Genomic data from a previous study of the NW and NC hybrid zones did not identify any hybrids among the individuals morphologically assigned to *I. graellsii* in the localities from the NW hybrid zone included in this study. However, in the localities from the NC hybrid zone included in this study, that proportion ranged from 0 to 30% of F₁-F₂ hybrids (details in Table 1) (Sánchez-Guillén et al. 2023). Therefore, the sample of *I. graellsii* individuals from the NC hybrid zone could potentially include F₁-F₂ hybrids. The specimens were preserved in ethanol (96%) and then stored at −20 °C. Dehydration/rehydration process does not deform the reproductive structures.

Geometric morphometric analyses

Since linear estimates do not capture the complexity of genital shape, we used a two-dimensional geometric morphometrics approach to investigate RCD of male and female genital traits. This approach breaks down the complexity in shape and CS of the reproductive structures of interest (see Klingenberg 2016; e.g., Showalter et al. 2014). Here, we investigated RCD of both shape and CS of male caudal appendages and female prothorax in two views (lateral and posterior) because these structures can influence the ability to achieve copulation and thus may contribute to defining species-specificity (Song 2009; Simmons and Garcia-Gonzalez 2011). Indeed, morphological differences in the male's caudal appendages and the female's prothorax are used for the morphological identification of these species (Askew 1988; Monetti et al. 2002).

Table 1. Details of the *I. elegans* and *I. graellsii* specimens used for the morphometric analysis.

Species	Locality	Distribution	Species proportion	Hybrids proportion	Males I. e.	Females I. e.	Males I. g.	Females I. g.	Year	Latitude	Longitude
<i>I. elegans</i>	Marais D'Orx, France	Allopatry	100%	-	25	10	-	-	2015	43°35'58"N	1°23'30"W
<i>I. elegans</i>	Sablère, France	Allopatry	100%	-	26	8	-	-	2016	46°49'53"N	1°51'53"W
<i>I. elegans</i>	St. Cyprien, France	Allopatry	100%	-	26	26	-	-	2016	42°37'07"N	3°00'16"W
<i>I. graellsii</i>	Gamillazo, Salamanca	Allopatry	100%	-	-	-	14	20	2021	40°57'22"N	5°17'21"W
<i>I. graellsii</i>	Saïdia, Morocco	Allopatry	100%	-	-	-	0	16	2009	35°05'27"N	2°15'57"W
<i>I. graellsii</i>	Alba, Galicia	Allopatry	100%	-	-	-	16	9	2003	42°26'30"N	8°38'05"W
<i>I. elegans</i>	Doniños, Galicia	Sympatry (NW)	100%	0%	24	23	0	0	2016	43°29'27"N	8°18'29"W
<i>I. elegans</i>	Laxe, Galicia	Sympatry (NW)	99–100%	30% Introg. I. e.	27	22	0	0	2018	43°12'44"N	8°59'43"W
<i>I. graellsii</i>	Cachadas, Galicia	Sympatry (NW)	99–100%	0%	0	0	31	28	2018–2019	42°27'12"N	8°50'52"W
<i>I. graellsii</i>	Xuño, Galicia	Sympatry (NW)	90–100%	20% Introg. I. g.	3	0	26	13	2005, 2013, 2016	42°37'59"N	9°02'19"W
<i>I. elegans</i>	Perdiguero, Rioja	Sympatry (NC)	71–90%	10% Introg. I. g. 10% F ₁ hybrid	19	20	0	1	2018	42°17'06"N	1°58'55"W
<i>I. elegans</i>	Plaiaundi, Rioja	Sympatry (NC)	99–100%	NA	15	0	1	0	2016	43°20'44"N	1°47'40"W
<i>I. elegans</i>	Villar, Rioja	Sympatry (NC)	74–100%	20% F ₁ hybrid	25	24	0	0	2018	42°24'03"N	2°42'15"W
<i>I. graellsii</i>	Valpierre, Rioja	Sympatry (NC)	95–100%	10% Introg. I. g.	2	1	28	21	2018	42°27'11"N	2°51'48"W
<i>I. graellsii</i>	Mateo, Rioja	Sympatry (NC)	95–100%	0%	0	0	21	27	2018	42°30'34"N	2°44'05"W

Species proportions refers to the proportion of individuals of each species in each sampled population. Hybrid index indicated the proportion of hybrids detected in a previous molecular study by using 381 species-specific SNPs to analyze genotypic composition (F₁ hybrid indicates first generation hybrids; Introg. I. e., indicates introgressed toward *I. elegans*; and Introg. I. g., indicates introgressed toward *I. graellsii* (see Sánchez-Guillén et al. 2023). N males and N females indicate the number of samples included in the morphometric analyses. The distribution indicates allopatric and sympatric populations: NW (North-west) and NC (North-central) hybrid zones.

Photographs of the posterior and lateral view of the male's caudal appendages and the female's prothorax were obtained under a stereoscopic microscope (Zeiss Stemi 305) with an integrated camera (Axiocam ERc5s). The posterior view of the male's caudal appendages was sampled using a combination of eight landmarks (identifiable points that have clear biological or anatomical significance) and 80 semi-landmarks (points placed along curves where there is no specific anatomical feature to serve as a fixed reference) on four curves (left and right sides), which were assigned above and below each of the cerci. The lateral view was sampled with seven landmarks and 36 semi-landmarks on two curves which were assigned on the cercus and the paraproct of the left side. The posterior view of the female's prothorax was sampled with a combination of four landmarks and 21 semi-landmarks on one curve which was assigned surrounding the pronotum. The left lateral view was sampled with 4 landmarks and 19 semi-landmarks on one curve, also surrounding the pronotum. Cartesian coordinates of each posterior and lateral view of males and females were recorded with tpsDIG2 version 2.04 (Rohlf 2006). The configuration of the landmarks on each of the male and female reproductive structures of both species is described in Supplementary Table 1.

We then used a Generalized Procrustes Analysis (Rohlf and Slice 1990) to superimpose landmark configurations; variation due to differences in translation, orientation, and scale was removed, followed by a thin-plate spline analysis Bookstein (1991) using the geomorph package version 4.0.4 (Adams et al. 2022). The GPA translates all samples to an origin, scales them to the same size, and rotates them by least squares to the coordinates of the corresponding points, aligning them as closely as possible (Adams et al. 2022). Semilandmarks were aligned using the Bending Energy minimization criteria, which optimize the semilandmark positions with the lowest deformation energy from a consensus curve of reference (Gunz and Mitteroecker 2013). Before performing GPA, the size estimator (CS) was obtained for each configuration, which was measured as the square root of the sum of the squared distances from each reference point to the centroid of the configuration (Dryden and Mardia 2016; Klingenberg 2020). Additionally, shape variation of both reproductive structures (male's caudal appendages and female's prothorax) was then evaluated graphically along the first two axes of a Principal Components Analysis (PCA) to facilitate anatomical description of morphological changes using the geomorph package version 4.0.4 (Adams et al. 2022).

Reproductive Character Displacement (RCD) in genital traits

We measured RCD in both hybrid zones and tested three predictions: 1) RCD will be detected in the *I. graellsii* males and the *I. elegans* females since they are involved in the heterospecific cross in which reinforcement has been detected (see Arce-Valdés et al. 2023); 2) RCD will be detected in the *I. elegans* males since the lock-and-key genital evolution will drive a genital coevolution to prevent hybridization (Sloan and Simmons 2019); 3) the signal of RCD will be stronger in the *I. elegans* females than the *I. graellsii* males since hybridization costs are usually higher for females than for males (Coyne and Orr 2004).

