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THE GENETIC BASIS OF DEVELOPMENTAL STABILITY IN *APIS MELLIFERA*: HETEROZYGOSITY VERSUS GENIC BALANCE

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Abstract.—The genetic basis for developmental stability in the haplo-diploid honeybee *Apis mellifera* was determined by comparing the level of asymmetry between diploid females and haploid males both among and within inbreeding levels. There was no significant relationship between the level of inbreeding and the level of fluctuating asymmetry for both females and males. It is therefore argued that the general level of genomic heterozygosity is not an important factor for the determination and maintenance of developmental stability in this system, but rather that the balance of genes within chromosomes plays the major role. The observation that males were generally more asymmetric than females suggests that developmental stability in females may also be influenced by additional factors such as gene dosage, sex-limited genes or cytoplasmic elements.

Key words.—*Apis mellifera*, developmental stability, fluctuating asymmetry, genomic coadaptation, inbreeding.

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Total phenotypic variation for a trait is a reflection of the underlying genetic and non-genetic variability associated with its development. One component of total phenotypic variation, which is often ignored, is the variation caused by developmental accidents that have neither a genetic nor environmental origin, so called developmental "noise" (Mather, 1953; Waddington, 1957; Thoday, 1958).

Developmental homeostasis or homeorhesis has been defined as the ability of an organism to withstand genetic and environmental disturbances encountered during development, via buffering mechanisms and to produce a predetermined phenotype (Waddington, 1942; Lerner, 1954). Such homeostasis can be viewed as having two principal components, viz., canalization and developmental stability (reviewed by Zakharov, 1989). The term canalization refers to the developmental processes by which consistent phenotypes are produced under a range of genetic and environmental conditions. On the other hand developmental stability refers to those processes that ensure common developmental outcomes under specified conditions. Canalization processes act to reduce the phenotypic variation associated with a particular trait, that might otherwise result from genetic and environmental variability, whereas developmental stability relates to processes that reduce the phenotypic variation resulting from developmental accidents. The biochemical and physiological processes underlying these homoestatic mechanisms are poorly understood although they are thought to operate via a form of negative feedback control (Lerner, 1954; Zakharov, 1989).

The development of the two sides of a bilaterally symmetrical organism is presumably under the influence of identical genetic and external environmental conditions. Thus any random and uncorrelated differences in the expression of a character represented on both sides of such an organism, so called fluctuating asymmetry (FA) (Van Valen, 1962), can be expected to result from developmental accidents occurring during development. As such, the level of fluctuating asymmetry of a character has

proved a useful means of assessing the efficiency of developmental stability associated with a variety of characters in a wide range of organisms (reviewed by Palmer and Strobeck, 1986; Zakharov, 1987, 1989).

The genetic basis of developmental stability has been the subject of much debate over the last fifty years among evolutionary and developmental geneticists. There are those who argue that developmental stability is affected by heterozygosity, with heterozygous individuals being developmentally more stable than their homozygous counterparts due to some inherent superior biochemical efficiency present in heterozygotes (Lerner, 1954; Soulé, 1979). Indeed, there are a number of studies which have shown a negative relationship between heterozygosity and FA (Soulé, 1979; Kat, 1982; Vrijenhoek and Lerman, 1982; Biémont, 1983; Leary et al., 1983, 1984). Others argue that developmental stability is a result of coadapted gene complexes, or genic balance, established over the evolutionary history of the organism via natural selection (Dobzhansky, 1950, 1970; Mather, 1953, 1973; Thoday, 1955). Studies have shown that the level of FA increases upon disruption of coadapted gene complexes (Zakharov, 1981; Graham and Felley, 1985; Leary et al., 1985; Clarke and McKenzie, 1987, 1992; McKenzie and Clarke, 1988). However, for both the heterozygosity and genic balance theories there are examples which fail to show any such relationships with fluctuating asymmetry (see Palmer and Strobeck, 1986 for review).

