Transgenerational effect of adult density through paternal effect in *Drosophila melanogaster*

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Abstract:

Notwithstanding recent evidences, paternal environment is thought to be a potential but unlikely source of fitness variation that can affect trait evolution. Here we studied transgenerational effect(s) of males' exposure to varying adult density in *Drosophila melanogaster* laboratory populations. We held sires at normal (N), medium (M) and high (H) adult densities for two days, before allowing them to mate with virgin females. We then collected eggs from the dams and measured the egg size. Finally we investigated the effect of the paternal treatment on juvenile and adult (male) fitness components. We found a significant treatment effect on juvenile competitive ability where the progeny sired by the H-males had higher competitive ability. Since we did not find the treatment to affect egg size, this effect is unlikely to be mediated through variation in female provisioning. Male fitness components were also found to have a significant treatment effect: M-sons had lower dry weight at eclosion, higher mating latency and lower competitive mating success. While being the first study to show both adaptive and non-adaptive effect of the paternal density in *Drosophila*, our results highlight the importance of considering paternal environment as important source of fitness variation.

Key words: Sire effect, juvenile competitive fitness, mating latency, male reproductive success, crowding adaptation

Introduction:

Parental environment has the potential to influence offspring traits and fitness through transgenerational effects. While maternal transgenerational effect (henceforth maternal effect) has been investigated in great detail due to its prevalence as well as prominence, examples of similar paternal effect is far fewer. However, there is a growing body of evidence showing the importance of the paternal trangenerational effect on fitness related traits of the offspring [1-4].It is therefore important to assess the extent of such paternal effect under various ecological scenarios and in different organisms.

There are many reports of environment dependent maternal effect mediated through variation in maternal provisioning in egg/offspring [5,6]. Females living under high density may suffer from adverse effects of crowding (such as, malnutrition) and may therefore struggle to allocate resources in maternal provisioning either in the form of stored resources in egg or lactation, which in turn would lead to poor quality progeny [7]. Alternatively, females raised in high density may strategically produce less number of eggs/progeny while investing more resources (e.g., yolk) in each of them – thereby giving the progeny a better start for the impending challenges of high density environment [8-11]. Generally, under fluctuating environment, such parental ability to optimize offspring phenotype has been conjectured to be adaptive [1, 12]. Guppy (*Poeciala reticulata*) females were found to produce larger offspring (a) under food limitation [13] and (b) when they experienced high level of competition – priming the offspring for better competitive ability [14]. The larger eggs produced by *D. melanogaster* females that grew in poor food, survive (egg to adult survivorship) better in poor food and give rise to smaller adults [11]. In contrast, Valtonen et al. (2012) found *D. melanogaster* females grown on poor quality food to produce larger offspring (adult) compared to those grown on normal food [15].

Note that much of the maternal effects discussed above are mediated through variation in resource provisioning by mothers. Not surprisingly, most of the reports of environment dependent paternal transgenerational effect (hereafter, paternal effect) come from animals with paternal provisioning

through nuptial gift transfer to the females during mating [16-20]. However, it is only recently that studies have started to address if similar paternal effects are also present in species without paternal provisioning. Males of a solitary Ascidian (*Styela plecata*) produced offspring phenotype corresponding to the population density experienced by the father [4]. Female Neriid flies (*Telostylenus angusticollis*) raised on richer diet produced larger eggs and offspring that developed faster, while males raised on richer diet sired larger offspring with better survival rate, especially under resource scarcity [3, 21]. Valtonen et al. (2012) reported that fathers fed on poor quality diet sire larger sons [15]. Our previous study showed that paternal experience of rivals' number adaptively affected offspring reproductive behaviour in *D. melanogaster* [22]. Islam et al. (1994) showed paternal social environment to have a significant impact on offspring behavioural traits [23]. Paternal experience of ambient temperature was also found to affect offspring fecundity in *D. melanogaster* [24]. Low temperature was found to affect offspring phenotype in two other species of Drosophila – *D. simulans* [25] and *D. serrata* [26]. Thus there is a growing body of evidence showing environment dependent paternal effect, however, the full impact of such effect on adaptation to the relevant ecological challenge is not yet clear.