Under RCD, reproductive characters may or may not differ between the species in allopatry (Slatkin 1980; Liou and Price 1994); rather, the key criterion to determine RCD is that the difference in distance between these reproductive characters is larger in sympatry than in allopatry. To test this, we first measured the difference in morphological distance between *I. elegans* and *I. graellsii* from allopatry and from each hybrid zone. When the difference in morphological distance was significantly larger in the hybrid zone compared to the allopatric zone (distance in sympatry - distance in allopatry > 0) this provided evidence of RCD. Second, in cases where RCD was detected—given its potential to occur in either one or both species (unilateral or

bilateral)—we conducted intraspecific comparisons to identify whether morphological measures of one or both species are significantly larger or smaller in allopatry than in sympatry. We inferred that RCD was unilateral when the difference in morphological distance was significant only in one of the intraspecific comparisons or when both species changed in the same direction (i.e., both species became larger or smaller), but the magnitude of change was larger in one of the species. We inferred that RCD was bilateral when the difference in morphological distance was significantly different in both intraspecific comparisons and the two species changed in opposite directions (one species became smaller and the other species became larger).

We performed the statistical analyses in the following manner. Firstly, to assess the statistical hypotheses investigating RCD patterns of shape variation and covariation for the set of (Procrustes-aligned) coordinates, we performed a Procrustes ANOVA model using the 'procrD.lm' function from the Geomorph package version 4.0.4 (Adams et al. 2022). This function calculates the Procrustes (morphological) distance variance explained by each factor in the model, which were the CS, geographic group (allopatric zone, NW hybrid zone, or NC hybrid zone) and their interaction. When the geographic group factor was statistically significant, we perform a contrast test with R base package (R core team 2019). When the interspecific differences in morphological distance were significantly larger in the hybrid zone(s) compared to the allopatric zone, we complemented this analysis with an intraspecific pairwise evaluation using the 'permudist' function from the 'Morpho' package version 2.8 (Schlager 2017). This function compares the distance between two group means to the distances obtained when observations are randomly assigned to groups (here 1000 permutations) (Schlager 2017). Secondly, to assess the statistical hypotheses investigating RCD patterns of CS variation we used the function 'lm.rpp' from the RRPP package (Collyer and Adams 2018, 2021) to perform linear models. When the geographic group factor showed statistical significance, we performed a contrast test with R base package (R core team 2019). When the interspecific differences in morphological distance were significantly larger in the hybrid zone(s) compared to the allopatric zone, we conducted intraspecific pairwise comparisons to detect which groups were statistically different using the 'pairwise' function. Differences were estimated as Euclidean distance between least-squares means to each group and the significance of distances was assigned with the permutation procedure previously described.

Character displacement in body size and correlation analysis

Populations frequently undergo divergence in morphological traits that are not directly related to acquiring resources or reproductive processes due to evolutionary correlations with traits that are under active selection (Conner and Hartl 2004). Generally, traits under sexual selection exhibit allometry (positive or negative), i.e., are proportionally larger/smaller in larger/smaller individuals while traits under natural selection are isometric, i.e., show constant proportion scaled in relation to body size (Darwin 1879; Eberhard 2009). We predict that genital traits (male caudal appendages and female prothorax) in both hybrid zones may exhibit different correlation patterns compared to those in allopatry due to an increase of linkage disequilibrium—associations among alleles at different loci—between genital morphology and body size (see Lexer et al. 2003) providing evidence for selection (reinforcement) against hybrids with intermediate morphologies (see Bridle and Butlin 2002).

To quantify phenotypic variation in adult body sizes, we used wing length as a proxy for body size (Nava-Bolaños et al. 2012, 2014; Córdoba-Aguilar et al. 2015). This approach was necessary due to abdominal deformation during preservation in ethanol. We included a subset of 10 males and 10 females from

each zone, and species: one allopatric population (Cyprien for *I. elegans* and Gamillazo for *I. graellsii*) and two populations from each hybrid zone (NW hybrid zone: Laxe for *I. elegans* and Cachadas for *I. graellsii*, and the NC hybrid zone: Perdiguero for *I. elegans* and Valpierre for *I. graellsii*). The wings were dissected using fine scissors and examined under a stereoscopic microscope (Zeiss Stemi 305). We measured the hindwing length from the arculus to the proximal corner of the pterostigma (Carchini et al. 2000) using IMAGEJ software (version 1.53k; Abràmoff et al. 2004). To avoid inter-observer variance, all hindwing length measurements were performed by a single person. First, to test for character displacement of body size (wing size) we used Generalized Linear Models (GLMs, Gamma distribution, and inverse link function), and the significance of the effects (zones: allopatry, NC and NW hybrid zones) was tested with the Chi-square distribution. Once we detected significant effects of the zone factor, we perform a contrast test with R base package (R core team 2019). When the interspecific differences in morphological distance were significantly larger in the hybrid zone(s) compared to the allopatric zone, we used contrast to test paired intraspecific differences. Second, we tested for allometry between the wing length (body size) and the CS of the superior caudal appendages of the males and the prothorax of the females. We performed a Pearson correlation between genital size (CS) and wing length. Both the CS data and the wing length data were log-transformed to linearize the relationship between genital traits and body size indicators. We used R software (R Core Team 2019; version 3.6.2) for statistical analysis.

Effect of environmental gradients on morphological variation

To discriminate phenotypic differences caused by sympatry from those caused by environmental and/or geographic factors we tested whether environmental and geographic factors and geographic group (allopatry, NW and NC hybrid zones) might be correlated with the morphological characters measured. We included five environmental variables (precipitation, annual mean temperature, maximum temperature, minimum temperature, and elevation), along with two geographic variables (latitude and longitude) in our analysis. These specific variables were selected based on their established significance in shaping the geographical distribution of *I. elegans* and their known involvement in odonate behavior, as environmental conditions like temperature and precipitation can influence habitat quality and resource availability (see Wellenreuther et al. 2012; Lancaster et al. 2015; Dudaniec et al. 2018, 2022). Lastly, the broad relevance of these variables in understanding species ecology and distribution makes them essential for comprehensively assessing the environmental and geographical factors associated with phenotypic variation in *Ischnura*.

Climate data were extracted from the bioclimatic parameters available in the Worldclim database version 1.4 (Fick and Hijmans 2017) (at 1 km cell resolution). Rasters from each variable were obtained using the function 'getData' from the 'raster' package. We explored possible correlations among environmental variables using the 'PerformanceAnalytics' package version 2.0.4 (Peterson and Carl 2020). For both environmental and geographic data, when two variables presented a strong correlation (absolute value of correlation coefficients $|r| > 0.7$), one of them was excluded from the model (see Dormann et al. 2013). Finally, we fitted separate Gaussian generalized linear models (GLMs) with an identity link function for the shape (described by the first principal component of shape variance for each geometric configuration, because the first principal component accounts for the greatest possible proportion of the total variance (Klingenberg 2016), and for the CS of male and female reproductive structures in each species. All models included the geographic variables (latitude and longitude) because in all data groups they showed correlation

coefficient values below 0.7 (see Supplementary Table 2). We then added the environmental variables that had a correlation coefficient of less than 0.7 absolute with both geographic variables. For males and females of *I. elegans* and for males of *I. graellsii*, the explanatory variables of the GLMs included one environmental variable (annual mean temperature), the two geographic variables (latitude and longitude), and the geographic group (allopatry, NW and NC hybrid zones) to test for zone variation in individual phenotypic responses to these environmental factors. For *I. graellsii* females, the explanatory variables used in the model were the elevation, the two geographical variables, and the geographic group (allopatry, NW, and NC hybrid zones). During the simplification of the models, the Corrected Akaike Information Criterion (AICc) was applied to select the best model using the “dredge” function from the “MuMIn” package version 1.47.5 (Bartoń 2023). All analyses were conducted using the statistical software R-project, version 3.6.2 (R Core Team 2019).

