**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2017

Behavioural mechanisms of reproductive isolation between two hybridizing dung fly species

Giesen, Athene ; Blanckenhorn, Wolf U ; Schäfer, Martin A

DOI: <https://doi.org/10.1016/j.anbehav.2017.08.008>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-143627>

Journal Article

Accepted Version

The following work is licensed under a Creative Commons: Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.

Originally published at:

Giesen, Athene; Blanckenhorn, Wolf U; Schäfer, Martin A (2017). Behavioural mechanisms of reproductive isolation between two hybridizing dung fly species. *Animal Behaviour*, 132:155-166.

DOI: <https://doi.org/10.1016/j.anbehav.2017.08.008>

1 **Behavioural mechanisms of reproductive isolation between two hybridising dung**
2 **fly species (*Sepsis cynipsea* and *S. neocynipsea*; Diptera: Sepsidae)**

3 Athene Giesen*, Wolf U. Blanckenhorn, Martin A. Schäfer

4 *Department of Evolutionary Biology and Environmental Studies, University of Zurich,*
5 *Switzerland*

6

7 [Animal Behaviour 10.1016/j.anbehav.2017.08.008](#)

8

9 athene.giesen@ieu.uzh.ch

10 martin.schaefer@ieu.uzh.ch

11 wolf.blanckenhorn@ieu.uzh.ch

12

13 **Abstract**

14 Characterization of the phenotypic differentiation and genetic basis of traits that can
15 contribute to reproductive isolation is an important avenue to understand the
16 mechanisms of speciation. We quantified the degree of prezygotic isolation and
17 geographic variation in mating behaviour among four populations of *Sepsis*
18 *neocynipsea* that occur in allopatry, parapatry, or sympatry with four populations of
19 its sister species *S. cynipsea*. To obtain insights into the quantitative genetic basis and
20 the role of selection against hybrid phenotypes we also investigated mating behaviour
21 of F₁ hybrid offspring and corresponding backcrosses with the parental populations.
22 Our study documents successful hybridisation under laboratory conditions, with low
23 copulation frequencies in heterospecific pairings but higher frequencies in pairings of
24 F₁ hybrids signifying hybrid vigour. Analyses of F₁ offspring and their parental
25 backcrosses provided little evidence for sexual selection against hybrids. Longer
26 copulation latencies in heterospecific pairings indicate species recognition, probably
27 due to surface or volatile chemicals. The frequency of male mating attempts did not
28 differ greatly among species or hybrid pairings, suggesting no male discrimination of
29 mating partners. Female shaking duration, signifying female choice and/or reluctance
30 to mate, differs strongly between the species and appears to contribute to avoiding
31 heterospecific males; this trait is partially maternal inherited. Importantly, females of
32 both species discriminated more strongly against males in areas of sympatry than
33 allopatry indicating reinforcement. Shorter copulations in heterospecific parental
34 pairings and longer copulations in F₁ hybrids suggest mechanistic difficulties with
35 sperm transfer. Overall, our study highlights an important role of character
36 displacement affecting mating behaviour of hybridising sepsid species in geographic
37 areas of co-existence.

38 **Highlights**

- 39 • Successful but low rates of hybridisation
- 40 • Females, but not males, discriminate against heterospecific mating partners
- 41 • Maternal inheritance of female mating behaviour
- 42 • Biogeographic variation in heterospecific mating behavior driven by
- 43 reinforcement
- 44 • Little evidence for sexual selection against hybrids

45

46 **Keywords:** hybridisation, speciation, reproductive isolation, prezygotic,

47 heterospecific, female discrimination, mate choice, inheritance, biogeography,

48 reinforcement, sexual selection, species recognition, Sepsidae, sepsid, Diptera, *Sepsis*

49 *cynipsea*, *Sepsis neocynipsea*

INTRODUCTION

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 hybridising species (Schluter, 2000, 2001). More interestingly, reproductive isolation may evolve through sexual selection leading to divergence in mate or gamete recognition systems (Kozak, Reisland & Boughmann, 2009; Svensson et al., 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 timescales (Panhuis et al., 2001; Kraaijeveld, Kraaijeveld-Smit & Maan, 2010). 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 geographic areas of co-existence (Lande, 1981; Gavrilets, 2000; Turelli, Barton & Coyne, 2001). In this context, several authors recently emphasized the necessity 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 mostly absolute barriers result from postzygotic isolation with reinforcement, fertilization problems, and hybrid male sterility (Hood, Egan & Feder, 2012; Reed & Markow, 2004; Wassermann &

Koepfer, 1977). Although reproductive isolation involves many different types of traits, behaviour is considered as a main driving force behind the evolution of reproductive barriers to gene flow (Gleason & Ritchie, 1998; Puniamoorthy et al., 2009; Shaw & Herlihy, 2000). For example, Puniamoorthy (2014) demonstrated for the neotropical 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 *S. 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 hybridise in nature. We here examine typical mating traits in con- vs. 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 likely indicating male assessment, as well as copulation frequency, latency and duration (Blanckenhorn et al., 2000; Parker, 1972a,b; 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 lowest hybridisation 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* (Diptera: Sepsidae) are two closely related species that exhibit clear morphological and behavioural differences (Pont & Meier, 2002) but limited variation in gene sequence data indicating differentiation (Su, Kutty, & Meier, 2008; Puniamoorthy, Su, & Meier, 2008; Baur et al., unpubl. data). *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 species similarly breed in fresh cowpats and are reproductively active from spring to late autumn (Eberhard, 1999; Parker, 1972a,b; Pont & Meier, 2002). While the mating system of *S. cynipsea* is well studied (Blanckenhorn et al., 1999, 2002; Hosken et al., 2003; Parker 1972a,b; Puniamoorthy et al., 2009; Rohner, Blanckenhorn & Puniamoorthy, 2016; Ward, 1983; Ward, Hemmi & Rösli, 1992), only little is known about its sister species *S. neocynipsea* (Eberhard, 1999; Puniamoorthy et al., 2009; Rohner, Blanckenhorn & Puniamoorthy, 2016).

Ethical Note and Maintenance of Flies

No legal regulations for scientific laboratory work with sepsid flies exist in Switzerland, the EU, or the USA such that we did not apply for any licenses or

permits. After 30+ years of experience working with dung flies, our ability to handle them carefully and appropriately is given. 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 1L transparent plastic containers with fixed Eppendorf tubes offering sugar and water *ad libitum*. Most other non-target 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 x 2.1 x 1.6 cm³) filled with fresh cow dung as oviposition substrate and some grains of sugar. Emerging F₁ offspring of single females were then transferred into 1 x 1 x 1.4 dm³ plastic containers with fresh cow dung, water *ad libitum*, and sugar for continuous propagation in the laboratory. The iso-female lines were subsequently held in these containers in a climate chamber at 24°C, 60% humidity, and 16h:8h light:dark cycle, with regular maintenance by supplying fresh cow dung every 14 days (rearing conditions detailed in Puniamoorthy, Schäfer and Blanckenhorn, 2012). Our experimental flies were derived from these iso-female lines that had been housed and propagated for up to 2 years before our experiment (see Rohner, Blanckenhorn and Puniamoorthy, 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 to 15 iso-female lines per population in the laboratory (Table 1). *Sepsis cynipsea* and *S. neocynipsea* were obtained from two areas in Switzerland

where the two species co-occur sympatrically (Zurich, Sörenberg). *S. cynipsea* were further collected from two other adjoining European regions where *S. neocynipsea* has not been observed (Ludwigshafen, Germany, and Stirling, Scotland). However, the literature documents neighbouring *S. neocynipsea* appearances in both regions (Pont & Meier, 2002; Ozerov, 2005), so that we classified these populations as parapatric. The other *Sepsis neocynipsea* originated from two allopatric North American populations where *S. cynipsea* does not exist (Fort Hall, Idaho, and Lamar Valley, Wyoming).

With these flies we thus could 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 to 20 pairing replicates derived from our iso-female lines. Potentially lower sample sizes in mating experiments with F_1 hybrid offspring were expected due to difficulties in obtaining hybrids. For backcrosses we targeted a sample size of six replicates per pairing, as we set up two reciprocal types (female hybrid with male parental – $F_1 \times P$, and female parental with male hybrid – $P \times F_1$) to detect possible sex specific effects. In the end we conducted observations for (1) con- and hetero-specific parental (P) pairings (mean sample size = 19.13, range 15 to 20), (2) F_1 hybrid (F_1) pairings using the offspring resulting from heterospecific pairings (mean sample size = 11.44, range 3 to 20), and (3) backcrosses (BC) of F_1 hybrid offspring with the parental species (mean sample size = 4.23, range 1 to 6). All pairings were done reciprocally.

Hybrid flies for our behavioural assessments of the F_1 and backcrosses were generated by randomly combining up to 30 flies of one sex from various iso-female lines of a given population and species with a roughly equal number of flies of the other sex from various iso-female lines of a given population of the other species (Table 2; again done reciprocally). Matings in this setting were necessarily heterospecific, and females were allowed to oviposit eggs into fresh cow dung to generate F_1 hybrid offspring for our experiments.

