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SIMULATING RANGE EXPANSION: MALE SPECIES RECOGNITION AND LOSS OF PREMATING ISOLATION IN DAMSELFLIES

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Prolonged periods of allopatry might result in loss of the ability to discriminate against other formerly sympatric species, and can lead to heterospecific matings and hybridization upon secondary contact. Loss of premating isolation during prolonged allopatry can operate in the opposite direction of reinforcement, but has until now been little explored. We investigated how premating isolation between two closely related damselfly species, *Calopteryx splendens* and *C. virgo*, might be affected by the expected future northward range expansion of *C. splendens* into the allopatric zone of *C. virgo* in northern Scandinavia. We simulated the expected secondary contact by presenting *C. splendens* females to *C. virgo* males in the northern allopatric populations in Finland. Premating isolation toward *C. splendens* in northern allopatric populations was compared to sympatric populations in southern Finland and southern Sweden. Male courtship responses of *C. virgo* toward conspecific females showed limited geographic variation, however, courtship attempts toward heterospecific *C. splendens* females increased significantly from sympatry to allopatry. Our results suggest that allopatric *C. virgo* males have partly lost their ability to discriminate against heterospecific females. Reduced premating isolation in allopatry might lead to increased heterospecific matings between taxa that are currently expanding and shifting their ranges in response to climate change.

KEY WORDS: ***Calopteryx*, environmental change, hybridization, male mate choice, premating isolation, reinforcement, sympatry.**

Closely related species are expected to show strong species discrimination abilities in sympatry due to selection against unfit hybrid offspring, or because of direct selection against costly heterospecific matings (i.e., reinforcement Dobzhansky 1937; Noor 1999; Servedio 2001; Coyne and Orr 2004; Servedio 2004a). However, when congeneric species have only recently become sympatric, they may lack sufficient premating isolation mechanisms to prevent hybridization (Croucher et al. 2007). Similarly, even if species co-occurred in the past and evolved species recognition abilities, such mate recognition ability might be lost during subsequent prolonged phases of allopatry. This is because alleles for mate discrimination against heterospecifics will not be beneficial in the allopatric region, and might consequently be lost as a result of selection or because of genetic drift. As a result of this, an increase in heterospecific matings and hybridization due to loss of premating reproductive isolation might be expected when formerly allopatric species come into secondary contact as a result of a change in the distributional range of one or both species, e.g., as a consequence of a changing climate (Hickling et al. 2005).

Climate is an important factor influencing the geographic range of many species. Increasing ambient temperatures are expected to force species to shift their distributions by expanding into new geographic areas and by trying to escape from areas that become climatically unsuitable (Andrewartha and Birch 1954; Moritz et al. 2008). In the northern hemisphere, range shifts in response to climate warming are observed through northward range expansions toward the cooler upper latitudinal limits, and through range contractions at the warmer and lower latitudinal limits of species' ranges (Parmesan 2006 and references therein). Numerous cases of such climate-driven distributional range shifts and extinctions have been recorded for both vertebrate and invertebrate taxa from various geographic regions around the world (e.g., Parmesan 1996; Parmesan et al. 1999; Hickling et al. 2005; Deutsch et al. 2008). In Europe, many documented cases of range shifts among insects have been attributed directly to increasing temperatures (Parmesan 2006). For example, of the 35 European butterfly species that were studied by Parmesan et al. (1999), 22 have shifted their ranges northwards by 35–240 km over the last century, whereas only two have shifted south. These, and other recent range expansions, are rapidly producing new areas of range overlap of formerly allopatric taxa, and they offer unique research opportunities to study the consequences of novel species interactions, such as interspecific sexual isolation between newly sympatric species.

The genus *Calopteryx* ("demoiselles"), together with many other odonates (dragonflies and damselflies), belongs to a group of insects that are strongly affected by the ongoing climate change in Europe (Hickling et al. 2005). The banded demoiselle (*Calopteryx splendens*) and the beautiful demoiselle (*C. virgo*) are two congeneric damselfly species (Misof et al. 2000) that inhabit freshwater streams in Europe and closely resemble each other ecologically and phenotypically (Askew 2004). *Calopteryx virgo* is abundant throughout most of continental Europe and on the Iberian Peninsula, whereas *C. splendens* occurs as far south as the Mediterranean Sea in France and has a northern range limit that coincides with the classical biogeographical boundary *Limes Norrlandicus* in central Sweden (Aguilar and Dommanget 1998; Askew 2004). In Fennoscandia (Scandinavian Peninsula and Finland), allopatric populations of *C. virgo* extend north of the *L. Norrlandicus* in central Sweden (Askew 2004) and north of 64°51′00 latitude in Finland (Karjalainen 2002), which is the upper distributional limit of *C. splendens* in Finland. Unlike the allopatric northern populations of *C. virgo*, the populations in southern Fennoscandia overlap in their distribution with *C. splendens* (see Fig. 1). However, within this southern sympatric zone, a microgeographic "mosaic" of allopatric and sympatric populations of both species occur, which are connected by the dispersal of individuals between geographically close populations (Stettmer 1996; Svensson et al. 2004). The northern distributional range limit of these two *Calopteryx* species has been suggested to be determined by species-specific temperature tolerances (Schütte and Schrimpf 2002; Rüppel et al. 2005), as has been demonstrated for other damselfly species (e.g., Zahner 1959; van Doorslaer and Stoks 2005). In a recent study, Hickling et al. (2005) showed that 23 of the 24 temperate odonate species in the United Kingdom, including *C. splendens* and *C. virgo*, significantly expanded their range size and northern range limit between 1960 and 1995. Together, these data strongly indicate that European populations of *C. splendens* and *C. virgo* are currently moving northwards. Consequently, it can be expected that the distribution of *C. splendens* will soon expand into the allopatric region of *C. virgo* in northern Sweden and Finland (Fig. 1).