Here we investigated the effect of paternal experience of population density on progeny behaviour and fitness components by using *D. melanogaster* laboratory adapted populations. As discussed previously, paternal effect has already been reported in these [22] and other populations of *D. melanogaster*, establishing them as a relevant system to investigate the paternal effect and its consequences on Darwinian fitness [27]. Further, laboratory adapted populations of *D. melanogaster* can be investigated for a range of environmental parameters. Among the many components of their ecology, population density (number of individuals per unit space) is particularly very important. Fruit flies naturally grow in ephemeral resource patches, such as rotting fruits. Since quality of resource patch is expected to vary a great deal – both within and across generations, crowding in transiently available rich patches is expected to be a key component of their natural ecology. Therefore density of adults in a patch not only determines the extent to which individuals have to compete for limiting space (e.g., oviposition substrate) but also for other resources, such as perishing

food and suitable mates. Increase in density also leads to an increase in the chance of disease transmission [28].In essence, density often determines the nature and intensity of selection acting on a population and is generally addressed within the broader premises of density dependent selection [29-31]. The laboratory adapted populations of the fruit flies have been extremely successfully used to investigate various components of the theories of density dependent selection (see [32] for a recent example and a review of some of the relatively older studies). Population density is known to affect the expression of a wide range of traits both within and across life-history stages [32-37]. However, little is known about its transgenerational consequences (but see [8]).

We subjected males to three adult density treatments and then allowed them to produce progeny by mating the treated males to untreated dams. We then investigated the effect of the paternal density treatment on progeny fitness components in juvenile (juvenile competitive fitness) and adult stages (males: mating ability, mating latency, copulation duration, courtship frequency, competitive mating success). We show that density experienced by the fathers affect both juvenile and adult fitness component.

Methods:

All the experiments were done using a set of laboratory adapted populations of *D. melanogaster* – BL. Full laboratory history of these populations can be found in [38]. Briefly, these are a set of five replicate populations (BL_{1-5}) maintained on standard Banana-Jaggery-Yeast food, under 14-day discrete generation cycle at 25 ^{O}C ambient temperature, 60-80% relative humidity, with population size ~2800. Larval density is maintained at ~70 per 6-8ml food per vial (25mm×90mm, diameter×height). Adult density is ~70 per vial for the first couple of days of their adult life and thereafter ~2800 individuals in a ~6.4 l cage (19cm×14cm×24cm). We also used a derived population, BL_{st} which was derived from BL_1 by introducing an autosomal recessive marker – scarlet eye (st) [22]. BL_{st} population is maintained under a set of conditions identical to the other BL populations.

Paternal treatment:

Sires and dams were generated from a BL population. The design of the protocol followed to generate the experimental flies is described in Figure 1. To generate the experimental sires and the dams, eggs were collected from a BL population and cultured under standard density (i.e., 70 per 6-8ml food per vial). 100 such vials were set up, of which 65 were used to collect the sires (= sire-vials) and the rest 35 for dams (dam vials). Dams were collected as virgins and held in single sex vials at a density of 25 per vial with ad lib food until the day of the sire-dam mating (see below). In the sire-vials, all the flies were allowed to eclose. These flies were used to set up three adult density treatments – normal (N: 70 individuals per vial), medium (M: 140 individuals per vial) and high (H: 210 individuals per vial). 10 vials were set up for each of the treatments, using flies that were approximately 1 day old. These vials were left undisturbed for 2 days, following which males from them were separated and used as sires in the subsequent step. Here and elsewhere throughout the study, all the fly sorting, including collection of virgins, were done under light CO_2-anaesthesia, unless mentioned otherwise.

Sire-dam mating:

Following the 2-day long conditioning, 25 males were randomly isolated from each adult density treatment vials, to be used as sires. They were then combined with dams (see previous section) in fresh food vials (25 sires + 25 dams in a vial) and allowed a time of one and half hour - sufficient time for a single round of mating. This method of ensuring single round of mating has been previously used [39]. In addition, mating was visually observed. 10 mating vials were set up per density treatment. After mating, the sires were discarded and the already inseminated dams from all 10 vials of a treatment (i.e., a total of 250 females) were transferred to a 2 litre plastic cage with food smeared with live yeast. Three such cages were thus set up – one for each density treatment. Two days later eggs were collected from these cages to set up the remainder of the experiments. To collect the eggs, a fresh food plate was introduced in the cage. The dams were allowed a short window (2-3 hours) for oviposition. Using a fine brush, eggs were counted on to a fine Agar-strip, which was then transferred to the culture vials (see below). These eggs are hereafter referred to as treatment eggs.

Measurement of egg-size:

To test if the sires influenced the size of the eggs laid by the dams [40], a subset of these eggs were frozen at -20°C and their size was measured. For this purpose, eggs were mounted on a glass slide on their dorsal side and photographed using Nikon (SMZ745T) and the area of the two-dimensional elliptical outline of the eggs were measured in ImageJ, software. This area was taken as a proxy for the size of each egg. A given egg was measured thrice and the average of these three measurements was taken as the unit of analysis. 50 eggs per treatment were measured for this purpose.

