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

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

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Abstract

Divergent ecological selection may diversify populations of the same species evolving in different niches. However, for adaptation to result in speciation, the ecologically divergent populations have to experience at least some degree of reproductive isolation. While ecological selection pressures in similar environments are expected to result in convergent adaptation, sexually selected traits may diverge in different directions in different locations. 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 adaptations to secondary sympatry with the alternate host race are consistent across contact zones. We found low, albeit significant, divergence between host races, but only a few traits, including the female ovipositor, were consistently different. Interestingly, co-existence with the other host race significantly increased the degree of morphological divergence, but the patterns of divergence were not consistent across the two sympatric contact zones. Thus, local stochastic fixation or reinforcement could generate trait divergence, and evidence from additional sources is hence needed to conclude whether divergence is adaptive.

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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 development 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). Ecological divergence does not necessarily lead to speciation, however, as divergence and specialization may be reversed in the absence of reproductive isolation (Taylor et al., 2006, Lackey & Boughman, 2017).

While there are numerous examples of strong ecological divergence between young species pairs, the early stages of speciation may be reversible if the drivers of reproductive isolation are behavioral, plastic or depend on environmental context (Taylor et al., 2006, Lackey & Boughman, 2017). The coupling of traits involved in ecological adaptation to traits causing reproductive isolation is crucial for speciation to occur, and is pivotal in models of speciation (Kirkpatrick & Ravigne, 2002). For this reason, speciation is most readily achieved under some degree of allopatry (Mayr, 1947, Coyne & Orr, 2004, Rundle & Nosil, 2005) or if genetic incompatibilities arise, causing postzygotic barriers (Hollinger & Hermisson, 2017). At early stages of divergence postzygotic isolation is generally weak, and behavioral barriers to maladaptive mating may be absent in allopatric populations (Nosil, 2012). Prezygotic barriers, arising more readily during the early stages of diversification (Mendelson, 2003, Sanchez-Guillen et al., 2014) may be needed for specialization to persist. These may be environment dependent (Funk et al., 2002, Nosil et al., 2002, Lamont et al., 2003). Models suggest that traits under natural selection may be co-opted as mating cues (Thibert-Plante & Gavrilets, 2013). However, sexually selected traits that are not co-opted ecological traits are instead predicted to conform to a mutation order scenario (Mendelson et al., 2014) where incompatible alleles are fixed in different populations and may drift together with mating preferences along a line of equilibrium (Uyeda et al., 2009).

It remains an empirical challenge to resolve whether ecological adaptations and sexually selected/mate recognition traits diverge to the same extent under parallel selection regimes. Few studies have explicitly compared parallelism in multiple traits under different selection regimes. A recent study of Bahamas mosquitofish (*Gambusia hubbsi*) suggests that only a few traits are highly predictable across parallel predation environments (Langerhans, 2018). In a series of studies of Skyros wall lizards (*Podarcis gaigeae*) traits under natural selection were found to evolve in parallel (Runemark et al., 2014, Runemark et al., 2015), whereas traits under sexual selection diverged stochastically (Runemark et al., 2011). Parallelism in responses to co-existence with congeners is less well understood, although the evolutionary divergence of both ecological and sexual traits may be affected also by the local presence or absence of the other incipient species (Amarasekare, 2003, Calabrese & Pfennig, 2020). If the reproductive isolation between the incipient species is incomplete, such secondary contact may break up important ecological adaptations, which may be particularly evident in e.g. insect host races inhabiting different discrete plant niches. Close congeners inhabiting different niches may be poorly adapted to the alternative environment (Rundle & Nosil, 2005, Nilsson et al., 2017, Cronemberger et al., 2020, Martin et al., 2020), which may lead to reinforcement (Servedio & 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 (Figure 1A), either parallel- or nonparallel character displacement (Figure 1B-C) or reduced divergence due to introgression (Figure 1D) could be expected for sexually selected traits that are not strongly coupled with host plant use.

To understand if ecological selection and selection to reduce maladaptive mating result in different patterns of divergence, there is a need for joint investigations of how traits mediating ecological specialization and sexually selected traits develop during adaptation to novel niches. Such studies should test the hypothesis that the direction of change in sexually selected traits, during the early stages of speciation, can be more arbitrary than the ecological traits, which are expected to diverge in parallel across populations of similar niches (Figure 1). In particular, studies asking how co-existence with congeners affects the sexually selected trait distribution, e.g. through parallel- or non-parallel character displacement (Figure 1), compared to the distribution of ecologically important traits, may provide insight into the nature of the selection pressures acting on those different trait types. Here, we address how ecologically and putatively sexually selected 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). Macroevolutionary analyses reveal that such host plant driven speciation is one of the main factors explaining the tremendous diversity of phytophagous insects, because many speciation events can be attributed to host plant shifts (Berlocher & Feder, 2002, Dres & Mallet, 2002, Nylin et al., 2014), and diversification rates are elevated in herbivorous insects compared to their non-herbivorous relatives (Mitter et al., 1988, Farrell, 1998, Wiens et al., 2015). For an insect specialist like *T. conura,* the host plant provides a discrete environment imposing a multidimensional selection pressure. Hence, insect host plant adaptations are predicted to affect multiple traits involved in e.g. phenological matching, female host preference and larval performance (Matsubayashi et al., 2010).The putatively strong selection pressures involved in host plant adaptation provides a strong prediction of parallelism in host-use related traits of phytophagous insects at early stages of the speciation process (Nosil et al., 2002, Meyers et al., 2020). However, the prediction is less straightforward for sexually selected traits, because these are not necessarily associated with host use.

Tephritis conura provides an excellent system for studying patterns of divergence in ecological and sexually selected traits, as it enables comparisons of trait variation across replicated sympatric and allopatric settings. The two recently established host races inhabit two geographically isolated sympatric zones with adjacent allopatric populations, one East and one West of the Baltic Sea (Figure 2A). *Tephritis conura* females of the two host races are ovipositing into buds of either *Cirsium heterophyllum* (the ancestral host; Figure 2B) or *C. oleraceum* (the novel host; Figure 2C), and larvae show host specific performance and survival (Diegisser et al., 2008). During courtship, *Tephritid* flies performs a mating ritual that includes elaborate wing movements from both sexes (Sivinski, 2000, but see Briceno & Eberhard, 2017). Hence, *T. conura* offers an opportunity to compare the diversification of traits that are likely to be under ecological (e.g. body size, ovipositor length) or sexual (wing size, wing morphology) across different zones of secondary contact. Specifically, we test the hypotheses (i) that there are morphological effects of a host plant shift in ecologically and sexually selected characters, (ii) that host race divergence in ecologically selected traits is parallel in the two geographic areas and (iii) that traits putatively under sexual selection also diverge in parallel in the two replicas. Given the consistent genetic differentiation between host races both in allopatry and sympatry (Diegisser et al., 2006a, Ortega et al., [*in prep.]*) we predict the host races to be morphologically divergent, and this divergence to be consistent across geographical settings. As larval survival is strongly reduced in the alternate host (Diegisser et al., 2008), we predict hybridization to be maladaptive, potentially resulting in reinforcement and character displacement between the host specialists in sympatry.