Finally, to test for the spatial autocorrelation in the morphological variation, we used a redundancy analysis of the spatially detrended morphological data against the Distance-based Moran's Eigenvector Maps (dbMEMs: Borcard et al. 2018). The redundancy analysis was done with the ‘rda’ function in the ‘cca’ R package. The spatially detrended morphological data were model residuals of regressing linearly morphological data against the geographic coordinates of the localities. dbMEMs allow the decomposition of the spatial relationships among study sites at different scales and detect spatial autocorrelation at various scales (Stoch et al. 2016; Borcard et al. 2018). For this purpose, we used the ‘dbmem’ function and the threshold was estimated with the function ‘give.thresh’ both functions in the ‘adespatial’ R package.

RESULTS

Reproductive character displacement

The results from the posterior view detected similar results to the lateral view but had less variation; we therefore simplify this section by providing in the main document results from the lateral view and the results from the posterior view in the supplementary material only.

Male caudal appendages

Shape variation: PCA plots showed a clear separation between species (Supplementary Fig. 1A–F). Procrustes ANOVA showed statistically significant differences between *I. elegans* and *I. graellsii* from allopatry ($F = 144.25$, $p < 0.001$), and from both hybrid zones (NW: $F = 89.06$, $p < 0.001$; NC: $F = 90.29$, $p < 0.001$; Supplementary Table 3). CS explained close values of shape variance (8 to 22%, $p < 0.01$), but the interaction between CS and groups was non-significant. Contrast tests indicated that interspecific morphological distance at any hybrid zone (NW: $d = 0.19$, NC: $d = 0.18$; Supplementary Table 4) were significantly smaller (NW: $t = -11.17$, $p < 0.001$; NC: $t = -12.13$, $p < 0.001$; Supplementary Table 5) than that in allopatry ($d = 0.23$; Supplementary Table 4) (Fig. 3A) (distance sympatry – distance allopatry > 0). Thus, interspecific morphological distances did not support the occurrence of RCD in shape either of the hybrid zones (see Table 2).

CS variation: The linear models showed statistically significant differences between *I. elegans* and *I. graellsii* in both hybrid zones (NW: $F = 13.629$, $p < 0.001$; NC: $F = 5.151$, $p = 0.018$), but not in allopatry ($F = 0.017$, $p = 0.894$) (Fig. 3B; Supplementary Table 6). Contrast tests indicated that interspecific morphological distance in both hybrid zones (NW: $d = 31.45$, NC: $d = 23.53$;

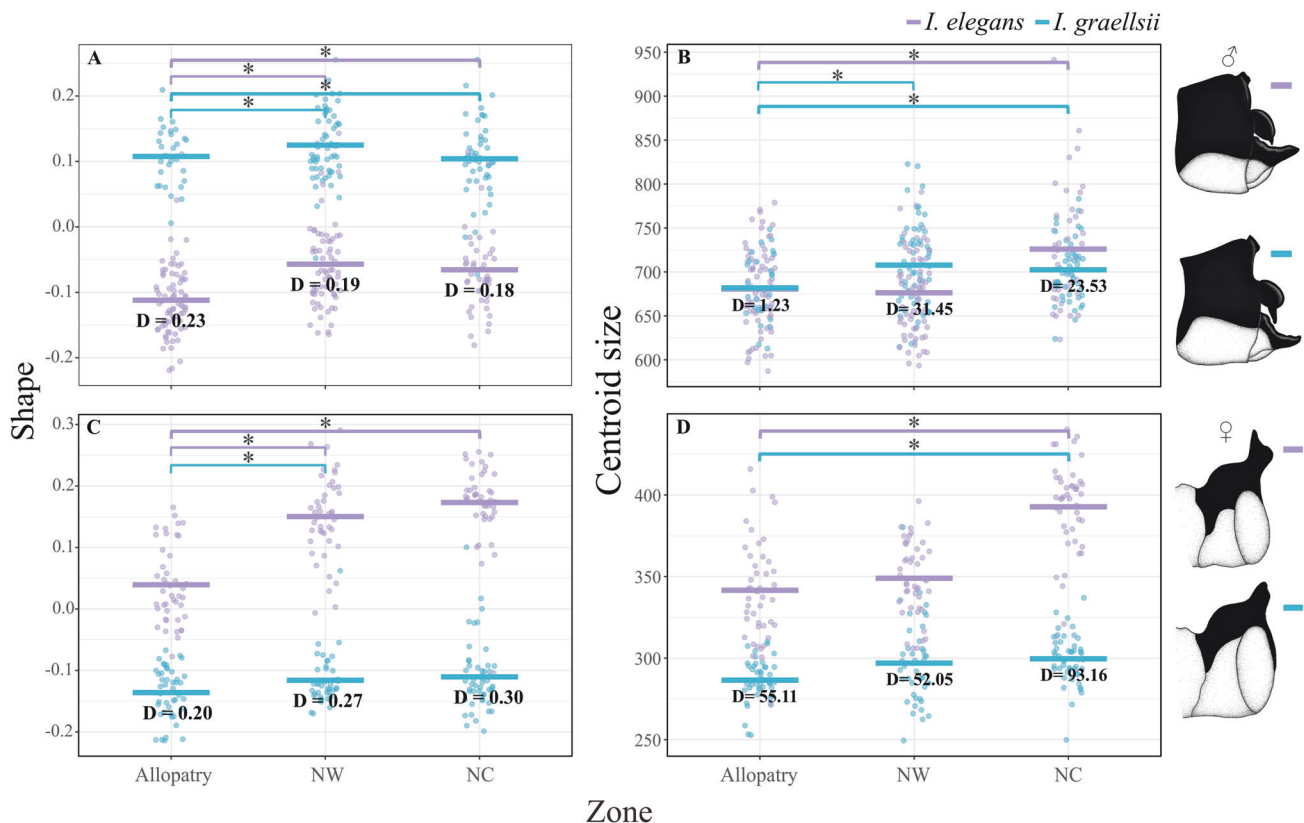


Fig. 3 RCD of male and female reproductive structures in *Ischnura damselflies*. RCD represented by median values of the shape and the centroid size (CS) for the lateral view of the male caudal appendages of *I. elegans* and *I. graellsii* (A, C, respectively) and median values of female prothorax of *I. elegans* and *I. graellsii* (B, D, respectively) from allopatry and both hybrid zones. *Above the plot indicate statistically significant differences at a significant level of $p < 0.05$.

