Title: Non-parallel morphological divergence following colonization of a new host plant

Authors: Kalle J. Nilsson[1]*, Jesús Ortega[2], Magne Friberg[1] & Anna Runemark[1]

* Corresponding author

1. Department of Biology, Lund University, Sölvegatan 37, 223 62 Lund, Sweden
2. Institute of Evolutionary Biology, Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

Running title: Non-parallel morphological divergence following colonization of a new host

Keywords: Parallelism, host plant adaptation, secondary sympatry, adaptation to co-existence

Word count: 4993

Number of tables and figures: 1 table and 4 figures

Number of items in appendix: 17

Abstract

Divergent ecological selection may diversify conspecific populations evolving into different niches, and may lead to speciation if gene flow is ceased, e.g, due to reinforcement leading to character displacement. Adaptation to the same niche is expected to be parallel. Whether selection against maladaptive hybridization in secondary sympatry also results in parallel divergence in traits that are not directly related to the ecological niches remains an empirical challenge, although such character displacement can be decisive for if ecotypes develop into new species. Here, we use a host shift in the phytophagous peacock fly *Tephritis conura*, with both host races represented in two geographically separate areas East and West of the Baltic Sea to investigate convergence in morphological adaptations. We asked i) if there are consistent morphological adaptations to a host plant shift and ii) if the response to secondary sympatry with the alternate host race is parallel across contact zones. We found surprisingly low and variable, albeit significant, divergence between host races. Only one trait, the length of the female ovipositor, which serves an important function in the interaction with the hosts, was consistently different. Instead, co-existence with the other host race significantly affected the degree of morphological divergence, but the divergence was largely driven by different traits in different contact zones. Thus, local stochastic fixation or reinforcement could generate trait divergence, and additional evidence is needed to conclude whether divergence is locally adaptive.

Introduction

Adaptation to novel niches through ecological selection has been identified as a major driver of speciation (Schluter 2008; Schluter 2009; Nosil 2012). Classic adaptive radiations including the Darwin Finches (Weiner 1994; Loo et al 2019), the Hawaiian tarweeds and silverswords (Baldwin et al 2003) and the recurrent evolution of limnetic-benthic stickleback pairs (Schluter 1993; Bay et al 2017) are driven by adaptations to novel diets and habitats. Speciation driven by expansion of novel dietary niches has potential to be rapid, like in *Rhagoletis* flies, where populations of hawthorn-feeding flies have adapted to use the introduced apple (*Malus domesticus*) as a host during the last 150 years (Bush 1969; Feder et al 1988; Linn et al 2004; Meyers et al 2020). It remains an empirical challenge to resolve to which extent morphological adaptations are parallel and predictable under parallel selection regimes (Bolnick et al 2018). Few studies have explicitly compared parallelism in multiple traits in such scenarios, but a recent study of Bahamas mosquitofish (*Gambusia hubbsi*) suggests that only a few traits are highly predictable across parallel predation environments (Langerhans 2018).

Parallelism in response to co-existence with congeners provide a further empirical challenge, as evolutionary divergence may be affected by the local presence or absence of the other incipient species (Amarasekare 2003; Calabrese and Pfennig 2020). Reducing gene flow to populations adapted to other niches is crucial for ecological specialization to result in diversification, as ecological divergence does not necessarily lead to speciation (Taylor et al 2006; Bolnick 2011; Lackey and Boughman 2017). Divergence and specialization may be reversed in the absence of reproductive isolation (Taylor et al 2006; Lackey and Boughman 2017). Hence, if reproductive isolation between the incipient species is incomplete, secondary contact may break up important ecological adaptations. This could be particularly evident

when different populations of the diverging species are inhabiting discrete niches, like in the case of insect host races. Close congeners inhabiting different niches may be poorly adapted to the alternative environment (Rundle and Nosil 2005; Nilsson et al 2017; Cronemberger et al 2020; Martin et al 2020), which may lead to reinforcement (Servedio and Noor 2003) and character displacement with stronger differences in traits involved in mate choice (Hinojosa et al 2020). While parallel divergence is expected for ecologically selected traits (Fig. 1a), either parallel- or nonparallel character displacement (Fig. 1b,c) or reduced divergence due to introgression (Fig. 1d) could be expected for traits that are not strongly coupled with host plant use.

To understand whether selection to reduce maladaptive mating in the presence of conspecifics results in parallel morphological divergence to the same extent as ecological adaptations to a novel niche, it is necessary to compare patterns of divergence in allopatry and sympatry across geographic regions. Such studies would test the hypothesis that morphological responses to sympatry can be more arbitrary than adaptations of ecological traits, which are expected to diverge in parallel across populations of similar niches (Fig. 1). In particular, studies asking how co-existence with congeners affects trait distributions, e.g. through parallel- or non-parallel character displacement (Fig. 1), may provide insight into the nature of the selection pressures exerted by co-existence of close congeners. Here, we address how morphological traits evolve and diversify in allopatric and sympatric areas using two host races of the fly *Tephritis conura.* This species has recently undergone a host shift resulting in host races specializing on utilizing different *Cirsium* thistle species as larval food plants (Diegisser et al 2006a; Diegisser et al 2006b; Diegisser et al 2007; Diegisser et al 2008).

80 Macroevolutionary analyses reveal that host plant driven speciation, as in the case of *T.*
81 *conura,* is one of the main factors explaining the tremendous diversity of phytophagous
82 insects as many speciation events can be attributed to host plant shifts (Berlocher and Feder
83 2002; Dres and Mallet 2002; Nylin et al 2014). Moreover, diversification rates are elevated in
84 herbivorous insects compared to their non-herbivorous relatives (Mitter et al 1988; Farrell
85 1998; Wiens et al 2015). For an insect specialist like *T. conura,* the host plant provides a
86 discrete environment imposing a multidimensional selection pressure. Hence, insect host plant
87 adaptations are predicted to affect multiple traits involved in e.g. phenological matching,
88 female host preference and larval performance (Matsubayashi et al 2010). The putatively
89 strong selection pressures involved in host plant adaptation provide a strong prediction of
90 parallelism in host-use related traits of phytophagous insects at early stages of the speciation
91 process (Nosil et al 2002; Meyers et al 2020). However, the prediction is less straightforward
92 for responses to sympatry, because the traits involved in putative reinforcement are not
93 necessarily associated with host use.
94
95 *Tephritis conura* provides an excellent system for studying parallelism in divergence in
96 response to ecological adaptation and co-existence with congeners, as it enables comparisons
97 of trait variation across replicated sympatric and allopatric settings. The two recently
98 established host races inhabit two geographically isolated sympatric zones with adjacent
99 allopatric populations, one East and one West of the Baltic Sea (Fig. 2a). *Tephritis conura*
100 females of the two host races are ovipositing into buds of either *C. heterophyllum* (the
101 ancestral host; Fig. 2b) or *C. oleraceum* (the novel host; Fig. 2c), and larvae show host
102 specific performance and survival (Diegisser et al 2008). Hence, the system enables
103 investigating response to sympatry across two geographically separated contact zones.
104 Specifically, we test the hypotheses (i) that there are consistent morphological responses to a

