Title: Evolution of dispersal syndrome and its corresponding metabolomic changes

Authors: Sudipta Tung[1], Abhishek Mishra[1], Navdeep Gogna[2], Mohammed Aamir Sadiq[1], P.M. Shreenidhi[1], V.R. Shree Sruti[1], Kavita Dorai[2] and Sutirth Dey[1*]

Affiliations:

1. Population Biology Laboratory, Biology Division, Indian Institute of Science Education and Research (IISER) Pune, Dr. Homi Bhabha Road, Pune, Maharashtra, India, 411008.
2. Department of Physical Sciences, Indian Institute of Science Education and Research Mohali, Sector 81 Knowledge City, Mohali, Punjab, India, 140306.

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* **Name and address of the corresponding author:**

Sutirth Dey
Associate Professor, Biology Division
Indian Institute of Science Education and Research, Pune
Dr. Homi Bhabha Road, Pune, Maharashtra, India 411 008
Tel: +91-20-25908054
Email: s.dey@iiserpune.ac.in

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Abstract

Dispersal is one of the strategies for organisms to deal with climate change and habitat degradation. Consequently, the effects of dispersal evolution on behavioural and life-history traits of natural populations are of considerable interest. Here we explore the same questions in the context of laboratory populations by subjecting four outbred populations of *Drosophila melanogaster* to selection for dispersal for more than 49 generations. Compared to the controls, the dispersal-selected populations had significantly greater locomotor activity, exploratory tendency and aggression but similar values of body size, fecundity and longevity. Untargeted metabolomics using NMR spectroscopy suggested that the selected flies had elevated levels of cellular respiration and octopamine, but lesser amount of fatty acids. All these observations correlate well with the behavioural data. Our results document how metabolic pathways respond to dispersal selection and help us to understand the consequences of dispersal evolution at the sub-organismal level.

1. Introduction

Dispersal affects several ecological and evolutionary processes (Clobert *et al.* 2012) and is one of the early responses of mobile organisms under environmental stresses. Faced with the spectre of global climate change and large-scale anthropogenic habitat destruction, fates of several natural populations depend, at least in part, on their dispersive abilities. Not surprisingly therefore, evolution of dispersal and its consequences on the organisms themselves have become major topics of investigation over the last two decades (reviewed in Ronce 2007).

There are two somewhat different ways by which this issue has been investigated in the dispersal literature. The first approach involves comparing the behavioural or life-history attributes of dispersers with those of the non-dispersers in a given population (e.g. Ronce & Clobert 2012). Such studies have led to the conclusion that very often (although not always) certain suites of behavioural and life-history traits are closely associated with dispersers (reviewed in Clobert *et al.* 2009). For example, in terms of behaviour, dispersers typically exhibit greater exploratory tendencies (Cote *et al.* 2010; Korsten *et al.* 2013) and are more aggressive (Duckworth & Badyaev 2007), whereas in terms of life-history, the dispersers are often larger in size (Dingle *et al.* 1980) and have greater fecundity (Ebenhard 1990). The primary motivation for investigating such suites (also called dispersal syndromes) is the assumption that associations between dispersal-related traits are expected to affect the genetic and demographic outcomes of dispersal (Clobert *et al.* 2009; Ronce & Clobert 2012). While the assumption is fairly intuitive, the robustness of the dispersal syndrome is not, a fact that has been amply noted in the literature (Ronce & Clobert 2012). This is primarily because dispersal is a complex process and what exactly evolves can be rather sensitive to differences in the nature of the selection pressure (Bonte *et al.* 2012). For example, in spider mites, selection applied through spatially correlated extinctions leads to an increase in the frequency of long distance dispersers (LDDs) even though the fraction of individuals dispersing (i.e. dispersal propensity) is reduced (Fronhofer *et al.* 2014). However, dispersal propensity does evolve when there is a direct selection on dispersal (Yano & Takafuji 2002). Evidently, there is no reason to expect the same set of behaviour or life-history traits to have evolved in these two experiments, even though some attribute of dispersal had evolved in both cases.

The second approach to study the consequences of dispersal evolution is experimental evolution, which seeks to exercise greater control over the various selection pressures affecting dispersal, even at the cost of simplifying some of the ecological interactions. From a large number of studies in the last few years (Yano & Takafuji 2002; Fronhofer *et al.* 2014; Matsumura & Miyatake 2015), it is clear that under most laboratory conditions, dispersal can evolve reasonably fast. Surprisingly though, most laboratory experimental evolution studies report that life-history traits like longevity and fecundity do not evolve (Li & Margolies 1994; Bitume *et al.* 2011) as a correlated response to selection for dispersal. Unfortunately, the experimental evolution studies have often not focused on the various behavioral attributes (although see Matsumura & Miyatake 2015; Matsumura *et al.* 2016), which makes it difficult to compare the observations with those from association studies.

Another major question in this context is the underlying mechanism of dispersal evolution. Although a lot is known about the anatomical or physiological changes associated with dispersal phenotypes (reviewed in Zera & Brisson 2012), we have relatively lesser understanding of what happens at the molecular level. Two genes that have been fairly conclusively shown to be related to dispersal in insects are the glycolytic enzyme Phosphoglucose isomerase (*Pgi*) in the Glanville fritillary butterflies (Niitepõld *et al.* 2009) and the cGMP-dependent protein kinase called foraging (*for*) gene in *Drosophila melanogaster* (Osborne *et al.* 1997). In *C. elegans*, three genes, namely G-protein coupled receptors *npr-1* (de Bono & Bargmann 1998) and *tyra-3* (Bendesky *et al.* 2011) and *rol-1* (Friedenberg 2003) have also been shown to have a connection with dispersal phenotypes. Although these are valuable insights, it is not always clear whether these genes would be the ones whose frequencies would change during dispersal evolution. More critically, given the complexity of dispersal, it is intuitive to assume that in any given species, dispersal evolution will probably involve changes in a fairly large number of genes and metabolic pathways. Therefore, a promising approach would be to look at the changes at the level of the metabolome and correlate that with the corresponding behavioural and life-history changes. Although this approach has been successfully used in the context of adaptation to stresses (Sørensen *et al.* 2017) or circadian profiles of metabolites (Gogna *et al.* 2015), to the best of our knowledge, it has never been attempted in the context of dispersal evolution.

Here we address some of these issues using four large, outbred populations of *Drosophila melanogaster* that have been selected for increased dispersal. These populations have evolved significantly greater dispersal propensity and ability (i.e. distance covered by the dispersers) and a larger fraction of Long-Distance Dispersers (LDDs) in the population (Tung *et al.* 2017). In this study, we investigate three correlated behavioural traits (locomotor activity, exploration and aggression) and three life-history traits (body size, fecundity and life-span). We find that while all three behavioural traits have evolved significantly, none of the life-history traits had been altered due to selection for dispersal. We then investigate the metabolomic changes in the selected flies using non-targeted NMR spectroscopy and connect them with the corresponding behavioural and life-history changes.

2. Materials and methods

The details of all assays are mentioned in the Appendix S1 in Supporting Information (Text S1) and only a brief description is presented here as an aid to appreciate the results and discussion.

Experimental populations:

We used eight large (breeding size of ~2400) laboratory populations of *Drosophila melanogaster*, four of which (VB_{1-4}) had been selected for greater dispersal ability and propensity for more than 49 generations and the remaining four (VBC_{1-4}) were their corresponding ancestry-matched controls (Tung *et al.* 2017). The details of the ancestry, maintenance and selection protocols of these populations can be found as Text S1.1. All assays were performed after rearing both VB and VBC populations for one generation under common conditions, to rule out the contributions of non-genetic parental effects.

