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RESEARCH ARTICLE

Experimental evolution of response to anoxia in *Drosophila melanogaster*: recovery of locomotion following CO_2 or N_2 exposure

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ABSTRACT

Many insects enter coma upon exposure to anoxia, a feature routinely exploited by experimentalists to handle them. But the genetic and physiological bases of anoxic coma induction and recovery are only partially understood, as are the long-term consequences for the animal's performance. We examined three populations of *Drosophila melanogaster* (designated B) that have been inadvertently under selection for rapid recovery from CO_2 exposure for nearly 40 years (around 1000 generations) resulting from routine maintenance practices. We contrasted CO_2 and N_2 (presumed a less reactive gas) knockdown and recovery times of these B flies with six populations of common ancestry (A and C populations) that were not exposed to CO_2 over the same period. We found that B populations showed faster and more consistent locomotor recovery than A or C populations after CO_2 knockdown, a result also observed with N_2 knockdown. A and C populations showed much higher variance in recovery time after CO_2 exposure than after N_2 exposure, suggesting gas-specific effects on pathways associated with locomotor recovery. Although these selection treatments result in considerable variation in life history attributes and body size, with the characteristic intermediacy of B populations, their superiority in resistance to gas exposure and locomotor recovery suggests that this is a direct consequence of prior repeated exposure to anoxia, broadly, and CO_2, specifically. Hence we describe a powerful new evolutionary model for the genetic and physiological investigation of anoxic coma in insects.

KEY WORDS: Anoxic coma, Laboratory selection, Carbon dioxide, Adaptation, Locomotor behaviour, Locomotor recovery, Time to immobilization

INTRODUCTION

Adult insects have the remarkable ability to survive extended periods of anoxia. Instrumental in this capacity is their ability to enter a reversible coma when oxygen concentrations fall (e.g. below 2% in *Drosophila*) (Dawson-Scully et al., 2010). This shut-down in neuromuscular activity has been described as a protective adaptation in locusts, which enter coma in response to thermal or anoxic stress (Rodgers et al., 2010). Whereas extreme temperatures may frequently be encountered by adult insects, anoxic conditions seem like a less plausible agent of selection. Hence little is known about whether this tolerance represents an evolved adaptation in the adult stage or is a cross-tolerance resulting from exposure to other stressors. However, the existence of naturally occurring strains (Dawson-Scully et al., 2010) and mutant allelic variation (Xiao and Robertson, 2016) has contributed to the potential power of *Drosophila* as a model system for delineating the genetic and molecular bases for tolerance of anoxic conditions; knowledge that could be important to research on animals that suffer pathological consequences from even short-term anoxia, such as mammals. Moreover, anoxic coma – most commonly induced using carbon dioxide – is exploited routinely as a convenient means of immobilization for sorting in fly laboratories worldwide, with a limited understanding of its physiological consequences. Here, we present further evidence for naturally occurring variation from a replicated selection experiment in *Drosophila melanogaster* that has been running for more than 37 years (over 1000 generations).

Although CO_2 is commonly used to knock out adult flies for sorting by sex or other specific phenotypes, its impact on the animal's physiology and subsequent performance remain poorly understood. A few reports indicate that flies that have been exposed to CO_2 knockdown display abnormal behavior and reduced reproductive or physiological performance. Perron et al. (1972) found that exposing newly emerged adult flies (within 3 h) to CO_2 caused increased mortality and reduced fecundity. Barron (2000) found that CO_2 exposure increased copulation latency, even when flies were given 20 h recovery from knockdown. CO_2 anaesthesia impairs subsequent climbing and flight activities (Bartholomew et al., 2015). Additionally, CO_2 exposure blocks synaptic transmission at the neuromuscular junctions, causing rapid immobilization and increased incidence of cardiac arrest in *Drosophila* larvae (Badre et al., 2005). In contrast, another study found no impact of CO_2 exposure on subsequent survival and fecundity of adult flies (Partridge et al., 1986), and it is tacitly assumed that there is no long-lasting effect if sufficient recovery time (i.e. 24 h+) is permitted (Colinet and Renault, 2012).

As part of an experiment aimed at understanding ageing, and life-history evolution in general, five large outbreeding populations have been incidentally subjected to CO_2 exposure each generation since 1980 (Burke et al., 2016; Rose, 1984), while 10 other populations descended from the same ancestor but maintained in population cages have not. In the selection treatment designated 'B', groups (vials) of 40–50 females lay eggs for a short time immediately post-recovery from CO_2 knockdown. We reasoned that natural selection would favour individual females with rapid recovery and subsequent fecundity. Rapid and more complete recovery from exposure to CO_2 by B females, compared with the populations lacking an evolutionary history of exposure (A and C populations) would be evidence for an adaptive response to exposure – either the general impact of anoxic coma or the specific effects of CO_2.

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We aimed to address differences in coma response between populations from these different selection treatments using behavioural analysis. In particular, using a high-throughput system, we examined individual locomotor performance before, during and after exposure to anoxia in populations from the A, B and C selection treatments. Our results, from measuring over 1800 flies, show that B populations (those with a selection history of CO_2 exposure) have faster and more consistent recovery from CO_2 knockdown than A or C populations. B populations also have fast and consistent locomotor recovery from nitrogen (N_2) knockdown, suggesting generalized adaptation to anoxia exposure as well as specific resistance to CO_2.

MATERIALS AND METHODS

Drosophila melanogaster experimental evolution system

The laboratory phylogeny of $ACO_{1–5}$, $B_{1–5}$ and $CO_{1–5}$ populations (here referred to as A, B and C) of *Drosophila melanogaster* Meigen 1830 has been previously described (Rose et al., 2004). These flies are all derived from the Ives base population (Rose, 1984) and have been subjected to selection for age-specific reproduction using discrete generations of 9, 14 and 28 days for A, B and C flies, respectively. Census population sizes of at least 1000 individuals per generation have been sustained to minimize genetic drift and inbreeding depression. Owing to generation time differences and different dates of foundation, by January 2018, the A treatment had been applied for approximately 1075 generations, while B had been applied for around 1000 generations and C for 380 generations.

