

Hybrid queen butterflies from the cross *Danaus chrysippus* (L.) \times *D. gilippus* (Cramer): confirmation of species status for the parents and further support for Haldane's Rule

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A cross between queen butterflies of the Palaeotropical species *Danaus chrysippus* and the Neotropical *D. gilippus* was achieved with difficulty in both directions. Only one progeny ($N=70$) was reared comprising sterile males and inviable females in a precisely 1:1 ratio. Both prezygotic and postzygotic barriers to gene flow are strong. The result supports Haldane's Rule, to which we propose a minor amendment. The F_1 hybrids were intermediate for background colour between the brown (genotype BB) of *gilippus* and orange (genotype bb) of *chrysippus*. Most F_1 pattern characters were also intermediate. In polymorphic *chrysippus* populations, because Bb heterozygotes are brown, or nearly so, we suggest the B allele may have evolved towards dominance in sympatry. Hybrid males show positive heterosis for body size. The close similarity of male genitalia between the allopatric, genetically distant species *chrysippus* and *gilippus*, compared to their divergence between *gilippus* and its largely sympatric sister species *eresimus*, suggest that reinforcement of sexual isolation or reproductive character displacement have evolved in sympatry. © 2002 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2002, 76, 535–544.

ADDITIONAL KEYWORDS: evolution of dominance – heterosis – prezygotic barriers – postzygotic barriers – reinforcement – reproductive character displacement – sexual isolation.

INTRODUCTION

The queen butterflies *Danaus* (*Anosia*) *chrysippus* in the Old World and *D. (A) gilippus* in the Americas have both enjoyed a high profile in butterfly studies; and yet their taxonomic status remains unresolved. In particular, Ackery & Vane-Wright (1984) say, “we have been unable to discover any apomorphies which characterize [*gilippus*] to the exclusion of *D. chrysippus*.” They go on to speculate, “although the Old World *chrysippus* can be defined by pattern characters . . . , it is so simi-

lar in all other known features to the New World *gilippus* that . . . the two may best be regarded as one species.” Our principal aim in attempting to hybridize *chrysippus* and *gilippus* is to test the Ackery/Vane-Wright hypothesis, which would be supported if fertile hybrids resulted from the cross.

The subgenus *Anosia* (queen butterflies) of the genus *Danaus* (Order Lepidoptera, Family Nymphalidae, Subfamily Danainae, Tribe Danaini, Subtribe Danaina) comprises four currently recognized species, three from the New World and one from the Old World (Ackery & Vane-Wright, 1984), as follows: *D. (A) eresimus* (Florida, Texas and Antilles south to the Amazon Basin), *D. (A) plexaure* (central South America, south of the Amazon, Bolivia and southern Brazil to Paraguay, Argentina and Uruguay), *D. (A) gilippus* (south-

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ern USA and Antilles south to Argentina and Uruguay) and *D. (A) chrysippus* (Afrotropical, Palaearctic and Indo-Pacific Regions). As *plexaure* lacks structural autapomorphies in relation to *eresimus*, it is doubtfully distinct and may best be treated as a vicariant subspecies of the latter (G. Lamas in Ackery & Vane-Wright, 1984).

Calculated from distribution data given by Ackery & Vane-Wright (1984), the taxon (*eresimus+plexaure*) is sympatric with *gilippus* over 95% of its range. Observation of high density mixed populations of *eresimus* and *gilippus*, over four weeks and two seasons, on Grand Cayman, West Indies (DASS, unpublished) suggests that sexual isolation between them must be strong. Indeed, *eresimus-gilippus* hybrids have never been recorded. Although interspecific courtship is sporadic, it is invariably brief and apparently rarely consummated. The biology of *plexaure* is entirely unknown but the ecology and behaviour of *eresimus* and *gilippus* appear to be very similar, and the two are, moreover, accurate Müllerian mimics. On Grand Cayman they may, however, differ in food plant preference, possibly for *Cynanchum angustifolium* and *Sarcostemma clausum*, respectively (DASS, unpublished), although in other parts of their range both butterflies will accept either or both plant genera (Brown & Heineman, 1972; de Vries, 1987; Schwartz, 1989; Smith *et al.*, 1994). Moreover, their pheromones probably differ in composition, although this has never been demonstrated. In contrast to *eresimus* and *gilippus*, *chrysippus* is separated from the New World *Anosias* by the Atlantic and Pacific Oceans. It is subdivided over its vast range into actually (sympatric) or potentially (allopatric) interbreeding subspecies or semispecies of uncertain status. The *chrysippus* complex is a syngameon within which crosses, even among geographically distant populations, such as from West Africa and Australia, readily produce F₁, F₂ and back-cross progenies that are fertile in both sexes (Clarke *et al.*, 1973), although incipient sexual isolation (Gordon, 1984; Smith, 1984) and disturbed segregations and sex ratios (Owen & Chanter, 1968; Gordon, 1984; Smith *et al.*, 1997, 1998; Smith, 1998) occur in hybrid zones.

MATERIAL AND METHODS

INSECT MATERIAL

Eggs of *D. gilippus* ssp. *bernice* were obtained on Grand Cayman, British West Indies, in January 1997, by placing wild females in muslin sleeves that enclosed shoots of the abundant local foodplant *Sarcostemma clausum* (Asclepiadaceae). Eggs and young larvae were taken by air to Kilifi, Coast Province, Kenya. Larvae were then sleeved (to protect

them from parasitoid wasps) on potted *Asclepias curassavica* (Asclepiadaceae) to complete their development. The first *gilippus* imago (female) eclosed early on 30 January 1997, an egg-adult development time of 19 days. Two more females, eclosed on 1 February 1997, were also observed closely. As the females used in the crossing experiments had to be virgin, *chrysippus* males of the local subspecies *dorippus*, two old individuals, collected in the wild, and four newly eclosed individuals, were used to mate with the *gilippus* females.