Behavioural assays:

Locomotor activity and resting behaviour of adult male flies of the VB and VBC populations were assayed using the *Drosophila* Activity Monitoring (DAM2) data collection system (Trikinetics Inc, Waltham, MA) following standard protocol (Chiu *et al.* 2010; see also Text S1.2.1). We recorded two kinds of activity/rest data - i) for six hours without any acclimatization period and ii) for 24 hours, after six hours of acclimatization. The first kind of data were recorded in presence and absence of food while the second was recorded only in the presence of food.

Exploratory tendency was measured following an earlier assay setup (Liu *et al.* 2007) using 32 flies of each sex from each VB and VBC population (Text S1.2.2).

Male-male aggression was estimated using 30 VB-VBC fights for each VB and VBC population based on standard protocol (Text S1.2.3). Following a previous study (Yurkovic *et al.* 2006), a winner was declared when one male chases-away the other male off the arena three consecutive times.

Life-history assays:

Dry weight was estimated on five batches of 20 males or 20 females from each of the four VB and VBC populations, after drying at 60ºC for 72 hours (Text S1.2.4).

For the fecundity assay, the number of eggs laid by 15-day old or 33-day old females was estimated as early-life and late-life fecundity respectively (Text S1.2.5). For each population × reproductive timing combination, we used 40 pairs (1 male + 1 female) of flies for estimating fecundity.

For the longevity assay, we monitored 10 sets of 10 flies of either sex per population daily, from eclosion till death. Longevity was scored as the number of days to death for each fly (Text S1.2.6).

NMR sample preparation and spectroscopy:

We investigated the evolution of the metabolome of the VB flies using NMR spectroscopy on one block of selected-control populations (VB_4-VBC_4). For each population, we used 11 replicates, each of which consisted of 30 males. Samples were prepared following established protocols (Gogna *et al.* 2015) and subjected to both 1D and 2D NMR experiments followed by metabolite fingerprinting using databases such as MMCD and BMRB (Text S1.2.7).

Statistical analyses:

VB_i and VBC_j that shared a subscript (i.e. $i = j$) were assayed and analysed together as a block as they were related to each other by ancestry. For locomotor activity, the fraction of time spent resting/sleeping (arcsine-square root transformed), average sleep bout, length of the longest sleep bout and female fecundity data, we used two factor mixed-model ANOVA with selection (VB and VBC) as fixed factor crossed with block (1-4) as a random factor. Three factor mixed-model ANOVA was performed for adult exploration, dry body weight and longevity data with selection (VB and VBC) and sex (male and female) as fixed factors and block (1-4) as a random factor, crossed with each other. For the aggression data, we used Mann-Whitney U tests to compare the performance of VB and the VBC males. The value of effect size (Cohen's *d*) was interpreted as large, medium and small when $d \geq 0.8$, $0.8 > d \geq 0.5$ and $d < 0.5$ respectively (Cohen 1988). All the above statistical analyses were performed using STATISTICA® v5 (StatSoft. Inc., Tulsa, Oklahoma).

The NMR spectral data were analysed using standard procedures (see Text S1.2.7). The spectral data were normalized to total area, Pareto scaled and subjected to Principal Component Analysis to identify and remove the outliers. This was followed by Orthogonal Projections to Latent Structure-Discriminant Analysis (OPLS-DA) to identify the metabolites responsible for separating VB and VBC flies. The significance test of the model was performed using CV-ANOVA (cross-validated ANOVA). Further, permutation analysis was performed on the best model using 1000 permutation tests with a threshold *P*-value of 0.05, which indicated that none of the results were better than the original one. The average level of the metabolites in the selected and control populations, were compared using Student's *t*-tests, followed by Bonferroni correction, thus restricting the family-wise error rate to < 0.05.

3. Results

VBs are restless but rest less

During the first six hours after set-up, the VB populations had significantly greater locomotor activity irrespective of the absence (Fig. 1A, $F_{1, 3}$=60.3, *P*=0.004) or presence (Fig. 1C, $F_{1,3}$= 423.3, *P*=0.0003) of food. Moreover, of the total duration of six hours, the VBs spent significantly less time in rest/sleep, both in the absence (Fig. 1B, $F_{1, 3}$=50.4, *P*=0.006) and presence (Fig. 1D, $F_{1, 3}$=386.9, *P*=0.0003) of food. Interestingly though, when assayed in the presence of food over a duration of 24-hours after the initial six hours of acclimatization, although the difference in activity persisted (Fig. 1E, $F_{1, 3}$= 59.9, *P*= 0.004), the VBs spent similar amount of time in rest/sleep as the VBCs (Fig. 1F, $F_{1, 3}$= 5.47, *P*=0.1). The length of average sleep bouts (Fig. S1A, $F_{1, 3}$=2.2, *P*=0.23) and maximum sleep bouts (Fig. S1B, $F_{1, 3}$=4.7, *P*=0.12) of the VBs and the VBCs were also comparable.

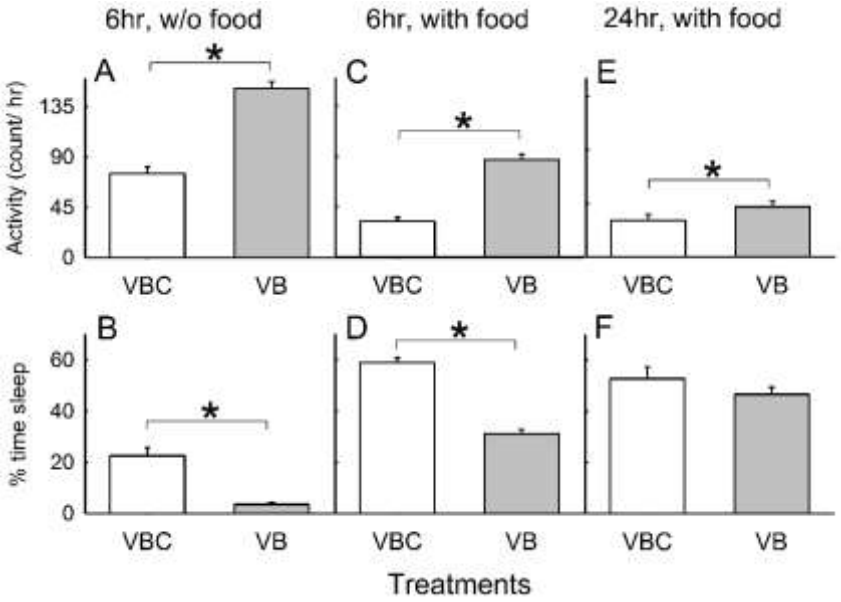

Figure 1. Locomotor activity-sleep profiles in the presence and absence of food. In the absence of food during the first six hours after introduction **(A)** Locomotor activity of VBs was significantly greater than the VBCs although **(B)** duration of rest was significantly lower. In the presence of food during the first six hours, similar results were obtained for **(C)** locomotor activity and **(D)** rest. After acclimatization for six hours, over the next 24 hours, the VBs had significantly greater (**E**) locomotor activity but similar levels of (**F**) sleep duration as the VBCs. The error bars represent standard errors around the mean (SEM) and * denotes $P < 0.05$.

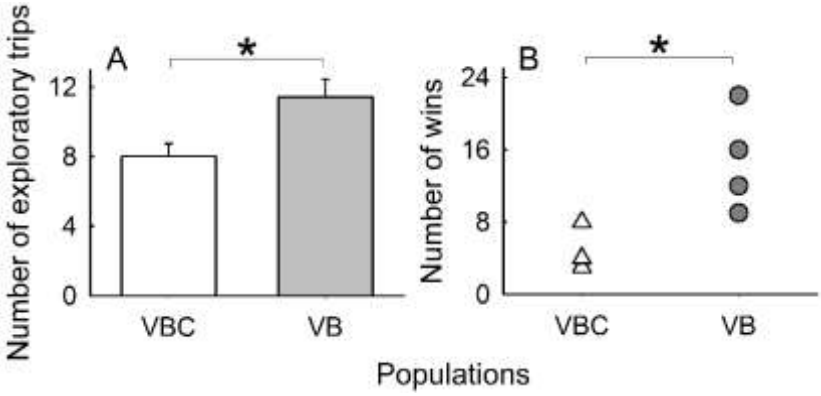

Figure 2. Exploration and aggression behavior of VBs and VBCs. (**A**) VB flies commenced significantly more number of exploratory trips than VBCs. The error bars represent standard errors around the mean (SEM). (**B**) VB males were more aggressive as they won significantly more number of fights against VBC males. Both these results were consistent across all the four blocks. * denotes $P<0.05$.