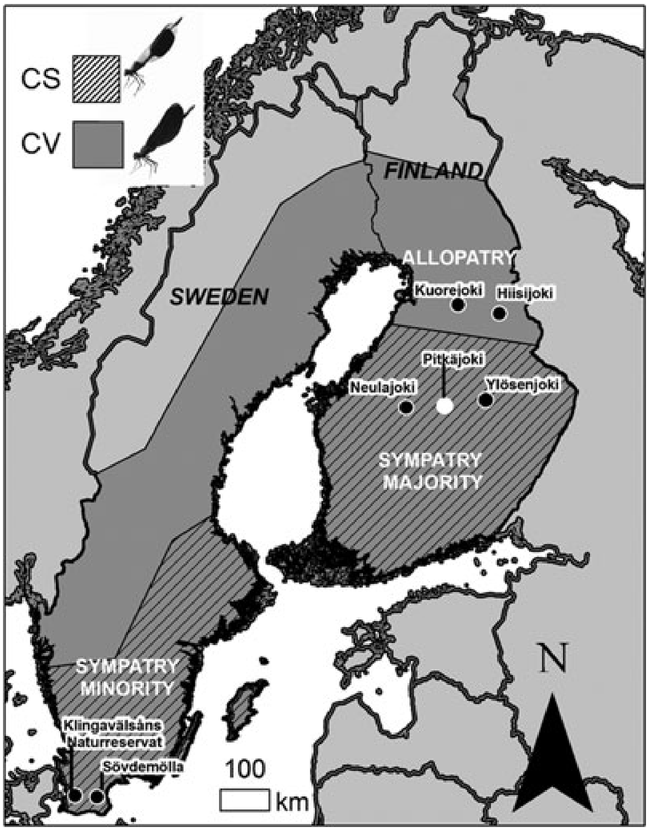

Figure 1. **Map showing the six study populations and the distributional range of Calopteryx *splendens* (CS, area with diagonal dark lines) and *C. virgo* (CV, dark gray area) in Sweden and Finland. The distributional ranges are based upon Karjalainen (2002) and Askew (2004). Black circles show the position of the study populations in Sweden and Finland, and the white circle shows the position of the population Pitkäjoki, which was used to obtain *C. splendens* females for the presentation experiments.**

In this study, we investigated the possible consequences of such a northward range expansion of *C. splendens* on the potential to mate and/or hybridize with *C. virgo* in northern Fennoscandia. Interspecific matings between the two *Calopteryx* species are relatively frequent and have been recorded in the sympatric zone in southern Sweden (Svensson et al. 2007), as well as in Finland (Tynkkynen et al. 2008). Despite the relative frequency of interspecific matings, the abundance of hybrids is low: 0.1% of all *C. splendens* males were found to be hybrids (Tynkkynen et al. 2008). However, if as a consequence of secondary allopatry the northern populations of *C. virgo* have partly or completely lost their ability to discriminate against *C. splendens*, an increased frequency of heterospecific matings might result if *C. splendens* expands northwards into this northern allopatric zone of *C. virgo*. Genetic and biogeographical data suggest that these two species and many closely related insect species occurred sympatrically in the Ponto-Mediterranean area during the last ice age (Sternberg 1998; Hewitt 1999, 2000). Hence, the ongoing northward extension of *C. splendens* is expected to create a situation of secondary sympatry. The current study differs from the more classical study systems of reinforcement and premating sexual isolation, where allopatry is the ancestral state, and sympatry is the derived state (Dobzhansky 1937; Noor 1999; Servedio 2001, 2004a). Therefore, we do not only address the specific issue of premating isolation in sympatry, but also the general issue of how long adaptations might be maintained when there is no longer selection in favor of them.

Our study also differs from previous studies in that we study male mating preferences toward conspecific and heterospecific females. We focus on male mate preferences and male species recognition for the following reasons: (1) although understudied, male mate choice is widespread among insects (Bonduriansky 2001) and in *Calopteryx* spp. males (Svensson et al. 2007), (2) *Calopteryx* spp. males successfully force copulations with conspecific and heterospecific females (Cordero 1999), and (3) heterospecific matings in areas of sympatry typically occur between females of the rare species and males of the common species (*C. splendens* females and *C. virgo* males in this case), but not vice versa (Wirtz 1999; Svensson et al. 2007; Tynkkynen et al. 2008). This latter point is due to the fact that in the presence of conspecific males, females reject heterospecific males and, consequently, a male in an environment of both heterospecific sexes is less likely to mate. In contrast, in the absence of conspecific males, females will sometimes accept fertilizations by males of other species, thus leading to hybrid matings between females of the rare species and males of the more common species (reviewed in Wirtz 1999). Forced copulations in *Calopteryx* spp. are particularly common when male density is high, and in these cases, female mate choice may have little bearing on which male they mate with (Cordero 1999; Cordero and Andrés 2002). The interplay of female mate choice and forced male copulations is, therefore, strongly dependent on the prevailing population conditions, such as the relative density of conspecific and heterospecific mates.

The potential consequences of a northward range expansion of *C. splendens* were investigated by comparing premating sexual isolation between *C. virgo* males and *C. splendens* females from sympatric populations in Sweden and Finland with populations in the allopatric zone in northern Finland. We translocated *C. splendens* from the sympatric into the allopatric region and then presented these females to local allopatric *C. virgo* males. Thereby we simulated the invasion of a “new” species to the northern allopatric region. We also presented *C. virgo* females to conspecific males in populations throughout Fennoscandia to control for potential differences in mating propensity among populations.