Assessment of Mating Behaviour

For each pairing replicate (cf. above) we combined five virgin female with five virgin male individuals (i.e. 5f:5m) into a round 50 mL (length 8 cm x diameter 2.5 cm) glass vial containing a smear of cow dung, all independently and randomly chosen from the various iso-female lines of a given population. This implies that some of the individuals in each replicate vial may have stemmed from the same iso-female line by chance. Virginity was guaranteed by separating flies by sex within 24 h after emergence. Flies were always aged 3 to 6 days after adult eclosion to ensure sexual maturity (Teuschl & Blanckenhorn, 2007). Due to errors, losses or deaths, effective group sizes varied between 3f:3m and 6f:6m. We thus followed Puniamoorthy (2014), who reported for *Archiseopsis diversiformis* (Diptera: Sepsidae) that hybrids were not produced when flies were confronted with only one mating partner of the other population/species, but only when confronting groups of flies, thus emulating the natural situation at cowpats and increasing interaction probabilities (Eberhard, 1999; Parker, 1972a,b).

Observation of mating behaviour started right after fly introduction and lasted for 30 min. We recorded (i) the total number of male mating attempts as an indicator

of male willingness to mate, i.e. jumps onto a female, (ii) the cumulative female shaking duration with a mounted male indicating mate assessment and/or reluctance to mate [recorded in seconds, in the Figures converted to min], and (iii) the average duration of all copulations [in min] (Blanckenhorn et al., 2000; Boake, Price & Andreadis, 1998; Ding & Blanckenhorn, 2002). We always scored the entire copulation duration, even if it exceeded the 30 min observation interval. From these assays we further derived, for final analysis, (iv) the time to first copulation (i.e. copulation latency) as an indicator of how fast mating ensues, and (v) the number of copulations realized per male mating attempt (copulation frequency). After the experiments all flies were frozen and stored in 100% ethanol at -20°C for any future work.

Statistical Analyses

We ultimately standardized all trait measurements (except copulation duration) for analysis to 30 minutes and one pair. The number of male mating attempts, female shaking duration, and copulation latency were $\log_{10}$ -transformed for better residual distribution in parametric statistical tests. Mating frequency was arcsine-transformed for analysis (logistic analyses with binomial errors yielded qualitatively similar results). All five traits were analysed separately, with and without the other traits as covariates because male and female behaviours interact to produce matings (only significant covariates are reported in the Results), with univariate GLMs in SPSS Statistics Version 23. For the parental pairings, a given behavioural trait was analysed as a function of species (*S. cynipsea*: C, *S. neocynipsea*: N, and CxN vs. NxN – female always named first), and biogeographic type nested within species (sympatry vs. parapatry in Europe vs. allopatry across continents) as fixed

factors, and population nested within biogeographic type within species as random effect. Particular pairings were additionally compared (planned comparisons): baseline behaviour of C vs. N; cross-continental vs. within-population N; and direction of heterospecific mating, i.e. CxN vs. Nx C. F₁ hybrid and backcrosses were analysed analogously but separately. We finally also performed two corresponding multivariate analyses subsuming, on the one hand, copulation frequency, male mating attempts, and female shaking (using all data including zeroes) and, on the other hand, copulation latency and copulation duration (only for the subset of replicates in which copulations occurred).

A separate additional analysis to investigate the inheritance of all behavioural traits compared the conspecific parental pairings (N, C) with the F₁ hybrid offspring in both directions (CxN, Nx C) using one-way univariate ANOVA with analogous nesting, fixed and random factors as above, followed by post-hoc Tukey's tests. This qualitatively tested for deviations from the null expectation of intermediate inheritance, i.e. whether a trait shows dominance or maternal/paternal inheritance instead.

RESULTS

Mean values for all traits and pairings with 95% confidence intervals are reported in Table 3. Detailed ANOVA statistics and covariate effects (*F*-statistics, *P*-values, β -slopes) are reported in Appendix Tables A2 to A7.

I. Baseline comparison of conspecific S. cynipsea and S. neocynipsea behaviour

Comparing the two species with four populations each as the baseline, *S. cynipsea* performed more successful copulations per mating attempt than *S.*

neocynipsea ($F(1,6) = 6.44$, $P = 0.044$; Fig. 1a; appendix Table A3). Lower copulation frequencies were associated with more male mating attempts (covariate effect: $F(1,150) = 12.18$, $P = 0.001$, $\beta = -0.365$; no other covariate had a significant effect). This variation in copulation frequency reflects corresponding species differences in mating interactions, as *S. neocynipsea* males performed more mating attempts per 30 minutes than *S. cynipsea* males ($F(1,6) = 9.64$, $P = 0.021$; Fig. 1b), while *S. cynipsea* females displayed much more cumulative shaking (i.e. rejection or assessment behaviour: $F(1,6) = 33.73$, $P = 0.001$; Fig. 1c). Both traits stimulate each other as more male mating attempts necessarily entail more cumulative female shaking if the female is unwilling to mate (covariate effect: $F(1,144) = 12.90$, $P < 0.001$, for females affecting males $\beta = 0.136$, for males affecting females $\beta = 0.606$). The first copulation started somewhat earlier in *S. cynipsea* than in *S. neocynipsea* ($F(1,6) = 5.36$, $P = 0.055$; Fig. 2a), and copulation duration was slightly longer in *S. cynipsea* ($F(1,6) = 4.48$, $P = 0.073$; Fig. 2b).

II. Baseline comparison of European and North American *S. neocynipsea* behaviour

Copulation frequency (Fig. 1a) in cross-continental *S. neocynipsea* pairings did not vary among parentals, F_1 hybrids or backcrosses, nor did cumulative female shaking behaviour (Fig. 1c) and copulation latency (Fig. 2a). The only differences to be reported here are that males in cross-continental parental *S. neocynipsea* pairings performed more mating attempts than males in conspecific pairings within populations ($F(1,6) = 12.09$, $P = 0.013$; violet vs. dark blue dots in Fig. 1b; appendix Tale A4). Likewise, males of the cross-continental F_1 (hybrid) generation performed more mating attempts than males in the conspecific parental pairings ($F(1,6) = 13.58$, $P = 0.009$; Fig. 1b). In both comparisons male mating attempts again covaried

positively with female shaking (covariate effect: $F(1,6) > 62.22$, $P < 0.001$, $\beta > 0.309$). Furthermore, copulation durations of F_1 hybrid pairings were longer than those of the parental conspecific pairings ($F(1,6) = 15.39$, $P = 0.007$; Fig. 2b). Lastly, backcross direction showed no significant effect for any trait except copulation frequency ($F(1,6) = 11.18$, $P = 0.012$), which was negatively affected by male mating attempts ($F(1,53) = 25.491$, $P < 0.001$, $\beta = -0.556$) as well as female shaking duration ($F(1,53) = 12.58$, $P = 0.001$, $\beta = -0.335$).

*III. Comparison of F_1 hybrid offspring with parental *S. cynipsea* and *S. neocynipsea* – inheritance of behavioural traits*

Comparing the F_1 offspring, as depending on hybrid direction (i.e. CxN vs. NxN), with the parental species permits inferences about the inheritance of a trait (Fig. 3). One-way GLM with pure (baseline) parentals plus the reciprocal F_1 hybrids (CxN, NxN) as main effect with four levels revealed significant variation for female shaking duration suggesting partial maternal inheritance ($F(3,12) = 23.33$, $P < 0.001$; Fig. 3a); a post-hoc Tukey's test further revealed significant differences between the parental species (appendix Table A1). Analogous one-way GLM further showed significant variation for copulation frequency suggesting dominance of *S. neocynipsea*'s low copulation pattern ($F(3,12) = 3.77$, $P = 0.038$; Fig. 3b), with the corresponding post-hoc Tukey's test shown in appendix Table A1. All other traits showed no such variation suggesting the default intermediate inheritance (appendix Fig. A1).

IV. Comparison of heterospecific vs. conspecific parental pairings, F_1 hybrid and backcross pairings

Heterospecific parental pairings never showed variation in crossing direction (CxN vs. NxN) in any trait, except for copulation latency ($F(1,4) = 6.93, P = 0.013$; appendix Table A4). As expected, conspecific parental pairings resulted in much higher copulation frequencies than heterospecific pairings ($F(3,12) = 18.01, P < 0.001$; Fig. 1a); copulation probability was additionally negatively related to the number of male mating attempts ($F(1,362) = 18.00, P < 0.001, \beta = -0.186$; appendix Table A2). Even though there was only slight variation in the number of male mating attempts in an analogous test ($F(3,12) = 3.32, P = 0.055$; Fig. 1b), females in conspecific pairings showed longer cumulative shaking duration than those in heterospecific pairings ($F(3,12) = 13.05, P = 0.001$; Fig. 1c), the two traits again being correlated positively with each other ($F(1,349) = 32.75, P < 0.001, \beta = 0.542$). Importantly, analogous multivariate analysis of all these three traits together also indicated overall significant variation among species and cross types (C, N, CxN, NxN: $F(9,36) = 13.19, P < 0.001$; cf. Fig. 1). Copulation latency was much longer ($F(3,10) = 9.46, P < 0.001$), and copulation duration significantly shorter in heterospecific pairings ($F(3,11) = 5.66, p = 0.006$; Fig. 2a,b). Again, the corresponding multivariate analysis was also significant ($F(6,21) = 6.29, P < 0.001$).

Variation in F_1 hybrid direction (CxN vs. NxN; right grey vs. blue dots in Fig. 1c) was only evident for female shaking duration, with less shaking observed when the mother was *S. neocynipsea* ($F(1,6) = 21.82, P < 0.001$; appendix Table A6). This comparison confirms the above-mentioned maternal inheritance pattern of this trait (Fig. 3a).

Backcrosses of F_1 hybrids with both parental species indicated no sex-specific variation for any of the studied behavioural traits, independent of whether the parental species was female or male (appendix Fig. A2; Table A7).

