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Phenotypic variation in *Heliconius erato* crosses shows iridescent structural colour is sex-linked and controlled by multiple genes

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Abstract

Bright, highly reflective iridescent colours can be seen across nature and are produced by the scattering of light from nanostructures. *Heliconius* butterflies have been widely studied for their diversity and mimicry of wing colour patterns. Despite iridescence evolving multiple times in this genus, little is known about the genetic basis of the colour and the development of the structures which produce it. *Heliconius erato* can be found across Central and South America, but only races found in Western Ecuador and Colombia have developed blue iridescent colour. Here, we use crosses between iridescent and non-iridescent races of *H. erato* to study phenotypic variation in the resulting F2 generation. Using measurements of blue colour from photographs, we find that iridescent structural colour is a quantitative trait controlled by multiple genes, with strong evidence for loci on the Z sex chromosome. Iridescence is not linked to the Mendelian colour pattern locus that also segregates in these crosses (controlled by the gene *cortex*). Small angle X-ray scattering data shows that spacing between longitudinal ridges on the scales, which affects the intensity of the blue reflectance, also varies quantitatively in F2 crosses.

1. Introduction

Structural colours are bright and highly reflective colours produced by the interaction of light with nanostructures. They can be seen across a range of taxa, including fish, birds, molluscs and insects, and have numerous functions covering visual communication and recognition, mate choice and thermoregulation [1–3]. Despite this, little is known about the genetic basis of structural colour, or how genetic variation translates into developmental differences of the nanostructures.

Examples of the different ways structural colour is produced can be seen across butterfly species. Multilayer reflectors produce the bright blue colour in *Morpho* butterflies [4], while *Callophrys rubi* have a highly connected gyroid structure contained within the upper and lower lamina [5]. Scales on butterfly wings are formed as a long, flattened extension of the cuticle. Generally, they are composed of longitudinal ridges which are linked transversely by cross-ribs (Figure 1). These nanostructures make up a variety of repeating elements which can vary in thickness and patterning, producing different visual effects. F-actin filaments are important in the development of wing scale cells, and appear to pre-pattern where the ridges will form [6].

The neotropical *Heliconius* butterflies (Nymphalidae) are well-known for the diversity in their wing colour patterns and mimicry between species [7]. Many of these colour patterns are formed by chemical pigments, but several species also exhibit structurally produced blue reflectance. *Heliconius* butterflies can produce structural colour by thin film interference using different features on their scales. Longwing *H. doris,* for example, display hindwing colour reflected by their lower lamina; the resulting colour can be blue or green depending on the absence or presence of the yellow pigment 3-OH-kynurenine [8]. Several other species, including *Heliconius erato,* produce iridescent colours, that change in both brightness and wavelength of peak reflectance with angle, using layered lamellae that make up their scale ridges. Density of the ridges, the curvature and layering of the lamellae affect the intensity of the structural colour, with denser ridge spacing producing higher reflectance [9].

Heliconius erato is found across Central and South America and has evolved more than 25 races with a diversity of colour patterns. These aposematic patterns are mimetic with *Heliconius melpomene* and are an example of Müllerian mimicry. Variation in pigment colour patterns has been found to map to a handful of loci that control a diversity of patterns in several distantly related species [10–13]. Despite iridescent colour evolving multiple times in *Heliconius*, the genetics of this trait have not been studied to the same extent as pigment colour patterns, likely due to the difficulty of measuring the trait. Iridescent *H. erato cyrbia* is found on the Western slopes of the Andes in Ecuador. *H. erato* races found further north in Panama lack this structural colour, and hybrid zones arise between the iridescent and non-iridescent races, where populations with intermediate levels of iridescence can be found. Previous researchers have noted that levels of iridescence vary in F2 hybrid crosses and appear to do so in a continuous manner [12,14] but have not attempted to quantify the variation. Continuous

variation in the F2 would suggest that the trait is controlled by multiple loci and therefore not controlled by the "tool kit" of major effect loci that regulate pigment colour patterns. The genes controlling variation in iridescence may perhaps be those directly controlling the formation of scale structure.

Experimental genetic crosses can be used to estimate the number of genes involved in controlling a trait by investigating the distribution of the phenotype across segregating generations [15]. Traits that are controlled by a single locus of major effect will segregate according to Mendelian ratios, with 50-100% of individuals in the F2 generation having phenotypes the same as one or other of their parents (depending on dominance of the alleles). The more individuals there are with intermediate phenotypes, the more loci are likely to be involved, as a greater number of allele combinations will be possible. We can also estimate positions of loci in the genome by looking for links to known loci which control other phenotypes, and by looking for patterns of sex linkage.

Here, we aim to determine whether iridescence in *Heliconius erato* is a quantitative trait controlled by multiple genes, and if any of these genes are sex-linked or linked to known colour pattern loci, by looking at the segregation of the trait in F2 crosses between different races. *H. erato demophoon* from Panama is black with red and yellow bands. This race was crossed to *H. erato cyrbia* from Ecuador, which has a similar colour pattern but has an iridescent blue colour instead of being matt black (Figure 2). The only major colour pattern differences between these races are the white margin on the hindwing of *H. erato cyrbia* and the yellow bar on the dorsal hindwing of *H. erato demophoon.* Based on previous crosses, these are likely to be controlled by alternative alleles of the *Cr* locus on linkage group 15, which is homologous to three tightly linked loci (*Yb, Sb* and *N*) in *H. melpomene* [10]*,* and corresponds to the gene *cortex* [16]. There are also differences in the size and position of the red forewing band between *cyrbia* and *demophoon*, likely controlled by the gene *WntA*, found on chromosome 10 [12,17,18]. We also use small angle X-ray scattering (SAXS) to quantify ridge spacing in broods. As several aspects of scale morphology are known to vary between the iridescent and non-iridescent races [9] it is possible that apparent continuous variation in the reflectance in the F2 could be due to independent segregation of these different features, each of which may be controlled by a major effect gene. Therefore, we also test whether ridge spacing shows continuous variation in the F2 generation.

2. Methods

2.1 Crossing Experiments

Experimental crosses were performed between geographical races of *Heliconius erato* at the insectary in Mashpi Reserve, Ecuador, over a period of 2 years. *H. erato demophoon* was collected from Gamboa, Panama (9.12º N, 79.67º W) in May 2014, then transported to Mashpi, Ecuador (0.17º N, 78.87º W), where they were kept as stocks. Iridescent *H. erato cyrbia* were collected from the area around Mashpi. *H. e. demophoon* were crossed with *H. e. cyrbia*, and the F1 generation crossed together, along with the addition of 2 backcrosses (BC) between the F1 and *cyrbia* (Figure 2). Crosses were reciprocal, so that in roughly half of the first generation crosses the female was the iridescent race and the male non-iridescent, and vice versa. In line with previous studies with intraspecific *Heliconius* hybrids [12,19], races readily hybridised and we did not observe any evidence of hybrid inviability or differing success between the reciprocal crosses. *Passiflora* species were provided as larval food plants and for oviposition, and butterflies were given *Lantana camara* and other locally collected flowers, plus sugar solution (10%) and pollen to feed. The bodies of the parents and offspring were preserved in NaCl saturated 20% dimethyl sulfoxide (DMSO) 0.25M EDTA solution to preserve the DNA, and the wings stored separately in glassine envelopes. A total of 302 individuals obtained from 14 crosses were used in the analysis (Table 1).