105 host plant shift across the Eastern and Western areas and (ii) that responses to sympatry are
106 parallel in the two contact zones. Given the consistent genetic differentiation between host
107 races both in allopatry and sympatry (Diegisser et al 2006a; Ortega et al *in prep*) we predict
108 the host races to be morphologically divergent, and this divergence to be consistent across
109 geographical settings. As larval survival is strongly reduced in the alternate host (Diegisser et
110 al 2008), we predict hybridization to be maladaptive, potentially resulting in reinforcement
111 and character displacement between the host specialists in sympatry.
112
113 **Methods**
114 **Study species and sampling**
115 The dipteran *T. conura* infest several species of the thistle genus *Cirsium* (Asteraceae)
116 (Romstock-Volkl 1997). Adult *T. conura* oviposit into thistle buds during early summer,
117 wherein the adolescent flies remain during larval development and pupation. *Tephritis conura*
118 infesting *Cirsium heterophyllum*, the melancholy thistle, have recently colonized and adapted
119 to *Cirsium oleraceum*, the cabbage thistle. Haplotype analyses suggest that a peripatric host
120 shift took place during the last ice age in the Alps (Diegisser et al 2006b). The two host races
121 are largely reproductively isolated, but there is evidence of some small amounts of gene flow
122 (Diegisser et al 2006b). The flies infesting *C. oleraceum* have adapted ecologically to the
123 smaller bud sizes (Diegisser et al 2007), and have a significantly shorter ovipositor to body
124 size ratio. Another variable character is wing pigmentation. Wing patterns in Tephritid flies
125 have been suggested to be under sexual selection, as males perform dances with their wings to
126 attract females (Sivinski and Pereira 2005). Tephritid males attempting to initiate copulation
127 situate themselves in front of female flies and posture with their wings (Sivinski 2000; but see
128 Briceno and Eberhard 2017). We therefore have reason to assume that ecological selection
129 pressures are more important for size traits in general, and for ovipositor length in particular,

130 while wing traits are more likely shaped by sexual selection. For simplicity, we refer to flies
131 infecting *C. heterophyllum* as CH-flies, while the flies infecting *C. oleraceum* will be denoted
132 CO-flies.
133
134 We used a parallel sampling design to examine phenotypic adaptation to a novel host plant
135 and effects of co-existence between host races, by sampling each host race both in sympatry
136 and allopatry on each side of the Baltic (Fig. 2a). We collected thistle buds infested by *T.*
137 *conura* larvae/pupae and allowed the adults to eclose in a common environment. CO-fly
138 larvae were sampled in Germany and Lithuania (allopatric areas), and both host races were
139 collected in sympatric areas in southern Sweden and Estonia. Allopatric CH-fly larvae were
140 sampled in central-Sweden and Finland (Fig. 2a, Table S1). All sampling took place during
141 June and July 2018. The sampling scheme enables examining to what extent patterns of
142 phenotypic divergence are explained by host plant adaptations, by co-existence with the other
143 host race, and if these patterns differ between the two transects. Typically, *C. heterophyllum*
144 and *C. oleraceum* do not grow in the same microhabitat. Thus, the sympatric and allopatric
145 definitions here refer to the presence of one or both thistle species in a region (Fig. 2a).
146
147 **Morphological measurements**
148 *Tephritis conura* adults eclosed from field-collected thistle buds in a common lab
149 environment, (see Supplementary material S1). One male and one female per bud were
150 euthanized by freezing a few days after emergence and subsequently included in the
151 morphological analysis. For each individual, we took magnified photographs using a
152 Celestron 44308 USB microscope. We photographed a lateral image of the fly body after
153 removal of the wings and a dorsal image of the right wing on a transparent background to
154 allow better visibility of the wing veins. Body length and ovipositor length were measured

digitally from lateral photographs (Fig. S1). We placed 14 landmarks, adapted from Pieterse et al. (2017), digitally on the dorsal wings (Fig. 2d,S2) for geometric morphometrics (Zelditch 2004). We added a landmark; number 15, to reflect the high variance in the proximal area on the wing. Digitization was performed in TPSDig2 v2.31(Rohlf 2017) and we used TPSUtil v1.76 (Rohlf 2018) for file handling. Wing melanisation was measured with an automated script without any user queries developed in MATLAB (Matlab 2017). As the wings do not display any chromaticity, analysis is based on the red spectral band only. The script extracted the intensity of the red spectral band for each pixel, and performed white calibration by division with the statistical mode corresponding to the white background of the images. Subsequently, images were inverted to represent absorbance rather than reflectance, so the melanised wing would extend from the white background (set to zero). The script identified the wing and separated melanised areas from non-melanised areas, and the size of these areas were divided to estimate the fraction of the wing that was melanised.

Statistical analysis

We used PAST3 v3.20 (Hammer et al 2001) to apply a Procrustes fit to the landmark data to align and scale the wings (Fig. 2d). To produce relative warps (i.e. principal components of shape) to compare shape between groups, a wing-shape principal component analysis (PCA) was performed with the Procrustes fitted data using PAST3 v3.20 (Hammer et al 2001) (Fig. S3). Based on the variance explained by the eigenvalues (Fig. S4) and the broken stick criterion (Jackson 1993), six relative warps, principal components of shape, which jointly explain 68% of the wing shape variance were identified (Fig. S5). These relative warps were included in subsequent analyses of phenotypic divergence to represent wing shapes. All subsequent statistical analysis were performed in the statistical software R (R Core Team 2019).

We quantified five morphological traits (body length, ovipositor length, wing length, wing width and melanisation ratio) in addition to wing shape (represented by relative warps produced from landmark analysis) for 583 flies. As an exploratory analysis to investigate whether host race was the major factor explaining variation in fly morphology, we performed a full PCA on the variables fly body length (mm), wing length (mm), wing width (mm), wing melanisation ratio (%) and relative warps 1-6 reflecting wing shape, excluding ovipositor length to be able to include both sexes. We identified four significant dimensions of variation from the full PCA analysis using the broken stick criterion (Jackson 1993) (Fig. S6). Collectively, these full PCA-axes explained 78% of the morphological variance in the dataset.

To formally test if the two host races were significantly differentiated, we applied a multivariate analysis of variance (MANOVA), with all variables measured included as response variables (body length, ovipositor length, wing width, wind length, wing melanisation and PC1-6 of wing shapes). To further test if the patterns of morphological adaptation were parallel in the Eastern and the Western transects, and explicitly address if co-existence affected host plant races in the same way in these replicates, we performed a full MANOVA with host race, co-existence and geographical setting and their 3-way and 2-way interactions as factors. The MANOVAs were performed separately on females and males as this enabled including the biologically important trait ovipositor length (Diegisser et al 2007) in analyses of females. This division of sexes reduced the multicollinearity of explanatory factors to below recommended values (Hair, 2010).