Methods

Sampling

The dipteran *T. conura* infest several species of the thistle genus *Cirsium* (Asteraceae) (Romstock-Volkl, 1997). Adult *T. conura* oviposit into thistle buds during early summer, wherein the adolescent flies remain during larval development and pupation. *Tephritis conura* infesting *Cirsium heterophyllum*, the melancholy thistle, have recently colonized and adapted to *C. oleraceum*, the cabbage thistle. Haplotype analyses suggest that a peripatric host shift took place during the last ice age in the Alps (Diegisser et al., 2006b). The two host races are largely reproductively isolated, but there is evidence of some small amounts of gene flow (Diegisser et al., 2006b). The flies infesting *C. oleraceum* have adapted ecologically to the smaller bud sizes (Diegisser et al., 2007), and have a significantly shorter ovipositor to body size ratio. Another variable character is wing pigmentation. Wing patterns in Tephritid flies have been suggested to be under sexual selection, as males perform dances with their wings to attract females (Sivinski & Pereira, 2005). Tephritid males attempting to initiate copulation situate themselves in front of female flies and posture with their wings (Sivinski, 2000, but see Briceno & Eberhard, 2017). We therefore have reason to assume that ecological selection pressures are more important for size traits in general, and for ovipositor length in particular, while wing traits are more likely shaped by sexual selection. For simplicity, we refer to flies infecting *C. heterophyllum* as CH-flies, while the flies infecting *C. oleraceum* will be denoted CO-flies.

We used a parallel sampling design to examine phenotypic adaptation to a novel host plant and effects of co-existence between host races, by sampling each host race both in sympatry and allopatry on each side of the Baltic (Figure 2A). We collected thistle buds infested by *T. conura* larvae/pupae and allowed the adults to eclose in a common environment. CO-fly larvae were sampled in Germany and Lithuania (allopatric areas), both host races were collected in the sympatric areas in southern Sweden and Estonia, and allopatric CH-fly larvae were sampled in mid-Sweden and Finland (Figure 2A, Table S1). All sampling took place during June and July 2018. The sampling scheme enables examining to what extent patterns of phenotypic divergence are explained by host plant adaptations, by co-existence with the other host race, and if these patterns differ between the two transects. Typically, *C. heterophyllum* and *C. oleraceum* do not grow in the same microhabitat. Thus, the sympatric and allopatric definitions here refer to the presence of one or both thistle species in the general area (Figure 2A).

Morphological measurements

Tephritis conura adults eclosed from field-collected thistle buds in a common lab environment, (see Supplementary material S1). One male and one female per bud were euthanized by freezing a few days after emergence and subsequently included in the morphological analysis. For each individual, we took magnified photographs using a Celestron 44308 USB microscope. We photographed a lateral image of the fly body after removal of the wings and a dorsal image of the right wing on a transparent background to allow better visibility of the wing veins. Body length and ovipositor length were measured digitally from lateral photographs (Figure S1). We placed 14 landmarks, adapted from Pieterse et al. (2017), digitally on the dorsal wings (Figures 2D and 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.

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 (Figure 2D). To produce relative warps (i.e. principal components of shape) to compare shape between groups, a principal component analysis (PCA) was performed with the Procrustes fitted data using PAST3 v3.20 (Hammer et al., 2001) (Figure S3). Based on the variance explained by the eigenvalues (Figure S4) and the broken stick criterion (Jackson, 1993), six principal components which jointly explain 67.6% of the variance were used in further analyses of wing shape. All subsequent statistical analysis were performed in R (R Core Team, 2019).

To investigate whether host race was the major factor explaining variation in fly morphology, we performed a 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. We quantified five morphological traits and wing shape for 583 flies. We found six significant dimensions of wing shape variation between flies (Figure S5), that were included in subsequent analyses of phenotypic divergence. We identified four significant dimensions of variation from a PCA-analysis using the broken stick criterion (Jackson, 1993) (Figure S6). Collectively, these PCA-axes explained 78.0% 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 of these variables included as response variables (body length, ovipositor length, wing width, wind length, wing melanisation and PC1-6 of wing shapes). To investigate both whether host races differed, and if co-existence with the other host race affected morphology, we ran a generalized linear model (GLM) including host origin, co-existence, and their interaction as explanatory factors. 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 final 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 ovipositor (Diegisser et al., 2007) in analyses of females. This also reduced the multicollinearity of explanatory factors to below recommended values (Hair, 2010). To find which combination of factors that best explained the data, we estimated and compared AIC-scores (Akaike, 1974) for all models.

To further investigate parallel and non-parallel differentiation of the host race pairs, we applied separate linear discriminant function analyses (LDAs) on the data from the Eastern and Western transects. We used host races and co-existence with the other host race as factors in the models. This analysis was performed on males and females separately. To test if the patterns of divergence differed significantly between the transects, we performed each LDA 10,000 times using the bootstrap R package ‘boot’ (Canty & Ripley, 2020) and used the confidence intervals to assess if the loadings differed between analyses. In addition, we assessed the proportion of divergence is shared among host races and unique among the populations in a nested MANOVA using size and shape variables against host race and transect following Langerhans & DeWitt (2004). We applied this test to sympatric populations separately from allopatric populations, to avoid confounding effects of co-existence.

Results

We found weak evidence for separation between host races along the two first major axes of divergence (Figure 2E), with CO-flies, on average had lower values of PC1 compared to CH-flies, reflecting predominantly size variables (GLM: $F_1 = 5.189$, $p = 0.023$; Figure 2E). This host race difference was small compared with the variation among populations.

We found multivariate divergence between host races (Pillai's trace = 0.237, $F_{11, 271} = 7.66$, $p<0.001$), as well as an effect of co-existence with the other host race (Pillai's trace = 0.257, $F_{11, 271} = 8.51$, $p < 0.001$), and a significant interaction between host race and co-existence (Table 1; Pillai's trace = 0.155, $F_{11, 271} = 4.52$, $p < 0.001$) in females. For males, co-existence (Pillai's trace = 0.165, $F_{10, 275} = 5.45$, $p < 0.001$) and the interaction between host race and co-existence (Pillai's trace = 0.141, $F_{10, 275} = 4.5$, $p < 0.001$) affected morphology, while host race alone did not significantly affect morphology (Table 1). The differences between males and females could potentially be explained by the strong divergence in ovipositor length, included only in the analyses of females.