Experiment 1: Juvenile fitness assay

Egg to adult survivorship was taken as a measure of Juvenile fitness. Survivorship of the treatment eggs were measured against a back ground of a common competitor (BL_{st}) under two conditions – crowded (C: 150 larvae per 1.5ml food in each vial) and un-crowded (UC: 70 larvae per 6ml food in each vial). During the assay, treatmenteggs generated in the previous step were cultured with eggs from common competitors in the ratio 1:4 (C: 30 target, 120 competitor; UC: 14 target, 56 competitor). These common competitors were collected from an untreated BL_{st} stock. On completion of development, it was possible to identify the target progeny from the competitor progeny based on eye colour – all progeny of the competitors were scarlet eyed whereas the target progeny were red eyed. 10 juvenile competition vials were set up for each of the three treatments (viz., N, M and H) and two assay conditions (i.e., 10 as C and 10 as UC for each treatment). They were left undisturbed till 12th day, by which time all surviving pupae finished eclosion. The adults were sorted based on eye colour and counted. Juvenile fitness score (w) was calculated for each vial following the formula:

$$w = \frac{numberofredeyedprogenyobserved}{numberofredeyedprogenyexpected}$$

The number of red eyed progeny expected was 14 and 30 for UC and C assay conditions respectively.

Experiment 2: Assay for behaviour and fitness of the sons

To investigate the effect of the treatment on the male progeny, the treatment eggs were cultured in food vials in the usual density (i.e., 70 per 6ml food in each vial) and the progeny were allowed to

develop. Upon onset of eclosion, males were collected as virgins (< 6 hours post-eclosion). Four assays were run with these males. (a)For each treatment, 50 males were immediately frozen at -20 ^{o}C and were later dried at 60 ^{o}C for 48 hours and weighed in groups of 5. (b) A separate set of males were similarly collected and held in groups of 5 per vial for further assays. Ten such vials, for each treatment, were set up and left undisturbed till they were 3days old. These males were then transferred to fresh food vials (hereafter referred to as mating vials) along with five age-matched, virgin females. Mating vials were set up without the use of anaesthesia. The females used in this step came from the same replicate BL population and were generated under their standard maintenance conditions, collected as virgins and held in groups of five per vial with ample food until the day of the experiment. 10 mating vials were set up for each of the three treatments. They were observed every two minutes starting from the time when the females were introduced and total number of mating pairs (n_x) was noted down at each time point (x = 0, 2, 4, 6…). This was done till all the flies finished mating. Mean mating latency (ML, time taken by a virgin pair to start mating) and mean copulation duration (CD, duration for which a pair mated) were calculated following an algorithm mentioned below.

$$ML = \frac{\sum(n_x - n_{x-2})x}{N}$$

For all values of x, until, $n_{x-2} \leq n_x$.

$$CD = \frac{\sum(n_{x-2} - n_x)x}{N} - ML$$

For all values of x, until, $n_{x-2} \geq n_x$.

Occasionally, some females/males did not mate within one hour long observation. These flies were excluded from the analysis. Mating ability (MA) is measured as the proportion of the sons successfully copulated. MA was calculated for every single vial.

(c) Courtship frequency was quantified for the 3day old (post-eclosion) sons of the three paternal density treatments by setting up similar mating vials as described in the previous section. Ten vials were set up for each treatment. Therefore a total of 30 vials were observed. After allowing the first mating, the courtship observation was initiated after a gap of approximately half an hour. Vials where

all the flies did not mate were removed from the assay. Every 45 minutes, each vial was observed for 30 seconds, during which the total number of courtship bouts (male to female) was noted down. A total of 8 observations were taken. In *Drosophila*, courtship behaviour includes chasing, tapping, courtship dance and song, genital licking and attempted mounting [41, 42]. Any of the above mentioned courtship behaviours, displayed by the five males in each vial was counted as one. The total number of independent male to female courtship displays was counted within the observation window [43].The treatment identities were unknown to the observers to avoid observer bias. (d) Another set of males were similarly collected and held, to be used for quantifying their mating success under competitive condition (CMS, Competitive mating success). This was done by setting up mating vials with five 3day old target males, five competitor males (BL_{st}) and five virgin females (BL_{st}). Ten such mating vials were set up for each of the three treatments. After allowing a single round of mating for all the females in a mating vial, the females were individually transferred to oviposition test tubes (12mm diameter × 75mm height) with ample food. The females were allowed to oviposit for 18 hours. Following oviposition, the females were discarded and the tubes were retained to allow the progeny to develop and eclose. For each female, the identity of their mate (whether target/competitor) was ascertained by observing the eye colour of the progeny. Progeny sired by target males were red eyed whereas those sired by competitors were scarlet eyed. For a given vial, average CMS of the five target males in the vial was calculated as the proportion of the females mated to target males (i.e., produced red eyed offspring).

