

Failure to detect pleiotropy of maternal traits in a rare honey bee mutant is not a test of ground plan hypothesis that explains origins of social behavior

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**Oldroyd and Beekman attempt to test the forager reproductive ground plan hypothesis (forager RGPH) using a selected strain of anarchistic worker honey bees. The authors conclude that their experiment does not support the forager RGPH, and they strongly criticize studies in support of the hypothesis that have used strains selected for high and low levels of pollen hoarding. We show here that the criticism is poorly founded. The results of Oldroyd and Beekman are flawed by contradicted assumptions about the reproductive behavior of anarchistic bees, by lack of variation in ovary phenotypes, by inference from insufficient sample sizes, and by the use of incomplete datasets from foraging specialists in their statistical analyses.**

Oldroyd and Beekman (2008) compared the ovary size, ovarian activation level, and foraging behavior of worker bees from commercial Australian stocks and a strain selected for abnormal reproductive behavior. In their study, they claim to test the RGPH of honey bee forager behavior proposed by Amdam et al. (2004, 2006). Normal worker honey bees are essentially sterile females. Yet the forager RGPH posits that variation among foragers in their age at foraging onset and in their bias toward collecting pollen (a protein source) or nectar (a carbohydrate source) is explained in part by variation in a gene network that can synchronize female foraging choice and maternal provisioning behavior with ovarian physiology. This pleiotropic regulatory network and its link to foraging behavior in workers derive from the reproductive biology of ancestral solitary insects (Amdam et al. 2004; 2006).

Support for the forager RGPH comes from a battery of association studies that used unselected “wild type” sources of bees (Pankiw & Page 2000; Pankiw 2003; Amdam et al. 2006; Tsuruda 2007) and honey bee strains disruptively selected for high or low levels of stored surplus pollen (pollen-hoarding, Amdam et al. 2004; 2006; 2007);

from honey bee genome mapping studies (reviewed by Hunt et al. 2007); and from several RNA interference mediated gene knockdown experiments performed on wild type as well as high and low pollen-hording strain bees (Amdam et al. 2007; Nelson et al. 2007; Ihle et al. 2008). This body of work shows that sucrose responsiveness, foraging onset, foraging preference for nectar or pollen, ovary size (ovariole filament number) and circulating vitellogenin (yolk) protein levels are linked in worker bees, and suggest that this pleiotropy can emerge through a gene regulatory network that includes insulin/insulin-like signaling (reviewed by Page et al. 2006; Page & Amdam 2007). Similar endocrine systems that coordinate sensory responses and feeding behavior with female reproductive traits are present in many taxa (Magnarelli 1978; Klowden 1990; Than et al. 1994; Atchley et al. 2005).

Compared to wild type bees, the anarchistic honey bees of Oldroyd and Beekman represent an extremely rare behavioral mutation (Oldroyd et al. 1999; Thompson et al. 2006) that lay eggs in the presence of a functional queen. Most workers can produce viable eggs, but reproductive maturation normally is suppressed when a queen is heading the colony (Winston 1987). The vast majority of wild type as well as high and low pollen-hording strain bees lay eggs only if the queen is lost. The selection for the anarchistic syndrome has, however, caused several changes in the reproductive behavior of workers (Oldroyd & Ratnieks 2000). Anarchistic behavior is, as a result, fundamentally different from the behavior of workers that lay eggs in queenless colonies (Montague & Oldroyd 1998; Oldroyd et al. 1999; Oldroyd & Osborne 1999). Selection experiments show that an anarchy phenotype in workers can arise via several routes (Oldroyd & Osborne 1999), and yet it may ultimately be controlled by a small number of genes, possibly as few as two (Montague & Oldroyd 1998; Barron et al. 2001; Thompson et al. 2006).

Ovarian activation (swelling of the ovarioles) normally is positively associated with ovariole number in wild type worker honey bees (Makert et al. 2006; Amdam et al. 2006). This also is true in comparisons of bees of the high and low pollen-hoarding strains; the high strain bees have more ovarioles and more activated ovaries (Amdam et al. 2006; 2007). Interestingly, and in accord with their rare mutant phenotype, anarchistic bees differ from this normal relationship and do not have more ovarioles than wild type

but sometimes have more activated ovaries (Oldroyd & Beekman 2008). Nonetheless, Oldroyd and Beekman propose that relative to wild type, anarchistic bees are “more reproductive” and should be characterized by the pleiotropic trait associations of high pollen-hoarding strain bees. They frame this prediction as a test of the forager RGPH, assuming that foraging behavior in mutant anarchistic bees is mediated pleiotropically by the same gene networks and hormonal cascades that link ovarian physiology to foraging behavior in wild type bees and bees from high and low pollen-hoarding strains. They find that anarchistic bees and wild type do not differ in their foraging preference for nectar or pollen; thereby they claim that the forager RGPH is not supported, and they conclude that “their study highlights the pitfalls of making general conclusions about the evolution of a behavior from particularly selected lines [i.e., the pollen-hoarding strains selected by Page & Fondrk (1995)] when the genetic mechanisms behind the behavior are poorly understood”. There are several flaws in their line of inference.