Table 2. Reproductive character displacement (RCD) for the lateral view in *I. elegans* and *I. graellsii* from both hybrid zones.

Dimension	Sex	Interspecific comparison difference in distance	Intraspecific comparisons difference in distance		RCD	
		<i>Symp vs. Allop</i>	<i>I. elegans Symp vs. Allop</i>	<i>I. graellsii ymp vs. Allop</i>	<i>I. elegans</i>	<i>I. graellsii</i>
<i>NW hybrid zone</i>						
Shape	♂	*Symp < Allop	NA	NA	No	No
	♀	*Symp < Allop	*Symp > Allop	*Symp > Allop	Unilateral	No
CS	♂	*Symp > Allop	Symp=Allop	*Symp > Allop	No	Unilateral
	♀	Symp < Allop	NA	NA	No	No
<i>NC hybrid zone</i>						
Shape	♂	*Symp < Allop	NA	NA	No	No
	♀	*Symp > Allop	*Symp > Allop	Symp= Allop	Unilateral	No
CS	♂	*Symp > Allop	*Symp > Allop	*Symp > Allop	Unilateral	No
	♀	*Symp > Allop	*Symp > Allop	*Symp > Allop	Unilateral	No

The first and second columns indicate whether analyses correspond to the shape or centroid size (CS), sexes (male or females). The third column refers to the interspecific comparisons; when the difference in morphological distance was significantly larger in the hybrid zone compared to the allopatric zone (distance in sympatry - distance in allopatry > 60) this provided evidence of RCD. The fourth column includes the intraspecific comparisons (in cases where RCD was detected—given its potential of occurring in either one or both species—we conducted intraspecific comparisons (distance in sympatry versus distance in allopatry)). The last column indicates whether RCD was unilateral or bilateral: RCD was unilateral when the difference in morphological distance was significant in only one of the intraspecific comparisons or when both species changed in the same direction (i.e., both species became larger or smaller) but the magnitude of change was larger in one of the species; RCD was bilateral when the difference in morphological distance was significant in both intraspecific comparisons and the two species changed in opposite directions (one species became smaller and the other species became larger). CS: centroid size, Lat: lateral view, Symp: sympatry zones, Allop: allopatry zones, *statistically significant comparisons.

Supplementary Table 7) was significantly larger (NW: $t = 8.21$, $p < 0.001$; NC: $t = -13.77$, $p < 0.001$; Supplementary Table 5) than that in allopatry ($d = 1.23$; Supplementary Table 7) (Fig. 3B) (distance sympatry - distance allopatry > 0). Thus, interspecific morphological distances supported RCD in shape in both hybrid zones, although the directions of the displacement were different between hybrid zones (Table 2).

To determine whether this RCD was unilateral or bilateral (i.e., due to changes in one or both species), we conducted intraspecific comparisons. In *I. elegans*, the morphological distance was significantly different among zones (allopatry, NW and NC) ($F = 15.194$, $p < 0.001$; Fig. 3B; Supplementary Table 8). Post hoc analysis showed that the caudal appendages of males from the NW hybrid zone were not significantly different compared to males from allopatry ($z = -0.378$, $p = 0.65$, $d = 4.33$), while males from the NC hybrid zone were significantly larger than those from allopatry ($z = 3.445$, $p < 0.001$, $d = 45.35$) (Fig. 3B; Supplementary Table 9). Similarly, in *I. graellsii*, morphological distances were significantly different among zones (allopatry, NW and NC) ($F = 4.080$, $p < 0.001$; Fig. 3B; Supplementary Table 8). Post hoc analysis showed that the caudal appendages of males from the two hybrid zones were significantly larger than those from allopatry (NW: $z = 2.219$, $p = 0.003$, $d = 25.89$; NC: $z = 1.809$, $p = 0.023$, $d = 20.58$; Fig. 3B Supplementary Table 9).

In summary, in the NW hybrid zone we detected unilateral RCD, driven by *I. graellsii*, while in the NC hybrid zone RCD was unilateral, driven by *I. elegans*, because although both species became larger (Table 2), this change was less pronounced in *I. graellsii*, and samples morphologically assigned to *I. graellsii* could include hybrids (which are intermediate between both species).

Female Prothorax

Shape variation: PCA plots showed a clear separation between species (Supplementary Fig. 1G–L). Procrustes ANOVA showed statistically significant differences between *I. elegans* and *I. graellsii* from allopatry ($F = 21.21$, $p < 0.001$), and in both hybrid zones (NW: $F = 42.90$, $p < 0.001$; NC: $F = 15.02$, $p < 0.001$ Supplementary Table 10). CS had small but significant effects in all the models (6 to 16% of shape variance, $p < 0.01$), and the lowest variances were

explained by the interaction between CS and groups (2 to 4%). Contrast tests indicated that interspecific distances in both hybrid zones (NW: $d = 0.27$; NC: $d = 0.30$; Fig. 3C; Supplementary Table 11) were significantly larger than distance in allopatry ($d = 0.20$; Fig. 3C; Supplementary Table 11) (NW: $t = -11.53$, $p < 0.001$; NC: $t = -12.34$, $p < 0.001$; Supplementary Table 5). Thus, interspecific morphological distances supported RCD in shape in both hybrid zones (Fig. 3C; Supplementary Table 11).

To investigate whether the RCD was unilateral or bilateral, we conducted intraspecific comparisons. In *I. elegans*, PC axis1 explained 43.6%, and PC axis2 21.7% of the shape variance (Supplementary Fig. 2C). Procrustes ANOVA showed statistically significant differences among zones (allopatry, NW and NC) ($F = 13.802$, $p < 0.001$; Fig. 3C; Supplementary Table 12). Post hoc pairwise tests showed statistically significant differences between *I. elegans* from the allopatric zone and both hybrid zones (NW: $d = 0.06$, $p = 0.036$; NC: $d = 0.11$, $p < 0.001$; Fig. 3C; Supplementary Table 13). Similarly, in *I. graellsii*, PC axis 1 explained 43.7% and PC axis 2 25.1% of the shape variance (Supplementary Fig. 2D). Procrustes ANOVA showed statistically significant differences among zones (allopatry, NW and NC) ($F = 2.18$, $p = 0.026$; Fig. 3C; Supplementary Table 12). Post hoc pairwise tests detected statistically significant differences between *I. graellsii* from the allopatric and NW hybrid zone ($d = 0.05$, $p = 0.011$), but not between *I. graellsii* from the allopatric zone and the NC hybrid zone ($d = 0.03$, $p = 0.144$) (Fig. 3C; Supplementary Table 13).

In summary, in the NW hybrid zone we detected unilateral RCD in *I. elegans*. In the NC hybrid zone, both species changed in the same direction, but RCD was much more pronounced in *I. elegans* than in *I. graellsii*, and samples morphologically assigned to *I. graellsii* could include hybrids, so we considered it unilateral, driven by *I. elegans* (see Table 2).

