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**Authors for correspondence:**
Janne Swaegers
e-mail: janne.swaegers@kuleuven.be
Bengt Hansson
e-mail: bengt.hansson@biol.lu.se

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# Restricted X chromosome introgression and support for Haldane's rule in hybridizing damselflies

Janne Swaegers[1,2], Rosa Ana Sánchez-Guillén[3], Pallavi Chauhan[1], Maren Wellenreuther[4,5] and Bengt Hansson[1]

[1]Department of Biology, Lund University, Ecology Building, Lund 22362, Sweden
[2]Evolutionary Stress Ecology and Ecotoxicology, KU Leuven, Leuven, Belgium
[3]Instituto de Ecología A.C., Xalapa, Veracruz, Mexico
[4]The New Zealand Institute for Plant and Food Research Ltd, Nelson, New Zealand
[5]School of Biological Sciences, University of Auckland, Auckland, New Zealand

JS, 0000-0003-1952-3170; RAS-G, 0000-0001-6024-8321; PC, 0000-0002-5160-6673; MW, 0000-0002-2764-8291; BH, 0000-0001-6694-8169

Contemporary hybrid zones act as natural laboratories for the investigation of species boundaries and may shed light on the little understood roles of sex chromosomes in species divergence. Sex chromosomes are considered to function as a hotspot of genetic divergence between species; indicated by less genomic introgression compared to autosomes during hybridization. Moreover, they are thought to contribute to Haldane's rule, which states that hybrids of the heterogametic sex are more likely to be inviable or sterile. To test these hypotheses, we used contemporary hybrid zones of *Ischnura elegans*, a damselfly species that has been expanding its range into the northern and western regions of Spain, leading to chronic hybridization with its sister species *Ischnura graellsii*. We analysed genome-wide SNPs in the Spanish *I. elegans* and *I. graellsii* hybrid zone and found (i) that the X chromosome shows less genomic introgression compared to autosomes, and (ii) that males are underrepresented among admixed individuals, as predicted by Haldane's rule. This is the first study in Odonata that suggests a role of the X chromosome in reproductive isolation. Moreover, our data add to the few studies on species with X0 sex determination system and contradict the hypothesis that the absence of a Y chromosome causes exceptions to Haldane's rule.

## 1. Introduction

Since Darwin's theory of evolution [1], it has become clear that speciation—the evolution of reproductive barriers between populations—is complex and continuous. It is already well established that due to independent assortment and recombination, genome regions have unique evolutionary histories. For example, alleles that are neutral or (generally) adaptive are expected to cross species boundaries, while alleles under divergent selection or associated with reproductive isolation are not [2]. Species boundaries can therefore be expected to be 'semipermeable'. The heterogeneity of genomic divergence is expected to be the result of the interplay between natural and sexual selection as well as gene flow, demography and recombination. However, characterizing the genomic architecture of barriers to gene exchange remains a key challenge in studies of speciation [3,4], especially in non-model species [4].

Contemporary hybrid zones—regions where species hybridize and introgress—offer fascinating opportunities to study speciation [5]. First, hybrid zones act as natural laboratories for the investigation of species boundaries and more generally the origin of species [6]. It is within these hybrid zones that divergent loci associated with reproductive isolation can be detected. This is in contrast to the comparison of allopatric (non-overlapping) parental species, where divergent loci can reflect different selection pressures and/or

random effects operating after speciation has been completed [7,8]. Second, hybrid zones may shed light on the role of sex chromosomes in facilitating species divergence, as indicated by less genomic introgression on the X (or Z) chromosome compared to autosomes during hybridization [6]. Moreover, studies in hybrid zones may provide insights into the observation that when two closely related species hybridize, it is often the heterogametic sex that suffers from fitness reduction (Haldane's rule [9]).

Indeed, loci that are showing divergence between species are often enriched on sex chromosomes (a pattern coined the large X-effect [10]). An explanation for this pattern is that recessive mutations that increase fitness in each of the diverging populations would accumulate faster on the X chromosome because of immediate exposure to selection in the heterogametic sex (males in XY systems) (faster-X theory [11,12]). During hybridization exposure of recessive mutations at the X chromosome may also results in deleterious effects in hybrids of the heterogametic sex due to negative gene interactions (epistasis) between these recessive X-linked mutations and dominant mutations at autosomes (the dominance theory of Haldane's rule [9,13]). Other non-mutually exclusive processes such as recombination, mutation and drift (due to effective population size differences between X chromosomes and autosomes), may add to these patterns [12,14].

Haldane's rule is widely supported by studies of hybridizing species of mammals and Diptera (XY), and birds and Lepidotera (ZW) [9,13]. Similarly, the large X-effect has strong support from many lineages [15], although the relative importance of the underlying processes (e.g. selection versus drift) has often been shown to be difficult to disentangle [16]. Nevertheless, the evidence for these patterns and processes is still sparse or lacking for many taxa and some sex chromosome systems. To further expand our knowledge on the role of sex chromosomes in speciation, more comprehensive knowledge is needed in a wider range of taxa and in other sex determination systems, such as the X0 and Z0 systems [4].

Here, we sought to clarify the role of the X chromosome in the origin of reproductive barriers in Odonata (dragonflies and damselflies), which is an insect order where some species have an X0 sex determination system and where both Haldane's rule and the large X-effect are yet to be investigated. More specifically, we focus on the recently established hybrid zone in Spain between the damselfly sister species pair *Ischnura elegans* and *I. graellsii* [17]. The hybrid zone is thought to be a consequence of the recent anthropogenic-driven range expansion of *I. elegans* into the northern and western regions of Spain [18]. Both species have been studied in exceptional detail for the last 20 years, providing access to a wealth of ecological and natural history data. Admixture analyses in the hybrid zone have revealed that the majority of *I. elegans* show levels of introgression similar to those expected for *I. elegans* backcrosses, and in a few cases, $F_1$ hybrids (first generation hybrids) [18]. Moreover, we have recently generated a scaffold-level genome assembly in *I. elegans*, and identified X-linked genes and their properties [19]. *Ischnura* damselfly females have one pair of X chromosomes (XX), whereas males have a single X chromosome (and no Y chromosome). Thus, females have a diploid sex chromosome karyotype (XX), whereas males are hemizygous for X (X0). To our knowledge, so far only two other studies have investigated introgression patterns between autosomes and the X chromosome in species with an X0 sex determination system (both in the insect order Orthoptera; [20,21]). Interestingly, the absence of a Y chromosome might relax several mechanisms that might contribute to Haldane's rule, such as incompatibilities between Y-linked and autosomal genes [22,23] and meiotic drive [24]. By studying species with an X0 sex determination system, we can explore whether these mechanisms are necessary for Haldane's rule to apply. The few existing case studies of X0 sex determination systems show incidentally rare exceptions to Haldane's rule [25].