Very few model systems exist in which it is possible to discriminate between hypotheses. Comparing levels of FA with levels of homozygosity within and between populations has the potential for assessing the influence of heterozygosity on FA. However, caution is needed in such studies without relevant information on the mechanisms associated with homozygote formation. Increasing homozygosity may also result in disruption of coadapted gene complexes (Mather, 1973). Some experiments have shown increased levels of FA upon inbreeding (Mather, 1953; Thoday, 1955, 1958; Beardmore, 1960; Brückner, 1976; Clarke et al., 1986), while others have shown no such relationship (Reeve, 1960; Waddington, 1960; Bader, 1965*a*, 1965*b*; Bailit et al., 1970; Bradley, 1980). Inbreeding may also result in the unmasking of deleterious recessive alleles which may impact on the level of developmental stability (Clarke et al., 1986). Similarly, disrupting genic balance, such as in the creation of F_1 hybrids in crosses between genetically dissimilar parental strains, will also affect the level of heterozygosity. Such hybridization studies have shown both increased (Zakharov, 1981; Graham and Felley, 1985; Leary et al., 1985) and decreased (Mather, 1953; Reeve, 1960; Leamy, 1984; Ferguson, 1986) levels of FA in F_1 hybrids when compared with parental strains. Others have shown no consistent pattern (Thoday, 1955; Jackson, 1973; Felley, 1980).

In order to differentiate between these alternative hypotheses, a system is needed in which it is possible to alter one parameter, either the level of heterozygosity or genic balance, without affecting the other. A haplo-diploid system has the potential to do this. The genic balance in a typically outbreeding diploid consists of two components, a relational balance between homologous chromosomes and an internal balance among genes within chromosomes (Mather, 1973). Inbreeding in such a species upsets the relational balance leading to a breakdown in coadapted gene complexes. In an inbreeding species such relational balance is relatively unimportant as each homologue is identical and the major form of balance is that among genes within chromosomes. Such a balance is a result of complex epistatic and dominance relationships among genes. In a haplo-diploid species, as one part of the population, the male sex, is always haploid, characteristics that are important to both sexes should not rely on any association between homologous chromosomes, but should depend on the internal balance within one set of chromosomes. In addition, no deleterious recessive alleles should be present in this system as any such alleles should be eliminated from the population via selection on the haploid part of the genome. Therefore, by inbreeding in such a system it should be possible to increase the level of homozygosity in the diploid part of the population without affecting the internal genic balance, and without the prob-

lems associated with the unmasking of deleterious recessive alleles. Thus it should be possible to assess which of these factors is important for the maintenance of developmental stability in this system. We acknowledge that in choosing a haplo-diploid system as a model caution may be required in extrapolating results to normal diploid systems.

We studied developmental stability in the honeybee, *Apis mellifera.* Sex determination in *A. mellifera* is controlled by multiple alleles at a single sex locus. Haploid males (drones) are derived from unfertilized eggs and are thus hemizygous at the sex locus. Diploid individuals heterozygous at the sex locus are females (workers and queens), whereas those homozygous at this locus develop into diploid drones (Mackensen, 1951). Such diploid males do not exist in normal colonies as they are removed from the hive early in development by workers (Woyke, 1963), resulting in brood loss. Inbreeding is normally avoided in honeybee colonies by the system of mating. However, it is possible to generate diploid individuals with different degrees of homozygosity through instrumental insemination of queens.

The aim of this experiment is to assess the effect of inbreeding on developmental stability in *A. mellifera,* as measured by fluctuating asymmetry. If the level of heterozygosity is important for the maintenance of developmental stability, the increase in homozygosity in diploid individuals upon inbreeding should result in a concomitant increase in the level of FA in females, while the level of FA in males should show no change. Conversely, if heterozygosity is unimportant there should be no such increase in FA upon inbreeding and males and females should display similar levels of FA, both within and among inbreeding levels. While such a result would not conclusively show that genic balance was the major factor contributing to stability it would certainly lend support to the argument.

