

Genetics of diapause in the comma butterfly *Polygonia c-album*

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Abstract. The processes of local adaptation and ecological speciation can be better understood by studying the genetic background of life-history decisions. The sex chromosomes host genes for many population differences in the Lepidoptera and therefore the inheritance of diapause determination in the butterfly *Polygonia c-album* may be hypothesized to be sex-linked. In the present study, *Polygonia c-album* (L.) from Spain and Sweden and hybrid offspring are raised under an LD 17 : 7 h photoperiod that induces most pure Swedish individuals to develop into the diapausing dark morph and most pure Spanish individuals into the light and directly-developing morph. If inheritance of the daylength threshold for diapause is X-linked, as is known to be the case for host-plant preferences, females should follow the developmental path of their male parents' populations. However, female hybrids instead have a diapause propensity intermediate to that of their parental stocks and, consequentially, diapause determination is not X-linked. However, male hybrids eclose as the diapausing morph to a higher extent than females and, moreover, this pattern is more pronounced in the Spanish female \times Swedish male cross than in the reciprocal cross. Hence, it is concluded that the genetic determination of the critical daylength for diapause is mainly autosomal but with some influence of sex-linked genes and/or parental effects, possibly as an effect of the importance of protandry for males. Such sex effects could provide a starting point for the evolution of population differences inherited on the sex chromosomes.

Key words. Autosomal inheritance, intraspecific hybridization, parental effect, photoperiod, seasonal polyphenism, sex effect, sex linkage.

Introduction

The genetic determination of a locally-adapted life-history trait, such as diapause and seasonal polyphenism, is of particular interest because it may influence the response of a population to climate change. In addition, such heritable differences constitute an important requirement for divergent evolution and eventually speciation. Because selection acts on phenotypes, it is important to understand how the genetic background interacts with phenotypic plasticity to determine the life cycle of insects (Bradford & Roff, 1995; Winterhalter & Mousseau, 2007).

Insects in temperate zones annually face the challenges of survival over winter. Diapause is a solution that provides important advantages in terms of minimized metabolism and maximized survival (Tauber *et al.*, 1986). During diapause,

certain genes are switched off, whereas others are expressed exclusively during this period of the insect's life (Denlinger, 2002) and, in some insect species, there are even different morphs depending on developmental pathway (seasonal polyphenism; Shapiro, 1976; Nylin, 1992). The synchronization between lifecycle and season is governed by hormones, which in turn are usually controlled by environmental cues indicating seasonal change, with the most important cues being photoperiod and temperature (Tauber *et al.*, 1986; Denlinger, 2002). For potentially multivoltine populations, there are trade-offs between entering diapause or trying to manage an additional generation before the arrival of winter and this can result in populations with partial voltinism within that area (Wiklund *et al.*, 1991).

Although the 'choice' of developmental pathway is by its nature plastic, there is also a clear genetic component behind it. In the case of photoperiodic determination of diapause, for example, the critical threshold day length is genetically determined and varies predictably among populations

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from different latitudes (Danilevskij, 1965; Bradshaw, 1976; Kato, 2005).

How geographical variation in diapause is genetically determined appears to be inconsistent between species. Differences in diapause response sometimes appear to be regulated by one or several autosomal alleles (Danilevskij, 1965; Tauber & Tauber, 1977; Han & Denlinger, 2009), although the results of other studies suggest that population differences in photoperiodic diapause regulation can be determined by one or several genes located on sex chromosomes (King, 1974; Rockey *et al.*, 1987a, b; Scriber, 1994).

In the Lepidoptera, females are the heterogametic sex (XY or ZW) and males are the homogametic sex (XX or ZZ). It is suggested that the sex chromosomes host a disproportionately high number of genes of importance for species differences in the Lepidoptera, indicating a role in speciation (Sperling, 1994; Prowell, 1998). In line with this general observation, previous studies show that genes located on the X-chromosome evidently can be of great importance for species and population differences in host plant preference in butterfly females because, in several species, female offspring inherit the host-plant preference of their male parents' populations (Thompson, 1988; Scriber *et al.*, 1991; Janz, 1998; Nygren *et al.*, 2006).