All flies were raised in vials (25×95 mm) at a density of 80–100 flies per vial on banana food (containing banana, inactivated yeast, sweeteners and agar) in an incubator at 25°C. A 12 h:12 h light:dark photoperiod with the lights on at 09:00 h and off at 21:00 h has been applied since establishment in the Chippindale lab in 2002 (B populations) and 2008 (A and C populations). Since 1980, B populations have been subjected to CO_2 exposure once per generation at day 14, at which time flies from different vials were mixed and placed in new vials. Flies were discarded when 80–100 eggs have been laid, typically less than an hour after knockdown. This treatment is expected to put females under selection for rapid egg production after recovery, and presumably allows little opportunity for mating. Thus, the selection pressure on male recovery time is presumed to be minimal. A and C populations, however, were kept in cages as adults and have not been exposed to CO_2 since the initial population branching in 1980.

The fact that B populations have experienced CO_2 exposure through their laboratory evolutionary history but also uniquely have a 14-day life cycle introduces a potential confound to using either of the other two treatments as matched controls. To counter this deficiency in the design, we bracketed the B selection treatment with the faster (A) and slower (C) demographic treatments to help rule out a correlation between either life history (development time, age at reproduction, etc.) or fly size and the characters of interest; A-selected flies are known to be faster developing and smaller than B-selected flies, while C-selected flies are slower developing and larger (Burke et al., 2010).

Fly collection

Three replicate populations of each type were arbitrarily selected ($A_{1,3,5}$, $B_{1,3,5}$ and $C_{1,3,5}$) and tested in this study. Note that whereas A_n and C_n are paired replicates, B_n is no more related than any other B replicate phylogenetically. However, the same-numbered replicates of all three selection treatments were handled together in our experiments, potentially creating parallel handling effects. Before the experimental generation, at least one generation of rearing at moderate density on a 2-week cycle was undertaken to synchronize rearing and reduce potential non-genetic age effects. Newly emerged flies were collected within 2 days and held in food vials at a density of 30 same-sexed flies per vial. We mouth-aspirated individuals to avoid the use of CO_2 or N_2 during collection. Flies were transferred into fresh food vials once every 3–4 days until testing. Experiments were performed during the light period between 12:00 and 17:00 h and tested flies from the different populations contrasted were all the same age within a trial; depending upon the specific trial, they were 3–7 days old.

Locomotor assay

The locomotor assay was performed as previously described (Xiao and Robertson, 2015). Briefly, flies were loaded individually into circular arenas (1.27 cm diameter, 0.3 cm depth) and their walking activities were recorded with a digital camera (C905, Logitech) for analysis. The experimental apparatus, which was designed specifically for flies to receive controlled CO_2 or N_2 exposure, allowed simultaneous assay of up to 128 individuals. After being loaded via mouth aspiration, flies were allocated 5 min to become accustomed to the arenas and an additional 5 min for the observation of activities prior to gas exposure. A 30 s CO_2 or N_2 exposure at 10 l min^{-1} was then applied. All the flies were knocked down during the exposure. A base flow of room air at 2 l min^{-1} was provided throughout the experiment except for the duration of CO_2 or N_2 exposure. Following restoration of air, recovery activities during the next 60 min were recorded.

Fly positions were tracked once every 0.2 s. A parameter, time to locomotor recovery from knockdown, was evaluated by following the protocol of Xiao and Robertson (2016). Other locomotor analysis (e.g. the calculation of path length per minute) and data visualization were conducted with R and several packages including gdata and tidyverse (https://www.r-project.org/).

Estimation of time to immobilization upon gas exposure

Upon exposure, the time to immobilization was defined as the beginning of a 10-s interval in which a fly displayed estimated path length below a fixed threshold. The threshold (2.68 mm) was determined based on two considerations: (1) allowing a diagonal relocation of one pixel per second (equivalent to 0.268 mm s^{-1}) to offset the potential wobbling effect of camera; and (2) pooling the path length from 10 consecutive seconds to avoid potential underestimation owing to transient activities (e.g. convulsion-like jumping).

Statistics

Levene's test was used to assess the equality of variance of data from different groups. Welch's *t*-test was performed for the analysis of recovery time between two groups of data. Tukey's HSD test was performed for the analysis of overall recovery time among selection treatments. Mixed-effects ANOVA was applied, with population number treated as a random factor to address common ancestry of same-numbered A- and C-selected populations and common handling of same-numbered replicates in the assays. Spearman rank correlation was applied for the analysis of correlation between body size and time to recovery from knockdown. Statistics were performed using R and the related packages gdata, tidyverse and ggridges. $P<0.05$ was considered statistically significant.

RESULTS

Recovery from anoxia: overall findings

In order to examine patterns of overall response to anoxia, we fitted a three-way mixed-effects model (replicate number, which reflects

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some potential ancestry and common handling effects, was included as a random factor). Both gases immobilized flies quickly (see below), but recovery from N_2 was approximately 25% faster than recovery from CO_2 across all populations employed (means of 624 and 827 s, respectively; $P<0.05$, Welch's *t*-test) (Fig. 1A,B; Fig. S1). There was a strong effect of selection as the three B populations (exposed to CO_2 throughout laboratory history) recovered 40% more rapidly than either A- or C-selected flies (means of 824, 487 and 869 s for A, B and C, respectively, $P<0.05$, Tukey's HSD; Fig. 1C), which did not differ in overall recovery time. However, there was a strong selection×gas interaction, which appears to reflect a rank-order reversal between A- and C-selection