To further investigate parallelism in differentiation of the host race pairs, we applied separate linear discriminant function analyses (LDAs) on the data from the Eastern and Western

205 transects. We used host races and co-existence with the other host race as factors in the
206 models. This analysis was performed on males and females separately. To test if the patterns
207 of divergence differed significantly between transects, we performed each LDA 10,000 times
208 using the bootstrap R package 'boot' (Canty and Ripley 2020) and used the confidence
209 intervals to assess if the loadings differed between analyses. In addition, we assessed the
210 proportions of divergence that is shared among host races and unique among the populations,
211 respectively, in a nested MANOVA using size and shape variables against host race and
212 transect following Langerhans & DeWitt (2004).
213
214 **Results**
215 We found no strong evidence for separation between host races along the two major axes of
216 divergence when including both sexes (Fig. 2e). CO-flies had, on average, lower values of
217 PC1 compared to CH-flies, reflecting smaller body and wing size, as predominantly size
218 variables load positively on PC1 (GLM: $F_{1, 581} = 5.189$, $p = 0.023$; Fig. 2e; Table S2). The
219 significant host race differences also hold true in a more complex model, including co-
220 existence and geographic origin (see below; Table 1). However, the difference between host
221 races was small compared with the variation among populations within host races. There was
222 no separation between host races along the second PC axis, with higher values representing
223 higher wing melanisation and lower representation of wings that are larger than average in the
224 distal and proximal areas of the wing, while being smaller in the posterior area, see relative
225 warp 5 in Fig. S5.
226
227 Intriguingly, host plant and co-existence affected female morphology differently East and
228 West of the Baltic Sea, as illustrated by a significant 3-way interaction (Pillai's trace = 0.117,
229 $F_{11, 277} = 3.22$, $p < 0.001$; Table 1). All main effects and 2-way interactions were also

230 significant in the full model (Table 1). These patterns hold true also for males (Pillai's trace =
231 0.092, $F_{10, 271}$ = 2.74, p = 0.003; Table 1). Hence, depending on geography, host race and co-
232 existence affected fly morphology differently. Co-existence with the other host race decreased
233 morphological divergence in both females and males (Pillai's trace = 0.295, $F_{11, 277}$ = 10.15, p
234 <0.001 and Pillai's trace = 0.194, $F_{10, 271}$ = 6.54, p <0.001 respectively), and flies from the
235 two transects differed significantly in morphology for both sexes (Pillai's trace = 0.374, $F_{10,}$
236 $_{271}$ = 14.52, p <0.001 for females and Pillai's trace = 0.393, $F_{10, 271}$ = 17.51, p <0.001 for
237 males).
238
239 Interestingly, host race differences in females depend on co-existence, with CO-fly females
240 becoming more similar in sympatry compared to CH-fly females (Pillai's trace = 0.218, $F_{11,}$
241 $_{277}$ = 6.79, p <0.001; Tables 1 and S3; Fig. 3 and S7). In contrast to females, co-existence
242 affected CO-fly males more strongly, with CO-fly males becoming more similar to CH-fly
243 males in sympatry whereas CH-fly males were more similar across all populations (Pillai's
244 trace = 0.179, $F_{10, 271}$ = 5.92, p <0.001; Tables 1 and S3; Fig. S8-S11). The differences
245 between host races varied between transects in females, with a stronger host race divergence
246 in the Western than the Eastern transect (Pillai's trace = 0.18, $F_{11, 277}$ = 6.79, p <0.001; Tables
247 1 and S3; Fig. 3 and S7; Tables 1 and S3; Fig. 3 and S7). This pattern holds true also for
248 males (Pillai's trace = 0.16, $F_{10, 271}$ = 5.12, p <0.001; Tables 1 and S3; Fig. S8-S11). Finally,
249 the differences between allopatric and sympatric populations are stronger in Western flies
250 compared to Eastern both in females (Pillai's trace = 0.163, $F_{11, 277}$ = 4.74, p <0.001; Tables 1
251 and S3; Fig. 3 and S7) and males (Pillai's trace = 0.131, $F_{10, 271}$ = 4.1, p <0.001; Tables 1 and
252 S3; Fig. S8-S11).
253

To assess how much of the female divergence was unique and shared between host races we also estimated Wilk's partial η^2. While a high share of the partial variance was explained by shared divergence between host races ($F_{11.273}$ = 4.96, 30.6%) and transect specific patterns of host race divergence ($F_{11.273}$ = 3.81, 25.3%), divergence between transects explained the highest percentage of partial variance ($F_{11.273}$ = 6.85, 37.8% (Table S4).

As host race affected morphology differently depending on geography, we further tested if the major axis of divergence separated host races in both transects, and if the same traits separated groups using a Linear Discriminant Analysis (LDA). This analytical approach revealed that the importance of host race for population separation differed between transects. In the Western LDA, host races separated along the first discriminant axis whereas the sympatric and allopatric populations separated along the second discriminant axis (Fig. 4). In the Eastern LDA, the first discriminant instead divided the two CO-fly populations and the second discriminant axis divided the two CH-fly host race populations (Fig. 4). These analyses also revealed that the host races from transects East and West of the Baltic had diverged in different sets of characters as bootstrap loadings from the two LDAs show that different characters loaded on the first discriminant axes (Table S5). For the Eastern transect, body length and wing warp 6 had positive loadings, and wing size, ovipositor length and wing warp 1 had negative loadings on LD1, whereas for the Western transect, LD1 had positive loadings for wing width and shape. In the Eastern transect, wing shape, body length and wing width had negative loadings, but wing length positive loadings on LD2, while for the Western transect body length, ovipositor length and wing lengths loaded positively on LD2. Wing warp 5 loaded positively on LD1 for both transects, but the loading was substantially stronger for Western populations.

Discussion

Contrary to previous studies that found clear differences between the *T. conura* host races (Diegisser et al 2007) we found low and variable morphological differentiation. The multivariate differentiation between host races was driven by subtle differences across many traits, and the traits that were divergent between host races differed in the two transects. Host races separated slightly but significantly along the main axis of variation, reflecting mainly ovipositor length differences, with CH-fly ovipositors being slightly longer than CO-fly ovipositors, but with unexpected substantial variation among populations. Previous work on the *T. conura* host races has reported consistent divergence for loci inferred to be involved in host plant adaptation (*Peptidase D* and *Hexokinase*; see (Diegisser et al 2006b), and recently obtained whole genome data (Ortega et al *in prep*) support the presence of discrete genetic clusters for each host race. Moreover, the flies also have poor performance on the alternate host plant (Diegisser et al 2008). In light of this, the utterly moderate and variable host race divergence in morphology was unexpected. However, our findings are in line with the observation that parallelism in fitness is typically higher than parallelism in both phenotypic divergence and genetic divergence (Bolnick et al 2018). Patterns of parallel divergence only in traits under very strong ecological selection is consistent with the findings of parallelism in response to different predation regimes in Bahamas Mosquitofish (Langerhans 2018), where only a few traits show highly predictable patterns of diversification. Hence, at early stages of diversification driven by ecological adaptation, parallelism may be high only for traits that are strongly coupled to the ecological factor that adaptively diversify the incipient species.

We find that two out of five female traits, ovipositor length and body length, match a parallel divergence scenario, and body length only in the Western transect (Fig. 1a). Hence, the degree of parallelism among host races in morphological divergence was lower than expected.

304 Ovipositor length was significantly diverged between host races, but the strength of
305 divergence differed between sympatric and allopatric populations as well as between the
306 Eastern and Western populations. In Western populations, the sympatric CH-fly population
307 has intermediate ovipositor lengths, whereas CO-flies and Eastern CH-flies show parallel
308 ecological divergence between sympatry and allopatry, corresponding to the expectations
309 under parallel ecological selection (Fig. 1a). The intermediate measurements for traits found
310 in the Western CH-flies could suggest selection for shorter ovipositors in sympatry, or reflect
311 introgression. We find host race divergence in body size, with clear host race differences in
312 body length in the Western transect (corresponding to Fig. 1a), while Eastern flies have a
313 shuffled distribution of body sizes. Size typically varies with temperature in insects (Atkinson
314 1994; but see Shelomi 2012). While the parallel sampling design would partly correct for
315 temperature effects, temperature could still differ between the Eastern and Western transects.
316
317 When we jointly investigated effects of co-existence, geographic origin and their interactions
318 on host race divergence, we found that both co-existence with the other host race and whether
319 the population originate from East or West of the Baltic Sea affected morphology (Fig. 3).
320 Hence, the low host race divergence may, to a high extent, be explained by interacting effects
321 of co-existence with the other host race, with non-parallel patterns of divergence in the two
322 transects, which could indicate a mutation order scenario (Fig. 1c) (Mendelson et al 2014). If
323 reinforcement due to maladaptive hybridization would have been a prominent force acting on
324 the *T. conura* host races, we would expect traits to be more divergent between host races in
325 sympatry compared to allopatry, i.e. for character displacement to arise (Comeault et al 2016;
326 Calabrese and Pfennig 2020; Kyogoku and Wheatcroft 2020; Fig. 1b,c). This was generally
327 not the case. Instead, the most common pattern was that traits in sympatric populations are
328 more similar between host races than traits in allopatry. Contrary to our expectations, based

