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Rugged fitness landscapes and by-product adaptation in experimental populations of *Drosophila melanogaster*

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ABSTRACT

Background: While the concept of the fitness landscape is central to evolutionary theory, empirical characterizations of fitness landscapes have remained difficult. Recently, a number of laboratory experiments using microbes have suggested that fitness landscapes are often rugged, though there is some variation across environments and species. However, there have been very few characterizations of fitness landscapes in sexual organisms, making it unclear whether the conclusions from studies of microbes are applicable to other groups.

Questions: Are fitness landscapes smooth or rugged in simplified laboratory environments for sexual organisms? How does landscape topography influence patterns of adaptation?

Methods: We conducted a series of experiments using replicate populations of *Drosophila melanogaster* adapted to either cadmium- or ethanol-enriched food to characterize the fitness and phenotypes of these populations in a simplified laboratory environment (ethanol-enriched media).

Results: We found that replicate populations adapted to different laboratory environments have diverged phenotypically in physiology, mating behaviour, and offspring production in alternate environments. However, both ethanol- and cadmium-adapted populations show high fitness in the ethanol-enriched environment relative to their founding population, and cadmium-adapted males actually outcompete ethanol-adapted males for mates in an ethanol environment.

Conclusions: Our data indicate that the simplified ethanol-enriched medium represents a rugged fitness landscape, and that alternately adapted populations occupy different fitness peaks on this landscape. Because cadmium-adapted populations were never exposed to ethanol previously, it appears that these populations adapted to ethanol as a by-product of adaptation to their cadmium-enriched environment. Therefore, even in simplified laboratory environments, we find evidence for rugged fitness landscapes, and the overlap of fitness peaks on the phenotypic landscape allowed for by-product adaptation.

Keywords: adaptation, ecological divergence, ethanol, mate competition.

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INTRODUCTION

The adaptive landscape (Wright, 1931, 1932; Simpson, 1944) has been a highly influential concept in evolutionary biology, providing a graphical metaphor for the roles of natural selection and genetic drift in adaptation and diversification (Schluter *et al.*, 1994; Gavrilets, 2004; Colegrave and Buckling, 2005; Svensson and Calsbeek, 2012). Adaptive and fitness landscapes feature two 'horizontal' axes representing aspects of either a population's or individual's genotype or phenotype, and a vertical axis that denotes the fitness of each genotype/phenotype. The resulting surface is envisioned to contain peaks of high fitness and valleys of low fitness, analogous to the contours of a physical landscape, with natural selection corresponding to a local hill climbing process at the population level. The number and location of 'adaptive peaks' can influence patterns of evolution and diversification (Arnold, 2003). A smooth adaptive landscape has only one adaptive peak and adaptation is therefore predictable because, given sufficient time and underlying variation, populations will converge on this peak no matter where they start. A rugged landscape, on the other hand, has two or more adaptive peaks and different populations may climb different peaks depending on their starting position, the order in which mutations arise, and the vagaries of genetic drift. Rugged landscapes also make it possible for populations to get 'stuck' on a sub-optimal (i.e. local) adaptive peak if a fitness valley lies between it and the global fitness optimum. This is because populations tend to climb the nearest rather than the highest peak. Therefore, the number, shape, and location of peaks relative to a population's position on the landscape will affect how predictable adaptation is, as well as the likelihood that it will reach the highest peak within an environment (Whitlock *et al.*, 1995; Colegrave and Buckling, 2005; Svensson and Calsbeek, 2012).

Despite their heuristic importance in evolutionary biology, adaptive landscapes are hard to measure, as doing so requires data on the fitness of a wide range of genotypes/phenotypes, many of which are unlikely to exist in nature. Insight is therefore often limited to estimating the shape of individual fitness surfaces in the immediate vicinity of the population mean genotype/phenotype, and we thus know very little about the broader-scale shape of adaptive landscapes. For example, while it is clear that multiple peaks can and often do exist in many environments, the extent of this ruggedness is unknown, leaving open simple questions such as whether populations adapting to the same environment will tend to follow the same evolutionary pathway.