Materials and Methods

STUDY SPECIES

The Eurasian *Calopteryx* group began its radiation around 6.2 million years ago (Dumont et al. 2005). The first product of this radiation, at around 5.3 million years, was the *C. virgo*-group; followed by the *C. splendens*-group, at around 3.7 million years (Dumont et al. 2005). Like many other insect species, the glacial refugia of *C. splendens* during the climatic oscillations of the last Pleistocene ice ages were situated around the Ponto-Mediterranean area (Sternberg 1998). Although little is known about the exact glacial distribution of *C. virgo*, it appears highly likely that they co-occurred sympatrically with *C. splendens* in the same refugial area because of their ecological similarity. This is because the pronounced ice sheets in northern and central Europe would have only allowed populations of *C. virgo* to survive that occupied the warmer, southern refugia in Europe around the Ponto-Mediterranean area (Hewitt 1999; Schmitt 2007). Allopatry of *C. virgo* in northern Fennoscandia is hence most likely a secondarily derived state rather than an ancestral condition.

Nowadays, *C. splendens* and *C. virgo* are common damselfly species along freshwater streams in most parts of Europe, although the abundance of both species has decreased markedly during the last decades as a result of habitat fragmentation (Askew 2004). Allopatric populations of *C. virgo* occur north of the *L. Norrlandicus*, while no truly allopatric region exists for *C. splendens* in northern Europe (Askew 2004). Both calopterygid species are ecologically and phenotypically similar, although males differ in the degree of wing melanization. These species interact considerably in sympatry, with vigorous male–male competition over access to egg-laying sites (Tynkkynen et al. 2006). As a result, interspecific matings and putative hybrids have been found in the wild (De Marchi 1990; Corbet 1999; Tynkkynen et al. 2008). Males and females of both species emerge between late May and early June in southern Sweden (Svensson et al. 2004) and toward the end of June in Finland (K. Tynkkynen, pers. obs.).

Newly emerged individuals are characterized by a soft exoskeleton, which hardens within the first week after emergence (Rüppel et al. 2005). After the exoskeleton has hardened, males and females return to the water where mating and oviposition takes place (Corbet 1999). Males are territorial and usually defend a patch of habitat suitable for oviposition. Some males mate with multiple females during their life span, although the majority of males obtain few or no matings (Corbet 1999).

Female *C. splendens* and *C. virgo* resemble each other in color except for wing coloration. Female wing coloration has been suggested to be a species recognition cue used by *Calopteryx* spp. males in mate selection (Waage 1975, 1979; Beukema 2004). Females *C. splendens* generally have green wings, whereas *C. virgo* females have brown wings, although females of both species can exhibit considerable intraspecific variation in color (Askew 2004; Dijkstra and Lewington 2006). *Calopteryx* males clasp females to form precopulatory tandems, and these tandems rarely break down without copulation (Cordero 1999; Cordero and Andrés 2002). Females from several *Calopteryx* species can also be induced to mate with heterospecific males through so-called “hand-pairing” techniques (E. I. Svensson and K. Tynkkynen, unpubl. data), which is done by pressing the anal appendages of a male against the prothorax of a female (Oppenheimer and Waage 1987).

GEOGRAPHIC REGIONS AND STUDY POPULATIONS

Female presentations were carried out at six populations in Sweden and Finland between 2007 and 2008 (Fig. 1). The two Swedish populations, Klingavälsån Naturreservat (55°63′84, 13°54′14) and Sövdemölla (55°60′45, 13°65′63), and the two central Finnish populations, Ylösenjoki (62°36′05, 26°53′41) and Neulajoki (62°26′07, 25°48′81) are sympatric sites, containing both *C. virgo* and *C. splendens.* We refer to the Swedish populations as “sympatry minority” and to the Finish populations as “sympatry majority,” to reflect the increase in the relative abundance of *C. virgo* with latitude (see results). The northern Finnish populations Hiisijoki (64°92′51, 29°25′02) and Kuorejoki (64°92′46, 27°60′99) are situated in the purely allopatric *C. virgo* region, ∼100 km north from the “sympatry majority” region (Fig. 1).

Calopteryx virgo females used in the presentations were always taken from the local population in which they were presented. Local *C. splendens* females were also used in the two Swedish populations, however, *C. splendens* females were taken from a single source population for presentations in the four Finnish populations (Pitkäjoki; 62°12′03, 26°05′34, Fig. 1). This was done because *C. splendens* females presented at the two allopatric *C. virgo* populations had to be caught in the sympatric zone, and the presentation of nonlocal females might potentially exert an additional effect on the mating response of *C. virgo* males (see Svensson et al. 2006). Thus, to unambiguously compare the presentations from the sympatric and allopatric populations in Finland, *C. splendens* females were obtained from the same source population in all the presentations in Finland. This design made it possible to rule out any effects due to unmeasured population differences between *C. splendens* females on the courtship responses of *C. virgo* males in the study populations in Finland.