<u>Experimental replications and data analyses:</u>

The entire study was carried out in three randomized blocks, using three different BL populations - BL_1, BL_3 and BL_5.The blocks were handled in separate days. Number of replications within each block has been mentioned in the previous sections along with the assay design. Except the egg size and dry body weight assay, all the experimental replication was done at the level of assay mating vials or juvenile competition vials. All the assays had 10 replicate vials. Vial means were used as the unit of analysis. For egg size assay, size of each egg was used as the unit of analysis. For dry body weight, weight of groups of 5 individuals was used as the unit of analysis. Data were analysed using mixed

model Analysis of Variance (ANOVA). Block was treated as random factor, while paternal density treatment and assay density (wherever applicable) were treated as fixed factors. Multiple comparisons were done using Tukey's HSD. All the analyses were done in Statistica, version 10 (Statsoft, Tulsa, OK, U.S.A.).

Results:

Variation in size of the eggs represents variation in maternal provisioning. The effect of the paternal density treatment on size of the eggs produced by the dams was not significant (Table 1, mean ±SE, μm^2, N: 80039.1 ±387.5; M: 79967.4 ±415.3; H: 79611.9 ±411.4). The juvenile competitive fitness assay quantified overall egg to adult survival of the target juveniles compared to the same of juveniles from a common background (common competitors). While the data from un-crowded assay condition reflects the baseline survivorship, those from crowded assay condition represents difference in juvenile competitive ability across the three paternal density treatments. Paternal density treatment had a significant effect on Juvenile fitness (Table 1). While there was no significant difference between N and M-treatments, H-treatment had 8.9% higher juvenile fitness compared to that of the N-treatment. This relative advantage of the H-treatment was only evident under larval crowding, i.e., C-assay density (Figure 2), indicating competitive superiority of that H-juveniles. However, the paternal treatment × assay density interaction was marginally non-significant (Table 1).

We only quantified the effect of the paternal density on male offspring. We found a significant effect of the treatment on dry body weight, ML and CMS (Table 1, Figure 3). Multiple comparisons using Tukey's HSD indicated that dry body weight of the M-sons were significantly less than that of the N-sons, with M-sons having 8.7% lower mean dry body weight. The difference between the dry body weight of the H and N-sons was not statistically significant. Hence the M-sons were significantly smaller compared to the other two treatments. In the mating assay, though we found some males to fail in acquiring mating, there was no effect of the treatment on mating ability of the sons (MA: mean ±SE, N: 0.91 ±0.04; M: 0.91 ±0.04; H: 0.93 ±0.04). The M-treatment sons showed significantly higher (approximately 35%) ML compared to that showed by the N-treatment sons. While H-

treatment also showed 16% higher ML compared to N-treatment, this difference was not significant. Therefore M-sons took longer time to start mating with virgin females indicating females' reluctance to accept them as mate due to either poor performance in courtship or small size. This relative disadvantage of the M-sons was also evident in terms of their competitive ability in mating competitions. Multiple comparisons on the CMS results indicated that the M-sons had significantly lower CMS compared to H and N-treatments. CMS of the M-sons was approximately 34% less than that of the N-sons. This is however, not due to a reduced courtship performance by the M-sons as we found the effect of the treatment on CF (mean ±SE, N: 6.7 ±0.6; M: 6.8 ±0.6; H: 7.6 ±0.8) to be non-significant. We also did not find any effect of the treatment on CD (mean ±SE, minutes, N: 18.6 ±0.4; M: 17.6 ±0.4; H: 17.9 ±0.4), potentially indicating the lack of the treatment effect on post-copulatory traits of the sons (Table 1).

Discussion:

Given that very few studies have shown the effect of paternal environment on offspring fitness components, there were two main objectives of the present study – (a) to assess if paternal exposure to varying population density affected progeny traits; if yes, then (b) to evaluate the adaptive significance of such effect. The results clearly showed that at sufficiently high density, males had an adaptive paternal effect on juvenile competitive fitness. As we did not find any effect of our treatment on size of the eggs produced by the dams, such paternal effect is unlikely to be mediated by variation in provisioning by the females. We further show that at intermediate density, males sire smaller sons which are inferior in acquiring mates. Interestingly, such maladaptive effect of paternal density on offspring adult fitness was not detected at high density.

In holometabolus insects like fruit flies, juvenile (larva and pupa) survival constitutes one of the most important components of fitness [31]. In addition, juvenile ecology may also have a major effect on the life-history and fitness components of the adult stage (Heat shock [44]; cold shock [45, 46];

crowding [47, 32, 36]. The observed paternal effect on juvenile competitive fitness therefore is extremely consequential. Some relatively recent studies have pointed out that evolving parental ability to optimize offspring fitness related traits can be adaptation to ecological challenges [48], including crowding [4]. Given that fruit fly natural ecology regularly involves adult and larval crowding, the observed paternal effect on juvenile competitive fitness can indicate males' adaptation to crowding. Interestingly, we observed the paternal effect on juvenile competitive fitness, only at the highest density, which may indicate a certain threshold density beyond which such paternal effect starts affecting offspring traits. In addition, when assayed under un-crowded condition the progeny from the three sire treatments do not show any measurable difference in their egg-to-adult survival. This suggests that the juvenile competitive ability rather than baseline juvenile viability was affected by the treatment. Since a number of traits (e.g., feeding rate, waste tolerance, development time etc.) affect juvenile competitive ability in these flies, it will be interesting to find out the trait responsible for better competitive ability of the H-sons in our study.