Females of the two host plant races differed in ovipositor length and in loadings of relative warp 5, reflecting wing shape (Tukeys post hoc tests; Table 2). Co-existence influenced a suite of characters, including ovipositor length, wing length, wing width, melanisation ratio, and wing shape. Moreover, body length, wing width, and several wing shape components were significantly affected by the interaction between host race and co-existence (Table 2; figures 3 and S7). Ovipositor length diverged as expected under ecological selection, especially in the Eastern transect (Figure 1A). In contrast, wing length showed tendencies to diverge as expected by character displacement in the Eastern transect (Figure 1B; Figure 3C). Male host races differed in fewer variables than females, with only relative warp 5 differing significantly between the host races (Table 2, figures S8-S11). Co-existence reduced male wing length with both host races having shorter wings in sympatric regions. Male wing width, melanisation ratio and wing shape also differed between populations that co-exist with the other host race and those that do not.

Host race and co-existence affected female morphology differently East and West of the Baltic Sea, as illustrated by a significant 3-way interaction (Pillai's trace = 0.117, $F_{11, 277}$ = 3.22, p <0.001; Table S2). All 2-way interactions and main effects were also significant in the full model (Table 1). This pattern holds true also for males (Pillai's trace = 0.092, $F_{10, 271}$ = 2.74, p = 0.0031; Table S3 and S4). Hence, depending on geography, host race and co-existence affects fly morphology differently.

To assess how much of the female divergence that was unique to a transect, shared between the host races and differentially affected by host race in the two transects, we estimated Wilk's partial η^2. We find that 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 S5).

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). We find that the importance of host race 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 (Figure 4). In the Eastern LDA, the first discriminant instead divided the two CO populations and the second discriminant axis divided the two CH populations (Figure 4). Moreover, different sets of characters separated populations in the two transects as bootstrap loadings show that different characters loaded on the first discriminant axes in the LDAs (Fig. 4C; Table S6).

Discussion

Contrary to previous studies that found clear differences between the host races (Diegisser et al., 2007) we found low and variable morphological differentiation between host races of *T. conura*. Multivariate differentiation between host races was driven by subtle differences across many traits. Host race separation reflected mainly ovipositor length differences with CH-fly ovipositors being longer than CO-fly ovipositors, although this varied among populations. Previous work report consistent divergence for two loci (Diegisser et al., 2006b), and whole genome data (Ortega et al., [*in prep.*]) support the presence of discrete genetic host races. Moreover, the flies also have poor performance on the alternate host plant (Diegisser et al., 2008). In light of this, the moderate host race divergence in morphology was unexpected, but this result is in line with the observation that parallelism in fitness is typically higher than for both phenotypic divergence and genetic divergence (Bolnick et al., 2018). Moreover, parallel divergence only in traits under strong ecological selection is consistent with the findings 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 the species is adapting to.

Two out of five female traits match a parallel divergence scenario, ovipositor length, and body length in the Western transect. Hence, the degree of parallelism among host races in morphological divergence was lower than expected. Intermediate ovipositor lengths in Western CH-flies could suggest selection for shorter ovipositors in sympatry, potentially due to introgression or to be able to utilize also *C. oleraceum* buds to a higher degree. Many of the traits measured are highly correlated with body size, a factor that typically varies with temperature in insects (Atkinson, 1994, but see Shelomi, 2012). While the parallel sampling design with allopatric regions both South and North of both sympatric zones would partly correct for such temperature effects, temperature could still differ between the Eastern and Western transects, potentially contributing to non-parallel patterns of divergence.

When we jointly investigated effects of co-existence, geographic origin and their interactions on host race divergence, we found that both co-existence with the other host race and transect affected morphology. Hence, the low host race divergence may be explained by interacting effects of co-existence with the other host race, with non-parallel patterns of divergence in the two transects. This pattern would be consistent a mutation order scenario (Figure 1C). If reinforcement due to maladaptive hybridization would have been a prominent force in sympatric populations, we would expect traits to be more divergent between the host races in sympatry compared to allopatry, i.e. for character displacement to arise (Comeault et al., 2016, Calabrese & Pfennig, 2020, Kyogoku & Wheatcroft, 2020; Figure 1B-C). This was generally not the case. While the degree of host race divergence differed between sympatry and allopatry, the most common pattern was that the traits in sympatric populations are more similar between host races than traits in allopatry, most prominent in wing morphology traits. This could be indicative by introgression (Figure 1D), consistent with findings by Ortega et al. [*in prep*.], or effects of a shared environment. Co-existence most strongly affects on wing size, with wing width being shorter in sympatry than in allopatry in the Western transect. This could be consistent with selection for shorter dispersal distances (Claramunt et al., 2012), potentially to avoid dispersal to the other host plant, reducing survival. We also found patterns consistent with character displacement in in wing length in the Eastern transect. Hence, contrary to reports of parallelism also in sexually selected traits in ecotypes in e.g. sticklebacks (Boughman et al., 2005), we find mixed evidence for parallelism in wing traits that putatively are under sexual selection. This could reflect a mutation order scenario, where the difference may be important but the specific trait or direction of the difference is arbitrary (Mendelson et al., 2014). Some degree of sexual selection may further differentiate populations under a mutation order scenario (Rundle & Rowe, 2018), underlining the need to further investigate if there is sexual selection at work in this system and identifying the traits under selection.

The degree of divergence differ between transects. While host races are separated by the main discriminant axis in the Western transect, host race was a less important predictor of divergence in the Eastern transect. The traits that differ between host races also differs between our two geographic replicates. A mutation order scenario (Mendelson et al., 2014) could explain the seemingly arbitrary divergence between the transects (Figure 1C). However, the differences in patterns between the transects East and West of the Baltic Sea could have several additional explanations related to demographic history, population size and the extent to which the host plant races co-exist locally. Possibly, one contact zone may be older than the other and populations in older sympatry would have had more time for character displacement to develop. Alternatively, if the Eastern transect has a higher proportion suitable thistle habitat, this could have increased both within- and between host race connectivity and potentially gene flow (Servedio & Noor, 2003). Genetic data and detailed analyses of introgression should be used to resolve whether selection against hybridization in allopatric regions could be expected.

Morphological differentiation does not always strongly reflect even crucial ecological adaptations. For instance, cultural evolution contributes to reproductive isolation in Cassia crossbills (*Loxia* sinesciuris) (Porter & Benkman, 2019) and *Rhagoletis pomonella* have adapted their phenologies to host fruits ripening at different times of the year (Filchak et al., 2000). A potential explanation to the low consistency of host race divergence may lie with the traits included in this analysis. Females were more diverged than males. The strong divergence in females likely reflects the inclusion of ovipositor length in the analyses, as ovipositor length was one of the traits that differed significantly between the host races, consistent with previous findings (Diegisser et al., 2007). These findings are similar to those of Jourdan et al. (2016) where divergence in female mosquitofishes (*Gambusia*) divergence was more parallel than male divergence. Potentially, the other traits measured are not important enough for host plant adaptation to result in strongly parallel divergence. Traits that have been shown to differ strongly between host races include female ovipositor length (Diegisser et al., 2007) and the larval ability to survive on the different host plant species (Diegisser et al., 2008), and these traits are more likely to show parallel divergence. Moreover, host plant preference may have a potential to act as a magic trait (Gavrilets, 2004, Thibert-Plante & Gavrilets, 2013) for *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 previously 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, with parallelism only in ecologically strongly selected traits, whereas there are 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 in putatively sexually selected traits and even character displacement in opposite directions in the two geographic replicas, suggesting that mutation order dependent divergence may lead to different solutions to avoid introgression in independent contact zones.