329 on the fact that *Tephritid* flies perform a mating ritual that includes elaborate wing
330 movements from both sexes during courtship (Sivinski 2000; but see Briceno and Eberhard
331 2017), this pattern was strongest for wing morphology traits. This is the opposite of what
332 would be predicted under a reinforcement scenario but consistent with introgression (Fig. 1d),
333 as found by Ortega et al. [*in prep*.], or effects of a shared environment.
334
335 Wing size was most strongly affected by co-existence. Interestingly, wing length was overall
336 significantly shorter in sympatry than in allopatry, consistent on both sides of the Baltic Sea.
337 Disproportionally shorter wing length in relation to body size could be consistent with
338 selection for shorter dispersal distances (Claramunt et al 2012), potentially to avoid dispersal
339 to the alternate host plant which could lead to maladaptive introgression. We also found
340 patterns consistent with character displacement, as sympatric flies have similarly narrower
341 wings than their respective allopatric counterparts in the Western transect. In contrast, Eastern
342 sympatric CH-flies have broader and sympatric CO-flies narrower wings. These observations
343 are consistent with a mutation order scenario, where the difference may be important but the
344 specific trait or direction of the difference is arbitrary (Mendelson et al 2014). Some degree of
345 sexual selection may further differentiate populations under a mutation order scenario
346 (Rundle and Rowe 2018), underlining the need to investigate if there is sexual selection at
347 work in this system and disentangling how it would operate.
348
349 Contrary to studies of parallelism in sexually selected traits in ecotypes in e.g. sticklebacks
350 (Boughman et al 2005), we find mixed evidence for parallelism. While shared host race
351 divergence constituted the main share of variation, and host races are separated by the main
352 discriminant axis in the Western transect, host race was a less important predictor of
353 divergence in the Eastern transect where the two CO-fly populations instead separate along

354 the first discriminant axis. The traits that differ between host races also differs between our
355 two geographic replicates. The differences in patterns between the transects East and West of
356 the Baltic Sea could have several additional or alternative explanations related to
357 demographic history, population size and the extent to which the host plant races co-exist
358 locally. Possibly, one contact zone may be older than the other and populations in older
359 sympatry would have had more time for character displacement to develop. Alternatively, if
360 the Eastern transect has a higher proportion suitable thistle habitat, this could have increased
361 both within- and between host race connectivity and potentially gene flow (Servedio and
362 Noor 2003). Genetic data and detailed analyses of introgression should be used to resolve
363 whether selection against hybridization could be expected.
364
365 Morphological differentiation does not always strongly reflect even crucial ecological
366 adaptations. For instance, cultural evolution contributes to reproductive isolation in Cassia
367 crossbills, *Loxia* sinesciuris (Porter and Benkman 2019) and *Rhagoletis pomonella* have
368 adapted their phenologies to host fruits ripening at different times of the year (Filchak et al
369 2000). Another potential explanation to the low consistency of host race divergence may be
370 the traits included in this analysis. Females were more diverged than males likely as
371 ovipositor length was differentiated, consistent with previous findings (Diegisser et al 2007).
372 These findings are similar to those of Jourdan et al. (2016) where divergence in female
373 mosquitofishes (*Gambusia*) divergence was more parallel than male divergence. Potentially,
374 the other traits measured are not important enough for host plant adaptation to result in
375 strongly parallel divergence.
376
377 Traits that have been shown to differ strongly between host races include female ovipositor
378 length (Diegisser et al 2007) and the larval ability to survive on the different host plant

species (Diegisser et al 2008). A performance experiment showed low viability of *T. conura* larvae adapted to *C. heterophyllum* when reared on *C. oleraceum* (Diegisser et al 2008). Thus, the larval ability to process plant tissue is likely under strong selection, and expected to show similar patterns across populations. Furthermore, host plant preference may have a potential to act as a magic trait (Gavrilets 2004; Thibert-Plante and Gavrilets 2013) in *T. conura*, separating the habitats of the populations and simultaneously providing reproductive isolation as these flies mate on their host plant (Diegisser et al 2007). Finally, we cannot rule out that other, unmeasured traits are important for sexual selection, as pheromones have been suggested to play a role in tephritid mate choice (Roriz et al 2019), as well as overexpression of antioxidants, which have been shown to increase male performance under certain conditions (Teets et al 2019). Regardless of the exact selection pressures acting on our set of study traits, our findings are important, because they show a context dependence for host race adaptation. Parallelism was found only in ecologically strongly selected traits, whereas we found non-parallel changes, some consistent with character displacement for other traits. These insights should guide the design and interpretation when studying ecologically driven divergence.

In conclusion, our work suggests that morphological responses to niche shifts can be highly context dependent. Co-existence with closely related congeners and demographic origin may affect easily measured morphological characters, potentially masking underlying parallelism in traits important for adaptation to specific niches. Moreover, we find intriguing patterns of non-parallel divergence to co-existence in the two geographic replicas, suggesting that mutation order dependent divergence potentially could lead to different solutions to avoid introgression in independent contact zones.

Acknowledgements

We thank Mikkel Brydegaard for help with automatizing wing color analysis, Jes Johannesen for helpful advice on *Tephritis conura* ecology, Øystein Opedal for helpful statistical discussions, Jodie Lilley, Emma Kärrnäs and Mathilde Schnuriger for help during field- and lab work, and the members of the Lund University research group on the Evolutionary Ecology of Plant-Insect Interactions for valuable discussions on earlier drafts of this manuscript. This study was financed by a Wenner-Gren Fellowship, a Crafoord grant and a Swedish Research Council grant to AR.

Declarations

Funding

This study was financed by a Wenner-Gren Fellowship, a Crafoord grant and a Swedish Research Council grant to AR.

Conflicts of interest

The authors declare no conflicts of interest.

Ethics approval

Not applicable

Consent to participate

All co-authors consent to participation.

Consent for publication

All co-authors consent to publication.

Availability if data and material

All data will be deposited in dryad (upon acceptance of the MS).

Code availability

The code used to analyse data is available at github.com/kalnil/tephritis.

Author contribution

A.R conceived and designed the study. KJ.N., J.O. and A.R. performed the field work. KJ.N. reared the flies, quantified the morphological traits and performed the statistical analyses, with advice from A.R. and M.F. KJ.N. wrote a draft of the manuscript. A.R. and M.F. helped writing the manuscript and all co-authors commented on and approved the final version of the manuscript.