2.2 Phenotypic Colour Analysis

All butterfly wings were photographed flat under standard lighting conditions using a mounted Nikon D7000 DSLR camera with a 40mm f/2.8 lens set to an aperture of f/10, shutter speed of 1/60 and ISO of 100. Lights were mounted at a fixed angle of 45 degrees to maximise the observed blue reflection from the iridescent wing regions. All photographs also included an X-Rite Colour Checker to help standardise the colour of the images. RAW format images were standardised using the levels tool in Adobe Photoshop CS2 (Version 9.0). Using the colour histogram plugin in ImageJ [20,21], red-green-blue (RGB) values were recorded from 2 sections of the wings and averaged (Figure 3). These areas were chosen because the scales on these sections of the wings close to the body tended to be the least damaged and worn, so a more accurate measurement of the colour could be taken, and the wing venation was used as a marker to allow the same areas to be measured each time.

Blue reflection from the iridescent wing regions was measured as variation in blue-red (BR) colour. This was calculated as $(B-R)/(B+R)$, with -1 being completely red and 1 being completely blue. The level of UV reflectance could not be measured from our photographs. Previous spectral measurements of the wing reflectance show that peak reflectance for *H. erato cyrbia* is just below the visible range at about 360-370nm, with much of the reflectance being within the human visible range, while *H. erato*

demophoon reflects very little but tends to show highest reflectance in the red-infrared range [9]. Therefore, the colour values will allow variation in colour and reflectance to be measured but will not represent butterfly visual systems. Repeatability of the colour measurements was tested using the repeatability equation of Whitlock and Schluter [22] by taking 5 measurements each on 5 randomly selected individuals. This estimates the fraction of total variance that is among groups in a random-effects ANOVA. We used the Castle-Wright estimator:

$$n_e = \frac{[\mu(P_1) - \mu(P_2)]^2 - Var[\mu(P_1)] - Var[\mu(P_2)]}{8Var(S)}$$

where *S = Var(F₂) – Var (F₁)*, to estimate the effective number of genetic loci (n_e) contributing to variation in the trait [15,23,24]. This is the difference between the mean BR values of the parental races squared, then the subtraction of the two variance terms, which corrects for sampling error of the estimates of the parental means (P_1 and P_2).

The genotype at the *Cr* locus was scored in 286 individuals based on the presence and absence of the white hind-wing margin and the dorsal hind-wing yellow bar, under the assumption that these pattern elements are controlled by alternative alleles of the *Cr* locus [10,25]. The *demophoon* genotype has the yellow bar present and is scored as $Cr^d Cr^d$, a white margin indicates the *cyrbia* genotype and this is scored as $Cr^c Cr^c$, and the $Cr^d Cr^c$ heterozygous genotype has neither of these elements (Figure 2). To look for association between variation in the red band and blue colour, we took four measurements of forewing band size in 71 F2 individuals and three further measurements to adjust for wing size (Figure 4), based on methods from Baxter *et al.* [26]. Using ImageJ, band measurements were carried out on the dorsal side of the wing and repeated for both the left and right wings. The average of these two measurements was divided by the average of the three standardising wing measurements. The three standardising wing measurements were also used to assess overall size of these individuals.

All statistical analyses were carried out in the R statistical package version 3.4.2 [27]. Welch's t-tests were used for analysis of differences between sexes and reciprocal crosses. ANOVA models were used to compare blue values to *Cr* genotypes. Yellow bar and white margin traits were tested for departures from the expected segregation ratios, based on the above hypothesis of the linkage and Mendelian inheritance, using a chi-squared test. Correlations between BR values and forewing red band measurements, ridge spacing, and cross-rib spacing (see below) were tested with the Pearson correlation coefficient.

2.3 Small angle X-ray scattering data collection

We estimated the size of the spacing between scale ridges and between cross-ribs (Figure 1) using small angle X-ray scattering (SAXS) carried out at the ID02 beamline at the European Synchrotron (ESRF), Grenoble, France [28]. The detector was a high-sensitivity FReLon 16M Kodak CCD with an

effective area of 2048 x 2048 pixels (24 µm pixel size). The X-ray wavelength λ was 0.0995 nm (12.45 keV), the beam was collimated to 50 µm x 50 µm and the accessible q-range was from 0.0017 to 0.07 nm^{-1} at 30.7m sample-to-detector distance. All 2D images were corrected for dark, spatial distortion, normalised by transmitted flux and masked to account for the beam stop and the edges of the detector. We azimuthally integrated the 2D images to obtain one dimensional patterns of scattered intensity I as a function of the momentum transfer vector q, where $q = (4\pi\ sin\ \theta)/\lambda$. Here, 2θ is the scattering angle. A typical scattering profile of a *Heliconius* scale is shown in Figure 5.

Wings were mounted in a frame that could be rotated to precisely align the samples. We collected between 33 and 113 measurements over 10-20 mm between two of the wing veins on the forewing (Figure 3) of 74 *H. erato* individuals: 8 *cyrbia*, 1 *demophoon*, 6 F1 (from 2 crosses in reciprocal directions) and 59 F2 (all from a single cross). In addition, we measured 4 *Heliconius erato hydara* individuals to be analysed alongside the *demophoon.* The *H. e. hydara* were also collected in Panama, do not have iridescent colour and differ from *demophoon* only in the lack of yellow hindwing bar. To obtain estimates of the ridge spacing, we fitted the peak positions in the one dimensional scattered intensity to a composite Lorentzian + linear profile using the lmfit Python module [29]. We then used the centre of each fitted profile to calculate ridge spacing using the expression $D = 2\pi/q$ and averaged these to obtain a single estimate per individual. The average distances between ridges are in good agreement with those previously reported for *H. erato* [9].

3. Results

3.1 Segregation of blue colour

Measurements of blue scores were shown to be repeatable, with 99% of variation due to differences between individuals and 1% due to measurement error ($R^2 = 0.99$, $F_{4,20} = 54159$, $p < 0.001$; Table S3). *H. erato demophoon* showed very little blue colour with an average BR value of -0.56 ± 0.08 compared to iridescent *H. erato cyrbia* which had a mean value of 0.97 ± 0.05 (Table 2). The mean for the F2 generation fell midway between the 2 parental races (Figure 6), suggesting additive effects of alleles. The mean of the F1 was slightly skewed towards *demophoon*, although the median was in a similar position to the F2 (0.13 and 0.14). The mean BR value of the backcrosses did not fall halfway between that of the F1 and the parental race, which they were crossed with, but were skewed towards *cyrbia*, the Ecuadorian race. This suggests that the effects of the alleles are not completely additive, and there may be some dominance of the *cyrbia* alleles or epistatic interactions between loci.

The lack of discrete groups in the F2 generation suggests that variation in the trait is controlled by more than one locus. Using the Castle-Wright estimator, with mean BR values and variances from only one cross direction to reduce variation due to sex linkage (see subsequent results), we obtained an estimate of 4.6 loci contributing to the trait. While this formula assumes that crosses started with inbred lines, it is generally robust to deviations from the assumptions [30]. However, it likely

underestimates the total number of loci as it assumes loci all have equal effects. It is therefore perhaps best interpreted as the likely number of loci with medium to large effects on the phenotype. In addition, the F1 individual wings that we measured were of varying age and condition, which may have increased the variance and decreased the mean value of blue reflectance seen in these individuals relative to the F2 individuals, which were all preserved soon after emergence. This could influence the estimation of the number of loci.

3.2 Sex linkage

Sex linkage leads to a difference in the trait between reciprocal crosses in the F1 generation, which is confined to the heterogametic sex, or a difference between reciprocal crosses in the F2 generation in the homogametic sex [31]. As in birds, female butterflies are the heterogametic sex; they have ZW sex chromosomes whereas males have ZZ. Differences would occur depending on which parent or grandparent the Z or W is inherited from (Figure 7). If the sex difference is present in the parental population, or the pattern is the same in reciprocal crosses, this would indicate a sex-limited trait (i.e. an autosomal trait that is expressed differently between the sexes).