CS variation. The linear models showed statistically significant differences between *I. elegans* and *I. graellsii* in allopatry ($F = 122.45$, $p < 0.001$) and from both hybrid zones (NW: $F = 100.34$, $p < 0.001$; NC: $F = 415.07$, $p < 0.001$) (Fig. 3D; Supplementary Table 14). Contrast tests indicated that interspecific

morphological distance in the NC hybrid zone (NC: $d = 93.16$, Supplementary Table 15) was significantly larger (NC: $t = -13.06$, $p < 0.001$; Supplementary Table 5) than in the allopatric zone ($d = 55.11$ Supplementary Table 15) while in the NW hybrid zone NW ($d = 52.05$; Supplementary Table 15) was not significantly different (NW: $t = 0.49$, $p = 0.62$; Supplementary Table 5) from the allopatric zone (Fig. 3D) (distance sympatry – distance allopatry > 0). Thus, interspecific distance supported RCD in CS in the NC hybrid zone (see Table 2).

To investigate whether the RCD in the NC hybrid zone was unidirectional or bidirectional, we conducted intraspecific comparisons. In *I. elegans*, the morphological distance was significantly different among zones (allopatry, NW, and NC) ($F = 45.857$, $p < 0.001$; Fig. 3D; Supplementary Table 16). Post-hoc analysis showed that the female prothoraxes from the NW hybrid zone were not significantly different from allopatry males ($z = -0.521$, $p = 0.326$, $d = 7.39$), whereas males from the NC hybrid zone were significantly larger than in allopatry ($z = 4.357$, $p < 0.001$, $d = 51.16$; Fig. 3D; Supplementary Table 17). Likewise, in *I. graellsii*, morphological distances were significantly different among zones (allopatry, NW and NC) ($F = 6.607$, $p = 0.004$; Fig. 3D; Supplementary Table 16). Post hoc analysis showed that the prothoraxes of females from both hybrid zones were significantly larger than in allopatry (NW: $z = 2.141$, $d = 10.45$, $p = 0.008$; NC: $z = 2.607$, $d = 13.12$, $p < 0.001$; Fig. 3D Supplementary Table 17).

In summary, in the NW hybrid zone we did not detect RCD, while in the NC hybrid zone, since both species changed in the same direction, RCD was unilaterally driven by *I. elegans*, because it was much more pronounced in *I. elegans* than in *I. graellsii* and samples morphologically assigned to *I. graellsii* could include hybrids (Table 2).

Character displacement in body size and correlation analysis

In males, the GLM showed significant effects of the interaction between species and region ($X^2 = 29.257$, d.f. = 2, $p < 0.001$; Supplementary Table 18). The contrast test revealed statistically significant differences between *I. elegans* and *I. graellsii* in allopatry ($t = -2.504$, d.f. = 55, $p = 0.015$) and from both hybrid zones (NW: $t = -10.229$, d.f. = 55, $p < 0.001$; NC: $t = -6.432$, d.f. = 55, $p < 0.001$) (Fig. 4A; Supplementary Table 19). Contrast tests indicated that interspecific distances in both hybrid zones (NW: $d = 1.97$; NC: $d = 1.30$; Supplementary Table 19) were significantly larger than distance in allopatry ($d = 0.48$; Supplementary Table 19) (NW: $t = 7.771$, $p < 0.001$; NC: $t = 8.728$, $p < 0.001$; Supplementary Table 20) (Fig. 4A) (distance sympatry – distance allopatry > 0). Thus, interspecific morphological distances supported RCD in wing size in both hybrid zones (Fig. 4A). Thus, interspecific morphological distance supported RCD in wing size in the NC and the NW hybrid zones. To investigate whether the RCD in the hybrid zones was unidirectional or bidirectional, we conducted intraspecific comparisons. In *I. elegans*, the wing length was significantly different between allopatry and both hybrid zones (NW: $t = 4.208$, d.f. = 55, $p < 0.001$; NC: $t = 3.662$, d.f. = 55, $p < 0.001$; Fig. 4A, Supplementary Table 19). However, in *I. graellsii* the wing length was significantly different only between allopatry and the NW hybrid zone (NW: $t = -3.503$, d.f. = 55, $p < 0.001$; NC: $t = -0.284$, d.f. = 55, $p < 0.778$; Fig. 4A; Supplementary Table 19).

In females, the GLM showed significant effects of the interaction between species and region ($X^2 = 22.502$, d.f. = 2, $p < 0.001$; Supplementary Table 18). The contrast test showed statistically significant differences between *I. elegans* and *I. graellsii* in allopatry ($t = -3.062$, d.f. = 53, $p = 0.003$) and from both hybrid zones (NW: $t = -9.229$, d.f. = 53, $p < 0.001$; NC: $t = -4.660$, d.f. = 53, $p < 0.001$) (Fig. 4B; Supplementary Table 21). Contrast tests indicated that interspecific distances in both hybrid zones (NW: $d = 1.54$; NC: $d = 0.79$; Supplementary Table 21) were significantly larger than distance in allopatry ($d = 0.50$; Supplementary Table 21) (NW: $t = -9.914$, $p < 0.001$; NC: $t = 11.560$, $p < 0.001$; Supplementary Table 20) (Fig. 4B) (distance sympatry – distance allopatry > 0). Thus,

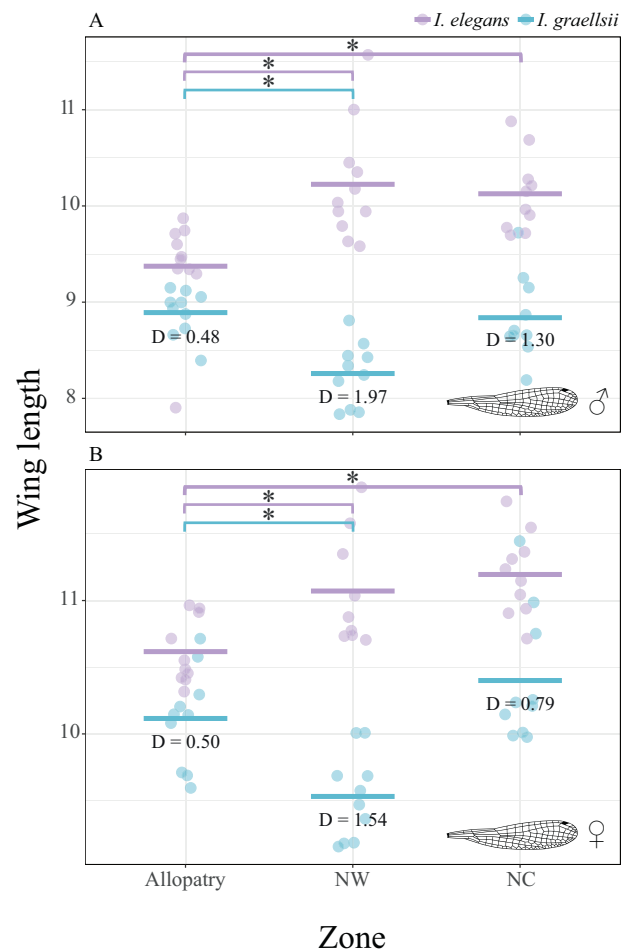


Fig. 4 RCD of wing size in male and female *Ischnura* species. RCD represented by median values of male (A) and female (B) wing size (body size) of *I. graellsii* and *I. elegans* from allopatry and both hybrid zones.

interspecific morphological distances supported RCD in wing size in both hybrid zones (Fig. 4B). To investigate whether the RCD in both hybrid zones was unidirectional or bidirectional, we conducted intraspecific comparisons. In *I. elegans*, the wing length in both hybrid zones was significantly different from the allopatry zone (NW: $t = 2.585$, d.f. = 53, $p = 0.013$; NC: $t = 3.358$, d.f. = 53, $p = 0.001$; Fig. 4B, Supplementary Table 21). However, in *I. graellsii*, we found that wing length was statistically different only between allopatry and NW zones (NW: $t = -3.768$, d.f. = 53, $p < 0.001$; NC: $t = 1.757$, d.f. = 53, $p = 0.085$; Fig. 4B; Supplementary Table 21).