Selection for dispersal leads to greater exploratory behaviour and aggression

Selection regime had significant effects on adult exploratory behaviour, when the flies were introduced into a novel environment and the VB flies had significantly greater tendency to explore a novel area than the VBC flies (Fig. 2A, $F_{1, 3}$=11.96, *P*=0.04). Although we failed to find a selection × sex interaction ($F_{1, 3}$=0.009, *P*=0.93), for both sexes, the VB flies exhibited greater exploratory behaviour than the corresponding VBC flies (Fig. S2). Furthermore, we found significant effect of selection on male aggression (Mann-Whitney *U*=0.0, *P*= 0.02) in all the four blocks: VB males were found to be significantly more aggressive than the VBC males with large effect size (*d* = 2.05) in one-to-one fight for food and mate present in the arena (Fig. 2B).

Selection for dispersal does not lead to changes in dry body weight, fecundity or longevity

The mean dry weight of VBs and VBCs were found to be comparable (Fig. 3A, $F_{1, 3}$= 0.76, *P*=0.45). Although *Drosophila* females are known to be heavier than males, we did not find any significant selection × sex interaction ($F_{1, 3}$= 2.1, *P*=0.24) in our study suggesting that selection did not affect the dry weight of the two sexes in VB and VBC flies differentially. There was no significant difference between the fecundity of the VB and VBC flies with respect to either early fecundity (Fig. 3B, $F_{1, 3}$= 0.25, *P*=0.65) or late fecundity (Fig. 3B, $F_{1, 3}$= 0.2, *P*= 0.68), indicating the absence of a trade-off between increased dispersal ability and reproductive output. We also did not find any trade-off between dispersal and longevity: the average life-span of VBs was found to be similar to that of the VBCs (Fig. 3C, $F_{1, 3}$= 4.9, *P*=0.11).

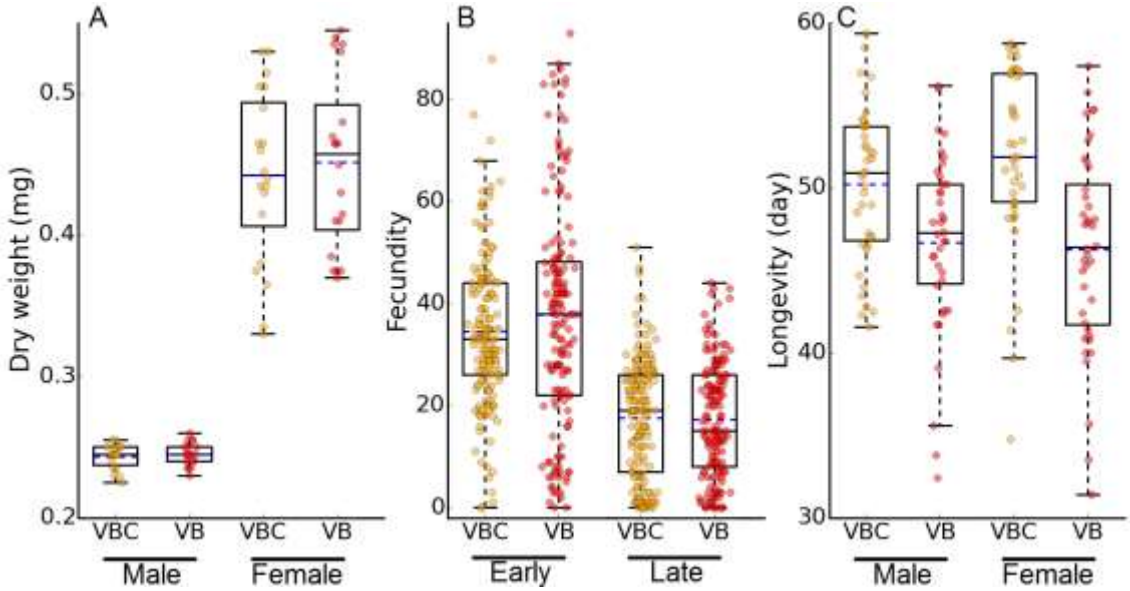

Figure 3. Life-history traits of VBs and VBCs. Cleveland-box plots show (**A**) dry body weight, (**B**) early-late fecundity and (**C**) longevity profiles of VB and VBC populations were not different from each other. The points represent the pooled data for all the replicates of VB and VBC populations with small random jitter along X-axis. The edges of the box denote 25th and 75th percentiles, while the black solid lines and blue broken lines represent the median and mean respectively.

Selection for dispersal leads to changes in the metabolome profile

OPLS-DA scores plot (Fig. S3) documents the differences in the metabolite profile of the selected and control flies. Fig. S4 shows the colour coded coefficient loadings plot used to identify the metabolites responsible for differentiating both VB and VBC flies. The variance explained by the model (R^2X) was 0.968 and the variance predicted by the model (Q^2) was 0.953, showing that the model was effective and had a good predictive accuracy. The credibility and robust nature of the model were also confirmed by testing the statistical significance of the model with CV-ANOVA (p-value < 0.01) and permutation test (p-value < 0.05). The metabolites, which were significantly different between the selected and control flies in *t*-test followed by Bonferroni correction, are tabulated in Table 1.

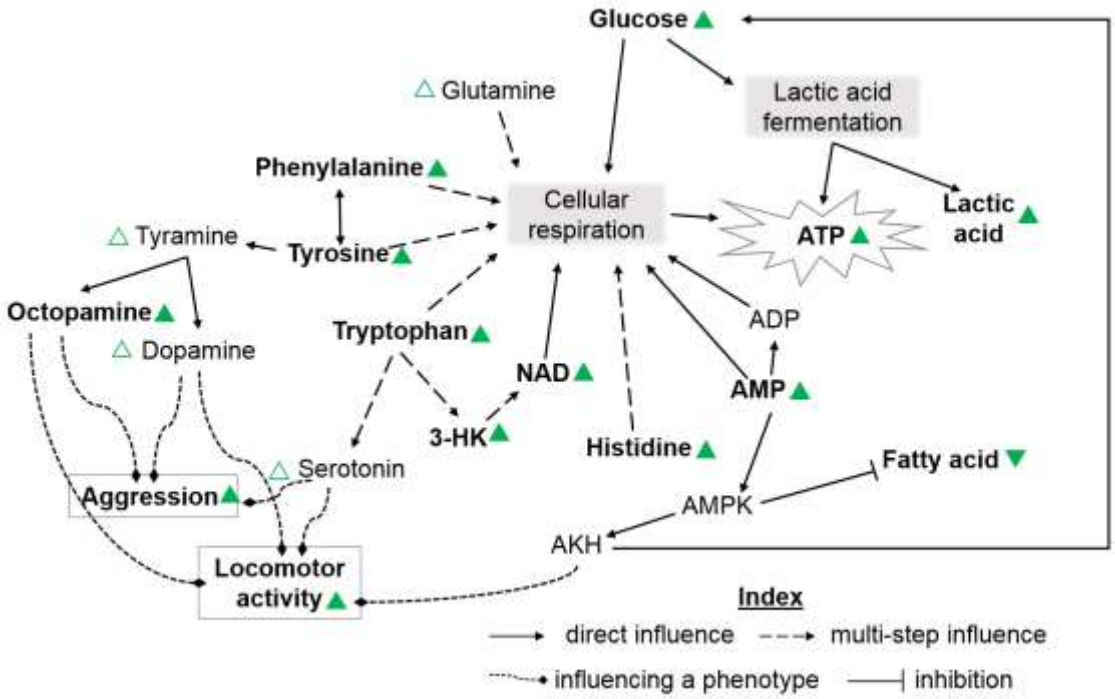

Figure 4. Alterations in metabolic pathways. Schematic diagram of the interactions between the metabolites that were found significantly changed in the VBs in the NMR data. Upright and inverted triangles adjacent to a metabolite denote whether its level had increased or decreased respectively. Filled and open triangles represent statistically significant and non-significant changes respectively (see Table 1 for values).