Today high-throughput sequencing technology provides unprecedented opportunities to study genomic evolutionary histories at hybrid zones [6] allowing exciting approaches to disentangle evolutionary processes across the speciation continuum [5]. Here, we analyse genome-wide distributed single-nucleotide polymorphisms (SNPs) in the Spanish *I. elegans* and *I. graellsii* hybrid zone to test whether the X chromosome shows less genomic introgression compared to autosomes. In addition, we tested whether X0 males are underrepresented among hybrids and backcrosses as predicted by Haldane's rule during hybridization caused by range expansion.

## 2. Methods

### (a) Sampling strategy

We sampled individuals from 15 localities in the hybrid zone along with five localities of allopatric *I. elegans* and four localities of allopatric *I. graellsii* (figure 1*a*; for details, see electronic supplementary material, table S1). Additionally, three closely related species from the *elegans-clade* (*I. fountaineae*, *I. genei* and *I. saharensis*) were also sampled (electronic supplementary material, table S1).

### (b) Library construction, RAD-seq analysis and filtering

Genomic DNA from the head and thorax of 253 individuals (244 samples of *I. elegans* and *I. graellsii* and nine samples of closely related *Ischnura* species that were used as outgroup samples in part of subsequent analyses, electronic supplementary material, table S1) was extracted with the DNeasy Blood & Tissue Kit (Qiagen). Extracted genomic DNA was quantified using Nanodrop and Qubit and DNA degradation was visually inspected through 1% agarose gel electrophoresis. In total, eight single-digest Restriction site-Associated DNA (RAD) libraries were constructed following the protocol described by Etter *et al*. [26] and modified by Dudaniec *et al*. [27]. Per library, 40 unique barcodes were used to label the samples (sourced from Metabion). Five of these libraries (containing 206 samples) were paired-end sequenced (2*100 bp) on separate lanes of an Illumina HiSeq 2500 at SNP&SEQ Technology Platform at Uppsala University, whereas the remaining three libraries (containing 61 samples) were paired-end sequenced (2*125 bp) on three lanes of an Illumina HiSeq 2500 at BGI (Hong Kong).

We used the bioinformatic pipelines in STACKS v. 2.2 [28,29] to process the sequences. Process_radtags was used to demultiplex the raw reads, and clone_filter to identify and discard PCR clones using default parameters. Next, sequence reads were aligned to the *I. elegans* draft genome assembly (genome size of 1.67 Gbp; [19]) using BOWTIE2 v. 2.3 (mismatch allowance per seed alignment of 1, maximum mismatch penalty of 6 and minimum of 2, maximum fragment length of 1000 bp and minimum of 100 bp, [30]). The aligned samples were processed with the ref_map pipeline to detect SNPs using default parameters (different runs were performed when including and excluding outgroup samples).

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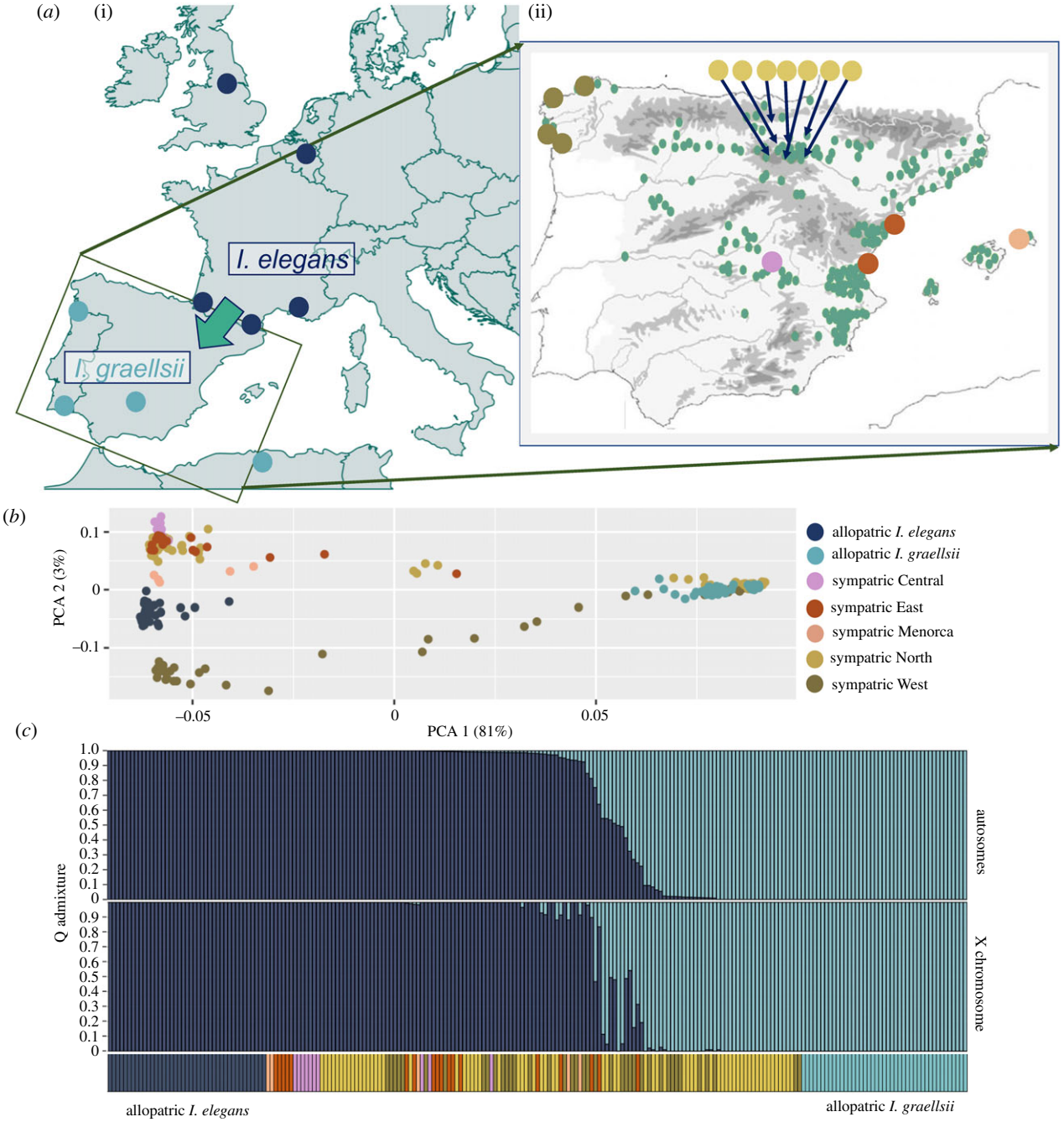


