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All-female broods in the butterfly *Hypolimnas bolina* (L.)

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[Plate 7]

It has been known since 1924 that a proportion of females in the butterfly *H. bolina* produce only daughters, whereas others produce a 1:1 sex ratio. The present results confirm this, and show also that occasionally a few males are produced. The production of broods with a disturbed sex ratio is inherited entirely through the female line. By sexing the embryos and larvae cytologically and observing mortality in the embryos and late pupal stage we have obtained evidence that the deficiency of males is due to their very high mortality in the pre-adult stage. It is suggested that the abnormal sex ratio is due to an infective cytoplasmic factor (the presence of spirochaetes, as in *Drosophila*, having been ruled out), in contrast to *Acraea encedon* where meiotic drive of the Y chromosome has been postulated. The view is put forward that the polymorphism in *H. bolina* is maintained by the 'infected' females being at a slight disadvantage and that their numbers are maintained by contagion from an unidentified reservoir species.

INTRODUCTION

All-female broods are known in a few species of Lepidoptera (*Abraxas grossulariata* (L.) (see, for example, Robinson 1971), *Hypolimnas bolina* (L.) (Poulton 1924), *Acraea encedon* (L.) (Poulton 1914; Owen 1970, 1971), and *Danaus chrysippus* (L.) (Owen & Chanter 1968), but the mechanisms determining them have never been fully worked out.

MATERIALS AND RESULTS

Since 1972 we have received stocks of *H. bolina* from Fiji, New Guinea, Sarawak, Sri Lanka, Hong Kong and Australia, in order to study the genetics of the polymorphism for colour pattern in the female. Among these wild stocks (and their progeny) we have obtained females giving all-female broods from Sarawak, Sri Lanka and Hong Kong but not from Australia and New Guinea, nor from Fiji, where Poulton (1924) originally reported them. Genetic investigations of the inheritance of wing pattern showed that our all-female broods were not the result of parthenogenesis but that the female parent had to be fertilized by a male.

Table 1 gives the origin of our wild stocks. The 11 bisexual broods produced 234 males and 247 females between them. Among the broods listed as 'unisexual' are three from Sarawak which between them produced 7 males in 107 insects. The other 7 broods (five of which were also from Sarawak) produced 165 females and no males. 139 females subsequently derived from these unisexual broods produced 3216 females, together with 21 males, at least seven of which were shown to be fertile. Even after four generations of crossing such females to males from bisexual stock, all broods contained to be 'unisexual'. In contrast, 189 females from bisexual broods, with one exception, produced bisexual progeny in an approximately 1:1 ratio (1713 males to 1820 females). The exceptional female, who had a sister producing a bisexual brood, herself produced 82 females only and no males. The one mated offspring of this female in its turn also gave rise to an all-female brood of 43 insects.

TABLE 1. BROODS FROM WILD FEMALES

origin	bisexual	unisexual
Fiji	2	0
Sarawak	1	8†
New Guinea	1	0
Sri Lanka	2	1
Hong Kong	0	1
Australia	5	0

Broods too small to determine the sex ratio and of which subsequent broods did not establish their status have been excluded.

† 3 of these 8 broods in fact produced 40 females and 2 males, 58 females and 4 males, and 1 male and 2 females respectively. This was in contrast to the bisexual broods where a good 1:1 ratio was obtained.

One of the questions that has to be asked is whether the abnormal females lay only fertile eggs destined to give rise to females, or whether embryos of both sexes are produced and there is differential mortality, as in *Drosophila* (Oishi 1971).

Smith (1945) has shown that in some Lepidoptera the sex of the larva can be identified by the presence of a heteropyknotic body in the interphase nuclei of the somatic cells in the female (the heterogametic sex). Furthermore, Scali & Masetti (1973) have shown that a similar spot can be demonstrated in embryos. We have used the method to investigate *H. bolina*, where the female but not the male has a heteropyknotic body. We found that in the progeny of females from all-female broods there was an approximately 1:1 ratio of males to females among the developing ova (judged by the absence or presence of a heteropyknotic body (figure 1, plate 7) as there was in the bisexual broods. Among the first instar feeding larvae in the unisexual broods there was a deficiency of males, and there were almost none by the second and later instars (1 in 77), again judged by the presence of the heteropyknotic body (table 2). In contrast, in the bisexual broods there was an approximately 1:1 ratio throughout development.

