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Ecology not genetic covariance explains correlated trait divergence during speciation

Authors: Clarissa F. de Carvalho[1,†,*], Nicholas P. Planidin[2], Romain Villoutreix[2], Víctor Soria-Carrasco[3], Rüdiger Riesch[2,4], Jeffrey L. Feder[5], Thomas L. Parchman[6], Jon Slate[1], Zachariah Gompert[7], Patrik Nosil[1,2,8,*]

Affiliations:
[1]*School of Biosciences, University of Sheffield; Sheffield, S10 2TN, UK*
[2]*CEFE, Univ Montpellier, CNRS, EPHE, IRD; Montpellier, France*
[3]*John Innes Centre; Norwich, NR4 7UH, UK*
[4]*Department of Biological Sciences, Centre for Ecology, Evolution and Behaviour, Royal Holloway University of London; Egham, TW20 0EX, UK.*
[5]*Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, 46556, USA*
[6]*Department of Biology, University of Nevada Reno; Reno, Nevada 89557, USA*
[7]*Department of Biology, Utah State University; Logan, Utah, 84322, USA*
[8]*SETE, Univ Université Paul Sabatier, CNRS, UMR-5321, F-09200 Moulis, France*†*Present address: Departamento de Ecologia e Biologia Evolutiva, UNIFESP; Diadema, 09972-270, Brazil*
**Corresponding authors*

Email: clarissa.carvalho@unifesp.br; patrik.nosil@sete.cnrs.fr

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Abstract

The formation of new species often involves the correlated divergence of multiple traits and genetic regions. However, the mechanisms by which such trait covariation builds up remain poorly understood. In this context, we consider two non-exclusive hypotheses. First, genetic covariance between traits can cause divergent selection on one trait to promote population divergence in correlated traits (a genetic covariation hypothesis). Second, correlated environmental pressures can generate selection on multiple traits, facilitating the evolution of trait complexes (an environmental covariation hypothesis). Here, we test these hypotheses using cryptic coloration (controlled by a likely incipient supergene) and chemical traits (*i.e.,* cuticular hydrocarbons, CHCs) involved in desiccation resistance and mate choice in *Timema cristinae* stick insects. We first demonstrate that population divergence in color-pattern is correlated with divergence in some (but not all) CHC traits. We show that correlated population divergence is unlikely to be explained by genetic covariation, as our analyses using genotyping-by-sequencing data reveal weak within-population genetic covariance between color-pattern and CHC traits. In contrast, we find that correlated geographic variation in climate and host plant likely generates selection jointly on color-pattern and some CHC traits. This supports the environmental covariation hypothesis, likely via the effects of two correlated environmental axes selecting on different traits. Finally, we provide evidence that misalignment between natural and sexual selection also contributes to patterns of correlated trait divergence. Our results shed light on transitions between phases of speciation by showing that environmental factors can promote population divergence in trait complexes, even without strong genetic covariance.

Introduction

Species formation entails the accumulation of phenotypic and genetic differences that generate reproductive isolation along a spectrum from weak to strong elimination of gene flow (Darwin, 1859; Dobzhansky, 1937; Seehausen et al., 2014). This process unfolds over extended time-scales, making it usually impractical to observe in its entirety. Thus, researchers often investigate varying levels of divergence among existing populations and species as proxies for different stages of the speciation process (*i.e.,* the speciation continuum) (Bolnick et al., 2023; Mallet, 2008; Nosil et al., 2009; Seehausen et al., 2014; Stankowski & Ravinet, 2021). As such, by examining trait differences and reproductive isolation among populations, it may be possible to infer the processes underlying the transition from weak to strong population divergence (*i.e.,* different phases of the speciation process).

Numerous processes can contribute to population divergence along the speciation continuum. For instance, distinct environmental conditions can exert divergent selective pressures that diminish the homogenizing effects of gene flow. This process can lead to ecological isolation that fosters population divergence (Nosil, 2012; Schluter, 2009). Consequently, divergent selection acting on a trait (or traits) can create statistical associations between adaptive alleles and multiple other loci, thereby facilitating the buildup of genetic differentiation that leads to speciation (Barton, 1983).

Given that local adaptation can be complex, numerous traits can be simultaneously under divergent selection. This way, patterns of statistical association and covariance will develop among these traits and their encoding genes as speciation proceeds (Feder et al., 2012; Smadja & Butlin, 2011). This process can lead to alternate coadapted gene complexes, comprised of distinct sets of traits and genes that differentially adapt populations to contrasting selection pressures (Dobzhansky, 1950; Mayr, 1954), As these complexes diverge, populations may evolve into ecotypes and species that exhibit partial or strong reproductive isolation (Lowry, 2012). From this perspective, a key challenge of studies on speciation is to elucidate the mechanisms that enable the adaptive divergence of traits and genes into differentially correlated complexes, the entities we recognize as ecotypes and species. We here investigate the mechanisms by which divergence in adaptive traits can become correlated (‘correlated trait divergence’, hereafter, Fig. 1).

Extensive research across diverse taxa has demonstrated numerous examples of correlated trait divergence. For example, certain phenotypic traits of threespine stickleback (*Gasterosteus aculeatus*) covary in diverging populations (*i.e.,* thus showing correlated divergence; Jones et al., 2012; Reimchen, 1983; Schluter, 1994). Specifically, variation in sticklebacks’ lateral armor plating can covary with body size and schooling behavior due to the pleiotropic effects of the *Eda* locus (Schluter et al., 2021). At the same time, lateral plate variation can also covary with pelvic spines, even though the latter are controlled by a gene located on a different chromosome from that of plates (*pitx1*; Chan et al., 2010; Shapiro et al., 2004). Similar patterns of correlated trait divergence have been observed in other taxa spanning fish other than stickleback (De-Kayne et al., 2022; Kautt et al., 2020; Reznick et al., 2001), mammals (Hager et al., 2022), mollusks (Koch et al., 2021), insects (Joron et al., 2011; Singer & McBride, 2010), and plants (Lee et al., 2017; Lowry & Willis, 2010; Todesco et al., 2020).