V. Effects of biogeographic type

Biogeographic type (sym-, para-, allopatry) in the parental heterospecific pairings systematically affected copulation frequency, female shaking duration (Fig. 1a,c) and copulation latency (Fig. 2a), while the other traits showed no such variation. Copulation frequency and shaking duration increased from sympatric via parapatric to allopatric cross-continental pairings ($F(4,6) = 11.78, P = 0.005$ and $F(4,6) = 4.97, P = 0.041$, respectively; left to right in Fig. 1a; appendix Table A2). Similarly, flies of the cross-continental allopatric parental pairings required less time until first copulation ensued than flies from the corresponding European parapatric and sympatric pairings, although this appeared to be the case primarily in the CxN but not the NxN subset of the data ($F(4,4) = 5.84, P = 0.001$; Fig. 2a). Crucially, the multivariate analysis subsuming all the traits presented in Fig. 1 also yielded a significant effect of biogeographic type ($F(12,18) = 3.49, P = 0.008$), which was not quite the case for the two copulation traits presented in Fig. 2 ($F(8,8) = 3.27, P = 0.057$).

Analogous analysis of the F1 hybrid generation only revealed systematic effects of biogeographic type on female shaking, however now showing a decrease in shaking duration from sympatric to allopatric pairings ($F(4,6) = 4.48, P = 0.042$; Fig. 1c; appendix Table A3), but not on any other traits.

DISCUSSION

Sepsis cynipsea and *S. neocynipsea* were previously determined as different species based on their COI genetic distances (Su, Kutty & Meier, 2008; Puniamoorthy, Su & Meier, 2008), and behavioural as well as morphological differences (Pont & Meier, 2002). The species show low conspecific population differentiation but high heterospecific genetic differentiation based on neutral genetic microsatellite markers (Baur et al., unpubl. data). Furthermore, North American and European populations of *S. neocynipsea* were recognized as the same species despite their geographical isolation and some morphological differences (Pont & Meier, 2002). We here documented quantitative differences in some precopulatory behavioural traits important for mating that are shared by both species, notably male mating attempts, female shaking behaviour, copulation frequency, latency and duration. Particularly female shaking when males are mounted on their back, a trait that is part of the general repertoire of sepsid flies (Puniamoorthy et al., 2009), is much more pronounced in *S. cynipsea* than in *S. neocynipsea*. Previous studies of *S. cynipsea* had identified this trait as contributing to female choice of mating partners and/or an expression of female reluctance to mate (Blanckenhorn et al., 2000; Parker, 1972 a,b; Ward, 1983; Ward, Hemi & Rösli, 1992); it also has been shown to evolve in response to mating system manipulations due to sexual selection and conflict (Martin & Hosken, 2003).

We were here able to detect that females across almost all pairings and generations respond with higher cumulative shaking to more male mating attempts, but this resulted in lower copulation frequencies, indicating reluctance to mate (Blanckenhorn et al., 2000). However, *S. cynipsea* females did shake longer in

conspecific pairings than did *S. neocynipsea* females resulting in higher copulation frequencies suggesting mate assessment. Overall, if the female is willing to mate, males do not need to attack her so often, indicating females willingness to copulate. Nevertheless, our evidence here that female shaking contributes to mate recognition, which could lead to reproductive isolation, is limited to significant variation in the expected direction between sym-, para- and allopatric heterospecific pairings (Fig. 1c, green vs. blue). It should be clear that traits merely differing quantitatively do not serve as well for reproductive isolation as do qualitatively different courtship traits (Puniamoorthy, 2014). Overall, our study revealed strong evidence for possible hybridisation of these two species, and also some evidence of species recognition and reproductive isolation at the precopulatory level, most apparent in terms of lower copulation frequencies and, particularly, in longer copulation latencies in heterospecific pairings. Under laboratory conditions, viable F₁ hybrid offspring had higher copulation success with each other and even with parental partners in backcrosses than occurred in the baseline conspecific parental pairings. This indicates hybrid vigour rather than outbreeding depression, facilitating hybridisation in nature (Todesco et al., 2016; Wolf, Takebayasi & Rieseberg, 2001). The extent to which hybridisation occurs in nature is currently being investigated at the genomic level.

Comparison of parental S. cynipsea and S. neocynipsea behaviour

Our study revealed anticipated but so far not quantified differences between the sister species in most assessed traits (Fig. 1), verifying on behavioural grounds that the two species are indeed separate but very closely related (Pont & Meier, 2002; Puniamoorthy, Su & Meier, 2008; Su, Kutty & Meier, 2008). Besides the prominent differences in female shaking behaviour discussed above, species differences in the

other four traits assessed here are less pronounced. *S. neocynipsea* males exhibited more mating attempts than *S. cynipsea* males. In nature, *S. cynipsea* is much more abundant than *S. neocynipsea* (and any other sepsid species) in places of co-occurrence in Europe, such as the Swiss Alps from where we sampled our populations (Pont & Meier, 2002). This implies that *S. cynipsea* males should more easily find conspecific mating partners, and therefore do not need to try hard to achieve copulations. Despite more potential harassment of *S. neocynipsea* females by males of the other species in nature, known as the rare female hypothesis (Noor, 1995; Yukilevich, 2012), the latter performed less shaking but nevertheless ended up maintaining lower copulation frequencies, indicating that whatever other means *S. neocynipsea* females have to fend off unwanted males are very effective. *Sepsis cynipsea* females, in contrast, showed stronger shaking nevertheless resulting in more copulation per mating attempt, re-emphasizing the role of this behaviour in mate assessment (Ward, 1983).

Male and female traits depended significantly on each other, showing that the cumulative female shaking duration was longer the more often males attempted to mate with a partner, most likely to fend off the constant harassment of males. In turn, copulation frequency depended significantly on male-female interactions. Copulation success per mating attempt was lower in *S. neocynipsea*, for which ~37% of all male mating attempts resulted in copulations, as opposed to ~84% for *S. cynipsea* (Table 3). We recorded more copulations in conspecific pairings when males needed fewer attempts likely because females seem to be more willing to mate, possibly facilitated by species recognition of the conspecific partner. As expected, copulation success in our forced heterospecific pairings was much lower (~11%). Moreover, conspecific *S. cynipsea* pairings showed longer copulation latencies, suggesting *S. cynipsea* females

spend overall more time assessing mates (by shaking more; Blanckenhorn et al., 2000), whereas *S. neocynipsea* females start copulating faster when mounted by a conspecific male. Finally, *S. cynipsea* showed slightly longer copulation durations (ca. 23 vs. 21 min), the biological significance of which is probably minor.

Perhaps surprisingly, *S. cynipsea* females shook less in heterospecific than conspecific pairings (Table 3). This lower shaking duration when facing heterospecific males could be a result of faster male dismounting or dislocation due to the species differences in the male armoured foreleg, which is an important male tool to cling on to the female's wing (Pont & Meier, 2002). In this context shaking appears effective for *S. cynipsea* females in assessing or rejecting mates (Blanckenhorn et al., 1999, 2000; Martin & Hosken, 2003; Ward, 1983, Ward et al., 1992), while *S. neocynipsea* females must have other means of assessing unwanted males: for instance surface or volatile hydrocarbons could be involved (Puniamoorthy, 2013). Other candidates could be subtle male courtship behaviour (e.g. circling around a female) or leg positions during pairing, which have been demonstrated in several sepsid species and many other insects (Eberhard, 1996; Puniamoorthy et al., 2009). Species recognition in heterospecific pairings here is most prominently expressed in longer copulation latencies compared to conspecific pairings. On the whole, the lower copulation frequencies, shorter copulation durations and longer copulation latencies in heterospecific pairings all signify strongly that these flies have more difficulties to mate, probably due to divergence in mate recognition systems.

Sepsis neocynipsea males in cross-continental, conspecific parental pairings performed more mating attempts, while not provoking more female shaking, perhaps because they dismounted faster on their own. On the other hand, similar female shaking durations, copulation frequencies and latencies, with only minor differences

in the other behavioural traits between the continents and across all generations, portend that *S. neocynipsea* from North America and Europe indeed recognize each other as the same species.

Trait inheritance in F₁ hybrids and hybrid vigour

Our study revealed no significant variation related to hybrid direction (CxN vs. NxN) for any behavioural trait. Accordingly, F₁ hybrid offspring showed intermediate phenotypes relative to the parental species, indicating intermediate and presumably mainly autosomal inheritance of most of the quantitative behavioural traits considered here. Shaking behaviour is a prominent exception, which appears to be at least partly maternally inherited because hybrids expressed shaking more similar to the maternal species (Fig. 3a). Copulation frequency further showed evidence for dominance, as the lower copulation probabilities of *S. neocynipsea*, mediated by whatever mechanism, seem to be inherited by all the hybrids (Fig. 3b). Although our study design was not suited to calculate heritabilities, we were able to detect these strong signs of maternal inheritance and dominance. Further work to explore the genetic basis underlying these mechanisms can be a central aim of future studies. Our results confirm that most mating traits considered here are quantitative and heritable, and can therefore evolve in response to natural and sexual selection (cf. Martin & Hosken, 2003; Mühlhäuser & Blanckenhorn, 2004).

Interestingly, hybrid vigour was evident in F₁ hybrid offspring, not so much for the male and female behavioural traits themselves, but certainly by virtue of increased copulation success relative to the heterospecific parental pairings (Fig. 1, Table 3; Baranwal et al., 2012). We can therefore conclude that hybridisation does not immediately lead to cessation of mating behaviour and copulation in this system,

although this may happen in later generations or further backcrosses. A first sign of mating barriers may be increased copulation durations of hybrids, suggesting some postmating but prezygotic difficulties such as disturbed sperm transfer (Arthur & Dyer, 2015). Whether reproductive success is depressed in hybrids or backcrosses despite the continuing mating success documented here will be investigated in future studies.