In addition, further information on the honeybee system is desirable, as the two previous studies on the effect of inbreeding on FA in *A. mellifera* have shown conflicting results, with one showing increased FA with increased inbreeding level (Brückner, 1976) and the other no change in FA (Clarke et al., 1986).

Materials and Methods

Breeding Program and Collection of Samples

Experiment 1.—We conducted an inbreeding program for six generations during 1988 in Burnley, Australia. The original queen was imported from another state, and naturally mated to indigenous drones (see pedigree in Fig. 1). Thus the inbreeding program started with a queen and colony with extremely high levels of heterozygosity. Approximately six instrumental inseminations were performed for each generation.

The inbreeding coefficients (F) of queens, workers and drones at each generation are given in Figure 1. The first five generations of the pedigree were backcrosses to the original queen. The sixth generation was the result of crosses between drones and virgin queens reared from a generation-four queen.

Progeny (workers and drones) of each queen were captured as soon as they began to emerge from the brood comb. These bees were stored in alcohol, and are referred to as 'maternal reared' (MR).

Oldroyd and Moran (1987) showed that the morphology of worker honeybees is influenced by the level of inbreeding of the workers caring for the brood. Colonies comprised of inbred workers tend to rear smaller bees than colonies comprised of workers that are not inbred, independent of the genotype of the brood. Rinderer et al. (1986) showed that the genotype of nurse bees affects the weight of the workers they rear. Experiments concerned with the effects of varying levels of inbreeding on morphology therefore need to standardize the rearing environment of experimental bees, in order to unambiguously determine the genetic (rather than environmental) effects of inbreeding on morphology. Therefore, when crossing was completed, all available queens were caged onto smaller worker brood combs, where they laid eggs. Combs of very young larvae were then transferred to a single colony for standardized rearing. Sealed brood combs were then transferred to an incubator. Emerging workers were matured for a few days, and then placed in alcohol. Work-