Despite these findings, the mechanisms by which divergence in different adaptive traits becomes correlated is less understood. In addition, it remains unclear to what extent these correlated trait complexes represent phenotypic polymorphisms (*i.e.*, morphs) with simple genetic bases (*e.g.*, major loci or chromosomal inversions) or divergent ecotypes with some degree of genome-wide differentiation and reproductive isolation. Consequently, the transition from morphs to ecotypes and species requires further study. This gap in our knowledge can be investigated by analyzing divergence in various traits across a continuum of population divergence.

We provide such a study here, based on wild stick insect populations spanning a wide range of divergence in adaptive traits and reproductive isolation. We consider two non-mutually exclusive hypotheses to test for and explain correlated trait divergence (Fig. 1). The first hypothesis posits that the genetic covariance of traits causes their correlated divergence (*i.e.,* a genetic covariation hypothesis; Fig. 1D). This is because genetic covariance arising from pleiotropy or linkage disequilibrium (*i.e.,* LD) can result in indirect correlated responses among traits, where selection acting on one trait impacts the response of other traits (Lande, 1979, 1982). This way, divergence among populations tends to occur along axes with greater genetic variation and covariation (Schluter, 1996). Thus, if the genetic covariation hypothesis holds true, we predict there will be substantial genetic covariance between locally adapted traits. If this pattern is due to physical linkage, it could also represent supergene evolution (Hager et al., 2022; Joron et al., 2011; Koch et al., 2021; Schwander et al., 2014; Todesco et al., 2020). We here use genetic mapping with partial genome sequences (*I.e*., genotyping-by-sequencing, GBS) to quantify genetic covariance and test this hypothesis.

The second hypothesis posits that the environmental variation across the landscape generates correlated selection on different traits, resulting in their correlated divergence (*i.e.,* an environmental covariation hypothesis)(Fig. 1E). This phenomenon can manifest itself in two ways. First, a single selective pressure can influence multiple traits concurrently, as may occur when high predation impacts multiple characters in guppies (*Poecilia reticulata;* Endler, 1995). Second, environmental gradients can be structured across the landscape in a manner that causes multiple environmental pressures to covary. The correlated selective pressures arising from this covariation can jointly affect traits with no genetic or functional relationship with each other (Armbruster & Schwaegerle, 1996). For example, higher predation intensity in guppy populations is often correlated with other abiotic factors such as higher temperatures and light intensity, which affect multiple traits in guppies (Endler, 1995). In any case, if the environmental covariation hypothesis holds true, we predict that the environmental factors exerting selective pressures on different traits will be correlated with each other and with trait co-divergence. This process could be further influenced by the alignment between different selective forces acting on the traits, such as different sources of natural or sexual selection (Endler, 1980; Reimchen, 1989; Safran & McGraw, 2004; Servedio & Boughman, 2017).

Study system

We investigate correlated trait divergence and test the two aforementioned hypotheses using *Timema cristinae* stick insects, an emerging model system for studying adaptation and genomics of speciation. These wingless, plant-feeding insects are distributed throughout the Santa Ynez mountains in California, USA (Vickery, 1993). *Timema cristinae* is primarily found on two host-plant species: *Adenostoma fasciculatum* (Rosaceae), and *Ceanothus spinosus* (Rhamnaceae) (Nosil & Crespi, 2006; Sandoval, 1994b). Divergent selection associated with the two host-plant species contributes to partial reproductive isolation between populations associated to them, leading to the *Adenostoma* and *Ceanothus* ecotypes (Nosil, 2007). Here, we specifically investigate two types of adaptive traits that vary between the ecotypes (Fig. 2): (1) cryptic color-pattern polymorphism; and (2) cuticular hydrocarbons.

Cryptic color-pattern morphs in *T. cristinae* are characterized by the presence versus absence of a white dorsal stripe. This trait is subject to divergent selection exerted by visual predators such as birds and lizards (Sandoval, 1994a, 1994b). The green-striped morph is more cryptic on the needle-like leaves of *Adenostoma* plants, while the green-unstriped morph is more cryptic on the broader leaves of *Ceanothus* (Fig. 2). Accordingly, the frequencies of the color-pattern morphs vary between host-plant ecotypes, with the striped morph being more prevalent on the *Adenostoma* ecotype and the unstriped morph predominating on the *Ceanothus* ecotype (Bolnick & Nosil, 2007; Nosil et al., 2018; Sandoval, 1994a, 1994b). Although *T. cristinae* has a third morph that is melanistic (dark) in body color and lacks a stripe (Comeault et al., 2015), its frequencies do not exhibit significant host-related divergence between populations (Comeault et al., 2015; Lindtke et al., 2017). Therefore, it is not the focus of this study. Despite the pronounced selection acting on the color-pattern morphs, a combination of gene flow and negative frequency-dependent selection prevents fixation of either the green-striped or green-unstriped morph in the populations. This creates a geographic mosaic in the degree of color-pattern divergence among populations (Nosil et al., 2018, 2024). Recent genomic research has revealed that color-pattern is strongly associated with two large regions of suppressed recombination on chromosome 8, referred to as the *Pattern* and *Mel-Stripe2* loci (Nosil et al., 2024). Together, these two loci thus likely function as an incipient supergene (see Nosil et al., 2024).

In addition to color-pattern, we examine cuticular hydrocarbons (CHCs, hereafter), which are waxy chemical compounds with roles in adaptation, sexual selection, and speciation in insects. For example, CHCs have been shown to contribute to maintenance of water balance, communication, and mate recognition in insects (Chung & Carroll, 2015; Otte et al., 2018). The functions played by CHCs vary according to some properties of these compounds, such as their carbon chain length (Chung & Carroll, 2015). In *T. cristinae*, it has been shown that CHCs are controlled by multiple loci across the genome, exhibiting non-zero but modest heritability (Chaturvedi et al., 2022; Riesch et al., 2017). A recent study has further reported that single nucleotide polymorphisms (SNPs) associated with CHC variation overlap with genetic regions associated with climate adaptation in different *Timema* species (Chaturvedi et al., 2022). This link between CHCs and climate adaptation is most likely related to their general role in maintaining water balance and preventing desiccation (Menzel et al., 2017, 2018; Neems & Butlin, 1995). It has also been shown that CHC profiles differ between the *T. cristinae* host-plant ecotypes (Riesch et al., 2017), but the extent to which this relationship reflects adaptation remains unclear. Most critically, greater divergence in female CHCs in *Timema* is associated with mate choice and sexual isolation, both within and between species (Riesch et al., 2017; Schwander et al., 2013). This was demonstrated by manipulative perfuming experiments, which have established a causal role for female CHCs in male mate choice within a population of *T. cristinae* and sexual isolation between a species pair (Riesch et al., 2017). These results prompted our focus on female rather than male CHCs in the current manuscript.