**Figure 1.** (*a*) Maps showing the allopatric and sympatric populations of *I. elegans* and *I. graellsii* that were studied. Green areas on the rightmost map indicate where *I. elegans* has expanded its range into Spain. (*b*) The first two axes of a principal component analysis (PCA) of all allopatric and sympatric individuals. The colours match the sample locations on the map. (*c*) Individual admixture proportions (*Q*-values) based on autosomal and X-linked SNPs, respectively. Samples have been ordered based on the *Q*-values from autosomal SNPs. (Online version in colour.)

We discarded 20 samples that had a mean depth <20× and also two *I. graellsii* samples from the population Seyhouse (Algeria) as exploratory analyses of population structure revealed possible hybridization in those samples with a third *Ischnura* species [31]. We generated three different SNP sets for subsequent analyses using 'populations' in STACKS: a first set including all SNPs detected among allopatric samples of *I. elegans* and *I. graellsii*; a second set with only diagnostic SNPs between the allopatric samples of *I. elegans* and *I. graellsii* (i.e. loci that are differentially fixed between these two groups), and a third set with outgroup samples included. For all three SNP sets, only SNPs with a minor allele frequency of >0.05 and an observed heterozygosity of <0.7 were retained. Moreover, loci had to occur in 80% of the individuals in a population. For the two non-diagnostic SNP sets, the locus had to occur in 80% of the individuals in a population and in 20 of the 25 (or 28 for the SNP set with outgroup samples included) populations to be included in the final SNP set. The SNP sets that did not include the outgroup samples were subsequently filtered to include only one random SNP per RAD-tag to create data without closely linked loci (using the write_random_snp option in STACKS). These SNP sets are hereafter referred to as the 'full SNP set', 'unlinked full SNP set' and the 'diagnostic SNP set', respectively, while the SNP set with the outgroup samples is referred to as the 'outgroup SNP set'. For all these SNP sets, we differentiated between SNPs that were located on autosomes versus the X chromosome based on an *I. elegans* reference genome assembly [19]. This genome assembly consists of 423 X chromosome scaffolds (4.7%) and 8565 autosomal scaffolds (95.3%) [19].

Next, we genotypically classified individuals as male or female based on observed homozygosity ($H_O$) at X-linked SNPs. As males

are hemizygous, we expect an $H_O = 1.0$ at X-linked SNPs for males, yet in practice, deviations are expected due to genotyping error. As females have two copies of the X chromosome, we expect lower $H_O$ in females compared to males. Accordingly, using data of X-linked SNPs at the full SNP set, we found that the $H_O$ values among all *I. elegans* and *I. graellsii* samples were bimodally distributed (electronic supplementary material, figure S1). We selected a cut-off value at the valley of the bimodal $H_O$ distribution (i.e. $H_O = 0.96$) to classify samples having $H_O < 0.96$ as females and samples having $H_O > 0.96$ as males. In this way, we genotypically classified the *I. elegans* and *I. graellsii* samples as 129 females and 94 males. As we used samples that were in many cases >10 years old, phenotypically sexing of individuals was not always straightforward. Among the *I. elegans* and *graellsii* samples that had been phenotypically classified as females, all 96 had $H_O < 0.96$ as expected, whereas 19 of 105 phenotypically classified as males had $H_O < 0.96$ (these were treated as females in the analyses). Among the samples that had not been phenotypically sexed, 14 were classified as females and eight as males based on $H_O$. The outgroup samples were genotypically classified as eight females and one male using $H_O$ at X-linked SNPs at the outgroup SNP set.

Finally, we filtered the X-linked SNPs further by retaining only those SNPs that were homozygous in all genotypically classified males. This was done for all SNP sets, giving the final SNP sets: the full SNP set with 50 733 SNPs of which 2469 are X-linked, the full unlinked SNP set with 7352 SNPs of which 390 are X-linked, the diagnostic SNP set with 1931 SNPs of which 111 are X-linked, and the outgroup SNP set with 64 452 SNPs of which 4603 are X-linked. When analyses are performed on only autosomal SNPs or only X-linked SNPs, we referred to these SNP sets as, e.g. the X-linked full SNP set or the autosomal diagnostic SNP dataset. The percentage of loci that was assembled in the diagnostic SNP set from the total of unlinked SNPs (26%) was in the order of magnitude that we expected from other similar studies (e.g. 11–23% in two previous RAD-seq hybridization studies [32,33]). Using the full SNP set, we calculated genetic diversity and differentiation indices within and between allopatric and sympatric regions (observed and expected heterozygosity, pi, $F_{ST}$ and divergence $d_{xy}$ in STACKS v. 2.2).

## (c) Population structure analysis

To discern population structure among the samples, we performed principal component analysis (PCA) using the PCA function in PLINK v. 1.9 [34]. For this analysis, we used autosomal SNPs from the full SNP set).