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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, performed the statistical analyses, with advice from A.R. and 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.

Conflict of interest statement

The authors have no conflict of interest to declare.

Data availability statement

All data sets will be uploaded to the Dryad Digital Repository (www.datadryad.org) upon acceptance.

References

Akaike, H. 1974. New look at statistical-model identification. *Ieee Transactions on Automatic Control* **AC19**: 716-723.

Amarasekare, P. 2003. Competitive coexistence in spatially structured environments: a synthesis. *Ecology Letters* **6**: 1109-1122.

Atkinson, D. (1994) Temperature and organism size - A biological law for ectotherms. *Advances in Ecological Research* **25**: 1-58

Baldwin, B. G., Carlquist, S. & Carr, G. D. 2003. *Tarweeds & Silverswords : evolution of the Madiinae (Asteraceae)*. Missouri Botanical Garden Press, St. Louis, Missouri.

Bay, R. A., Arnegard, M. E., Conte, G. L., Best, J., Bedford, N. L., McCann, S. R., . . . Peichel, C. L. 2017. Genetic Coupling of Female Mate Choice with Polygenic

Ecological Divergence Facilitates Stickleback Speciation. *Current Biology* **27**: 3344-3349.

Berlocher, S. H. & Feder, J. L. 2002. Sympatric speciation in phytophagous insects: Moving beyond controversy? *Annual Review of Entomology* **47**: 773-815.

Bolnick, D. I., Barrett, R. D. H., Oke, K. B., Rennison, D. J. & Stuart, Y. E. (2018) (Non)Parallel Evolution. *Annual Review of Ecology, Evolution, and Systematics* **49**: 303-330

Boughman, J. W., Rundle, H. D. & Schluter, D. 2005. Parallel evolution of sexual isolation in sticklebacks. *Evolution* **59**: 361-373.

Briceno, R. D. & Eberhard, W. G. 2017. Copulatory Dialogues Between Male and Female Tsetse Flies (Diptera: Muscidae: *Glossina pallidipes*). *Journal of Insect Behavior* **30**: 394-408.

Bush, G. L. 1969. Sympatric host race formation and speciation in frugivorious flies of genus *Rhagoletis* (Diptera, Tephritidae). *Evolution* **23**: 237-251.

Calabrese, G. M. & Pfennig, K. S. 2020. Reinforcement and the Proliferation of Species. *Journal of Heredity* **111**: 138-146.

Canty, A. & Ripley, B. D. (2020) boot: Bootstrap R (S-Plus) Functions. R package version 1.3-25.

Claramunt, S., Derryberry, E. P., Remsen, J. V., Jr. & Brumfield, R. T. 2012. High dispersal ability inhibits speciation in a continental radiation of passerine birds. *Proceedings of the Royal Society B-Biological Sciences* **279**: 1567-1574.

Comeault, A. A., Venkat, A. & Matute, D. R. 2016. Correlated evolution of male and female reproductive traits drive a cascading effect of reinforcement in *Drosophila yakuba*. *Proceedings of the Royal Society B-Biological Sciences* **283**.

Coyne, J. A. & Orr, H. A. 2004. *Speciation*. Sinauer Associates, Sunderland, Mass.

Cronemberger, A. A., Aleixo, A., Mikkelsen, E. K. & Weir, J. T. 2020. Postzygotic isolation drives genomic speciation between highly cryptic Hypocnemis antbirds from Amazonia. *Evolution* **74**: 2512-2525.

Diegisser, T., Johannesen, J. & Seitz, A. 2006a. The role of geographic setting on the diversification process among *Tephritis conura* (Tephritidae) host races. *Heredity* **96**: 410-418.

Diegisser, T., Johannesen, J. & Seitz, A. 2008. Performance of host-races of the fruit fly, *Tephritis conura* on a derived host plant, the cabbage thistle *Cirsium oleraceum*: Implications for the original host shift. *Journal of Insect Science* **8**.

Diegisser, T., Seitz, A. & Johannesen, J. 2006b. Phylogeographic patterns of host-race evolution in *Tephritis conura* (Diptera : Tephritidae). *Molecular Ecology* **15**: 681-694.

Diegisser, T., Seitz, A. & Johannesen, J. 2007. Morphological adaptation in host races of *Tephritis conura*. *Entomologia Experimentalis Et Applicata* **122**: 155-164.

Dres, M. & Mallet, J. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society B-Biological Sciences* **357**: 471-492.

Farrell, B. D. 1998. "Inordinate fondness" explained: Why are there so many beetles? *Science* **281**: 555-559.

Feder, J. L., Chilcote, C. A. & Bush, G. L. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. *Nature* **336**: 61-64.

Filchak, K. E., Roethele, J. B. & Feder, J. L. 2000. Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. *Nature* **407**: 739-742.

Funk, D. J., Filchak, K. E. & Feder, J. L. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. *Genetica* **116**: 251-267.

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

Hammer, Ø., Harper, D. A. T. & P. D., R. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologica Electronica* **4**: 9.

Hinojosa, J. C., Koubinova, D., Dinca, V., Hernandez-Roldan, J., Munguira, M. L., Garcia-Barros, E., . . . Vila, R. 2020. Rapid colour shift by reproductive character displacement in *Cupido* butterflies. *Molecular Ecology* **29**: 4942-4955.

Hollinger, I. & Hermisson, J. 2017. Bounds to parapatric speciation: A Dobzhansky-Muller incompatibility model involving autosomes, X chromosomes, and mitochondria. *Evolution* **71**: 1366-1380.

Jackson, D. A. 1993. Stopping rules in principal components-analysis - A comparison of heuristic and statistical approaches. *Ecology* **74**: 2204-2214.

Jourdan, J., Krause, S. T., Lazar, V. M., Zimmer, C., Sommer-Trembo, C., Arias-Rodriguez, L., . . . Plath, M. 2016. Shared and unique patterns of phenotypic diversification along a stream gradient in two congeneric species. *Scientific Reports* **6**: 38971.

Kirkpatrick, M. & Ravigne, V. 2002. Speciation by natural and sexual selection: Models and experiments. *American Naturalist* **159**: S22-S35.

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

Lackey, A. C. R. & Boughman, J. W. 2017. Evolution of reproductive isolation in stickleback fish. *Evolution* **71**: 357-372.