In a wide range of species including *Drosophila melanogaster*, maternal exposure to high density or poor nutrition has been found to affect offspring fitness components [8, 11, 15]. Such effects can either be beneficial [10, 11, 14, 49, 50] or detrimental [51] depending on the component of the fitness under consideration and the prevailing condition. As maternal provisioning and other maternal effects play vital roles in offspring survival and performance, such maternal density/nutrition effect is not surprising. However, what is not intuitive is the paternal density to have similar impact on offspring fitness, as our results suggest, given that *Drosophila* males do not pass on any nutrition to the offspring. It is well known in the *Drosophila* literature that even the laboratory populations harbour heritable genetic variation in survival under crowding both as adults and juveniles (see [32] and the references therein for an updated review). Therefore, one possibility is that genetically superior males, which are better at surviving under high density, may produce offspring which are better both as juveniles, explaining at least part of our observations. Though larval competitive ability is known to respond to experimental evolution, indicating heritable genetic variation [30, 31], such heritable variation is very unlikely to have led to the observed treatment effect on juvenile competitive fitness.

This is because (a) in our assay, we recorded very little mortality in males during the treatment, indicating negligible hard selection. In addition, we also ensured that there was no soft selection by randomly picking the set of males from the treatment vials to use them as sires. (b) Even if there was selection in the current experimental design, the selection is likely to be weak. Such weak selection is unlikely to explain the observed differences in some of the traits (viz., 8.9% increase in juvenile competitive fitness, 35% increase in mating latency), especially within one generation. Alternatively, males may alter maternal provisioning and thereby indirectly affect offspring fitness components [8, 11]. We, however, did not find any measurable difference in the size of the eggs produced by females mated to the males belonging to the three treatments, making variation in maternal provisioning an unlikely explanation. Therefore, although sire-effect on the quality of the eggs produced by the females cannot be completely ruled out, our results tentatively point at non-genetic paternal effect [1] as the potential cause behind the observed effect of the treatment.

While we found adaptive paternal effect on juvenile performance, adult performance however, was found to have a significant maladaptive effect of paternal density. Males that experienced intermediate density were found to sire sons which (a) are smaller, (b) take longer time to start mating and (c) have lower mating success. Since we did not find any effect of the treatment on courtship frequency, reduced mating success and increased mating latency was a likely outcome of females' reluctance to accept relatively smaller males as their mates, a known fitness consequence of reduced size in *Drosophila* males [52, 53]. Body size has been reported to be affected by transgenerational paternal effect in another Dipteran – *Telostylinus angusticollis* [21]. Unlike the maladaptive effect found in our study, the paternal effect on body size reported by Bonduriansky and Head (2007)[21], however, was adaptive, especially under certain prevailing conditions. Though prima facie, the observed body size reduction appears to be maladaptive, it will be interesting to investigate its fitness consequence under varying adult density. Interestingly, this effect was found only at intermediate density and not in the high density treatment. At this point, it is, however, difficult to suggest any reason for such specific expression of the paternal effect at intermediate density.

As variation in population density and crowding related ecological challenges are common in almost all organisms, including fruit flies, paternal effect of the nature reported here is important to understand. Though paternal ability to optimize offspring traits is likely to be adaptive, especially under fluctuating environment, the results reported here show that paternal effect can be both adaptive and maladaptive. To the best of our knowledge this is the first evidence of the effect of paternal density on juvenile and adult fitness components in *D. melanogaster*. Importantly, our results emphasize the importance of considering paternal effect as a source of variation in fitness related traits. The full impact of such paternal effect in the evolution of life-history traits and the underlying mechanisms are emerging as an important topic of discussion, which is likely to see an increasing attention in years to come.

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Author contribution: BN conceived the idea. BN, PD and SS designed the experiments. PD, SS, TV and AA performed the experiments. Analysis of the data was done by BN and PD. Manuscript was written by BN with assistance from PD and SS.