## (d) Individual ancestry coefficients

We compared the ancestry of individuals to allopatric *I. elegans* and *I. graellsii* between autosomes and the X chromosome by calculating individual ancestry coefficients (*Q*-values) using both the autosomal and X-linked diagnostic SNP set in ADMIXTURE v. 1.3.0 [35]. ADMIXTURE was run using the supervised learning mode with the allopatric *I. elegans* and *I. graellsii* individuals as reference samples meaning 100% ancestry is assumed for the respective species. For the X-linked diagnostic SNP set, hemizygosity was accounted for by setting the haploid flag for all males. We tested whether individuals are more admixed at autosomal SNPs than X-linked SNPs by comparing the frequencies of sympatric individuals which have *Q*-values of 0 of 1 ('pure' individuals) with those that have *Q*-values >0 and <1 ('admixed' individuals) using a $\chi^2$-test.

## (e) Introgression analysis

We used two different approaches to infer whether introgression patterns are different between autosomes versus the X chromosome. First, we employed a Bayesian genomic clines (BGC) analysis of Gompert & Buerkle [36,37], which makes use of Markov chain Monte Carlo to estimate genomic cline parameters within a Bayesian genomic cline model. The per locus probability of being inherited from a given parental population ($\phi$) is calculated, which is then compared to the genome-wide average probability, i.e. the hybrid index. Two parameters, $\alpha$ and $\beta$, summarize this probability and hence the pattern of introgression between the parental populations that are nearly fixed for the focal markers. For this analysis, we used the autosomal and X-linked diagnostic SNP set. In our case, the parameter $\alpha$ measures the directional movement of alleles from *I. graellsii* into *I. elegans* ($\alpha > 0$) or movement from *I. elegans* into *I. graellsii* ($\alpha < 0$), while the $\beta$ parameter, measures the strength of the barrier to gene flow between the two species. Higher positive values of the $\beta$ parameter describe steeper clines and a greater strength of the gene flow barrier. We ran 5 independent chains in BGC using the genotype certainty model, each for 50 000 steps with a burn-in of 25 000 and thinning samples by 20. We combined the output for both $\alpha$ and $\beta$ using ClineHelpR (available at https://github.com/btmartin721/ClineHelpR). To test whether X-linked SNPs displayed higher $\beta$ values than the autosomes, we generated 10 000 permuted datasets by sampling without replacement from the autosomal $\beta$ value distribution. For each dataset, we sampled 111 times, i.e. the number of X-linked diagnostic SNPs, to generate equal sample sizes between autosomal and X-linked datasets. Subsequently, we compared the median of $\beta$ values of the X-linked distribution to the median of each permuted autosomal dataset and considered a greater gene flow barrier on X-linked SNPs compared to autosomal SNPs if the X-linked observed median exceeded the median in >95% of the permuted datasets [38].

Second, we made use of ABBA–BABA statistics which are based on the relative frequency of shared alleles between three focal groups, along one outgroup to determine which allele is ancestral. In our case, we compare (i) the frequency of shared alleles between sympatric *I. elegans* and allopatric *I. graellsii* (ABBA) compared to shared alleles between allopatric *I. elegans* and allopatric *I. graellsii* (BABA), and (ii) the frequency of shared alleles between sympatric *I. graellsii* and allopatric *I. elegans* (ABBA) compared to shared alleles between allopatric *I. graellsii* and allopatric *I. elegans* (BABA). If introgression occurs in sympatry, higher frequencies of ABBA than of BABA are expected. Patterson's D is the original test statistic used to measure this but is now often used in parallel with related test statistics $f_d$ and $f_{dM}$ that are less biased when, for example, used in sliding windows frameworks [39]. We here report the results using $f_{dM,}$ yet similar results were found with test statistics D and $f_d$ (results in electronic supplementary material, table S5). For this analysis, we used the outgroup SNP set. We ran Dsuite [39] to measure these test statistics along the genome using a sliding window approach. More specifically, we ran the function Dinvestigate with a window size of 50 informative SNPs and a step of 5 SNPs. As outgroup, we used three samples each from congeneric species *I. genei*, *I. fountaineae* and *I. saharensis*. We calculated the introgression parameters both for introgression from allopatric *I. graellsii* into sympatric *I. elegans* and from allopatric *I. elegans* into sympatric *I. graellsii*. Below we show which samples were used as 'P1', 'P2' and 'P3' for both analyses ('P4' is the outgroup). As we wanted to include sympatric individuals that can be considered to be genomically *I. elegans* or *I. graellsii*, respectively, in this analysis, but did not know how incorporating individuals of more recent hybrid ancestry will affect the results, we used different autosomal *Q* admixture cut-off values to decide which sympatric individuals can be considered to be either genomically *I. elegans* or *I. graellsii*. (i) $Q = 0$ for sympatric *I. elegans* and $Q = 1$ for sympatric *I. graellsii*, (ii) $Q < 0.1$ for sympatric *I. elegans* and $Q > 0.9$ for sympatric *I. graellsii*, (iii) $Q < 0.25$ for sympatric *I. elegans* and $Q > 0.75$ for sympatric *I. graellsii*. We ran one analysis for each of these chosen cut-off values per species (six analyses in total).

Analogously to the BGC analysis, we generated permuted datasets from the distributions of test statistics of the autosomal