Previous research in *T. cristinae* has shown that the effects of color-pattern divergence on genetic differentiation are restricted to chromosome 8, while divergence in female CHC is associated with greater genome-wide differentiation (Riesch et al., 2017). However, these patterns were analyzed independently, leaving unanswered questions regarding the potential cumulative effects of differentiation in each trait, the extent to which they show correlated trait divergence, and why.

Therefore, we seek to bridge this gap here by exploring correlated trait divergence between color-pattern and female CHCs among populations.

In our study, we first tested whether color-pattern and female CHC traits show correlated divergence among populations spanning different degrees of trait divergence. We subsequently used GBS data to test the genetic covariation hypothesis, by testing the prediction that there is within-population genetic covariance between color-pattern and CHCs. We then tested the environmental covariation hypothesis by exploring the relationship between the environmental conditions that exert selection on color-pattern (*e.g.*, host plants) and on chemical traits (*e.g.*, climate) across *T. cristinae* populations. Specifically, we assessed the prediction that these environmental axes are correlated. Given the importance of CHCs in sexual isolation, we took advantage of abundant experimental data on mate choice in *T. cristinae* to also explore whether sexual selection may affect patterns of correlated divergence between color-pattern and CHCs. Finally, we integrated our results with existing knowledge on differentiation and reproductive isolation among *Timema* species, generating broader understanding of transitions between phases of speciation and on the role of genomic divergence in speciation.

Materials and Methods

Association between divergence in color-pattern and CHCs

We began our investigation by estimating population divergence in color-pattern and CHCs to then test whether these sets of divergence are correlated. To this end, we used phenotypic data from Riesch et al. (2017) (https://dx.doi.org/10.5061/dryad.nq67q), encompassing seven populations of *T. cristinae* at different stages of divergence (*i.e.*, 21 population pairs, Table S1). We assessed divergence in color-pattern among populations based on the frequency of the striped morph in the population (*i.e.,* green-striped versus green-unstriped), for this set of 21 population pairs.

Divergence in female CHC traits also was analyzed using data from Riesch et al. (2017) on females from the same seven populations. Briefly, Riesch et al. (2017) collected wild individuals, cold-euthanized, and extracted their CHCs by immersing them in 1mL of hexane before preserving the specimens in 95% ethanol. Gas chromatography was used to quantify 26 CHC compounds in each insect. We quantified the abundance of each CHC by measuring the area under its chromatographic peak, which corresponds to the amount of the compound represented by that peak. These areas were then compared to the area of a known quantity of an internal standard, which was added to each sample prior to gas chromatography to ensure accurate quantification. Because CHC abundances vary with body size, we standardized the data by converting absolute amounts into relative proportions. For each sample, we divided the quantity of each CHC by the total sum of all quantified CHCs. To remove the non-independence among CHCs, proportional abundances were log-contrast transformed using 5-methylheptacosane (5Me27) as a reference, resulting in 25 transformed values per individual. The 25 CHC compounds were categorized into three classes based on their carbon chain length: eight pentacosanes, seven heptacosanes, and ten nonacosanes (*i.e.*, chains of 25, 27, and 29 hydrocarbons, respectively).

In this study, we analyzed this categorized dataset of 25 CHC compounds, treating each of the three CHC classes as a distinct trait (*i.e.,* pentacosanes, heptacosanes, and nonacosanes). Following Riesch et al., (2017), we reduced data dimensionality by performing separate principal component analyses (PCA) for each CHC trait, based on covariance matrices with promax rotation. We then retained the principal component axes with an eigenvalue larger than the mean eigenvalue. In total, we retained two PCs for pentacosanes, one for heptacosanes, and three for nonacosanes (Table S2). These steps were conducted in IBM SPSS Statistics software (v29.0.2.0), following Riesch et al. (2017). We estimated the mean of the scores for each principal component for each CHC trait for each population, and then estimated the pairwise Euclidean distances between populations in R.

The data from Riesch et al. (2017) comprised a broad spectrum of diversity across all traits. For instance, with respect to color-pattern, the striped morph frequency within populations range from as low as 1% (PRC) to as high as 86% (LA). We used these distances in color-pattern and CHCs to test for correlated population divergence among these traits. We used Mantel tests to estimate the correlations between color-pattern and each CHC trait separately and evaluated their significance. Mantel tests were performed in the *vegan* R package v2.6-4 (Oksanen et al., 2022) based on 10,000 permutations. To complement these analyses, we used Bayesian linear mixed models to estimate the degree of association between color-pattern divergence and CHC divergence (BLMM, see Supplemental Information for details).

Genetic covariance between color-pattern and CHCs using genotyping-by-sequencing

To test whether the genetic covariation hypothesis can explain the observed correlated trait divergence, we used genomic prediction analyses to estimate the genetic covariance between color-pattern and each of the CHC traits within a single population (FHA population, 34.518 N, -119.801 W). Specifically, we estimated breeding values (BVs) using genotype-by-sequencing data and the results of multilocus (polygenic) genome-wide association (GWA) mapping for color-pattern and CHC traits, applying Bayesian sparse linear mixed models (BSLMM; Zhou et al., 2013). These BVs were then used to estimate the genetic covariance between these types of traits.

We opted for genomic prediction over traditional approaches such as QTL mapping or breeding experiments because it is particularly well-suited for our study system and objectives. Genomic prediction performs well for both polygenic traits (e.g., CHCs), which are influenced by many loci with small and uncertain individual effects (Chaturvedi et al., 2022; Riesch et al., 2017), and traits primarily determined by one or a few major-effect loci (e.g., color-pattern; Gompert et al., 2019; Nosil et al., 2024; Zhou et al., 2013). While QTL-based approaches are powerful for identifying major effect loci, they can struggle to capture the contribution of traits controlled by many small-effect loci. Similarly, breeding experiments provide valuable insights into heritability and inheritance patterns, but typically do not explicitly account for major-effect loci (Aase et al., 2022; Gienapp et al., 2017). In *T. cristinae,* controlled breeding experiments are particularly challenging, given this species has only one generation per year and rearing individuals in laboratory conditions is difficult. Genomic prediction with BSLMM provides a feasible alternative, allowing us to estimate genetic parameters directly from field-collected individuals while accounting for both polygenic and major-effect contributions, and it further enables the estimation of the realized (rather than expected) kinship matrix.