Comparing generations – are hybrids recognized?

Our data revealed little variation in most traits across the generations (parental, F_1 , backcrosses), highlighting no breakdown over generations of important traits that potentially could reduce mating success. Instead, hybridisation may merely be disrupted by the difficulties in sperm transfer indicated by prolonged copulation durations of F_1 hybrids. Invariant male mating attempts also indicate that males do not discriminate strongly against heterospecific partners, as can be expected because sperm are relatively cheap, while effort in achieving a mating is substantial in any case (Birkhead & Moller, 1998). The mating system of both species is best described as scramble competition, with few if any aggressive interactions among males and a paramount role of female choice, by whatever mechanism (Blanckenhorn et al., 2000).

Comparing the biogeographic range across sym-, para- and allopatry

Strongest precopulatory isolation is often demonstrated in sympatric species pairs, indicating reinforcement (Coyne & Orr, 1989, 2004; Yukilevich, 2012). A biogeographic effect in the parental pairings was detected for copulation frequency, latency and female shaking behaviour (Figs. 1, 2). Heterospecific pairings from

European populations in either sym- or parapatry exhibited longer latencies to copulation than flies in the cross-continental allopatric pairings, suggesting reinforcement of species recognition in areas where the two species co-occur. High conspecific gene flow may maintain this pattern throughout Europe (Fig. 1; Table 3). In contrast, heterospecific parental pairings showed stronger female shaking in allopatric pairings across continents and little shaking in sympatric pairings, though this pattern may be equally explained by faster dismounting of unwanted mates and species recognition of males in sympatric pairings. Reinforcement through stronger female shaking behaviour in areas of sympatry is also reflected in the F_1 hybrid offspring, for which sympatric pairings showed more shaking than allopatric pairings (Fig. 1; Table 3).

CONCLUSIONS

We documented successful hybridisation under forced laboratory conditions between the close sister species *S. cynipsea* and *S. neocynipsea*. Female mate choice and species recognition can explain the low frequency of heterospecific relative to conspecific copulations realized per male mating attempt as well as their longer copulation latencies. The observed pattern of F_1 hybrids and backcrosses showing lower copulation frequencies, longer copulation latencies and durations than the conspecific parentals, while at the same time achieving more copulations than flies in heterospecific pairings, could result from hybrid vigour mediated by mixture of genes from both species permitting species recognition (Baranwal et al., 2012). We also observed heterospecific parental pairings with lower and F_1 and backcross pairings with higher copulation durations as the parental species, indicating possible

difficulties with sperm transfer. Copulations do not necessarily imply successful fertilization, however, so offspring production needs to be documented to reveal possible mechanisms of post-copulatory isolation such as e.g. male sterility according to Haldane's rule (Haldane, 1922).

ACKNOWLEDGEMENTS

We thank Manuela Bizzozzero, Martina Ramel, Isabel Schumacher, the students of the biology of reproduction course 2013 (BIO361) and especially Christina Ebner for help with collecting the data. Thanks also to the dung fly group at the University of Zurich for their support and help in maintaining fly cultures, and Patrick Rohner, Nalini Puniamoorthy, Anders Kjaersgaard and Cait Dmitriew for collecting samples. The University Research Priority Program 'Evolution in Action' of the University Zurich and the Swiss National Foundation Grant No. 31-143787 funded this research.

REFERENCES

- Arthur N.J., & Dyer K.A. (2015). Asymmetrical sexual isolation but no postmating isolation between the closely related species *Drosophila suboccidentalis* and *Drosophila occidentalis*. *BioMed Central Evolutionary Biology*, 15(38).
Retrieved from: <http://dx.doi.org/10.1186/s12862-015-0328-y>
- Baranwal V.K., Mikkilineni V., Zehr U.B., Tyagi A.K., & Kapoor S. (2012). Heterosis: emerging ideas about hybrid vigour. *Journal of Experimental Botany*, 63(18), 6309-6314.
Retrieved from: <http://dx.doi.org/10.1093/jxb/ers/291>
- Birkhead T.R., & Møller A.P. (1998). *Sperm competition and sexual selection*. San Diego, CA, U.S.A.: Academic Press.
- Blanckenhorn W.U., Morf C., Mühlhäuser C., & Reusch T. (1999). Spatio-temporal variation in selection on body size in the dung fly *Sepsis cynipsea*. *Journal of Evolutionary Biology*, 12, 563-576.
Retrieved from: <http://dx.doi.org/10.1046/j.1420-9101.1999.00050.x>
- Blanckenhorn W.U., Mühlhäuser C., Morf C., Reusch T., & Reuter M. (2000). Female Choice, Female Reluctance to Mate and Sexual Selection on Body Size in the Dung Fly *Sepsis cynipsea*. *Ethology*, 106, 577-593.
Retrieved from: <http://dx.doi.org/10.1046/j.1439-0310.2000.00573.x>
- Boake C.R.B., Price D.K., & Andreadis D.K. (1998). Inheritance of behavioral differences between two interfertile, sympatric species *Drosophila silvestris* and *D. heteroneura*. *Heredity*, 80, 642-650.
Retrieved from: <http://www.ncbi.nlm.nih.gov/pubmed/9650282>
- Coyne J.A., & Orr H.A. (1989). Patterns of speciation in *Drosophila* revisited. *Evolution*, 51, 295-303.
Retrieved from: <http://dx.doi.org/10.2307/2410984>.

569

570 Coyne J.A., & Orr H.A. (2004). *Speciation*. Sunderland M.A., U.S.A.: Sinauer

571 Associates Inc.

572

573 Ding A., & Blanckenhorn W.U. (2002). The effect of sexual size dimorphism on

574 mating behavior in two dung flies with contrasting dimorphism. *Evolutionary Ecology*

575 *Resources*, 4, 259-273.

576 Retrieved from: <http://www.evolutionary-ecology.com/issues/v04n02/kkar1377.pdf>

577

578 Dobzhansky T. (1951). *Genetics and the origin of species* (3rd ed.). New York N.Y.,

579 U.S.A.: Columbia University Press.

580

581 Eberhard W.G. (1999). Mating systems of sepsid flies and sexual behavior away from

582 oviposition sites by *Sepsis neocynipsea* (Diptera: Sepsidae). *Journal of the Kansas*

583 *Entomological Society*, 72(1), 129-130.

584

585 Eberhard W.G. (1996). *Female control: sexual selection by cryptic female choice*.

586 Princeton, N.J., U.S.A.: Princeton University Press.

587

588 Gavrilets S. (2000). Rapid evolution of reproductive barriers driven by sexual

589 conflict. *Nature*, 403, 886-889.

590 Retrieved from: <http://dx.doi.org/10.1038/35002564>

591

592 Gleason J.M., & Ritchie M.G. (1998). Evolution of courtship song and reproductive

593 isolation in the *Drosophila willistoni* species complex: do sexual signals diverge the

594 most quickly? *Evolution*, 52, 1493-1500.

595 Retrieved from: <http://dx.doi.org/10.2307/2411319>

596

597 Haldane J.B.S. (1922). Sex ratio and the unisexual sterility in animal hybrids. *Journal*

598 *of Genetics*, 12, 101-109.

599 Retrieved from <http://dx.doi.org/10.1007/BF02983075>

600

601 Hood G.R., Egan S.P., & Feder J.L. (2012). Evidence for sexual isolation as a
602 prezygotic barrier to gene flow between morphologically divergent species of
603 *Rhagoletis* fruit flies. *Ecological Entomology*, 37(6), 521-528.
604 Retrieved from: <http://dx.doi.org/10.1111/j.1365-2311.2012.01392.x>
605
606 Hosken D.J., Martin O.Y., Born J., & Huber F. (2003). Sexual conflict in *Sepsis*
607 *cynipsea*: female reluctance, fertility and mate choice. *Journal of Evolutionary*
608 *Biology*, 16(3), 485-490.
609 Retrieved from: <http://ncbi.nlm.nih.gov/pubmed/14635848>
610
611 Kozak G.M., Reiland M., & Boughmann J.W. (2009). Sex differences in mate
612 recognition and conspecific preference in species with mutual mate choice. *Evolution*,
613 63, 353-365.
614 Retrieved from: <http://dx.doi.org/10.1111/j.1558-5646.2008.00564.x>
615
616 Kraaijeveld K., Kraaijeveld-Smit F.J.L., & Maan M.E. (2010). Sexual selection and
617 speciation: the comparative evidence revisited. *Biological Reviews*, 86(2), 367-377.
618 Retrieved from: <http://dx.doi.org/10.1111/j.1469-185X.2010.00150.x>
619
620 Lande R. (1981). Model of speciation by sexual selection on polygenic traits.
621 *Proceedings of the National Academy of Sciences*, 78(6), 3721-3725.
622 Retrieved from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC319643/>
623
624 Martin O.Y., & Hosken D.J. (2003). The evolution of reproductive isolation through
625 sexual conflict. *Nature*, 423, 979-982.
626 Retrieved from: <http://dx.doi.org/10.1038/nature01752>
627
628 Mayr E. (1942). *Systematics and the origin of species*. New York, N.Y., U.S.A.:
629 Columbia University Press.
630
631 Mühlhäuser C., & Blanckenhorn W.U. (2004). The quantitative genetics of sexual
632 selection in the dung fly *Sepsis cynipsea*. *Behaviour*, 141(3), 327-341.
633 Retrieved from: <http://dx.doi.org/10.1163/156853904322981888>

