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The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*

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Abstract

Carbon dioxide gas is used as an insect anesthetic in many laboratories, despite recent studies which have shown that CO₂ can alter behavior and fitness. We examine the effects of CO₂ and anoxia (N₂) on cold tolerance, measuring the rapid cold-hardening (RCH) response and chill coma recovery in *Drosophila melanogaster*. Short exposures to CO₂ or N₂ do not significantly affect RCH, but 60 min of exposure negates RCH. Exposure to CO₂ anesthesia increases chill coma recovery time, but this effect disappears if the flies are given 90 min recovery in air before chill coma induction. Flies treated with N₂ show a similar pattern, but require significantly longer chill coma recovery times even after 90 min of recovery from anoxia. Our results suggest that CO₂ anesthesia is an acceptable way to manipulate flies before cold tolerance experiments (when using RCH or chill coma recovery as a measure), provided exposure duration is minimized and recovery is permitted before chill coma induction. However, we recommend that exposure to N₂ not be used as a method of anesthesia for chill coma studies.

Keywords

Anesthesia; Cold tolerance; Nitrogen; Carbon dioxide; Chill coma recovery

1. Introduction

Although the physiological study of insect cold tolerance has focused largely on the ability (or otherwise) of insects to survive internal ice formation (Salt, 1961), the vast majority of insects are neither freeze tolerant nor freeze avoiding, but die at some temperature before they freeze (“non-freezing cold injury” or “pre-freeze mortality,” see Sinclair et al. (2003); Baust and Rojas (1985)). A number of insects that suffer from non-freezing cold injury are nevertheless able to rapidly alter their tolerance of acute cold exposure by rapid cold-hardening (RCH; Lee et al., 1987), whereby low temperature tolerance is greatly enhanced by a short pre-exposure to a milder low temperature. For example, Czajka and Lee (1990) showed that adult *Drosophila melanogaster* will die (but not freeze) when exposed to −5 °C for 2 h, but if chilled at 5 °C for as little as 2 h prior to the subzero treatment, most will survive.

Although RCH has been described in many species, the mechanisms are not well understood (Chown and Nicholson, 2004). Lee et al. (1987) found an increase in concentration of the common carbohydrate cryoprotectant glycerol after 0 °C pre-treatments in the flesh fly (*Sarcophaga crassipalpis*) to 81.3 mM, nearly three times the levels found in prechilled organisms. Although the changes were not large enough to have a colligative effect (i.e. by depressing the supercooling point), glycerol may have non-colligative effects like protecting

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membranes from phase transition or proteins from denaturation. While glycerol and Hsp70 concentrations did not increase with RCH pre-treatments in *D. melanogaster* (Kelty and Lee, 2001), Overgaard et al. (2005) found an increase in a polyunsaturated fatty acid, linoleic acid, and decreases in monounsaturated and saturated fatty acids after an RCH pre-treatment of 5 h at 0 °C, leading to an overall decrease in membrane saturation. Desaturation will lower the temperature at which cell membranes undergo a liquid-crystal to gel phase transition, allowing function at lower temperatures (Hochachka and Somero, 2002).

At low (but non-lethal) temperatures, insects lose motor activity in a reversible state known as chill coma (Lee, 1991). Both onset of chill coma (CT_{min} or knockdown) and recovery of activity upon rewarming (chill coma recovery) have been used to assess cold tolerance (David et al., 1998, 2003; Gibert et al., 2001; Sinclair and Roberts, 2005). Although the mechanisms of chill coma onset are poorly understood, Goller and Esch (1990) suggest that it results from a loss of function of the ion channels necessary for maintaining the membrane potential, leading to voltage equilibration and a loss of muscle cell excitability. Since resting potentials in insects are mostly maintained by a Na^+/K^+ ATPase (Huddart and Wood, 1966; Rheuben, 1972), Hosler et al. (2000) suggest that its activity is temperature dependent, and coordination recovery after return to room temperature is dependent upon functional restoration of this pump. However, the underlying causes of chill coma recovery variation have not been investigated, it is unclear whether the mechanisms are shared with chill coma onset and the mechanisms permitting modification of chill coma recovery are almost certainly different to those involved in RCH or basal cold tolerance (Sinclair and Roberts, 2005).