We selected the FHA population to perform the genetic covariance analyses because its large population size enables appreciable sample sizes and robust genetic mapping of color-pattern and CHC traits, which was not feasible in other single populations (Comeault et al., 2015; Riesch et al., 2017). We note that previous studies have shown that the genetic basis for color-pattern is conserved across *T. cristinae* populations (Lindtke et al., 2017; Nosil et al., 2024), making it likely that our estimates of genetic covariance are representative of other populations (see below also for results testing this assumption using phenotypic covariances). We thus used the results of the GWA mapping of color-pattern in FHA from (Nosil et al., 2024) (https://zenodo.org/records/11050621), and performed a new GWA mapping for the different CHC traits (*i.e.,* pentacosanes, heptacosanes and nonacosanes) using the same sample set. Because color-pattern was only mapped in green-unstriped and green-striped individuals (*i.e.,* both sexes but excluding melanistic individuals) and CHCs were only mapped in females (*i.e.,* all morphs but no males), the sample sizes were different between the two sets of data (pattern n = 538, CHC n = 197), which only partially overlapped (n for both traits = 183).

Prior to BLSMM analysis, we tested and found no evidence for population structure in the FHA genomic dataset. The first two principal components (PC1 and PC2) explained only 0.87% and 0.80% of the total variance, respectively, with the top ten PCs explaining a cumulative of 7.51%. This low proportion of variance explained, along with the lack of distinct genetic clusters in the PCA plot (Fig. S1-S2), suggests no detectable population structure. This is not surprising considering that the FHA population inhabits a relatively small patch of *Adenostoma* habitat (< 1 km^2, Comeault et al., 2014). Additionally, the kinship matrix estimated using BSLMM in *gemma* (Zhou et al., 2013) showed no significant effects of relatedness among the samples, with most being distantly related (mean standardized relatedness = 0.00, -0.02–0.01, 95% confidence interval, Fig. S3). Only one pair of samples showed a high standardized relatedness value (0.54, the maximum observed), but, overall, just 0.04% of pairwise comparisons exceeded 0.05 (Fig. S3). This makes this dataset ideal for genetic covariance analyses.

We did the GWA mapping for each CHC trait using the BSLMM in *gemma* (Markov chain Monte Carlo, MCMC, for each trait: 10 chains, sampling steps: 1,000,000; burn-in: 200,000; minor allele frequency threshold: 0; Zhou et al., 2013). The hyper-parameters of the GWAS runs for each trait were summarized in Table S3. We next estimated the BVs based on the model-averaged effect estimates for each SNP, which includes the possible sparse/main effect and polygenic effect of each SNP. We calculated genetic correlations of the BVs across traits to compute the standardized genetic covariance matrix (*i.e.,* the standardized G-matrix). Confidence intervals on the Pearson correlations were calculated using bootstrap re-sampling of the individuals (1000 replicates each). The heritability values (the diagonal in genetic correlation matrices) were taken as the proportion of variance explained (PVE) from *gemma* outputs. We evaluated the consistency of this estimate of genetic covariance based on genomic prediction across traits using jackknife re-sampling procedure (see Supplementary Information for details).

Because we did not have large enough sample sizes to conduct GWA and obtain BVs to estimate the genetic covariance in other *T. cristinae* populations, we estimated the phenotypic covariance between color-pattern and CHC traits in FHA and several additional populations (n=10). Given that phenotypic covariance often mirrors genetic covariance (Lande & Arnold, 1983), this analysis provided insights into whether genetic covariance exists between color-pattern and CHCs in other *T. cristinae* populations. We began this analysis by estimating the phenotypic covariance between color-pattern morphs and variation in each CHC trait in the FHA population. We used phenotypic data from Riesch et al. (2017), which included color-pattern morphs and the proportional abundances of each CHC trait in the FHA population (*i.e.*, the same phenotype used for GWA mapping). We estimated the phenotypic covariance between color-pattern and the proportional abundances for each CHC trait, then assessed the significance of each analysis with permutation tests (n=1,000 permutations) in R v4.3.2 (Core team, 2023).

Subsequently, we expanded this analysis to additional *T. cristinae* populations with available CHC data and individual photographs for assessing color-pattern variation (Table S2, Riesch et al., 2017). Individuals were classified into green-striped, green-unstriped or melanistic morphs, following previously established, highly repeatable classifications (Nosil, 2004). We selected 10 populations based on two criteria: (1) the presence of both striped and unstriped individuals; and (2) a sample size of approximately 20 individuals per population (including both sexes to boost statistical power). Melanistic morphs were excluded from the analyses. Following the steps described earlier, we reduced CHC data dimensionality by performing PCA on the log-contrast transformed CHC abundances from Riesch et al. (2017), yielding PC scores for pentacosanes, heptacosanes and nonacosanes. Since CHC profiles are known to vary significantly by sex, we extracted sex-specific residuals for each PC. We then estimated phenotypic covariance between color-pattern morphs and the corrected CHC PCs within each population. To assess statistical significance, we performed permutation tests in R (1,000 permutations each; Core team, 2023). These results were consistent with analyses conducted using Bayesian hierarchical linear models, which allowed for information-sharing across the 10 populations while accounting for population-level random effects on the association between color-pattern and CHC variation (see Supplementary Information for details).