MATING ENCLOSURE

For courtship and mating, we used a large (4 m × 6 m × 3 m (high)), partially shaded, outdoor flight cage that was densely planted with potential food plant flowers, *Calotropis gigantea* (Asclepiadaceae), and nectar-bearing flowers, including *Stachytarpheta indica* (Verbenaceae), a cosmopolitan species that occurs in the natural habitat of both *chrysippus* and *gilippus* (personal observations). To stimulate pheromone production in the males, we also introduced bunches of dried *Heliotropium steudneri* (Boraginaceae) as a rich source of pyrrolizidine alkaloids (PAs). In order to attract females, *Anosia* males must have access to PAs, which they metabolize into the active pheromone principle, danaidone (Pliske & Eisner, 1969; Edgar *et al.*, 1973; Schneider *et al.*, 1975). F₁ hybrid eggs were obtained by sleeving (presumed) mated *gilippus* females on *A. curassavica*. The hybrid larvae were raised on *A. curassavica* and/or *C. gigantea*. [Note, *C. procera*, a congener of the latter, is a locally abundant alien on Grand Cayman, where it is avoided by both *gilippus* and *eresimus*. All attempts to persuade females of both species to oviposit in sleeves on *C. procera*, or to force larvae of either species to feed upon it, were unsuccessful.]

In January 1998 we attempted to obtain the reciprocal cross, i.e. between *gilippus* males from Grand Cayman and virgin *chrysippus* females from Kenya, again in a no-choice design. In this case courtship was not closely observed but, as 12 months previously, after more than 7 days, one mating was eventually achieved and the female laid more than 100 eggs. Unfortunately, all were accidentally lost to predatory ants.

The hybrid specimens that eclosed in England have been deposited in the Hope Department of Entomology, Oxford University Museum of Natural History.

MORPHOLOGICAL CONFIRMATION OF THE BUTTERFLIES USED

Male genitalia from each of *chrysippus*, *gilippus*, *chrysippus* × *gilippus* F₁ hybrids and *eresimus* were examined 'blind' by DG. The number of spines on the

aedeagus provided data for a morphometric comparison of the three species and the hybrids.

RESULTS

OBSERVATIONS ON COURTSHIP BETWEEN *GILIPPUS* FEMALES AND *CHRYSIPPUS* MALES

The behaviour of the butterflies in the flight cage was observed over seven consecutive days, 30 January to 5 February 1997, for periods of 30–120 min and a total of 16 h. As male queen butterflies rarely initiate courtship before 11.00 (DASS, unpublished), most observation was done in the afternoon. Recording began at 15.00 h on 30 January 1997 when one newly eclosed virgin *gilippus* female and two wild *chrysippus* males, one old and tattered, the other younger, were introduced into the flight cage. Over the next two days the number of *gilippus* females rose to three and of *chrysippus* males, with the addition of four newly eclosed individuals, to six. All the butterflies remained in the cage until the experiment was terminated on 21 February 1997, although from 6 February to 21 February 1997 they were largely unobserved.

On 30 January 1997, for the first 30 min several male–female encounters occurred, but without recognition. Then, the males started to approach the female closely when she was in flight, although never when perched with closed wings. Over the same period the males briefly but repeatedly courted one another. However, neither male extended his hairpencils, either in homosexual or heterosexual encounters. On the second day, there were several periods of intense courtship (1–2 min), mainly involving the older male. Although he expanded his hairpencils, in most encounters he failed to achieve contact with the female's antennae and, hence, to transfer pheromone to her (Pliske & Eisner, 1969; Seibt *et al.*, 1972). The female invariably took evasive action and eventually settled with closed wings. Although, on one occasion the male achieved antennal contact, the female, once settled, consistently signalled refusal with open wings. By the third day, now with three females and six males in the cage, intense courtship activity, both homosexual (by both sexes) and heterosexual (including soliciting of males by females) was ceaseless. All the males were using their hairpencils in all types of interaction although the young ones may have had insufficient access to the PA source to produce effective pheromone transfer particles (Schneider *et al.*, 1975; Smith, 1975). On the fourth day, for the first time a courted female permitted a male to alight beside her. He bent his abdomen towards hers until they touched, whereupon she flew away. This sequence, which was to be repeated many times, was the closest any of the authors came to observing copulation.

COPULATION AND REARING OF PROGENY

After observations were concluded on 5 February 1997, the stand-off between the sexes must have continued for a further 16 days, as it was not until 21 February 1997 that one of the three sleeved *gilippus* females laid eggs on *A. curassavica*; between 21 and 23 February 1997 this female laid a total of 77 eggs, of which 70 hatched, developed and pupated successfully. The brood was then split; 40 pupae were reared in Kenya and 30 were sent to eclose in England. All pupae eclosed between 13 and 17 March 1997 to produce perfectly formed F₁ hybrid imagines, 35 of each sex. The two *gilippus* females that failed to oviposit may not have mated.

HYBRID FITNESS

The hybrid males flew successfully, imbibed nectar and appeared capable of normal sexual behaviour. This was confirmed when four hybrid males mated successfully with virgin *chrysippus* females at Kilifi. These four females laid several hundred eggs but all failed to hatch. The hybrid male fathers were therefore presumed sterile. The hybrid females, though apparently perfectly formed, were unable to fly or even to hold onto a plant stem. They fell to the floor and all died within 2 days without feeding. Hence, it was impossible to obtain either F₂ or backcross broods.

THE F₁ HYBRID PHENOTYPE

Variable characters among the parent species and F₁ hybrids are summarized in Table 1 and most are also illustrated in Figure 1. Comments on each character are here listed in the order shown in Table 1.

