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Drosophila melanogaster males evolve increased courtship as a correlated response to larval crowding

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A competitive resource-based environment such as larval crowding is an important stress factor in the life history of holometabolous insects such as *Drosophila melanogaster* and can have major consequences for adult fitness through its direct effects on body size. We investigated precopulatory sexual behaviour, which is an important subset of adult fitness, using *D. melanogaster* populations adapted to larval crowding for 100+ generations. We found that selected males evolved significantly higher courtship frequency than their ancestral controls. While we found a negative correlation between larval density and adult courtship frequency using low, moderate and high larval rearing densities, we also found that supplementation of live yeast, which is an important dietary component in their life cycle, caused a significant rise in courtship frequency. We further dissected out the male and female behavioural components that contribute to overall courtship activity by factorially combining selected and ancestral male and female types, reared in both low and high larval densities, and found that courtship frequency of selected males was higher with selected females than with control females. We also quantified the mating frequency, a crude measure for mating success, and found that increased courtship frequency in selected populations did not lead to increased mating success. From the results of this and a previous study, we conclude that precopulatory sexual activity in these populations is not traded for life span.

Larval crowding is an important ecological stressor in the life history of holometabolous insects such as *Drosophila melanogaster*. It can potentially impose nutritional limitation by affecting the quality and quantity of resources that can be acquired by the larvae. Resource acquisition at the larval stage plays a very important role in determining adult fitness components (Chippindale, Leroi, Kim, & Rose, 1993). Adults emerging from crowded larval cultures are typically small and have less stored resources. Larvae from crowded cultures are also exposed to toxic metabolic wastes such as urea and ammonia. Tolerance to such nitrogenous wastes requires energy (Borash, Gibbs, Joshi, & Mueller, 1998), limiting the resources available for development and other functions. Thus, larval crowding can have major consequences for adult fitness, especially through its effects on body size. Recent studies show that larval activity in the food can also have positive effects on fitness by affecting the composition of yeast species in the food (Stamps, Yang, Morales, & Boundy-Mills, 2012). At high densities, it is possible that larvae transplant yeasts and facilitate their growth by churning up the food at a higher rate (Stamps et al., 2012; also see Thibert, Farine, Cortot, & Ferveur, 2016).

Larval density is known to affect both pre- and postcopulatory success of males and females in *Drosophila*. Males that develop at low larval density emerge as larger individuals and have higher success in mating: they mate at a faster rate and remate more often than smaller males that emerge from crowded cultures (Partridge, Ewing, & Chandler, 1987; Partridge & Farquhar, 1981, 1983; Partridge, Green, & Fowler, 1987; Turiegano, Monedero, Pita, Torroja, & Canal, 2013; Wigby, Perry, Kim, & Sirot, 2015). Similarly, females reared at low larval densities are larger, mate faster and remate more quickly than smaller females that emerge from high larval densities (Amitin & Pitnick, 2007; Wigby et al., 2015).

Larval crowding can also affect patterns of postcopulatory sexual selection. Bangham, Chapman, and Partridge (2002) looked at

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the correlations between adult body size, accessory gland and testis size on postcopulatory success in *D. melanogaster* and found that larger males have higher postcopulatory success. Larger males tend to transfer greater quantities of ejaculate (Simmons, Parker, & Stockley, 1999), including absolute quantities of sex peptide, to females during mating (Wigby et al., 2015), and have greater sperm competitive ability (Amitin & Pitnick, 2007). However, increased fitness of larger males comes at a cost of female fitness. When females are mated to large males, they lay fewer eggs and have a shorter life span (Friberg & Arnqvist, 2003; Pitnick and Garcia-Gonzalez 2002). The harmful effects of males are mediated through transfer of seminal proteins (Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995), mating (Cohet & David, 1976; Partridge, Green et al., 1987) and persistent courtship (Long, Pischedda, Nichols, & Rice, 2012; Turiegano et al., 2013). Such antagonistic interactions are expected to select for female strategies that reduce the harmful effects of males, for example, by avoiding mating with aggressive males (Friberg, 2006). Accordingly, it has been seen that females from low-density cultures are better at moving away from males during courtship, exhibiting reluctance to mate (Turiegano et al., 2013).

Thus it is clear that larval density can affect a range of reproductive traits including courtship and mating. In the wild, *Drosophila* populations are known to exist at a range of densities (Atkinson, 1979; Grimaldi & Jaenike, 1984) and hence larval density is likely to play an important role in the evolution of courtship and mating behaviour. However, all the above-mentioned studies on the effect of larval density/adult body size on adult pre/postcopulatory reproductive behaviour involved manipulation of larval density over a single generation. Hence their results represent phenotypic correlations that need not mirror the underlying genetic correlations. To the best of our knowledge, no study has addressed the evolution of these traits in populations that experience larval crowding consistently over generations.