Environmental covariation

We next assessed the environmental covariation hypothesis by testing the prediction that there is a correlation between the primary axes of selection for color-pattern versus CHC traits. Prior studies have demonstrated that host-plant species exert strong selective pressures on color-pattern morphs (Sandoval et al., 1994; Nosil et al., 2006, 2018), while CHCs in *Timema* species, including *T. cristinae*, are known to correlate with climatic variation and exhibit signatures of selection across the genome (Chaturvedi et al., 2022). Therefore, these environmental variables provided a meaningful proxy for selection pressures acting on these traits. A*s* such, we here estimate the covariation between host-plant species and climate (see Figs. S4-S5 for association between climate and CHCs)

To test this prediction, we used data from Nosil et al. (2018) (https://doi.org/10.5061/dryad.v1q13). This dataset comprised n=206 populations, n=98 on *Adenostoma* and n=108 on *Ceanothus* host plants, with information on elevation and 19 data layers from WorldClim (Harris et al., 2014). We performed Wilcoxon signed-rank tests between all climatic variables and host plants (Table S5) and among elevation and host plants. We performed Pearson correlations between climate and elevation, using every climate layer with the exception of ‘precipitation of driest month’, which was invariant (*i.e.,* zero) for all populations. Additionally, we performed PCAs to summarize these data and conducted the correlation analysis with elevation, as well as using the principal components as latent climate variables. The first two principal components summarizing the bioclimatic data corresponded to 92.8% of the variation explained (PC1=67.9%, PC2=24.9%). The variables that contributed most to PC1 were the precipitation variables, as well as other temperature variables such as annual mean temperature (see Table S5).

We further used climate and elevation data to estimate the association between these variables and CHC traits using the data from Riesch et al. (2017). To obtain more robust results we used data from 15 populations whose localities were also present in the dataset containing climate/elevation information (Table S2). We then summarized the different CHC traits according to their chain length (*i.e.,* pentacosanes, heptacosanes, or nonacosanes) using PCA in the IBM SPSS Statistics software (v29.0.2.0), as described above. We estimated the relationship between the first principal component axis describing CHCs variation (PC1 = 50.3%, 88.5%, and 92.0% of the variance explained for pentacosanes, heptacosanes and nonacosanes, respectively) and the first principal component axis describing climatic variation, as well as elevation (see Figs. S4-S5). All the statistical analyses were performed in R (R Core Team, 2023).

Misalignment between natural and sexual selection

Because divergence in color-pattern did not significantly correlate with divergence in certain CHC traits (in particular pentacosanes, see results below), we tested an additional hypothesis that sexual selection may explain the observed disparate pattern. We specifically asked if sexual selection or CHC-based mate choice could explain the the weak correlation between population divergence in some CHC traits and in color-pattern. In *Timema,* female CHCs causally affect mate choice within populations and species (*i.e.,* sexual selection), and correlate with sexual reproductive isolation between populations and species (Riesch et al., 2017). Thus, it is plausible that sexual selection is shaping the divergence of CHCs across populations. Natural and sexual selection pressures could thus be simultaneously driving variation in female CHCs in different ways for different CHC traits (Endler, 1980; Safran & McGraw, 2004; Servedio & Boughman, 2017). Therefore, we posited a scenario where a misalignment between natural and sexual selection pressures could disrupt environmental covariation from driving correlated trait divergence (Fig. 3). If this hypothesis held true, we could predict that the divergence in pentacosanes should exhibit a detectable regression relationship with sexual isolation levels among populations.

To test whether sexual selection underlies patterns of CHC divergence, we estimated the regression relationship between divergence in each CHC trait and sexual isolation. We used the pairwise Euclidean CHC distances calculated above and the distances in sexual isolation between the 21 population pairs derived from the seven populations. We used the data from Riesch et al. (2017), who calculated the pairwise index of sexual isolation (I_{PSI}) based on mating propensity derived from no-choice mating trials from Nosil & Hohenlohe (2012). While previous research has assessed the correlation between female CHCs and sexual isolation by aggregating all CHC traits (Riesch et al., 2017), we here for the first time estimated distances for each CHC trait separately. We fitted Bayesian linear mixed models (BLMM) to estimate the degree of association between CHC divergence and sexual isolation, including random effects accounting for the pairwise nature of the variables (Clarke et al., 2002; Gompert et al., 2014). The Bayesian approach uses a Markov chain Monte Carlo framework to estimate the regression coefficients and deviance information criterion (DIC) for model selection. The model was fitted via the *rjags* R package (Plummer, 2018), including linear models ($y \sim x$), and quadratic models ($y \sim x^2 + x$), where divergence in each CHC class was the explanatory variable and sexual isolation was the response variable. The variables were scaled and centered before the analyses. We ran three chains of the model, with 10,000 iterations, a burn-in of 2,000 iterations, and a thinning interval of 5. The results are all represented in Table S6. Additionally, we assessed the combined effects of the CHC traits on sexual isolation. We also included geographical distances in the model, which were calculated using the geodesic distance between coordinate points and then logarithmically transformed (Rousset, 1997). We ran BLMM using the same parameters described above. The results are represented in Table S7. All the statistical analyses were again performed in R (R Core Team, 2023).

Results

Divergence in color-pattern is correlated with divergence in some CHC traits

We find that patterns of co-divergence between color-pattern and CHCs across populations differed among CHC traits. Our analyses indicated that population divergence in color-pattern was strongly correlated with divergence in nonacaosanes (r=0.82, P<0.001, Mantel test; Fig. 4), and was modestly correlated with divergence in heptacosanes (r=0.38, P=0.10, Mantel test). However, color-pattern divergence among populations exhibited no significant correlation with female pentacosanes (r=0.11, P=0.27, Mantel test; Fig. 4). These results were supported by BLMM, showing a strong association between color-pattern divergence and nonacosane divergence (BLMM β = 0.80, 95% equal tail probability interval, 95% equal-tail probability interval, ETPI = 0.52 – 1.06), but less so for heptacosane (BLMM β = 0.33, 95% ETPI = -0.16 – 0.78) and pentacosane divergence (BLMM β = 0.13, 95% ETPI = -0.34 – 0.59). We next evaluate the results of the hypothesis testing to explain the causes of this variation.

Weak genetic covariance between color-pattern and CHCs within the same population

We assessed the genetic covariation hypothesis by testing the prediction for within-population genetic covariance between color-pattern and CHCs. Our results showed modest genetic covariance values (at best) between color-pattern and all CHC traits, being r = 0.10 for nonacosanes (Pearson correlation, 95% CIs = -0.04 – 0.24, Fig. 5A), r = -0.06 for heptacosanes (Pearson correlation, 95% CIs = -0.20 – 0.09), and r = -0.16 for pentacosanes (Pearson correlation, 95% CI = -0.32 – 0.00, Fig. 5A). Jackknife re-sampling procedures yielded consistent estimates with relatively narrow confidence intervals: -0.16 for color-pattern and pentacosanes (95% CI: -0.19, -0.08); -0.06 for pentacosanes (95% CI: -0.10, -0.022); and 0.10 for nonacosanes (0.046, 0.139).

