



Behavioural mechanisms of reproductive isolation between two hybridizing dung fly species



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Characterization of the phenotypic differentiation and genetic basis of traits that can contribute to reproductive isolation is an important avenue to understand the mechanisms of speciation. We quantified the degree of prezygotic isolation and geographical variation in mating behaviour among four populations of *Sepsis neocynipsea* that occur in allopatry, parapatry or sympatry with four populations of its sister species *Sepsis cynipsea*. To obtain insights into the quantitative genetic basis and the role of selection against hybrid phenotypes we also investigated mating behaviour of F₁ hybrid offspring and corresponding backcrosses with the parental populations. Our study documents successful hybridization under laboratory conditions, with low copulation frequencies in heterospecific pairings but higher frequencies in pairings of F₁ hybrids signifying hybrid vigour. Analyses of F₁ offspring and their parental backcrosses provided little evidence for sexual selection against hybrids. Longer copulation latencies in heterospecific pairings indicate species recognition, probably due to surface or volatile chemicals. The frequency of male mating attempts did not differ greatly between species or hybrid pairings, suggesting no male discrimination of mating partners. Female shaking duration, signifying female choice and/or reluctance to mate, differed strongly between the species and appears to contribute to avoiding heterospecific males; this trait is partially maternally inherited. Importantly, females of both species discriminated more strongly against males in areas of sympatry than allopatry indicating reinforcement. Shorter copulations in heterospecific parental pairings and longer copulations in F₁ hybrids suggest mechanistic difficulties with sperm transfer. Overall, our study highlights an important role of character displacement affecting mating behaviour of hybridizing sepsid species in geographical areas of coexistence.

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Speciation proceeds gradually from restricted levels of gene flow at early stages to complete reproductive isolation at later stages (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1942). In many cases ecological, spatial or temporal niche differentiation prevents interbreeding between hybridizing species (Schluter, 2000, 2001). More interestingly, reproductive isolation may evolve through sexual selection leading to divergence in mate or gamete recognition systems (Kozak, Reiland, & Boughmann, 2009; Svensson, Karlsson, Friber, & Eroukhmanoff, 2007; Via, 2001). While theoretical studies have established sexual selection as an important potential agent in driving the evolution of reproductive isolation (Gavrilets, 2000; Lande, 1981; Turelli, Barton, & Coyne, 2001), supporting empirical data remain scarce and largely restricted to

phylogenetic species comparisons over long evolutionary time-scales (Kraaijeveld, Kraaijeveld-Smit, & Maan, 2010; Panhuis, Butlin, Zuk, & Tregenza, 2001). As a consequence, for many taxa it is unclear whether sexual selection alone causes reproductive isolation independent of species composition within habitats, or whether it acts in a more punctuated manner as predicted for reproductive character displacement in geographical areas of coexistence (Gavrilets, 2000; Lande, 1981; Turelli et al., 2001). In this context, several authors have recently emphasized the need to better understand the relationship between micro-evolutionary mechanisms causing trait divergence and macro-evolutionary patterns among lineages showing some degree of reproductive isolation.

Behavioural, morphological (i.e. mechanical) or olfactory differences between incipient species can lead to strong prezygotic isolating barriers, which, however, may remain incomplete. The main, and therefore the strongest, barriers result from postzygotic isolation with reinforcement, fertilization problems and hybrid male sterility (Hood, Egan, & Feder, 2012; Reed & Markow, 2004;

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Wassermann & Koepfer, 1977). Although reproductive isolation involves many different types of traits, behaviour is considered to be one of the main driving forces behind the evolution of reproductive barriers to gene flow (Gleason & Ritchie, 1998; Puniamoorthy, Ismail, Tan, & Meier, 2009; Shaw & Herlihy, 2000). For example, Puniamoorthy (2014) demonstrated for the neotropical fly *Archiseptis diversiformis* that qualitatively different courtship behaviours contributed to reproductive isolation between two geographically separated populations otherwise presenting only minor morphological and molecular differentiation.

The closely related sister species *Sepsis cynipsea* and *Sepsis neocynipsea* (Diptera: Sepsidae) offer great opportunity to investigate behavioural mechanisms and underlying evolutionary forces leading to reproductive isolation at early stages of speciation (Via, 2009). Based on their partially sympatric distribution in the Swiss Alps and strong similarities in morphology and behaviour we suspected that these two species might hybridize in nature. In this study, we examined typical mating traits in conspecific versus heterospecific parental pairings, F₁ hybrids and backcrosses between Swiss sympatric, European parapatric and North American allopatric populations, focusing on behavioural traits common to both species: male mating attempts by jumping on a partner; female shaking during pairing, here probably indicating male assessment; and copulation frequency, latency and duration (Blanckenhorn, Mühlhäuser, Morf, Reusch, & Reuter, 2000; Parker, 1972a, 1972b; Ward, 1983). Although the reluctance and assessment functions of female shaking can be hard to distinguish in practice (Blanckenhorn et al., 2000), we expected more pronounced female mate choice in heterospecific pairings following male assessment and species recognition, eventually resulting in reluctance to mate. We further expected the lowest hybridization rates and strongest (i.e. reinforced) behavioural differentiation in the European sympatric populations of the Swiss Alps, and some differentiation between European and North American *S. neocynipsea* due to their spatial separation.