1–2. Forewing measurements on the parental forms are taken from wild specimens of the same subspecies, collected in the same locations as the experimental parents; after 3 weeks in an insectary the parents themselves were too battered to measure. The *chrysippus* and *gilippus* males do not differ significantly in size ($t_{21}=1.7$; $0.10 > P > 0.05$). However, F₁ males show highly significant positive heterosis, compared to males of both parental species combined ($t_{37}=4.2$; $P < 0.001$). The *chrysippus* and *gilippus* females do not differ significantly in forewing length ($t_{14}=2.0$; $0.10 > P > 0.05$). Moreover, as they are ranked for size (downwards) in the order *chrysippus* > F₁ > *gilippus*, there is no heterosis for forewing length in females. As previously found in all populations of *D. chrysippus* (Smith, 1980), the mean forewing length of male *chrysippus* and *gilippus* used in this study, and their hybrids, exceeds that of females by 1–3 mm.

3. The ground colour of *chrysippus* ssp. *dorippus* is a uniform tawny orange (Fig. 1a,b), whereas *gilippus* is a

Table 1. Phenotypic characters scored for *Danaus chrysippus dorippus*, *D. gilippus berenice* and F₁ hybrids

Character	<i>D. chrysippus</i>	<i>D. gilippus</i>	F ₁ hybrids
1 Mean male fw length ± SE (mm)	40.7±0.45 (N=14)	39.6±0.43 (N=9)	42.4±0.38 (N=16)
2 Mean female fw length ± SE (mm)	39.8±0.75 (N=12)	36.8±1.40 (N=4)	39.0±0.44 (N=13)
3 Ground colour	Tawny orange	Nutbrown	Intermediate*
4 White spots in fw spaces <i>M</i> ₁ & <i>M</i> ₂ (d & v)	Absent	Present	93.1% penetrance (N=29)*
5 Postmedial white spots in fw spaces <i>Cu</i> ₁ , <i>Cu</i> ₂ & 2 <i>A</i> (d & v)	Absent	Present	Absent
6 Submarginal white spots in fw spaces <i>r</i> ₅ -2 <i>A</i> (d & v)	Absent	Present	Absent
7 White spot(s) (externae) in fw space <i>r</i> ₄ (d & v)	Single in 85.3% (N=35)	Double	Single in 34.5% (N=29)*
8 Three black spots on hw <i>m-cu</i> crossveins (d & v)	Present	Absent	Intermediate on d & v *
9 Colour of hw long veins (v)	Pale	Broadly and densely black	Narrowly black*
10 Width of hw black border at vein <i>Cu</i> ₁ (v)	Narrow (2.5 mm)	Broad (4.5 mm)	Intermediate (3.5 mm)*
11 Submarginal white spots on hw (v)	Single (outer) row of 13	Double row, 13 outer and 14 inner	Double row, 13 outer 7–10 small inner*

Notation: SE, standard error; fw, forewing; hw, hindwing; d, dorsal; v, ventral. *see text for more detail.

rich chocolate brown (Fig. 1c,d). The hybrids are predominantly orange with more or less heavy brown shading, on the dorsal side, in the basal areas of both wings and extending along the costal margin of the forewing to between one-third and two-thirds of its length (Fig. 1e–h). Although the extent of brown is variable, submarginal and marginal areas of both wings (excepting the narrow black borders) are tawny orange in all specimens.

4. The subapical, white spots in forewing spaces *M*₂ and *M*₃ of *D. gilippus* are homologous with the much larger ones found in most *D. chrysippus* subspecies; the spots are absent only from subspecies *dorippus*. This character is penetrant in the F₁ hybrids, as small pale spots on the forewing underside (93.1%, *N*=29) and, as faint marks, on the upper side (69.0%, *N*=29). Expressivity of the character, when present, in the hybrids is essentially uniform.

5–6. The 11–13 conspicuous, 'extra', white spots, 3 in the postmedial area and 8–10 in the submarginal area, on both forewing surfaces (spaces 2*A*–*R*₅) of *gilippus*, all of which are lacking in *chrysippus* ssp. *dorippus*, are also absent in the hybrids.

7. All *gilippus* have a pair of white spots (externae) in forewing space *R*₄, whereas in most *chrysippus* there is a single, larger spot (Ackery & Vane-Wright, 1984). In the *chrysippus* specimens we have examined (*N*=35), 85.3% have a single spot that often clearly results from fusion of two spots; the remainder, unlike *gilippus* where the two spots are well separated, has a

pair of closely adjacent spots. The latter description applies to 65.5% of hybrids examined (*N*=29), whereas the minority (34.5%) has a single 'chrysippus' spot.

8–9. The presence of three black spots on the *m-cu* crossveins of the hindwing, on both surfaces, is a pattern autapomorphy in *D. chrysippus* (Fig. 1a,b) in relation to other *Anosias*. These spots may be vestiges of the heavy, black scaling that highlights the long veins on the ventral side of the hindwing of all other *Danaus* (Ackery & Vane-Wright, 1984). In the hybrids, indistinct spots are visible on the dorsal surface (Fig. 1e,f), but they are more apparent on the ventral side, where they take the form of bulges in the broad lines of black scales on the veins bordering the cell (Fig. 1g,h). The expression of characters 8–9 in the hybrids is almost precisely intermediate between the parents.