Statistically significant correlations were only detected for the posterior view, specifically in *I. graellsii* males and *I. elegans* females from both hybrid zones (Supplementary Figs. 3–6; Supplementary Table 22). The allometric analysis of the posterior view showed that log10-transformed wing size and the CS of the male caudal appendages were significantly correlated in *I. graellsii* from the NW (Cachadas: $r = 0.851$; $p = 0.001$) and from the NC (Valpierre: $r = 0.636$; $p = 0.047$) hybrid zones, and their relationships were allometric (Cachadas: slope = 1.17; Valpierre: slope = 0.43) (Supplementary Fig. 3B, C, Supplementary Table 22). However, in the allopatric population of *I. graellsii* (Gamillazo: $r = -0.066$; $p = 0.855$) that correlation was not detected (Supplementary Fig. 3A). Similarly, log10-transformed wing size and female prothorax CS were significantly correlated in *I. elegans* from the NW (Laxe: $r = 0.657$; $p = 0.039$) and from the NC (Perdiguerro: $r = 0.749$; $p = 0.012$) hybrid zones, and their

relationships were allometric (Laxe: slope=1.06; Perdiguero slope= 1.14) (Supplementary Fig. 3E, F, Supplementary Table 22). However, in the allopatric population of *I. elegans* (Cyprien: $r = 0.354$; $p = 0.315$) that correlation was not detected (Supplementary Fig. 3D).

Therefore, *I. graellsii* male caudal appendages and *I. elegans* female prothorax size increased at the same rate as body size in both hybrid zones, but not in the allopatric zones.

Effect of environmental gradients on morphological variation

We found that phenotypic variation in the shape and CS of both male caudal appendages and female prothorax were significantly explained by geographic group (hybrid zones and allopatry), environmental factors, or both. Given that results from both views were similar, results from the posterior view were given in the supplementary material (Supplementary Table 23). Table 3 and Supplementary Fig. 7 includes results of the GLM for the lateral

Table 3. Influence of ecological (latitudinal/longitudinal gradients), environmental, and sampling zone on shape and CS (lateral view) of male and female reproductive characters of *Ischnura elegans* and *I. graellsii* damselflies.

Dimension	Sex	Environmental/ geographical factors	<i>I. elegans</i>			
			Estimate	Std. Error	t-value	P-value
CS	♂	AMT + Longitude + Zone				
		AMT	−10.578	7.012	−1.509	0.133
		Longitude	−11.331	3.216	−3.523	< 0.001
		Allopatry Zone	835.793	101.203	8.259	< 0.001
		NW Zone	−119.910	28.588	−4.194	< 0.001
		NC Zone	1.757	10.845	0.162	0.871
	♀	Longitude + Zone				
		Longitude	−4.148	1.869	−2.219	0.028
		Allopatry Zone	346.823	4.696	73.848	< 0.001
		NW Zone	−33.720	19.389	−1.739	0.084
		NC Zone	36.018	8.897	4.048	< 0.001
Shape (PC1)	♂	AMT + Longitude + Zone				
		AMT	−0.025	0.011	−2.317	0.021
		Longitude	0.011	0.005	2.141	0.033
		Allopatry Zone	−0.032	0.017	−1.854	0.065
		NW Zone	0.129	0.043	2.988	0.003
		NC Zone	0.365	0.152	2.405	0.017
	♀	AMT + Zone				
		AMT	0.021	0.011	1.874	0.063
		Allopatry Zone	−0.136	0.016	−8.573	< 0.001
		NW Zone	−0.265	0.157	−1.685	0.094
		NC Zone	0.062	0.021	2.989	0.003
Dimension	Sex	Environmental/ geographical factors	<i>I. graellsii</i>			
			Estimate	Std. Error	t value	P-value
CS	♂	AMT + Longitude + Zone				
		AMT	14.159	8.823	1.605	0.111
		Longitude	18.650	8.255	2.259	0.025
		Allopatry Zone	626.276	74.48	8.409	< 0.001
		NW Zone	41.805	12.375	3.378	< 0.001
		NC Zone	−40.329	28.256	−1.427	0.156
	♀	Zone				
		Allopatry Zone	286.467	2.723	105.207	< 0.001
		NW Zone	10.454	3.944	2.651	0.009
		NC Zone	13.116	3.771	3.478	< 0.001
Shape (PC1)	♂	Zone				
		Allopatry Zone	0.010	0.010	1.007	0.316
		NW Zone	−0.002	0.013	−0.184	0.855
		NC Zone	−0.023	0.013	−1.794	0.075
	♀	Latitude + Longitude				
		Latitude	0.010	0.003	3.593	< 0.001
		Longitude	0.005	0.002	2.263	0.025

Estimated regression parameters, standard errors, *t*-values, and *P*-values for the Gaussian GLMs applied to the shape and the CS of reproductive structures. The models presented in the table were the best supported models based on the corrected Akaike Information Criterion (AICc); the AICc scores for the other models tested are provided in the supplemental materials. CS Centroid size, AMT Annual mean temperature, Allopatry Allopatric zone, NW Northwest hybrid zone, NC North-Central hybrid zone. Significant effects are marked in bold font.