Three anesthetics are commonly used to anesthetize *D. melanogaster* in laboratories: ether, chilling (short exposures to 0 °C) and carbon dioxide (Ashburner and Thompson, 1978), which all result in a reversible loss of mobility and coordination. Badre et al. (2005) showed that the effects of CO₂ on *D. melanogaster* larvae were not due to decreased hemolymph pH, but found that CO₂ decreased sensitivity to glutamate at the neuromuscular junction, leading to loss of motor ability. For anesthetics to be useful, it must be assumed that they have no effect on traits being measured in the laboratory. However, CO₂ exposure has been shown to reduce longevity, fecundity (Perron et al., 1972) and mating success (Barron, 2000) in *D. melanogaster*. Additionally, CO₂ anesthesia increased development time and impaired locomotion and feeding behavior in the German cockroach, *Blattella germanica* (Tanaka, 1982; Branscome et al., 2005). David et al. (1998) did not find a significant anesthesia effect when measuring chill coma recovery after CO₂, but they did not control for the length of exposure, nor did they examine the effects of different lengths of recovery time after the anesthesia.

In this study, we examine the effects of CO₂ anesthesia on chill coma recovery and induction of the RCH response in adult *D. melanogaster*. Because the current model of the mechanism of CO₂ anesthesia suggests that CO₂ affects physiological processes at the neuromuscular junction, while the RCH response seems to depend on decreased membrane saturation, we hypothesize that CO₂ will not significantly affect the ability of *D. melanogaster* to induce the RCH response. However, because the current hypothesis explaining chill coma centers on muscular excitability, we hypothesize that reduced glutamate sensitivity by CO₂ saturation will increase chill coma recovery time.

2. Materials and methods

2.1. Origin and handling

D. melanogaster (collected in 1998 from Terhune, NJ, USA) were mass-bred in cages with plates of *Drosophila* medium (Tucson *Drosophila* Stock Center recipe: 0.9% agar, 2.4% cornmeal, 3.9% sugar, 1.4% dried yeast (w/v), 0.3% (v/v) propionic acid) as an oviposition substrate. They were reared in a 14 h:10 h L:D photoperiod in an incubator at 22 °C (approx.

20–25% RH), in 25 × 95 mm vials containing 10 mL of the same *Drosophila* medium as a food source. After 9 days of development, the adults were removed from the vials daily to ensure approximate equal ages among groups. Newly emerged flies were sorted by sex under light CO₂ anesthesia (< 10 min), and males were placed in fresh food vials, 10 individuals per vial. These individuals were used for experiments when 4-days old, allowing at least 48 h of recovery from the CO₂ before any treatments. All gas treatments were conducted in food vials, which were flushed with 60 s of the appropriate gas from a compressed canister or an air pump (for controls), using a regulator with a 1.3 mm diameter stainless steel needle tip. The tubes were sealed with rubber stoppers and parafilm and then left undisturbed for the assigned time. To account for the large number of vials and the 60 s treatment, all treatments were handled in blocks of 20 vials and handling was offset at 1 min intervals. We estimate that timing of recovery and treatment is therefore accurate to ± 15 s. All temperature treatments were conducted in 2 ml, waterproof, low temperature vials containing a small piece of white paper with labeling information, 10 individuals per vial. Recovery was assessed in 6-well culture plates containing a small piece of *Drosophila* medium. After experiments, survival or recovery was only accepted when the fly could stand up and walk in a coordinated fashion.

2.2. Anesthesia recovery time experiments

Food vials, each containing ten 4-day-old adult males were randomly assigned into one of three groups: a CO₂ treatment, a N₂ treatment (anoxic control, not expected to cause pH changes as seen with CO₂), or an air (handling control) group. The vials were flushed with gas for 60 s and sealed for a randomly assigned time between 5 and 120 min, using 5 min intervals ($n = 10$ vials per treatment-time combination). After the treatment, the vials were flushed with air for 60 s, recapped with a cotton plug, and recovery was scored every 60 s until all were recovered.

2.3. RCH experiments

RCH was assessed using a 2-h exposure to a discriminating temperature (−5.2 °C) expected to result in 80% mortality, but able to be mitigated by an RCH pre-treatment (Fig. 1). Flies were randomly assigned to CO₂, N₂ or control groups, and then into one of the three exposure times (10, 30 or 60 min; Fig. 2). Within each of these groups, the flies were arbitrarily assigned to receive direct transfer (directly from room temperature to −5.2 °C for 2 h) or a pre-treatment (0 °C for 2 h before −5.2 °C for 2 h). Flies were immediately transferred to −5.2 °C after treatment; no recovery from anesthesia or chill coma was allowed. After the subzero exposure, flies were transferred to a well of a 6-well flat-bottomed culture plate with a wedge of *Drosophila* food, and scored for survival after 24 h. There were four groups of 10 flies per treatment/time/gas combination, and the experiment was repeated on 3 consecutive days. RCH assays were repeated on 3 consecutive days, using flies that were all collected as eggs on the same day, but which took different periods of time to develop to eclosion. Thus, we have a ‘development time’ factor in our analysis of RCH.