10–11. The width of the black border on the hindwing ventral side, at the point where it crosses vein *Cu*₁, is 4.5 mm in *gilippus* (Fig. 1c), compared to 2.5 mm in *chrysippus* (Fig. 1a); as the species do not differ significantly in body size, the difference cannot be allometric. In the hybrids the border is intermediate at 3.5 mm (Fig. 1g,h). Whereas the broad, black border of *gilippus* accommodates two rows of submarginal, white spots, the narrower border of *chrysippus* has only one row; the latter is a pattern autapomorphy in all *chrysippus* compared to all other *Danaus*. The hybrids are intermediate for this character, with a complete outer row of 13 spots and an incomplete inner row of 7–10 spots (compared to the *gilippus*

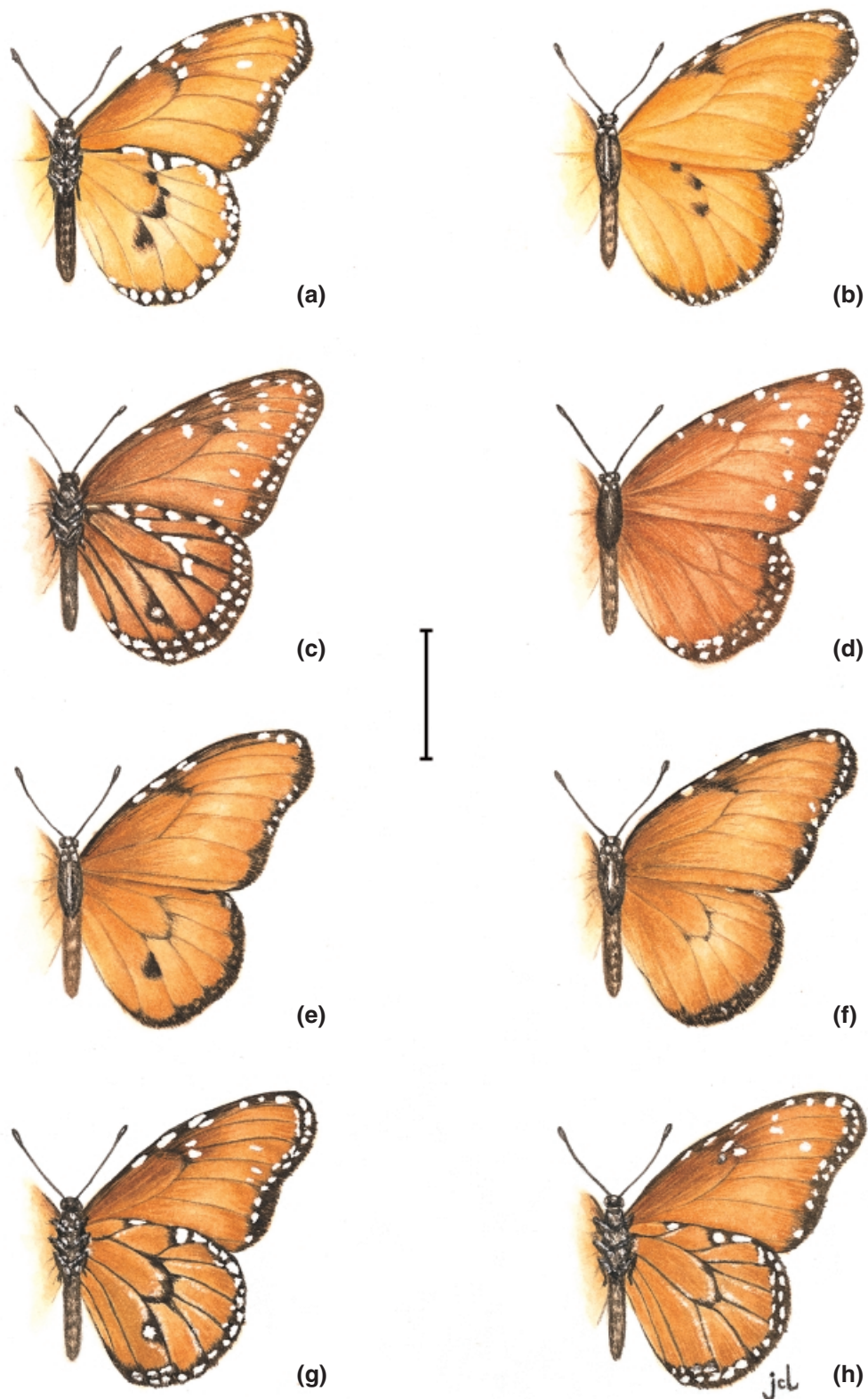


Figure 1. (a) *Danaus chrysippus dorippus* male (ventral). (b) *D. chrysippus dorippus* female (dorsal). (c) *D. gilippus berenice* male (ventral). (d) *D. gilippus berenice* female (dorsal). (e) F₁ male (dorsal) from the cross *chrysippus* (male) × *gilippus* (female). (f) F₁ female (dorsal). (g) F₁ male (ventral). (h) F₁ female (ventral). Scale bar = 2 cm.

Table 2. Analysis of the number of spines on the aedeagi of male *Anosia* butterflies

Species	Mean number of spines \pm standard error				N
	Left (L)	Right (R)	Total	Ratio L/R	
<i>D. chrysippus dorippus</i>	5.1 \pm 0.27	6.7 \pm 0.44	11.8 \pm 0.56	0.72 \pm 0.05	23
<i>D. gilippus berenice</i>	4.6 \pm 0.53	6.2 \pm 0.28	10.8 \pm 0.61	0.75 \pm 0.09	13
<i>chrysippus</i> \times <i>gilippus</i> F ₁	5.3 \pm 0.25	6.3 \pm 0.95	11.5 \pm 0.95	0.89 \pm 0.11	4
<i>D. eresimus tethys</i>	3.1 \pm 0.28	4.4 \pm 0.51	7.5 \pm 0.54*	0.84 \pm 0.14	10