In this study, our aim was to investigate the evolution of courtship and mating behaviour in response to larval crowding. We used replicate populations of *D. melanogaster* selected for adaptation to larval crowding for more than 100 generations. We grew selected and control populations at different larval densities and specifically asked whether courtship activity and mating are affected by (1) adaptation to larval crowding and (2) selection history of males and females. Two of the most important components of the male's environment in our study were larval density and the type of female with which the male was housed, both of which we altered.

In a previous study using these populations, we showed that the males and females from the selected populations had evolved decreased adult body weight and increased adult longevity compared to the flies from the control populations (Shenoi, Ali, & Prasad, 2016). One possible reason for the evolution of higher longevity might be that these flies have evolved reduced courtship/mating behaviour. Previous studies show that courtship activity is costly to males and reduces their longevity (Cordts & Partridge, 1996). On the other hand, in females, copulation per se is costly and repeated copulation reduces their lifetime fecundity (Kuijper, Stewart, & Rice, 2006) and longevity (Fowler & Partridge, 1989). Hence we assayed the frequency of courtship and mating in the selected and control populations.

METHODS

Fly Populations Used

The fly populations used in this study are the same as those described in detail in Shenoi et al. (2016). Briefly, there are eight populations: MCU 1–4 (selected for adaptation to larval crowding; maintained at 800 eggs/vial density in 1.5 ml of food) and MB 1–4 (ancestral controls, maintained at 60–80 eggs/vial density in 6–8 ml of food). They have been maintained on standard cornmeal–charcoal food for more than 110 generations at 25 °C (24:0 light:dark period) and 50% humidity on a 21-day generation cycle. The populations (MCUs = *D. melanogaster* crowded as larvae, uncrowded as adults; MBs = *D. melanogaster* baseline, uncrowded as larvae, uncrowded as adults) were named according to the larval and adult densities at which they were maintained. MCUs connected to MBs by the same replicate numbers are the direct descendants and hence more closely related to each other. Hence they were treated as statistical blocks (representing ancestry) in analyses.

Standardization and Generation of Experimental Flies

To counter nongenetic parental effects (Rose 1984), the fly populations were passed through one generation of a common rearing regime. We call this process ‘standardization’ and the flies thus generated are called standardized flies (Shenoi et al., 2016). For the assays, eggs were collected from standardized flies that were at least 8 days old but not more than 12 days old, as adults (i.e. on or before day 21 after egg collection). The flies were fed on cornmeal–charcoal food supplemented with ad libitum live yeast (supplied as paste, with water) for 48 h, followed by an egg-laying window of 6 h. In earlier experiments (Shenoi et al., 2016), we had found that at high larval density (800 eggs per vial, in 5 ml of food), only ca. 5% of MBs survived, in contrast to ca. 30–40% survivors in MCUs. Hence the ‘optimum’ highest larval density treatment for this study was also set at 600 eggs per vial in 5 ml of food. As there were considerable differences in survivorship of MCUs and MBs when reared at 600 eggs per vial, we collected more vials for generating MBs than MCUs and thus made sure that the adult density across all treatment cages was constant during the pre-experimental period. Owing to the positive correlation of developmental time with larval rearing density (Santos, Borash, Joshi, Bounlutay, & Mueller, 1997), eclosion peaks broaden when larval densities are high. To account for this, we collected eggs for different density treatments on different days (300 and 600 eggs per vial: from 10- and 8-day-old standardized flies, respectively; 60 eggs per vial: from 12-day-old standardized flies). Hence, regardless of their larval densities, the experimental flies had a ‘synchronized’ peak eclosion period.

Quantifying Precopulatory Sexual Activity

Courtship frequency

We visually observed and counted male to female (heterosexual) courtship events. Each observation lasted for 60 s, which was timed by a stop watch. Rather than scoring different stages of courtship behaviour displayed by the same male (viz. orientation, chasing, wing flapping, licking and attempted copulation), we counted them collectively. In other words, any of these five behaviours, if observed, were scored as one event. We employed this strategy because these behaviours are subsets of courtship activity and one type of behaviour may be succeeded by another, not necessarily in the above order. The first observation was recorded only 2 h after the entire experimental set-up (see Experimental design) was ready. *Drosophila* females judge the quality of a courting male using various sensory cues before consenting to mating (Villella & Hall, 2008) or choose to resist the courtship of a male through rejection behaviour such as ovipositor extrusion, kicking or simply running away (Connolly & Cook, 1973; Ejima, Nakayama, & Aigaki, 2001; Spieth & Ringo, 1983). We did not