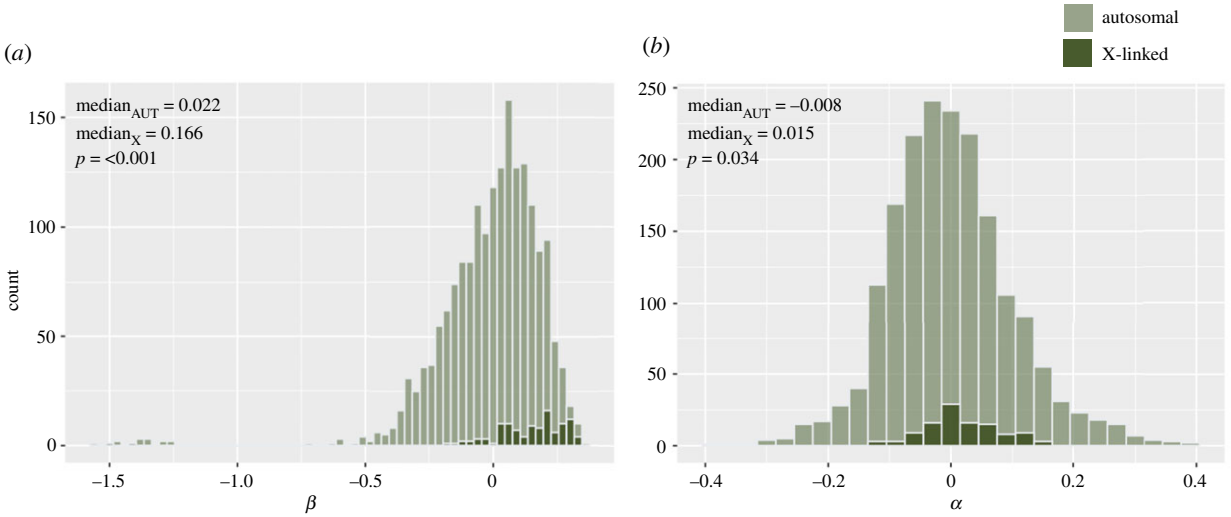


**Figure 2.** Results of BGC analysis of females in sympatric populations of *I. elegans* and *I. graellsii*. Shown are the (*a*) $\beta$ and (*b*) $\alpha$ distributions. The medians of the distributions measured autosomal and X-linked SNPs, respectively, are given in each panel, as well as the *p*-value from a permutation test comparing these medians. (Online version in colour.)

windows and compared the medians of these to the median of the test statistics of the observed X-linked distribution. This was done for all six analyses with the given autosomal *Q* admixture cut-off value. Note that using the autosomal *Q* admixture cut-off value is a conservative approach to compare introgression levels between autosomal and X-linked windows. We considered there to be less introgression on the X chromosome compared to autosomes if the X-linked observed median was less than the median in >95% of the permuted datasets.

As it is not possible to analyse males as hemizygous at the X chromosome in BGC and Dsuite, we ran these analyses using a subset of the data containing only the genotypically classified female individuals. However, we reran the analyses (using the same SNP sets) including both males and females (which did not change the results qualitatively; see below).

To assess whether X-linked SNPs that had a positive $\beta$ in the BGC analysis, or negative $f_{dM}$ value in the ABBA–BABA analyses (three analyses for *I. elegans* and three for *I. graellsii*), were clustered on particular X scaffolds, we assessed the SNP density (number of SNPs/kbp) of these putative 'reproductive barrier' SNPs on all associated X scaffolds. Moreover, we assessed the position of the top 20 X-linked SNPs with highest $\beta$ values and lowest $f_{dM}$ values (ABBA–BABA analyses) and explored the annotated function of genes that were found on the shared associated scaffolds of these SNPs between the BGC analysis and either the *I. elegans* or *I. graellsii* ABBA–BABA analyses.

### (f) Haldane's rule

To test whether males were underrepresented among sympatric admixed individuals, we tested for associations between sex and proportion admixture for three different autosomal and X-linked *Q* admixture cut-off values using the full SNP set. As only females were sampled in the western sympatric region ('sympatric West', figure 1*a*), we excluded all individuals in this region from the analysis. The following cut-off values were used to differentiate between admixed and non-admixed individuals: (i) $Q = 0$ or 1 (non-admixed individuals) and $0 < Q < 1$ (lowly to highly admixed individuals), (ii) $0.1 > Q > 0.9$ (non- to lowly admixed individuals) and $0.1 < Q < 0.9$ (moderately to highly admixed individuals) and (iii) $0.25 > Q > 0.75$ (non- to moderately admixed individuals) and $0.25 < Q < 0.75$ (highly admixed individuals). Fisher's exact tests were used to test whether males and females differed in numbers of non-admixed and admixed individuals for each *Q*-value cut-off based on autosomal and X-linked SNPs, respectively.

## 3. Results

### (a) Genetic structure

A PCA of all allopatric and sympatric *I. elegans* and *I. graellsii* individuals based on autosomal SNPs at the full SNP set clearly separated the allopatric populations at the first axis (PC1) which explained much of the variation (figure 1*b*). By contrast, some of the sympatric populations in the hybrid zone spread out along PC1, and separated partly along the minor second axis, PC2 (figure 1*b*). An admixture analysis confirmed these patterns by grouping individuals in allopatric populations in separate clusters, while some sympatric samples had intermediate admixture proportions (*Q*-values; figure 1*c*). Interestingly, more individuals had intermediate *Q*-values using autosomal SNPs compared to when using X-linked SNPs. At X-linked SNPs, sympatric individuals were more often showing *Q* admixture values closer to the values of allopatric individuals (figure 1*c*; see also electronic supplementary material, figure S2). Indeed, admixed individuals ($0 < Q < 1$) were significantly underrepresented at X-linked SNPs, compared to autosomal SNPs ($p < 0.001$).

Observed and expected heterozygosity and pi are shown in electronic supplementary material, table S2a for allopatric and sympatric populations of each species. $F_{ST}$ and divergence calculations are shown in electronic supplementary material, table S2b-c.

### (b) Bayesian genomic clines

We tested the strength and direction of allele movements between species using the diagnostic SNP set in females. We found that $\beta$ values were significantly higher at X-linked SNPs compared to autosomal SNPs (permutation test, $p < 0.001$; figure 2*a*). Also, the $\alpha$ parameter was higher at X-linked compared to autosomal SNPs ($p = 0.034$; figure 2*b*). In other words, X-linked SNPs showed steeper clines (and hence a greater strength of the gene flow barrier) with alleles more likely to move from *I. graellsii* into *I. elegans*