**eresimus* has significantly fewer spines than (*chrysippus*+*gilippus*), $t_{44}=4.7$; $P<0.001$.

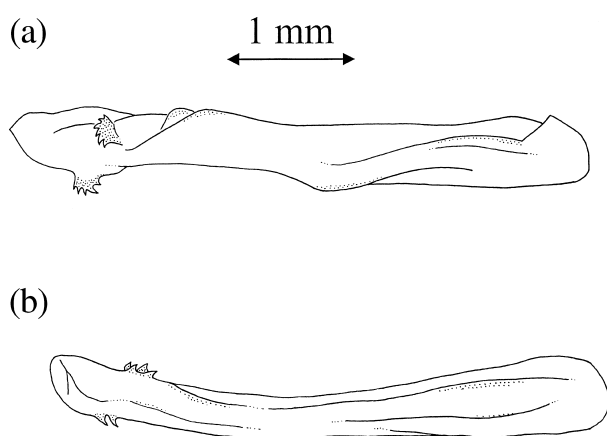


Figure 2. Aedeagi of (a) *Danaus chrysippus dorippus* and (b) *D. eresimus tethys*, dorsal views, with posterior end to the left. Note the two diagnostic bunches of spines (Table 2), the left hand group slightly anterior to the right. The aedeagi of *D. gilippus* and the *chrysippus* \times *gilippus* F₁ hybrids are indistinguishable from (a).

inner row of 14 spots). Moreover, the inner spots of the hybrid are smaller than in the *gilippus* parent and irregularly rectangular, rather than circular in outline.

MALE GENITALIA

Because, on the one hand, it has been suggested that *chrysippus* and *gilippus* might be conspecific (Ackery & Vane-Wright, 1984), whereas, on the other hand, *eresimus* (which may include *plexaure*) has always been recognized as a distinct species, we decided to examine the male genitalia of all three species and the *chrysippus*-*gilippus* hybrids. We find the male genitalia of *chrysippus*, *gilippus* and their F₁ hybrid to be qualitatively and quantitatively indistinguishable (Table 2), while the aedeagus of (*chrysippus*+*gilippus*) is readily

distinguished from *eresimus* by the presence of a pair of dorso-lateral tubercles, the left one slightly anterior to the right, on the apex of each being mounted a bunch of spines: in *eresimus* two groups of smaller spines, similarly positioned, arise from the flat surface of the aedeagus (Fig. 2; for *gilippus* and *plexaure*, see Ackery & Vane-Wright, 1984; figs 101–2). The number of spines in *eresimus* is very significantly fewer than in (*chrysippus*+*gilippus*) (Table 2).

DISCUSSION

THE F₁ HYBRID PHENOTYPE

Heterosis for body size (character 1), confined to F₁ males (Table 1), is also widespread in crosses among subspecies of *D. chrysippus* (Smith (1980). The occurrence of heterosis only in the homogametic (ZZ) males might be explained by monosomy for Z-linked genes in the heterogametic (ZW) females coupled, either with the apparent absence of dosage compensation for Z-linked genes in butterflies (Johnson & Turner, 1979), or with a disturbed Z-autosome effect (Coyne & Orr, 1989) in the hybrid females.

Ground colour (character 3) in *D. chrysippus* is either nutbrown or tawny orange and is controlled by the B locus with two alleles, *B* and *b*; some subspecies are brown (*BB*) and others, such as *dorippus* orange (*bb*) (Fig. 1a,b), while polymorphic populations are frequent, especially in central and east Africa (Smith, 1975, 1998; Smith *et al.*, 1997, 1998). The colour genetics of *D. gilippus* has not been investigated, but the phenotype of ssp. *berenice* (Fig. 1c,d) suggests it must be *BB*. The *B* allele is variably dominant to co-dominant in crosses between *chrysippus* subspecies. Ground colour in our F₁ butterflies is intermediate between the parents (Fig. 1e–h) and somewhat resembles the brownish, otherwise *dorippus*-like, f. *klugii* Butler of *D. chrysippus*, that results from the cross f. *dorippus* (orange, *bb*) \times f. *chrysippus* (brown, *BB*) (Smith, 1998). However, our hybrids have more orange

than is usual in *klugii* or other *Bb* heterozygotes in *D. chrysippus*, where brown is often fully dominant. This suggests that, when *Bb* heterozygotes result from crosses between sympatric *BB* and *bb* morphs in a polymorphic population, but not otherwise, dominance of the *B* allele may have been favoured by natural selection (Fisher, 1930).

The white spots in forewing spaces M_2 and M_3 of *D. chrysippus* are absent from *dorippus* (*CC*) but are expressed as white spots on a black background (*cc*) in all other forms of *D. chrysippus*. In *chrysippus Cc* heterozygotes, the two spots are variably penetrant (Smith, 1975, 1998), the most frequent expression taking the form of two pale, oblong marks, usually visible only on the underside (*f. transiens* Suffert). Penetrance is significantly higher (74.4%, $N=173$) on a *BB* background compared to *bb* (45%, $N=129$) (Smith, 1975). The phenotype of the hybrids strongly suggests that the two much smaller white spots in spaces M_2 and M_3 of *gilippus* are homologous with those in *chrysippus*. Penetrance of these spots in the hybrids (93.1%, $N=29$) is significantly higher than in *CC*×*cc* crosses in the polymorphic *chrysippus* population at Dar es Salaam (Smith, 1998): comparing the interspecific hybrids ($N=29$) with the F_1 of *BBcc*×*bbCC* crosses within *D. chrysippus* ($N=220$), $\chi^2_1=4.8$; $0.05>P>0.02$ whereas for a similar comparison with the F_1 of *bbCC*×*bbcc* crosses ($N=127$), $\chi^2_1=20.2$; $P<0.001$. Therefore, comparing *Cc* hybrids from parents of allopatric and sympatric origins, the frequency of *C* allele dominance is significantly higher in the latter: thus, the *C* locus evidence also supports the hypothesis that dominance has evolved in sympatry, perhaps by accumulation of modifier genes (Fisher, 1930; Ford, 1955; Clarke & Sheppard, 1960).