Metabolites	*p* value	Effect size (Cohen's *d*)	Fold change in VB (VB/VBC)
Tryptophan	1.15E-06*	3.77	3.27
AMP	6.93E-06*	3.16	2.63
3-HK	9.54E-07*	3.86	2.5
Octopamine	5.81E-08*	3.88	2.26
Phenylalanine	1.50E-06*	3.58	1.95
NAD	4.04E-08*	4.8	1.82
Tyrosine	6.19E-08*	3.96	1.73
Histidine	2.73E-05*	2.72	1.71
Glucose	2.41E-07*	4.18	1.65
ATP	1.06E-06*	3.69	1.57
Sucrose	2.15E-04*	2.39	1.48
Citric acid	1.57E-04*	2.15	1.36
Lactic acid	6.89E-05*	2.26	1.35
Glutamine	9.71E-04	1.94	1.27
Tyramine	5.57E-03	1.46	1.15
Serotonin	1.58E-02	1.16	1.15
Dopamine	1.63E-01	0.73	1.1
Fatty acid	2.14E-04*	2.49	0.77

Table 1: List of metabolites altered during the course of selection for increased dispersal. Only those metabolites are shown that were either significantly different between the VBs and the VBCs or the fold change was >1. The *p*-values for a given metabolite were obtained from *t*-tests between the VBs and VBCs. * indicates that the differences were statistically significant even after Bonferroni correction at the 0.05 level. All effect size values are large (i.e. $d > 0.8$), except for dopamine, where it is medium (i.e. $0.5 < d < 0.8$). Note that the AMP: ATP ratio for the VBs and VBCs were 0.43 and 0.26 respectively.

4. Discussion

4.1 Selection for dispersal leads to similar patterns of activity but different patterns of rest in the short and long time-scale

During the first six hours after introduction to a new environment (same duration for which the flies are allowed to disperse during selection), the selected populations had greater locomotor activity than the controls (Fig. 1A and 1C), and spent lesser time in rest (Fig. 1B and 1D). This observation is consistent with previous results (Hanski *et al.* 2006; Matsumura *et al.* 2016) and also with the fact that the VBs were under intense selection to reach a new environment within the first six hours of introduction to the source (Tung *et al.* 2017). Consequently, maximizing the amount of activity and minimizing the resting period during that time would be of obvious advantage to the VB flies. More interestingly though, we found similar activity/rest patterns in the absence and presence of food (*cf* Figs 1A with 1C and 1B with 1D), which suggests that the increased activity is independent of starvation or desiccation cues. This is again consistent with

earlier observations that the dispersal propensity and ability differences between VBs and VBCs were observed irrespective of the presence or absence of food (Tung *et al.* 2017). However, when we measured the activity of these flies over 24 hours, after allowing a period of six hours to acclimatize, although the VB males were found to be significantly more active than VBC males (Fig. 1E), the percentage of time spent resting was not different between these two lines (Fig. 1F). In other words, the VB flies rest lesser during the period that corresponds to the time when they face selection, but revert to normal levels of rest once that phase is over. During the latter period, the quality of the rest/sleep of the flies, as measured by the length of average bout of sleep (Fig. S1A) and maximum bout of sleep (Fig. S1B), during 24 hours, was also found to be similar in VBs and VBCs. Thus, although the VBs are more active, it seems unlikely that they would face negative effects of rest-deprivation in the long-run. To the best of our knowledge, this is the first demonstration that dispersers also modulate their rest-patterns temporally in way that could reduce the negative effects of rest-deprivation (Huber *et al.* 2004; Kayser *et al.* 2015). Increased activity of dispersers can positively correlate with another important behavioural trait, namely exploratory tendency (Cote *et al.* 2010) which is what we investigated next.

4.2 The evolution of dispersal led to simultaneous evolution of exploratory behaviour

Dispersers often have greater exploratory tendency (Cote *et al.* 2010; Korsten *et al.* 2013) which is thought to be beneficial for finding new habitats. In our selection protocol, there was no sensory cue in the path connecting the source to the destination. Therefore, only those flies (of either sex) could disperse successfully that took the risk of getting into the path and then continuing along it, implying that exploratory tendency was under strong positive selection. Therefore, it was no surprise that the dispersal-selected VBs were more exploratory in nature than the VBCs (Fig. 2A), and the result was consistent across both sexes (Fig. S2).

Elevated exploratory tendency can be important during range expansion as the individuals present at the range edges are more likely to experience environments different from their native and/or previously introduced habitats. For example, Kenyan house sparrows that were present at a range expansion front were found to be significantly more exploratory (Liebl & Martin 2012). Interestingly, in many species, exploration is also found to be strongly related to invasion (Rehage *et al.* 2005; Cote *et al.* 2010; Russell *et al.* 2010), which involves conflict/confrontation with the native species. Consequently, aggression is another behavioural trait strongly correlated with exploration (Verbeek *et al.* 1996; Dingemanse & de Goede 2004) and often closely related to personality-dependent dispersal (Cote *et al.* 2010). Thus we next investigated the effect of dispersal evolution on aggression.

4.3 Male-male aggression evolved as a correlated response to selection for dispersal

Aggression is an important trait that influences an individual's ability to retain resources and mates or gain new ones (O'Riain *et al.* 1996). Not surprisingly, several studies have reported a strong association between dispersal tendencies and aggression (Wahlström 1994; Duckworth & Badyaev 2007) which is consistent with our observations (Fig. 2B). However, while enhanced aggression might be a factor for the dispersal success in some of the natural populations (Duckworth & Badyaev 2007), in our system, the dispersing flies have no obvious fitness advantage for being more aggressive, as they did not have to compete with any native individuals at the destination. Thus, in our experiment, aggression evolved as a correlated response of dispersal evolution, most likely due to changes at the biochemical / physiological levels.

4.4 Dispersal-selected lines have comparable body size as that of the controls

One life history trait that could potentially explain the increased levels of dispersal, locomotor activity and aggression in the VBs is adult body size. Bigger organisms are expected to have greater energy reserves and, in general, body size is positively correlated with dispersal (Dingle *et al.* 1980; Sutherland *et al.* 2000, although see Gu & Danthanarayana 1992). Moreover, in *Drosophila melanogaster*, it is known that larger males win significantly more aggressive encounters compared to smaller males (Partridge & Farquhar 1983). However, we failed to find a significant difference in body weight, a proxy for body size, between VBs and VBCs (Fig. 3A) which suggests that the increased aggression and locomotor activity in VBs were not mere artefacts of differences in body sizes between the two populations. This result also highlights that how a trait evolves (here, no change in body size) due to a particular selection pressure may not always be inferred from existing trait-associations (here, the general observation that body size and dispersal are positively correlated).