These results were further supported by the lack of significant phenotypic covariance between color-pattern and the CHC traits in FHA. Specifically we found no association between color-pattern and proportional abundances of each CHC trait (pentacosanes, heptacosanes and nonacosanes). Phenotypic covariance was zero in all comparisons, with permutation p-values of 0.07, 0.40, 0.22, respectively.

We also found no significant association (i.e., phenotypic covariation) between color-pattern and CHC trait variation within any of the other 10 populations analyzed (summarized in Table S2). Bayesian hierarchical linear models further supported these findings, indicating that color-pattern morphs did not credibly explain variation in the different CHC traits (Table S8). In all analyses, the credible intervals included zero. Additionally, random intercepts for each population showed only minor deviations from the overall population mean (Table S9). These results imply that it is unlikely that there would be significant genetic covariance between color-pattern and the different CHC traits. Thus, our findings do not support for the genetic covariation hypothesis.

Host plant and climate are associated with correlated trait divergence

To assess the environmental covariation hypothesis, we tested the prediction for a correlation between the primary axes of selection (i.e., environmental variation) for color-pattern versus CHC traits, *i.e.,* between host-plant species and climate. We found a robust association between host-plant species and nearly all 19 bioclimatic variables from WorldClim except for the 'maximum temperature of the warmest month' (Table S5). The association was exemplified by the highly significant correlation between host-plant species and the first principal component axis summarizing the climatic variables ($W = 7872$, $P < 0.001$, Wilcoxon signed-rank test; Fig. 5B). We found that the relationship stems from the common association of climate and host plant species with elevation ($r = 0.96$, $P < 0.001$, Pearson correlation; $W = 7932$, $P < 0.001$, Wilcoxon signed-rank test; respectively). This alignment of climatic variables and host plants could explain the positive correlation observed between divergence in color-pattern and nonacosanes and perhaps heptacosanes.

Sexual selection can explain weak correlated trait divergence

We proposed a scenario where a potential misalignment between natural and sexual selection could disrupt the environmental covariation from driving correlated trait divergence of traits (Fig. 6). If true, then pentacosanes are predicted to exhibit a strong relationship with sexual isolation levels among populations rather than with color-pattern. Consistent with this hypothesis, we found a strong positive relationship between divergence in pentacosanes and sexual isolation, but with a quadratic model providing a better fit than the linear alternative (BLMM linear β=0.76, 95% ETPI = 0.49 – 1.03; quadratic β = 0.40, 95% ETPI = 0.07 – 0.73; Fig. 6; Table S6). Furthermore, BLMM revealed that divergence in female pentacosanes explain sexual isolation slightly better than geographical distance (Table S7). In contrast, heptacosanes and nonacosanes did not exhibit significant associations with sexual isolation (BLMM β=0.09, 95% ETPI = -0.38 – 0.56; β=0.01, 95% ETPI = -0.45 – 0.48; respectively. Linear models provided a slightly better fit than quadratic models for these relationships with heptacosanes and nonacosanes (Table S6). Thus, sexual selection could be exerting effects mainly on pentacosanes, potentially in misalignment with the effects of natural selection associated with climate.

Discussion

Speciation is often a complex process involving the divergence of various genetic loci and traits. When multiple traits co-diverge, their synergistic interaction can lead to enhanced phenotypic and genetic differentiation, potentially strengthening isolating barriers between populations. Therefore, understanding correlated divergence in adaptive traits and the mechanisms underlying it can be crucial for understanding the processes by which population divergence and genome-wide differentiation can build up throughout different phases of speciation. In this study, we demonstrate that the correlated divergence of adaptive traits in *T. cristinae* is driven more by environmental covariation than by genetic covariation. Furthermore, our findings suggest that the effects of sexual selection contrast with the effects of natural selection, affecting correlated trait divergence. Our analyses and results on the mechanisms that underpin correlated trait divergence contribute to knowledge concerning transitions between phases of speciation (Corl et al., 2010; Peccoud et al., 2009) and the role of genomic divergence in speciation (Nosil & Feder, 2013). Discussion of our main findings follows.

Environmental covariation drive trait co-divergence, despite weak genetic covariation

In this study, we report significant correlated divergence between color-pattern and one class of female CHCs (*i.e.,* nonacosanes, and, to lesser extent, heptacosanes). We subsequently used a method that applies GWA to estimate a quantitative genetics measure (*i.e.,* genetic covariance), thus integrating frameworks from two different traditions in speciation research (Stinchcombe & Hoekstra, 2008). Our analyses show weak genetic covariance between color-pattern and all CHC traits analyzed within the FHA population, which challenges the hypothesis of a strong genetic link between these traits. We also found that there is not significant phenotypic covariance between color-pattern and the different CHC traits across 10 additional populations (Table S2, S8, S9). While this suggests that genetic covariance between these traits may be weak or absent in these populations, definitive conclusions would require direct estimation of genetic covariance using genomic data within these focal populations. Future studies with larger sample sizes and family-based designs could resolve to what extent phenotypic and genetic covariances mirror one another.

These genetic covariance results are consistent with the fact that color-pattern and CHCs are governed by distinct genetic architectures in *T. cristinae*. While color-pattern is under the control of an incipient supergene with high heritability (Comeault et al., 2015; Nosil et al., 2018, 2024), CHCs are influenced by multiple genetic regions with modest heritability (Chaturvedi et al., 2022; Riesch et al., 2017). We acknowledge that genomic prediction in GWAS faces challenges, particularly with modest sample sizes, sparse markers, and for traits with polygenic architectures such as CHCs in our study. These factors may affect the precision of individual SNP effect estimates and polygenic predictions, and we caution against over-interpreting their absolute values. Nevertheless,our primary conclusions rely on genetic covariances, which tend to be more robust than individual SNP effects. We found that there does not appear to be shared loci of large effect pleiotropically influencing both traits (*i.e.*, loci with effects on CHCs do not appear to be clustered or preferentially located within the supergene region governing color-pattern). Consequently, our study implies that selection acting on one of these traits may not necessarily drive evolutionary changes in the other, indicating that their genetic covariance is unlikely to contribute to their correlated divergence. Future work with larger samples, denser genomic coverage (e.g., whole-genome sequencing), and independent validation could refine these estimates.