Only two explanations seem possible: (1) that all the embryos in the unisexual broods are destined to be female but only half of them develop a heteropyknotic body before the late first instar stage, or (2) that there is differential mortality of males, the vast majority having died by the late first instar, leaving mostly females.

TABLE 2. THE NUCLEAR SEX OF SAMPLES OF EMBRYOS AND LARVAE FROM BROODS DESTINED TO GIVE RISE ONLY TO FEMALES

brood	embryo		1st instar		2nd instar		3rd and later instars	
	males	females	males	females	males	females	males	females
12590	15	18	—	—	—	—	—	—
12730	13	12	10	24	0	20	—	—
12740	—	—	3	12	1	9	0	26
12758	13	6	12	29	0	18	0	4
totals	41	36	25	65	1	47	0	30

Since the unisexual stock has to be mated to males in every generation, there can be no overall difference in the genetic constitution of the two except with respect to genes associated with the Y chromosome (the female being the heterogametic sex in those Lepidoptera adequately tested, and *H. bolina* is likely to be no exception since a heteropyknotic body is apparently the manifestation of a Y chromosome (Traut & Rathjens 1973). Even here, there should be no segregation for such factors within the female stock. Consequently it is difficult on genetic grounds to explain an early and a late developing heteropyknotic spot within the unisexual broods but not the bisexual ones. The difficulty of explaining on genetic grounds the presence of two kinds of female in the unisexual broods strongly suggests that Smith's diagnostic method is applicable to embryos in *H. bolina.* This view is strengthened by examination of fourth and fifth instar larvae, which can be sexed both by the heteropyknotic body and by dissection. This showed a complete correlation between the two methods in both types of brood. In fourth and fifth instar female larvae and adult females we could detect no morphological difference between the individuals of the unisexual and bisexual broods, and this extended to the structure and egg content of the ovarioles. Thus there is no evidence for two kinds of female among those in unisexual broods nor between these and those giving rise to bisexual broods. Therefore, the difference between the two types of brood is likely to be due to a difference in the mortality pattern as between the sexes. This was originally suggested by Simmonds (1930).

Chanter & Owen (1972) studying the butterfly *A. encedon*, suggested that all-female broods in that species were due to meiotic drive acting on the Y chromosome, because the number of adults produced by the two kinds of female was approximately the same. Our results are consistent with their findings, the unisexual broods where at least one adult was produced having a mean size of 23.3

and the bisexual ones of 18.7. Furthermore, the proportion of mated females that produced no adult progeny was not significantly different between the two kinds of female (unisexual broods 32 in 171, bisexual broods 55 in 244, $P = 0.39$). However, the approximate equality of family size (among all those that had offspring) from our two kinds of female could well be due to a negative relationship between family size and survival rate, or, alternatively, to differential fecundity between the two kinds of female. We have been able to get no reliable figures on either of these points because of the huge variance between broods. But we have been able to obtain more evidence of differential mortality between the sexes in unisexual broods.

In order to demonstrate this rigorously it would be necessary for the unisexual and bisexual broods to be otherwise genetically identical, and for the larvae to be raised separately to randomize environmental factors affecting mortality. It would be desirable to use equal numbers of fertile eggs from each female, and equal numbers of females from the two kinds of brood, if an analysis of variance is to be used. Because of the refractory nature of the material, the only one of these conditions met in this preliminary study is that the larvae were raised individually. If differential mortality is to account for the unisexual broods, on average 50 % or more of the embryos must fail to reach sexual maturity. Because the cytological evidence suggested that many of the deaths occurred before the second instar, we have divided both kinds of brood into those with an observed mortality of 50 % or greater before the second instar, and those with less, as judged by a sample of eggs segregated and treated individually. Of the ten samples from unisexual broods, all had a mortality of 50% or greater, whereas of the 24 bisexual broods 10 had an observed mortality of 50 % or greater. Thus there is a significant difference ($P = 0.0014$ (one-tailed test)). Since the broods were not genetically identical in other respects, and were not necessarily raised contemporaneously, this result has to be treated with some caution.

In order to take the matter further, we investigated, retrospectively, mortality in the late pupal stage. Although most differential mortality appears to take place before the second instar (table 2), we have no evidence that it does not continue into later stages and we therefore compared the number of adults produced with the number which died in the pupa at a stage late enough for the sex to be recognizable (table 3).