Comparing the F1 offspring of reciprocal crosses suggested some sex linkage (Figure 8; Table 3). Offspring of crosses with a male *cyrbia* parent had significantly higher blue values than those which had a female *cyrbia* parent. Separated by sex, there was no difference between the males from reciprocal F1 crosses, which had a mean of 0.23 and 0.25 respectively (t(11) = -0.19, p = 0.85). The variation was amongst the female offspring which had means of -0.03 and 0.26 (t(44) = -5.55, p<0.001; Table 4). This pattern would be expected if there were one or more loci controlling iridescence on the Z chromosome. In each case, males will be receiving one Z chromosome from an iridescent parent, and the other from a non-iridescent parent. The female offspring, in contrast, will only receive a Z chromosome from their father (Figure 7). To confirm that these results were not biased by a particular cross, individual crosses were plotted and the same pattern was found (Figure S1). We did not find any difference in blue score between the sexes in pure *H. erato cyrbia* (Table 3), demonstrating that the difference between the sexes in the crosses is not due autosomally mediated sexual dimorphism.

If blue colour was controlled only by genes on the Z chromosome, we would expect that females from crosses with a non-iridescent father would have the same phenotype as *demophoon* females. However, they are significantly bluer than wild *demophoon*, supporting the hypothesis that the colour is controlled by multiple loci on different chromosomes (-0.03 ± 0.2 and -0.56 ± 0.1, t(25) = -10.6, p<0.001).

In the F2 generation, sex linkage would be shown as males with an iridescent maternal grandfather being more blue than those with an iridescent maternal grandmother. The results point towards this pattern, however the differences between the male groups are not significant, possibly due to small

sample sizes in the first group (Figure 9; Table 4). There was little difference in females. Overall, however, offspring with an iridescent maternal grandfather were bluer than those with black maternal grandfather. This is consistent with sex linkage, due to the greater number of "*cyrbia*" Z chromosomes present in the F2 offspring with an iridescent maternal grandfather (Figure 7). Within the offspring with an iridescent maternal grandfather, males were bluer than females, while this was not the case for crosses with a black maternal grandfather, also supporting Z linkage (Table 3). In summary, F1 females were bluer when they had an iridescent father, and males were bluer in the F2 when they had an iridescent maternal grandfather. There were no differences in BR values between males and females in the parental races, *H. e. demophoon* and *H. e. cyrbia*. These results support the presence of loci controlling iridescence in the Z chromosome.

3.3 Links to other colour pattern loci

In *H. erato*, the *Cr* locus controls the presence of a yellow forewing bar in *demophoon* and a white margin in *cyrbia*. There were 3 observed phenotypes in the F2 generation – yellow bar present, white margin present, and both absent (Figure 2). Consistent with the hypothesis that these two features are controlled by recessive, tightly linked loci or are alternative alleles of the same locus, we did not find any individuals that had both a yellow dorsal bar and a white margin present. The ratio of these traits was also consistent with a 1:2:1 ratio as expected under the assumption that the individuals lacking both features were heterozygous at this locus ($x^2 = 2.1$, d.f. = 2, p = 0.35). There was no significant difference in BR values between individuals with different *Cr* genotypes ($F_{2,107} = 2.05$, p = 0.133) (Figure 10), suggesting that *cortex* is not one of the genes controlling iridescence, nor are there any major effect loci linked to this region on *Heliconius* chromosome 15. In the F2, there were also no significant correlations between blue colour and any of the standardised linear measurements used to determine shape of the red forewing band (Table 5, Figure S2), showing iridescence is unlikely to be linked to *WntA* on chromosome 10.

3.4 Nanostructure variation

As we expected, there was a negative correlation between longitudinal ridge spacing and BR values (r = -0.52, p<0.001; Figure 11), indicating that blue reflectance increases with increasing density of ridges on the scale. The strength of this correlation shows that ridge spacing is only one factor which is affecting the intensity of iridescence, and that other aspects of scale morphology that determine blue reflectance may segregate somewhat independently in the crosses. BR values also declined with increasing cross-rib spacing, although not significantly (r = -0.20, p = 0.09; Figure 11). Ridge spacing and cross-rib spacing were highly correlated with each other (r = 0.34, p = 0.002; Figure 11) suggesting a genetic correlation between these traits. Therefore, the correlation between cross-rib spacing and BR value is likely due to this association between ridge and cross-rib spacing, as we do not expect the cross-ribs to directly affect colour.

Consistent with previous findings [9], *H. erato cyrbia* had closer ridge spacing than *H. erato demophoon* (Table 6). Like the BR values, measurements of ridge spacing in the F2 generation fell between the parental races (Figure 12) and were fairly continuous, consistent with the action of multiple genes. Interestingly, ridge spacing in the F1 generation was highly variable between individuals. This could indicate variation in epistatically acting alleles in the parental populations that segregate in the F1 generation, or may suggest environmental effects. However, the phenotyped F1 individuals in this comparison were from two different reciprocal crosses, with apparent differences between these two groups. Therefore, some of the variation that is observed may be due to cross-specific genetic effects and possibly sex-linkage, but we have data from too few individuals to fully dissect these effects.

Cross-rib spacing in the F2 generation appears to extend beyond the range of the parental races (Figure 13), again possibly indicating epistatically acting alleles in the parental populations, although not all parental individuals were measured. Large variation in cross-rib spacing may be expected as it is not predicted to have an effect on colour, so may be under weaker selection. In the F2 generation, males had narrower longitudinal ridge spacing than females, which was similar to the differences seen in this generation in blue values, and may suggest sex linkage of loci controlling ridge spacing ($t(57) = 3.80$, $p < 0.001$; Figure 14). Cross-rib spacing was also smaller in males ($t(43) = 4.95$, $p < 0.001$), supporting the idea that ridge spacing and cross-rib spacing may be genetically correlated. However, in this case we cannot rule out a contribution of autosomally mediated sexual dimorphism because we only have data from one F2 cross. There was not a significant difference in ridge spacing between sexes in the parental populations (*hydara/demophoon* $t(2.3) = 0.34$, $p = 0.77$; *cyrbia* $t(4.4) = 0.53$, $p = 0.63$), but this may be due to small sample sizes and the differences were in the same direction as in the F2, with females having larger spacing on average (Figure 14). There was a significant difference in cross-rib spacing between sexes in *cyrbia* ($t(5.6) = 3.42$, $p = 0.02$) but not *hydrara/demophoon* ($t(1.4) = 1.35$, $p = 0.36$). Nevertheless, the differences in ridge spacing seen within the parental races are smaller than those seen in the F2 generation, supporting a role for sex linkage. Using the wing measurements, there was not a significant difference in wing size between males (11.2 ± 0.5mm) and females (10.9 ± 0.6mm) in the F2 ($t(68) = -1.82$, $p = 0.07$), and in fact males tended to be larger, suggesting that the increased ridge and cross-rib spacing in females is not due to overall sexual size dimorphism. Overall, ridge spacing appears to have a very similar genetic architecture to that of the BR colour values, suggesting that it is also controlled by multiple loci.

4. Discussion

Our phenotypic analysis of crosses between iridescent and non-iridescent races shows that iridescence is controlled by multiple loci in *H. erato* with convincing evidence for loci on the Z chromosome. There is an extensive history of using experimental crosses in *Heliconius* to investigate the genes

controlling colour and pattern, but although iridescence had been shown to segregate in crosses, the trait has not been investigated due to the difficulty of quantifying the continuous phenotype and measuring the number of different features affecting the colour. We show that standardised photographs and the BR ratio is an effective method of estimating variation in blue iridescent reflectance. As expected, iridescent *H. erato cyrbia* gave the highest blue values, and non-iridescent *H. e. demophoon* the lowest. BR values correlated with longitudinal ridge spacing, which has previously been shown to have an effect on the brightness of the blue iridescent colour [9]. The distribution of blue values in the F2 generation suggests that variation in the trait is not controlled by a single locus.