Much of our understanding of adaptive landscapes, and of adaptive processes in general, comes from laboratory evolution experiments (Garland and Rose, 2009; Cooper, 2012; Kassen, 2014). Laboratory settings are greatly simplified compared with natural environments (Huey and Rosenzweig, 2009), as they generally minimize interspecific interactions and resource heterogeneity, among other things. It is easy to imagine that such simplified environments will reduce the ruggedness of the adaptive landscape and, therefore, may bias our insight into this issue. However, rugged landscapes can arise in the simplest one- or two-locus genetic models (Whitlock *et al.*, 1995; Colegrave and Buckling, 2005), meaning the ruggedness may persist even in simplified environments. Indeed, empirical attempts to characterize adaptive landscapes (mostly using microbes) suggest ruggedness is common even in highly simplified laboratory environments (Travisano *et al.*, 1995; Burch and Chao, 2000; Colegrave and Buckling, 2005; Lachapelle *et al.*, 2015; Nahum *et al.*, 2015; Lachapelle and Colegrave, 2017; Simões *et al.*, 2017), although indications of smooth landscapes have been inferred in some systems (Fragata *et al.*, 2014) and heterogeneity has been found among studies in very similar environments (Teotónio and Rose, 2000; Melnyk and Kassen, 2011).

An additional understudied topic concerns landscape overlap, and in particular the extent to which genotypic and/or phenotypic adaptive peaks are shared among environments. This is related to the extent to which adaptation promotes specialization in which an increase in fitness in one environment comes at a cost to fitness in other environments. Empirical studies have tended to focus on the response to abiotic stressors, with several laboratory experiments demonstrating that adaptation to one stress often increases resistance to others (Hoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005; Telonis-Scott *et al.*, 2006). Such results suggest that adaptation need not entail strict specialization such that adaptive peaks can, to some extent, be shared among environments, and that populations can increase fitness in one environment as a by-product of adaptation to another. Whether such by-product adaptation is reciprocal has rarely been addressed, although Bubliy and Loeschcke (2005) demonstrated that while adaptation to desiccation stress in several *Drosophila* populations increased their resistance to cold, heat, and starvation stress as a by-product, the reverse was not necessarily true. This lack of adaptive reciprocity suggests that there are multiple adaptive phenotypes, and therefore multiple fitness peaks indicative of a rugged adaptive landscape, with some increasing fitness to a range of stresses (i.e. fitness peaks are shared) while others are specific to the stress in question (i.e. the peak is unique).

In a previous experiment using the fruit fly *Drosophila melanogaster* (Arbuthnott *et al.*, 2014), we took advantage of ten replicate populations that have been adapting to two separate novel environments, ethanol- or cadmium-enriched larval food, originally created by Aneil Agrawal (University of Toronto) and later moved to the Rundle lab (University of Ottawa). To test for adaptation to these environments, we performed a reciprocal transplant experiment that measured the number of adult offspring produced by replicate male–female pairs from each population (ethanol, cadmium, and the common ancestral stock population from which they were all derived) on ethanol and cadmium food (for details, see Arbuthnott *et al.*, 2014). Each of the 20 experimental populations had higher fitness than the ancestral stock in the environment in which it had evolved, indicating adaptation. However, while adaptation to ethanol resulted in decreased fitness in cadmium compared with the ancestral stock population, adaptation to cadmium actually increased fitness in ethanol to the extent that the cadmium-evolved populations performed as well in ethanol as the ethanol-evolved populations (Fig. 1). Our results were therefore similar to previously observed increases in resistance to novel stresses in fruit fly populations adapting to a separate stressor (Hoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005; Telonis-Scott *et al.*, 2006). The different patterns in the performance of the cadmium and ethanol populations across the two environments (Fig. 1) indicate they are phenotypically divergent and thus suggest that cadmium populations may have climbed a separate adaptive peak in ethanol that was inaccessible to the ancestor. Here we present a series of additional experiments that strengthen the inference of a rugged fitness landscape in ethanol and suggest partial overlap between fitness peaks in these two environments.

MATERIALS AND METHODS

Experimental populations

A laboratory stock population was founded in A. Agrawal's lab (University of Toronto) in September 2005 from a large collection of flies from the Similkameen Valley, British Columbia (Yeaman *et al.*, 2010). In 2007, 20 independent populations were established from this

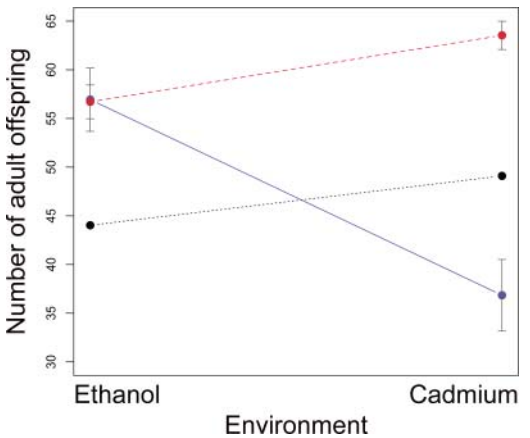

Fig. 1. Average number of adult offspring produced by single male–female pairs from ten ethanol-evolved populations (blue dots, solid line), ten cadmium-evolved populations (red dots, dashed line), and the single ancestral stock population (black dots, dotted line) as measured in a two-generation reciprocal transplant assay involving ethanol- and cadmium-supplemented food. Points represent the mean (± among-population standard error) of the ten replicate populations within each environment.