There is no significant difference between females of the two kinds of brood, as judged by a two-tailed test ($P = 0.72$), or between males and females in the bisexual broods ($P = 0.23$), or between the males from the bisexual broods and the females from the unisexual broods ($P = 0{\cdot}31$). However, there is a significant difference between the males and females from the unisexual broods ($P < 10^{-7}$) and the males from the bisexual and unisexual broods ($P < 10^{-7}$).

We subsequently attempted to get more data by making a special effort to dissect dead pupae in 9 further unisexual broods. No males emerged and none were found dead in the pupa case. However, the 'mortality' among the females appeared to be the same as that among both the males and females of the 80 further bisexual broods examined ($\chi^2_2 = 0.51$, $P > 0.6$). The actual number of dead insects sexed in the pupa was greater in the prospective than in the retrospective count because of the special care taken in studying the dead insects. Thus there is no evidence for a difference in mortality between the females in the unisexual broods and the males and females in the bisexual broods. However, these results do suggest that even late in pre-adult life there is increased mortality among the few males found in the unisexual broods. This result also needs treating with caution, since few all-female broods actually produced any recognizable males so that these families contributed heavily to the estimate of mortality. Nevertheless, the mortality in early life and that in the pupal stage taken together suggest rather strongly that there is a much enhanced mortality rate throughout pre-adult life among males in the unisexual broods.

TABLE 3. MORTALITY IN THE LATE PUPAL STAGE

		no. of broods	unisexual		no. of broods	bisexual	
			♂♂	♀♀		♂♂	♀♀
retrospective	emerged	130	21	3030	109	1161	1285
	died in pupa		26	60		17	28
prospective	emerged	9	0	186	80	552	535
	died in pupa		0	8		24	28

In *A. encedon*, Chanter & Owen (1972) did not compare the early mortality in unisexual and bisexual broods, nor look at the heteropyknotic body in ova and young larvae. However, they did look at overall larval mortality in a few broods with equivocal results, and were therefore prepared to accept the hypothesis of meiotic drive disturbing the sex ratio, despite the fact that, as they were aware, the complete absence of one class as the result of meiotic drive must be a very rare phenomenon. If we had not investigated the sex ratio and mortality in the early stages, meoitic drive could conceivably have been an explanation of the unisexual broods in *H. bolina*, since the female is the heterogametic sex and the absence of males was not complete.

In *Drosophila*, where the female is the homogametic sex, meiotic drive due to a gene on the Y chromosome cannot be the explanation of unisexual broods 'inherited' as in *A. encedon* and *H. bolina*. In some *Drosophila* species spirochaetes and viruses have been implicated in the differential mortality of the sexes in the all-female broods (see for example, Oishi & Poulson 1970; Oishi 1971).

Because our results give good evidence that the disturbed sex ratio is due to the high mortality of the males, as in *Drosophila*, we initiated an investigation into the possibility of the presence of either spirochaetes or viruses in insects destined to give rise to two sorts of broods. A preliminary study of our larvae by Mr I. Heuch, and a more detailed one of larvae, pupae and adults by Dr C. D. Alergant, Director of Venereology, University of Liverpool, failed to detect spirochaetes in either the unisexual or bisexual stocks. Furthermore, Dr T. W. Tinsley and Dr K. Harrap of the Unit of Invertebrate Virology at Oxford have failed to detect virus at any stage in either type of brood. Dr Harrap did however detect what appeared to be a microsporidian both in a unisexual and a bisexual brood. These results do not rule out the presence of a virus, but show that if it is present it is very difficult to detect.

Our genetic investigations, nevertheless, suggest that there is an as yet unidentified cytoplasmic factor in the all-female broods which is almost lethal to the male *H. bolina.* It is possible however that a Y-autosome translocation could explain the mortality of the males if the homologue to the autosome is usually lost during meiosis (Ursula Mittwoch, personal communication). Professor Suomalainen is examining the meiotic chromosomes of females of the two types in order to investigate this possibility. However, if a translocation were responsible one would have to postulate either that the female from a bisexual brood which gave rise only to unisexual progeny was a contaminant or that a Y-autosome translocation with effectively the same properties as the original one has arisen in our stock. In addition it is difficult to see how the translocation mechanism could be maintained as a polymorphism in the population and furthermore, if *H. bolina* behaves like *Ephestia kühniella* Z. one would expect multiple heteropyknotic bodies in the presence of a Y-autosome translocation (Traut & Rathjens 1973). These have not been observed (figure 1, plate 7)

Discussion

The widespread occurrence of unisexual broods in *H. bolina* poses an interesting ecological problem. Unlike the situation postulated by Chanter & Owen (1972) in *A. encedon,* where they suggested that meiotic drive coupled with inbreeding could help to explain the sex ratio polymorphism, no similar mechanism can be applied to *H. bolina,* since here we know that mortality of the males is involved.