The differences in blue values found between sexes in the F1 reciprocal *erato* crosses suggest that there could be a major effect locus involved in iridescent colour on the Z chromosome. We may expect that genes on the sex chromosomes will control sexually selected traits [32]. Reinhold [33] calculated that in *Drosophila* around a third of phenotypic variation in sexually selected traits was caused by X-linked genes, and that X-linked genes only influenced traits classified as under sexual selection. Iridescent structural colours are used as sexual signals in many butterfly species [2,34,35]. Work with *Colias* butterflies has found many wing pattern elements are sex-linked, including melanisation, UV reflectance and yellow wing pigmentation [36,37]. These studies found that sex linkage was important in prezygotic isolation and species differentiation. Therefore, sex linkage of iridescence in *Heliconius* may have contributed to the differentiation of this trait between geographical races.

Unlike some Lepidoptera, *Heliconius* do not show complete sex chromosome dosage compensation. Analysis of *H. cydno* and *H. melpomene* gene expression showed a modest dosage effect on the Z chromosome, and overall reduced expression compared to autosomes [38]. Our results are also consistent with a lack of complete dosage compensation, with some evidence for expression of both Z chromosome alleles in males. A lack of dosage compensation could also favour the build-up of sexually selected or sexually antagonistic loci on the Z chromosome, as these will automatically be expressed differently between the sexes.

The three *erato* phenotypes controlled by the *Cr* locus did not show any correlation with iridescent colour values. The gene *cortex*, found in this genomic region, has been shown to underlie these colour pattern differences [16]. There are several reasons why major colour patterning genes could have been hypothesised to also control structural colour variation in *Heliconius*. Knockouts of one of the genes that control colour pattern in *Heliconius*, *optix*, in *Junonia coenia* butterflies resulted in a change in pigmentation, and the gain of structural colour [39], although this was not observed in the same tests with *Heliconius erato*. In addition, linkage between divergently selected loci would be expected under 'divergence hitchhiking', in which genomic regions around key divergently selected loci are protected

from recombination during speciation [40]. Hitchhiking regions can be small in natural populations unless recombination is reduced, but in Lepidoptera there is no recombination in the female germ-line. Furthermore, for highly polygenic traits we would expect many loci to be distributed throughout the whole genome, so that for any genetic marker there will be some phenotypic association. Individuals with homozygous *Cr* phenotypes, for example, will have inherited an entire chromosome 15 from either an iridescent or non-iridescent grandparent, due to the lack of female recombination. Therefore, any combination of a single major effect locus or multiple smaller effect loci on chromosome 15 would have been seen as a difference in iridescence between individuals with different *Cr* phenotypes. The fact that we find no association with *Cr* suggests that structural colour is not highly polygenic, but controlled by a moderate number of loci, none of which are located on chromosome 15. It is also consistent with it being controlled independently of colour pattern. Similarly, we see no association with variation in forewing red band size, which is largely determined by the gene *WntA*. This region on chromosome 10 controls forewing band shape in multiple races of *H. erato*, as well as other *Heliconius* species [12].

In *Heliconius* pigment colour patterns, a small set of major effect genes have been well studied but a larger set of "modifier" loci have also been found which adjust colour pattern [12]. It is possible that the iridescence genes have a similar distribution of effect sizes, with a small number of major effect genes, including one on the Z chromosome, and a distribution of other smaller effect genes. This supports the existing evidence of the importance of major effect loci in adaptive change [10–12]. Future work with the co-mimic of *erato*, *Heliconius melpomene*, will allow us to compare the genetic basis of iridescence between the two species. Following the two-step process of Müllerian mimicry described by Turner [41,42], a large effect mutation, such as the one we have found on the Z chromosome, allows an adaptive phenotypic change large enough for the population to resemble those in the mimicry ring and survive, then smaller changes will produce incremental improvements in mimicry.

Longitudinal ridge spacing also appears to have a polygenic architecture. The continuous variation that is observed in blue colour in the F2 broods does not seem to be due to major effect loci with discrete effects on different aspects of scale structure. Rather it seems that multiple interacting genes are involved in controlling scale morphology. The correlation between ridge and cross-rib spacing suggests that some of these loci produce correlated effects on various aspects of scale morphology. However, the fact that we do not see a perfect correlation between these and blue colour suggests that there is some independent segregation of other aspects of scale morphology that contribute to the colour. Measurements of other aspects of scale morphology, such as ridge curvature and layering, will be needed to confirm this.

Conclusions

Crosses are ideal for investigating the genetic basis of colour and pattern as traits will segregate in following generations. Crossing iridescent and non-iridescent *Heliconius erato* has allowed us to quantify variation in the colour and determine that it is sex-linked and controlled by multiple loci.

Data accessibility

SAXS data (doi:10.15131/shef.data.6839315) and accompanying code (doi: 10.15131/shef.data.6837905) have been uploaded to an online repository. Colour measurements, repeatability measurements and cross pedigree information can be found as part of the electronic supplementary material.

Authors' contributions

MNB collected the colour data, performed the genetic analysis and wrote the manuscript. JER analysed the SAXS data under the supervision of AJP. AJP co-ordinated collection of the SAXS data. The crosses were performed by PAS, CM, DC, MNB, NJN and EVC. The SAXS data was collected by AJP, TZ, EVC, NJN and MNB. Wing size and red band measurements were performed by HEB. The study was devised and co-ordinated by NJN. All authors read and commented on the manuscript.

Competing Interests

We declare we have no competing interests.

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Figures

Figure 1. Scanning Electron Microscope image showing the structures on a *Heliconius* wing scale. Longitudinal ridges, composed of overlapping lamellae, are connected by cross-ribs.
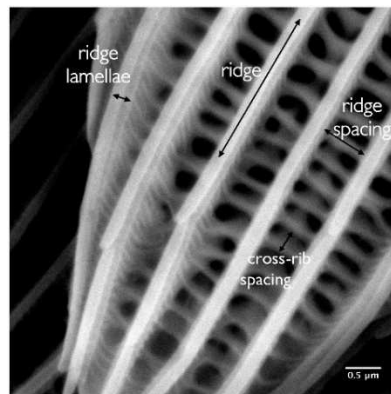
Figure 2. Cross design and examples of colour pattern variation in *H. erato* F1, F2, and backcross generations. Examples of the *Cr* genotypes are shown in the F2 generation.