DATA COLLECTION AND FIELD EXPERIMENTS

Field work was carried out between 1000 and 1700 h, which is the main mating period of *Calopteryx* species in northern Europe (Svensson et al. 2004). No field work was carried out in days with heavy rain and/or strong wind, because *Calopteryx* damselflies are very sensitive to these weather conditions and remain inactive under such circumstances. Population density counts were done on separate days to obtain representative measures of conspecific and heterospecific densities (number of replicated counts 2–19, mean 5.5). At each population, multiple population censuses were conducted along a 100 m riparian stretch of representative habitat that was measured with a distance measuring wheel (York Survey Supply Centre Ltd, York, UK). All *C. splendens* and *C. virgo* individuals along the stretch were counted and all counts were performed by the same person (M. Wellenreuther) to avoid interobserver biases.

Calopteryx splendens females were caught with hand-nets and subsequently stored in an aerated holding container that was stored in a dark place and cooled with ice-blocks. Only fully mature females with paper-like, crisp wings and firm veins were used in the experiments (following Svensson et al. 2004), whereas immature females were immediately released after capture. Before each presentation, a single female was taken out of the holding container and tied around the thorax with a thread that was also attached to a 1.5 m long bamboo stick. All tethered *C. splendens* females were presented to five heterospecific males, and the courtship responses of males toward females were recorded. *Calopteryx virgo* females were also presented to conspecific males at each population to determine the baseline courtship propensity in these populations, using the same procedures as outlined above. Only males that were found in the area immediately adjacent to the river and were mature were chosen for the presentation experiments. To quantify male courtship responses, a four-degree nominal scale was used where 0 represented the male escaping or attacking the female, (1) a male approach without further courtship effort, (2) a male courtship, and (3) a male clasping and attempt to form a tandem with the focal female. At each population, a minimum of 17 *C. splendens* (mean 18.2 ± 0.70 SE) and 15 *C. virgo* (mean 16.2 ± 0.91 SE) females were presented.

STATISTICAL ANALYSES

To investigate the geographic pattern of species abundance, multiple (replicated) counts were used to estimate the density and average percentage of both species in each of the two populations in the three geographic regions. Population estimates of density and percentage abundance were then compared between regions using a nested analysis of variance (ANOVA) (populations nested within regions) to investigate the relationship between geographic region ("sympatry minority," "sympatry majority," and "allopatry") and the relative presence of *C. splendens* and *C. virgo.*

The difference in courtship that *C. virgo* males directed toward conspecific and heterospecific females was used to estimate premating isolation. Only the highest courtship score from the five males toward a single female was used for subsequent analyses. This was done because females in the wild encounter many males during the day and previous research on *Calopteryx* males has shown that most claspings lead to successful copulations (Cordero 1999), and the primary interest of this study is to quantify the likelihood of heterospecific matings between species. In this sense, this measure of courtship response gives a direct estimate of the likelihood of hybridization for a female and is thus most suitable to the question of this study. To estimate differences in male courtship response in relation to female species identity and population, a factorial ANCOVA in a general linear model (GLM) framework was used. Male courtship response was used as the dependent variable, population (the six study populations) and species and an interaction term between population and species as categorical predictors (*C. splendens* and *C. virgo*), and the morphological traits of females were included as covariates. Inspection of the courtship scores showed that they were slightly skewed, however, the residuals of the GLM model were normally distributed, the homogeneity of variances was even and there was no evidence for a mean–variance relationship, indicating that the use of parametric tests was justified. Finally, the overall frequencies of the four types of male courtship responses were compared between the three regions using a chi-square test.

Lastly, overall premating isolation between *C. splendens* and *C. virgo* in the three geographic regions ("sympatry minority," "sympatry majority," and "allopatry") was calculated for each population as the average heterospecific courtship response divided by the average conspecific courtship response. The population values for each geographic region were averaged to obtain an overall index of premating isolation for each region. All significant posterior differences between groups were obtained from Tukey's post hoc tests (HSD), and these were carried out in all cases for the whole group, i.e., each population was compared to all other populations in a group. All statistical tests were performed using Statistica (version 7.0, StatSoft, Tulsa, OK).

Results

The two study populations in southern Sweden—Klingavälsån Naturreservat, and Sövdemölla, were both characterized by a higher proportion of *C. splendens* than *C. virgo*. In particular, only 16% of the individuals at Klingavälsån Naturreservat and 14% of the individuals at Sövdemölla were *C. virgo*. The two sympatric populations in Finland, Neulajoki and Ylösenjoki, however, had a comparatively higher ratio of *C. virgo* in the populations, with 88% of the population in Neulajoki and 40% of the population in Ylösenjoki consisting of *C. virgo*. The remaining two populations, Kuorejoki and Hiisijoki, were situated in the allopatric *C. virgo* region >100 km north of the geographical range (Karjalainen 2002) and consequently comprised of 100% *C. virgo*.