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Trait	Effect	SS	DF	MS	DF Den	MS Den	F	p
Egg size	Paternal density (PD)	1.75×10^{7}	2	8.75×10^{6}	4.02	3.53×10^{6}	2.48	0.20
	Block	9.84×10^{8}	2	4.92×10^{8}	4.03	3.54×10^{6}	139.11	**<0.01**
	PD×Block	1.41×10^{7}	4	3.53×10^{6}	438.00	2.25×10^{7}	0.16	0.96
Juvenile fitness	Paternal density (PD)	0.21	2	0.11	4.03	0.01	9.93	**0.03**
	Assay density (AD)	0.97	1	0.97	2.00	0.10	9.31	0.09
	Block	0.40	2	0.20	1.58	0.09	2.15	0.35
	PD×AD	0.23	2	0.11	4.01	0.02	5.42	0.07
	PD×Block	0.04	4	0.01	4.00	0.02	0.51	0.74
	AD×Block	0.21	2	0.10	4.00	0.02	4.96	0.08
	PD × AD × Block	0.08	4	0.02	148.00	0.02	1.24	0.30
Dry body weight	Paternal density (PD)	2.67×10^{-7}	2	1.34×10^{-7}	4.00	6.22×10^{-9}	21.49	**< 0.01**
	Block	7.78×10^{-7}	2	3.89×10^{-7}	4.00	6.22×10^{-9}	62.50	**< 0.01**
	PD×Block	2.49×10^{-8}	4	6.22×10^{-9}	80	1.27×10^{-8}	0.49	0.74
Mating latency	Paternal density (PD)	41.810	2	20.90	4	1.12	18.59	**0.01**
	Block	51.158	2	25.58	4	1.12	22.75	**0.01**
	PD×Block	4.477	4	1.12	77	4.59	0.24	0.91
Copulation duration	Paternal density (PD)	14.43	2	7.21	4	1.81	3.98	0.11
	Block	80.87	2	40.44	4	1.81	22.32	**0.01**
	PD×Block	7.22	4	1.81	77	5.51	0.33	0.86
Mating ability	Paternal density (PD)	0.009259	2	0.00	4	0.04	0.10	0.90
	Block	0.046678	2	0.02	4	0.04	0.52	0.63
	PD×Block	0.179963	4	0.04	77	0.02	2.64	**0.04**
Courtship frequency	Paternal density (PD)	13.28	2	6.64	4.03	10.79	0.62	0.58
	Block	500.78	2	250.39	4.00	10.81	23.16	**0.01**
	PD×Block	43.24	4	10.81	73.00	7.36	1.47	0.22
Competitive mating success	Paternal density (PD)	0.64	2	0.32	4.03	0.02	19.47	**0.01**
	Block	0.09	2	0.05	4.06	0.02	2.86	0.17
	PD×Block	0.07	4	0.02	75.00	0.04	0.41	0.80

Table 1: Summary of results of mixed model ANOVA on the various traits under investigation. Paternal density and assay density (wherever applicable) were considered as fixed factor, while block as random factor. All tests were done considering α=0.05 and significant p-values are mentioned in **bold** fonts.