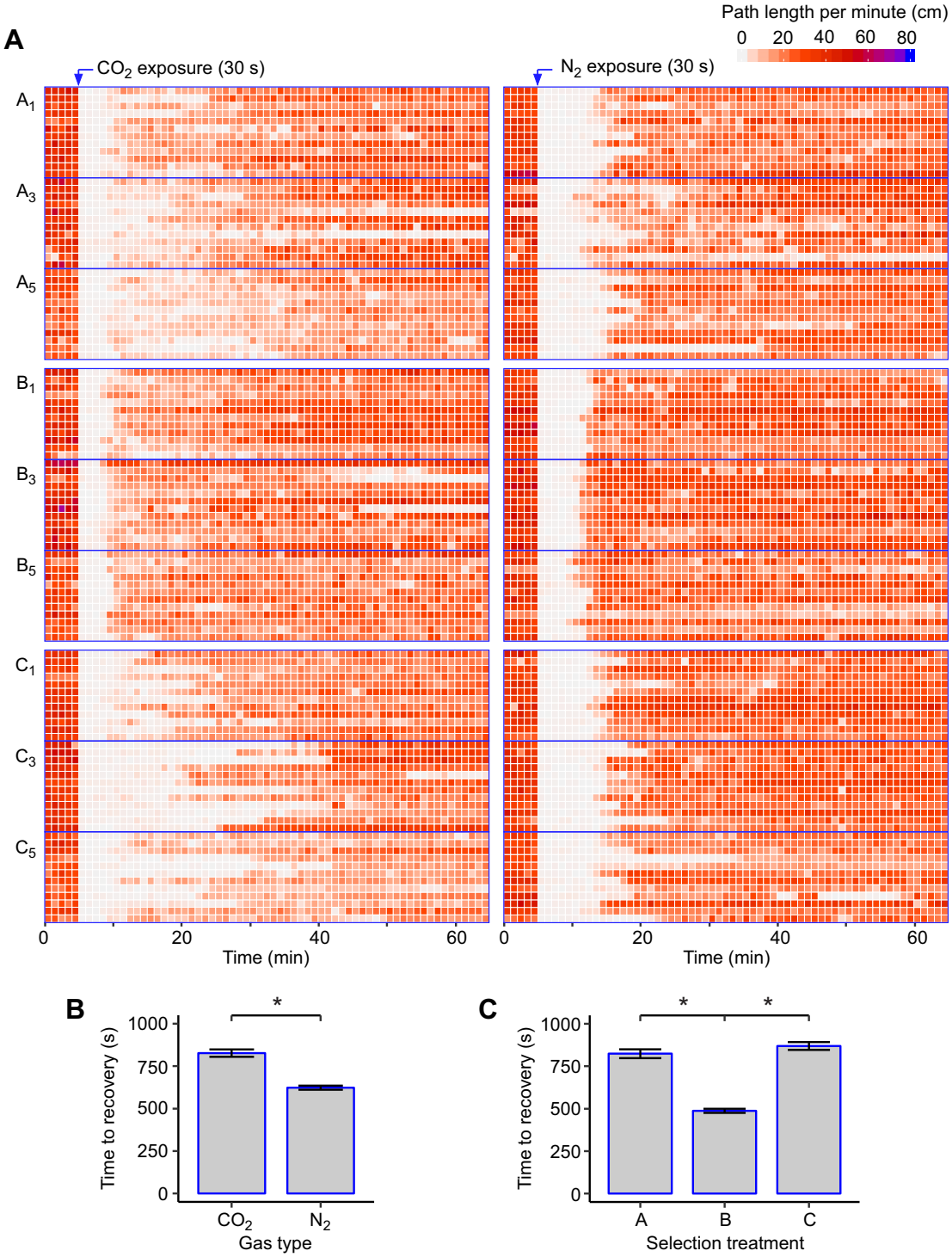

Fig. 1. Locomotor activities of A-, B- and C-selected flies subject to CO_2 or N_2 exposure. (A) Path lengths per minute (cm) of female adult flies in circular chambers were calculated and are shown as a heatmap. Each unit of coloured squares represents the path length travelled within a minute. Each line of squares represents the activities of a single fly. Time durations for gas exposure (CO_2 or N_2) and overall test are indicated. A gradient colour key is provided. (B) Time to recovery of locomotion upon different gas exposure. Asterisk (*) denotes $P<0.05$ by Welch's *t*-test. (C) Time to recovery of locomotion upon selection treatments. Asterisk (*) denotes $P<0.05$ by Tukey's HSD test.

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treatments across the two gas treatments, as well as a nearly significant three-way interaction. These interactions motivated us to describe results for the two gases separately to gain insight into the responses (see below). The selection×sex interaction appears to reflect the following reversal – A<C in males and A>C in females – although *post hoc* tests revealed that no A or C differences were significant statistically; all B versus A or C differences were significant (P<0.05, Tukey's HSD). The sexes did not differ in recovery time, although below we note some suggestive sex-specific differences with respect to the female selection hypothesis.