Lamont, B. B., He, T., Enright, N. J., Krauss, S. L. & Miller, B. P. 2003. Anthropogenic disturbance promotes hybridization between *Banksia* species by altering their biology. *Journal of Evolutionary Biology* **16**: 551-557.

Langerhans, R. B. 2018. Predictability and Parallelism of Multitrait Adaptation. *Journal of Heredity* **109**: 59-70.

Langerhans, R. B. & DeWitt, T. J. 2004. Shared and unique features of evolutionary diversification. *American Naturalist* **164**: 335-349.

Linn, C. E., Darnbroski, H. R., Feder, J. L., Berlocher, S. H., Nojima, S. & Roelofs, W. L. 2004. Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: Reduced response of hybrids to parental host-fruit odors. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 17753-17758.

Loo, W. T., Garcia-Loor, J., Dudaniec, R. Y., Kleindorfer, S. & Cavanaugh, C. M. 2019. Host phylogeny, diet, and habitat differentiate the gut microbiomes of Darwin's finches on Santa Cruz Island. *Scientific Reports* **9**: 12.

Martin, B. T., Douglas, M. R., Chafin, T. K., Jr, J. S. P., Birkhead, R. D., Phillips, C. A. & Douglas, M. E. 2020. Contrasting signatures of introgression in North American box turtle (*Terrapene* spp.) contact zones. *Molecular Ecology* **29**: 4186-4202.

Matsubayashi, K. W., Ohshima, I. & Nosil, P. 2010. Ecological speciation in phytophagous insects. *Entomologia Experimentalis Et Applicata* **134**: 1-27.

Mayr, E. 1947. Ecological factors in speciation. *Evolution* **1**: 263-288.

Mendelson, T. C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae : *Etheostoma*). *Evolution* **57**: 317-327.

Mendelson, T. C., Martin, M. D. & Flaxman, S. M. 2014. Mutation-order divergence by sexual selection: diversification of sexual signals in similar environments as a first step in speciation. *Ecology Letters* **17**: 1053-1066.

Meyers, P. J., Doellman, M. M., Ragland, G. J., Hood, G. R., Egan, S. P., Powell, T. H. Q., . . . Feder, J. L. 2020. Can the genomics of ecological speciation be predicted across the divergence continuum from host races to species? A case study in *Rhagoletis*.

Philosophical Transactions of the Royal Society of London B Biological Sciences **375**: 20190534.

Mitter, C., Farrell, B. & Wiegmann, B. 1988. The Phylogenetic Study of Adaptive Zones - Has Phytophagy Promoted Insect Diversification? *American Naturalist* **132**: 107-128.

Nilsson, P. A., Hulthen, K., Chapman, B., Hansson, L. A., Brodersen, J., Baktoft, H., . . . Skov, C. 2017. Species integrity enhanced by a predation cost to hybrids in the wild. *Biology Letters* **13**.

Nosil, P. 2012. *Ecological speciation*. Oxford University Press, Oxford.

Nosil, P., Crespi, B. J. & Sandoval, C. P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* **417**: 440-443.

Nylin, S., Slove, J. & Janz, N. 2014. Host Plant Utilization, Host Range Oscillations and Diversification in Nymphalid Butterflies: a Phylogenetic Investigation. *Evolution* **68**: 105-124.

Ortega, J., Chao, Y., Nilsson, KJ., Runemark, A. 2021. Genomic landscape of *Tephritis conura*. [*in prep.*].

Pieterse, W., Benitez, H. A. & Addison, P. 2017. The use of geometric morphometric analysis to illustrate the shape change induced by different fruit hosts on the wing shape of *Bactrocera dorsalis* and *Ceratitis capitata* (Diptera: Tephritidae). *Zoologischer Anzeiger* **269**: 110-116.

Porter, C. K. & Benkman, C. W. 2019. Character displacement of a learned behaviour and its implications for ecological speciation. *Proceedings of the Royal Society B-Biological Sciences* **286**: 10.

R Core Team (2019) R: A language and environment for statistical computing. pp. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

Rohlf, J. F. (2017) tpsDig2: Digitize coordinates of landmarks and capture outlines. pp., Ecology & Evolution and Anthropology, Stony Brook University.

Rohlf, J. F. (2018) tpsUtil: Utility program useful when working with tps files. pp., Ecology & Evolution and Anthropology, Stony Brook University.

Romstock-Volkl, M. 1997. Host race formation in *Tephritis conura*: determinants from three trophic levels. *Ecological Studies* **130**: 21-38.

Roriz, A. K. P., Japyassu, H. F., Caceres, C., Vera, M. T. & Joachim-Bravo, I. S. 2019. Pheromone emission patterns and courtship sequences across distinct populations within *Anastrepha fraterculus* (Diptera-Tephritidae) cryptic species complex. *Bulletin of Entomological Research* **109**: 408-417.

Rundle, H. D. & Nosil, P. 2005. Ecological speciation. *Ecology Letters* **8**: 336-352.

Rundle, H. D. & Rowe, L. 2018. The contribution of sexual selection to ecological and mutation-order speciation. *Evolution* **72**: 2571-2575.

Runemark, A., Brydegaard, M. & Svensson, E. I. 2014. Does relaxed predation drive phenotypic divergence among insular populations? *Journal of Evolutionary Biology* **27**: 1676-1690.

Runemark, A., Gabirot, M. & Svensson, E. I. 2011. Population divergence in chemical signals and the potential for premating isolation between islet- and mainland populations of the Skyros wall lizard (*Podarcis gaigeae*). *Journal of Evolutionary Biology* **24**: 795-809.

Runemark, A., Sagonas, K. & Svensson, E. I. 2015. Ecological explanations to island gigantism: dietary niche divergence, predation, and size in an endemic lizard. *Ecology* **96**: 2077-2092.

Sanchez-Guillen, R. A., Cordoba-Aguilar, A., Cordero-Rivera, A. & Wellenreuther, M. 2014. Rapid evolution of prezygotic barriers in non-territorial damselflies. *Biological Journal of the Linnean Society* **113**: 485-496.

Schluter, D. 1993. Adaptive Radiation in Sticklebacks - Size, Shape and Habitat Use Efficiency. *Ecology* **74**: 699-709.

Schluter, D. 2008. *The ecology of adaptive radiation*. Oxford Univ. Press, Oxford.

Schluter, D. 2009. Evidence for Ecological Speciation and Its Alternative. *Science* **323**: 737-741.

Servedio, M. R. & Noor, M. A. F. 2003. The role of reinforcement in speciation: Theory and data. *Annual Review of Ecology Evolution and Systematics* **34**: 339-364.

Shelomi, M. 2012. Where Are We Now? Bergmann's Rule Sensu Lato in Insects. *American Naturalist* **180**: 511-519.

Sivinski, J. 2000. *Breeding habits and sex in families closely related to the Tephritidae: Opportunities for comparative studies of the evolution of fruit fly behavior*. CRC Press, Boca Raton.