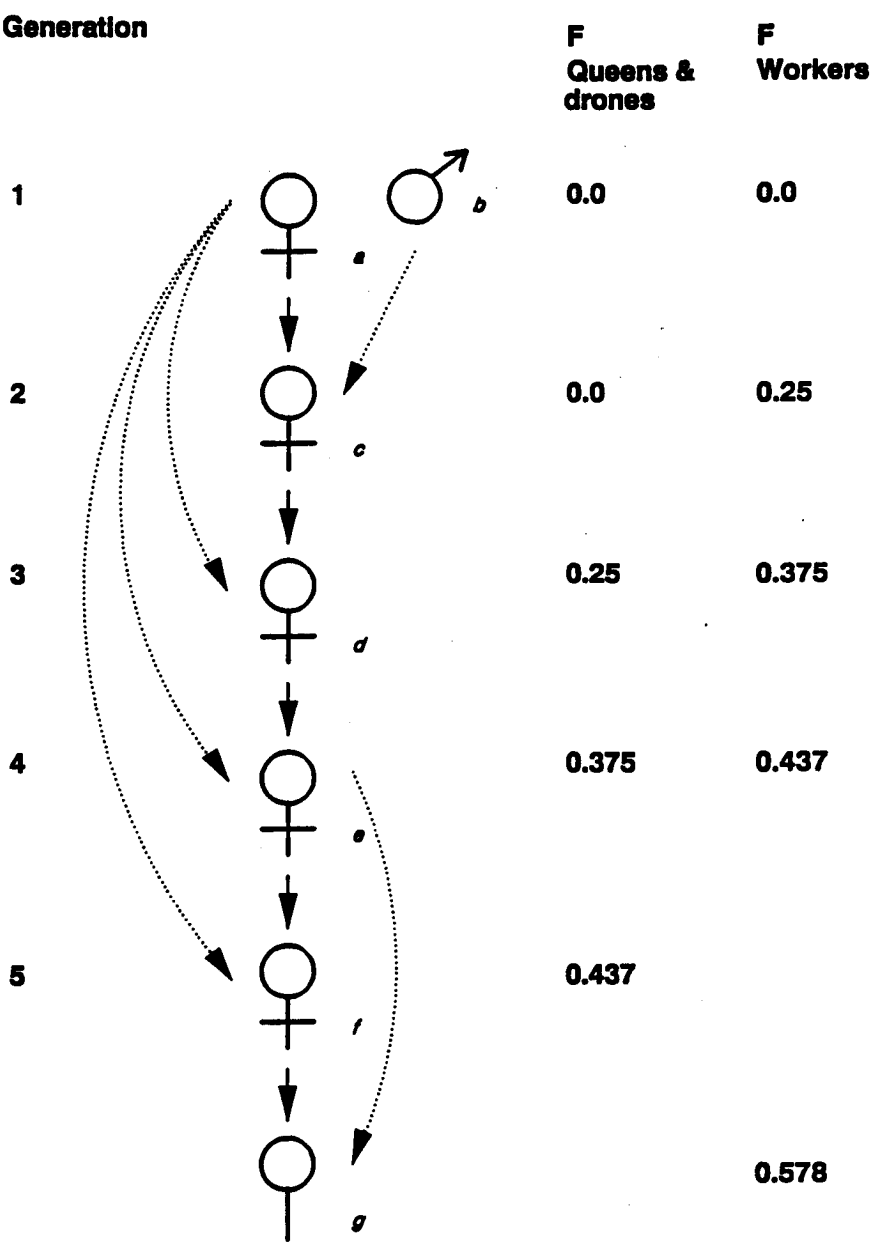

FIG. 1. Inbreeding program. Solid lines represent eggs, broken lines sperm [see Laidlaw and Page (1986) or Crow and Roberts (1950) for details of methodology]. The queen *a* was open mated to unknown drones (*b*). Queen (*c*) was reared from queen *a* and inseminated with semen collected from brother drones (sons of queen *a*). Daughter queens (*d*) were reared from queen *c*, and again backcrossed to sons of queen *a*. Daughter queens (*e*) were reared from queen *d* and backcrossed to sons of queen *a*. Daughter queens (*f*) were reared from *e* and inseminated with brother drones (sons of queen *e*) to produce the final generation of workers, *g*. The inbreeding coefficient, *F*, of workers is always one generation ahead of the inbreeding coefficient of queens and drones. Generation numbers refer to queens heading the colonies. Methods for calculating inbreeding coefficients in bees are given by Laidlaw and Page (1986).

ers of this kind are referred to below as 'standard reared' (SR).

Experiment 2.—A second inbreeding program was conducted in Baton Rouge, USA, commencing in the spring of 1989. The aim of this experiment was to generate higher levels of inbreeding than that achieved in the first inbreeding series. This program involved two generations of selfing of an *A. m. carnica* stock, followed by a single drone insemination of a virgin queen by her brother. This produced a colony in which the queen, workers, and haploid drones had an inbreeding coefficient of $F = 0.75$. Daughter queens were reared from this colony and instrumentally inseminated with