The reasons for the bias towards sex-linked population and species differences are still unclear (Janz, 2003). Compared with autosomal loci, recessive genes generally evolve more rapidly if sex-linked because they can be exposed to selection in the heterogametic sex (Prowell, 1998). They may also be more protected from recombination and introgression (Janz, 2003). These factors probably interact to produce more rapid differentiation in sex-linked traits, a process that sometimes aids speciation.

Depending on how diapause decisions are inherited, different evolutionary implications and different results from population hybridization events may be expected. The shape and dynamics of the hybrid zone between two closely-related species (or subspecies) of butterflies in eastern North America (*Papilio glaucus* and *Papilio canadensis*) is hypothesized to be controlled by interaction among sex-linked loci (Hagen & Scriber, 1989). The differences in both host-plant preference and diapause control between these taxa are governed by genes on the X-chromosome (Scriber, 1994). Hybrids between the two taxa even appear to have given rise to another third population with a 'false second' generation (*Papilio appalachensis*), which is isolated in time from the two mother populations (Scriber & Ordning, 2005). Possibly, the X-linked inheritance helps to speed up these incipient speciation events.

The comma butterfly *Polygonia c-album* (L.) (Nymphalidae; Nymphalinae) is a polyphagous butterfly species that is widely distributed over Eurasia. *Polygonia c-album* hibernates in the adult stage, is potentially multivoltine and can appear in two different adult morphs: a light directly developing summer morph and a darker one destined for winter diapause. Morphs differ in allocation of resources (Karlsson *et al.*, 2008), anti-predator structures (Wiklund & Tullberg, 2004) and are distinguished quite easily because the diapausing form of both sexes has a much darker brown or black colour on the underside of the wings than the directly developing form, which

may appear almost yellow. Morph and diapause are not perfectly linked but are still well correlated (Nylin, 1992); the frequencies of colour morphs is used in the present study as a convenient proxy for the propensity to enter reproductive diapause. The development into either of the two morphs is a plastic response to (mainly) photoperiod during the larval stages (Nylin, 1989), although it has a genetic background where the critical day length required for the development of the summer morph differs among populations according to latitude (Nylin, 1989). In Norway and Sweden, not even very long daylengths are enough to induce the summer morph when daylength is constant. Instead, increasing daylengths are necessary, and spring is so late that daylengths already start to decrease before the offspring of hibernating individuals reach pupation (Nylin, 1989). Thus, only the diapausing morph, and thereby a single generation per season, normally occurs in these regions. The directly developing summer morph (and hence additional partial or complete generations per year) can be found further south in Europe, North Africa and Asia.

In *P. c-album*, population differences in host plant preference and specificity are strongly linked to the X-chromosome (Janz, 1998; Nygren *et al.*, 2006). How diapause in *P. c-album* is regulated genetically, however, remains unknown. Because variation in host plant specificity in this species shows latitudinal patterns associated with voltinism (Nylin, 1988; Nylin *et al.*, 2009), it is speculated that host use and diapause control could both be sex-linked and thus inherited together as co-adapted gene complexes, as in the *P. glaucus* species group.

If diapause is linked to the X-chromosome, it is expected that, in a reciprocal cross between Swedish and Spanish *P. c-album* raised under near-critical conditions for diapause decisions (i.e. conditions where the outcome varies strongly among individuals), the majority of females with Swedish male parents will enter diapause, whereas females with Spanish male parents are expected to develop directly. If diapause control is instead inherited in an additive autosomal fashion or with incomplete dominance, an intermediate response is expected in both sexes and in both of the reciprocal crosses between males and females of the two populations.