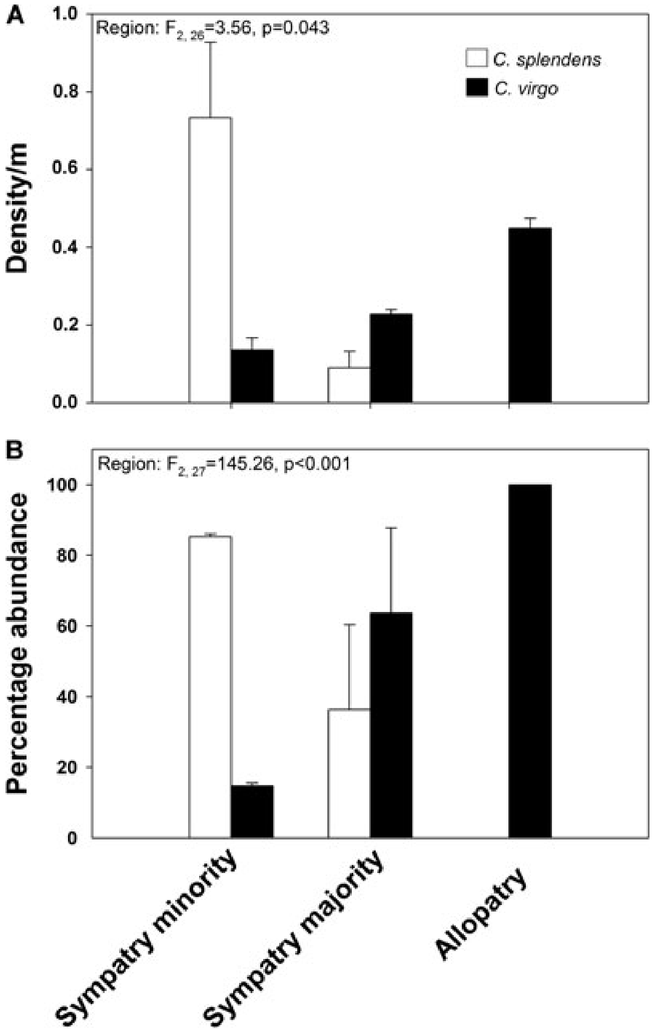

Figure 2. (A) Density (number of individuals/m) of *C. virgo* and *C. splendens* in "sympatry minority," "sympatry majority," and "allopatry." (B) Relative abundance of *C. splendens* and *C. virgo* as percentages of total population in "sympatry minority," "sympatry majority," and "allopatry," Error bars show standard error for the two populations per region.

Data on species density and relative species abundance are summarized for the three geographic regions "sympatry minority," "sympatry majority," and "allopatry" (Fig. 2). The nested

ANOVA on the relative density of the two *Calopteryx* species between the three regions showed significant differences between regions ($F_{2,26} = 3.56$, $P = 0.043$), but not between populations within regions ($F_{3,26} = 0.19$, $P = 0.904$). Post hoc tests showed the significant variation was due to differences between the two regions "sympatry minority" and "allopatry," with the "sympatry minority" region having a higher density of *C. splendens* and a lower density of *C. virgo*, whereas "sympatry majority" did not differ significantly from these two regions. Similarly, the nested ANOVA on the relative abundance of the species between regions was also significant ($F_{2,27} = 145.26$, $P < 0.001$), and posthoc test revealed that this was caused by a steady increase in the abundance of *C. virgo* from "sympatry minority" to "allopatry" (sympatry minority <sympatry majority <allopatry, $P < 0.05$).

When comparing the relative abundance of species between regions, populations nested within regions were also significantly different ($P < 0.05$), and this difference was attributable to the relative abundance of *C. virgo* in the two "sympatry majority" populations. In particular, the relative abundance of *C. virgo* in Neulajoki was similar to the two allopatric populations (Hiisijoki and Kuorejoki), whereas the abundance of *C. virgo* in Ylösenjoki was grouped with the two populations from the "sympatry minority" region (Klingavälsån Naturreservat and Sövdemölla).

Comparisons of conspecific and heterospecific courtship responses of the six populations showed that the courtship responses of *C. virgo* males were not affected by female morphology ($P > 0.05$ for all eight morphological traits, data not shown), but differed significantly between both species and populations (species: $F_{1,186} = 458.63$ $P < 0.001$; population: $F_{5,186} = 9.47$ $P < 0.001$). Species differences arose because *C. virgo* females were predominantly courted and clasped by their conspecific males, whereas *C. splendens* females obtained claspings less often (*C. virgo* females: 2.8 ± 0.03SE, $n = 132$ and *C. splendens* females: 1.0 ± 0.09SE, $n = 121$). Population differences were mainly due to variation in courtship responses toward *C. splendens* females in the "sympatry majority" region, and the allopatric region in northern Finland (Fig. 3). There was also a significant interaction between population origin and female species identity, demonstrating that the relative courtship response of *C. virgo* males toward conspecific and heterospecific females differed between populations ($F_{5,186} = 6.83$, $P < 0.001$, Fig. 3). Tukey's posthoc tests detected six homogenous groups (Fig. 3) that were significantly different from one another ($P < 0.05$). The first group consisted of *C. virgo* females from all six study populations, further indicating that male courtship response toward conspecific *C. virgo* females was relatively similar across all populations (Fig. 3). Group two to five were characterized by increasing courtship responses of *C. splendens* females with heterospecific males with increasing latitude (see Fig. 3 for detailed posthoc test results).

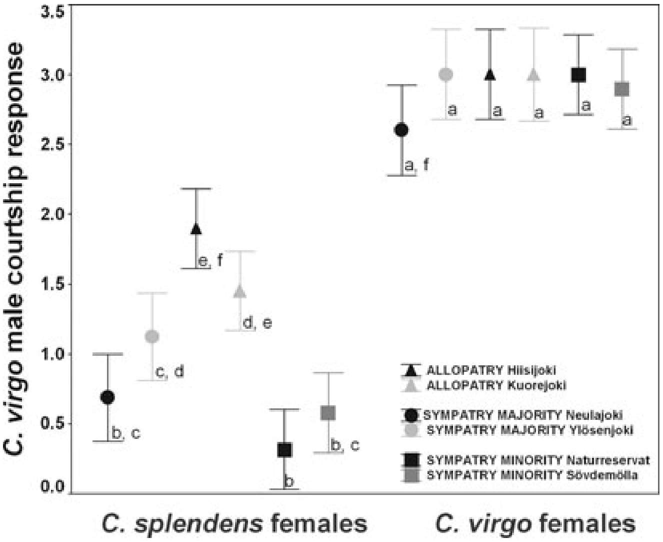

Figure 3. Courtship response of *C. virgo* males toward *C. splendens* and *C. virgo* females in six study population (ANOVA: $F_{5,211} = 5{,}16$, $P < 0.001$). Different letters next to each population denote groups that are statistically different according to Tukey's HSD posthoc tests ($P < 0.05$). Bars around means show 95% confidence intervals.