Owen (1974) has suggested that the production of all-female broods in which the brood size is not reduced might be an adaptation to the sudden increase in the availability of food, since he believes that the population would then build up quickly. However, he does not explain how such females can be maintained in the population just because their presence is an advantage. In our view it still needs some form of inter- or intra-population frequency dependent selection to maintain both types of female in the population if they have different fertility with respect to the production of females.

The frequencies of the two kinds of female need not change from generation to generation if their relative fertilities are such that each contributes on average the same number of female progeny to the next generation. However, this stability will only be neutral. To explain how the females producing the all-female broods

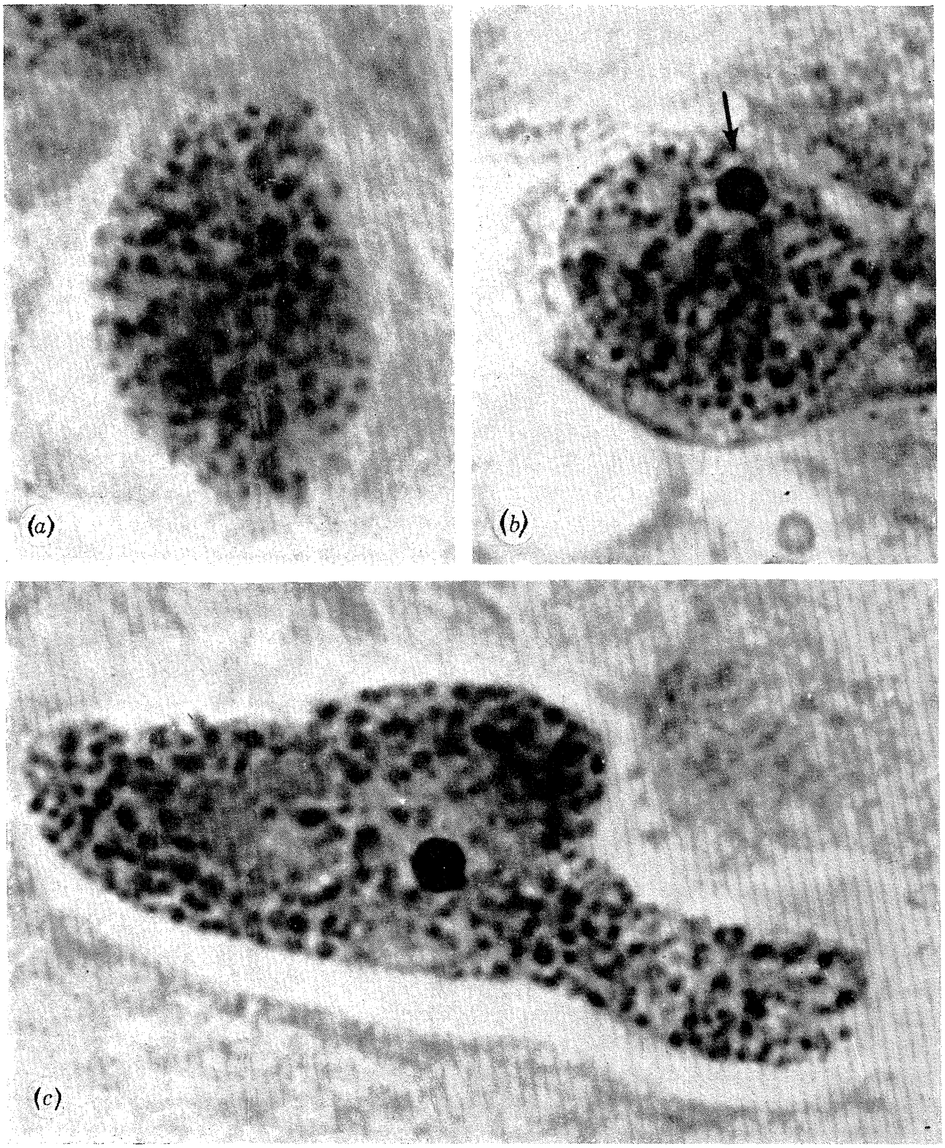

FIGURE 1. Aceto-orcein squashes (magn. × 1560). (*a*) Nucleus from male embryo, showing no sex chromatin; (*b*) nucleus from female embryo, showing sex chromatin indicated by arrow, and (*c*) nucleus from female third instar larva, showing sex chromatin.