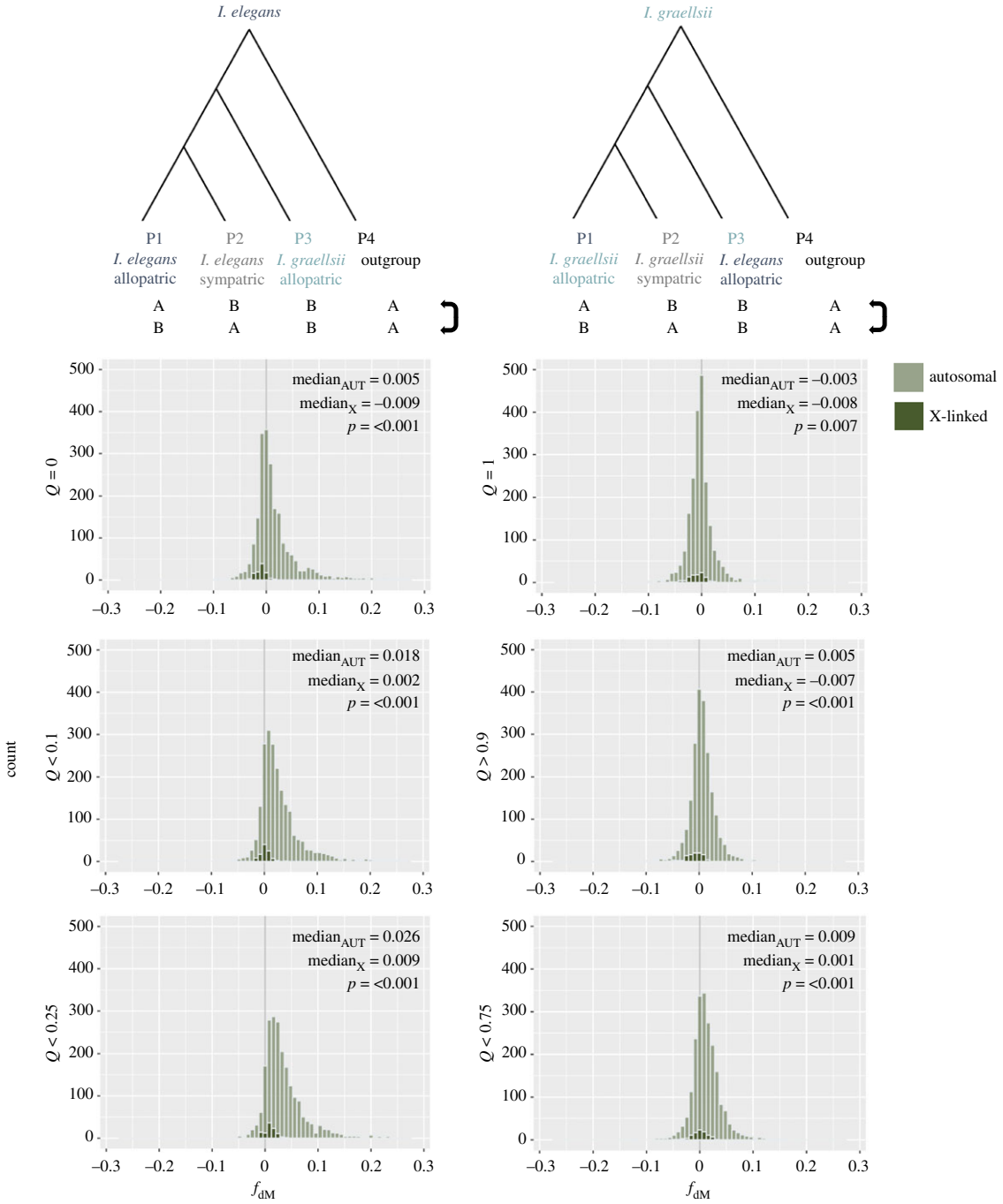


**Figure 3.** Results of ABBA–BABA analysis of females in sympatric populations of *I. elegans* and *I. graellsii*. Shown are the $f_{dM}$ distributions. The medians of the distributions measured with autosomal and X-linked SNPs, respectively, are included in each panel, as well as the *p*-value from a permutation test to compare these medians. The left panels show results from sympatric *I. elegans*, and the right panels are sympatric *I. graellsii*. (Online version in colour.)

compared to the autosomal SNPs. Indeed, 87% of the X-linked SNPs showed positive $\beta$ values compared to 56% of the autosomal SNPs and 59% showed positive $\alpha$ values compared to 46% in the autosomes. Similar results were found in analysis that included both females and males (electronic supplementary material, table S3).

## (c) ABBA–BABA

Figure 3 shows the distributions of $f_{dM}$ statistics between autosomal windows and windows located on the X chromosome. This statistic has the advantage of being symmetrically distributed around zero under the null hypothesis of no introgression and quantifies shared variation between P2 and P3 (positive values; ABBA) or between P1 and P3 (negative values; BABA) equally. For most $Q$ admixture cut-off values used to include sympatric individuals (i.e. $Q = 0$ or 1; $Q < 0.1$ or >0.9; $Q < 0.25$ or >0.75), X-linked SNPs showed significantly less introgression ($f_{dM}$ values distributed close to 0) between allopatric *I. graellsii* and sympatric *I. elegans* (*I. elegans* panel), and between allopatric *I. elegans* and sympatric *I. graellsii* (*I. graellsii* panel), than autosomal SNPs ($f_{dM}$ biased towards positive values; permutation test, $p \leq 0.01$ in.all six analyses; figure 3). Overall, the bias towards more introgression of autosomal than X-linked SNPs was more apparent for introgression into sympatric *I. elegans* (*I. elegans* panel). From figure 3, it can also be concluded that overall introgression occurs more frequently from allopatric *I. graellsii* into sympatric *I. elegans* than from allopatric *I. elegans* into sympatric *I. graellsii* (Wilcoxon rank-sum test,