The nested ANOVA on the three geographic regions (with population nested within geographic regions) showed that courtship success of *C. splendens* females varied significantly between the three geographic regions ($F_{2,103} = 20.88$, $P < 0.001$, Fig. 4), but not between populations within regions ($F_{3,103} = 1.95$; $P = 0.127$). In particular, the courtship response of *C. virgo* males toward *C. splendens* females differed significantly between allopatry and the two sympatric regions (Fig. 4). The courtship response of *C. virgo* males toward conspecific females also differed significantly between the three geographic regions ($F_{2,91} = 5.45$, $P < 0.005$, Fig. 4), and posthoc test showed that this was due to a lower courtship response toward *C. virgo* females in the "sympatry majority" region, compared to the two other geographic regions. Closer inspection of the results by population showed that this significant difference was solely driven by lower courtship responses toward *C. virgo* females in the Finnish population Neulajoki (mean courtship 2.6, whereas the courtship success in all other populations ranged from 2.9 to 3.0, $F_{3,91} = 7.35$; $P < 0.001$).

A direct analysis of the distinct courtship behaviors showed that there was a clear difference in the frequencies of these behaviors in the three geographic regions (Fig. 5). In particular, the two male response categories "escape" and "approach" decreased from "sympatry minority" to "sympatry majority" and "allopatry," whereas the two positive response categories "courtship" and "clasping" increased ($\chi^2_{6,109} = 38.90$, $P < 0.001$, Fig. 5). Most notably, although no males in the region of "sympatry

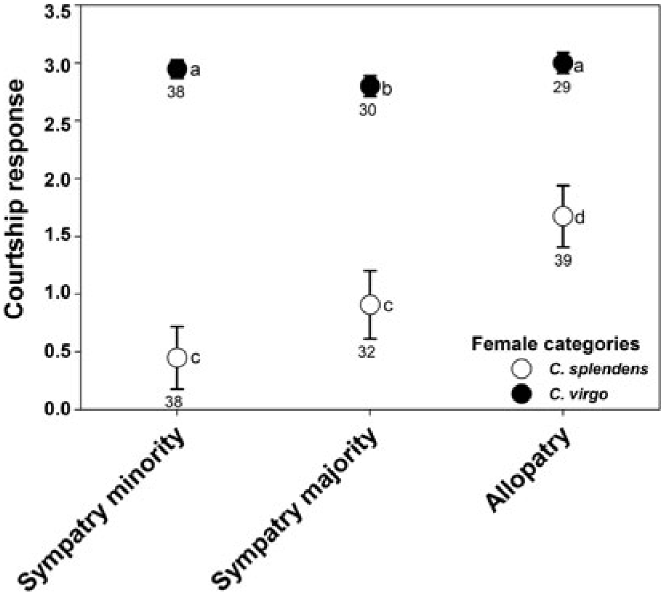

Figure 4. Male courtship responses toward *C. splendens* and *C. virgo* females across three regions: "sympatry minority," "sympatry majority," and "allopatry" (two populations per region). Nested ANOVAs (populations nested within regions) of courtship responses of *C. virgo* males toward *C. splendens* females showed significant differences between regions ($F_{2,103} = 20.88$; $P < 0.001$) but not between populations within regions ($F_{3,103} = 1.95$; $P = 0.127$). For *C. virgo* females, both regional category ($F_{2,91} = 5.45$; $P < 0.006$) and populations within categories ($F_{3,91} = 7.35$; $P < 0.001$) were significant. Tukey's post hoc tests showed that this difference was entirely driven by the population Neulajoki. Different letters next to each category indicate groups that are statistically different according to posthoc tests ($P < 0.05$).

minority" clasped any heterospecific females, the number of *C. virgo* males that clasped *C. splendens* females increased to two in the "sympatry majority" region and to 13 in the "allopatry" region.

Premating isolation between *C. splendens* and *C. virgo* was highest (value = 0.78) in the "sympatry minority" region in southern Sweden. Premating isolation decreased to 0.68 in the sympatric Finnish region (sympatry majority), and further decreased to 0.49 in the allopatric zone in Finland (allopatry).

Discussion

Premating isolation between closely related species can become weak or lost after prolonged periods of allopatry, because selection to maintain isolation is no longer present (Noor 1999; Servedio 2004b). When species come into secondary contact due to range expansions or range shifts, lost species discrimination ability might lead to an increase in the number of heterospecific copulations and the rate of hybridization, which could result in