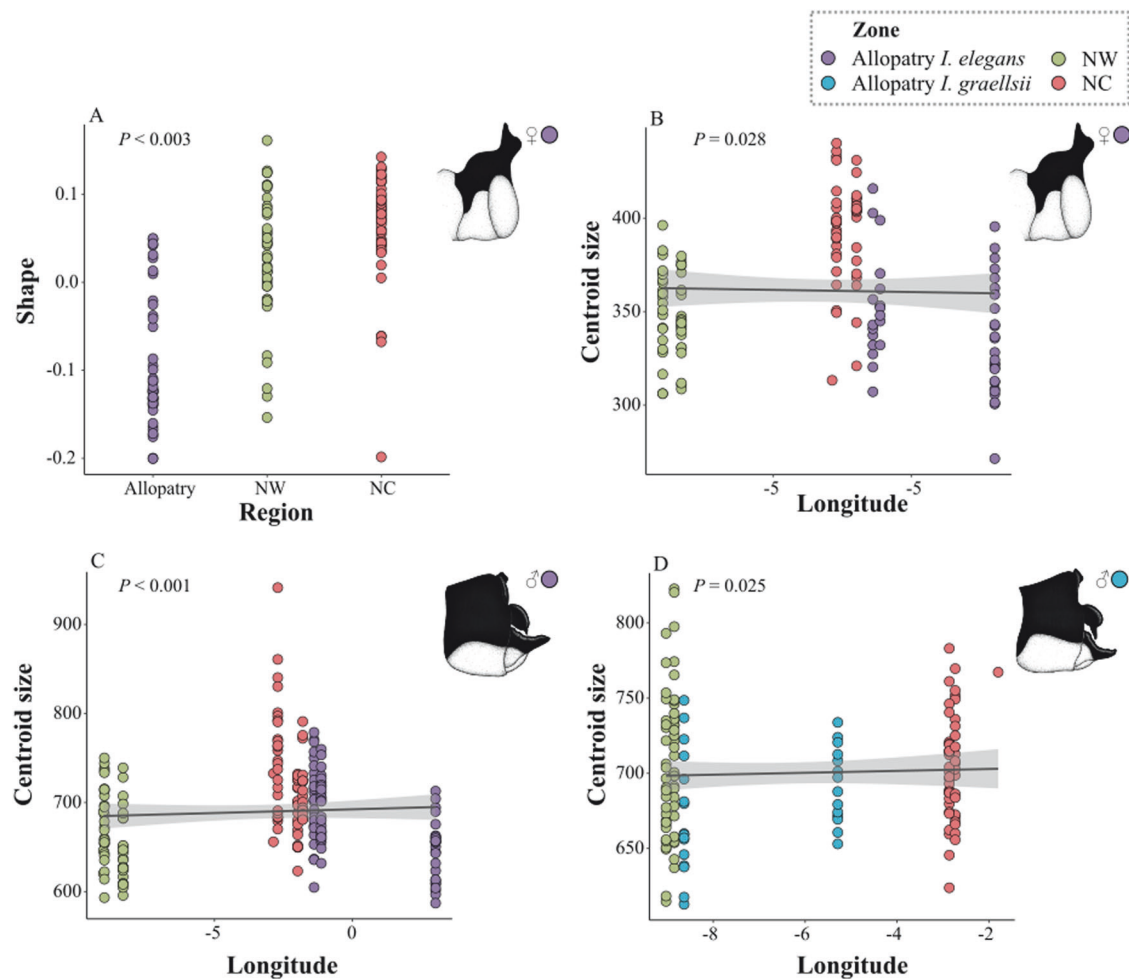


Fig. 5 GLM analysis of environmental gradients and morphological variation in *Ischnura* species. Relationship and confidence intervals between geographic group and the shape (lateral view) for *I. elegans* females (**A**); longitude and the centroid size (lateral view) for *I. elegans* females (**B**); *I. elegans* males (**C**), and *I. graellsii* males (**D**). The line was fit with a generalized linear regression model (GLM).

view, and Supplementary Table 24 includes all scores of the best-fitting models according to the AICc.

The GLM results indicated that RCD in the shape of the female prothorax of *I. elegans* from the two hybrid zones was influenced only by the geographic group (Fig. 5A, Table 3; Supplementary Table 24). Post-hoc comparisons indicated that both hybrid zones differed from allopatry in the shape (Supplementary Table 25). Additionally, GLM results indicated that RCD in CS of the female prothorax and of the male caudal appendages of *I. elegans* from the NC hybrid zone were positively correlated with longitude and the geographic group in both sexes (Fig. 5B, C, Table 3, Supplementary Table 24). Post-hoc comparisons indicated that the CS in the NC hybrid zone differed from allopatry (Supplementary Table 25).

Similarly, in *I. graellsii*, GLM results indicated that RCD in CS of the male caudal appendages from the NW hybrid zone were positively correlated with longitude and the geographic group (Fig. 5D, Table 3; Supplementary Table 24). Post-hoc comparisons indicated that CS in the NW hybrid zone differed from allopatry (Supplementary Table 25).

Spatial structure using dbMEMs

The analysis using dbMEMs did not reveal significant spatial patterns in the variables studied for none of the species or the

views (lateral and posterior), indicating the absence of spatial autocorrelation (see Supplementary Table 26).

DISCUSSION

Reinforcement, a form of RCD, reduces the prevalence of alleles linked to weakened heterospecific discrimination; this reduces the likelihood of paying hybridization costs, enhances prezygotic isolation, and leads to the development of greater phenotypic divergence in sympatry than allopatry (Pfennig and Pfennig 2009). We investigated RCD in the traits crucial for tandem formation in populations experiencing reinforcement of the mechanical barrier that prevents the tandem formation (Arce-Valdés et al. 2023). In the hybrid zones (sympatric distributions), we detected a larger divergence in genital traits between than in allopatry. The observed pattern was consistent with RCD by asymmetric reinforcement, i.e. only in the cross between the *I. graellsii* males and *I. elegans* females: in NW hybrid zone we detected RCD in the genital traits of the *I. graellsii* males (CS) and the *I. elegans* females (shape), with a stronger signal observed in females than in males. Meanwhile, in the NC hybrid zone we detected RCD in both females (CS and shape) and males (CS) of *I. elegans*, in line with the lock-and-key hypothesis of genital evolution through female choice for conspecific males, here also with a stronger signal in females than in males. Alternative explanations for RCD in the CS

such as the geographic variable (longitude), are ruled out in the discussion.

Reproductive character displacement by reinforcement

The RCD pattern can be asymmetric or unilateral, occurring more strongly or exclusively in one of the species (e.g. Nishimura et al. 2022); it can also be asymmetric between sexes, generally more pronounced in females (Coyne and Orr 2004). Our results differed from, for example, the beetles *Odontolabis mouhoti* and *O. cuvera*, where RCD in body size, genitalia length, and coloration was due to shifts by both species—a bilateral pattern of RCD (Kawano 2003). Asymmetric (or unilateral) RCD can be explained by several factors, such as unequal hybridization costs, biases in likelihood of hybridization, asymmetrical effects of reproductive interference, evolutionary constraints, or historical accidents (see Cooley 2007). Asymmetric patterns of RCD can be produced by differences in the abundance of the parental species (since the rarer species have a greater chance of encountering/mating with the common species than vice versa; Peterson et al. 2005; Hochkirch et al. 2007), and the level of gene flow with the parental populations (homogenization by gene flow) (Liou and Price 1994; Pfennig and Ryan 2006). In our system, *I. elegans* is the rarer species, which has expanded their distribution into the *I. graellsii* distribution in western and central Spain, and thus, has a greater chance of encountering with the common *I. graellsii* than vice versa, and is therefore more frequently at risk of heterospecific matings (Arce-Valdés et al. 2023; Sánchez-Guillén et al. 2023). In a similar scenario, *Pterostichus thunbergi* and *P. habui* beetles show unilateral RCD pattern of genital morphology. *Pterostichus thunbergi* differed more from *P. habui* at sympatry than at allopatry, likely because *P. habui* arrives at the contact zone in small numbers, and thus, would experience stronger selection pressures than *P. habui* (Kosuda et al. 2016).