B flies showed fast recovery time from CO_2 and N_2 knockdown

B flies recovered rapidly from CO_2 exposure, with the first animals waking up at around 4 min (Fig. 1), whereas A or C flies recovered more slowly and variably, taking 76% and 96% longer, respectively

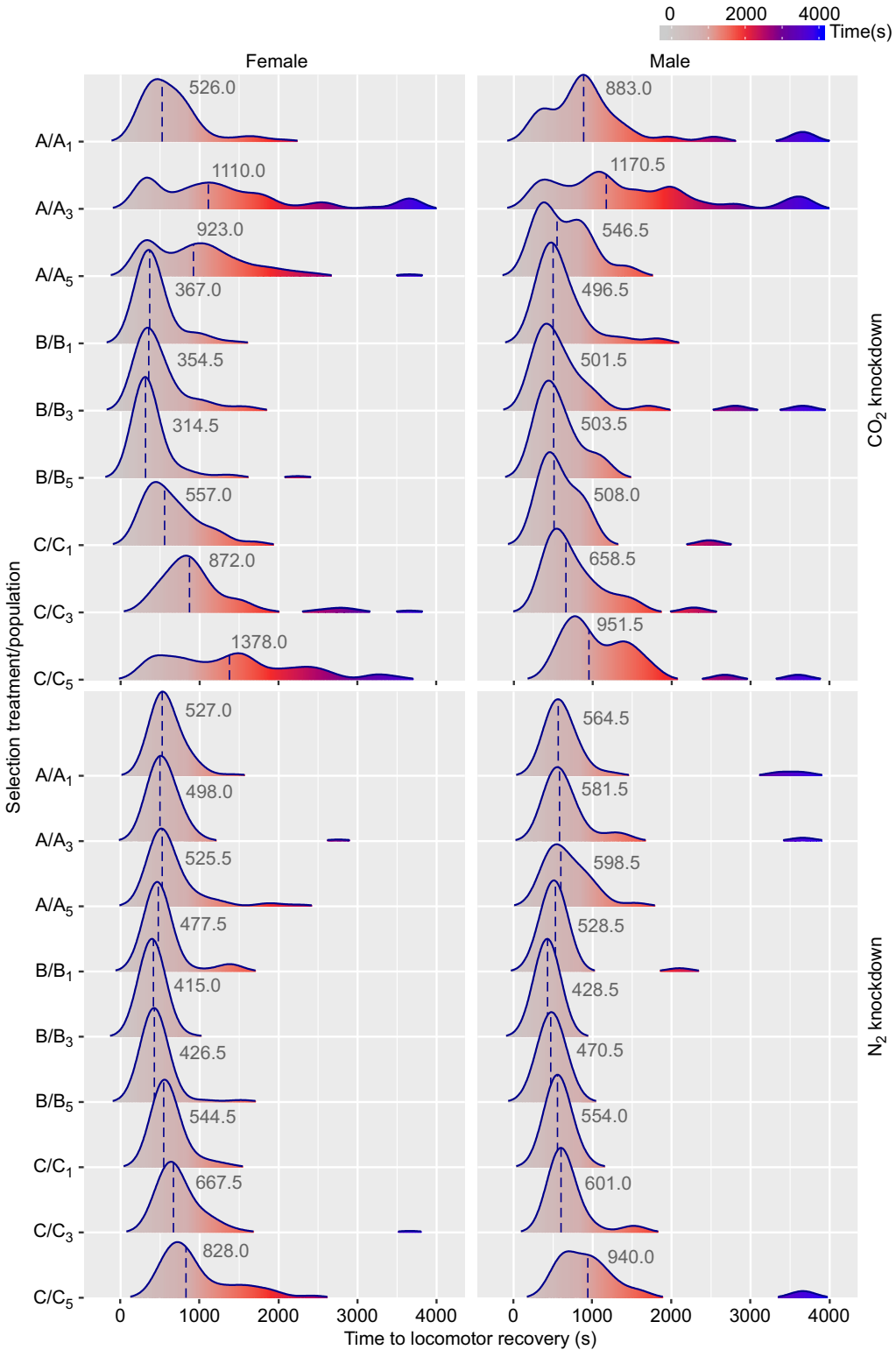

Fig. 2. Density plots of time to locomotor recovery in A, B and C flies. The times to locomotor recovery from nine populations are plotted as density curves. Gradient colours are used for the visualization of variance of data. Medians (blue dashed lines) of time to locomotor recovery are shown. A colour key is provided. The R package ggridges was used for analysis.

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(B<A and B<C, P<0.05, Tukey's HSD). Density plots of time to recovery from CO_2 knockdown revealed a considerable degree of variation in all populations (Fig. 2). A and C females showed similar recovery curves, both of which had either multiple peaks or long and lumpy tails. B females had, however, a greatly increased fast peak and a negligible tail of late waking individuals. B females had shorter recovery time (medians 367.0, 354.5 and 314.5 s for B_1, B_3 and B_5, respectively) than A females (526.0, 1110.0 and 923.0 s) or C females (557.0, 872.0 and 1378.0 s) (Fig. 2; also see Table S1). B females recovered noticeably (30%) faster than B males did, although there was no significant effect of sex detected with the *post hoc* test. These data suggested that selection for CO_2 recovery in B populations has operated, at least partially, through the elimination of genetic or physiological variants causing prolonged recovery time.

With N_2 knockdown, the overall variance of recovery time was reduced compared with CO_2 knockdown (Fig. 2). As with CO_2, B-selected populations showed relatively fast recovery. B females showed shorter recovery time (medians 477.5, 415.0 and 426.5 s) than A females (527.0, 498.0 and 525.5 s) or C females (544.5, 667.5 and 828.0 s) (Fig. 2; also see Table S1). Overall, male flies did not significantly differ from females in recovery performance. Interestingly, in contrast to the greater difference with CO_2, in the B-selected populations males were just 7% slower to recover than females were.

Thus, unlike the typical intermediacy of B flies in the body size or other observed facets of life history (Burke et al., 2010), the recovery times of B flies were not intermediate between A and C flies. These data also indicated a carry-over of rapid recovery from CO_2 exposure to N_2 exposure because B flies had never been exposed to N_2 throughout their laboratory evolution. And the data hint at some sex specificity for CO_2 adaptation, as B females are particularly fast to regain locomotor function after knockdown.