437 **References**

438 Amarasekare P (2003) Competitive coexistence in spatially structured environments: A
439 synthesis. Ecology Letters 6:1109-1122
440 Atkinson D (1994) Temperature and organism size - A biological law for ectotherms. In:
441 Begon M & Fitter AH (eds) Advances in Ecological Research, vol 25. Academic Press
442 Ltd-Elsevier Science Ltd, London, pp1-58
443 Baldwin BG, Carlquist S & Carr GD 2003 Tarweeds & silverswords: evolution of the
444 *Madiinae (Asteraceae)*. Missouri Botanical Garden Press, St. Louis, Mo.
445 Bay RA, Arnegard ME, Conte GL, Best J, Bedford NL, McCann SR, Dubin ME, Chan YF,
446 Jones FC, Kingsley DM, Schluter D & Peichel CL (2017) Genetic coupling of female
447 mate choice with polygenic ecological divergence facilitates stickleback speciation.
448 Current Biology 27:3344-3349
449 Berlocher SH & Feder JL (2002) Sympatric speciation in phytophagous insects: Moving
450 beyond controversy? Annual Review of Entomology 47:773-815
451 Bolnick DI (2011) Sympatric speciation in threespine stickleback: Why not? International
452 Journal of Ecology 2011:e942847
453 Bolnick DI, Barrett RDH, Oke KB, Rennison DJ & Stuart YE (2018) (Non)Parallel evolution.
454 Annual Review of Ecology, Evolution, and Systematics 49:303-330
455 Boughman JW, Rundle HD & Schluter D (2005) Parallel evolution of sexual isolation in
456 sticklebacks. Evolution 59:361-373
457 Briceno RD & Eberhard WG (2017) Copulatory dialogues between male and female tsetse
458 flies (*Diptera: Muscidae: Glossina pallidipes*). Journal of Insect Behavior 30:394-408
459 Bush GL (1969) Sympatric host race formation and speciation in frugivorious flies of genus
460 *Rhagoletis* (*Diptera, Tephritidae*). Evolution 23:237-251

461 Calabrese GM & Pfennig KS (2020) Reinforcement and the proliferation of species. Journal
462 of Heredity 111:138-146
463 Canty A & Ripley BD (2020) boot: Bootstrap R (S-Plus) functions. R package version 1.3-25.
464 Claramunt S, Derryberry EP, Remsen JV, Jr. & Brumfield RT (2012) High dispersal ability
465 inhibits speciation in a continental radiation of passerine birds. Proceedings of the
466 Royal Society B-Biological Sciences 279:1567-1574
467 Comeault AA, Venkat A & Matute DR (2016) Correlated evolution of male and female
468 reproductive traits drive a cascading effect of reinforcement in *Drosophila yakuba*.
469 Proceedings of the Royal Society B-Biological Sciences 283:20160730
470 Cronemberger AA, Aleixo A, Mikkelsen EK & Weir JT (2020) Postzygotic isolation drives
471 genomic speciation between highly cryptic *Hypocnemis* antbirds from Amazonia.
472 Evolution 74:2512-2525
473 Diegisser T, Johannesen J & Seitz A (2006a) The role of geographic setting on the
474 diversification process among *Tephritis conura (Tephritidae)* host races. Heredity
475 96:410-418
476 Diegisser T, Johannesen J & Seitz A (2008) Performance of host-races of the fruit fly,
477 *Tephritis conura* on a derived host plant, the cabbage thistle *Cirsium oleraceum*:
478 Implications for the original host shift. Journal of Insect Science 8:1-6
479 Diegisser T, Seitz A & Johannesen J (2006b) Phylogeographic patterns of host-race evolution
480 in *Tephritis conura (Diptera: Tephritidae)*. Molecular Ecology 15:681-694
481 Diegisser T, Seitz A & Johannesen J (2007) Morphological adaptation in host races of
482 *Tephritis conura*. Entomologia Experimentalis Et Applicata 122:155-164
483 Dres M & Mallet J (2002) Host races in plant-feeding insects and their importance in
484 sympatric speciation. Philosophical Transactions of the Royal Society B-Biological
485 Sciences 357:471-492

Farrell BD (1998) "Inordinate fondness" explained: Why are there so many beetles? Science 281:555-559

Feder JL, Chilcote CA & Bush GL (1988) Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. Nature 336:61-64

Filchak KE, Roethele JB & Feder JL (2000) Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. Nature 407:739-742

Gavrilets S 2004 Fitness landscapes and the origin of species. Princeton University Press, Princeton, N.J.

Hammer Ø, Harper DAT & P. D. R (2001) PAST: Paleontological statistics software package for education and data nalysis. Palaeontologica Electronica 4:9

Hinojosa JC, Koubinova D, Dinca V, Hernandez-Roldan J, Munguira ML, Garcia-Barros E, Vila M, Alvarez N, Mutanen M & Vila R (2020) Rapid colour shift by reproductive character displacement in *Cupido* butterflies. Molecular Ecology 29:4942-4955

Jackson DA (1993) Stopping rules in principal components-analysis - A comparison of heuristic and statistical approaches. Ecology 74:2204-2214

Jourdan J, Krause ST, Lazar VM, Zimmer C, Sommer-Trembo C, Arias-Rodriguez L, Klaus S, Riesch R & Plath M (2016) Shared and unique patterns of phenotypic diversification along a stream gradient in two congeneric species. Scientific Reports 6:38971

Kyogoku D & Wheatcroft D (2020) Heterospecific mating interactions as an interface between ecology and evolution. Journal of Evolutionary Biology 33:1330-1344

Lackey ACR & Boughman JW (2017) Evolution of reproductive isolation in stickleback fish. Evolution 71:357-372

Langerhans RB (2018) Predictability and parallelism of multitrait adaptation. Journal of Heredity 109:59-70

511 Langerhans RB & DeWitt TJ (2004) Shared and unique features of evolutionary
512 diversification. American Naturalist 164:335-349
513 Linn CE, Darnbroski HR, Feder JL, Berlocher SH, Nojima S & Roelofs WL (2004)
514 Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: Reduced
515 response of hybrids to parental host-fruit odors. Proceedings of the National Academy
516 of Sciences of the United States of America 101:17753-17758
517 Loo WT, Garcia-Loor J, Dudaniec RY, Kleindorfer S & Cavanaugh CM (2019) Host
518 phylogeny, diet, and habitat differentiate the gut microbiomes of Darwin's finches on
519 Santa Cruz Island. Scientific Reports 9:18781
520 Martin BT, Douglas MR, Chafin TK, Jr JSP, Birkhead RD, Phillips CA & Douglas ME
521 (2020) Contrasting signatures of introgression in North American box turtle
522 (*Terrapene* spp.) contact zones. Molecular Ecology 29:4186-4202
523 Matlab (2017) 9.3.0.713579 (R2017b).
524 Matsubayashi KW, Ohshima I & Nosil P (2010) Ecological speciation in phytophagous
525 insects. Entomologia Experimentalis Et Applicata 134:1-27
526 Mendelson TC, Martin MD & Flaxman SM (2014) Mutation-order divergence by sexual
527 selection: Diversification of sexual signals in similar environments as a first step in
528 speciation. Ecology Letters 17:1053-1066
529 Meyers PJ, Doellman MM, Ragland GJ, Hood GR, Egan SP, Powell THQ, Nosil P & Feder
530 JL (2020) Can the genomics of ecological speciation be predicted across the
531 divergence continuum from host races to species? A case study in *Rhagoletis*.
532 Philosophical Transactions of the Royal Society of London B-Biological Sciences
533 375:20190534
534 Mitter C, Farrell B & Wiegmann B (1988) The phylogenetic study of adaptive zones - Has
535 phytophagy promoted insect diversification? American Naturalist 132:107-128