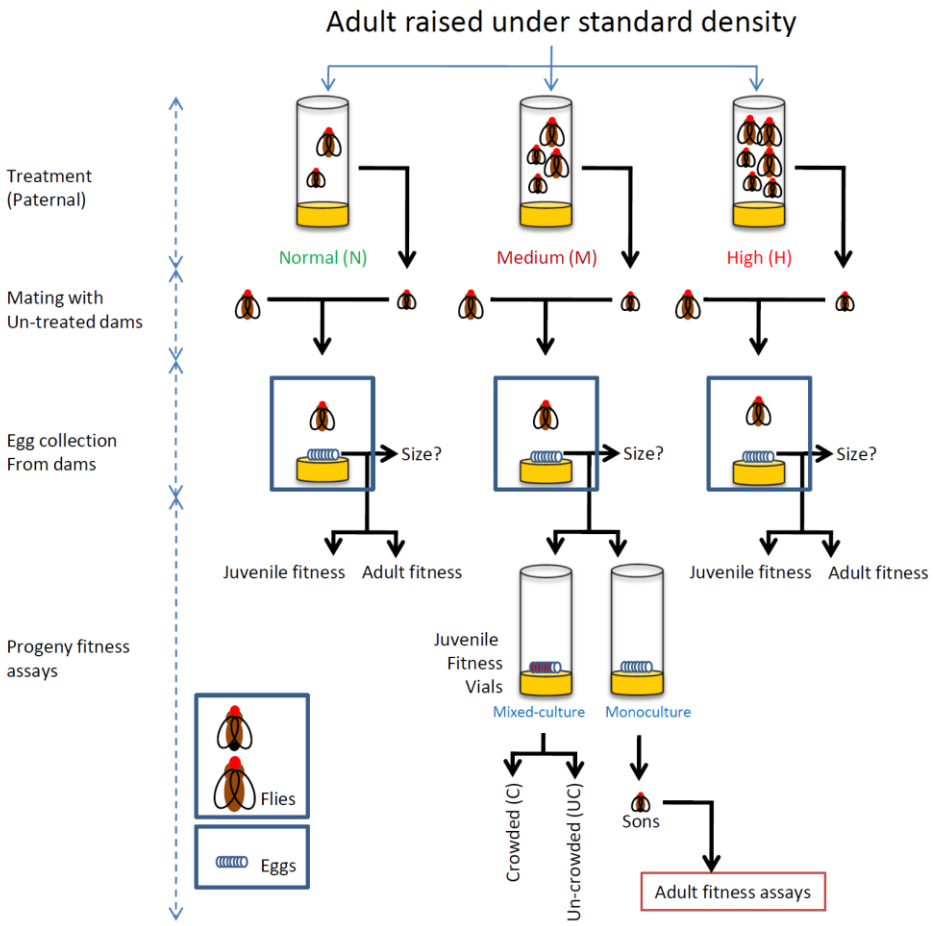

Figure 1: The design of the assay. The schematic diagram shows the design of the entire study, which spanned two generations. Treatment was given in the paternal generation. Untreated dams were mated to the treated sires, followed by the collection of eggs from the dams. Assays were done with the eggs and the offspring emerging out of the eggs. Some eggs were subjected to mixed culture (along with competitor eggs) and juvenile competitive fitness vials were set up. Some eggs were cultured as monocultures (without any competitor eggs) – male progeny emerging from these vials were used for further assays, such as, mating ability, mating latency, copulation duration, competitive mating success and courtship frequency.

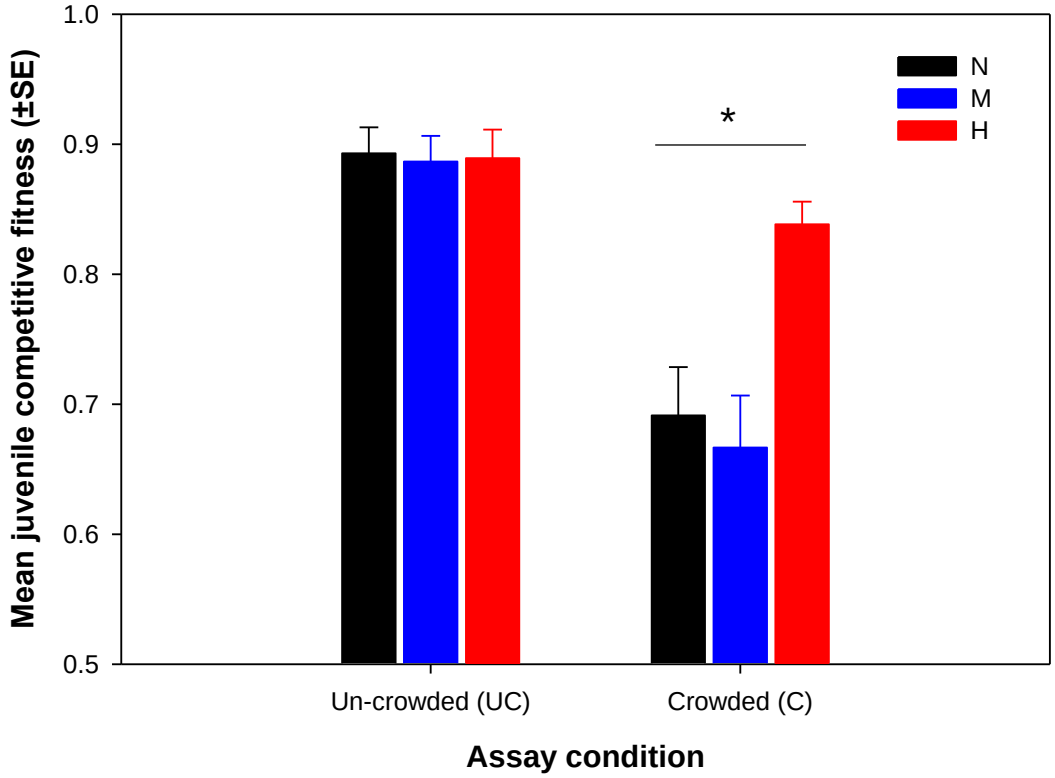

Figure 2: Effect of the paternal density treatment on juvenile competitive fitness, under crowded and un-crowded assay conditions. Target eggs (eggs produced by dams mated to treatment sires) were cultured with competitor eggs (eggs produced by untreated females and males) in juvenile competition vials – under un-crowded and crowded conditions. Proportion of the target progeny successfully emerging as adults is considered as the measure of juvenile competitive fitness. Black, blue and red colour coding represent the progeny of N, M and H males respectively. Paternal treatment was found to have a significant effect only when the assay was carried out under crowded condition (represented by *asterisk). The entire experiment was done following a randomized block design and the data were analysed using three factor mixed model ANOVA with paternal treatment and assay condition as fixed factors and block as random factor.