METHODS

Study Organisms

Sepsis cynipsea and *S. neocynipsea* are two closely related species that exhibit clear morphological and behavioural differences (Pont & Meier, 2002) but limited variation in gene sequence data indicating differentiation (Baur, Schäfer, Blanckenhorn, & Giesen, 2017; Puniamoorthy, Su, & Meier, 2008; Su, Kutty, & Meier, 2008). *Sepsis cynipsea* is the most common sepsid species in north-central Europe, while populations of *S. neocynipsea* are present in Europe only in the Alps and other mountainous regions, whereas in North America they abound also at low altitudes, there occupying the ecological niche of the absent *S. cynipsea* (Pont & Meier, 2002). Both similarly breed in fresh cowpats and are reproductively active from spring to late autumn (Eberhard, 1999; Parker, 1972a, 1972b; Pont & Meier, 2002). While the mating system of *S. cynipsea* is well studied (Blanckenhorn, Morf, Mühlhäuser, & Reusch, 1999; Ding & Blanckenhorn, 2002; Hosken, Martin, Born, & Huber, 2003; Parker, 1972a, 1972b; Puniamoorthy et al., 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016; Ward, 1983; Ward, Hemi, & Rösli, 1992), little is known about its sister species *S. neocynipsea* (Eberhard, 1999; Puniamoorthy et al., 2009; Rohner et al., 2016).

Ethical Note and Maintenance of Flies

No legal regulations for scientific laboratory work with sepsid flies exist in Switzerland, the EU or the U.S.A. and no licences or permits were needed. We caught wild individuals by swiping a

butterfly net over fresh cowpats. Sepsid flies were extracted from the net using an aspirator and transferred into 1-litre transparent plastic containers with fixed Eppendorf tubes offering sugar and water ad libitum. Most other nontarget insects so collected were released again on site. Collected live flies were brought or sent to our laboratory, where they were identified by sex and species according to differences in male armoured foreleg morphology. Male flies were stored as voucher specimens in 100% ethanol at -20°C , and gravid females were isolated into round 50 ml glass vials including a rectangular plastic dish ($4.2 \times 2.1 \times 1.6 \text{ cm}^3$) filled with fresh cow dung as oviposition substrate and some grains of sugar. Emerging F₁ offspring of single females were then transferred into $1 \times 1 \times 1.4 \text{ dm}^3$ plastic containers with fresh cow dung, water ad libitum and sugar for continuous propagation in the laboratory. Isofemale lines were subsequently held in these containers in a climate chamber at 24°C , 60% humidity and 16:8 h light:dark cycle; fresh cow dung was provided every 14 days (rearing conditions are detailed in Puniamoorthy, Schäfer, & Blanckenhorn, 2012). We identified species in isofemale lines according to their male F₁ offspring. Our experimental flies were derived from isofemale lines that had been housed and propagated for up to 2 years before our experiment (see Rohner et al., 2016, for more details). After experiments we froze all flies in 100% ethanol at -20°C .

Fly Origin and Pairing Scheme

Wild-caught gravid females were collected from six sites (i.e. populations) to ultimately establish 5–15 isofemale lines per population in the laboratory (Table 1). *Sepsis cynipsea* and *S. neocynipsea* were obtained from two areas in Switzerland where the two species are sympatric (Zurich, Sörenberg). *Sepsis cynipsea* were further collected from another two European sites, where we did not observe *S. neocynipsea* (Ludwigshafen, Germany, and Stirling, U.K.). However, there are records of *S. neocynipsea* near these sites (Ozerov, 2005; Pont & Meier, 2002), so we classified these populations as parapatric. The other *S. neocynipsea* originated from two allopatric North American populations where *S. cynipsea* does not exist (Fort Hall, ID, and Lamar Valley, WY).

With these flies, we could thus form reciprocal heterospecific parental pairings of three biogeographical types with two population replicates each: European sympatry, European parapatry and cross-continental allopatry (Table 2). In parallel, we performed conspecific parental pairings within each of the four populations per species as the baseline for comparison, as well as two reciprocal population replicates of European with North American *S. neocynipsea* as conspecific allopatric cross-continental pairings (Table 2). In all cases, one population replicate consisted of 15–20 pairing replicates derived from our isofemale lines. Potentially

Table 1
Biogeographical origin of isofemale lines per population of the study species

| Biogeographical origins (code) | | |
|---|---|----------------------------------|
| <i>S. cynipsea</i> | <i>S. neocynipsea</i> | Coordinates |
| Switzerland, Zurich (CH ₁) | Switzerland, Zurich (CH ₁) | 47°24'0.60"N, 8°34'23.97"E |
| Switzerland, Sörenberg (CH ₂) | Switzerland, Sörenberg (CH ₂) | 46°49'23.72"N, 8°1'54.59"E |
| U.K., Stirling (EU ₁) | | 56°6'59.47"N, -3°56'12.83"W |
| Germany, Ludwigshafen (EU ₂) | | 49°28'41.25"N, 8°22'21.65"E |
| | Idaho, Fort Hall (NA ₁) | 43°1'59.69"N, -112°26'17.91"W |
| | Wyoming, Lamar Valley (NA ₂) | 44°52'6.67"N, -110°10'28.72"W |

EU = Europe; CH = Switzerland; NA = North America.

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