The absence from the hybrids of the 'gilippus' post-medial and submarginal, forewing, white spots (characters 5–6) is presumably due to a dominant gene carried in the *chrysippus* parent. All the characters 7–11 are intermediate between the parents; the expression of characters 8–11 in the hybrids is also correlated with suppression of black pigment in *chrysippus* compared to *gilippus*. As evidence from segregation in F_2 or backcross progenies is neither available nor obtainable, it is impossible to distinguish between alternative, equally feasible, control mechanisms for 'reduction of black', i.e. by a major gene, with two co-dominant alleles, or by polygenes.

SEXUAL ISOLATION

The weakness of the mate recognition system between male *chrysippus* and female *gilippus* and the sterility/inviability of the F_1 establishes the status of *gilippus* and *chrysippus* as species according to both the recognition species concept (RSC) (Paterson, 1985, 1993)

and the biological species concept (BSC) (Dobzhansky, 1937; Mayr, 1963). The stand-off between the sexes lasted at least one week, and possibly up to three! (As copulation was not observed, and females were sleeved to stimulate oviposition only intermittently, we cannot be more precise about the timing.) It is clear, however, that the single verified pairing (of a possible three) was achieved only by denial of choice and escalating sexual frustration. The results of the mating experiment suggest that premating recognition (RSC) between *chrysippus* (males) and *gilippus* (females) is so weak that, were populations of the two species to come into secondary contact in nature, heterotypic matings would occur, if at all, only as a result of grossly distorted sex ratios or severe and synchronous population bottlenecks. Even in eventualities such as these, however, as hybrid males are sterile and females inviable, postmating isolation would be complete and gene flow zero (BSC).

Nevertheless, despite the strong evidence for sexual isolation, three caveats should be entered: (i) although the F_1 male×*chrysippus* female backcross is sterile, the alternative backcross, F_1 male×*gilippus* female, was not carried out; (ii) we have no hybrid sterility/inviability data from the reciprocal F_1 cross, male *gilippus*×female *chrysippus*; we know only that copulation and oviposition are hard to achieve; and (iii) we are aware that our data are limited to one, albeit large, F_1 hybrid brood. However, we are certain that chronic failure of sexual recognition (RSC) jeopardises, not only the cross described, but also its reciprocal. Three other points worthy of note are: (i) as two of the three experimental females failed to oviposit when sleeved, the mother of our brood was probably the only one that mated; (ii) no eggs were laid, either on the possibly unacceptable *C. gigantea* plant growing in the flight cage, or on *A. curassavica* in a sleeve, for 3 weeks; and (iii) virgin *gilippus* and *chrysippus* females, courted by and mated to experienced males of their own species, invariably achieve *copula* in their first afternoon and oviposit within 24 h when sleeved on *A. curassavica* (DASS, IJG, unpublished).

The courtship behavioural sequences in *gilippus* (Brower *et al.*, 1965) and *chrysippus* (Seibt *et al.*, 1972) are identical. Therefore, the barriers to successful courtship between the species are probably not behavioural *per se*, but rather to be explained by discordant visual and/or olfactory cues (Silberglied & Taylor, 1978; Ritchie & Phillips, 1998). For some 15 min, the males simply failed to recognize the females as potential mates. As the colour phenotypes of the species are highly contrasted (Fig. 1), the initial barrier was probably visual, a conclusion that is supported by data from polymorphic *D. chrysippus* populations in Ghana and Tanzania that mate assortatively for orange and brown colour, determined mainly by female choice

(Gordon, 1984; Smith, 1984). After 15 min, the females began to excite male interest, but only when in flight. Males could have been attracted by the gliding flight, characteristic of danaines and identical in the two species, or possibly by an olfactory stimulus. Although female danaine pheromones have not been demonstrated, Boppré (1984) remarked "A variety of observations and experimental results strongly suggest the occurrence of female pheromones in butterflies, and perhaps chemical signals for close-range communication may be widespread or even typical. [However,] ... the lack of both conspicuous 'scent organs' and odours detectable to humans [but see Rothschild *et al.* (1984)] make the study of female chemical signals particularly difficult."

It was not until the second day that male pursuits, with vigorous use of the hairpencils, and female evasions and refusals became hectic and prolonged. Again, refusals by females may have resulted from inappropriate cues, either visual or olfactory. No major differences between the chemical compositions of *chrysippus* and *gilippus* male pheromones are known, and the active principle in both species is the PA derived heterocyclic ketone, danaidone (Meinwald *et al.*, 1969, 1971; Schneider *et al.*, 1975). Moreover, as one or more of only three dihydropyrrolizines, hydroxy-danaidal, danaidal and danaidone, are active principles in all danaine hairpencil scents examined, they are unlikely to play any part in species recognition systems (Boppré, 1984). On the other hand, subtle interspecific variation among a cocktail of up to 33 accessory pheromone components (Meinwald *et al.*, 1974) is impossible to rule out and is, indeed, highly probable. It is clear that, given the scarcity of structural and pattern apomorphies in most danaine genera (e.g. *Danaus*, *Tirumala*, *Parantica*, *Ideopsis*, *Amauris* and *Euploea*), renewed work on pheromone chemistry, ideally accompanied by allozyme or DNA studies, is a prerequisite for further taxonomic resolution of the subfamily.

HALDANE'S RULE AND HYBRID FITNESS

Haldane (1922) stated as follows

When in the F₁ offspring of two different animal races one sex is absent, rare or sterile, that sex is the heterozygous [heterogametic] one.