H. erato demophoon   *H. erato cyrbia*

F1

H. erato cyrbia

F2

Cr^dCr^d Cr^dCr^c Cr^cCr^c **Backcross**
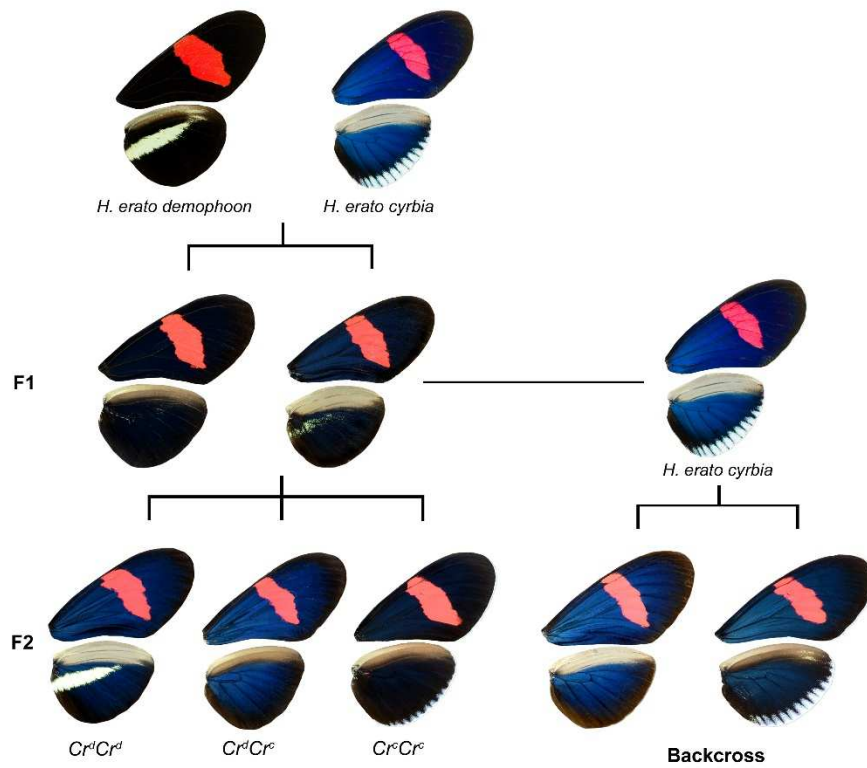
Figure 3. RGB values were measured in the hatched areas highlighted on the right wings and averaged for each butterfly. Left wings were used when the right side were too damaged. Small angle X-ray scattering (SAXS) measurements were taken along the dotted line shown on the left forewing.

1 cm

Figure 4. Four measurements of forewing band width were taken (bold arrows) along with three further measurements to standardise wing size (dotted arrows), using wing veins as points of reference.
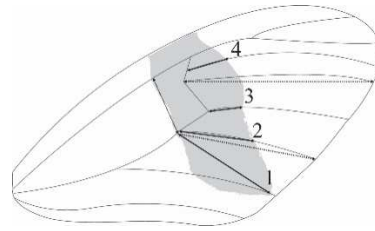

Figure 5. Representative small angle X-ray scattering patterns for a single frame of a male *H. e. cyrbia* parent. (A) The 2D pattern reveals approximately perpendicular scattering intensity from scale features. From their orientation, length scales of the scattered intensity and previous interpretations, we infer that they correspond to the spacing between ridges and cross-ribs. (B) Full azimuthal integration of the scattered intensity as a function of the magnitude of the momentum transfer vector q. The peaks corresponding to ridge and cross-rib spacing are indicated together with the measurements in real space.
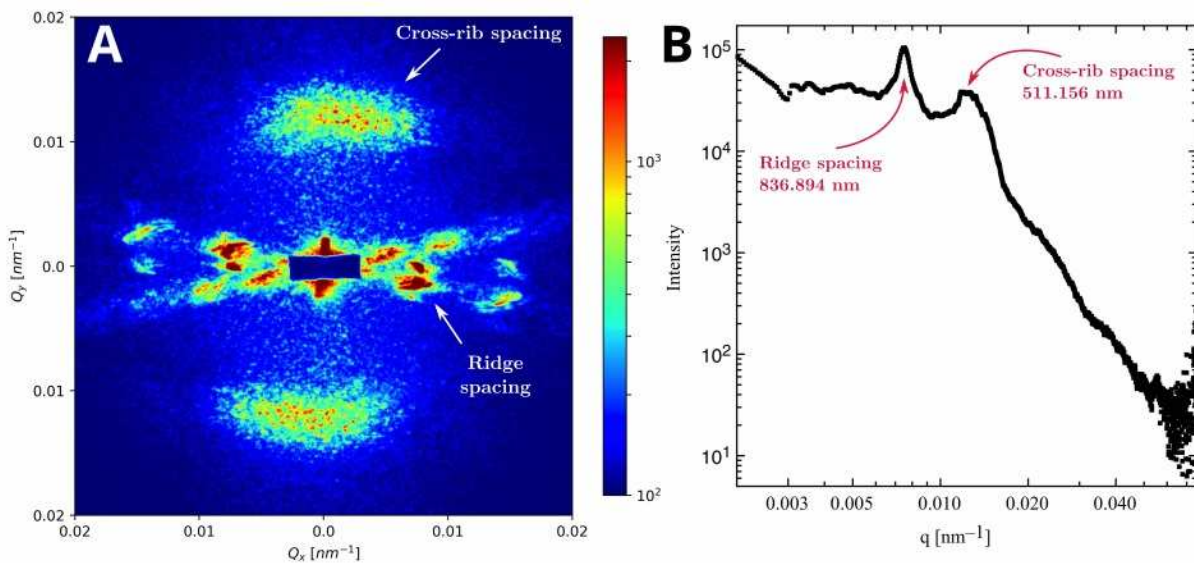
Figure 6. Mean BR values across *H. erato* generations. F1 and F2 individuals largely fall between the parental *demophoon* and *cyrbia* races. The backcross generation (BC) are highly skewed towards *cyrbia*, which is the race they were crossed with.
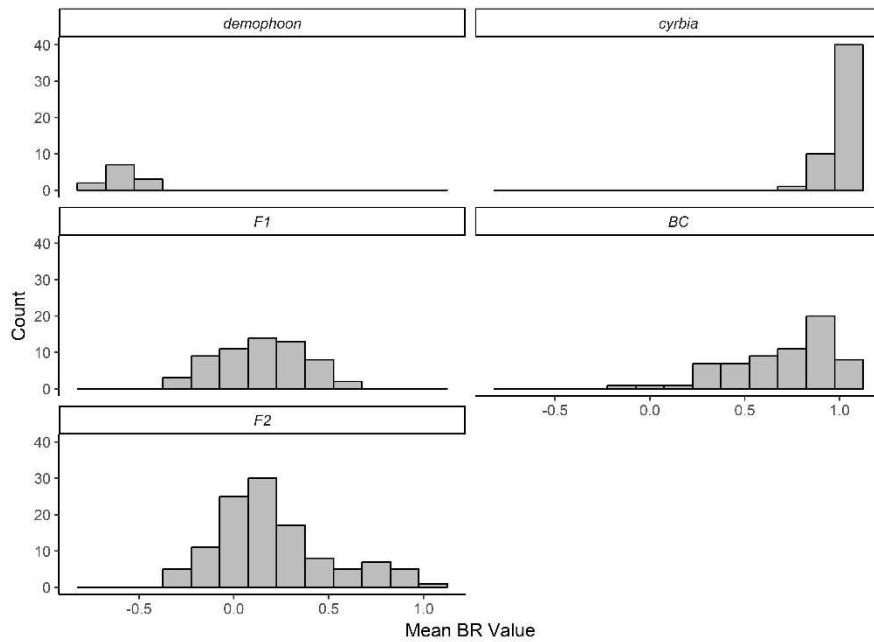