Variation within selection treatments

Consistency of phenotypes across populations under a common selection regime is evidence for their parallel or convergent evolution. With CO_2 knockdown, A_1 females recovered faster than A_3 or A_5 females (Fig. 3). C_1 females recovered faster than C_3 or C_5 females. No population differences were observed in B females. Similarly, male A and C flies showed population differences in recovery time, whereas B males had statistically the same recovery times. Therefore, B flies evolved high consistency in the recovery time from CO_2 knockdown. With N_2 knockdown, B and C flies (both females and males) showed population differences in recovery time, whereas A flies (both females and males) had the same recovery times among populations (Fig. 3).

Correlation between fly size and recovery time

B flies have been noted to have dry masses intermediate to A and C flies (Burke et al., 2010). Here, we estimated the fly size (defined as the length of the major body axis) and analyzed the correlation between fly size and recovery time from knockdown.

Fly sizes of B females (medians 3.20, 3.10 and 3.09 mm) were indeed in a range between those of A females (2.87, 2.83 and 2.88 mm) and C females (3.37, 3.39 and 3.37 mm) (Fig. 4). Fly sizes of B males (2.75, 2.78 and 2.71 mm) were also intermediate between those of A males (2.56, 2.57 and 2.62 mm) and C males (2.85, 2.88 and 2.80 mm). Typically for this species, males were found to be approximately 12% smaller than females, but the model also showed a curious interaction between sex and selection. This interaction appears to reflect differences in sexual size dimorphism, with the A males being 9% smaller than the females, the B males 12% and the C males 15%. *Post hoc* (Tukey's HSD) tests revealed significant differences between all selection treatments and sex combinations except that A females were not different in size from C males. Thus we have a broad and continuous range of sizes, with fly sizes in the order of A<B<C within each sex, the same order as developmental time and age at reproduction. This order was different from that of recovery time (i.e. B flies recovered faster than A or C flies).

We further examined the relationship between recovery time and fly size in each population. With CO_2 knockdown, there was no significant correlation between recovery time and fly size in any

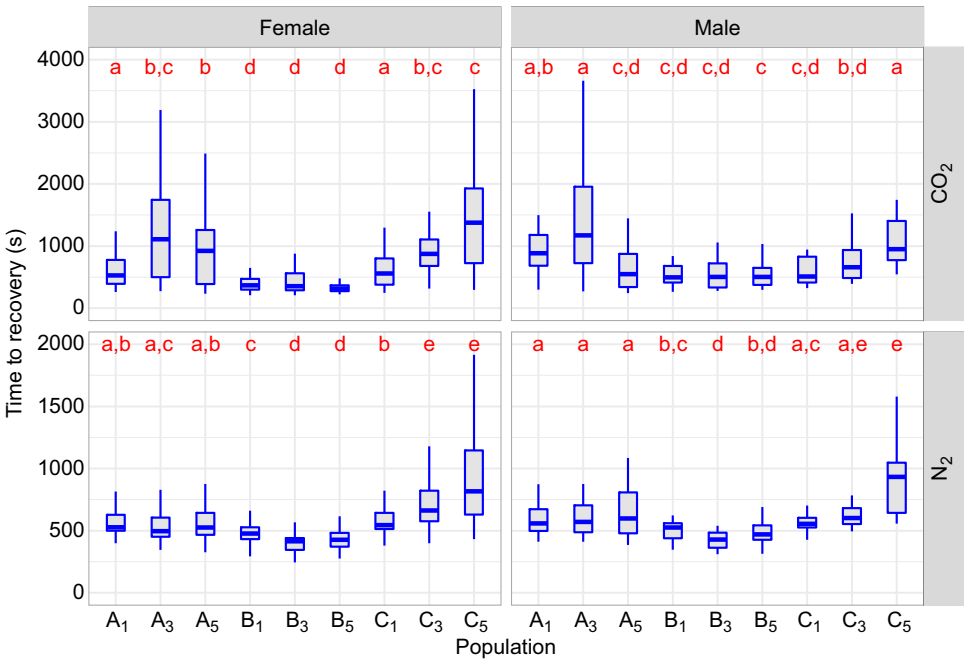

Fig. 3. Time to locomotor recovery in nine populations. Observations are plotted by sex and gas type. Different red letters denote statistical significance (Tukey's HSD; P<0.05).