Materials and methods

Insect collection and rearing

Female comma butterflies from Spain were collected in the mountain region above Barcelona (Can Liro and El Puig; three individuals) in mid April 2008. Swedish females were collected in early May 2008 in the vicinity of Stockholm (Riala and Margretelund; four individuals). Females of both populations had already mated in the wild and were placed in cages (50 × 50 × 45 cm) for oviposition. The roof consisted of transparent plastic and cages were lit from above by a 100-W tungsten light bulb (LD 8 : 16 h). A net covered the front of cages and the remaining sides consisted of green fabric. The floor was covered with moist paper towels. In the centre of each cage stood a pillar (height 30 cm), upon which a sponge with 10% aqueous sucrose was placed for females to feed. Females

were presented to stinging nettle (*Urtica dioica* L.), placed in glass bottles, for females to oviposit on. Eggs were collected and offspring of both broods were reared on *U. dioica*. Both populations were kept under directly developing conditions: larvae were initially reared in under an LD 12 : 12 h photoperiod at 17 °C, until molting into the third instar, when they were transferred to an LD 22 : 2 h photoperiod at 23 °C. To synchronize the emergence of adult butterflies for mating, F_1 Swedish and F_2 Spanish generations were used.

For the population crossings, approximately 25 adult males and 25 females were put in each cage for mating. Insects from the two populations were marked individually and divided among cages: two cages contained only Swedish males and females (Sw); two cages contained Spanish females and Swedish males ($Sp_{\text{♀}} \times Sw_{\text{♂}}$); two cages contained Swedish females and Spanish males ($Sw_{\text{♀}} \times Sp_{\text{♂}}$); and one cage contained only Spanish butterflies of both sexes (Sp). During mating, the couple sit *in copula* for 2–5 h and could then be carefully handled and put in covered glass jars (diameter 9 cm) on the bottom of the cages to complete the fertilization undisturbed. After mating, males were released into the cages again, whereas females were placed in separate cages for oviposition. The Swedish pure stock control group had four ovipositing females, which resulted in four families of larval offspring, whereas the Spanish control resulted in five families. The $Sp_{\text{♀}} \times Sw_{\text{♂}}$ cross resulted in four families, whereas five families could be reared in the reciprocal cross ($Sw_{\text{♀}} \times Sp_{\text{♂}}$).

For each family, 20 newly-hatched larvae were put individually in jars containing *U. dioica*. Jars consisted of two parts: the upper one containing the larva and the host, the stem of which was placed in the water-filled lower part through a small opening. The jars were placed in climate chambers (KB5410-L; Termaks AS, Norway; LD 17 : 7 h at 23 °C). These light and temperature conditions were chosen by extrapolating from previous finding regarding photoperiodic thresholds in Swedish and English populations (Nylin, 1989) and from pilot studies conducted in 2007. The aim was to induce diapause (dark morph) in most pure stock Swedish individuals and direct development (light morph) in Spanish stock. Plants were changed when needed as a result of withering or feeding damage. Water was sprayed over the jars once a day to increase humidity. Jar positions were changed daily and randomly to exclude positional effects. Two days after pupation, the sex of each individual was determined under a stereomicroscope ($\times 50\times$ magnification; M5-40728; Wild Heerbrugg, Switzerland). The morph of hatched adults was determined a few hours after emergence from the pupa.

Statistical analysis

Data was analyzed in STATISTICA, version 8.0 (Tulsa, Oklahoma) using generalized linear models (GLM), with Morph (dark or light; signalling the state of diapause) as a binomial response variable. Two types of models are available in STATISTICA: type 1 and type 3. Type 1 adds factors sequentially to the complete model, whereas type 3 specifically tests hypotheses about differences between subpopulation means. According to

the STATISTICA online textbook (<http://www.statsoft.com>), the type 3 model performs better with unbalanced designs as long as there are no missing cells. Type 3 was used, except for one case where there were no individuals in one cell.

Males of this species are known to have a higher propensity for diapause under near-critical conditions (Wiklund *et al.*, 1992; Wedell *et al.*, 1997), so males and females were analyzed separately, as well as together.

Family was also initially included as a factor (i.e. variation in diapause propensity among families within each population and cross to investigate genetic variation within categories). However, when this had been explored, the factor Family was subsequently removed from the statistical analyses because families are nested within the four different categories and because some families had few surviving representatives, which were not always of both sexes. Instead, Sex was considered to be a more important factor and thus included in the analysis. All data are given as the mean \pm SE.