Figure 7. If there are loci of interest on the Z chromosome, F1 females with an iridescent *cyrbia* father will be bluer than those with a non-iridescent *demophoon* father because they inherit an "*cyrbia*" Z chromosome. In the F2, males always inherit a complete, non-recombined Z chromosome from their maternal grandfather, so if he is iridescent they will be bluer than offspring from the reciprocal cross.
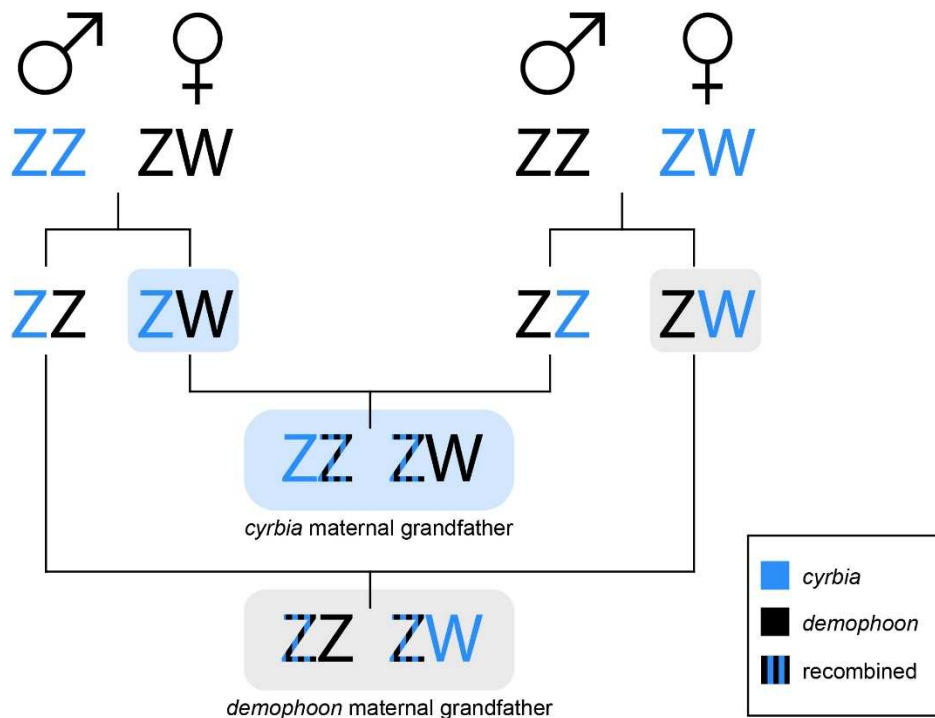
Figure 8. F1 females with an iridescent *cyrbia* father were significantly bluer than those with a *demophoon* father. There were no differences in males.
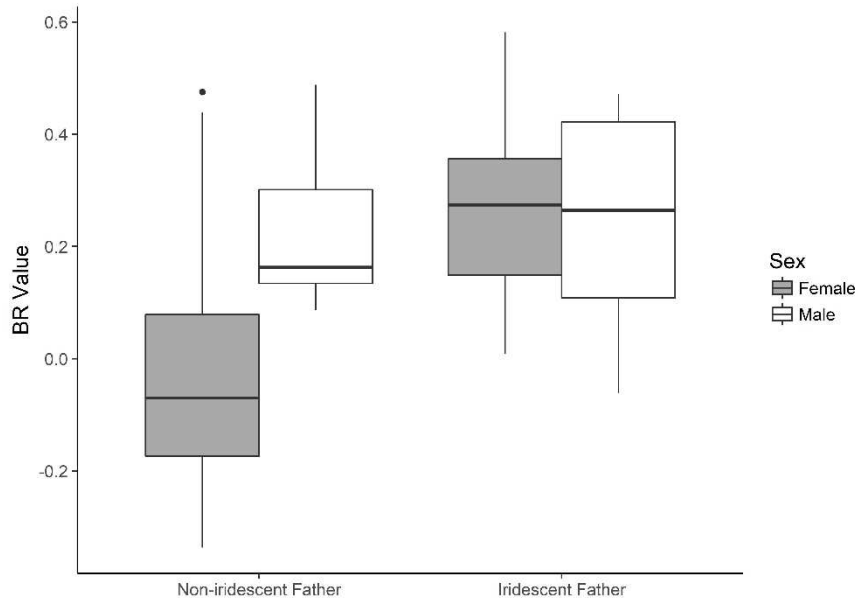

Figure 9. Mean BR values for F2 males with an iridescent maternal grandfather (MGF) were higher than those with an iridescent maternal grandmother, although not significantly. Females in both groups had similar BR values.
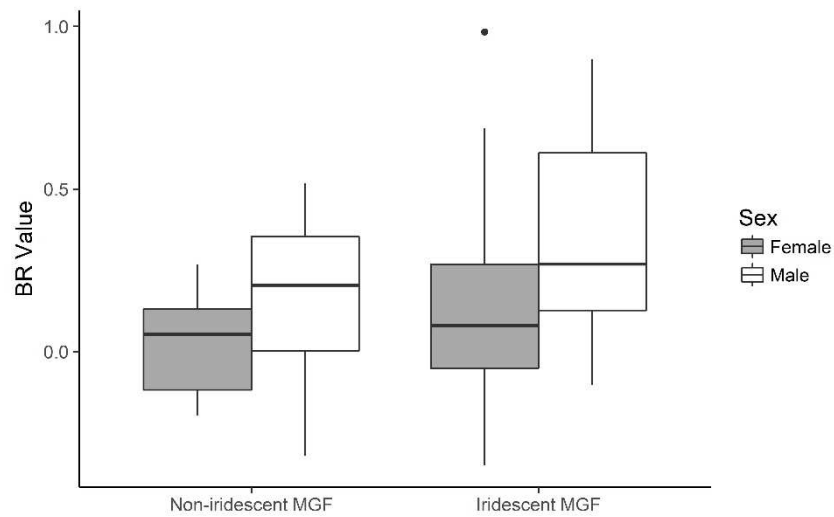
Figure 10. In the F2 generation, BR values did not differ with the different Cr phenotypes. $Cr^d Cr^d$ represents the *demophoon* genotype with the yellow bar present, and $Cr^c Cr^c$ is the *cyrbia* genotype with the white margin. $Cr^d Cr^c$ is heterozygous and has neither of these elements.
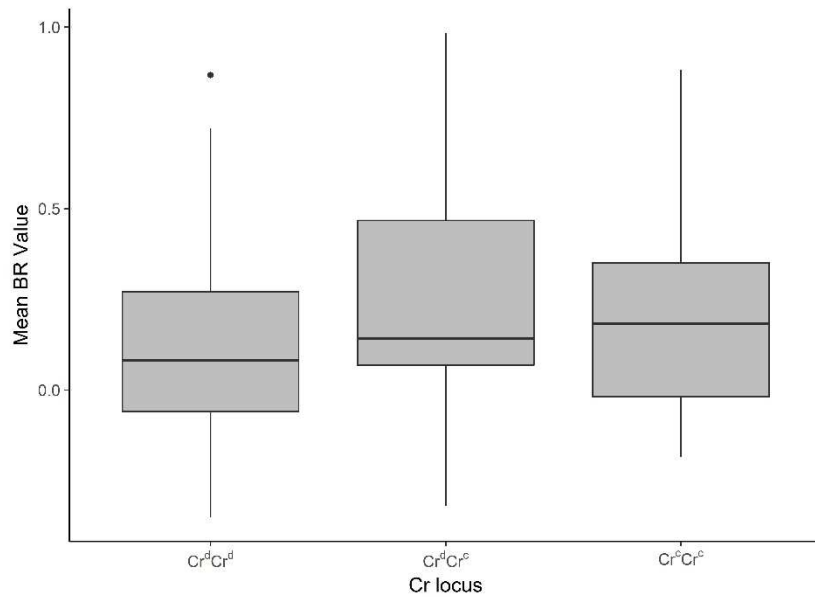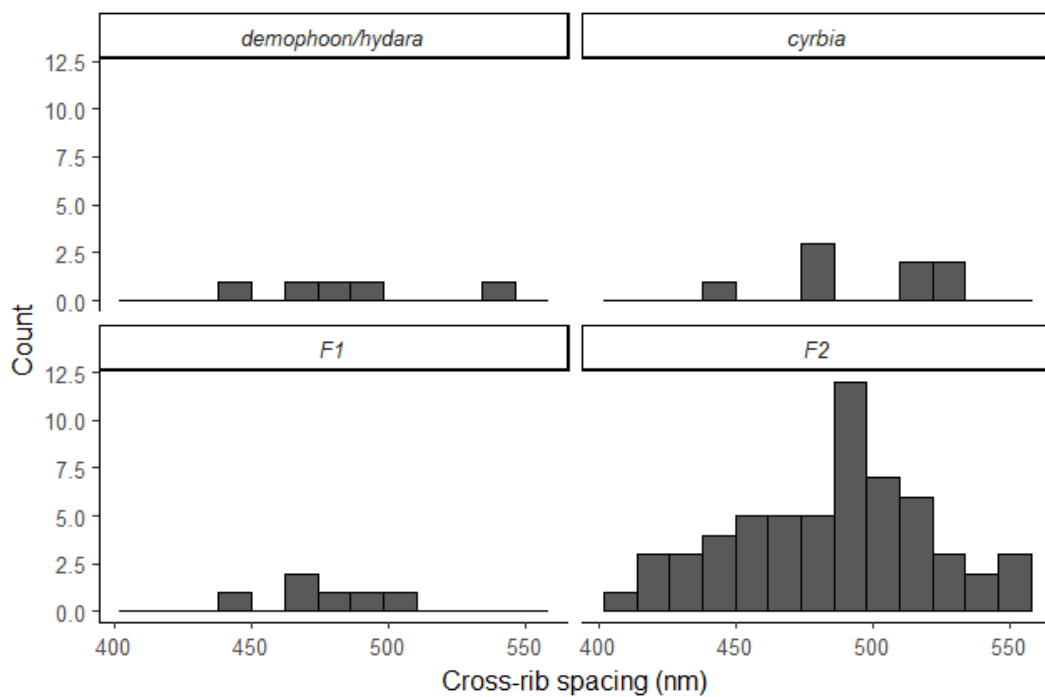