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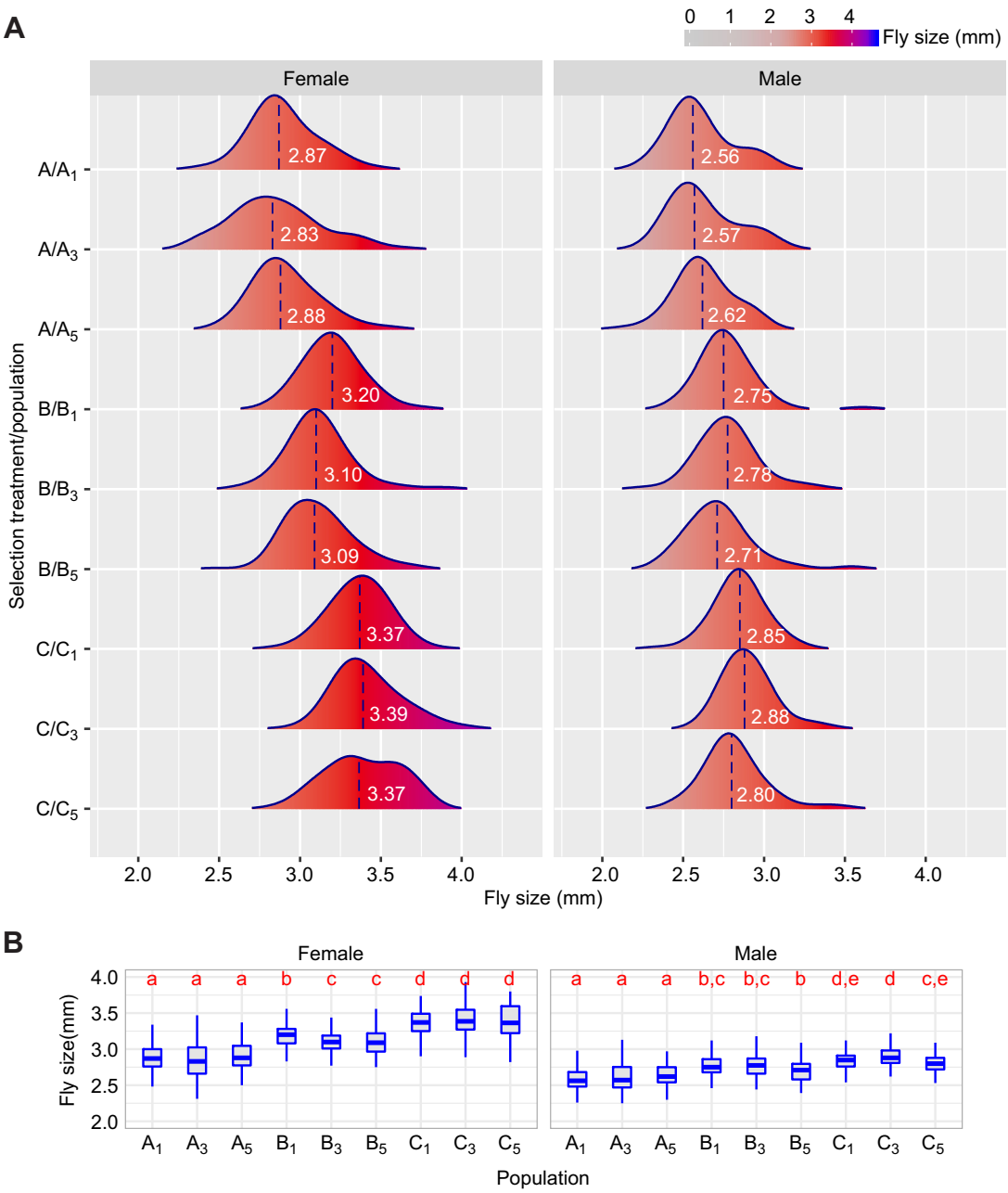

Fig. 4. Fly sizes of nine populations. (A) Density plots of fly sizes. Medians are indicated as dashed lines and values. (B) Tukey's HSD analysis of fly sizes in nine populations. Different letters denote statistical significance (P<0.05).

population of A, B or C in either sex (Table S1). With N_2 knockdown, there was no significant relationship between these two traits in most populations except for B_5 females (r=0.327, P=0.014). Taken together, we concluded that there was no significant correlation between recovery time and fly size.

Gas-specific locomotor immobilization in A, B and C flies

CO_2 or N_2 exposure generated an anoxic environment and led to behavioural immobilization. Because CO_2 exposure is known to cause acidification of the haemolymph (Badre et al., 2005), unlike the more inert gas N_2, there was a possibility that gas-specific physiological processes in addition to anoxia contributed to the knockdown. We explored the time course for locomotor cessation as flies entered the immobile state.

Both male and female flies became immobile in ∼10 s with CO_2 exposure (Fig. 5, Fig. S2). After slowing and stopping, many flies displayed violent, convulsion-like behaviour before immobilization. However, with N_2 exposure, flies maintained or even slightly increased activities for the first 6–7 s of exposure before slowing and stopping for several seconds, followed by convulsion and immobilization. The differences in the initial response and time course of behaviours suggest that CO_2 exposure caused unique physiological effects beyond simple oxygen deprivation.

An ANOVA of the same design used for recovery time was applied. Time to immobilization under N_2 was significantly greater than with CO_2 (∼15 s; *post hoc* Student's *t*-test, P<0.05) for both males and females, which did not differ from one another (Fig. 6A). The ANOVA indicated significant interactions between selection

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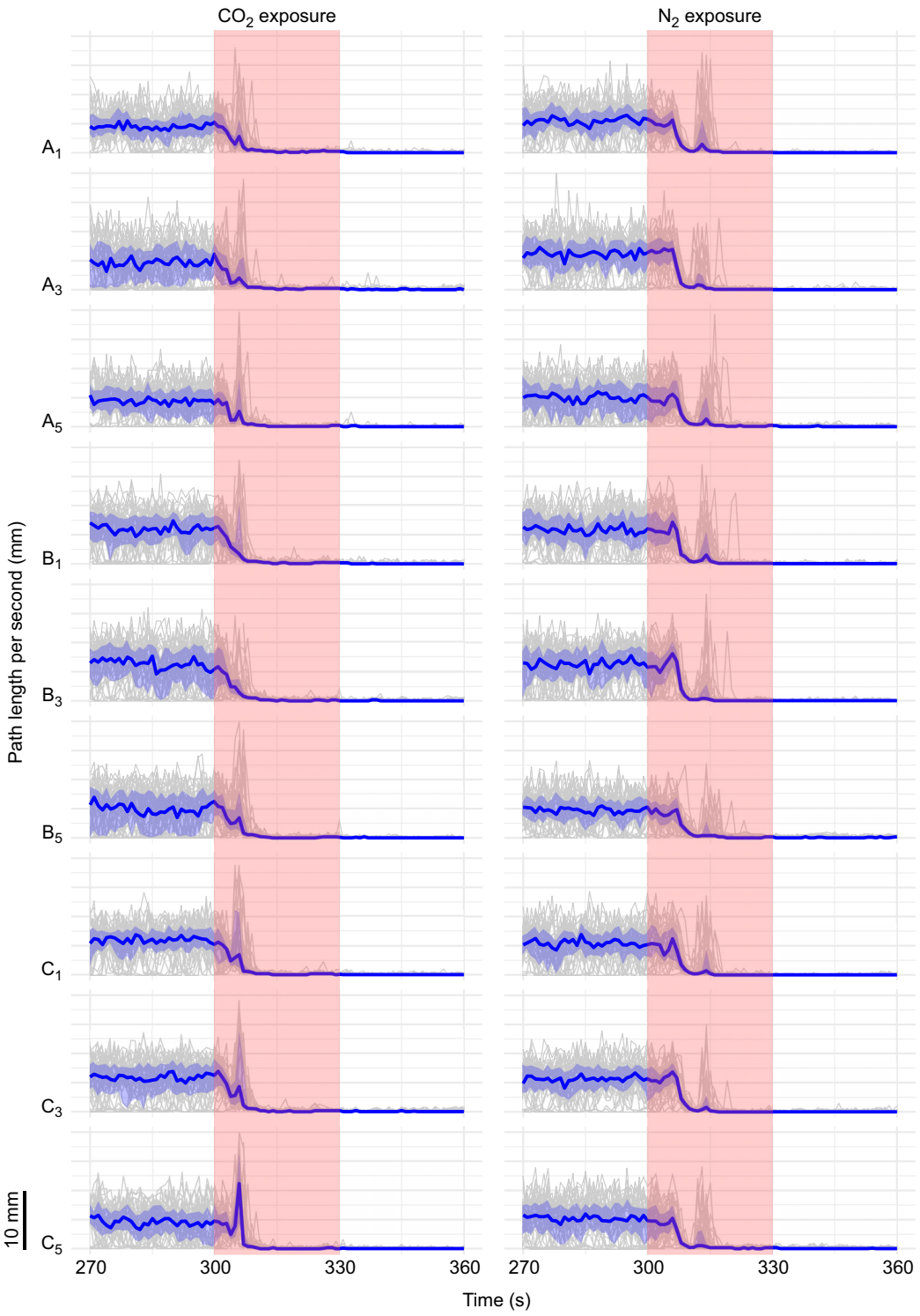