2.4. Chill coma recovery experiments

2.4.1. Varying exposure times—Eighteen groups of 10 vials, each containing 10 flies, were arbitrarily assigned into CO₂, N₂ or air treatment groups. Each group was then randomly divided into nine time groups from 0 to 120 min, using 15 min intervals, with 10 replications (vials) per time. Each vial was flushed with 60 s of gas and then left undisturbed for its assigned time. After the gas treatment, the flies were immediately transferred to 2 ml vials and placed into a 0 °C ice water bath for 4 h to induce chill coma before being removed to room temperature for recovery, which was assessed every 60 s.

2.4.2. Varying recovery times—The varying recovery time experiment followed the same basic protocol as the varying exposure time study. However, instead of using varying treatment

times, a constant 60 min gas exposure was used and flies were allowed time in air to recover from the anesthesia before the induction of chill coma. The vials were randomly assigned to three groups, 10 replications per group: immediate transfer (no recovery time), all awake by observation (approximately 30 min), and all awake+1 h (approximately 90 min). After the 60 min gas treatment, the vials were flushed with air for 60 s, re-capped with cotton plugs and allowed to sit undisturbed for the appropriate recovery time. The flies were then transferred to 2 ml vials, immersed in an ice-water slurry (0 °C) for 4 h before recovery was assessed as above.

2.5. Statistical analysis

Survival or recovery data were analyzed using Generalized Linear Models (PROC GENMOD) in SAS/STAT (v. 9.1, the SAS Institute, Cary, North Carolina), using a binomial error distribution and logit link function (survival) or poisson distribution with log link (recovery) and scaled deviance if deviance/df > 5. Significant differences between individual treatment groups were detected through non-overlapping 95% confidence intervals on least-squares means.

3. Results

3.1. Anesthesia recovery time experiments

Flies exposed to gas treatments for 5–120 min required significantly longer recovery times than control flies at every time point (Wald $\chi^2 = 8477.68$, df = 2, $p < 0.0001$; Fig. 3). Additionally, increasing gas treatment time generally increased recovery time from both CO₂ and N₂ (Wald $\chi^2 = 1107.33$, df = 13, $p < 0.0001$). Recovery values after N₂ treatments were significantly longer than CO₂ treatments at every time point except 40, 45, 90 and 120 min (Wald $\chi^2 = 627.32$, df = 26, $p < 0.0001$).

3.2. RCH experiments

Groups given a 2 h 0 °C RCH pre-treatment showed significantly better survival at −5.2 °C for 2 h than those directly transferred from room temperature (Wald $\chi^2 = 53.07$, df = 3, $p < 0.0001$). Treatment with CO₂ or N₂ had no significant effects on RCH or cold tolerance at short treatment times (10–50 min), but decreased survival at 60 min, so that survival in the pre-treated groups did not differ from the directly transferred ones (Fig. 4). There were significant differences in both basal cold tolerance and RCH response between flies of differing development time (Wald $\chi^2 = 11.09$, df = 2; $p = 0.004$, Fig. 4), particularly under CO₂ treatment, with later-developing flies having increased survival.

3.3. Chill coma recovery experiments

3.3.1. Varying exposure times—We measured chill coma recovery in flies exposed to CO₂, N₂ or air for 15–120 min. Exposure to any duration of CO₂ or N₂ increased chill coma recovery time compared the to control groups (Wald $\chi^2 = 4714.95$, df = 2, $p < 0.0001$; Fig. 5). Increasing exposure times generally increased recovery times, except between 30–45, 60–90 and 105–120 min with CO₂ and between 60 and 90 min with N₂, where recovery times proceeded along a plateau (Wald $\chi^2 = 1725.91$, df = 8, $p < 0.0001$). Additionally, N₂ treatment resulted in significantly longer chill coma recovery times than CO₂ at every time point (Wald $\chi^2 = 1019.59$, df = 16, $p < 0.0001$).

3.3.2. Varying recovery times—We measured chill coma recovery in flies exposed to CO₂ or N₂ for 60 min and allowed 0–90 min recovery in air before the induction of chill coma. Exposure to CO₂ or N₂ resulted in significantly higher chill coma recovery times than control groups (Wald $\chi^2 = 868.64$, df = 2, $p < 0.0001$; Fig. 6). Increasing recovery time from anesthesia

significantly decreased chill coma recovery time at every time point (Wald $\chi^2 = 777.97$, df = 2, $p < 0.0001$). Additionally, increasing anesthesia recovery time to 90 min negated the CO₂ effect, while chill coma recovery times of flies treated with N₂ were still significantly, but only slightly, higher than the control group (Wald $\chi^2 = 399.56$, df = 4, $p < 0.0001$).