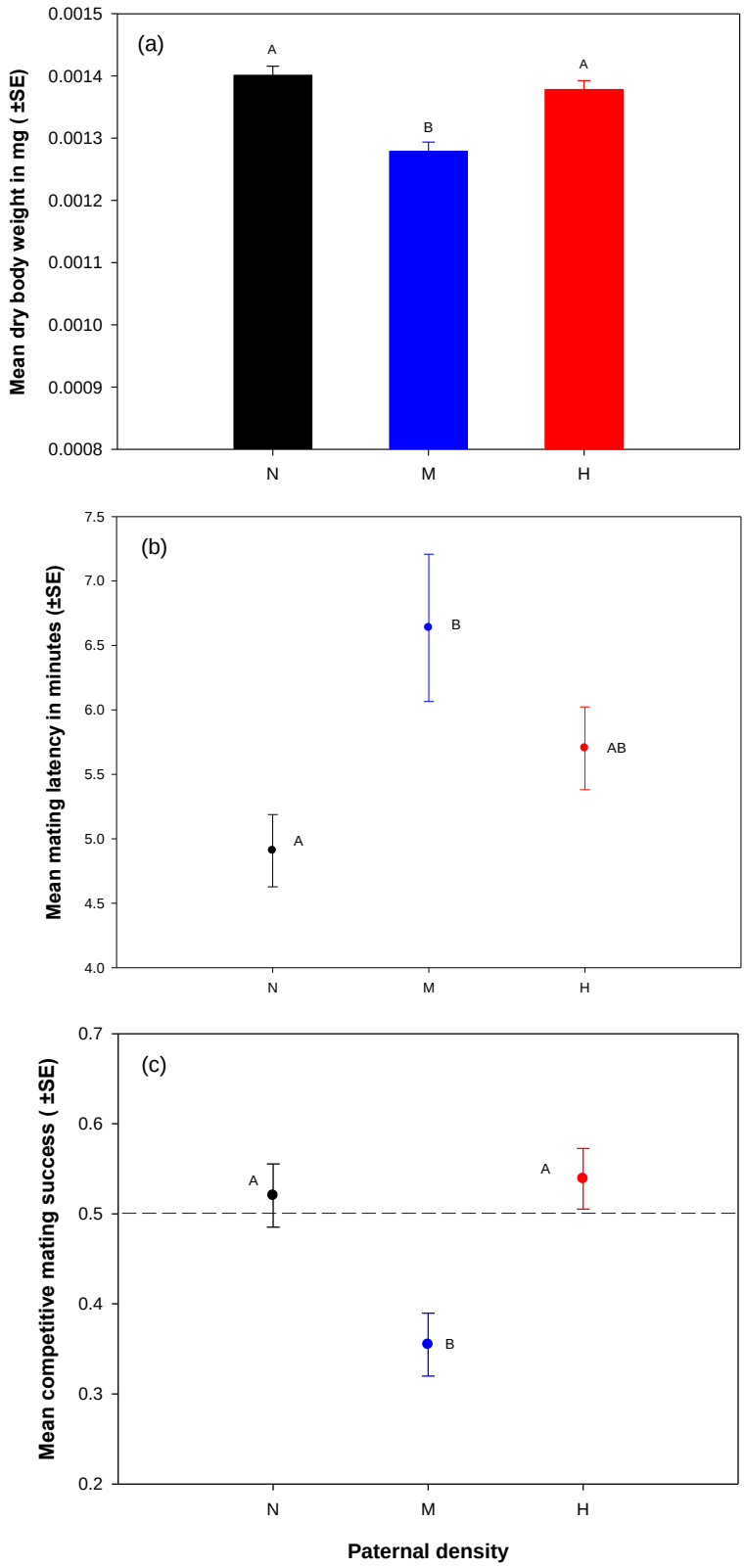

Figure 3: Effect of the paternal density treatment on male offspring. (a) Dry body weight at eclosion of the M-sons was found to be significantly less than that of the other treatments; (b) Mean mating

latency (time taken by a virgin male-female pair to start copulation) of the was significantly higher in M-sons; (c) When subjected to competition with (untreated) rival males, the competitive mating success of the M-sons was found to be significantly less than those of the other treatment sons. The blue broken line indicates the expected value of CMS if there is no mating bias. Black, blue and red colour coding represent data from the progeny of N, M and H males respectively. Treatments not sharing common alphabet were found to be significantly different from each other. The entire experiment was done following a randomized block design and the data were analysed using three factor mixed model ANOVA with paternal treatment and assay condition as fixed factors and block as random factor.