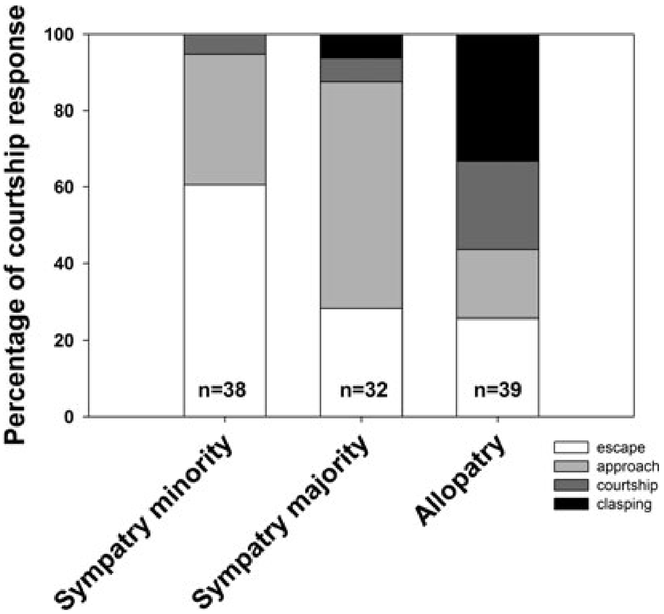

Figure 5. Percentage occurrence of the four types of male responses toward heterospecific *C. splendens* females in the three geographic regions in Fennoscandia. The chi-square test showed that the courtship response frequencies of *C. virgo* males toward *C. splendens* females differed significantly between regions (chi-square 26,109 = 38.90, $P < 0.001$).

local extinctions (Servedio and Noor 2003; Servedio 2004b). In this study, comparisons of male mating responses toward conspecific and heterospecific females across sympatric and allopatric populations were used to infer the potential consequences of future range expansions of *C. splendens* into the currently allopatric area of *C. virgo*.

This study showed that male courtship responses of *C. virgo* toward heterospecific *C. splendens* females differed significantly between populations (Fig. 3) and geographic regions (Fig. 4). Across the three geographic regions, the mean courtship responses of *C. virgo* males toward females of *C. splendens* increased from 0.45 in southern Sweden (sympatry minority), to 0.91 in southern Finland (sympatry majority) to 1.67 in northern Finland (allopatry). Thus, sexual isolation between *C. virgo* males and *C. splendens* females shows geographic variation. Sexual isolation was highest in the area of "sympatry minority" in southern Sweden, where *C. virgo* had the lowest relative abundance (14 and 16%), whereas it was significantly reduced in the allopatric zone of *C. virgo* in northern Finland (Figs. 3 and 4). Notably, the courtship responses of *C. virgo* males toward heterospecific females in allopatry were only slightly lower compared to those directed toward conspecific females (Figs. 3 and 4). These regional differences strongly suggest that the absence of *C. splendens* in northern Fennoscandia has resulted in the partial loss of species discrimination ability in allopatric *C. virgo* males.

Reinforcement theory predicts that premating isolation between hybridizing species is enhanced in response to selection against interspecific matings, regardless of whether hybrids are themselves unfit (indirect selection) or there are direct costs to females as a result of heterospecific matings (Servedio 2001; Servedio and Noor 2003; Servedio 2004a). This definition also includes the latter stages of sympatric speciation, where hybrids are ultimately selected against because of the adaptive phenotypic differences between the diverging parental populations (Schluter 2000; Kirkpartrick and Ravigné 2002). Although early models indicated that reinforcement of mating preferences would be severely constrained by gene flow and recombination (Felsenstein 1981), there is now accumulating empirical evidence that reinforcement operates in several taxa (see Coyne and Orr 2004 and references therein; Mallet 2007). If species ranges only partially overlap, however, then the absence of one species in allopatry can dilute the effects of reinforcement if there is a cost to maintain species recognition abilities. The partial loss of premating isolation between *C. virgo* and *C. splendens* in northern Fennoscandia hence indicates that species discrimination ability is costly and currently maintained by selection between these two species in sympatry.

There are several examples in which the geographic range expansion of one species into the range of another species has resulted in hybridization and extinction of the formerly allopatric species (reviewed in Rhymer and Simberloff 1996). For example, habitat expansion has led to geographic range overlap between Mallards (*Anas platyrhynchos*), Mexican ducks (*A. diazi*), and American black ducks (*A. rubripes*) (Rhymer and Simberloff 1996). Similarly, habitat expansion of the blue-winged (*Vermivora pinus*) and golden-winged (*V. chrysoptera*) warbler has led to frequent hybridization. *V. pinus*, which is expanding its range northward, frequently encounters *V. chrysoptera*, and the latter species has declined greatly (Gill 1980, 1987). The decline of the formerly allopatric species has been linked to a combination of both direct competition and hybridization (Gill 1980, 1987), which are factors that could also potentially affect the fate of *C. virgo* populations in northern Europe. However, the degree to which heterospecific matings might affect future *C. virgo* populations in northern Fennoscandia also depends on several other factors. One such factor is the length of the reproductive window. The flight season in northern Fennoscandia is with around 5 weeks (K. Tynkkynen, pers. obs.) significantly shorter than in southern Sweden, where *Calopteryx* damselflies can reproduce for around 2 months, from June to the beginning of August (M. Wellenreuther, pers. obs.). The shorter reproductive window in northern Scandinavia might select against time-consuming and costly mate discrimination in *C. virgo* males, which would explain the partial loss of male species discrimination against *C. splendens* females (Figs. 3 and 4). Although the expected future period of hybridization within the contact zone of these species might be transitory, it can potentially have an impact on the small and depauperate allopatric *C. virgo* populations, unless *C. virgo* males rapidly adjust to the presence of a new species.