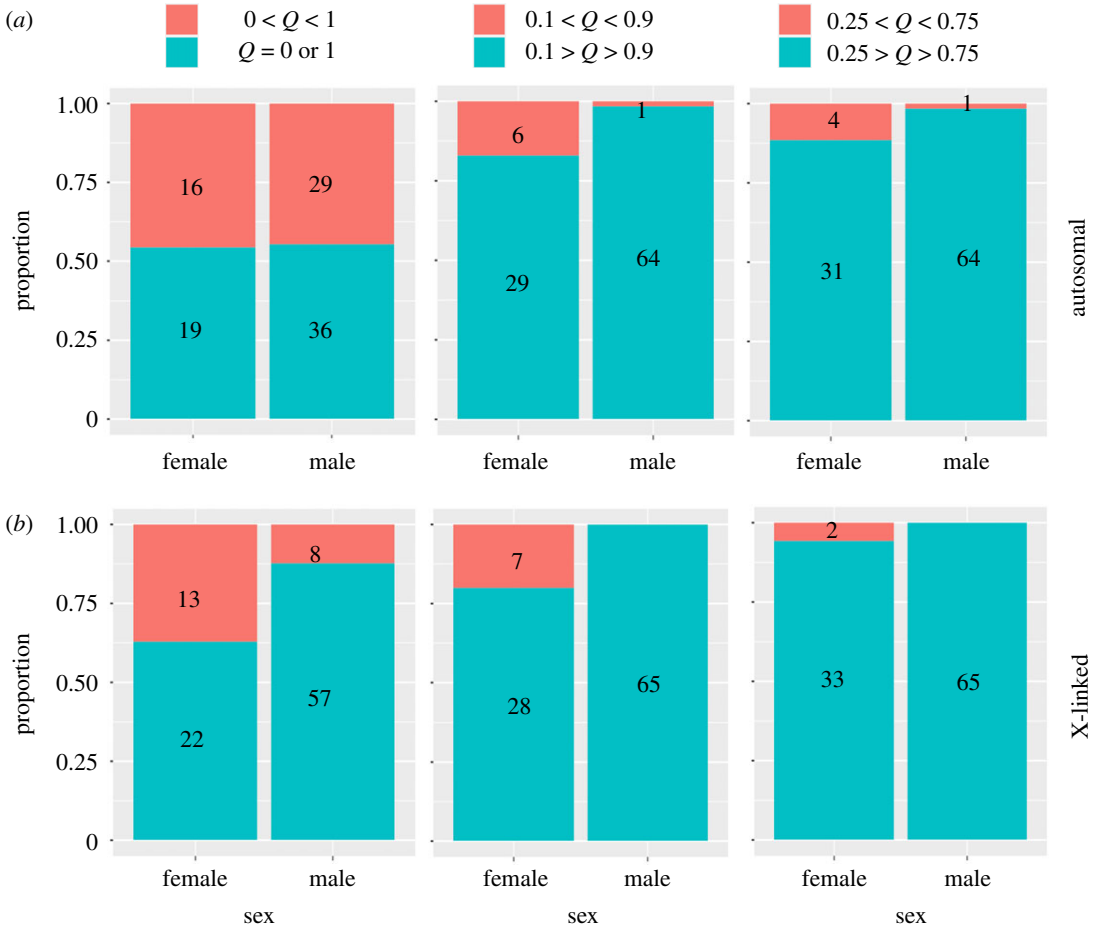


**Figure 4.** The proportion of admixed and non-admixed individuals in females and males, respectively, when using three different *Q* admixture cut-off values and either (*a*) autosomal and (*b*) X-linked SNPs. In all cases, males were underrepresented in the admixed (or highly admixed) category compared to females (Fisher's exact test; (*a*) $p = 1$, 0.007, 0.050, respectively; (*b*) $p = 0.009$, <0.001, 0.120, respectively). (Online version in colour.)

$p < 0.001$ in all three comparisons, $Q = 0$ versus $Q = 1$; $Q < 0.1$ versus $Q > 0.9$; $Q < 0.25$ versus $Q > 0.75$). These above results are for analysis with females only, but similar results were found in analyses including also males (electronic supplementary material, table S4).

## (d) Distribution of 'reproductive barrier' SNPs along X-linked scaffolds

The number of X-linked scaffolds with at least one putative 'reproductive barrier' SNP ranged between 16 and 73 among the BGC and the ABBA–BABA analyses (electronic supplementary material, table S6). Overall longer scaffolds hold more SNPs (Pearson correlation test: all $p < 0.05$ and one analysis $p = 0.059$), and no scaffolds were identified with a deviant SNP density of putative 'reproductive barrier' SNPs. Thus, there does not appear to be any strong clustering of candidate SNPs to one or a few specific scaffolds on the X chromosome. The top 20X-linked SNPs with highest $\beta$ values (BGC analysis) and lowest $f_{dM}$ values (ABBA–BABA analyses) were located on 16 scaffolds for the BGC analysis, on 11 scaffolds in all three *I. elegans* ABBA–BABA analyses, and on 11 scaffolds in all three *I. graellsii* ABBA–BABA analyses. Five of these, 16 BGC-scaffolds were in turn the same as at least one of the 11 *I. elegans*- or the 11 *I. graellsii*-ABBA–BABA-scaffolds. All annotated genes on these 5 scaffolds are listed in electronic supplementary material, table S7.

## (e) Haldane's rule

Admixed males were overall underrepresented within the hybrid zone, but the degree of underrepresentation differed for autosomal and X-linked diagnostic SNPs, and when different admixture cut-off values were used to categorize individuals as admixed or non-admixed (figure 4). For autosomal SNPs, males were significantly underrepresented in the admixed category both when individuals with *Q*-values between 0.1 and 0.9 ($0.1 < Q < 0.9$; Fisher's exact test, $p < 0.007$), and between 0.25 and 0.75 ($0.25 < Q < 0.75$; $p = 0.050$), were categorized as admixed (figure 4, upper panels). However, when *Q*-values between 0 and 1 ($0 < Q < 1$) were used to categorize admixed individuals, males were not significantly underrepresented among admixed individuals ($p = 1$). For X-linked SNPs, males were significantly underrepresented among admixed individuals when individuals with *Q*-values between 0 and 1 ($0 < Q < 1$; $p = 0.009$), and between 0.1 and 0.9 ($0.1 < Q < 0.9$; $p < 0.001$), were categorized as admixed (figure 4, lower panels). For *Q*-values between 0.25 and 0.75 ($0.25 < Q < 0.75$), males were not significantly underrepresented among the admixed individuals ($p = 0.120$), but it should be noted that the numbers of sampled highly admixed individuals was very low (figure 4).

# 4. Discussion

In this study, we analysed genome-wide distributed SNPs in the Spanish *I. elegans* and *I. graellsii* hybrid zone and found (i)

that the X chromosome showed less genomic introgression compared to autosomes and (ii) that males are under-represented among hybrids and backcrosses as predicted by Haldane's rule.