Results

In the pure Spanish control population, there was no difference in diapause propensity between families ($Sp_{\text{♀}} \times Sp_{\text{♂}}$: $\chi^2 = 31.11$, d.f. = 6, $P = 0.41$). In the pure Swedish control population, morph was however influenced by family ($Sw_{\text{♀}} \times Sw_{\text{♂}}$: $\chi^2 = 17.12$, d.f. = 4, $P < 0.001$). The source of this difference was one family where most individuals developed into the light morph, which distinguishes it from the other three families where the proportion of light individuals was low. Importantly, there was no significant difference among families in any of the two hybrid crosses ($Sw_{\text{♀}} \times Sp_{\text{♂}}$: $\chi^2 = 2.46$, d.f. = 4, $P = 0.65$; $Sp_{\text{♀}} \times Sw_{\text{♂}}$: $\chi^2 = 2.80$, d.f. = 3, $P = 0.42$). Thus, although there is an indication of intrapopulation genetic variation in diapause propensity (of more importance under near-critical conditions for diapause determination; Nylin, 1992; Nylin *et al.*, 2005), the important results are mainly independent of family under the experimental conditions. Variation among families was consequently ignored in the subsequent analyses to permit closer investigation of the influence of sex.

To illustrate the general patterns with respect to sex, another binomial GLM was performed separating males and females of the four categories (controls and the two crosses; Fig. 1). This analysis again showed significant differences in diapause propensity between the categories (log-likelihood type 3: $\chi^2 = 46.9$, d.f. = 3, $P < 0.001$). The proportion of light morph individuals was high in the Spanish stock (95%) and low in the Swedish stock (24%), and overall intermediate in the hybrids ($Sp_{\text{♀}} \times Sw_{\text{♂}}$: 49%; $Sw_{\text{♀}} \times Sp_{\text{♂}}$: 52%) (Fig. 1). Sex was not significant as main effect ($\chi^2 = 0.48$, d.f. = 1, $P = 0.49$) but the interaction category sex was significant ($\chi^2 = 12.95$, d.f. = 3, $P < 0.01$).

Of more interest, however, is to compare only the two control categories in a separate binomial GLM (i.e. to investigate whether suitable conditions for the experiment had been found) and then only the two reciprocal crosses (i.e. to investigate sex linkage).

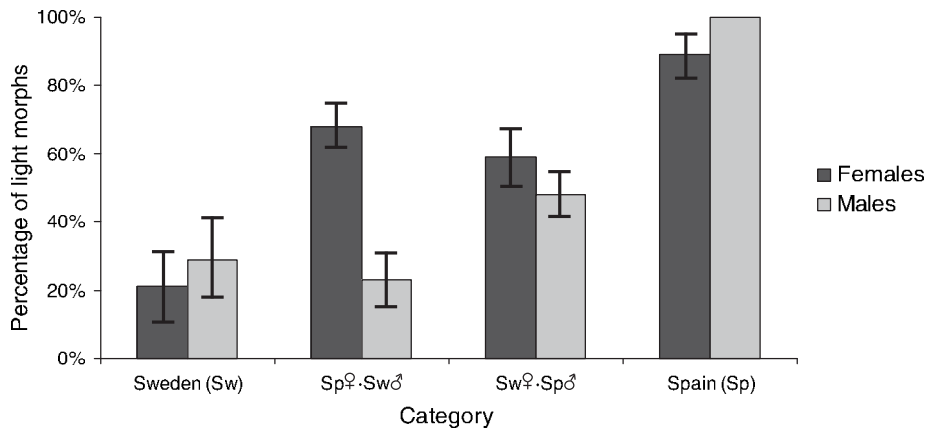


Fig. 1. The percentage of the light (directly developing) summer morph among adult males and females of *Polygonia c-album* in Swedish and Spanish stock, as well as the reciprocal hybrids, when larvae had been reared under an LD 17 : 7 h photoperiod at 23 °C. The error bars were derived from a 'weighted plot' of the results of a generalized linear model with Morph as the dependent factor (see text). The percentage of light morph individuals were 21% ± 10% for Swedish stock females ($n = 19$), 29% ± 13% for Swedish stock males ($n = 13$), 68% ± 7% for females of the Sp♀ × Sw♂ cross ($n = 44$), 23% ± 8% for males of the Sp♀ × Sw♂ cross ($n = 31$), 59% ± 9% for females of the Sw♀ × Sp♂ cross ($n = 33$), 48% ± 7% for males of the Sw♀ × Sp♂ cross ($n = 46$), 89% ± 7% for Spanish stock females ($n = 19$), and 100% ± 0% for Spanish stock males ($n = 18$).