It is possible that the presentation of nonlocal *C. splendens* females to *C. virgo* males in the four Finnish populations could simply reflect male discrimination against immigrant females, similar to the discrimination by females against immigrant males as was found by Svensson et al. (2006). There are two lines of evidence, however, that suggest that such a general discrimination against immigrants does not entirely explain the results of this study. First, there was no consistent male response toward immigrant *C. splendens* females in the two populations within the "sympatry majority" and "allopatry" region, but instead, premating isolation varied considerably between the two regions. Second, if geographic or ecological distance between populations (latitude, temperature, rainfall etc.) would have an impact on the magnitude of the discrimination against immigrants, then it would be expected that the geographically closer, and presumably more similar populations, would discriminate less strongly against immigrants than the more geographically distant and presumably more dissimilar, allopatric populations (Figs. 2 and 3). The results from this study show the opposite pattern, that is, immigrant heterospecific females received higher courtship scores in the geographically more distant and allopatric populations. For these reasons, it seems likely that the reduction in premating isolation between *C. virgo* males and *C. splendens* females in the allopatric region might at least be partly caused by a reduction in the ability of *C. virgo* males to discriminate against heterospecific females.

The finding that allopatric *C. virgo* males can still discriminate against heterospecific females, albeit to a lesser degree when in sympatry (Figs. 3 and 4), is also interesting because it provides some insight into the role of learned mate recognition in damselflies. In the North American damselfly *Enallagma civile*, male mate recognition appears to be predominately learned rather than innate (Miller and Fincke 1999). Additional support for a partially learned mate recognition ability has also been found in *C. splendens* females from southern Sweden (E. I. Svensson and A. Runemark, unpubl. data). Learned species discrimination ability could either be caused by "discrimination learning," for which a continuous presence of heterospecifics is necessary, or by "association learning," which does not require the presence of heterospecifics. Although we can rule-out any role for discrimination learning in the allopatric region, it is possible that the northern *C. virgo* males have learned to associate the phenotype of conspecific *C. virgo* females as suitable mates, but not the differently colored *C. splendens* females. A recent study examined the learning ability of allopatric *C. virgo* males in Finland (K. Tynkkynen et al., unpubl. data). *Calopteryx splendens* and *C. virgo* females were repeatedly presented to allopatric *C. virgo* males, and males were able to learn over time to recognize *C. splendens* females as heterospecifics. However, in comparison with a previous study on the learning ability of *C. splendens* females (E. I. Svensson and A. Runemark, unpubl. data), learning in *C. virgo* males was significantly less pronounced, which suggest that species recognition in *C. virgo* might also have an innate component. Recent models on the role of learning in evolution suggest that learned mate preferences might be as efficient, or even more efficient, than genetic mate preferences in mediating reproductive isolation and completing speciation (Price 2007; Brodin and Haas 2008; Servedio et al. 2009). The role of learning in maintaining species integrity, e.g., when species change their distributional ranges in response to climate change, is almost completely unknown and clearly deserves increased attention in the future. Ongoing studies in our laboratories aim to elucidate the relative importance of learning versus genetic mate preferences in species recognition among males and females of these two taxa.

Three other lines of evidence also indicate that heterospecific matings and/or hybridization between *C. virgo* males and *C. splendens* females species could increase near the northern range margins. First, in areas of secondary overlap, females of the rare species are expected to mate with males of the more common species (Wirtz 1999), because, for example, females may be less resistant to heterospecific claspings if they cannot find conspecific mates. Second, in many species of damselflies, male mating success is considerably more variable than female mating success, with most males obtaining very few or no matings, and only a minority of males (10–20%) obtaining multiple matings (Plaistow and Siva-Jothy 1996). Hybrids of *C. splendens* and *C. virgo* have been detected in natural populations in Finland (Tynkkynen et al. 2008), and heterospecific matings are also relatively frequent in southern Sweden (Svensson et al. 2007). The rate of interspecific matings will, however, be counteracted to some degree by the fact that *Calopteryx* spp. females typically mate with multiple partners, and also oviposit repeatedly during their life span (Rüppel et al. 2005). Multiple matings could reduce the overall cost associated with hybridization, because males and females that mate with heterospecifics could also mate with conspecifics and hence produce at least some nonhybrid offspring (Veen et al. 2001; Marshall et al. 2002). Third, females of many odonate species show conspecific sperm precedence (Córdoba-Aguilar et al. 2003), which has the ability to directly affect the relative contribution of each male following multiple copulations.

Range shifts and northward range expansions are currently affecting many species around the globe, and modifying species interactions in newly formed contact zones (Parmesan 1996, 2006; Parmesan et al. 1999; Thomas et al. 2001). Our study shows that species might partly lose the ability to discriminate against heterospecifics during prolonged periods of secondarily derived allopatry, which in turn could result in increased rates of interspecific matings upon secondary contact. If heterospecific matings are costly to male and/or female fitness, or result in the production of sterile offspring or high offspring mortality, such costs could lead to different outcomes. First, sexual isolation might re-evolve or strengthen (Figs. 3 and 4), or second, the costs might instead affect the persistence and local survival of populations in the new area of overlap. These effects will be magnified by characteristics such as small population sizes and short temporal windows of reproduction, both of which are commonly found in populations near the range.

Ecologists and evolutionary biologists are increasingly realizing the conservation implications of range expansions and their concomitant effects on hybridization risk, which can lead to local extinctions of a species (Rhymer and Simberloff 1996; Allendorf et al. 2001). The indirect effects of climate change resulting from changed species distributions, such as hybridization and introgression, have important implications for species coexistence. Our study highlights the need to consider the role of interspecific interactions, such as increased risks of heterospecific matings, in the evolution of species ranges. Although interspecific interactions are incorporated in some models of species range distributions and shifts (Case and Taper 2000), they are left out in other models (e.g., Kirkpatrick and Barton 1997). Our results further suggest that heterospecific matings can play a role in the organization of species distributions. Sexual isolation and other interspecific interactions might thus be important to consider if we wish to understand the future evolutionary consequences of the ongoing range shifts in response to climate change.

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