In *Drosophila*, body size is generally considered to be a good proxy for the total amount of resources available to an organism. Our results suggest that the selected flies have similar levels of resources compared to the controls (Fig. 3A), but at the same time, display elevated levels of activity (Figs 1A, 1C and 1E). Given that the kind of nutrients available to both populations are the same, one way for the selected flies to manage this feat would be to alter the pattern of resource allocation among the various traits (van Noordwijk & de Jong 1986). To investigate this possibility, we measured two crucial life-history traits, namely fecundity and longevity.

4.5 Selection for dispersal does not affect fecundity or longevity

The relationship between dispersal and fecundity has been somewhat controversial in the literature. On one hand, flight ability/ dispersal has been shown to be negatively correlated with fecundity in several insects including *Drosophila* (Roff 1977), long-winged crickets (Roff & Fairbairn 2007) and aphids (Dixon *et al.* 1993). This is thought to be due to energy limitation, as allocation of resources to the muscles reduces the availability of the same for reproductive functions. On the other hand, several investigators have reported a positive correlation between dispersal and fecundity (Hanski *et al.* 2006, reviewed in Rankin & Burchsted 1992), which is possible if the dispersers are also the physically superior organisms of the population who abound in resources (Bonte & de la Peña 2009). However, our results differed from both these expectations and there was no significant difference between the fecundity of the VB and the VBC populations either in early life or late life (Fig. 3B), which is consistent with an earlier dispersal evolution study on spotted-mites (Fronhofer *et al.* 2014). The lack of difference in fecundity between the VBs and the VBCs might be explained if we assume that the selected flies allocate a fixed amount of resources to reproductive functions and this amount has not been affected by selection for dispersal. While there are several theoretical studies in support of both assumptions (de Jong & van Noordwijk 1992; Mezey & Houle 2005), it is difficult to conclusively demonstrate them using our data. Therefore, we shifted our attention to a crucial body maintenance trait, namely longevity.

In Glanville fritillary butterflies, higher dispersal and mobility correlate strongly with higher flight metabolic performance (Hanski *et al.* 2004). Since a strong negative correlation between life-span and metabolic rate has been reported across various taxa (reviewed in Rattan 2008), dispersers are expected to have a shorter life-span, which was actually observed in a previous

study on tropical butterflies (Tufto *et al.* 2012). However, in our study, we did not find any significant difference in longevity between the dispersal-selected and the control populations, a result which is consistent over all four populations and both the sexes (Fig. 3C). One reason for this might be the nutrient-rich laboratory conditions under which the flies were reared during selection, which ensured that resources were never limiting, and therefore did not lead to the expected trade-offs between dispersal ability and fecundity or longevity. If true, then this would suggest that the patterns of trait correlation that would evolve under selection for dispersal would depend closely on, *inter alia*, the resource availability, which would obviously vary greatly across populations under natural conditions. Thus, even for the same species, it might be difficult to predict the outcomes of dispersal evolution under various scenarios.

4.6 Selected flies have elevated levels of cellular respiration

There was a clear difference between the overall metabolite profiles of the VB and VBC flies (Fig. S3 and S4) and the levels of 14 metabolites were significantly different between these two populations (Table 1). Most notably, the glucose levels of VBs were significantly higher than the VBCs and glucose is the primary proximate source of energy in the cell through the process of cellular respiration. Moreover, the VBs had greater levels of citric acid, nicotinamide adenine dinucleotide (NAD) and adenosine monophosphate (AMP), all of which are critically associated with cellular respiration (Fig. 4). Finally, the VBs also had significantly greater amounts of lactic acid. It is known that when the demand for energy is more than what cellular respiration can generate (e.g. during intense muscular activity) glucose undergoes anaerobic oxidation via lactic acid fermentation to produce ATP. True to this observation, the ATP levels in VBs was significantly higher than the VBCs (Table 1). However, the AMP: ATP ratio, which is an indicator of levels of cellular energy crunch (Hardie & Hawley 2001), is much higher for VBs. This suggests that, in spite of the greater levels of ATP, the VBs are in a more energy-depleted state than the VBCs (Table 1). Taken together, these results suggest that the VBs have elevated levels of both aerobic and anaerobic cellular respiration and produces significantly more ATPs, which is consistent with the fact that they disperse to longer distances (Tung *et al.* 2017) and have greater locomotor activity (Figs 1A, 1C and 1E).

4.7 Selected flies have elevated levels of octopamine and precursors for other neurotransmitters

The VBs also had increased levels of four amino acids, namely histidine, phenylalanine, tyrosine and tryptophan (Table 1). The metabolic break-down products of all these amino acids form intermediates of citric acid cycle or their precursors and therefore play a role in energy production (Voet & Voet 2011). More interestingly, phenylalanine and tyrosine act as precursors for octopamine and dopamine (Brandau & Axelrod 1972), while tryptophan is a precursor for serotonin (Stone & Darlington 2002). In *Drosophila*, increased levels of octopamine not only enhances aggression (Zhou *et al.* 2008) but also leads to greater activity (Yellman *et al.* 1997). This was consistent with the observation that octopamine levels had significantly increased in VBs (Table 1). Similarly, serotonin levels are also known to be positively correlated with activity (Yellman *et al.* 1997) and aggression (Dierick & Greenspan 2007). Dopamine can elevate the activity level in flies (Yellman *et al.* 1997) although its relationship with aggression is not as straightforward as for the other molecules (Alekseyenko *et al.* 2010). Combining these evidences with the fact that the VBs are more active (Fig. 1A, 1C and 1E) and aggressive (Fig. 2B),

strongly suggests that the levels of serotonin and dopamine have also increased in course of evolution for dispersal. We did detect an increase in the levels of both these neurotransmitters (Table 1), although the increase was not statistically significant after Bonferroni correction.

It should be noted here that apart from the three neurotransmitters discussed above, there are many more which can also potentially modulate fly behaviour. For example, it is known that increased levels of insulin (Belgacem & Martin 2006; Luo *et al.* 2014) and tachykinin (Asahina *et al.* 2014) can either reduce aggression or activity or both. Unfortunately, although a comprehensive investigation of the changes in the levels of the various neurotransmitters in these flies would be of immense interest, it is also outside the scope of the present study.

4.8 Selected flies have reduced levels of fatty acids

The end-product of the main route of tryptophan metabolism is nicotinamide (Stone & Darlington 2002), which subsequently produces NAD, a key element of cellular respiration (Khan *et al.* 2007). One of the main intermediates of the tryptophan-NAD pathway, 3-hydroxykynurenine (3-HK), is also found to be significantly higher in VBs, suggesting that the pathway has been enhanced in these flies. 3-HK is associated with free-radical generation and neural degeneration in flies (Savvateeva *et al.* 2000) which is consistent with the slightly lower (although not significant) longevity of the VB flies (Fig. 3C). Histidine, another amino acid with elevated levels in the VBs, is known to be coregulated with AMP (Rébora *et al.* 2005). Abundant supply of AMP and depletion of ATP (Table 1) increases the AMP: ATP ratio, which in turn is expected to activate AMP-activated protein kinase (AMPK) (Hardie & Hawley 2001). AMPK typically functions to facilitate the depletion of fat storage (Sinnett & Brenman 2016) which is corroborated by the observation that VBs had significantly lower levels of fatty acid (Table 1). AMPK also controls the normal secretion of adipokinetic hormone (AKH) (Braco *et al.* 2012). AKH in turn stimulates locomotor activity and helps in maintaining a hyperglycaemic state in the body (Bharucha *et al.* 2008): two facts that are consistent with our observations on VBs.

Taken together, it is evident that the changes at the behavioural and life-history level are well correlated with the underlying metabolomic changes (Fig. 4).