634

635 Panhuis T.M., Butlin R., Zuk M., Tregenza T. (2001). Sexual selection and
 636 speciation. *Trends in Ecology & Evolution*, 16(7), 364-371.
 637 Retrieved from: [http://dx.doi.org/10.1016/S0169-5347\(01\)002160-7](http://dx.doi.org/10.1016/S0169-5347(01)002160-7)
 638

639 Parker G.A. (1972a). Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera:
 640 Sepsidae). I. A preliminary analysis of the reproductive strategy and its associated
 641 behaviour patterns. *Behaviour*, 41, 172-206.
 642 Retrieved from: <http://dx.doi.org/10.1163/156853972X00257>
 643

644 Parker G.A. (1972b). Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera:
 645 Sepsidae) II. The significance of the precopulatory passive phase and emigration.
 646 *Behaviour*, 41, 242-250.
 647 Retrieved from: <http://dx.doi.org/10.1163/156853972X00031>
 648

649 Pont A.C., & Meier R. (2002). *The Sepsidae (Diptera) of Europe*. Leiden, N.E.:
 650 Fauna entomologica Scandinavia, 37.
 651

652 Puniamoorthy N., Ismail M.R.B., Tan D.S.H., & Meier R. (2009). From kissing to
 653 belly stridulation: comparative analysis reveals surprising diversity, rapid evolution,
 654 and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera:
 655 Sepsidae). *Journal of Evolutionary Biology*, 22:11, 2146-2156.
 656 Retrieved from: <http://dx.doi.org/10.1111/j.1420-9101.2009.01826.x>
 657

658 Puniamoorthy N., Schäfer M.A., & Blanckenhorn W.U. (2012). Sexual selection
 659 accounts for the geographic reversal of sexual size dimorphism in the dung fly *Sepsis*
 660 *punctum* (Diptera: Sepsidae). *Evolution*, 66:7, 2117-2126.
 661 Retrieved from: <http://dx.doi.org/10.1111/j.1558-5646.2012.01599.x>
 662

663 Puniamoorthy N. (2013). Morphology, molecules and mating behavior: an integrative
 664 study of population divergence and speciation in widespread sepsid flies (Sepsidae:
 665 Diptera) (Doctoral thesis). Zurich, C.H.: University of Zurich.
 666

Puniamoorthy N. (2014). Behavioural barriers to reproduction may evolve faster than sexual morphology among populations of a dung fly (Sepsidae). *Animal Behaviour*, 98, 139-148.

Retrieved from: <http://dx.doi.org/10.1016/j.anbheav.2014.10.005>

Reed L.K., & Markow T.A. (2004). Early events in speciation: polymorphism for hybrid male sterility in *Drosophila*. *Proceedings of the National Academy of Sciences*, 101, 9009-9012.

Retrieved from: <http://dx.doi.org/10.1073/pnas.0403106101>

Rohner P.T., Blanckenhorn W.U., & Puniamoorthy N. (2016). Sexual selection on male size drives the evolution of male-biased sexual size dimorphism via the prolongation of male development. *Evolution*, 70(6), 1189-1199.

Retrieved from: <http://dx.doi.org/10.1111/evo.12944>

Schluter D. (2000). *The ecology of adaptive radiation*. Oxford, U.K.: Oxford University Press.

Schluter D. (2001). Ecology and the origin of species. *Trends in Ecology and Evolution*, 16, 372-380.

Retrieved from: [http://dx.doi.org/10.1016/S0169-5347\(01\)02198-X](http://dx.doi.org/10.1016/S0169-5347(01)02198-X)

Shaw K.L., & Herlihy D.P. (2000). Acoustic preference functions and song variability in the Hawaiian cricket *Laupala cerasina*. *Proceedings of the Royal Society of Biological Sciences*, 267, 577-584.

Retrieved from: <http://dx.doi.org/10.1098/rspb.2000.1040>

Su K.F.Y., Kutty S., & Meier R. (2008). Morphology versus molecules: the phylogenetic relationships of Sepsidae (Diptera: Cyclorrhapha) based on morphology and DNA sequence data from ten genes. *Cladistics*, 24, 902-916.

Retrieved from: <http://dx.doi.org/10.1111/j.1096-0031.2008.00222.x>

699 Svensson E.I., Karlsson K., Friber M., & Eroukhmanoff F. (2007). Gender differences
700 in species recognition and the evolution of asymmetric sexual isolation. *Current*
701 *Biology*, 17, 1943-1947.
702 Retrieved from: <http://dx.doi.org/10.1016/j.cub.2007.09.038>
703
704 Teuschl Y., & Blanckenhorn W.U. (2007). The reluctant fly: what makes *Sepsis*
705 *cynipsea* females willing to copulate? *Animal Behaviour*, 73, 85-97.
706 Retrieved from: <http://dx.doi.org/10.1016/j.anbehav.2006.03.028>
707
708 Todesco M., Pascual M.A., Owens G.L., et al. (2016). Hybridization and extinction.
709 *Evolutionary Applications*, 9(7), 892-908.
710 Retrieved from: <http://dx.doi.org/10.1111/eva.12367>
711
712 Turelli M., Barton N.H., & Coyne J.A. (2001). Theory and speciation. *Trends in*
713 *Ecology & Evolution*, 16(7), 330-343.
714 Retrieved from: [http://dx.doi.org/10.1016/S0169-5347\(01\)02177-2](http://dx.doi.org/10.1016/S0169-5347(01)02177-2)
715
716 Via S. (2001). Sympatric speciation in animals: the ugly duckling grows up. *Trends in*
717 *Ecology and Evolution*, 16, 381-390.
718 Retrieved from: [http://dx.doi.org/10.1016/S0169-5347\(01\)02188-7](http://dx.doi.org/10.1016/S0169-5347(01)02188-7)
719
720 Via S. (2009). Natural selection in action during speciation. *Proceedings of the*
721 *National Academy of Sciences*, 106.
722 Retrieved from: <http://dx.doi.org/10.1073/pnas.0901397106>
723
724 Wassermann M., & Koepfer H.R. (1977). Character displacement for sexual isolation
725 between *Drosophila mojavensis* and *Drosophila arizonensis*. *Evolution*, 31(4), 812-
726 823.
727 Retrieved from: <http://dx.doi.org/10.2307/2407442>
728
729 Ward P.I. (1983). The effects of size on the mating behaviour of the dung fly *Sepsis*
730 *cynipsea*. *Behavioral Ecology and Sociobiology*, 13, 75-80.
731 Retrieved from: <http://dx.doi.org/10.1007/BF00295078>

732

733 Ward P.I., Hemi J., & Rösli T. (1992). Sexual conflict in the dung fly *Sepsis cynipsea*.
734 *Functional Ecology*, 6, 649-653.

735 Retrieved from: <http://www.jstor.org/stable/2389959>

736

737 Wolf D.E., Takebayasi N., & Rieseberg L. H. (2001). Predicting the risk of extinction
738 through hybridization. *Conservation Biology*, 15(4), 1039-1053.

739 Retrieved from: <http://dx.doi.org/10.1046/j.1523-1739.2001.0150041039.x>

740

741 Yukilevich R. (2012). Asymmetrical patterns of speciation uniquely support
742 reinforcement in *Drosophila*. *Evolution*, 66, 1430-1446.

743 Retrieved from: <http://dx.doi.org/10.1111/j.1558-5646.2011.01534.x>

744

745 **Table 1**

746 Biogeographic origin of *S. cynipsea* and *S. neocynipsea* populations used.

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

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

748

Table 2

Inter- (three groups) and intra-specific (one group) pairing scheme of three biogeographical types (female x male). All pairings were reciprocal.

Biogeographical type	Pairings	Populations
Sympatry in Europe	EU <i>S. neocynipsea</i> x EU <i>S. cynipsea</i>	(CH1) x (CH1)
		(CH2) x (CH2)
Parapatry across Europe	EU <i>S. neocynipsea</i> x EU <i>S. cynipsea</i>	(EU1) x (CH1)
		(EU2) x (CH2)
Allopatry across continents:		
inter-specific	NA <i>S. neocynipsea</i> x EU <i>S. cynipsea</i>	(NA1) x (CH1)
		(NA2) x (CH2)
intra-specific	NA <i>S. neocynipsea</i> x EU <i>S. neocynipsea</i>	(NA1) x (CH1)
		(NA2) x (CH2)

753 **Table 3**

754 Mean values ($\pm$ 95% CI) of female and male mating behaviour (female x male) for parental, F₁ hybrid pairings and backcrosses.