became common in the first place but are still found as a polymorphism in many populations requires discussion. If the females producing only female progeny are at an advantage, then the polymorphism will only be transient and should long since have disappeared unless it is of very recent origin, which is unlikely because of the wide geographic distribution of such females. Alternatively if one postulates that the stability is neutral, random processes might produce a polymorphism of longer duration in some populations but ultimately one or other of the forms of female would disappear. Finally, the polymorphism may be stable in which case one requires a mechanism which will put the commoner type of female, whichever that might be, at a disadvantage.

Such a mechanism might exist even if the females producing all-female broods were at an advantage. Thus if the species forms discrete populations and the females destined to produce only daughters produce more of them than do the bisexual ones, then the frequency of the former would increase if one of them were introduced into a colony. Such a population would ultimately consist only of females and therefore die out, and the locality could only be recolonized from a bisexual population. This would help to explain the presence of both kinds of female, since those producing only daughters would ultimately be at a disadvantage (though at an advantage when rare) since the population in which they were present would die out. This recolonization hypothesis appears attractive, since our breeding data, taken at their face value, suggest that females giving unisexual broods give rise to twice as many female progeny as those producing bisexual ones. However, it is difficult to invoke some form of inter-population selection as the stabilizing factor since the butterflies are apparently highly migratory (Woodhouse 1950) even in populations where unisexual females occur. Thus in Sri Lanka the population of the whole island is probably effectively a single genetic unit.

Neither a neutral nor a selective hypothesis seems adequate to explain the presence of two kinds of female. In our view the most likely explanation of the polymorphism is that the cytoplasmic factor is infectious but has a low infectivity. This possibility is supported by one of our broods in which a female from a bisexual brood produced a unisexual line. If there were a reservoir of the cytoplasmic factor in some other species in which it did not affect sex ratio, and the unisexual females in *H. bolina* were at a slight disadvantage, a stable polymorphism could be maintained. A constant ratio of the two types of female would occur when the proportion of newly infected females was balanced by the loss due to the reproductive disadvantage of infected females. Such a reservoir in another species, if it existed, would probably have a patchy distribution and thus explain the apparently patchy distribution of populations with females producing only female progeny.

Owen (1971) noted that in *A. encedon* populations with an abnormal sex ratio and a heavy excess of females appear to be associated with 'disturbed' ground. If in fact the abnormal sex ratios are due to a differential mortality of the males rather than Y drive, the situation might be explained by the 'reservoir species' being particularly common in such places.

We have investigated the matter in *H. bolina* by asking those who collected for us to describe the kind of locality from which our stocks came, and also those in Fiji where Simmonds (Poulton 1924) collected. Unlike *A. encedon*, there is absolutely no evidence for all-female broods being associated with cultivation, even though the distribution of populations with females giving a disturbed sex ratio is geographically patchy as in *A. encedon*.

It seems appropriate to concentrate on studying the ecology of *H. bolina* in Sri Lanka because the species is highly migratory there, females producing unisexual broods are found and the country is an island. As a first step one must know the relative frequencies of the two kinds of female in different localities and at different seasons of the year. As the result of our own observations and information supplied by Mr Karunaratne, there seems to be an excess of females, anyhow at some periods of the year, and also a fair proportion of the females that have been caught in the wild have been unmated, as judged by the absence of a spermatophore. However, to investigate the sex ratio in the wild it will be necessary to carry out mark-release-recapture experiments since the males and females have different habits.

The polymorphism, apart from its own ecological interest, may have important implications in the field of pest and vector control (Owen, Owen & Chanter 1973) since suggestions have often been made that methods involving the disturbance of the sex ratio might be effective. The information so far gleaned from *H. bolina* suggests that at least some of these methods are likely to be unrewarding.

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