536 Nilsson PA, Hulthen K, Chapman B, Hansson LA, Brodersen J, Baktoft H, Vinterstare J,
537 Bronmark C & Skov C (2017) Species integrity enhanced by a predation cost to
538 hybrids in the wild. Biology Letters 13:20170208
539 Nosil P 2012 Ecological speciation. Oxford University Press, Oxford
540 Nosil P, Crespi BJ & Sandoval CP (2002) Host-plant adaptation drives the parallel evolution
541 of reproductive isolation. Nature 417:440-443
542 Nylin S, Slove J & Janz N (2014) Host plant utilization, host range oscillations and
543 diversification in nymphalid butterflies: A phylogenetic investigation. Evolution
544 68:105-124
545 Ortega J (*in prep*) Genomic landscape of *Tephritis conura*
546 Pieterse W, Benitez HA & Addison P (2017) The use of geometric morphometric analysis to
547 illustrate the shape change induced by different fruit hosts on the wing shape of
548 *Bactrocera dorsalis* and *Ceratitis capitata (Diptera: Tephritidae)*. Zoologischer
549 Anzeiger 269:110-116
550 Porter CK & Benkman CW (2019) Character displacement of a learned behaviour and its
551 implications for ecological speciation. Proceedings of the Royal Society B-Biological
552 Sciences 286:20190761
553 R Core Team (2019) R: A language and environment for statistical computing.
554 Rohlf JF (2017) tpsDig2: Digitize coordinates of landmarks and capture outlines.
555 Rohlf JF (2018) tpsUtil: Utility program useful when working with tps files.
556 Romstock-Volkl M (1997) Host race formation in *Tephritis conura*: Determinants from three
557 trophic levels. Ecological Studies 130:21-38
558 Roriz AKP, Japyassu HF, Caceres C, Vera MT & Joachim-Bravo IS (2019) Pheromone
559 emission patterns and courtship sequences across distinct populations within

560 *Anastrepha fraterculus (Diptera-Tephritidae)* cryptic species complex. Bulletin of
561 Entomological Research 109:408-417
562 Rundle HD & Nosil P (2005) Ecological speciation. Ecology Letters 8:336-352
563 Rundle HD & Rowe L (2018) The contribution of sexual selection to ecological and
564 mutation-order speciation. Evolution 72:2571-2575
565 Schluter D (1993) Adaptive radiation in sticklebacks - Size, shape and habitat use efficiency.
566 Ecology 74:699-709
567 Schluter D 2008 The ecology of adaptive radiation. Oxford Univ. Press, Oxford
568 Schluter D (2009) Evidence for ecological speciation and its alternative. Science 323:737-741
569 Servedio MR & Noor MAF (2003) The role of reinforcement in speciation: Theory and data.
570 Annual Review of Ecology Evolution and Systematics 34:339-364
571 Shelomi M (2012) Where are we now? Bergmann's rule sensu lato in insects. American
572 Naturalist 180:511-519
573 Sivinski J (2000) Breeding habits and sex in families closely related to the *Tephritidae*:
574 Opportunities for comparative studies of the evolution of fruit fly behavior. In: Aluja
575 M & Norrbom AL (eds) Fruit Flies (*Tephritidae*). Boca Raton, pp23-37
576 Sivinski J & Pereira R (2005) Do wing markings in fruit flies *(Diptera: Tephritidae)* have
577 sexual significance? Florida Entomologist 88:321-324
578 Taylor EB, Boughman JW, Groenenboom M, Sniatynski M, Schluter D & Gow JL (2006)
579 Speciation in reverse: morphological and genetic evidence of the collapse of a three-
580 spined stickleback *(Gasterosteus aculeatus)* species pair. Molecular Ecology 15:343-
581 355
582 Teets NM, Dias VS, Pierce BK, Schetelig MF, Handler AM & Hahn DA (2019)
583 Overexpression of an antioxidant enzyme improves male mating performance after

stress in a lek-mating fruit fly. Proceedings of the Royal Society B-Biological Sciences 286:20190531

Thibert-Plante X & Gavrilets S (2013) Evolution of mate choice and the so-called magic traits in ecological speciation. Ecology Letters 16:1004-1013

Weiner J 1994 The beak of the finch: A story of evolution in our time. Jonathan Cape, London

Wiens JJ, Lapoint RT & Whiteman NK (2015) Herbivory increases diversification across insect clades. Nature Communications 6:8370

Zelditch ML 2004 Geometric morphometrics for biologists. Academic Press, Amsterdam

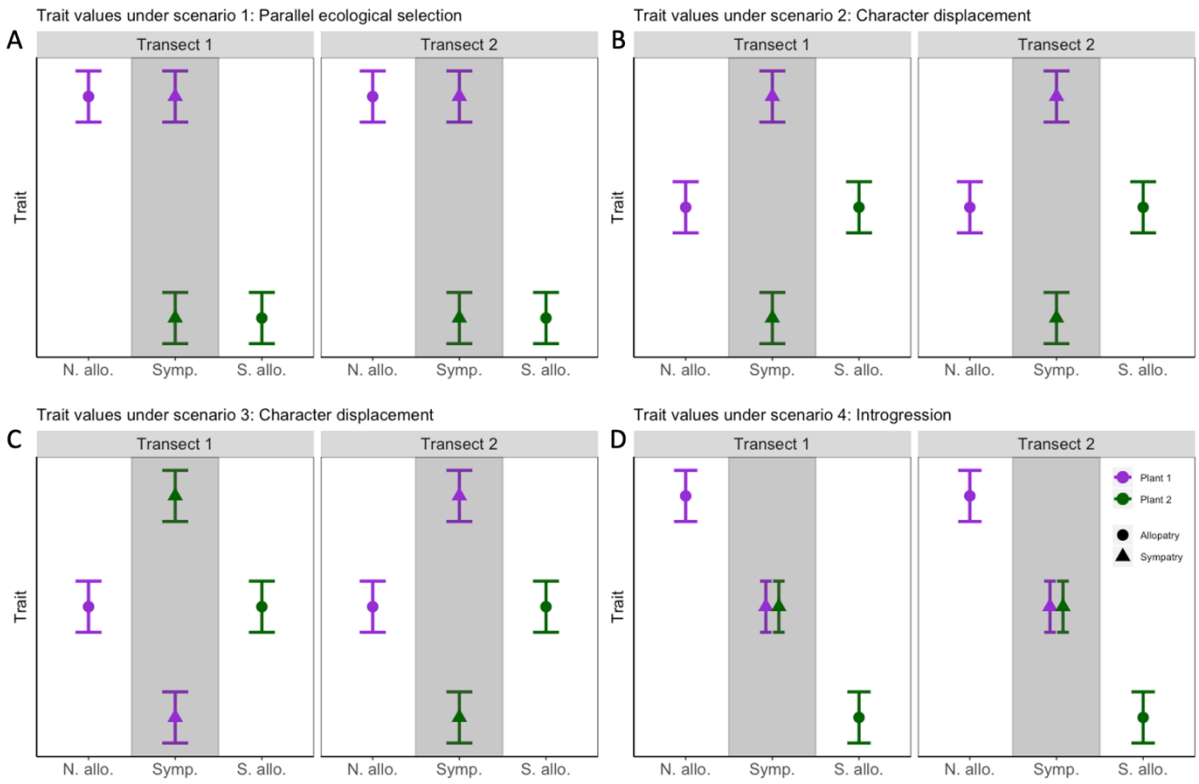

Figure 1 **Predictions for morphological divergence under different scenarios.** Four possible scenarios for population differentiation depending on the ecological and/or sexual selection pressures at play. Bars represent mean values of morphological traits, with one population being represented by each bar. While the white areas of the graph depict allopatric populations, the grey area includes co-existing populations. 'N. allo.' stands for northern allopatry, 'Symp.' stands for sympatry and 'S. allo.' stands for southern allopatry. Two hypothetical transects are depicted side by side. **A:** Scenario 1**,** parallel ecological selection between host races. This scenario would be expected for ecologically important traits where natural selection imposed by the environment provided by the host differ. **B**: Scenario 2, character displacement in a shared direction across transects. Should a sexual character be reinforced by maladaptive hybridization consistently across transects, this scenario where traits involved in mate recognition diverge further in sympatry due to character displacement would be expected. **C**: Scenario 3, character displacement in different directions across

transects. This would be expected in a sexual character which is reinforced by maladaptive hybridization, similarly to Scenario 2, however in this scenario the direction of adaptation is arbitrary, which would be expected under a mutation order regime. **D:** Scenario 4, hybridization in sympatric areas is causing introgression resulting in reduced divergence in these areas.