Generation	Pairing	Male attempts [No.]	mating Female shaking duration [min]	Copulation frequency [proportion]	Copulation latency [min]	Copulation duration [min]
Parental	C*	3.61 $\pm$ 0.78	6.90 $\pm$ 1.62	0.84 $\pm$ 0.16	4.26 $\pm$ 1.41	22.92 $\pm$ 1.18
	C x N	0.47 $\pm$ 0.08	3.15 $\pm$ 0.85	0.10 $\pm$ 0.07	10.04 $\pm$ 2.63	16.92 $\pm$ 3.04
	N x C	0.57 $\pm$ 0.09	1.33 $\pm$ 0.51	0.13 $\pm$ 0.12	11.43 $\pm$ 2.55	18.59 $\pm$ 2.48
	N*	5.02 $\pm$ 0.76	0.69 $\pm$ 0.35	0.37 $\pm$ 0.11	3.26 $\pm$ 0.96	20.68 $\pm$ 1.01
	N _{EU} *	4.53 $\pm$ 0.96	0.88 $\pm$ 0.66	0.41 $\pm$ 0.56	5.99 $\pm$ 2.29	20.46 $\pm$ 1.78
	N x N	10.45 $\pm$ 4.21	0.54 $\pm$ 0.15	0.23 $\pm$ 0.13	6.97 $\pm$ 1.81	24.10 $\pm$ 2.11
	N _{NA} *	5.49 $\pm$ 1.15	0.53 $\pm$ 0.30	0.33 $\pm$ 0.13	4.34 $\pm$ 1.96	20.87 $\pm$ 1.68
F ₁	C x N	5.68 $\pm$ 1.42	4.03 $\pm$ 1.34	0.47 $\pm$ 0.12	5.10 $\pm$ 1.73	23.84 $\pm$ 1.61
	N x C	5.04 $\pm$ 1.42	0.93 $\pm$ 0.44	0.40 $\pm$ 0.10	5.26 $\pm$ 2.04	22.28 $\pm$ 1.45
	N x N	8.57 $\pm$ 1.45	0.42 $\pm$ 0.12	0.36 $\pm$ 0.11	5.95 $\pm$ 1.70	24.71 $\pm$ 0.99

755 C = *S. cynipsea*; N = *S. neocynipsea*; EU = Europe; NA = North America; Asterix* denoting intraspecific pairings.

756 **Table 3 ff.**

757 Mean values ($\pm$ 95% CI) of female and male mating behaviour (female x male) for parental, F₁ hybrid pairings and backcrosses grouped together in different
758 combinations.

Generation	Pairing	Male mating attempts [No.]	Female shaking duration [min]	Copulation frequency [proportion]	Copulation latency [min]	Copulation duration [min]
Parental	Conspecific	4.31 $\pm$ 0.55	3.79 $\pm$ 0.94	0.60 $\pm$ 0.10	3.86 $\pm$ 0.96	21.97 $\pm$ 0.87
	Heterospecific	5.77 $\pm$ 0.55	2.20 $\pm$ 0.50	0.12 $\pm$ 0.07	9.79 $\pm$ 2.04	17.96 $\pm$ 2.02
	Sympatry	0.43 $\pm$ 0.11	0.92 $\pm$ 0.54	0.04 $\pm$ 0.01	12.20 $\pm$ 6.02	18.75 $\pm$ 5.97
	Parapatry	0.54 $\pm$ 0.11	1.89 $\pm$ 0.74	0.04 $\pm$ 0.03	12.86 $\pm$ 4.45	16.86 $\pm$ 4.35
	Allopatry	0.61 $\pm$ 0.10	3.72 $\pm$ 1.08	0.25 $\pm$ 0.19	8.44 $\pm$ 2.38	18.02 $\pm$ 7.06
F ₁	Sympatry	0.55 $\pm$ 0.13	3.46 $\pm$ 1.46	0.42 $\pm$ 0.15	7.77 $\pm$ 2.58	24.51 $\pm$ 2.18
	Parapatry	0.68 $\pm$ 0.12	1.99 $\pm$ 0.97	0.44 $\pm$ 0.17	6.93 $\pm$ 2.59	25.99 $\pm$ 1.73
	Allopatry	0.49 $\pm$ 0.10	1.91 $\pm$ 1.20	0.44 $\pm$ 0.11	7.56 $\pm$ 1.90	20.78 $\pm$ 1.41
Backcrosses	F ₁ x P	5.77 $\pm$ 0.86	1.24 $\pm$ 0.55	0.26 $\pm$ 0.09	5.98 $\pm$ 1.46	26.53 $\pm$ 1.40
	P x F ₁	5.54 $\pm$ 0.92	0.78 $\pm$ 0.37	0.29 $\pm$ 0.12	7.50 $\pm$ 2.17	24.91 $\pm$ 1.98

759

760

761 **Figure 1.** (a) Copulation frequency, (b) number of male mating attempts, and (c) cumulative female shaking duration (mean $\pm$ 95% CI population means) for
 762 parental conspecific, heterospecific, and F₁ hybrid offspring, representing all replicates (zeroes included). Conspecific parental pairings on the left are denoted
 763 in black for *S. cynipsea* and dark blue for *S. neocynipsea* populations. Heterospecific parental and F1 hybrid pairings for CxN are in grey, for NxN in light
 764 blue, while conspecific cross-continental *S. neocynipsea* (NxN) pairings are indicated in violet. Sympatric pairings are colored lighter than parapatric and
 765 allopatric, the latter being darkest.

766

767 **Figure 2.** (a) Copulation latency and (b) copulation duration (mean $\pm$ 95% CI population means) for parental conspecific, heterospecific, and F₁ hybrid
 768 offspring, representing only replicates with at least one copulation. Conspecific parental pairings on the left are denoted in black for *S. cynipsea* and dark blue
 769 for *S. neocynipsea* populations. Heterospecific parental and F1 hybrid pairings for CxN are in grey, for NxN in light blue, while conspecific cross-continental
 770 *S. neocynipsea* pairings (NxN) are indicated in violet. Sympatric pairings are colored lighter than parapatric and allopatric, the latter being darkest.

Figure 3. (a) Female shaking duration (indicating maternal inheritance) and (b) copulation frequency (indicating dominance) compared between conspecific *S. cynipsea* (black), conspecific *S. neocynipsea* (dark blue) and F₁ hybrid offspring (grey for CxN, light blue for NxN) pairings for evaluating the inheritance pattern of behavioural traits (mean $\pm$ 95% CI).

Figure A1. (a) Male mating attempts, (b) copulation duration, and (c) copulation latency compared between conspecific *S. cynipsea* (black), conspecific *S. neocynipsea* (dark blue), and F₁ hybrid offspring (grey for CxN, light blue for NxN) pairings for evaluating the inheritance pattern of behavioural traits (mean $\pm$ 95% CI).

Pairings

Figure A2a. Comparing male and female behavioural traits of parental and backcross pairings (mean $\pm$ 95% CI). Backcrosses with CN hybrids are denoted in green, those with NC hybrids in blue. Continental *S. neocynipsea* (NN) backcrosses are in pink.

785

786

787

Figure A2b. Comparing copulation traits of parental and backcross pairings (mean $\pm$ 95% CI). Backcrosses with CN hybrids are denoted in green, those with NC hybrids in blue. Continental *S. neocynipsea* (NN) backcrosses are in pink.

APPENDIX

Table A1: Post-hoc paired Tukey's tests following the one-way ANOVA comparing a) female shaking duration [min] and b) copulation frequency of parental conspecific (C, N) and F₁ hybrid pairings from evaluating the inheritance pattern of behavioural traits, calculated by the difference between the pairings and standard errors.

a) Female shaking duration [min]

Species	P C	F ₁ C x N	F ₁ N x C	P N
P C	-	0.262 ± 0.132 p = 0.195	0.992 ± 0.128 p < 0.001	1.205 ± 0.115 p < 0.001
F ₁ C x N	-0.262 ± 0.132 p = 0.195	-	0.730 ± 0.142 p < 0.001	0.943 ± 0.131 p < 0.001
F ₁ N x C	-0.993 ± 0.128 p < 0.001	-0.730 ± 0.142 p < 0.001	-	0.213 ± 0.127 p = 0.986
P N	-1.205 ± 0.115 p < 0.001	-0.943 ± 0.131 p < 0.001	-0.213 ± 0.127 p = 0.338	-

b) Copulation frequency

Species	P C	F ₁ C x N	F ₁ N x C	P N
P C	-	0.270 ± 0.0077 p = 0.003	0.326 ± 0.0074 p < 0.001	0.373 ± 0.072 p < 0.001
F ₁ C x N	-0.270 ± 0.077 p = 0.003	-	0.056 ± 0.078 p = 0.891	0.103 ± 0.077 p = 0.537
F ₁ N x C	-0.326 ± 0.074 p < 0.001	-0.056 ± 0.078 p = 0.891	-	0.047 ± 0.074 p = 0.921
P N	-0.373 ± 0.072 p < 0.001	-0.103 ± 0.077 p = 0.537	-0.470 ± 0.074 p = 0.921	-

C = *S. cynipsea*, N = *S. neocynipsea*, female x male: C x N, N x C

797 **Table A2.** Univariate analyses of variance results for all behavioural traits in all GLMs performed.