Instead, our results show that environmental covariation across the landscape where *T. cristinae* lives can better explain the correlated divergence observed between color-pattern and CHC traits. Our results reveal an alignment of the environmental axes that exert correlated selection on each trait. Specifically, host-plant species and climatic variables strongly correlate in space due to their association with elevation. For color-pattern, this relationship reflects a difference in the abundances of hosts with elevation, while for the CHC traits nonacosanes and heptacosanes it may reflect differences in temperature and precipitation. In this regard, nonacosanes, whose divergence showed the strongest covariance with color-pattern, contain the longest carbon chains. Longer chained CHCs tend to have higher melting temperatures and, as a result, are generally associated with a greater ability to maintain insect water balance (Chung & Carroll, 2015; Gibbs & Pomonis, 1995). Thus, the findings for nonacosanes are consistent with a role for these CHCs in creating a water-proof layer that varies in an elevation-dependent manner.

In summary, our results align with the concept of environmental covariation generating correlated trait divergence, even in the absence of strong genetic covariance. Our results challenge a prevailing view in the literature that emphasizes genetic and developmental causes for trait covariation compared to the environment (as debated in Armbruster & Schwaegerle, 1996; Endler, 1995). Nonetheless, the mixed support and role for genetic covariance in adaptive divergence has been highlighted in a past meta-analysis on several taxa (Agrawal & Stinchcombe, 2009), and our results are consistent with this past work. Furthermore, our findings refine the current emphasis on the role of specific genetic architectures that suppress recombination in evolution, implying that such architectures can facilitate adaptation and supergene evolution without necessarily contributing to genome-wide differentiation and speciation (Schwander et al., 2014; Yeaman, 2013). Our results highlight the important role of understanding complex environmental variation and covariation to further inform results obtained on genomic analyses to improve our understanding of population divergence and speciation.

Contrasting effects of natural and sexual selection

Our results also reveal that divergence in pentacosanes is not correlated with divergence in color-pattern. That is, although pentacosanes are influenced by climatic conditions and are likely subjected to their natural selection pressures (Figs. S4-S5) (Chaturvedi et al., 2022), the environmental covariation between climate and host-plants seems to be insufficient to generate correlated divergence between pentacosanes and color-pattern. Instead, divergence in pentacosanes is more correlated to sexual isolation among populations. This association is quadratic, potentially indicating the nonlinear impacts of trait evolution on mate choice and reproductive barriers (Bailey, 2008; Blais et al., 2004; Kingsolver et al., 2001). That is, our results show a rapid evolution of sexual isolation once populations reach threshold levels of divergence in female pentacosanes. Conversely, neither heptacosanes nor nonacosanes show significant association with sexual isolation, suggesting that the results found in Riesch et al. (2017) were mainly linked to variation in pentacosanes. These results align with studies showing that shorter-chained CHCs are more associated with attractiveness and sexual selection than longer-chained ones, typically associated with desiccation resistance (Berson et al., 2019; Ingleby et al., 2014; Mitchell et al., 2023; Simmons et al., 2014; Steiger et al., 2013). Shorter-chained CHCs exhibit lower melting points and higher volatility, likely enhancing the detection of signaling compounds and increasing their prominence in sexual selection (Menzel et al., 2017).

Collectively, our findings imply that the interplay between natural and sexual selection may lead to contrasting evolutionary trajectories for pentacosanes. While natural selection may drive the optimization of CHCs for climatic adaptation, sexual selection may favor specific chemical profiles for successful mate attraction and recognition (Thomas & Simmons, 2010). This scenario has been previously described in crickets, whose CHC profiles contrasted significantly under the effects of natural versus sexual selection (Mitchell et al., 2023), and may occur more generally for traits that experience natural and sexual selection (Endler, 1980; Reimchen, 1989; Safran & McGraw, 2004; Servedio & Boughman, 2017). As such, our results suggest that, although sexual selection on *T. cristinae* pentacosanes contributes to the evolution of sexual isolation among populations, it may disrupt the correlated selective pressures that lead to correlated trait divergence with color-pattern. To further elucidate this phenomenon, future research in the system should quantify the strength and form of natural and sexual selection targeting different classes of female CHCs.

Evolution from morphs to ecotypes to species

This study provides insight into the processes that promote the transition from morphs to ecotypes, with variable levels of population differentiation to the more divergent units often considered species. In terms of ecotypes, recent studies in *Timema* have shown how fluctuation can occur around very different equilibria, resulting in ‘balanced ecotypes' that represent elements of polymorphism but also intermediate stages of ecological speciation (Nosil et al., 2018, 2024). Here, we build upon past work by showing a pivotal role for environmental covariation in driving correlated and more pronounced population divergence in *T. cristinae*. Despite this divergence, reproductive isolation among these populations is still far from complete (Nosil, 2007). In this context, why has divergence of *T. cristinae* populations not progressed further towards speciation?

To shed light on this question, we consider features that characterize distinct *Timema* species. Here, we highlight two key phenomena: genome-wide genomic differentiation and strong divergence in CHCs (Fig. S6) (Riesch et al., 2017; Schwander et al., 2013). These two factors may be required to create new *Timema* species, likely facilitated by periods of geographic isolation. In contrast, notable differences in body color or color-pattern do not always occur between *Timema* species and thus do not appear essential for species divergence. As such, we speculate that if correlated trait divergence does play a role in the later stages of speciation, it is more likely to involve traits other than body color or color-pattern, such as CHCs and intrinsic post-mating isolation. Therefore, the path to greater population divergence may sometimes lead to more complete speciation, as observed in *Rhagoletis* flies (Powell et al., 2013), but in other cases as reported here, it may not. We speculate that the outcome depends on the traits involved and their effects on adaptation versus reproductive isolation, as well as their interaction and relationship with environmental variation. Further studies are now required to test these hypotheses, using an even wider range of the divergence process such that the entire path to new species can be reconstructed along the speciation continuum.

