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 maternal inherited. Importantly, females of both species discriminated more strongly against males in areas of sympathy 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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Although reproductive isolation involves many different types of traits, behaviour is considered to be
one of the main driving forces behind the evolution of repro-
ductive barriers to gene flow (Gleason & Ritchie, 1998; Puniamoorthy, Ismail, Tan, & Meier, 2009; Shaw & Herlihy, 2000).
For example, Puniamoorthy (2014) demonstrated for the neotrop-
ical fly Archisepsis 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 investi-
gate 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 be-
tween 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; fe-
male shaking during pairing, here probably indicating male
assessment; and copulation frequency, latency and duration
(Blankenhorn, Mühläuser, Morf, Reusch, & Reuter; 2000; Parker,
1972a, 1972b; Ward, 1983). Although the reluctance and assess-
ment functions of female shaking can be hard to distinguish in
practice (Blankenhorn et al., 2000), we expected more pro-
nounced female mate choice in heterospecific pairings following
male assessment and species recognition, eventually resulting in
reductance 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 indi-
cating differentiation (Baur, Schafer, Blankenhorn, & 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
(Blankenhorn, Morf, Mühläuser, & Reusch, 1999; Ding &
Blankenhorn, 2002; Hosken, Martin, Born, & Huber, 2003; Parker,
1972a, 1972b; Puniamoorthy et al., 2009; Rohner, Blankenhorn,
& Puniamoorthy, 2016; Ward, 1983; Ward, Hemli, & Rüslø, 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 ac-
cording 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 × 2.1 × 1.6 cm²) filled with
fresh cow dung as oviposition substrate and some grains of sugar.
Emerging F1 offspring of single females were then transferred into
1 × 1 × 1.4 dm³ 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, Schafer, & Blankenhorn, 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 ex-
periments 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 pop-
ulation 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 Stir-
ling, 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 popula-
tion replicates each: European sympatry, European parapary 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>
<thead>
<tr>
<th>Biographical origins (code)</th>
<th>S. cynipsea</th>
<th>S. neocynipsea</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Switzerland, Zurich (CH1)</td>
<td>Switzerland, Zurich (CH1)</td>
<td>47° 24’ 0.60” N, 8° 34’ 23.97” E</td>
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</tr>
<tr>
<td>Switzerland, Sörenberg (CH2)</td>
<td>Switzerland, Sörenberg (CH2)</td>
<td>46° 49’ 23.72” N, 8° 15’ 54.59” E</td>
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<tr>
<td>U.K., Stirling (EU1)</td>
<td>U.K., Stirling (EU1)</td>
<td>56° 6’ 59.47” N, –3° 58’ 12.83” W</td>
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<tr>
<td>Germany, Ludwigshafen (EU2)</td>
<td>Germany, Ludwigshafen (EU2)</td>
<td>49° 28’ 41.25” N, 8° 22’ 21.65” E</td>
<td></td>
</tr>
<tr>
<td>Idaho, Fort Hall (NA1)</td>
<td>Idaho, Fort Hall (NA1)</td>
<td>43° 1’ 59.69” N, –112° 26’ 17.91” W</td>
<td></td>
</tr>
<tr>
<td>Wyoming, Lamar Valley (NA2)</td>
<td>Wyoming, Lamar Valley (NA2)</td>
<td>44° 52’ 6.67” N, –110° 10’ 28.72” W</td>
<td></td>
</tr>
</tbody>
</table>

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