Generation and Pairing	Comparison	Trait	df	F	p	β
Parental	<i>S. cynipsea</i> vs. <i>S. neocynipsea</i>	Male	1, 6	9.641	0.021	
		WITH female	1, 6	12.900	<0.001	0.136
		WITH latency	1, 6	1.047	0.309	-0.086
		WITH female AND latency FOR latency	1, 6	1.149	0.287	-0.092
		WITH female AND latency FOR female	1, 6	2.811	0.097	0.083
		Female	1, 6	33.727	0.001	
		WITH male	1, 6	12.900	<0.001	0.606
		WITH latency	1, 6	0.483	0.489	0.123
		WITH male AND latency FOR male	1, 6	2.811	0.097	0.349
		WITH male AND latency FOR latency	1, 6	0.740	0.392	0.151

798 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

799 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	<i>S. cynipsea</i> vs. <i>S. neocynipsea</i>	Copulation	1, 6	6.437	0.044	
		WITH male	1, 6	12.281	0.001	-0.365
		WITH female	1, 6	0.493	0.484	0.037
		WITH male AND female FOR male	1, 6	20.832	<0.001	-0.493
		WITH male AND female FOR female	1, 6	4.106	0.045	0.104
		WITH male AND female AND latency FOR male	1, 6	52.157	<0.001	-0.830
		WITH male AND female AND latency FOR female	1, 6	0.956	0.331	-0.055
		WITH male AND female AND latency FOR latency	1, 6	0.411	0.523	-0.062
		Latency	1, 6	5.356	0.055	
		WITH male	1, 6	1.047	0.309	-0.121
		WITH female	1, 6	0.483	0.489	0.041
		WITH male AND female FOR male	1, 6	1.149	0.287	-0.131
		WITH male AND female FOR female	1, 6	0.740	0.392	0.052
		Duration	1, 6	4.475	0.073	

800 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

801 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Heterospecific Pairings	Male	1, 6	0.502	0.505	
		WITH female	1, 6	19.946	<0.001	0.173
		WITH latency	1, 4	1.141	0.296	-0.314
		WITH female AND latency FOR female	1, 4	0.014	0.908	0.011
		WITH female AND latency FOR latency	1, 4	1.044	0.317	-0.309
		Female	1, 6	4.533	0.077	
		WITH male	1, 6	19.946	<0.001	0.514
		WITH latency	1, 4	0.464	0.502	-0.434
		WITH male AND latency FOR male	1, 4	0.014	0.908	0.052
		WITH male AND latency FOR latency	1, 4	0.395	0.536	-0.418

802 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

803 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Heterospecific Pairings	Copulation	1, 6	0.077	0.791	
		WITH male	1, 6	9.084	0.003	-0.108
		WITH female	1, 6	2.067	0.152	0.033
		WITH male AND female FOR male	1, 6	17.242	<0.001	-0.164
		WITH male AND female FOR female	1, 6	7.102	0.008	0.061
		WITH male AND female AND latency FOR male	1, 4	50.795	<0.001	-0.782
		WITH male AND female AND latency FOR female	1, 4	0.086	0.772	-0.015
		WITH male AND female AND latency FOR latency	1, 4	0.024	0.878	-0.026
		Latency	1, 4	6.933	0.013	
		WITH male	1, 4	1.141	0.296	-0.139
		WITH female	1, 4	0.464	0.502	-0.042
		WITH male AND female FOR male	1, 4	1.044	0.317	-0.135
		WITH male AND female FOR female	1, 4	0.395	0.536	-0.039
		Duration	1, 6	0.260	0.621	

804 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

805 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Parental full model, con- vs. hetero-specifics	Male	3, 12	1.156	0.367	
		WITH female	1, 12	32.976	<0.001	0.158
		WITH latency	1, 10	1.777	0.185	-0.111
		WITH female AND latency FOR female	1, 10	2.201	0.141	0.064
		WITH female AND latency FOR latency	1, 10	1.734	0.190	-0.112
		Female	3, 12	12.035	0.001	
		WITH male	1, 12	32.752	<0.001	0.542
		WITH latency	1, 10	0.117	0.733	0.061
		WITH male AND latency FOR male	1, 10	2.201	0.141	0.280
		WITH male AND latency FOR latency	1, 10	0.261	0.610	0.091

806 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

807 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Parental full model, con- vs. hetero-specifics	Copulation	3, 12	18.005	<0.001	
		WITH male	1, 12	18.000	<0.001	-0.186
		WITH female	1, 12	1.860	0.173	0.034
		WITH male AND female FOR male	1, 12	32.455	<0.001	-0.266
		WITH male AND female FOR female	1, 12	9.182	0.003	0.076
		WITH male AND female AND latency FOR male	1, 10	83.917	<0.001	-0.820
		WITH male AND female AND latency FOR female	1, 10	1.026	0.313	-0.044
		WITH male AND female AND latency FOR latency	1, 10	0.529	0.469	-0.061
		Latency	3, 10	6.933	0.013	
		WITH male	1, 10	1.177	0.185	-0.126
		WITH female	1, 10	0.117	0.733	0.016
		WITH male AND female FOR male	1, 10	1.734	0.190	-0.128
		WITH male AND female FOR female	1, 10	0.261	0.610	0.024
		Duration	3, 10	6.558	0.004	

808 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

809 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
<i>S. neocynipsea</i>	Parental: cross-continental vs. continental	Male	1, 6	12.085	0.013	
		WITH female	1, 6	62.985	<0.001	0.297
		WITH latency	1, 6	0.675	0.414	-0.080
		WITH female AND latency FOR female	1, 6	24.958	<0.001	0.266
		WITH female AND latency FOR latency	1, 6	6.063	0.016	-0.192
		Female	1, 6	2.065	0.201	
		WITH male	1, 6	62.985	<0.001	1.033
		WITH latency	1, 6	0.560	0.456	0.121
		WITH male AND latency FOR male	1, 6	24.958	<0.001	0.884
		WITH male AND latency FOR latency	1, 6	3.289	<i>0.073</i>	0.262

810 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

811 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Parental full model, con- vs. hetero-specifics	Copulation	1, 6	3.037	0.132	
		WITH male	1, 6	17.938	<0.001	-0.325
		WITH female	1, 6	1.264	0.263	-0.052
		WITH male AND female FOR male	1, 6	15.369	<0.001	-0.390
		WITH male AND female FOR female	1, 6	1.419	0.236	0.064
		WITH male AND female AND latency FOR male	1, 6	36.095	<0.001	-0.649
		WITH male AND female AND latency FOR female	1, 6	4.368	0.040	-0.124
		WITH male AND female AND latency FOR latency	1, 6	0.003	0.956	0.004
		Latency	1, 6	2.172	0.188	
		WITH male	1, 6	0.675	0.414	-0.100
		WITH female	1, 6	0.560	0.456	0.056
		WITH male AND female FOR male	1, 6	6.063	0.016	-0.362
		WITH male AND female FOR female	1, 6	3.289	0.073	0.149
		Duration	1, 6	4.616	0.073	

812 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

813 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Cross-continental <i>S. neocynipsea</i>	Full model: all generations	Male	1, 16	0.935	0.441	
		WITH female	1, 16	137.439	<0.001	0.357
		WITH latency	1, 16	2.320	0.130	-0.097
		WITH female AND latency FOR female	1, 16	71.892	<0.001	0.343
		WITH female AND latency FOR latency	1, 16	7.220	0.008	-0.135
		Female	1, 16	0.354	0.786	
		WITH male	1, 16	137.439	<0.001	1.004
		WITH latency	1, 16	0.004	0.949	-0.006
		WITH male AND latency FOR male	1, 16	71.892	<0.001	0.889
		WITH male AND latency FOR latency	1, 16	1.982	0.161	0.116

814 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

815 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Parental	Full model: all generations	Copulation	3, 16	0.469	0.689	
		WITH male	1, 16	54.226	<0.001	-0.412
		WITH female	1, 16	8.438	0.004	-0.110
		WITH male AND female FOR male	1, 16	44.414	<0.001	-0.485
		WITH male AND female FOR female	1, 16	2.150	0.144	0.064
		WITH male AND female AND latency FOR male	1, 16	71.114	<0.001	-0.612
		WITH male AND female AND latency FOR female	1, 16	4.955	0.027	-0.100
		WITH male AND female AND latency FOR latency	1, 16	0.458	0.499	0.032
		Latency	3, 16	0.466	0.708	
		WITH male	1, 16	2.320	0.130	-0.141
		WITH female	1, 16	0.004	0.949	-0.004
		WITH male AND female FOR male	1, 16	7.220	0.008	-0.311
		WITH male AND female FOR female	1, 16	1.982	0.161	0.103
		Duration	3, 16	4.495	0.012	

816 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

817 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Cross-continental <i>S. neocynipsea</i>	F ₁ vs. conspecifics	Male	1, 6	13.579	0.009	
		WITH female	1, 6	62.217	0.001	0.309
		WITH latency	1, 6	5.313	0.024	-0.187
		WITH female AND latency FOR female	1, 6	21.897	<0.001	0.250
		WITH female AND latency FOR latency	1, 6	7.684	0.007	-0.202
		Female	1, 6	4.242	0.078	
		WITH male	1, 6	62.217	<0.001	1.136
		WITH latency	1, 6	0.142	0.707	0.059
		WITH male AND latency FOR male	1, 6	21.897	<0.001	0.914
		WITH male AND latency FOR latency	1, 6	2.562	0.114	0.230

818 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

819 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Cross-continental <i>S. neocynipsea</i>	F ₁ vs. conspecifics	Copulation	1, 6	0.005	0.947	
		WITH male	1, 6	13.034	<0.001	-0.370
		WITH female	1, 6	1.698	0.195	-0.073
		WITH male AND female FOR male	1, 6	12.493	0.001	-0.450
		WITH male AND female FOR female	1, 6	0.987	0.323	0.066
		WITH male AND female AND latency FOR male	1, 6	32.003	<0.001	-0.739
		WITH male AND female AND latency FOR female	1, 6	3.940	0.051	-0.135
		WITH male AND female AND latency FOR latency	1, 6	0.316	0.576	-0.048
		Latency	1, 6	1.120	0.328	
		WITH male	1, 6	5.313	0.024	-0.349
		WITH female	1, 6	0.142	0.707	0.032
		WITH male AND female FOR male	1, 6	7.684	0.007	-0.466
		WITH male AND female FOR female	1, 6	2.562	0.114	0.145
		Duration	1, 6	15.392	0.007	