In the first of these two binomial GLMs, comparing controls, as expected, there was an effect of population, where Spanish butterflies developed directly to a much higher extent than Swedish ones (Fig. 1) (Spanish females: 89% ± 7%; Spanish males: 100% ± 0%; Swedish females: 21% ± 10%; Swedish males: 29% ± 13%; binomial GLM, log-likelihood type 1: $\chi^2 = 41.17$, d.f. = 1, $P < 0.001$; type 1 was used because there were no dark morph males within the Spanish population.) Under these conditions, where variation was low within populations, there was no significant difference between the sexes ($\chi^2 = 1.34$, d.f. = 1, $P = 0.25$), nor was there any significant interaction between sex and population ($\chi^2 = 1.74$, d.f. = 1, $P = 0.19$). These results indicate that the experimental set-up was successful in that, under the chosen conditions, the majority of the Spanish individuals developed directly, whereas Swedish individuals were destined for diapause, regardless of sex.

When the two reciprocal crosses were compared, there was a significant effect of sex, showing that hybrid females had a higher proportion of directly developing individuals than hybrid males (Fig. 1) (binomial GLM: $\chi^2 = 12.10$, d.f. = 1, $P < 0.001$). There was no significant main effect of the direction of the cross ($\chi^2 = 0.96$, d.f. = 1, $P = 0.33$), although there was an interaction of cross × sex ($\chi^2 = 5.29$, d.f. = 1, $P < 0.05$). However, females did not have the diapause propensity expected from their paternal source populations; instead, females of both crosses showed an intermediate response between the two parental populations (Fig. 1). Thus, the main pattern of inheritance does not appear to be X-linked, although the significant interaction term suggests that a sex-linked genetic factor or parental effects influence the propensity to enter diapause in hybrids.

Figure 1 shows that hybrid males with Swedish male parents had a similar low propensity to develop as light morphs as did the pure Swedish male stock, whereas the reciprocal

hybrids developed the light colouration to a higher extent. To investigate this pattern in more detail, a chi-square test was made for males and females, respectively. A difference was seen in the percentage of directly developing males: 48% of the males in the Sw♀ × Sp♂ cross were of the light morph compared with only 22% of the Sp♀ × Sw♂ cross ($\chi^2 = 4.40$, d.f. = 1, $P < 0.05$). By contrast, no significant difference could be detected between the females of the reciprocal crosses, as would have been expected under X-linkage (Sw♀ × Sp♂: 58%; Sp♀ × Sw♂: 68%; $\chi^2 = 2.67$, d.f. = 1, $P = 0.10$). The tendency towards a difference was also in the opposite direction to that expected.

Discussion

Females do not follow the diapause determination thresholds of their fathers, in contrast to the predictions if inheritance is X-linked. Instead, after population crosses, females show a propensity to enter diapause intermediate to that of their parents. This suggests that the daylength response is mainly controlled by autosomal genes and is not inherited via sex chromosomes.

Still, there are effects of sex, both general and more specific. The propensity to enter diapause is higher for males than for females in hybrids. Pure stock male and female *P. c-album* is reported to differ in diapausing propensity under near-critical conditions for diapause (Wiklund *et al.*, 1992; Wedell *et al.*, 1997) and, in the present study, the same tendency is instead seen in the population crosses. This is because the present experimental set-up was designed for detecting genetic variation in diapause in the crosses, in contrast to the situation with respect to pure stock. It is of interest to note that the higher propensity for males to enter diapause is strong enough to override even the genetic differences in photoperiodic thresholds between populations in hybrids. This suggests that the often

observed differences in diapause propensity between the sexes are independent of the exact genetic background and are best seen as a secondary sexual characteristic. A practical consequence of this result is that it is of importance to distinguish between the sexes in studies of the genetic architecture behind thresholds for diapause induction.