stock, with ten of these maintained on food supplemented with 12% ethanol and the other ten on food containing 70 $\mu g \cdot mL^{-1}$ cadmium chloride. The single ancestral stock population continued to be maintained on its standard food. All populations were maintained in cages with overlapping generations on a 12/12 hour light/dark cycle at 25°C and 50% relative humidity. Each population cage received fresh food every 14 days for the cadmium populations and the stock, and every 21 days for the ethanol populations. In April 2011, these populations were transferred to the Rundle lab (University of Ottawa) and maintained following the same protocol. These populations were previously used in a study of the ecology of sexual conflict, which included the reciprocal transplant experiment described in the Introduction (Arbuthnott *et al.*, 2014). Conducted in July 2011 (i.e. after almost four years in their novel environments), results of this assay revealed that adaptation to cadmium also conferred increased fitness in ethanol to the extent that the performance of cadmium-adapted populations equalled that of the ethanol-evolved populations in ethanol (Fig. 1). Here we focus on further characterizing the fitness landscape in the ethanol environment.

Experiment 1: Male mating success in ethanol

In June 2012, we measured male mating success of the ethanol and cadmium populations in ethanol, as this has been shown to be an important fitness component for *D. melanogaster* populations during adaptation to ethanol in particular (Bokor and Pecsenye, 2000). Adult virgin males were collected using light CO_2 anaesthesia (as in all subsequent collections) from all 20 experimental populations when raised in ethanol-enriched media and held in vials containing ethanol food in groups of seven. At the same time, ancestral stock females were raised in regular food, collected as virgins, and stored in vials in groups of ten on ancestral

food. The experimental populations were randomly matched into ten unique pairs for the mating trials such that ethanol males from one population always competed against cadmium males from another population for mating with a limited number of stock females. The evening prior to the start of the mating trials, all virgin males were marked either blue or red using coloured yeast paste in a balanced design such that, for each population pair, the cadmium-adapted males were blue in half of the mating trials and red in the other half. Such marking has been used extensively in *D. melanogaster* (Rundle *et al.*, 1998, 2007; Mooers *et al.*, 1999) with no detectable effect on male mating behaviour or female mate choice. Consistent with this, mating rates did not differ significantly by colour in the current experiment (51% of mating males were blue; one sample *t*-test, $t_{123} = 0.695$, $P = 0.49$).

Flies were 2–4 days post-eclosion when used in the mating trials. For each trial, we first introduced 20 ethanol- and 20 cadmium-adapted males into a $14 \times 14 \times 14$ cm Plexiglas cage. Each cage contained a 9-cm diameter petri dish of ethanol food to ensure there was evaporated ethanol in the mating environment, as there would be in the population cages of ethanol-evolved populations. Twenty virgin stock females were then introduced and the cage was covered with white paper for 10 minutes and left undisturbed. Following this interval, we collected the first ten mating pairs in the cage and identified the mating males by colour. For each ethanol–cadmium population pair, we performed 12–14 replicate trials. To compare the mating success of the two types of males, we subtracted the number of mating cadmium-adapted males from the number of mating ethanol-adapted males for each cage. We would expect consistently positive values if ethanol-adapted males outperform cadmium-adapted males in ethanol. Significance was determined using a one-sample *t*-test on the average difference score, treating population pairs as replicates.

In addition to the above assay, we exposed a previously ethanol-evolved population to cadmium for several generations and then measured male mating success. In 2012, a single ethanol population was used to found two 'daughter' populations, one of which was maintained as it had previously been in the ethanol environment, while the other was maintained in the cadmium environment. After 20 weeks of exposure to these environments, males from two populations were raised for one generation in ethanol, collected as virgins, and then used to compete in a mating trial for a limited number of virgin stock females as described above. Nine replicate mating trials (i.e. cages) were performed using these populations.