5. Conclusions

Our study shows that in terms of relationship between dispersal and behavioural traits, there is excellent correspondence between the insights derived from association studies on field populations and experimental evolution studies. One reason for this might be that active dispersal is intimately related to locomotion which shares common control mechanisms with aggression and exploration via neurotransmitters like octopamine and serotonin. This automatically leads to the prediction that in passively-dispersing organisms, this trait-association is likely to breakdown. Incidentally, we also show that in terms of life-history traits, the correspondence between field and laboratory studies is poor. One reason for life-history traits not evolving in experimental evolution studies might be the fact that nutrition is typically non-limiting under laboratory conditions and therefore the organisms can increase expenditure in energy-intensive activities without paying major life-history costs. If true, then one can predict that artificial selection for dispersal under nutrient-limiting conditions would lead to a very different pattern of changes in life-history traits. Whether these changes would mimic the ones from field studies

remains to be seen. Finally, our study gives the first glimpses of the metabolome-level changes that accompany dispersal evolution. This is best thought of as an over-view of the myriad changes that can occur when dispersal evolves and the complex ways by which they can affect the various traits of the organism. Establishing the robustness of these metabolic level changes (particularly under field conditions) and connecting them to the corresponding genes is going to be one of the next big challenges in dispersal ecology.

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Online Supporting Information

Appendix S1: Detailed materials and methods

Text S1.1 Experimental populations

1. Ancestry and maintenance regime

In this experiment, we used eight large (breeding size of ~2400) outbred laboratory populations of *Drosophila melanogaster*, four of which were subjected to selection for increased dispersal over 49 generations (called VB_{1-4}) and the other four populations served as corresponding controls (VBC_{1-4}). VB and VBC populations that share a numerical subscript (e.g. VB_1 and VBC_1) were related by ancestry (Tung *et al.* 2017), and hence were always assayed together and treated as blocks in statistical analyses.

Both VBs and VBCs were maintained on a 15-day discrete generation cycle at 25°C and constant light conditions. In each generation, ~60-80 eggs were collected in clear plastic vials containing ~6 mL of standard banana-jaggery food (following Sheeba *et al.* 1998). For each VB and VBC population, we collected eggs in 80 and 40 vials respectively. After 12 days from the day of egg collection, the adults were collected and subjected to the selection protocol (see Text S1.2). Immediately after this, the adults of a given population were transferred to a plexi-glass cages (25 cm × 20 cm ×15 cm) and provided with yeast supplement along with standard banana-jaggery food. After ~40 hours, eggs were collected for the next generation. The adults were discarded after oviposition, thus ensuring that individuals of two successive generations never co-exist.

2. Selection procedure

The apparatus used for selection has three components- *a source, a path and a destination* (see (Tung *et al.* 2017 for detailed description). In order to impose selection on VBs, the adults collected from 40 vials (~2400) were introduced into a *source* container. Thus, for each VB population, two *source* containers were used. Each *source* was then connected to a *path* consisting of a plastic tube of inner diameter ~1 cm, which led to the *destination* container. The source and path did not contain any source of food or moisture, and the flies dispersed through the *path* into the *destination*, which contained a strip of moist cotton as a source of moisture. The process was continued until ~50% (visual estimation) of the population reached the *destination*

or for six hours (whichever happened earlier). The arbitrary cut-off of six hours was chosen based on the result of a prior assay in the lab that under such condition, the flies do not die due to desiccation stress during the first six hours (S. Tung personal observations).
Similar to VBs, adults coming from the 40 vials of each VBC populations were maintained in separate *source* containers but they were not allowed to disperse. They were also provided with a moist cotton plug, after 25% of the VBs reached their destination or 3 hours (whichever was earlier).
At the end of this process, for VBCs, all the flies from respective source containers were transferred to the cages, thus ensuring that there was no selection for dispersal. For each VB population, only the flies which reached the *destination* in the two selection setups were transferred into a cage and allowed to breed for the next generation. Thus, the breeding population size of VBs and VBCs remained comparable in each generation.
Starting from 2 m, the length of the path was increased intermittently over generations in order to intensify the selection for grater dispersal ability. By generation 67, when the last set of assays were performed, the path length had reached 20 m.

Text S1.2 Assays:

Prior to any assay, both VB and VBC populations were maintained under identical rearing conditions for one generation to ameliorate the influence of phenotypic plasticity or non-genetic parental effects. The progeny of these flies were used for the assays. Moreover, for all the assays, egg density was always maintained at ~50 eggs on ~6mL food in each vial to avoid any confounding effect of larval crowding on the life-history and behavioural traits measured.

1. Locomotor activity assay:

After 49 generations of selection, locomotor activity of the selected and control lines were checked both in the presence and absence of food using *Drosophila* Activity Monitoring (DAM2) data collection system (Trikinetics Inc, Waltham, MA). The activity for a given fly was estimated as the average number of times the fly crossed the IR beam of *Drosophila* activity monitor per hour, while, continuous inactivity for five minutes or more was considered as sleep/rest (Hendricks *et al.* 2000; Chiu *et al.* 2010). For each VB/VBC population, we measured the activity of only male flies as in females laying of eggs on the tubes could affect the accurate measurement of locomotor activity (Chiu *et al.* 2010). We performed this assay both in presence and absence of food. For the activity assay in the presence of food, on the 11th day from the day of egg collection, between 1830 h -1930 h, single adult male flies were aspirated into glass activity tubes of 5 mm diameter, containing standard banana-jaggery food at one end. We preferred aspiration over CO_2 anaesthesia to avoid any lingering effects of anaesthetization on the activity of the flies (van Dijken *et al.* 1977). Details of the preparation of the tubes and the cleaning of the same can be found elsewhere (Chiu *et al.* 2010). The selected populations were always assayed along with their matched control populations (i.e. VB_1 was assayed with VBC_1 and so on) and there were 30-32 replicates for each population. Activity data were collected for 30 hours and were divided into two parts - i) first 6 hours and ii) next 24 hours. The first set captured the activity-rest pattern immediately after introduction of the flies in the tube, while the next set measured the steady state activity-rest pattern, after 6 hours of acclimatization, for a

complete 24-hour cycle. For the entire duration of recording, the monitors containing the activity tubes were kept undisturbed inside an incubator maintained at 25 °C and constant light.

Locomotor activity assay in the absence of food was similar to the one mentioned above except that no food was provided and both ends of the activity tubes were secured with clean, dry cotton plugs. The setup for this assay was done on the 12th day from the day of egg collection between 1200 h-1300 h which roughly corresponds to the time at which selection was imposed during the regular maintenance of VBs. Moreover, in the no-food case, locomotor activity was recorded only for 6 hours from the time of setup as, after this period, the flies become stressed and slowly start dying due to desiccation. 30-32 flies were assayed for each of the VB and VBC populations.

For each of these datasets, average number of activity counts per hour was calculated as a measure of the activity level of the flies and the fraction of time the flies did not show any activity count was computed as an indicator of the sleeping/resting duration. In other words, the activity level of a fly is the total number of activity counts while it was not resting/sleeping. Thus, mathematically, the activity level of a fly was independent of the fraction of time it spent in resting/sleeping. In order to assess the quality of rest/sleep, for the 24-hour dataset, we computed the average length of uninterrupted rest/sleep bout and duration of the longest rest/sleep bout for each of the flies.

2. Exploration assay

This assay was performed after 53 generations of selection. For each VB or VBC population, we used 10 replicate vials each containing around 50 eggs / 6ml of banana-jaggery food. The assay was performed on the 12th day after egg collection. The male flies were aspirated from the egg-collection vials and introduced into the experimental arena (modified from Soibam *et al.* 2012) where their activity was recorded using a video camera. The experimental arena was made of a clear polycarbonate petri dish lid with an inner diameter of 10 cm. A small hole was drilled into the centre of the lid to introduce flies into the setup. The lid was placed on top of a blank sheet of paper which contained the traces of two concentric circles. The outer circle was of the same diameter as the petri dish lid while the inner circle divided the total area of the lid into two zones: the outer containing one third of the total area while the inner one enclosing two-thirds of the area (Liu *et al.* 2007) of the lid. 32 replicates were assayed for each sex of all the VB and VBC populations. After the flies were introduced into the arena, they were given one minute to acclimatize to the new environment. They were then observed for the next 10 minutes and the number of times they entered the inner zone, considered as the number of exploratory trips, was recorded.