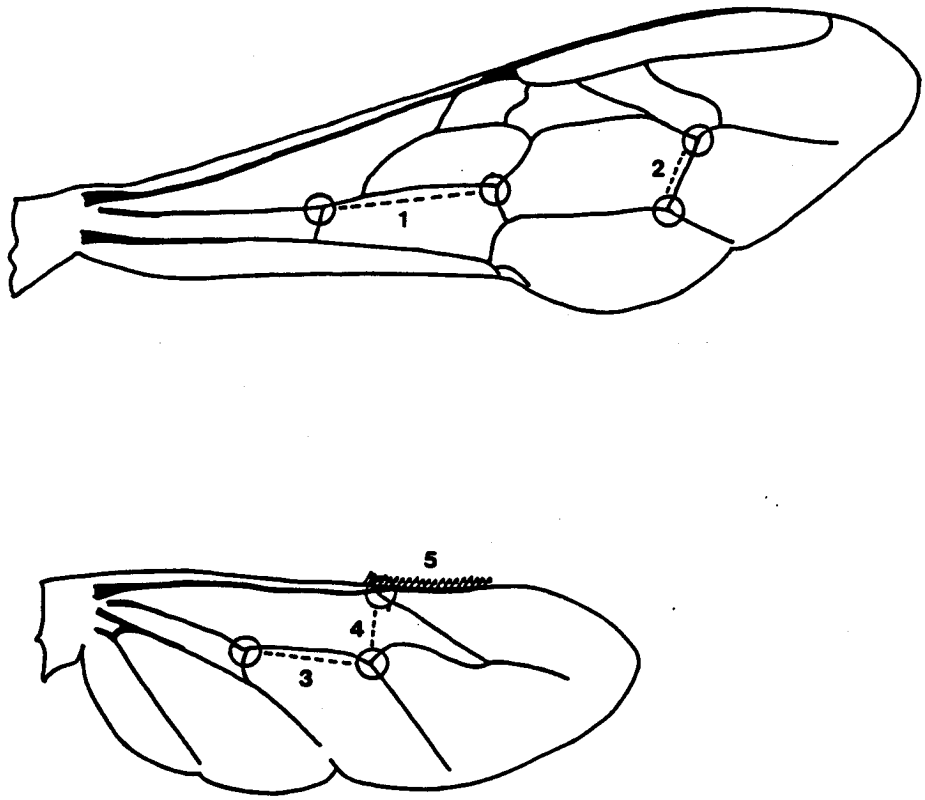

FIG. 2. Fore and hind wings showing the five characters examined in *A. mellifera*.

semen from unrelated sources. Comparisons of FA are between workers of the daughter queens ($F = 0$) and worker daughters of the inbred queen ($F = 0.75$).

Dissection and Scoring

Fore- and hind wings were removed and mounted on glass slides using either Hoyers fixative or Euparal. Four morphometric characters (wing veins) and one meristic character (number of hamuli) were measured (Fig. 2). Measurements were done with the aid of a ×25 dissecting microscope fitted with an eye-piece graticule. A minimum of 30 individuals was scored from each replicate colony in experiment 1 and 20 individuals were scored per replicate colony in experiment 2.

Statistical Methods

Asymmetry Estimate.—For each replicate colony, asymmetry values for each character were calculated as the sum of the squared signed differences between sides divided by the number of bees sampled from that replicate colony, i.e., $\Sigma (L_i - R_i)^2/N$. This is equivalent to Index 5 of Palmer and Strobeck (1986) which they state is best able to discriminate true differences in FA. As an estimate of measurement error duplicate measurements for each character were made on a sample of 30 individuals. The maximum recorded variance was 0.033. Given that this value is approximately 7% of the average asymmetry value observed for the metric characters it is reasonable to assume that measurement error does not contribute significantly to the asymmetry estimate.

Preliminary Steps.—Directional asymmetry was assessed by testing the departure of signed L − R differences from zero for each character for each replicate colony using *t*-tests. Of 200 such tests 6% showed significant positive deviation (skewed right) and 4% significant negative deviation (skewed left) from zero. These skewed distributions occurred randomly across characters, sex, and inbreeding levels. Omitting these distributions from the analyses had no significant effect on the results and have been included in the analyses that follow. The shape of the frequency distribution of signed L − R differences for each character were not assessed statistically. It has been argued extensively in the literature that fluctuating asymmetry is defined statistically in terms

TABLE 1. Asymmetry values $[\Sigma(L_i - R_i)^2/N]$ for different inbreeding levels (F) for males and females in *Apis mellifera*.