Experiment 2: Ethanol tolerance

Here we sought to determine whether the experimental populations had diverged in physiological tolerance of ethanol. To do this we carried out a knock-down assay (Weber and Diggins, 1990) that measured the time to losing consciousness in the presence of ethanol vapour. For this assay, we selected the four ethanol and four cadmium populations with the highest fitness in their respective environments [the same populations used in Arbuthnott *et al.* (2014)], as well as the ancestral stock population. The assay involved adding six males or six females to a standard (50-mL) empty *Drosophila* glass vial that was sealed with a permeable foam plug and then placed within a 300-mL glass bottle. The bottle had 50 mL of 99% ethanol in the bottom and after the addition of the vial was subsequently sealed. The vial was observed and we recorded the time it took each individual fly to lose consciousness. We calculated the average time to unconsciousness of the six individuals within a vial. Sixteen replicate vials of each sex were performed for each population. Variation in the knock-down

time was analysed using a general linear mixed model with selection environment, sex, and their interaction as fixed effects, and population (nested within selection environment) and population × sex interactions as random effects. The model was fit via ML and significance was tested based on partial (i.e. Type 3) likelihood ratio tests (LRT) for terms of interest. Because the ancestral population was unreplicated, it could not be included in statistical analyses but was used for qualitative comparisons.

Experiment 3: Hybrid fitness

Lastly, we measured the fitness, in the ethanol environment, of within- and between-environment 'hybrids'. If ethanol- and cadmium-evolved populations occupy separate fitness peaks in ethanol, it is possible that hybrids between populations adapted to different environments, if they possess intermediate phenotypes, may fall in a fitness valley between these adaptive peaks. For each of the eight populations used in Experiment 2 (i.e. four ethanol- and four cadmium-evolved), virgin males and females were collected from low-density (<50 eggs per vial) ethanol-food vials and then held separately by sex on ethanol food for 2 days (10/7 females/males per vial). We then created four replicate groups of five males and five females for all 64 possible combinations of male and female population and placed them in vials containing ethanol food. These flies were allowed to mate and lay eggs for one day before the adults were removed.

From these vials we collected virgin offspring of both sexes, ensuring that within each male × female population combination the males and females came from different vials to eliminate any possibility of full- or half-sibling pairings in the next stage. The offspring were held separately by sex for two days, as above. We then paired individual males and females from the same population combination in vials containing ethanol medium, allowing pairs to mate and lay eggs for two days before being removed. Twenty replicate male–female pairs were created for each of the 64 population combinations, split evenly between two blocks separated by a single day. We counted the number of emerging adult offspring in each vial, counting every second day from days 11 to 17 post egg laying, and used this as our measure of fitness. This measure encompasses the fertility and fecundity of inter-population hybrids, as well as the egg-to-adult survival of their offspring in the ethanol environment. To control for 'hybrid' status (i.e. within- vs. between-population crosses), we excluded the eight combinations representing pairings of males with their own females. As we are concerned with fitness effects at the level of environment, we ignored specific population-level effects and analysed offspring production with a generalized linear model with paternal and maternal environment (i.e. the environment in which that parent had evolved), their interaction, and block as fixed effects. Offspring production data showed an overabundance of pairs that produced no offspring. As a result, we found evidence of overdispersion for both Poisson and negative binomial distributions. We therefore analysed these data using a zero-inflated negative binomial distribution. We used the full model above to test for both the effects on the inflation of zeroes in the data as well as the non-zero offspring counts within our data. The significance of each effect on the non-zero count data was evaluated with partial (i.e. Type 3) likelihood ratio tests of the full model vs. a reduced model without the effect under consideration.

RESULTS

Male mating success

While there was variation among population pairs in relative mating success (Fig. 2), despite being raised and competing in the ethanol environment, the cadmium-adapted males outcompeted ethanol-adapted males for mating with stock females in the majority of population pairs. This resulted in a significantly higher average mating success in ethanol for cadmium- over ethanol-evolved males ($t_9 = 2.65$, $P = 0.027$). Furthermore, when we exposed a previously ethanol-evolved population to cadmium for 20 weeks, males from this newly cadmium-evolved population outcompeted their 'ancestral' ethanol-evolved males for access to a limited number of stock females in the presence of ethanol ($t_8 = 2.75$, $P = 0.025$); on average, 64% (±5%) of matings in these mate competition cages were with cadmium-adapted males.