A similar pattern is reported in the face fly (*Musca autumnalis*), where diapause propensity appears to be intermediate to the parents, although partially sexually-linked inheritance and incomplete dominance complicates the development choice because male flies are found to diapause to a higher extent than females (Kim *et al.*, 1995). Interestingly, a polygenic system with some influence of sex chromosome expression appears to be a recurring phenomenon in diapause regulation within Diptera (Riihimaa & Kimura, 1989; Kim *et al.*, 1995; Kimura & Yoshida, 1995; Takeda, 1998; Mathias *et al.*, 2007).

Such sexual differences in diapause propensity may ultimately be explained by sexual conflicts: it is likely that males and females do not share the same optimal lifecycle (Wiklund *et al.*, 1992). Females profit from larger body size because size is correlated with fecundity, although this might imply a longer development time (Nylin & Gotthard, 1998; García-Barros, 2000). Males, on the other hand, often gain higher fitness if development time is shorter than in females (Abrams *et al.*, 1996). In the speckled wood butterfly, *Pararge aegeria*, intraspecific hybridization shows that development time (including diapause development) is polygenic and is regulated by autosomal genes, as well as by modifying genetic factors on the Y-chromosome (Nylin *et al.*, 1994). In addition, males of *P. aegeria* are also found to enter diapause more readily than females. This pattern probably occurs because late or slow-growing male individuals, which would emerge too late for protandry in the direct developing generation, instead emerge in the spring, when they have a better chance to compete for females (Wiklund *et al.*, 1992; Wedell *et al.*, 1997).

Sexually antagonistic genes, which comprise those that are advantageous for one sex but disadvantageous for the other, may eventually become so strongly connected to sex that they become sexually linked (Bergero & Charlesworth, 2009). It is probable that the general sex effect on diapause control in *P. c-album* and other butterflies has evolved because of differential selection as a result of such sexual antagonism. In such cases, it is possible that population and species differences in diapause expression will become increasingly determined by genes on the sex chromosomes and eventually completely sex-linked, although inheritance at the early evolutionary stage of differentiation addressed in the present study is more complicated.

In the reciprocal crosses, there is a significant interaction between direction of cross and sex. This is partly the result of a tendency for females with Spanish mothers to develop directly at a higher frequency than those with Swedish mothers; if this is a real pattern, it could involve a Y-linked gene with relatively minor effect. However, the difference between crosses among males contributes more strongly to the interaction: hybrid males with Swedish fathers follow the diapause propensity of Swedish males, whereas males with Spanish fathers display a diapause propensity more intermediate between the parental populations. This pattern is enigmatic and cannot

easily be explained by classical genetics; instead, it suggests some form of parental effect (i.e. when a trait is not affected only by the genotype or environment of the individual but is influenced by the genotype and/or environment provided by the mother or father). Although the mechanisms are most often unknown, parental effects are known to affect diapause response in insects (Denlinger, 2002), and there are indications of such effects also within the *Polygonia* genus (Hidaka & Takahashi, 1967).

The findings of the present study imply that, although host specificity and thresholds for diapause both show latitudinal patterns, they are not inherited together as a co-adapted gene complex. Still, the combination of diapause response and host plant effects on other life-history traits has a great impact on local adaptations in a way that make some combinations more favourable than others. For example, high host specificity for stinging nettle, a host plant that permits high larval growth rate, combines well with a threshold for diapause resulting in multivoltinism (Nylin *et al.*, 2009). If there are reproductive barriers, such local adaptations can co-adapt with yet other traits and eventually aid speciation. *P. c-album* is a fairly mobile species (Shreeve, 1981) and thus has a relatively open population structure (Nylin *et al.*, 2005), although the maintenance of population differences is possible in allopatric populations or if populations are separated by time of the season. The effects of subsequent intraspecific hybridization events can vary depending on how phenotypes are inherited. If different traits have different modes of inheritance, this might often lead to a mismatch between genes, resulting in suboptimally performing hybrids (Nygren *et al.*, 2006).

In conclusion, the results of the present study suggest that the threshold for diapause induction in *P. c-album* is inherited in a mainly autosomal and additive fashion, although there is also a strong general as well as specific influence of sex. This is possibly a general phenomenon for sexually antagonistic traits, and may be related to speciation in Lepidoptera, because the often observed sex-linked differences between closely-related species in this taxon probably arose originally in this fashion.

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