Fig. 5. Time to immobilization upon gas exposure in female flies. Shown are the path lengths per second versus the time during which the 30 s CO_2 or N_2 exposure (red-shaded) is applied. Blue lines denote connected medians for each second, whereas grey lines are activities of individual flies. A scale bar (10 mm) is provided.

and both sex (P=0.004) and gas type (P=0.003). Tukey's HSD testing revealed that A-selected populations succumbed to N_2 more rapidly than B- or C-selected populations did (P<0.05), whereas B and C did not differ. The interactions appear to be driven by earlier knockdown of A-selected flies: for females by N_2 and for males by both gases (Tukey's HSD, P<0.05) compared with B and C flies, which did not differ, and the particularly early immobilization of A-selected males specifically under N_2 knockdown (Fig. 6B).

DISCUSSION

Our results show that flies with a long-term history of CO_2 exposure (the B populations) recover more rapidly and more consistently from

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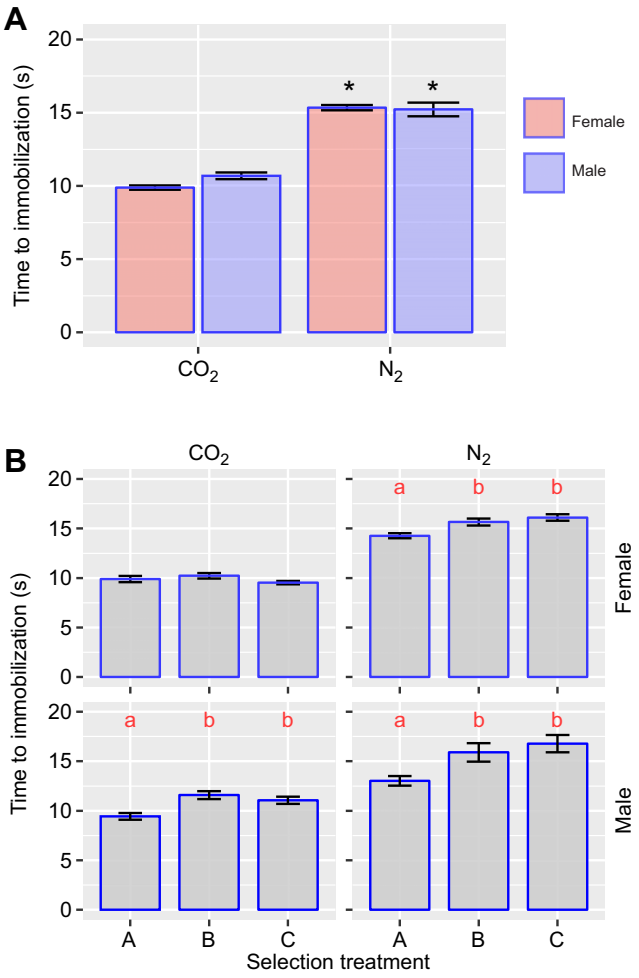

Fig. 6. Time to immobilization upon gas exposure in A, B and C flies. (A) Time to immobilization of flies exposed to CO_2 and N_2. Fly sexes are indicated. Asterisk (*) indicates $P<0.05$ between gases by Student's *t*-test. (B) Time to immobilization of flies with different selection treatments. The difference in red letters indicates $P<0.05$.

a gas-induced coma than flies that have not historically experienced this treatment (A and C populations). We posited that this selection would be specific to B females based upon the deduction that there was no direct selection on males. Indeed, because mating takes 15–20 min and interferes with oviposition, there is likely quite strong selection against re-mating after coma recovery for B females. Anecdotal observations (by A.K.C.) of vials during this period of time suggest that mating is rare or wholly absent. B-selected females were indeed fastest to recover, especially following CO_2 knockdown. Although suggestive, the females were not statistically different from the males in this selection treatment, and the still quick recovery by B males suggests genetic correlation across the sexes. In addition, the inconsistent contribution of sex to recovery time also helps address a potential confounding factor: body size. *Drosophila melanogaster* displays sexual size dimorphism whereby females are roughly 12% larger in body mass than males from the same population at eclosion (Testa et al., 2013; data herein).