4. Discussion

We found that the required time for recovery from anesthesia is proportional to the time exposed. Additionally, we showed that CO₂ anesthesia and N₂ (as an anoxic control) appear to interact with the RCH response. Both CO₂ and N₂ increased chill coma recovery time, but we found that the anoxia control resulted in an increased chill coma recovery time relative to the CO₂ treatment. This increase is minimized, although remains significant with N₂ treatments, if flies are given 90 min in air between anesthesia and induction of chill coma.

4.1. Anesthetic effects on cold tolerance and RCH

Membrane desaturation is the currently hypothesized mechanism of RCH (Overgaard et al., 2005) and the persistence of the RCH response after exposure to CO₂ and N₂ suggests that these treatments do not disrupt this mechanism. Although not significant, there is a trend in the CO₂ treated groups for pre-treatment survival to decrease with increasing gas times (Fig. 4), which could explain the elimination of the RCH effect at 60 min. Decreased hemolymph pH, a consequence of CO₂ exposure (Badre et al., 2005), could be expected to decrease the fluidity of membranes without altering saturation (Hochachka and Somero, 2002; Hazel et al., 1992), and it is possible that pH changes resulting from long exposure to CO₂ are counteracting the desaturation effects of the RCH response.

Flies exposed to N₂ tended towards an increase in basal cold tolerance with increasing gas treatment times, leading to an apparent loss of the RCH effect—survival of pre-treated groups at 60 min does not differ from that of their unexposed counterparts (Fig. 4). Anoxia, caused here by N₂, induces a 10³-fold increase in Hsp70 mRNA transcription in *D. melanogaster* (Ma and Haddad, 1997). Although Hsp70 is not thought to be responsible for the RCH effect in *Drosophila* (Overgaard et al., 2005), it is a dominant molecular chaperone of the cellular stress response and can therefore increase survival at high and low temperatures (Chown and Nicholson, 2004). In particular, Burton et al. (1988) showed that heat shock can provide some protection against cold injury in *D. melanogaster*. If Hsp70 production (induced by anoxia) does increase basal cold tolerance, but this effect is independent of the RCH response, it could explain the results we observed with exposure to N₂. However, anoxia exposure was not shown to increase cold tolerance in the pretreated groups, nor did it increase survival in the groups exposed to CO₂, which should also create an anoxic environment, in addition to its pH effects. This suggests that the protection offered by Hsp70 might be minimized by RCH pretreatments and a low pH, or a different mechanism altogether is occurring. Measurement of Hsp70 transcript with anoxia and RCH pretreatments in this experimental design and/or the use of Hsp70 induction mutants (Welte et al., 1993; Feder et al., 1996; Roberts and Feder, 2000) will allow this hypothesis to be tested. Yocum and Denlinger (1994) found that anoxia (also administered with N₂) blocked RCH without affecting basal cold tolerance in *S. crassipalpis*. Although we found essentially the opposite response, it is probably premature to interpret this as evidence of differing mechanisms of RCH in *D. melanogaster* and *S. crassipalpis*.

We found significant differences in cold tolerance and RCH between flies differing in development time (Fig. 4). Eggs were collected and placed in food vials simultaneously (after 24 h of laying), but adult collections were made over a series of 3 days. Consequently, the adults collected for the latter days of the experiments required 1 or 2 additional days of development compared to those used on the first day of the experiment. The RCH experiments were distributed over 3 days, and flies used on the first day (which had the fastest development)

had consistently lower survival under both CO₂ and N₂ treatments. Laboratory selection for shorter development time has been shown to decrease body size and fecundity (Nunney, 1996) and temperature is known to have significant effects on developmental time and fitness in *D. melanogaster* (Nunney and Cheung, 1997). Chen et al. (1987) manipulated development time by rearing *S. crassipalpis* at different temperatures, and found that lower rearing temperatures (i.e. increased development time) resulted in increased cold tolerance, although this is a possible effect of acclimation, rather than development time per se. Our results suggest that (temperature-independent) development time may account for some variation in the magnitude of the RCH response, and we suggest that this relationship could well be relevant to individual survival in the field.