F	N*	Character 1	Character 2	Character 3	Character 4	Character 5
MR males						
0	5 (215)	0.6326	0.4093	0.6977	0.9349	2.4279
0.25	5 (168)	0.6071	0.4226	0.6488	0.5655	2.3571
0.375	1 (33)	0.4242	0.4848	1.2727	0.8485	1.9697
MR females						
0	2 (98)	0.3163	0.2347	0.4082	0.3878	2.3265
0.25	3 (95)	0.2737	0.2421	0.8000	0.4526	1.8211
0.375	6 (213)	0.4460	0.3239	0.5399	0.4225	1.9624
0.437	2 (94)	0.3617	0.3511	0.7766	0.5213	2.1489
SR females						
0	3 (110)	0.3909	0.2455	0.6091	0.3455	2.2182
0.25	2 (80)	0.3750	0.3375	0.5500	0.4000	2.1375
0.375	4 (150)	0.4133	0.3267	0.6067	0.3267	2.5867
0.437	2 (80)	0.4375	0.1875	0.6125	0.3250	1.9000
0.578	5 (191)	0.4084	0.2094	0.6021	0.3822	2.3927
Experiment 2 females						
0	5 (100)	0.3900	0.3500	0.5600	0.4800	2.1200
0.75	2 (40)	0.3000	0.2750	0.4000	0.4750	2.5750

* N = Number of replicate colonies. Numbers in parentheses are the total number of bees measured for the stated level of inbreeding. MR = Material Reared; SR = Standard Reared.

of signed L − R differences being normally distributed around a mean of zero. There is considerable debate taking place at the present time about the necessity that the distribution be normally distributed. In cases where symmetry is the norm, the distribution can be expected to depart from normality towards leptokurtosis. In fact, such a distribution is commonly observed in FA analysis. In addition it has been shown that platykurtic, and even bimodal, distributions can represent fluctuating asymmetry (G. M. Clarke, unpubl. data; R. F. Leary, pers. comm.).

Regression analyses (not presented) between absolute left minus right differences and mean character size [i.e., $|L_i - R_i|$ versus $(L_i + R_i)/2$] showed that asymmetry values for each character were independent of character size across the range of sizes observed for both sexes. In addition, there was no significant correlation in signed L − R differences among characters. In some cases there was significant phenotypic correlation of mean value $[(L_i + R_i)/2]$ between characters. In addition the shape of the distribution of signed L − R differences for the meristic character (No. 5) was observed to be different than that observed for the four morphometric characters in that it had a significantly greater variance. Thus, in order to avoid confounding the results due to such correlation between characters and differing patterns of variance, asymmetry values were not summed across characters as is commonly done. As such, all characters were analyzed separately.

Statistical Analyses.—As the index used to estimate asymmetry is a variance, differences between and among samples were tested for significance using tests for homogeneity of variances. In experiment 1 differences in asymmetry values among inbreeding levels within each sex were tested using Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1981). Differences in asymmetry values between males and females and between SR and MR females were tested using two-tailed F-tests to test H_0: $\sigma_1^2 = \sigma_2^2$. In experiment 2, differences in asymmetry values between $F = 0.75$ and $F = 0.0$ females were tested using one-tailed tests to test $H_1: \sigma_1^2 > \sigma_2^2$.

No significant differences between replicate colonies were observed using tests for homogeneity of variances. Therefore, data from replicate colonies were pooled and asymmetry values calculated for each inbreeding level as $\Sigma (L_i - R_i)^2/N$, where N is the total number of bees sampled for each level of inbreeding.

Regression analyses were used to test for

TABLE 2. Results of tests for homogeneity of variances testing for differences between inbreeding levels for males and females in *Apis mellifera*. Bartlett's test and one-tailed *F*-tests were used for experiments 1 and 2 respectively. The values given are the probability that the observed differences in asymmetry values among levels of inbreeding are due to chance.