Ethanol tolerance

Flies from ethanol-evolved populations took longer to lose consciousness in the presence of ethanol vapour than did those from the cadmium-evolved populations (LRT: $\chi^2_1 = 5.75$, $P = 0.016$; Fig. 3) and this effect was consistent across the sexes (sex × treatment interaction: $\chi^2_1 = 0.33$, $P = 0.57$). Males and females also did not differ in their average knock-down times (sex effect: $\chi^2_1 < 0.01$, $P = 0.96$). Ethanol tolerance of the cadmium-evolved populations was similar to that of the ancestral stock, providing little indication that cadmium-evolved populations exhibited any increase in their tolerance to ethanol vapour.

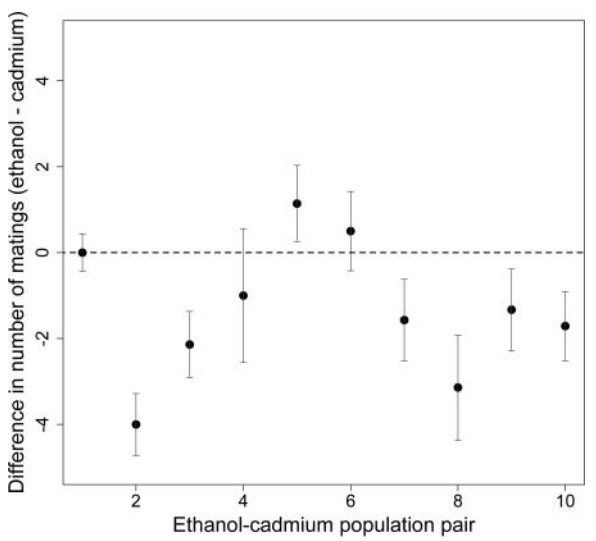

Fig. 2. Competitive mating success of males when reared and tested in ethanol-supplemented food. Ethanol- and cadmium-evolved populations were competed directly in pairs (arbitrarily numbered 1–10). Points represent the mean (± standard error) from 12–14 replicate trials per population pair of the difference in the number of females mated by the two types of males (ethanol-evolved males minus the cadmium-adapted males). Positive values denote that ethanol-evolved males outcompeted cadmium-evolved males, while negative values indicate the opposite.

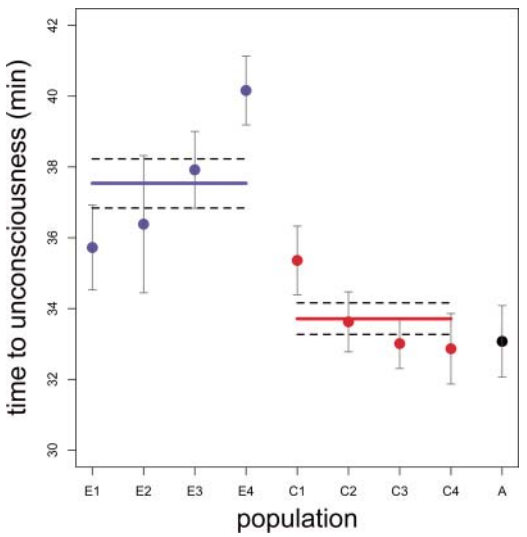

Fig. 3. Results of a knock-down assay measuring physiological resistance to ethanol for individuals from four ethanol-evolved populations (E1–E4, blue dots), four cadmium-evolved populations (C1–C4, red dots), and the ancestral stock population (A). Points represent the mean (± standard error) time to unconsciousness in the presence of ethanol vapour for 16 replicate vials containing six individuals each. Solid blue and red horizontal lines represent means among populations adapted to ethanol and cadmium, respectively, with dashed lines denoting standard errors treating populations as replicates.

Hybrid fitness

Maternal and paternal environment had opposite effects on hybrid fitness. Specifically, hybrid pairs produced more offspring if they had a cadmium-adapted mother and/or if they had an ethanol-adapted father (Fig. 4). We used a zero-inflated negative binomial distribution in our test of the effects of parental environmental adaptation on the fitness of hybrid offspring. First, from the count data, the mother's selection environment significantly impacted the hybrid pair's fitness (LRT: $\chi^2_2 = 6.05$, $P = 0.048$), as did the father's selection environment ($\chi^2_2 = 15.94$, $P = 0.0003$), though their interaction did not ($\chi^2_2 = 2.78$, $P = 0.25$). Block also had a significant effect ($\chi^2_2 = 9.54$, $P = 0.008$), though the effects of the mother's and father's environmental adaptation remained between blocks. Second, with respect to the overabundance of zeroes, there was a non-significant trend for a greater probability for a hybrid pair's offspring production to be zero if their mother was adapted to ethanol ($z_2 = -1.76$, $P = 0.08$). Neither the adaptive history of the father ($z_2 = -0.47$, $P = 0.64$) nor the interaction between the mother's and father's evolutionary history ($z_2 = 1.55$, $P = 0.12$) impacted the overabundance of zeroes in offspring production.