Haldane's Rule is widely applicable to hybrids between animal races that breed sexually (Coyne & Orr, 1989). Species in which the male is the heterogametic (XY) sex (e.g. mammals and *Drosophila*) contribute most of the evidence. However, where the female is heterogametic (ZW), as in birds (Haldane, 1932; Gray, 1958) or Lepidoptera (Grula & Taylor, 1980), Haldane's Rule also holds. Because, in the *chrysippus* × *gilippus* F₁, one sex is sterile and the other

inviably, our data are not strictly encompassed by Haldane's Rule as stated above. Nevertheless, although both sexes have zero Malthusian fitness, the heterogametic female is the more dysfunctional one as befits Haldane's Rule. Hence, to embrace crosses such as *chrysippus* × *gilippus* and *Drosophila americana* × *D. montana* (Coyne & Orr, 1989), that produce no fertile offspring of either sex, we suggest the following extension of the Rule:

When in the F₁ offspring of two different animal races, one sex is absent, rare, sterile or inviable, or, when one sex is sterile and the other absent, rare or inviable, the latter sex is the heterogametic one.

EVIDENCE FROM MITOCHONDRIAL DNA

Preliminary analyses by GL (unpublished) of sequence data from the 12S rRNA and cytochrome-c subunit I mitochondrial genes for large samples ($N \geq 8$) of *eresimus*, *gilippus* and *chrysippus* indicate that the two former, collected from the same location on Grand Cayman, West Indies, are genetically almost identical, whereas both species are separated from African, Asian and Australian *chrysippus* by substantial genetic distances. If confirmed, these data suggest that (*eresimus* + *gilippus*) and *chrysippus* ancestral stocks bifurcated several million years ago, whereas *eresimus* and *gilippus* have speciated only within the last few thousand years. Thus, the molecular evidence indicates that it is *eresimus* and *gilippus*, rather than *gilippus* and *chrysippus* (Ackery & Vane-Wright, 1984), that enjoy the sister relationship within the subgenus *Anosia*.

REINFORCEMENT OR REPRODUCTIVE CHARACTER DISPLACEMENT

In a detailed comparison of evolutionary rates for pre- and postmating isolation, Coyne & Orr (1989) concluded that both evolved at a similar rate in *Drosophila*, although premating isolation appeared most quickly between sympatric species (such as *eresimus* and *gilippus*). On the other hand, in allopatric species, such as *gilippus* and *chrysippus*, that probably speciated by dispersal across oceans and are now separated by substantial genetic and geographical distances, sexual isolation cannot result from selection. Hence, while the potentially complete sexual isolation of *gilippus* and *chrysippus* is no surprise, it is probably a pleiotropic consequence of independent genetic drift in long-separated populations (Ritchie & Phillips, 1998) rather than of selection. It is intriguing therefore that the (*eresimus* + *plexaure*) aedeagus differs qualitatively (Fig. 2) and quantitatively (Table 2) from that of (*gilippus* + *chrysippus*), whereas, among the latter pair

and their F₁ hybrid, aedeagi are indistinguishable. In the latter, the aedeagus has two bunches of spines mounted on a pair of lateral tubercles, whereas in the former, the spines are smaller, fewer and unmounted (Fig. 2; Ackery & Vane-Wright, 1984). The divergence of male genitalia in sympatry, but not in allopatry, probably results either from reinforcement of premating isolation (Dobzhansky, 1937, 1940; Blair, 1955) or, alternatively, from reproductive character displacement (Butlin, 1987, 1989). In either case, the evolution of sexual isolation between the sympatric sister-species pair *eresimus* and *gilippus* is expected to result from strong natural selection against hybrids.

This case warns against the widespread and somewhat uncritical practice in insect taxonomy of relying on male genitalia characters alone to establish relationship. It also serves to remind us that although genitalic difference, as with *eresimus* from *gilippus*, generally indicates sexual isolation, similarity, as between *chrysippus* and *gilippus*, is *per se* inadequate evidence for conspecificity.