820 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

821 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
<i>S. neocynipsea</i>	Backcrosses vs. conspecifics	Male	1, 9	16.860	0.004	
		WITH female	1, 9	86.660	<0.001	0.348
		WITH latency	1, 9	3.952	0.050	-0.167
		WITH female AND latency FOR female	1, 9	35.125	<0.001	0.304
		WITH female AND latency FOR latency	1, 9	7.284	0.009	-0.192
		Female	1, 9	2.127	0.189	
		WITH male	1, 9	86.660	<0.001	1.166
		WITH latency	1, 9	0.302	0.584	0.085
		WITH male AND latency FOR male	1, 9	35.125	<0.001	1.013
		WITH male AND latency FOR latency	1, 9	3.645	<i>0.060</i>	0.254

822 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

823 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
<i>S. neocynipsea</i>	Backcrosses vs. conspecifics	Copulation	1, 9	0.361	0.564	
		WITH male	1, 9	15.836	<0.001	-0.378
		WITH female	1, 9	2.837	0.095	-0.092
		WITH male AND female FOR male	1, 9	14.122	0.095	-0.462
		WITH male AND female FOR female	1, 9	1.060	0.305	0.069
		WITH male AND female AND latency FOR male	1, 9	27.634	<0.001	-0.683
		WITH male AND female AND latency FOR female	1, 9	4.314	0.041	-0.148
		WITH male AND female AND latency FOR latency	1, 9	0.241	0.624	0.042
		Latency	1, 9	0.649	0.442	
		WITH male	1, 9	3.952	0.050	-0.279
		WITH female	1, 9	0.302	0.584	0.044
		WITH male AND female FOR male	1, 9	7.284	0.009	-0.439
		WITH male AND female FOR female	1, 9	3.645	0.060	0.174
		Duration	1, 9	9.445	0.004	

824 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

825 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 CN vs. NC conspecifics	Male	1, 6	0.354	0.571	
		WITH female	1, 6	6.405	0.013	0.134
		WITH latency	1, 6	0.022	0.884	0.016
		WITH female AND latency FOR female	1, 6	6.037	0.017	0.141
		WITH female AND latency FOR latency	1, 6	0.460	0.501	-0.091
		Female	1, 6	27.445	<0.001	
		WITH male	1, 6	6.405	0.013	0.507
		WITH latency	1, 6	2.324	0.133	0.474
		WITH male AND latency FOR male	1, 6	6.037	0.017	0.726
		WITH male AND latency FOR latency	1, 6	2.729	0.104	0.492

826 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

827 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 CN vs. NC conspecifics	Copulation	1, 6	0.001	0.978	
		WITH male	1, 6	16.610	<0.001	-0.447
		WITH female	1, 6	0.770	0.383	-0.061
		WITH male AND female FOR male	1, 6	10.700	0.002	-0.434
		WITH male AND female FOR female	1, 6	0.002	0.968	-0.003
		WITH male AND female AND latency FOR male	1, 6	47.985	<0.001	-0.901
		WITH male AND female AND latency FOR female	1, 6	0.864	0.357	0.053
		WITH male AND female AND latency FOR latency	1, 6	1.812	0.184	-0.171
		Latency	1, 6	0.214	0.657	
		WITH male	1, 6	0.022	0.884	0.018
		WITH female	1, 6	2.324	0.133	0.087
		WITH male AND female FOR male	1, 6	0.460	0.501	-0.095
		WITH male AND female FOR female	1, 6	2.729	0.104	0.100
		Duration	1, 6	0.796	0.399	

828 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

829 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 vs. conspecifics	Male	1, 9	0.067	0.802	
		WITH female	1, 9	33.229	<0.001	0.207
		WITH latency	1, 9	0.878	0.351	-0.076
		WITH female AND latency FOR female	1, 9	15.898	<0.001	0.172
		WITH female AND latency FOR latency	1, 9	4.345	0.040	-0.182
		Female	1, 9	36.638	0.001	
		WITH male	1, 9	33.229	<0.001	0.824
		WITH latency	1, 9	3.310	0.072	0.374
		WITH male AND latency FOR male	1, 9	15.898	<0.001	0.850
		WITH male AND latency FOR latency	1, 9	6.059	0.016	0.474

830 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

831 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 vs. conspecifics	Copulation	1, 9	0.242	0.635	
		WITH male	1, 9	20.269	<0.001	-0.386
		WITH female	1, 9	1.161	0.283	-0.053
		WITH male AND female FOR male	1, 9	14.888	<0.001	-0.396
		WITH male AND female FOR female	1, 9	0.324	0.570	0.029
		WITH male AND female AND latency FOR male	1, 9	66.394	<0.001	-0.912
		WITH male AND female AND latency FOR female	1, 9	0.077	0.782	-0.014
		WITH male AND female AND latency FOR latency	1, 9	1.892	0.172	-0.133
		Latency	1, 9	2.169	0.175	
		WITH male	1, 9	0.878	0.341	-0.098
		WITH female	1, 9	3.310	0.072	0.091
		WITH male AND female FOR male	1, 9	4.345	0.040	-0.245
		WITH male AND female FOR female	1, 9	6.059	0.016	0.129
		Duration	2, 9	3.871	0.053	

832 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

833 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 vs. heterospecifics	Male	3, 12	0.533	0.668	
		WITH female	1, 12	26.509	<0.001	0.162
		WITH latency	1, 12	0.114	0.736	-0.035
		WITH female AND latency FOR female	1, 12	4.184	0.044	0.100
		WITH female AND latency FOR latency	1, 12	0.850	0.359	-0.115
		Female	3, 12	6.110	0.007	
		WITH male	1, 12	26.509	<0.001	0.512
		WITH latency	1, 12	0.938	0.336	0.275
		WITH male AND latency FOR male	1, 12	4.184	0.044	0.503
		WITH male AND latency FOR latency	1, 12	1.306	0.257	0.319

834 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

835 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	F1 vs. heterospecifics	Copulation	3, 12	10.763	0.001	
		WITH male	1, 12	21.432	<0.001	-0.198
		WITH female	1, 12	0.051	0.822	0.006
		WITH male AND female FOR male	1, 12	23.785	<0.001	-0.227
		WITH male AND female FOR female	1, 12	2.652	0.105	0.043
		WITH male AND female AND latency FOR male	1, 12	88.687	<0.001	-0.948
		WITH male AND female AND latency FOR female	1, 12	0.288	0.593	0.022
		WITH male AND female AND latency FOR latency	1, 12	1.620	0.207	-0.128
		Latency	3, 10	3.343	0.043	
		WITH male	1, 10	0.114	0.736	-0.032
		WITH female	1, 10	0.938	0.336	0.042
		WITH male AND female FOR male	1, 10	0.850	0.359	-0.093
		WITH male AND female FOR female	1, 10	1.306	0.257	0.051
		Duration	3, 10	8.013	0.001	

836 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

837 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	backcrosses	Male	7, 16	1.728	0.163	
		WITH female	1, 12	12.813	0.001	0.175
		WITH latency	1, 10	7.315	0.009	-0.328
		WITH female AND latency FOR female	1, 10	3.777	0.058	0.125
		WITH female AND latency FOR latency	1, 10	6.690	0.013	-0.323
		Female	7, 12	1.574	0.216	
		WITH male	1, 12	12.813	0.001	0.719
		WITH latency	1,10	0.000	0.983	-0.006
		WITH male AND latency FOR male	1, 10	3.777	0.058	0.632
		WITH male AND latency FOR latency	1, 10	0.438	0.511	0.199

838 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

839 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p	beta
Hybrid	backcrosses	Copulation	7, 16	1.003	0.459	
		WITH male	1, 16	6.133	0.015	-0.231
		WITH female	1, 16	0.016	0.899	0.008
		WITH male AND female FOR male	1, 16	15.139	<0.001	-0.482
		WITH male AND female FOR female	1, 16	2.302	0.133	0.092
		WITH male AND female AND latency FOR male	1, 16	46.178	<0.001	-0.883
		WITH male AND female AND latency FOR female	1, 16	4.275	0.045	-0.119
		WITH male AND female AND latency FOR latency	1, 16	3.964	0.053	0.230
		Latency	7, 10	1.600	0.223	
		WITH male	1, 10	7.315	0.009	-0.396
		WITH female	1, 10	0.000	0.983	-0.002
		WITH male AND female FOR male	1, 10	6.690	0.013	-0.408
		WITH male AND female FOR female	1, 10	0.438	0.511	0.0505
		Duration	7, 10	1.521	0.243	

840 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

841 **Table A2 ff.**

Generation and Pairing	Comparison	Trait	df	F	p
Biogeographic pairings	Heterospecific	Male	4, 6	1.232	0.389
		Female	4, 6	5.779	0.030
		Copulation	4, 6	2.503	0.151
		Latency	4, 4	5.844	0.001
		Duration	4, 6	0.921	0.502
	F ₁	Male	4, 6	1.129	0.421
		Female	4, 6	4.509	0.035
		Copulation	4, 6	0.468	0.759
		Latency	4, 6	0.271	0.887
		Duration	4, 6	4.025	0.055
Inheritance one-way ANOVA	F1 vs. Parental	Male	3, 12	1.881	0.183
		Female	3, 12	23.312	<0.001
		Copulation	3, 12	3.766	0.038
		Latency	3, 12	1.585	0.237
		Duration	3, 12	2.938	0.069

842 df = degree of freedom, F = F-statistics, p = p-value, β = covariate coefficient

843

844