Sivinski, J. & Pereira, R. 2005. Do wing markings in fruit flies (Diptera : Tephritidae) have sexual significance? *Florida Entomologist* **88**: 321-324.

Taylor, E. B., Boughman, J. W., Groenenboom, M., Sniatynski, M., Schluter, D. & Gow, J. L. 2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology* **15**: 343-355.

Teets, N. M., Dias, V. S., Pierce, B. K., Schetelig, M. F., Handler, A. M. & Hahn, D. A. 2019. 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**: 9.

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

Uyeda, J. C., Arnold, S. J., Hohenlohe, P. A. & Mead, L. S. 2009. Drift Promotes Speciation by Sexual Selection. *Evolution* **63**: 583-594.

Weiner, J. 1994. *The beak of the finch : evolution in real time : an extraordinary scientific adventure story about birds, biology and the origin of species*. Jonathan Cape, London.

Wiens, J. J., Lapoint, R. T. & Whiteman, N. K. 2015. Herbivory increases diversification across insect clades. *Nature Communications* **6**: 7.

Zelditch, M. L. 2004. *Geometric morphometrics for biologists*. Academic Press, Amsterdam.

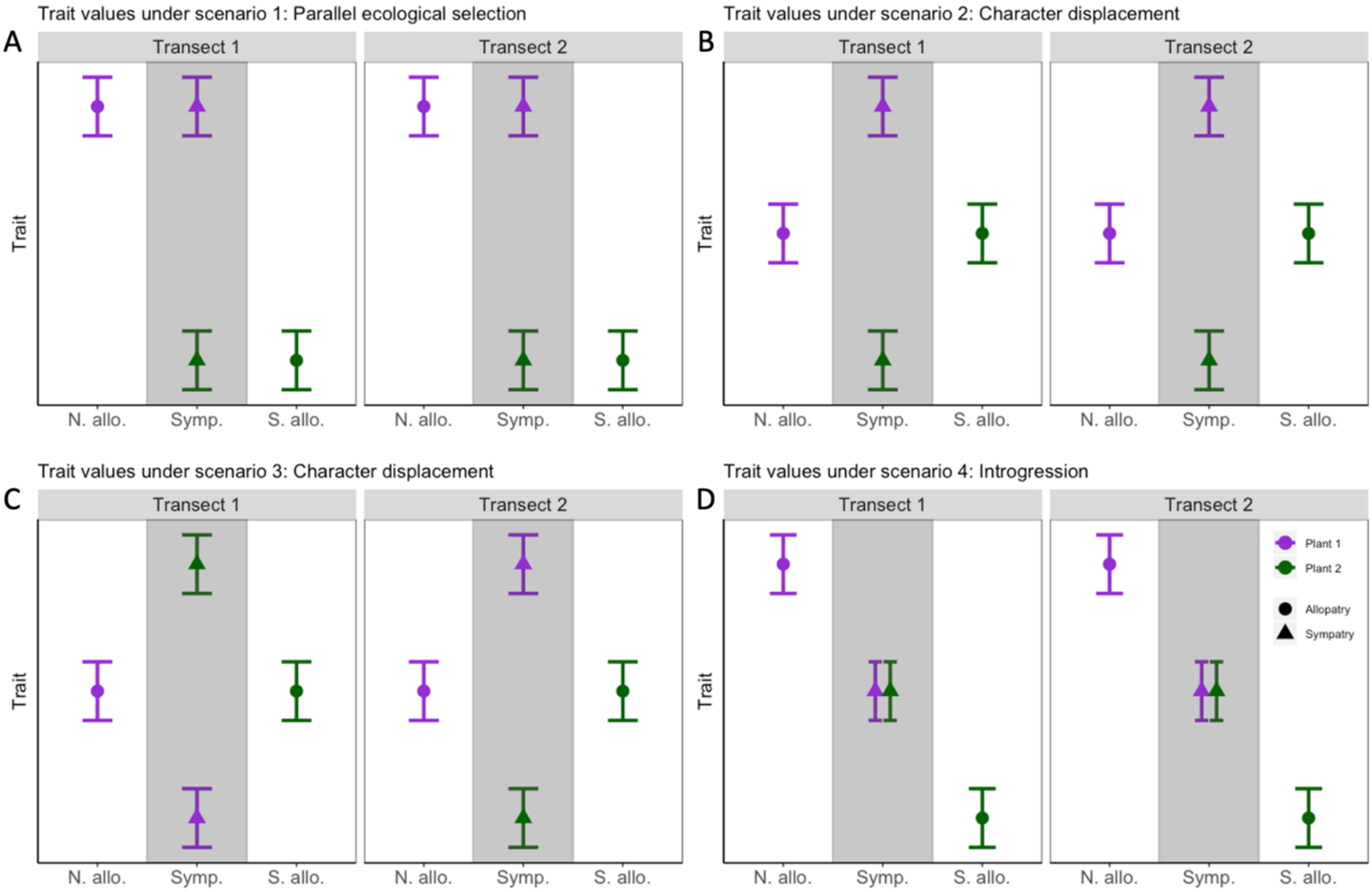

Figure 1: **Predictions to adaptation.** Four possible scenarios populations may follow when faced by ecological or sexual selection under a secondary contact of host races. **A:** Scenario 1**,** parallel ecological selection between host races. This scenario would be expected for ecologically important 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 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 intermediary traits.