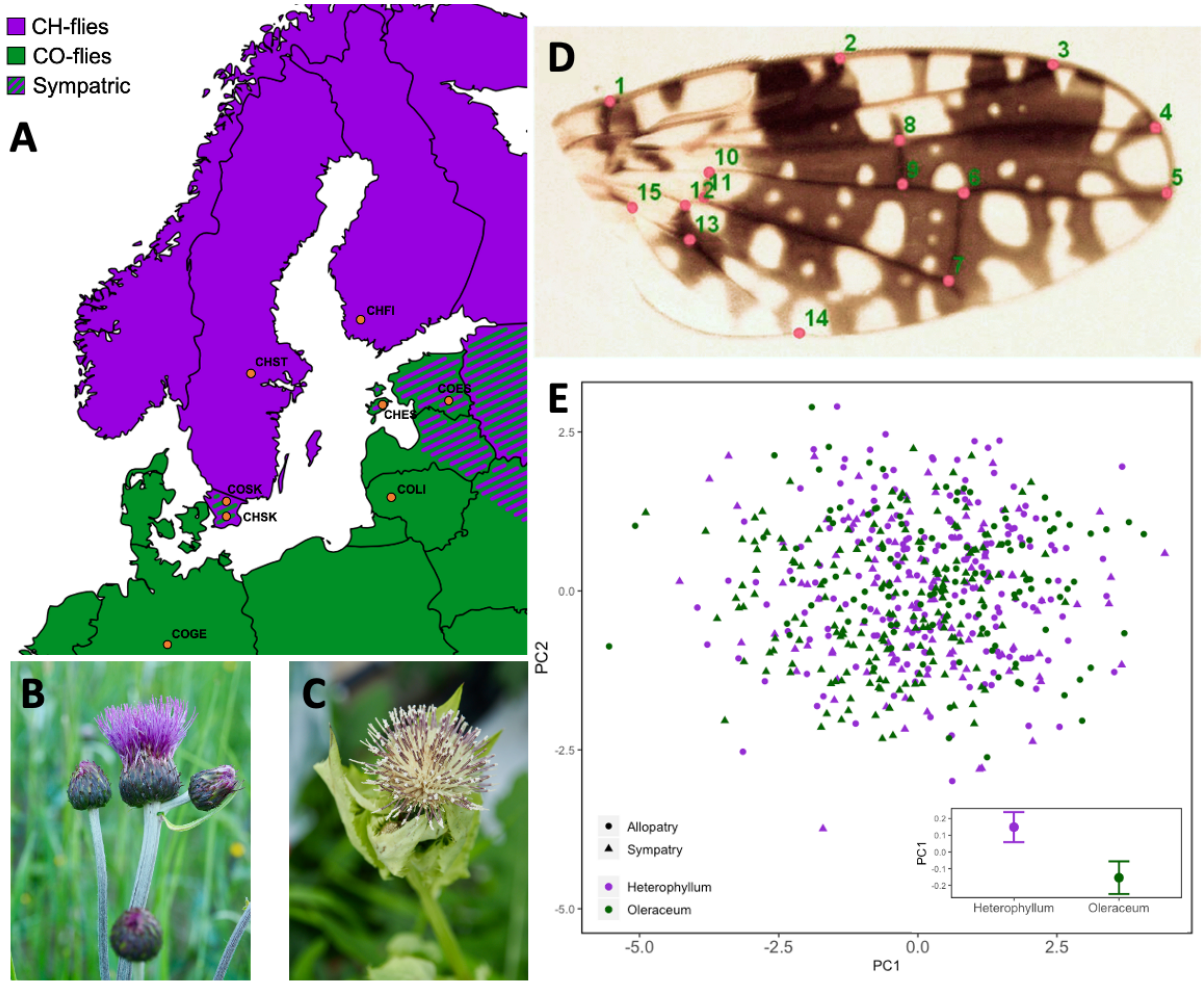

Figure 2: **Methods. A**: Map showing distribution of the *T. conura* host races, with the distribution of CH-flies infesting *Cirsium heterophyllum* depicted in purple and that of CO-flies infesting *Cirsium oleraceum* depicted in green. Sampling locations are indicated by red circles. **B**: *Cirsium heterophyllum*. **C**: *Cirsium oleraceum*. **D**: Dorsal photograph of a *T. conura* wing annotated with 15 landmarks used to analyze wing shape. **E**: Principal component analysis of *T. conura* morphology based on females. The first two principal components explain 37.84% of the variation. Insert shows the mean and standard error of the mean of PC1 in the two host races.

Table 1: **MANOVA.** Addressing the effect of fly host race, co-existence, transect and the interaction of these factors on multivariate morphological divergence in five morphological and six wing shape traits for females and four morphological traits and six wing shapes for males.

MANOVA	df	Pillai	Approx. F	num df	den df	P	
Females							
Host plant	1	0.258	8.42	11	267	< 0.001	***
Co-existence	1	0.295	10.15	11	267	< 0.001	***
Baltic Sea	1	0.374	14.52	11	267	< 0.001	***
Host plant x Co-existence	1	0.218	6.79	11	267	< 0.001	***
Host plant x Baltic Sea	1	0.18	5.31	11	267	< 0.001	***
Co-existence x Baltic Sea	1	0.163	4.74	11	267	< 0.001	***
Host plant x Co-existence x Baltic Sea	1	0.117	3.22	11	267	< 0.001	***
Residuals	277						
Males							
Host plant	1	0.078	2.29	10	271	0.014	*
Co-existence	1	0.194	6.54	10	271	< 0.001	***
Baltic Sea	1	0.393	17.51	10	271	< 0.001	***
Host plant x Co-existence	1	0.179	5.92	10	271	< 0.001	***
Host plant x Baltic Sea	1	0.16	5.12	10	271	< 0.001	***
Co-existence x Baltic Sea	1	0.131	4.1	10	271	< 0.001	***
Host plant x Co-existence x Baltic Sea	1	0.092	2.74	10	271	0.003	**
Residuals	280						