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REFERENCES

- Ackery PR & Vane-Wright RI. 1984.** *Milkweed butterflies*. London: British Museum (Natural History).
- Blair WF. 1955.** Mating call and stage of speciation in the *Microhyla olivacea*-*M. carolinensis* complex. *Evolution* **9**: 469-480.
- Boppré M. 1984.** Chemically mediated interactions between butterflies. In: Vane-Wright RI, Ackery PR, eds. *The biology of butterflies*. Symposia of the Royal Entomological Society of London, **11**. London: Academic Press, 259-275.
- Brower LP, Brower JvZ, Cranston FP. 1965.** Courtship behavior of the queen butterfly, *Danaus gilippus berenice*. *Zoologica, New York* **50**: 1-39.
- Brown FM & Heineman B. 1972.** *Jamaica and its Butterflies*. London: Classey.
- Butlin RK. 1987.** Species, speciation and reinforcement. *American Naturalist* **130**: 461-464.
- Butlin RK. 1989.** Reinforcement of premating isolation. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland: Sinauer, 158-179.
- Clarke CA & Sheppard PM. 1960.** The evolution of dominance under disruptive selection. *Heredity* **14**: 73-87.
- Clarke CA, Sheppard PM, Smith AG. 1973.** The genetics of fore and hindwing colour in crosses between *Danaus chrysippus* from Australia and from Sierra Leone (Danaiidae). *Journal of the Lepidopterists' Society* **27**: 73-77.
- Coyne JA & Orr HA. 1989.** Two rules of speciation. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland: Sinauer, 180-207.
- Dobzhansky T. 1937.** *Genetics and the origin of species*. New York: Columbia University Press.
- Dobzhansky T. 1940.** Speciation as a stage in evolutionary divergence. *American Naturalist* **74**: 312-321.
- Edgar JA, Culvenor CCJ, Robinson GS. 1973.** Hairpencil dihydropyrrolizines of Danainae from the New Hebrides. *Journal of the Australian Entomological Society* **12**: 144-150.
- Fisher RA. 1930.** *The genetical theory of natural selection*. Oxford: Clarendon Press.
- Ford EB. 1955.** Polymorphism and taxonomy. *Heredity* **9**: 255-264.
- Gordon IJ. 1984.** Polymorphism of the tropical butterfly *Danaus chrysippus* L. in Africa. *Heredity* **53**: 583-593.
- Gray AP. 1958.** *Bird hybrids*. Farnham Royal, UK: Commonwealth Agricultural Bureaux.
- Gruha JW & Taylor OR. 1980.** Some characteristics of hybrids derived from the sulfur butterflies *C. eurytheme* and *C. philodice*. *Evolution* **34**: 673-687.
- Haldane JBS. 1922.** Sex-ratio and unisexual sterility in hybrid animals. *Journal of Genetics* **12**: 101-109.
- Haldane JBS. 1932.** *The causes of evolution*. London: Longmans, Green & Co.
- Johnson MS & Turner JRG. 1979.** Absence of dosage compensation for a sex-linked enzyme in butterflies. *Heredity* **43**: 71-77.
- Mayr E. 1963.** *Animal species and evolution*. Cambridge: Belnap Press.
- Meinwald J, Boriack CJ, Schneider D, Boppré M, Wood WF, Eisner T. 1974.** Volatile ketones in the hairpencil secretion of danaid butterflies (*Amauris* and *Danaus*). *Experientia* **30**: 721-722.
- Meinwald J, Meinwald YC, Mazzochi PH. 1969.** Sex pheromone of the queen butterfly: chemistry. *Science* **164**: 1174-1175.
- Meinwald J, Thompson WR, Eisner T, Owen DF. 1971.** Pheromones VII. African monarch: major components of the hairpencil secretion. *Tetrahedron Letters* **1971(38)**: 3485-3488.
- Owen DF & Chanter DO. 1968.** Population biology of tropical African butterflies. 2. Sex ratio and polymorphism in *Danaus chrysippus* L. *Revue Zoologique et Botanique Africaines* **78**: 81-97.
- Paterson HEH. 1985.** The recognition concept of species. In: Vrba ES, ed. *Species and speciation*. Pretoria: Transvaal Museum, 21-29.
- Paterson HEH. 1993.** *Evolution and the recognition concept of species*. Baltimore: The John Hopkins University Press.
- Pliske TE & Eisner T. 1969.** Sex pheromone of the queen butterfly: biology. *Science* **164**: 1170-1172.
- Ritchie MG & Phillips SDF. 1998.** The genetics of sexual isolation. In: Howard DJ, Berlocher SH, eds. *Endless forms: species and speciation*. Oxford: Oxford University Press, 291-308.

- Rothschild M, Moore BP, Vance Brown W. 1984.** Pyrazines as warning odour components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera *Zygaena* and *Amata* (Lepidoptera). *Biological Journal of the Linnean Society* **23**: 375–380.
- Schneider D, Boppré M, Schneider H, Thompson WR, Boriack CJ, Petty RL, Meinwald J. 1975.** A pheromone precursor and its uptake in male *Danaus* butterflies. *Journal of Comparative Physiology* **97**: 245–256.
- Schwartz A. 1989.** *The butterflies of Hispaniola*. Gainesville: University of Florida Press.
- Seibt U, Schneider D, Eisner T. 1972.** Duftpinsel, Flugelfaschen und Balz des Tagfalters *Danaus chrysippus*. *Beihefte Zeitschrift für Tierpsychologie* **31**: 513–530.
- Silberglied RE & Taylor OR. 1978.** Ultraviolet reflection and its behavioural role in the courtship of the sulfur butterflies, *Colias eurytheme* and *C. philodice*. *Behavioural Ecology and Sociobiology* **3**: 203–243.
- Smith DAS. 1975.** Genetics of some polymorphic forms of the African butterfly *Danaus Chrysippus* L. (Lepidoptera: Danaidae). *Entomologica Scandinavica* **6**, 134–144.
- Smith DAS. 1980.** Heterosis, epistasis and linkage disequilibrium in a wild population of the polymorphic butterfly *Danaus chrysippus*. *Zoological Journal of the Linnean Society* **69**: 87–109.
- Smith DAS. 1984.** Mate selection in butterflies: competition, coyness, choice and chauvinism. In: Vane-Wright RI, Ackery PR, eds. *The biology of butterflies*. Symposia of the Royal Entomological Society of London, 11. London: Academic Press, 225–244.
- Smith DAS. 1998.** Non-Mendelian segregation and variable penetrance of colour genes in the polymorphic butterfly *Danaus chrysippus* (L.). *Heredity* **80**: 474–480.
- Smith DAS, Gordon IJ, Depew LA, Owen DF. 1998.** Genetics of the butterfly *Danaus chrysippus* (L.) in a broad hybrid zone, with special reference to sex ratio, polymorphism and intragenomic conflict. *Biological Journal of the Linnean Society* **65**: 1–40.
- Smith DS, Miller LD, Miller JY. 1994.** *The Butterflies of the West Indies and south Florida*. Oxford: Oxford University Press.
- Smith DAS, Owen DF, Gordon IJ, Lowis NK. 1997.** The butterfly *Danaus chrysippus* (L.) in East Africa: polymorphism and morph-ratio clines within a complex, extensive and dynamic hybrid zone. *Zoological Journal of the Linnean Society* **120**: 51–78.
- de Vries J. 1987.** *The butterflies of Costa Rica*. Princeton: Princeton University Press.