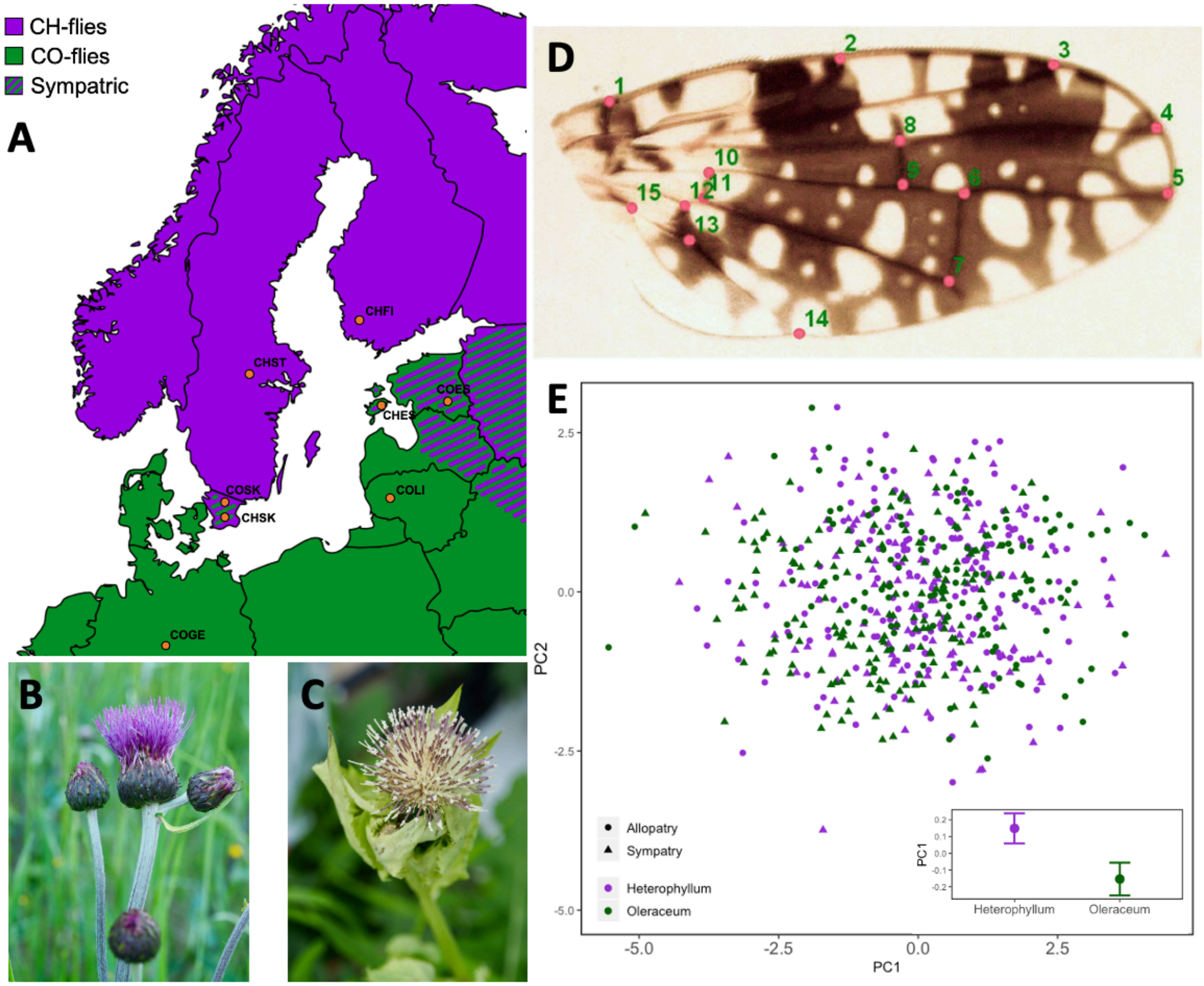

Figure 2: **Methods. A**: Map showing distribution of *T. conura* host races and our sampling locations. **B**: *Cirsium heterophyllum*. **C**: *Cirsium oleraceum*. **D**: Dorsal photograph of a *T. conura* wing annotated with 15 landmarks used to analyze wing shape. **E**: Principal component analysis of *T. conura* individuals measured. The first two principal components explain 37.84% of all variation. Insert shows the mean and standard error of the mean of PC1 in the host races.

Table 1: **Results from MANOVA analysis.** On factors explaining fly host race, whether the flies were allopatric or sympatric and the interaction of factors.

MANOVA	df	Pillai	Approx. F	num df	den df	P	
Host plant	1	0.237	7.66	11	271	< 0.001	***
Co-existence	1	0.257	8.51	11	271	< 0.001	***
Host plant x Co-existence	1	0.154	4.42	11	271	< 0.001	***
Residuals	281						

Table 2: **Post-Hoc ANOVA analyses.** Showing only traits which significantly affected differences between groups in the above MANOVA.

ANOVA	Independent	Dependent	F	num df	den df	P	
Females	Host plant	Ovipositor length	43.25	1	281	< 0.001	***
		Wing shape, warp 5	8.87	1	281	0.003	**
	Co-existence	Ovipositor length	12.28	1	281	< 0.001	***
		Wing length	9.05	1	281	0.003	**
		Wing width	17.45	1	281	< 0.001	***
		Melanisation ratio	9.26	1	281	0.003	**
		Wing shape, warp 2	10.84	1	281	0.001	**
		Wing shape, warp 3	6.50	1	281	0.011	*
		Wing shape, warp 5	12.09	1	281	< 0.001	***
	Interaction	Body length	6.37	1	281	0.012	*
		Wing width	5.61	1	281	0.018	*
		Wing shape, warp 1	7.42	1	281	0.007	**
		Wing shape, warp 3	6.80	1	281	0.01	**
		Wing shape, warp 5	6.92	1	281	0.009	**
Males	Host plant	Wing shape, warp 5	6.93	1	284	0.009	**
	Co-existence	Wing length	13.79	1	284	< 0.001	***
		Wing width	14.35	1	284	< 0.001	***
		Melanisation ratio	7.14	1	284	0.008	**
		Wing shape, warp 2	12.69	1	284	< 0.001	***
		Wing shape, warp 3	8.75	1	284	0.003	**
		Wing shape, warp 5	5.97	1	284	0.015	*
	Interaction	Body length	11.47	1	284	< 0.001	***
		Wing shape, warp 1	5.48	1	284	0.02	*
		Wing shape, warp 3	9.17	1	284	0.003	**
		Wing shape, warp 6	7.32	1	284	0.007	**