DISCUSSION

In a long-term evolution experiment in *D. melanogaster* involving adaptation to two novel environments consisting of ethanol- or cadmium-supplemented food, we previously presented evidence suggestive of a rugged adaptive landscape in ethanol (Arbuthnott *et al.*, 2014). In

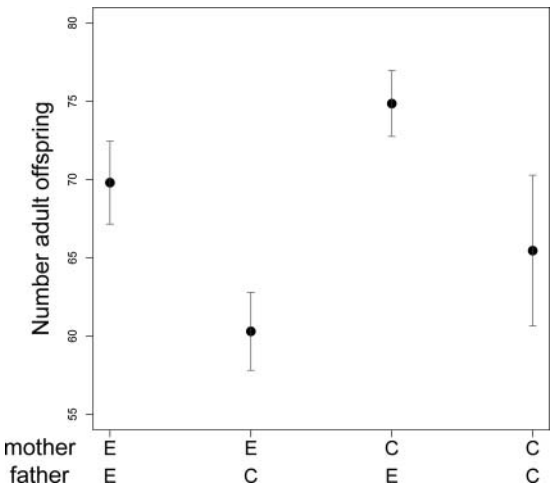

Fig. 4. The number of adult offspring produced for inter-population 'hybrids' when raised in an ethanol environment. Crosses are distinguished by the environment in which the mother and the father had previously evolved (E = ethanol, C = cadmium). Points represent the mean (± standard error) number of offspring from each male × female line combination among eight lines (20 replicates per line combination).

particular, a reciprocal transplant experiment that measured the number of adult offspring produced by male–female pairs demonstrated that both ethanol- and cadmium-evolved populations showed increased performance in the environment in which they had evolved compared with the ancestral stock. However, patterns of performance for ethanol- and cadmium-evolved populations differed across the two environments (Fig. 1), indicating phenotypic divergence between them, and the cadmium-evolved populations performed as well in ethanol as the ethanol-evolved populations, suggesting a possible second adaptive peak in ethanol that was shared with cadmium. Our current study was designed to further explore this possibility. We provide additional evidence for genetic/phenotypic divergence between populations evolved in these two environments by their differing performance in a competitive mating assay (Fig. 2), their differing physiological resistance to the effects of ethanol in a knock-down assay (Fig. 3), and the variable performance of F1 hybrids between them (Fig. 4). More importantly, male mating success was a major fitness component missing in our previous assay, and is one that may be of particular importance in ethanol (Bokor and Pecsenye, 2000). The fact that cadmium-evolved males outcompeted ethanol-evolved males for mating with stock females in our current study further suggests that the cadmium-evolved populations occupy a separate adaptive peak in ethanol that is of similar or possibly higher fitness and that is inaccessible via evolution of the ancestor in ethanol itself. Consistent with this also was the increase in mating success in ethanol of males from an ethanol-evolved population after short-term evolution in cadmium.

The inference of a rugged adaptive landscape would be further strengthened by the direct demonstration in ethanol of a fitness valley between cadmium- and ethanol-evolved phenotypes. The poor performance of the ancestral stock population in ethanol shows that there are areas of low fitness within the broader genotypic/phenotypic space relevant to this set of populations, but it does not necessitate separate adaptive peaks. Empirically demonstrating

fitness valleys is difficult, as adaptive 'edges' (or 'ridges' in higher-dimensional space) may exist such that populations can move between apparent peaks without ever traversing a valley (Whitlock *et al.*, 1995; Gavrilets, 2004). Nevertheless, to provide some insight into the landscape between the cadmium- and ethanol-evolved populations, we generated F1 hybrids between them, assuming that these would be intermediate in phenotype. While F1 offspring from the mating of ethanol-adapted females to cadmium-adapted males did show decreased fitness in ethanol, offspring of the reciprocal cross had a similar fitness in ethanol relative to the ethanol- and cadmium-evolved populations (Fig. 4).