The asymmetric reinforcement in response to maladaptive hybridization in hybrid zones can lead to asymmetric patterns of RCD in traits that are involved in premating isolation in sympatry (Howard and Harrison 1993; Coyne and Orr 2004). In a previous study in the NW hybrid zone, Arce-Valdés et al. (2023) detected asymmetric reinforcement of the prezygotic reproductive barrier to “tandem formation,” which involves both male caudal appendages and the female prothorax. However, the reinforcement is asymmetric, affecting only *I. graellsii* male–*I. elegans* female crosses. In the NC hybrid zone, the strength of the mechanical barrier to “tandem formation” was comparable to that of the barrier in the NW hybrid zone (Sánchez-Guillén et al. 2023), suggesting a potential reinforcement of this mechanical barrier in the NC hybrid zone. Consistent with the asymmetric reinforcement of the mechanical isolation affecting only *I. graellsii* male–*I. elegans* female crosses in the NW hybrid zone (Arce-Valdés et al. 2023), and the potential reinforcement of this mechanical barrier in the NC hybrid zone, RCD was also asymmetric between reciprocal crosses in both hybrid zones. In the NW hybrid zone, we detected RCD in the CS of the *I. graellsii* male caudal appendages and in the shape of the *I. elegans* female prothorax, while neither the shape nor the CS of the *I. graellsii* females and *I. elegans* males differed more than in allopatry. In fact, the male (shape) and the female (CS) genital traits increased in *I. graellsii* from the NW hybrid zone (decreasing the interspecific difference, see Fig. 3A, D). The interspecific gene flow between *I. elegans* and *I. graellsii* (Sánchez-Guillén et al. 2023) could explain the elongation of the male and female genital traits of *I. graellsii* in the NW hybrid zone. This could also be due to the inclusion of hybrids in captures, as their genital traits are intermediate to those of the two species (Monetti et al. 2002). Hybrids are much more likely to be misidentified as *I. graellsii* than *I. elegans* due to their morphological resemblance to *I. graellsii* (Sánchez-Guillén et al. 2023). Similarly, in the NC hybrid zone, the CS of the *I. elegans* male caudal appendages and the shape and CS of the *I. elegans*

female prothorax differed more than in allopatry, while neither the shape nor the CS of the *I. graellsii* females differed more than in allopatry. In fact, the measured genetic divergence between *I. elegans* and *I. graellsii* in the NW hybrid zone was lower than in the NC hybrid zone and in allopatry, indicating a higher level of interspecific gene flow in the NW than in the NC hybrid zone (Sánchez-Guillén et al. 2023). A similar finding was detected in the *Ohomopterus* beetles *O. maiyasanus* and *O. iwawakianus*, in which hybrid genital traits were intermediate to those of the two species, thus explaining elongation of the genital traits of *C. iwawakianus* (Sasabe et al. 2007).

Correlated evolution and evidence of the lock-and key. Populations may often also diverge in traits that are not directly involved in resource acquisition or reproduction owing to correlated evolution with traits that are targeted by selection (sensu Conner and Hartl 2004). For example, spadefoot toads have evolved smaller body sizes in the presence of their heterospecific competitor as a by-product, rather than as a direct target of character displacement (Pfennig and Pfennig 2005). Body size (wing size) and CS of genital traits showed a positive allometric correlation (only in *I. graellsii* males and *I. elegans* females) in both hybrid zones, but not in allopatry. This pattern aligns with the reinforcement of RI detected in the cross involving *I. graellsii* males and *I. elegans* females in the NW hybrid zone (Arce-Valdés et al. 2023), and putative reinforcement in the NC hybrid zone. However, the pattern of character displacement in body size differed from that on genital traits. Unlike the pattern of RCD detected in the genital traits, in the *I. graellsii* males from the NW hybrid zone and the *I. elegans* males and the females from the NC hybrid zone, the pattern of character displacement detected in body size was detected in males and females of both species from the NW hybrid zone, but only in males and females of *I. elegans* from NC hybrid zone. Thus, the pattern of character displacement observed in body size might be due to other factors, such as for instance resource competition (Kawano 1995), rather than correlated evolution.

Moreover, the detection of RCD in the males and the females of *I. elegans* is consistent with lock-and-key genital evolution by female choice for conspecific males. This kind of evidence has been considered to support lock-and-key genital evolution in other studies. For instance, Nishimura et al. (2022) considered the presence of RCD in both sexes of the ground beetle *Carabus maiyasanus* as evidence for the lock-and-key hypothesis of genital evolution. This pattern suggests that linkage disequilibrium arising from selection (reinforcement) against hybrids with intermediate morphologies may be at play. When selection targets phenotypic traits, linkage disequilibrium can lead to extensive intercorrelations among them (Lexer et al. 2003). This finding is consistent with both stabilizing forces, reinforcement and the lock-and-key, acting to enhance reproductive isolation between species. Thus, the observed proportionality between body size and genital size may reflect an increase of linkage disequilibrium between genital morphology and body size. This process may also result in bimodal variation, providing evidence for selection against intermediates (See Bridle and Butlin 2002).

Ruling out alternative explanations. Ecological changes in habitat or resource use may generate changes in the species that promote RCD (Goldberg Emma and Lande 2006; Pfennig and Pfennig 2009; Johanet et al. 2009). Earlier studies in odonates have shown that phenotypic variables, such as body size or wing size and shape show latitudinal variation (Johansson 2003; Hassall et al. 2008). Environmental and geographic variables can also influence the shape and size of the genitalia, since specific environmental conditions and local adaptations can lead to variations in mating behaviors and reproductive strategies, ultimately resulting in differences in the shape and size of genital

traits to optimize reproductive success within a given environment. The RCD in the shape of the *I. elegans* females was exclusively explained by the geographic group (hybrid zones and allopatry), with no apparent influence of any of the environmental or geographic factors included. On the other hand, the RCD in the CS of the genital traits of *I. graellsii* males and *I. elegans* males and females was explained by the geographic group and the longitude. However, although the longitude had an influence on the CS of the genital traits (larger at higher longitudes), this cannot explain the larger size of these traits in *I. graellsii* from the NW (which has lower longitude than the NC and the allopatric distributions), nor the larger size of these traits in *I. elegans* males and females from the NW, (lower longitude than the allopatric distribution). Thus, the geographic zones explained the RCD in the CS and shape in both hybrid zones and species. Our results highlight the importance of considering ecological and environmental factors when testing RCD.

CONCLUSION

Our study presents novel results in two non-territorial species of damselflies. We demonstrated RCD in both male and female genital traits (crucial for tandem formation) of the damselflies *I. elegans* and *I. graellsii*, which is consistent with an increase in mechanical isolation via reinforcement as detected in populations from the NW and NC hybrid zones experiencing reinforcement of the mechanical barrier preventing the tandem formation (Arce-Valdés et al. 2023) (and putative reinforcement in the NC hybrid zone). Additionally, our results are consistent with the lock-and-key genital evolution and linkage disequilibrium arising from reinforcement against hybrids. Our study adds to previous empirical studies that have together led to consensus on the taxonomically widespread role of RCD in preventing reproductive interactions between species (see Goldberg Emma and Lande 2006). Our study highlights the importance of using geometric morphometrics to deal with the complexity of reproductive structures especially that of females and controlling for environmental and geographic factors when investigating RCD. Our study contributes valuable insights into the dynamics of reproductive isolation mechanisms and genital coevolution, shedding light on the intricacies of damselfly population interactions in hybrid zones.

DATA AVAILABILITY

All datasets used in this manuscript were uploaded to DRYAD at: <https://doi.org/10.5061/dryad.jh9w0vtm9>.

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AUTHOR CONTRIBUTIONS

RAS-G designed the study. AVB-G and RAS-G did field samplings. Data were analyzed by AVB-G and RG. AVB-G, SMO-G and RAS-G designed analyses. AVB-G and RAS-G wrote the first draft of the manuscript. AVB-G made scientific illustrations. All authors read, reviewed, and approved the final version of the submitted manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The study was designed to minimize disruption to local ecosystems and to avoid the unnecessary harm or destruction of insect populations. All necessary permits to RAS-G were obtained from relevant authorities before the collection of insect specimens.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41437-024-00719-9>.

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