3. Male-male aggression assay

The aggression assay was performed after 52 generations of selection. For this assay, the flies were reared under low levels of larval crowding (~50 eggs in 6-8ml banana-jaggery food). Freshly eclosed males were collected and reared in social isolation (i.e. one male per vial) till the day of assay (following Yurkovic *et al.* 2006). We used 6 wells of a twelve-well culture plate (Corning®, NY, USA) as the assay apparatus, where each well served as the enclosure for one replicate of the aggression assay. A small plastic cup containing regular banana-jaggery medium was affixed at the centre of each well. A freshly decapitated female was stuck to the middle of the food cup using yeast paste. The food and the female served as defendable resources and

potential reasons for conflict. Following an earlier protocol, VB and VBC males were colour-coated with daylight fluorescent pigments (DayGlo) for easy identification (Dickens & Brant 2014). On the 12th day from egg collection, two males (one VB and one VBC) were introduced into the setup and their interaction was recorded for 45 minutes using a video camera. 30 such replicates were assayed for each of the four populations of VBs and the corresponding VBCs. Individual wells were visually isolated from each other using cotton to ensure no visual cues were being exchanged between replicates. Uniform lighting and constant temperature (25°C) were maintained.

The scoring for aggression was done after an initial five-minute acclimation period. For each of the replicates, the number of successful chase-aways from the food cup was recorded. A successful chase-away is defined as one in which one male completely chases the other male away from the top surface of the food cup (Yurkovic *et al.* 2006). Earlier studies have shown that in *Drosophila* a male that manages to complete three consecutive successful chase-aways usually manages to successfully chase away the other male in all future encounters (Yurkovic *et al.* 2006). Therefore, we used this criterion to identify the winner of each fight from all the blocks.

4. Body size assay

Dry body weight of the adults was measured as a proxy for body size after 49 generations of selection. For a given population, ~50 eggs were introduced into food vials containing ~6-8 mL of standard banana-jaggery medium. After 12 days from the day of egg collection, the adult flies were collected, sorted by sex, killed by flash freezing and stored at -80 °C till weighing. The flies were then dried at 60°C for 72 hours and (after thawing to room temperature) weighed to the nearest 0.1 mg in batches of 20 males or 20 females. Five batches of males and females were weighed for each of the four VB and VBC populations.

5. Female fecundity assay

Female fecundity of the selected (i.e., VB_{1-4}) and the control (i.e. VBC_{1-4}) populations were assayed after 53 generations of selection. Female fecundity was assayed both during early and late life. For early life fecundity, we used 15-day (post egg collection) old females, which is the same age at which the eggs are collected for the selection lines. Late life fecundity was measured on 33-day (post egg collection) old females. This is because, in *Drosophila*, it is known that during this time female fecundity reduces substantially due to aging but does not plateau out (Hanson & Ferris 1929). On the day of assay, flies were anaesthetized under mild CO_2 and one male and one female each were introduced into a 50 mL centrifuge tube containing a food cup. The tube had provision for aeration and the food in the food cup provided a surface for laying eggs. 40 such replicate setups were made for each VB_i and VBC_i (where $i \in$ 1-4) population. The setups were left undisturbed for 12 hours in a well-lit environment maintained at 25ºC and ambient humidity. At the end of 12 hours, the flies were discarded and the eggs laid on the food were counted under a stereo microscope. Since fecundity is largely determined by the body size of the females (Honěk 1993) which is in turn critically dependent on larval density (Prout & McChesney 1985), we maintained a constant egg density (~50 eggs per vial containing ~6-8 mL of standard banana-jaggery food), while collecting eggs for generating the flies for this assay.

6. Longevity assay

Longevity assay was performed after 51 generations of selection in a constantly lit environment maintained at 25ºC. We initiated the assay by introducing 10 freshly eclosed, unmated individuals of the same sex into a food vial containing ~6 mL standard banana-jaggery food. 10 such replicate setups were prepared for males and females separately from each of the VB/VBC populations. Thus in total, we measured the life-span of 1600 flies in this assay. The alive flies were counted daily at a particular time (arbitrarily set at 1500 h) and every alternate day, they were transferred into fresh food vials, till the last individual died. Flies that escaped or died during transfers were not included in the analysis.

7. Metabolomic study using NMR spectroscopy

Sample preparation: After 67 generations of selection, NMR spectroscopy was performed on one block of selected-control populations (VB_4-VBC_4). For each VB/VBC population, we used 11 replicates, each of which comprised of 30 males. Samples were prepared following established protocols (Gogna *et al.* 2015). The flies were first flash-frozen in liquid nitrogen, transferred to pre-labelled microfuges containing 0.5 mL of 50% acetonitrile solution, homogenized using a battery run homogenizer and centrifuged at 10000 rpm for 10 min at 4ºC. The supernatant was then transferred to another set of pre-labelled microfuge tubes, lyophilized and stored at -80ºC, to be used for NMR experiments. Prior to the NMR experiments, the samples were rehydrated in 500 ml of 50 mM phosphate buffer prepared using D_2O (pH 7.4), containing 1 mg/ ml of 3-(trimethylsilyl)-propionic acid-D4, sodium salt (TMSP) as a chemical shift reference and transferred to 5mm NMR tubes.

NMR spectroscopy: NMR spectra were recorded on a Bruker Biospin 600 Avance-III spectrometer operating at a ^{1}H frequency of 600.13 MHz at 300 K using a 5 mm QXI probe. Gradient shimming was performed prior to signal acquisition to optimize magnetic field homogeneity. 1D ^{1}H NMR spectra were acquired using the water suppressed Car–Purcell–Meiboom– Gill (CPMG) spin-echo pulse sequence optimized with a spin -echo delay *t* of 300 ms and n= 400 and a total spin–spin relaxation delay (2n*t*) time of 240 ms to achieve attenuation of fast-relaxing broad signals from larger molecules. The proton spectra were collected with a 90-degree pulse width of 9.15 ms, a relaxation delay of 2 s, 16 scans, 16 K data points and a spectral width of 7211.54 Hz. Data were zero-filled by a factor of 2 and the FIDs were multiplied by an exponential weighting function equivalent to a line broadening of 1 Hz prior to Fourier transformation. For resonance assignment and metabolite identification, two - dimensional NMR spectra were recorded, including ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H– ^{13}C heteronuclear and homonuclear single quantum coherence spectroscopy (HSQC, HMQC). 2D ^{1}H–^{13}C HMQC and HSQC spectra were obtained with a spectral width of 12 ppm and 200 ppm in the proton and carbon dimensions respectively, 1 K data points, 32 scans, 256 t1 increments and a recycle delay of 1.5 s. The COSY spectra were acquired with a spectral width of 12 ppm in both dimensions, 2 K data points, 32 scans and 128 t_1 increments. Metabolite fingerprinting for the *Drosophila* NMR spectra was done by checking identified metabolite peaks with standard NMR metabolite data deposited in databases such as MMCD (http://mmcd.nmrfam.wise.edu) and BMRB (http://www.bmrb.wise.edu). The NMR chemical shift assignments of several significant metabolites were further confirmed by recording the NMR spectra of pure compounds. For analysis of metabolites, single peak integrals for individual metabolites were

chosen with minimal overlaps with peaks from other compounds.