Figure 11. An increase in longitudinal ridge spacing correlated with a decrease in BR values. Blue colour slightly decreased with cross-rib spacing, but ridge spacing and cross-rib spacing were also highly correlated. The cross-hairs show the standard error from the 33 to 133 SAXS point measurements for each individual. Blue lines indicate the fitted linear regression, with the dotted lines showing the 95% confidence interval.
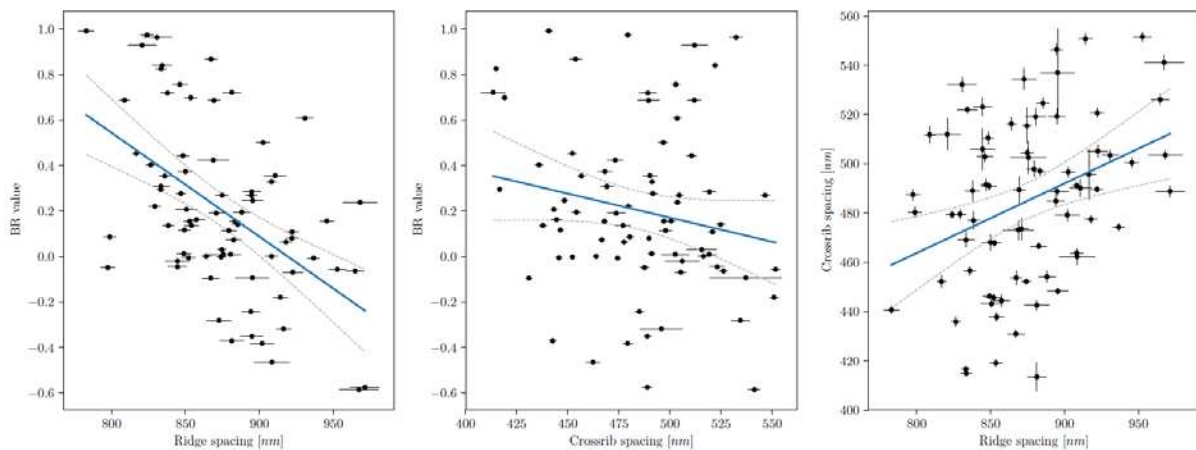
Figure 12. Variation in longitudinal ridge spacing in the F2 suggests it is controlled by multiple genes. In the F1, those with an iridescent father had lower ridge spacing, reflecting the higher BR values seen in this cross.
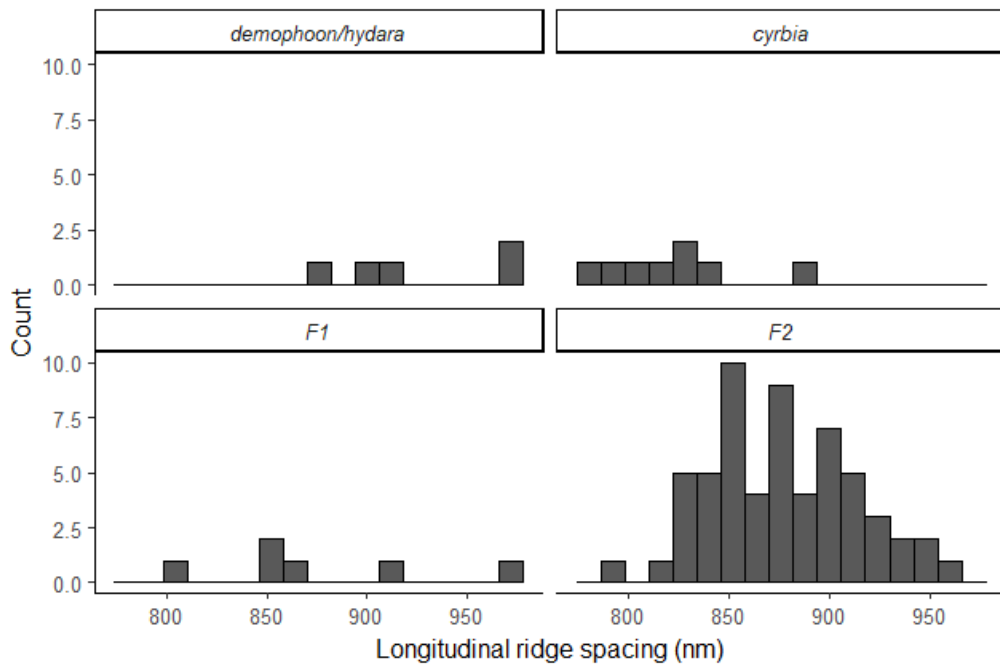
Figure 13. Cross-rib spacing also shows continuous variation in the F2 generation, and extremes extended beyond the values of the few parental individuals which were measured.