This asymmetry in hybrids' fitness, although not predicted, does shed some light on the genetic mechanisms underlying adaptation to ethanol and cadmium environments, and potentially about the adaptive landscape in ethanol. Such asymmetric inviability of hybrids has been termed Darwin's corollary to Haldane's rule (Turelli and Moyle, 2007), and indicates that genetic factors of large effect on the sex chromosomes or mitochondria control incompatibilities between isolated populations or species (Muller, 1942; Presgraves, 2010). Adaptation to ethanol- and/or cadmium-supplemented food has therefore apparently involved one or more loci of large effect on the sex chromosomes or mitochondria. In agreement with our results, factors increasing cadmium resistance have been attributed to the X chromosome in *D. melanogaster* (Shirley *et al.*, 1999; Nguyen *et al.*, 2014), although no such factors on the X chromosome have been identified for ethanol tolerance.

Incompatibilities between populations, including those observed here, are indicative of a rugged adaptive landscape in that they produce additional areas of low fitness (Orr, 2006), demonstrating further ruggedness among even genetically and phenotypically quite similar populations in a highly simplified laboratory environment. When we adapted a previously ethanol-evolved population to cadmium for 20 weeks, mating success of males from this population was found to have significantly increased when tested in ethanol, showing that in the ethanol-evolved populations there was genetic variation for increased mating success in ethanol. Although this result was unreplicated, it further suggests the presence of a fitness valley between the adaptive peaks in ethanol to explain this lack of a response in ethanol given the apparent presence of standing genetic variance.

In addition to suggesting a rugged adaptive landscape in ethanol, our data also imply overlap in adaptive peaks between the cadmium and ethanol environments, whereby populations occupying a fitness peak in cadmium also occupy a fitness peak in ethanol as a by-product. Adaptation to cadmium in *Drosophila* is often associated with an increased production of metallothionein, a protein used in the sequestration and accumulation of cadmium in the midgut (Shirley *et al.*, 1999). Populations adapted to cadmium also tend to show higher rates of cadmium secretion than do non-adapted populations (Nguyen *et al.*, 2014). Sequestration and secretion are ineffective solutions to high ethanol exposure, as ethanol is difficult to concentrate in the body given its efficiency in penetrating cell membranes (Fry, 2014). Instead, adaptation to ethanol in fruit flies is often associated with mechanisms used to detoxify it, such as through increased activity of alcohol dehydrogenase (ADH) (Geer *et al.*, 1991; Mercot *et al.*, 1994). Wild populations regularly exposed to high concentrations of ethanol accumulate less ethanol than non-adapted populations (Fry, 2014). Given these divergent approaches to addressing environmental toxins, it is not surprising that our experimental populations show divergent responses to ethanol and cadmium (low survival and productivity in the presence of cadmium for ethanol-adapted lines, low ethanol vapour tolerance in cadmium-adapted lines). However, it is surprising that cadmium adaptation, likely entailing sequestration and excretion of toxins, provides an efficient strategy for surviving non-lethal

concentrations of ethanol. Given that previous studies have inferred very disparate routes by which *D. melanogaster* processes these different stressors, we would not predict by-product adaptation. Adaptation to one stress has been shown to increase resistance to other stresses (by-product adaptation) in previous experiments (Hoffmann and Parsons, 1993; Hoffmann and Harshman, 1999; Bubliy and Loeschcke, 2005; Telonis-Scott *et al.*, 2006), but not to stressors that show such disparate strategies for toxin resistance. Our data therefore suggest adaptation to a non-exposed environment via a shared route that has not been previously identified, in that cadmium-adapted sequestration and excretion of toxins is an efficient way to survive increased ethanol exposure. Additionally, such incidental toxin resistance has important implications for the adaptive landscape that have remained unexplored in previous demonstrations of by-product adaptation.

Our results are in agreement with several studies in microbes and fruit flies suggesting not only that adaptive landscapes can be rugged, but also that there can be significant overlap in the adaptive peaks of separate landscapes, facilitating by-product adaptation such as we observed. Rugged landscapes, with separate adaptive peaks, appear to be common even in simplified laboratory environments in studies of microbes (Travisano *et al.*, 1995; Burch and Chao, 2000; Colegrave and Buckling, 2005; Lachapelle *et al.*, 2015; Nahum *et al.*, 2015; Lachapelle and Colegrave, 2017). In addition, even though their results were not always framed in this context, studies of multicellular organisms such as fruit flies have suggested that adaptive landscapes may be rugged, leading to idiosyncratic changes among populations adapting to the similar environments (Teotónio and Rose, 2000; Bubliy and Loeschcke, 2005; Vijendravarma and Kawecki, 2015; Simões *et al.*, 2017). For example, how 'reversible' evolution is when populations are moved to an ancestral environment depends on the environment to which populations adapted (Teotónio and Rose, 2000), suggesting that populations may have traversed an adaptive valley, which inhibits a return to ancestral phenotypes. Furthermore, as with our results, several studies have reported that populations may adapt to non-selected stresses in several populations (Hoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005; Telonis-Scott *et al.*, 2006), and that such by-product adaptation is not always reciprocal (Bubily and Loeschcke, 2005). Taken together, these data indicate that there are several separate adaptive phenotypes for stress resistance, some of which are stress-specific, while others act to increase resistance to several stresses. These separate lines of evidence from microbes and fruit flies are in agreement with our findings of rugged adaptive landscapes, and the potential importance of by-product adaptation in such rugged landscapes.