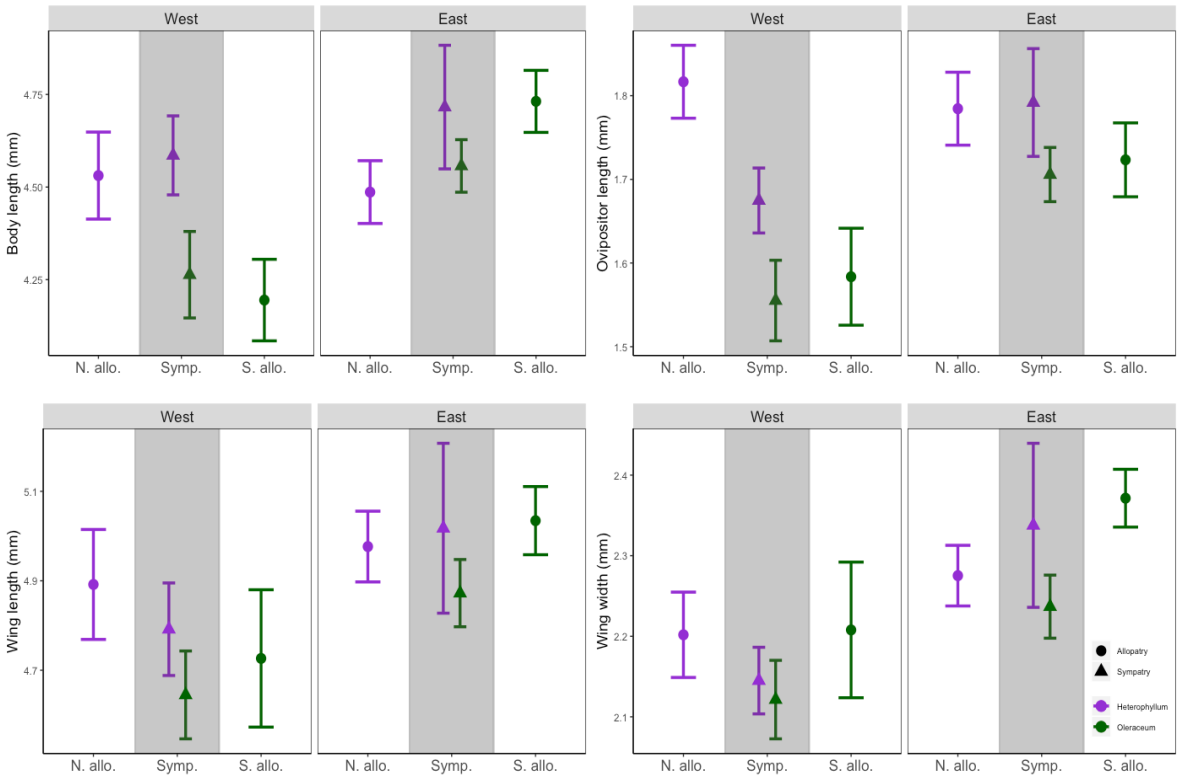

Figure 3: **Trait measurements of female *T. conura* divided by host race, co-existence and geographic setting.** Host race is illustrated by color, with purple corresponding to CH-flies infesting purple melancholy thistle and green to CO-flies infesting white cabbage thistle. The grey shaded area in the center contains the sympatric populations (denoted by triangles). X axis labels represent which state of co-existence the fly population is in. 'N.allo.' stands for Northern allopatric, 'Symp.' stands for sympatric and 'S. allo.' stands for Southern allopatric. West and East headers represent from which side of the Baltic Sea the populations are sampled. **A**: Mean values of female *T. conura* body length per population. **B:** Mean values of female *T. conura* ovipositor length per population. **C:** Mean values of female *T. conura* wing length per population. **D:** Mean values of female *T. conura* wing width per population. All plots portray mean trait values with 95% confidence interval bars of the mean.

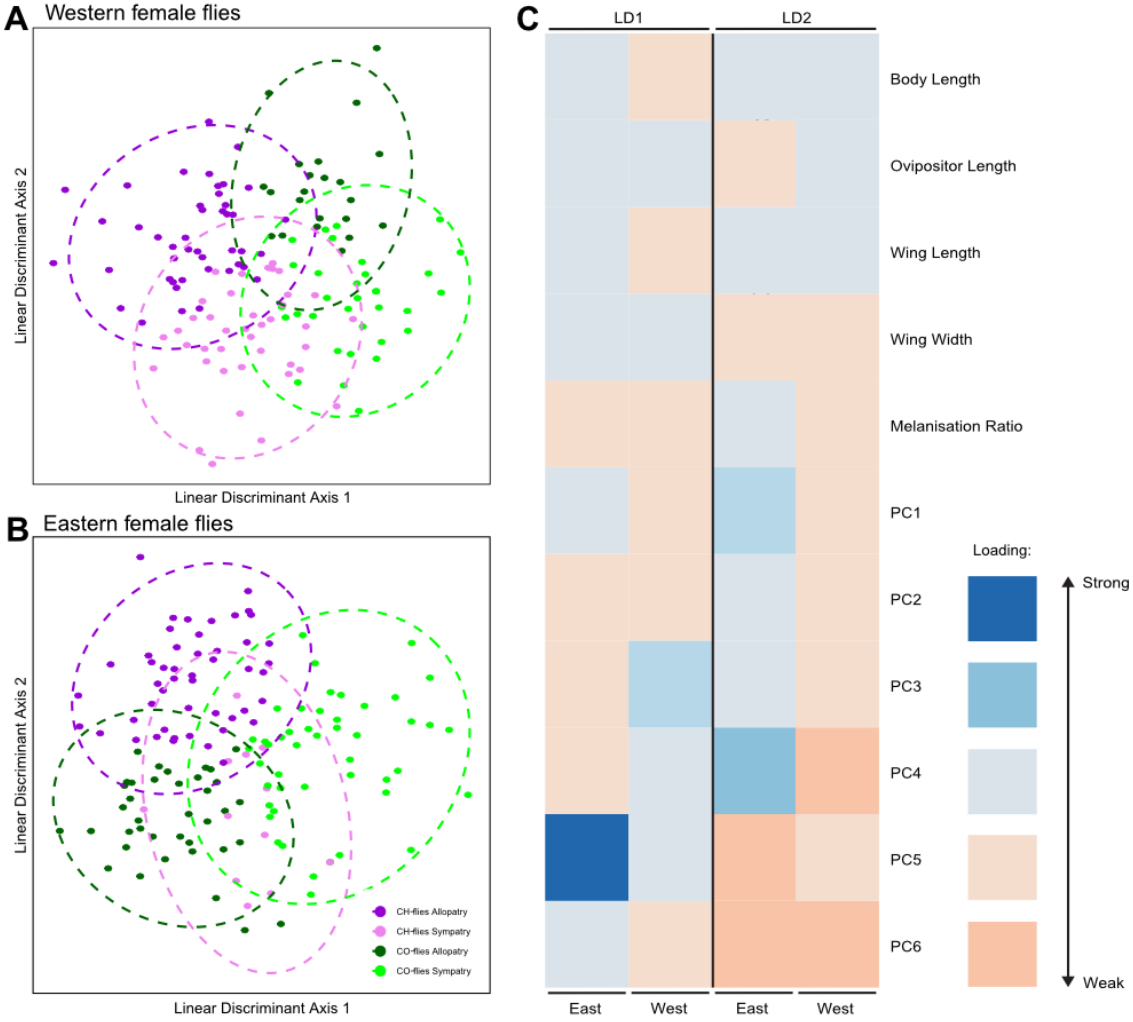

Figure 4: **Linear discriminant function analyses and bootstrapped loadings. A and B**: LDAs illustrating differences in how the host races group along the first two linear discriminant axes in Western (A) and Eastern (B) flies. **C:** The morphological traits loading on the two first discriminant axes for Eastern and Western flies. Colors illustrate how much the standard error diverges from zero based on 100 000 bootstrap replications. Loading that surpass zero are depicted in red colors whereas loadings significantly lower than zero are colored in blue. Plots and analyses are based on female fly morphology.