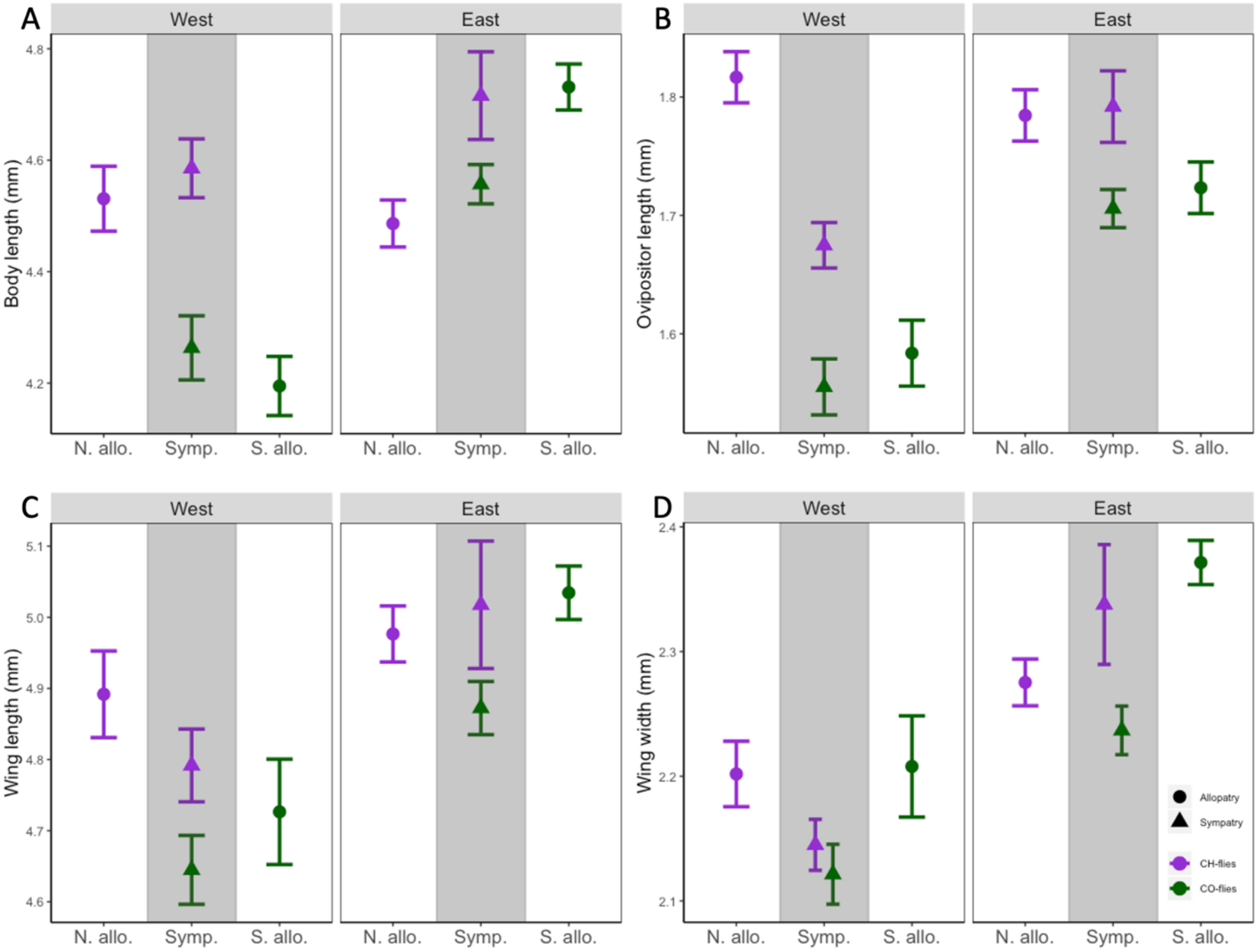

Figure 3: **Trait measurements:** All plots portray mean trait values with SE bars of the mean. Only female *T. conura* are included. 'West' and 'East' represents from which side of the Baltic Sea the population is sampled. X axis labels represent which state of co-existence the fly population is in. 'N.allo.' stands for Northern allopatric, 'Symp.' stands for sympatric and 'S. allo.' stands for Southern allopatric. **A**: Mean values of *T. conura* body length separated by population. **B:** Mean values of *T. conura* ovipositor length separated by population. **C:** Mean values of *T. conura* wing length separated by population. **D:** Mean values of *T. conura* wing width separated by population.

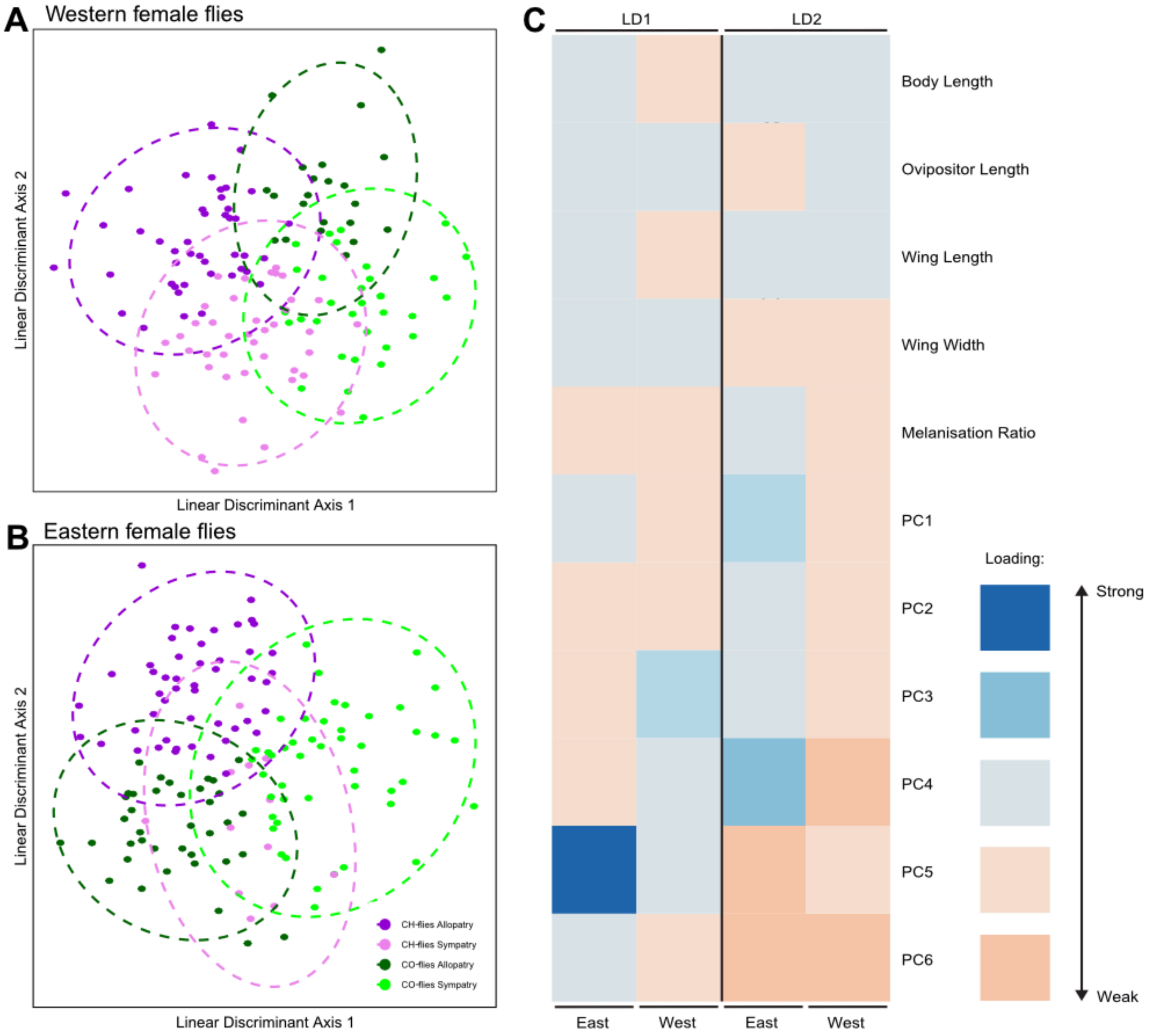

Figure 4: **Linear discriminant function analyses and bootstrapped loadings.** All female *T. conura* included**. A and B**: LDAs showing a different structure when comparing Eastern and Western flies **C:** Bootstrapped loadings colored from blue to red based on how much standard error surpasses zero or not, where loadings with standard errors which never surpassing zero are colored blue. Bootstrap performed with 100 000 replications.