Conclusion

Here, we report correlated divergence between color-pattern morph variation and certain CHC traits, primarily attributed to environmental covariation across the landscape rather than to strong genetic covariance. Our results thus underscore a pivotal, but often understudied, role for environmental covariation in the speciation process. Interestingly, environmental variation at a smaller scale, that is the colors of leaves versus stems within plant individuals, has been implicated in the evolution of color morphs in the *Timema* system (Nosil et al., 2020; Villoutreix et al., 2023), opening the potential for further work to even more fully investigate the evolution of morphs to ecotypes to species. A weak correlation between color-pattern and other CHC traits provided an opportunity to explore alternative explanations for weak correlated trait divergence, shedding light on how a misalignment between sexual and natural selection might affect trait divergence and speciation. Our study highlights the potential power of investigating multiple dimensions that drive population divergence and reproductive isolation, offering valuable insights into the processes that can facilitate or limit population and genomic divergence and transitions between phases along the speciation continuum. Comparable joint tests of the roles of genetics and environment in other taxa will likely continue to shed light into the general processes driving and constraining speciation.

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Data availability: This manuscript does not contain new data, all of which is publicly archived as described in the Methods section. All scripts and code used for analysis has been archived at Zenodo (https://doi.org/10.5281/zenodo.11204264).

Link to access scripts:
https://zenodo.org/records/11204264?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjcxN2M1NDgzLTM2YjYtNDcwZS1iOTZjLWNlMGY5OGI2MDJkMiIsImRhdGEiOnt9LCJyYW5kb20iOiJiOGQzMmM0YmIzZTA2NGEzNTZhNDg0YWNiMzlhMDZkZiJ9.-HmmKLaReuaRrInOfaKnE_5vgsfRF3PskS-x3JdA0aL9SVlSpLbRCxABb-faBrjCRA8QUdkUQ4P1sk8p2oKRgw

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Figure Legends

Figure 1. Hypotheses to explain correlated trait divergence. (**A**) Phenotypes for this hypothetical example. (**B**) Example of when population divergence in wing and in body color are not correlated. (**C**) Example of correlated trait divergence among populations. Greater divergence in wing size is correlated with greater divergence in body color. Two general and not mutually exclusive hypotheses are proposed to explain correlated trait divergence. (**D**) The 'genetic covariation' hypothesis predicts that genetic covariance between traits leads to correlated trait divergence. In this example, linkage disequilibrium (LD) between the gene controlling body color (BC) and the three genes controlling wing size (WS1-3) generates positive genetic covariance. (**E**) The 'environmental covariation' hypothesis predicts that correlated selective pressures lead to correlated trait divergence. In this example, background color influences body color, while precipitation levels (precip.) influence wing size across populations.

Figure 2. *Timema cristinae* ecotypes and the traits investigated in this study. (**A**) Host-plant species which define the ecotypes. (**B**) Color-pattern morphs in *T. cristinae*, mainly selected by host-plant species. (**C**) Female cuticular hydrocarbons (CHCs), with functions in chemical communication and water balance. Cuticular hydrocarbons are associated with climatic adaptation and they affect mate choice, thus are also influenced by sexual selection. (**D**) The relative abundance of different host-plant species and climatic variables both tend to vary with elevation across the landscape where *T. cristinae* is found. (**E**) Map of the populations used in this study. The association between host-plant species and climate was taken into account in the selection of study sites (*i.e.*, populations on *Adenostoma* and *Ceanothus* across different elevations).

Figure 3. Hypothesis of misalignment between natural (NS) and sexual (SS) selection to explain lack of correlation between trait divergence. In this example, wing size is influenced by NS and SS (pink and purple, respectively). When these two pressures are aligned (**A**), correlated trait divergence can occur between wing size and body color. However, when NS and SS are misaligned (**B**), the phenotypic means can be shifted away from natural selection's optimal adaptive peak. Consequently, SS disrupts the correlated NS effects in wing size and body color, resulting in their divergence to not be correlated.

Figure 4. Correlated divergence between color-pattern and female CHC traits. Population divergence between color-pattern and pentacosanes (r=0.11, P=0.27), heptacosanes (r=0.38, P=0.10), and nonacosanes (r=0.82, P<0.001, Mantel tests). Among the CHC traits, nonacosanes are the class with strongest correlated divergence with color-pattern.

Figure 5. Processes explaining correlated trait divergence between color-pattern and CHC traits, especially nonacosanes. (**A**) Genetic covariance between color-pattern and the three different female CHC traits (yellow), represented by Pearson correlation coefficients. Based on these results, genetic covariance is unlikely to explain correlated trait divergence between color-pattern and CHC traits. Heritability in color-pattern (the diagonal of the correlation matrix) is represented in red. (**B**) Environmental axes influencing color-pattern (host-plant species) and CHCs (climate). Host-plant species are strongly influenced by climate, represented by the first principal component axis summarizing 19 WorldClim variables (PC1=67% of variance explained; $W = 7872$, $P < 0.001$, Wilcoxon signed-rank test). This relationship is explained by the common association between each environmental variable and elevation (host-plant species and elevation; $W = 7932$, $P < 0.001$, Wilcoxon signed-rank test; PC1 summarizing climatic variables and elevation; $r = 0.96$, $P < 0.001$, Pearson correlation). These correlated selective pressures can explain correlated trait divergence. Abbreviations: A= *Adenostoma*, C=*Ceanothus.*

Figure 6. Association between the different CHC traits and sexual isolation. Divergence in pentacosanes shows strong association with the degree of sexual isolation beween population pairs, estimated using experimental mating trials (Bayesian linear mixed models, BLMM, linear β=0.76 [0.49, 1.03, 95% ETPI], quadratic β=0.40 [0.07, 0.73, 95% ETPI]), suggesting a role for sexual selection. Meanwhile, divergence in heptacosanes (β=0.09 [-0.38, 0.56, 95% ETPI]) and nonacosanes (β=0.02 [-0.45, 0.48, 95% ETPI]) are not significantly correlated with sexual isolation (see Table S6 for best models' fit in BLMM). The strong correlation between pentacosane divergence and sexual isolation suggests that a misalignment between sexual and natural selection (the latter exerted by climate) could be disrupting the effects of the environmental covariation, resulting in uncorrelated divergence between pentacosanes and color-pattern. Abbreviations: pop. = population, penta = pentacosanes; hepta = heptacosanes; nona = nonacosanes.