Sample	Character 1	Character 2	Character 3	Character 4	Character 5
Experiment 1					
MR males	0.3653	0.8109	0.0258	0.0030	0.7485
MR females	0.0298	0.0888	0.0017	0.4997	0.6272
SR females	0.9660	0.0042	0.9878	0.7454	0.5843
Experiment 2					
$F = 0.75$ vs $F = 0.0$	0.8211	0.8005	0.8815	0.5000	0.2219

MR = Material Reared; SR = Standard Reared.

any association between inbreeding level and asymmetry (Steel and Torrie, 1960). For these analyses individual replicate colony data were not pooled thus giving multiple estimates of asymmetry for each inbreeding level within each sex.

RESULTS

Asymmetry values for pooled replicate colony data are given in Table 1. Significant differences in asymmetry values among inbreeding levels were observed in a number of cases (Table 2). Of these cases, none showed any significant relationship with inbreeding level as revealed by regression analyses (Table 3). An examination of the variance values for these cases shows that in all instances the level of asymmetry is lower in at least one sample with a higher inbreeding value than in samples less inbred.

For experiment 2, there were no significant differences between $F = 0.75$ and $F = 0.0$ females (Table 2). In fact, for four of the five characters the asymmetry value was lower in the inbred sample than the outbred control.

In no cases was there a significant relationship between asymmetry and inbreeding level (Table 3). An examination of the sign of the regression lines indicates that within each sex both positive and negative regressions were observed.

Males displayed greater levels of asymmetry than females in 87% of cases (Table 1) of which 62% were significant (Table 4). Females were never observed to be significantly more asymmetric than males.

Of 20 comparisons between MR and SR females, MR females displayed higher levels of asymmetry in 50% of cases of which 2 cases were significant. SR females were significantly more asymmetric than MR females in a single case (Table 4).

DISCUSSION

The results show that inbreeding has no effect on developmental stability in *A. mellifera* as measured by fluctuating asymmetry. Increasing the level of homozygosity in the diploid part of the genome (females) up to levels at which 75% of the genome was homozygous resulted in no significant change in the level of fluctuating asymmetry from that observed in outbred material.

For this haplo-diploid system, the general level of genomic heterozygosity does not appear to be an important factor for the maintenance of developmental stability. This result is perhaps not surprising, as haploid males, which are effectively 100% homozygous, still need to possess a sufficient level

TABLE 3. Results of regression analyses testing for relationship between inbreeding level and asymmetry for each character. Values given are the probabilities that the slopes of the regression differ from zero due to chance. Signs in parentheses indicate negative or positive regression.

Sample	Character 1	Character 2	Character 3	Character 4	Character 5
MR males	(−) 0.4712	(+) 0.8083	(+) 0.4676	(−) 0.1304	(−) 0.8599
MR females	(+) 0.1406	(+) 0.0638	(+) 0.2511	(+) 0.5482	(−) 0.5582
SR females	(+) 0.8501	(−) 0.3940	(+) 0.7969	(+) 0.6183	(+) 0.8773

MR = Maternal reared; SR = Standard Reared.

TABLE 4. Results of two-tailed *F*-tests comparing the level of asymmetry within inbreeding levels (*F*) between MR males and MR females and between MR and SR females. Values given are the probabilities that the observed differences in asymmetry values are due to chance.

F	Character 1	Character 2	Character 3	Character 4	Character 5
Males vs females					
0	<0.0001	0.0022	0.0032	<0.0001	0.8320
0.25	<0.0001	0.0032	0.2406	0.2348	0.1688
0.375	0.9052	0.1008	0.0004	0.0040	0.9364
MR females vs SR females					
0	0.2876	0.8232	0.0450	0.5566	0.8180
0.25	0.1430	0.1224	0.0872	0.5732	0.4548
0.375	0.6222	0.9478	0.4344	0.0940	0.0650
0.437	0.3766	0.0046	0.2706	0.0318	0.5754

MR = Maternal Reared; SR = Standard Reared.

of developmental stability or homeostasis to buffer development against 'accidents' and to ensure the production of optimum phenotypes. As such, it would seem incongruous if the maintenance of developmental stability, which is equally vital for both haploid and diploid individuals, relied on genomic heterozygosity which is obviously only achievable in diploids.