Figure 14. Males have narrower longitudinal ridge spacing than females in the F2. This difference is less pronounced and not significant in the parental races. Significant differences in cross-rib spacing were seen in *cyrbia* and in the F2, with males again having narrower spacing. These results are consistent with the finding that males have higher measures of blue colour.
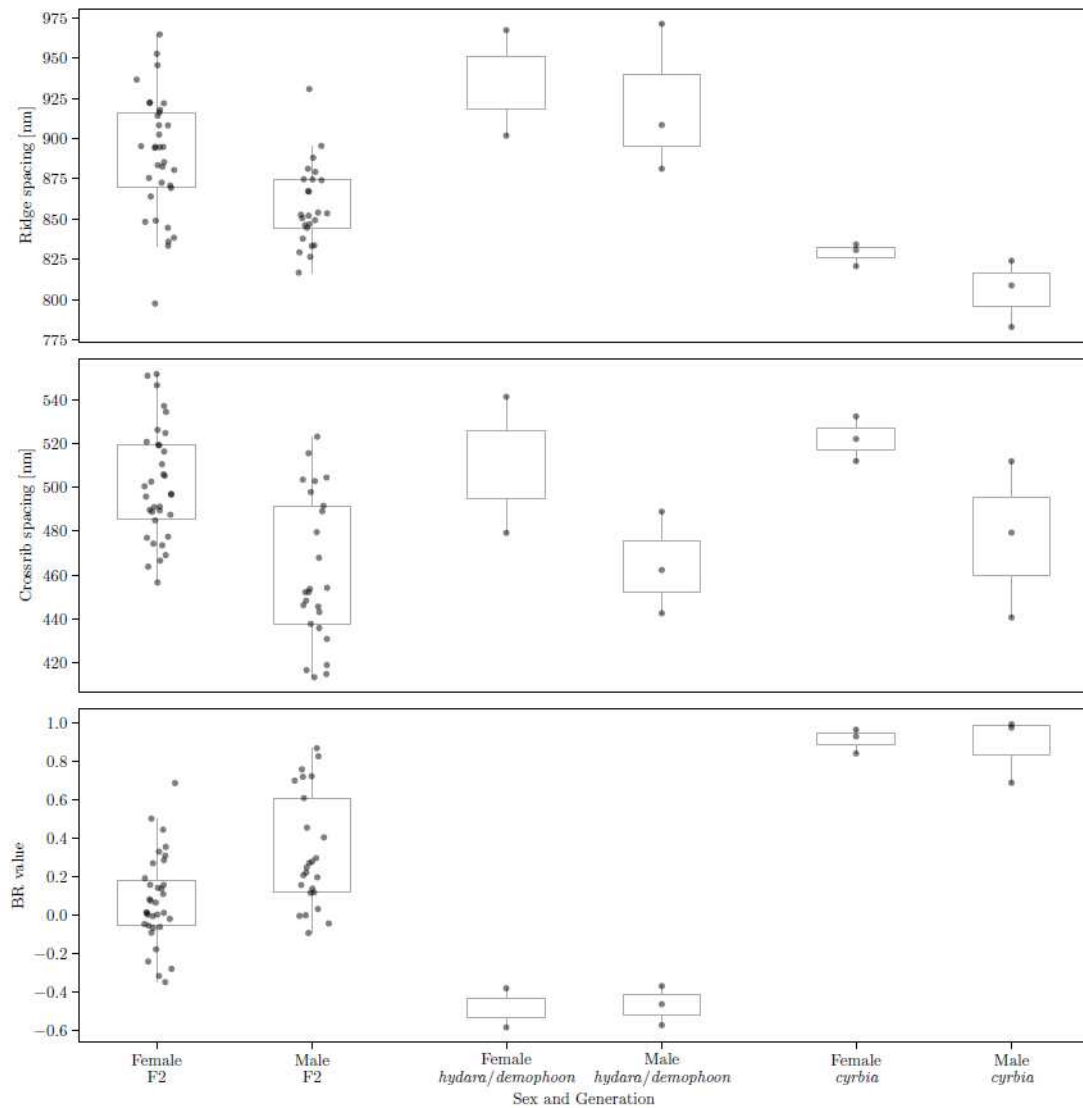
Tables

Table 1. *H. erato* crosses performed and the number of offspring produced from each. See supplementary Table S2 for details of each cross.

Cross type	Number of crosses	Number of offspring phenotyped for blue values	Number of offspring phenotyped for ridge spacing
F1: *demophoon* ♂ x *cyrbia* ♀	2	37	3
F1: *cyrbia* ♂ x *demophoon* ♀	3	33	3
F2: *cyrbia* maternal grandfather	3	100	59
F2: *demophoon* maternal grandfather	3	14	0
Backcross: *cyrbia* ♂ x (*demophoon* ♂ x *cyrbia* ♀)	2	16	0
Backcross: *cyrbia* ♀ x (*cyrbia* ♂ x *demophoon* ♀)	1	49	0

Table 2. Summary statistics for BR values in each generation of *H. erato*.

Generation	Mean BR value	Standard deviation	Variance	Sample size
demophoon	-0.56	0.08	0.01	12
F1	0.13	0.23	0.05	60
Backcross	0.69	0.28	0.08	65
F2	0.21	0.30	0.09	114
cyrbia	0.97	0.05	0.00	51

Table 3. Comparison of BR values (± SD) between females and males in each *H. erato* generation. Males are bluer than females in crosses with a *demophoon* father or *cyrbia* maternal grandfather (MGF). Males are also bluer in backcrosses with a *cyrbia* MGF. There are no differences in the parental races.

Generation	Female BR value	Female sample size	Male BR value	Male sample size	t statistic	d.f.	p-value
demophoon	-0.56 ± 0.1	6	-0.56 ± 0.1	6	-0.06	9.0	0.955
All F1	0.10 ± 0.3	46	0.24 ± 0.2	14	-2.37	28.9	0.025*
F1 *cyrbia* father	0.26 ± 0.2	21	0.25 ± 0.2	7	0.17	8.4	0.872
F1 *demo.* father	-0.03 ± 0.2	25	0.23 ± 0.1	7	-3.80	13.3	0.002*
All F2	0.10 ± 0.3	63	0.33 ± 0.3	51	-4.28	96.4	<0.001*
F2 *cyrbia* MGF	0.12 ± 0.3	53	0.35 ± 0.3	47	4.00	92.1	<0.001*
F2 *demo.* MGF	0.02 ± 0.2	10	0.15 ± 0.4	4	-0.72	3.5	0.512
All BC	0.60 ± 0.3	35	0.79 ± 0.2	30	-2.93	62.9	0.005*
BC *cyrbia* MGF	0.58 ± 0.3	24	0.83 ± 0.2	25	-3.86	42.7	<0.001*
BC *demo.* MGF	0.65 ± 0.4	11	0.62 ± 0.4	5	0.16	7.6	0.877
cyrbia	0.98 ± 0.02	16	0.97 ± 0.1	35	0.79	48.2	0.431

Table 4. Comparison of BR values for offspring from reciprocal F1 crosses, which had either an iridescent mother or iridescent father, and for F2 crosses, which had either an iridescent maternal grandfather or grandmother. Mean values and sample sizes are shown in Table 3.

F1 *cyrbia* or *demophoon* father				F2 *cyrbia* or *demophoon* maternal grandfather			
	t	d.f.	p-value		t	d.f.	p-value
Female	-5.55	43.6	<0.001*	Female	-1.64	19.5	0.118
Male	-0.19	10.8	0.850	Male	-1.06	3.4	0.357
All	-4.67	56.8	<0.001*	All	-2.53	20.2	0.020*

Table 5. There are no significant correlations between the forewing red band measurements and BR colour in the F2 generation. Measurements are ratios of band measurements to wing size. Degrees of freedom = 69. N = 71.

Standardised measurement	Mean	Standard deviation	t	r	p-value
Linear 1	0.76	0.08	-1.65	-0.20	0.10
Linear 2	0.55	0.06	-1.41	-0.17	0.16
Linear 3	0.35	0.05	-1.69	-0.20	0.10
Linear 4	0.41	0.05	0.38	0.05	0.71

Table 6. Mean spacing (±SD) between longitudinal ridges and between cross-ribs. The narrower ridge spacing in *cyrbia* results in a brighter iridescent colour. The mean values for the F1 and F2 generations fell between the values for the parental races.

Generation	Mean longitudinal ridge spacing (nm)	Mean cross-rib spacing (nm)	Sample size (Male, Female)
demophoon/hydara	926.05 ± 40.1	482.87 ± 37.1	5 (3, 2)
F1	875.64 ± 57.8	476.66 ± 20.0	6 (4, 2)
F2	876.25 ± 36.0	484.46 ± 35.0	59 (25, 34)
cyrbia	822.55 ± 30.8	494.82 ± 30.1	8 (5, 3)