Our inference about the ruggedness of the adaptive landscape in ethanol relies on the demonstration of both ethanol- and cadmium-adapted populations showing high fitness in ethanol-supplemented food. While our assays were designed to capture the key components of fitness for a *Drosophila* laboratory population (i.e. female fecundity and larval survival to emergence in the reciprocal transplant; competitive male mating success in the mating assay), we have not measured others (e.g. adult longevity, male sperm competitive ability). In addition, our assays involve conditions that deviate somewhat from the environment in which the ethanol populations evolved. For example, our previous reciprocal transplant (Arbuthnott *et al.*, 2014) and the hybrid fitness assay in this study measured offspring production of single male–female pairs over 24 hours in a vial, while the ethanol populations have been evolving in cages in which individuals were given the opportunity to reproduce several times throughout their lives and larvae competed for resources at higher density in bottles. Similarly, in our mating trials a set of similar-age virgin males were given a short period to compete for a limited number of similar-age virgin females, a scenario that differs from the

mating environment males likely encounter in higher density cages with overlapping generations and in which virgin females are likely extremely rare. Measuring fitness in an evolutionarily relevant scenario is challenging even with laboratory populations (Rice *et al.*, 2005), and this is especially so for those maintained in population cages with overlapping generations and unregulated densities. It is therefore possible that our inferences would change if we measured fitness in a way that reflects the selective history of these populations.

A second potential issue is that the 'ancestral' population was unreplicated and had an evolutionary history that was independent of the experimental lines. *Drosophila* cannot be frozen or otherwise kept in stasis like some laboratory organisms (e.g. plant seeds, bacteria, and fungi), and it is therefore possible that the stock population evolved decreased fitness in ethanol over the course of the evolution experiment, whether by drift or as a by-product of selection in the environment they experienced. However, our inference of ruggedness would only change if the apparent adaptation to ethanol by both ethanol- and cadmium-evolved populations (e.g. Fig. 1) was entirely an artefact of the decreased fitness of the stock in this environment (i.e. neither the ethanol- nor cadmium-evolved populations adapted to ethanol). Such a scenario is unlikely given the increased physiological tolerance to ethanol exhibited by the ethanol-evolved populations. While splitting a stock population into multiple independent replicates at the start of an evolution experiment would greatly reduce the likelihood of drift-induced changes in the control confounding the interpretation of the results, it would not address changes driven by selection in the control environment. Such changes are more likely when the control populations experience a novel environment, whether intentional (e.g. a change in maintenance routine) or not. In our case, there was no intentional change in the way the stock was maintained such that it experienced the same conditions during the evolution experiment as it had since being collected approximately two years earlier.

With the above caveat in mind, our results are suggestive of a rugged adaptive landscape within a highly simplified laboratory environment. While additional studies are not possible in our case because the populations no longer exist, our data demonstrate that useful insight can be gained into the question of landscape ruggedness and overlap via laboratory studies, and are consistent with a growing body of work suggesting that adaptive landscapes in the laboratory can be multi-peaked (Colegrave and Buckling, 2005; Kassen, 2014). Our data also suggest that there is an adaptive peak that to some extent is shared between ethanol and cadmium. Both are toxins and, despite them being qualitatively different, show that populations can reach adaptive peaks as a by-product of adapting to a separate environment with overlap in the location of adaptive peaks. Such adaptive peak overlap is surprising in this case, and would not have been predicted given what is known about the physiological bases of resistance to each of these toxins. Rugged adaptive landscapes and overlap of adaptive peaks across separate environments demonstrate that adaptation does not necessarily entail specialization for all genotypes/phenotypes or environments. Our results suggest that even simplified laboratory environments may possess rugged adaptive landscapes, and that these landscapes and adaptive walks on them can be studied in the laboratory even for multicellular organisms like *Drosophila*. We hope that such enticing results may spur further laboratory studies of adaptive landscapes in multicellular organisms.

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