Selection on life history has resulted in several divergent phenotypes, including differences in development time, dry mass, starvation resistance and longevity (Burke et al., 2010). Most strikingly, the A-selected populations have extremely rapid development and reduced body mass in comparison with the other populations. C-selected flies, with late reproduction and relatively relaxed selection for rapid development, are somewhat larger and slower growing than are B flies. The intermediacy of B populations for a wide range of characters, including body size, is further evidence that their rapid locomotor recovery and reduced sensitivity to gas exposure are not a spurious correlated effect of selection on life history or size: a ranking of B<A<C was observed for mean recovery time, whereas the ordering A<B<C was seen for mean fly size. Furthermore, there was no consistent correlation between recovery time and fly size of individuals within each of the populations or between the sexes, as noted above. Time to immobilization, however, was most rapid for the small, rapid developing A-selected flies, and particularly the males. It is therefore possible that body size affects knockdown time, but we cannot rule out other possible reasons for sensitivity to anoxia.

B populations may have evolved CO_2-specific recovery mechanisms, or they may have responded to anoxia exposure more generally. Because CO_2 is likely to have more reactive properties than some other potential agents of anoxia, such as causing acidification of the haemolymph (Badre et al., 2005), we exposed flies to an equivalent period of anoxia generated by 100% N_2 at the same flow rate. B flies, once again, returned to locomotion earlier and more consistently than A or C flies. These results indicate that B flies have developed fast and consistent locomotor recovery not only from CO_2 exposure but also from exposure to anoxia more generally. Anoxia induces neural responses such as an extracellular potassium surge in the brain (Armstrong et al., 2011). Adult flies are more sensitive to the initial insult than to the subsequent repetitive anoxia with a 4-min interval (Armstrong et al., 2011). Apparently, flies develop adaptive neural responses rapidly as a form of plasticity. Not only the recovery time but also the time to immobilization are associated with the B selection treatment. It is therefore possible that the selection response of B flies to anoxic conditions represents a form of genetic assimilation of a plastic trait (*sensu* Waddington, 1953).

Although B populations were relatively consistent in returning to locomotion after CO_2 exposure, incomplete return and delayed recovery were observed in many flies in A or C populations; this was rare after N_2 exposure. These data suggest different physiological responses to CO_2 and N_2 exposure, with CO_2 being more disruptive to recovery in flies not historically exposed to that gas. Evolution in the B selection treatment appears to have eliminated genotypes that have this delayed return to normal locomotion, as reflected in the sharply reduced variance and skewness in recovery times.

We also examined the time course to locomotor cessation upon gas exposure. Nitrogen exposure was associated with an approximately 50% longer time to immobilization than was CO_2 exposure. Many flies displayed a violent, convulsion-like behaviour just before the immobilization with both gases, but the initial behavioural response to N_2 was, if anything, an increase in locomotor activity. With N_2 exposure, flies gradually slowed down, then stopped for several seconds, followed by convulsion-like behaviour and subsequent immobilization, and these changes occurred in an orderly manner in every tested fly. Most flies withstood N_2 exposure for more than 10 s, but all of them were knocked down within 30 s; notably, animals slowed to a standstill, standing motionless for several seconds before convulsing. Altogether, the extended withstanding time and sequentially recognizable components of locomotion were unique to N_2 exposure, whereas rapid knockdown was consistent with CO_2 exposure. These data suggest that CO_2 has physiological effects beyond the simple absence of oxygen.

Anoxia directly affects synaptic transmission at neuromuscular junctions (Badre et al., 2005), but the more rapid impact of CO_2 indicates additional physiological responses. Flies detect hypoxia via the oxygen sensor, a family of atypical guanylyl cyclases, which activates downstream signalling cascades and leads to locomotor responses (Vermehren-Schmaedick et al., 2010). The time spent from oxygen sensing to signalling pathway activation and behavioural responses could be longer than the time required for the CO_2-evoked direct effect, which is known to result from decreased sensitivity to glutamate at the neuromuscular junctions (Badre et al., 2005; Milton and Partridge, 2008). CO_2 also has an acidifying effect on the haemolymph (Badre et al., 2005), which may be linked to a number of downstream consequences. Additionally, physiological mechanisms mediating locomotor recovery from coma could be different between CO_2 and N_2 exposure. For example, the classic eye-colour gene *white* in *Drosophila* has a pleiotropic function in promoting fast and consistent locomotor recovery from N_2 knockdown (Xiao and Robertson, 2016). A, B, and C flies all carry the *white*$^+$ gene and display relatively consistent and complete locomotor recovery from N_2 exposure. The delayed, incomplete and inconsistent locomotor recovery from CO_2 exposure suggests the coexistence of multiple restorative processes.

We demonstrate adaptation to CO_2 exposure in *D. melanogaster* as an unintended consequence of routinely applied culturing methods. We show that rapid, consistent recovery from CO_2 exposure reflects both generalized adaptation to anoxia and CO_2-specific components, suggesting a polygenic basis. Considerable inter-individual variation in recovery from both gases in populations not exposed to CO_2 for many generations may be reflective of relaxed selection in the laboratory for stresses encountered in the wild. For example, the controlled and moderate larval densities may make submersion during feeding and resultant exposure to hypoxia a rarity. Or there may be cross-tolerance with other stresses such as heat, which is not encountered in the laboratory. Such questions await further study, and this experiment in life history has serendipitously provided a well-controlled, replicated laboratory system to undertake genetic and physiological investigations.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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Data availability

Data are available from the Dryad digital repository (Xiao et al., 2019): dryad.tk23st2

Supplementary information

Supplementary information available online at
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