Oldroyd and colleagues concluded previously that anarchistic behavior is fundamentally different from the normal reproductive behavior of workers (see references above). Thus, their more current assumption (Oldroyd and Beekman 2008) that the abnormal reproductive behavior of mutant anarchistic workers is mediated pleiotropically by the same systems that affect the foraging preference of wild type bees and pollen-hoarding strains is not supported. Rather, their previous published work strongly suggests that anarchistic bees are poorly suited as a test for the forager RGPH.

Oldroyd and Beekman inexplicably trimmed out the bees with the greatest foraging biases in their analysis of foraging behavior by not including subsets of workers that collected only pollen or only nectar: The bees that collected only pollen should have been considered nectar foragers with zero nectar loads, and those with only nectar should have been considered pollen foragers with zero pollen loads (Pankiw & Page 2000; Pankiw & Page 2001; Rueppell et al. 2004; Amdam et al. 2006). Bees that were completely empty should also have been analyzed (Pankiw & Page 2000), as we recently found, in accord with the forager RGPH, that they are the foraging group with the fewest ovarioles (Page et al. 2008).

Unfortunately, it is unlikely that the dataset of Oldroyd and Beekman will provide conclusive results even if it is correctly analyzed. This is because ALL bees used in the

experiment had small ovaries (anarchistic and wild type bees had about 2.0 and 2.3 ovarioles per ovary, respectively,  $n = 2$  colonies, Oldroyd and Beekman 2008). These average sizes are about 30% smaller than that of low pollen-hoarding bees and an average for commercial honey bees of the U.S (2.9 and 3.0 ovarioles per ovary, respectively,  $n = 12$  colonies), and they are less than half the average ovary sizes of high pollen-hoarding strain bees and wild-caught U.S. Africanized honey bees (5.6 and 4.5 ovarioles per ovary, respectively,  $n = 12$  colonies; R.E. Page, M.K. Fondrk, O. Kaftanoglu, A. Siegel, unpublished data; Amdam et al. 2006; 2007). Therefore, without sufficient variance in ovary size, Oldroyd and Beekman are unable to properly test for the effects of ovary size on foraging behavior, the basis of the forager RGPH.

The small sample size of foragers, for which ovary size was determined, adds to the statistical limitations of the Oldroyd and Beekman study ( $n$  is less than 160 for their analysis of the effect of ovariole number on foraging behavior). To fully illustrate the relationship between these shortcoming and their negative results: We previously analyzed workers from 4 sources of wild type bees prescreened to represent a broad distribution of ovary sizes and processed more than 550 bees. Average ovariole number ( $\pm$  SD) was  $4.4 \pm 2.4$  for successful foragers in our experiment ( $n = 314$ ), which documented a significant association (ANOVA,  $p < 0.00001$ ) between ovary size and foraging behavior (Amdam et al. 2006). Yet if we artificially curtail this dataset to represent a mean ovariole number and sample size similar to that of Oldroyd and Beekman ( $2.7 \pm 0.9$ ,  $n = 171$ , respectively), the significant relationship can no longer be detected (ANOVA,  $p = 0.52$ ). This exemplifies how variance in the independent variable is critical to regression-based analyses, especially with small sample sizes.

Finally, despite their rejection of the forager RGPH, the results of Oldroyd and Beekman confirm several relationships outlined by the framework of the reproductive ground plan hypothesis, as we defined it (Amdam et al. 2004, 2006):

1. There is a genotypic component to the age of foraging onset. Wild type bees foraged at younger ages than did anarchistic bees
2. Genotypes with larger ovaries (more ovarioles) forage earlier in life. The wild type bees had marginally larger (though still small) ovaries.

3. There is a general correlation between ovary size and ovarian activation. Bees with more ovarioles also were more likely to have activated ovaries.

In conclusion, we can only agree with Oldroyd and Beekman, their study highlights the pitfalls of making general conclusions about the evolution of a behavior from particularly selected lines, especially lines that have not been adequately characterized.

### Acknowledgement

We thank M. K. Fondrk, K. E. Ihle, A. Siegel, D. Münch and F. Wolschin for helpful comments and discussions.

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