It would be more reasonable to assume that developmental stability in such a system depends on some factor which is common to both haploid and diploid parts of the genome. It thus seems probable that the major factor contributing to maintenance of developmental stability in this system is the internal balance of genes within chromosomes, likely to involve a series of complex epistatic and dominance relationships.

The fact that males were consistently more asymmetrical than females cannot be attributed to differences in size between the sexes. While males are larger than females, regression analyses performed on asymmetry values across the range of character sizes observed in both sexes demonstrated that the asymmetry value was independent of mean character size. This difference in asymmetry between the sexes suggests that developmental stability in haploid and diploid parts of the genome may be under the control of different mechanisms and that females (diploids) are developmentally more stable than males (haploids). It has been conjectured that the differences in the level of developmental stability between haploid an diploid honeybees may be attributable to gene dosage, in that diploids, by virtue of having two copies of each gene, regardless of the allelic state at each locus, are better able to maintain normal development than haploids with only a single copy (Lee, 1969, 1974). It has also been postulated that haplo-diploid systems contain genes that are limited to the diploid part of the population (Kerr, 1976). That specific genes or gene complexes can play a vital role in developmental stability has previously been shown in a diploid species, *Lucilia cuprina* (Diptera: Calliphoridae) (Clarke and McKenzie, 1987, 1992; McKenzie and Clarke, 1988).

In addition there may also be factors not related to the nuclear genome, i.e., cytoplasmic components, present in diploids which may be important for developmental stability (Moritz, 1986).

An examination of the level of fluctuating asymmetry of diploid males has the potential to provide information on the importance of diploidy to developmental stability in honeybees. We have made several attempts to generate diploid drones using the methods of Woyke (1963). Unfortunately, all attempts to date have been unsuccessful.

These results are consistent with those of Clarke et al. (1986) who also found no relationship between inbreeding and the level of asymmetry in this system. However, they contrast with those of Brückner (1976) who reported that inbred females were significantly more asymmetric than outbred females. The reasons for the differences between these studies are unclear as the same characters were examined. Brückner used different methods of analysis to those used in this study. We have reanalyzed our data using her methods, which in essence used a

variance value pooled across characters as an index of asymmetry, and obtained results identical to our original analyses, i.e., no significant increase in asymmetry with increased inbreeding. Thus, the differences between these two studies is unlikely to reflect differences in statistical methods. All three studies have observed that males were generally significantly more asymmetric than females.

Environmental conditions within inbred hives are likely to differ from those within outbred hives (Oldroyd and Moran, 1987). Nonetheless there were very few significant differences in asymmetry between maternal and standard reared females. Thus the increased levels of homozygosity associated with inbred workers does not significantly reduce the efficiency of canalization processes to buffer development against environmental disturbances.

Results presented here, if they can be generalized, have implications for the observed negative relationship between the levels of heterozygosity and fluctuating asymmetry reported for other diploid organisms (e.g., Soulé, 1979). Such a relationship may reflect a breakdown in the genic balance during homozygote formation rather than any innate superiority of heterozygous individuals over their homozygous counterparts.

The relationship between genic balance and developmental stability is well documented (Mather, 1953, 1973; Thoday, 1955; Zakharov, 1981; Graham and Felley, 1985; Leary et al., 1985; Clarke and McKenzie, 1987, 1992; McKenzie and Clarke, 1988). The level of developmental stability has been shown to be reduced upon the disruption of coadapted gene complexes following introgression of novel genetic material into the population through hybrid formation or mutation.

It is likely that the genetic basis and control of developmental stability may differ between organisms with different breeding systems and genetic organization. As such it must be emphasized that caution is needed when trying to establish causal relationships for observed patterns of fluctuating asymmetry in situations where the genetic structure of the population under examination is unknown.

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