### (a) Introgression patterns

Through two different approaches and SNP sets (BGC using the 'diagnostic SNP set' with only *I. elegans* and *graellsii* samples, and ABBA–BABA using the 'outgroup SNP set' which also included outgroup samples), we detected a lower level of introgression at the X chromosome compared to autosomes. Indeed, both methods measure introgression, yet BGC is a model-based approach while ABBA–BABA measures statistics proportional to the effective migration rate [40]. The similar results should be considered as complementary evidence for restricted introgression at the X chromosome compared to autosomes between the two species in the hybrid zone. This is the first study in Odonata that suggests a role of the X chromosome in reproductive isolation. Although at this point direct evidence is lacking, our results suggest that a large X-effect may have contributed to an accumulation of reproductive barrier genes on the X chromosome. Thus, our result in an X0-system is in line with the large body of research in XY and ZW systems that systematically have shown greater differentiation at the sex chromosomes than at the autosomes between closely related species (reviewed in [15]). To our knowledge, only two previous studies have investigated introgression patterns between autosomes and the X chromosome in hybrid zones of species with an X0 sex determination system (both in insect order Orthoptera [20,21]). Both these studies support that large X evolution has contributed to an accumulation of reproductive isolating genes on the X chromosome, as was also suggested in the current damselfly system.

Interestingly, both introgression analyses suggest that the direction of introgression is in general biased towards introgression from allopatric *I. graellsii* into sympatric *I. elegans*. This can be explained by three processes. First, from previous research in western Spain, we know that there is asymmetry in the strength of the reproductive barriers between reciprocal crosses. Male *I. elegans* can more easily mate and produce hybrids with female *I. graellsii* and female hybrids, than the other way around [17,41]. Curiously, this was also reflected here by the fact that the only sampled male F1 hybrid (autosomal SNP *Q*-value: 0.5) had inherited its X chromosome from an *I. graellsii* mother. Overall weaker reproductive barriers in *I. elegans* would imply easier introgression into this species. Second, it could be expected that, in this case, alleles from *I. graellsii* rather than from *I. elegans* confer higher fitness in hybrid individuals [5]. This hypothesis is based on the rational that alleles from the native *I. graellsii* are expected to contribute more to local adaptation than those from *I. elegans* (which is relatively new to this region) [42]. Note that even when reproductive barriers are strong between two species, adaptive introgression is possible [5]. Third, it seems reasonable to assume that the expanding *I. elegans* had reduced effective population size ($N_E$) during the initial colonization phase due to founder effects. Low $N_E$ would increase the magnitude of genetic drift and the likelihood that weakly deleterious alleles originating from *I. graellsii* will stochastically increase in frequency and become introgressed in sympatric *I. elegans* [43]. Both mechanistic asymmetry and adaptive introgression, as well as genetic drift, could have acted simultaneously to the observed asymmetric introgression from *I. graellsii* to *I. elegans*.

The ADMIXTURE plots and PCA show geographical variation in the levels of admixture between regions and populations (e.g. all admixed individuals from the western region originated from one population 'Louro'; data not shown). This geographical variation is to a high extent driven by variation in the time since the colonization of *I. elegans* which differs between eastern and western populations, as well as between populations within regions [31]. However, we believe that these geographical patterns are less relevant in the context of the detected large X-effect because the analysis of the level of introgression among X-linked and autosomal SNPs is conducted within individuals, implying that any geographical variation in the overall level of introgression in different populations should to a large extent be controlled for.

### (b) Evidence for Haldane's rule

When we compared the proportion of admixed versus non-admixed individuals between the sexes, we found fewer males than females among the admixed individuals. This pattern was pronounced at low levels of admixture of the X chromosome but not of the autosomes. Lower survival of males carrying hybrid and backcrossed X chromosomes is in accordance with the expectations from Haldane's rule. Our data hence suggest that Haldane's rule is valid in this insect order. An increased rate of mortality among hybrid and backcrossed males could be caused by the expression of recessive X-linked mutations and associated incompatibilities in X0 male hybrids. These incompatibilities are hypothesized to be caused by deleterious epistatic effects between X-linked and autosomal alleles with different species ancestry [13]. The observed pattern of lower level of introgression between the two studied species at the X chromosome as compared to the autosomes further supports the presence of X-linked incompatibilities. Interestingly, we note that two of the five shared X scaffolds that were associated with top 'reproductive barrier' SNPs have genes that are associated with DNA-binding or methyltransferase. These GO terms have been linked to meiotic recombination and hybrid male sterility in mice [44].

This is one of the rare studies using a natural system in which the study species do not have a Y chromosome, and our results imply that neither incompatibilities between Y-linked and autosomal genes, nor meiotic drive, are necessary to cause the deleterious effects in male hybrids. Thus, our study does not support the suggestion that the absence of a Y chromosome constitutes an exception to Haldane's rule [25].

Interestingly, the overall lower survival of males in the hybrid zone could impact sex-ratios and hence sexual conflict [45]. In the current species, sexual conflict over optimal mating rates is extensively studied [46], and our results hence warrant further investigation on the effects of hybridization on sexual conflict.

## 5. Conclusion

As predicted by theory, we here demonstrate that X-linked SNPs introgress less than autosomal SNPs in *I. elegans* and *I. graellsii* in the contemporary hybrid zone in Spain. Moreover, our data also suggest that Haldane's rule is valid in Odonata

and contradict the hypothesis that the absence of a Y chromosome causes exceptions to Haldane's rule. Thus, this is the first study in this insect order that suggests a role of the X chromosome in reproductive isolation. Future work is needed to establish if this also extends to other odonates and is thus a general rule. Expanding knowledge in the area of reproductive barriers, and mechanisms that fuel admixture, is urgently needed to predict biodiversity consequences under a scenario of climate induced range shifts that will increase the encounters of closely related species, and consequently the likelihood of introgressive hybridization [47]. Moreover, deciphering the relative contributions of X chromosomes and autosomes in keeping species together or not is shedding important fundamental insights into genome function and the evolutionary processes at play that contribute to speciation.

Data accessibility. All raw reads were deposited in the NCBI Short Read Archive (SRA) Database under SRA accession PRJNA850104. VCF files and metadata were deposited on Dryad (https://doi.org/10.5061/dryad.gqnk98sp8) [48].

Electronic supplementary material is available online [49].

Authors' contributions. J.S.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation, visualization, writing—original draft; R.A.S.-G: conceptualization, funding acquisition, resources, writing—review and editing; P.C.: resources, writing—review and editing; M.W.: conceptualization, writing—review and editing; B.H.: conceptualization, investigation, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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