Data Analysis: Multivariate statistical analysis was performed using SIMCA14.0 software (Umetrics, Umea, Sweden). Prior to analysis, all the spectra were converted into the ASCII format and imported into MATLAB for alignment using the Icoshift algorithm (Savorani *et al.* 2010). Spectral regions between 4.6 and 4.8 ppm were excluded from the analysis, to prevent errors due to any residual peak from the suppressed water signal. Data were normalized to the total area to compensate for possible differences in signal-to-noise ratios between spectra and to prevent separation due to variations in the amounts of sample.

After importing the data into SIMCA, the data was Pareto-scaled and first analysed using the unsupervised pattern recognition method of principal component analysis (PCA), which helped to remove outliers, defined in the data as observations located outside the 95% confidence region of the Hotelling's T^2 ellipses in the PCA score plots. Such outliers were excluded from further analysis. PCA was followed by the supervised pattern recognition method of orthogonal projections to latent structure-discriminant analysis (OPLS-DA), which maximizes the class discrimination. The OPLS-DA scores and loadings plots were used to identify the metabolites responsible for separating VB and VBC flies. The quality of the model was described by R^2X and Q^2 values, explaining the variance explained (indicating goodness of fit) and variance predicted by the model (predictability) respectively. The significance test of the model was performed using CV- ANOVA (cross-validated ANOVA) in the SIMCA software, where a p -value of 0.01 was considered to be statistically significant to validate the OPLS-DA model. Permutation analysis was also performed on the best model using 1000 permutation tests with a threshold p-value of 0.05 indicating that none of the results were better than the original one. t-tests coupled with Bonferroni corrections (to limit the family-wise error rate to 0.05) were performed to check for statistical significance of the differences in the metabolite levels between the VB and VBC flies.

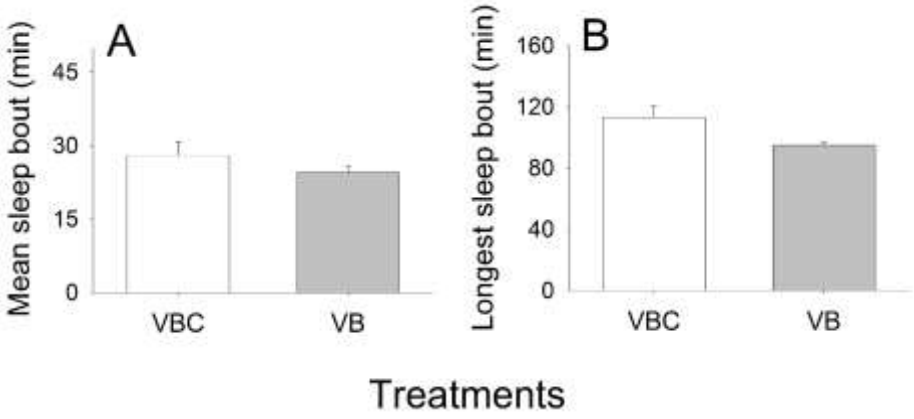

Figure S1. Average and maximum sleep bout over 24 hours post acclimatization. Over 24 hours in presence of food, (**A**) average sleep bout and (**B**) maximum sleep bout, are similar for both VBs and VBCs. The error bars represent standard errors around the mean (SEM) and * denotes $P<0.05$.

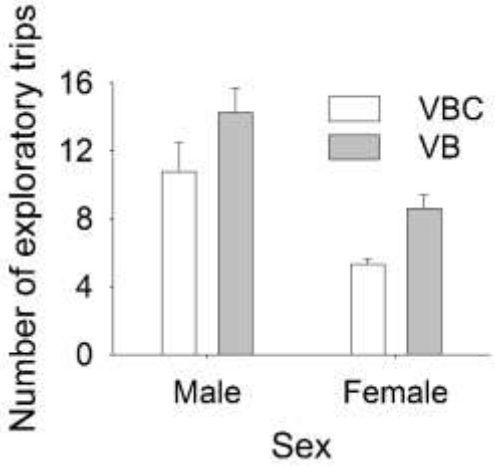

Figure S2. Exploratory behavior of males and females in VBs and VBCs. For both sexes, VBs performed more exploratory trips than VBCs. Error bars= SEM around the mean.

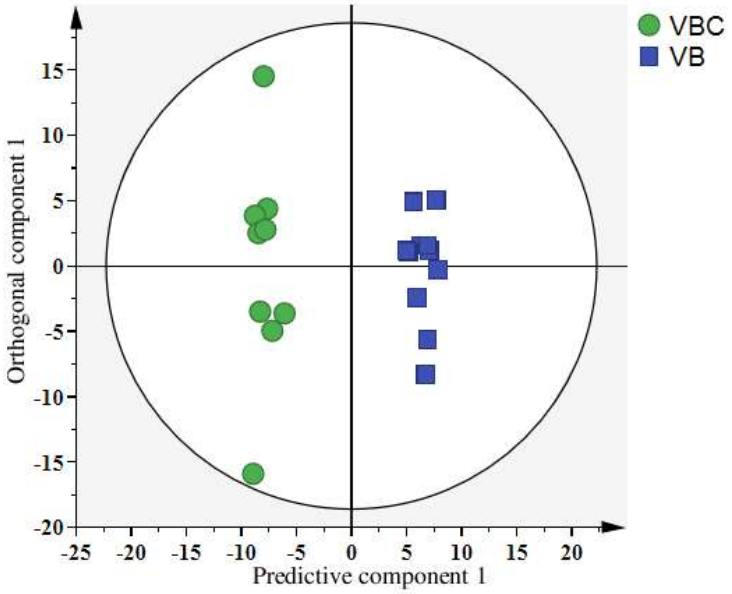

Figure S3. OPLS-DA score plot derived from 1D ^{1}H NMR spectra of VBs and VBCs.

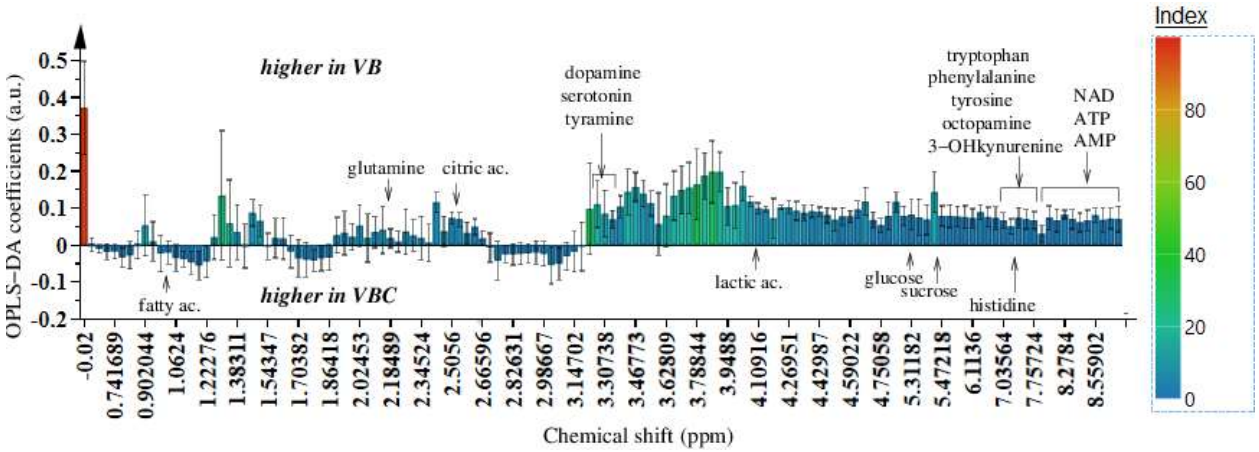

Figure S4. OPLS-DA loading plot obtained from the analysis of 1D ^{1}H NMR spectra of VBs and VBCs. Metabolites that are indicated above and below the baseline are present in higher quantity in VB and VBC flies respectively.

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