4.2. Anesthetic effects on chill coma recovery

Badre et al. (2005) showed that CO₂ blocks signal transduction at the neuromuscular junction resulting in anesthesia, while the onset of chill coma is thought to result from unexcitable muscle cells due to a loss of function of the Na⁺/K⁺ ATPase, leading to equilibration of membrane ions and loss of the membrane potential (Goller and Esch, 1990; Hosler et al., 2000). Given that nothing is currently known about the mechanisms of recovery from chill coma or anesthesia in insects, we here tentatively assume that recovery is a simple reversal of these events. Since both chill coma and CO₂ anesthesia act directly on the transmission of neuronal signals, although at different locations, it is not unexpected that chill coma recovery times increased with duration of CO₂ exposure (Fig. 5). Both glutamate sensitivity and muscle excitability are required for coordinated muscular activity (measured in 'chill coma recovery'), and it appears here that restoring both mechanisms results in some form of interaction, leading to longer recovery times.

Exposure to N₂ also increased chill coma recovery times as compared to controls. Anoxia causes hyperpolarization of *Drosophila* neurons, decreasing their excitability (Gu and Haddad, 1999). If chill coma recovery is a function of re-establishing a resting membrane potential, hyperpolarization alone would not be expected to increase recovery time, as the effects of N₂ and chill coma could cancel one another out. However, if N₂ affects the ability of the Na⁺/K⁺ ATPase to repolarize the cell after chill coma, exposure to anoxia could explain the observed increase in recovery time. Thus, it seems that the effects of anoxia (and/or chill coma recovery) are not simply expressed in terms of polarization/depolarization, and we suggest that further work on the physiological basis of recovery from chill coma, CO₂ anesthesia and anoxia are necessary to understand the mechanisms underlying the widely used phenotype of chill coma recovery.

Recovery from CO₂ before chill coma induction decreases recovery time to closely resemble control values. This suggests that the effects of CO₂ are offset by a relatively short recovery time at room temperature, which is in contrast to the longer-term effects on fitness and behavior implied by previous studies (Perron et al., 1972; Barron, 2000). By contrast, the effects of N₂ on chill coma recovery decline, but persist after 90 min of recovery. The decline in recovery time, however, also implies that the effects of N₂ exposure might also be reversible with sufficient recovery time.

5. Conclusions

Here we show that it is acceptable to use CO₂ anesthesia in cold tolerance experiments when using RCH or chill coma recovery as a cold tolerance measure. However, when measuring chill coma recovery, it is necessary to allow all flies to recover from the anesthesia and regain coordination, plus a minimum of an additional 60 min before cold treatments, in order to get results close to control values. This corroborates David et al.'s (1998) results, which showed no significant effects on chill coma recovery after 3 h of anesthesia recovery. Therefore, care

must be taken when using CO₂ anesthesia, but it does seem to be an acceptable way to handle *D. melanogaster* before cold tolerance experiments. By contrast, until further information about recovery from N₂ exposure is available, we recommend that this not be used as a method of anesthesia for chill coma studies.

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Fig 1.

Survival of 4-day old adult male *Drosophila melanogaster* after 2 h exposure to a subzero temperature, with or without a 2 h 0 °C RCH pre-treatment. Data points are means $\pm$ SE, $n = 3$ replications of 10 flies.

Fig 2.

Experimental design of the RCH experiment. Adult male flies were either exposed or not to a gas treatment, and then either given a 2 h pre-treatment at 0 °C (RCH) before treatment at -5.2 °C, or transferred directly into -5.2 °C for 2 h. Each treatment group contained four replicates of 10 flies and was repeated on 3 consecutive days.

Fig 3.

Time required for 80% recovery after an exposure to CO₂, N₂ or air. Data points are mean $\pm$ SE. $n = 10$ replicates of 10 flies. N₂ and CO₂ treatments differ significantly where marked with *. See text for details of statistics.

Fig 4.

Survival after a defined length of N_2 or CO_2 exposure and a 2 h treatment at $-5.2^\circ C$, with (pre-treatment) or without (direct transfer) a 2 h $0^\circ C$ pre-treatment. Data points are means $\pm$ SE. $n = 4$ replicates of 10 flies. Pre-treatments differ from controls where marked with *. See text for details of statistics. Development time refers to time to develop from egg to adult.

Fig 5. Time required for recovery from chill coma (4 h at 0 °C) after a defined length of CO₂ or N₂ exposure. Data points represent mean $\pm$ SE. $n = 10$ replicates of 10 flies. N₂ and CO₂ treatments differ significantly where marked with *. See text for details of statistics.

Fig 6.

Time required for recovery from chill coma (4 h at 0 °C) after 60 min of gas treatment, with varying amounts of recovery time from anesthesia. Data points represent means $\pm$ SE. $n = 10$ replicates of 10 flies. N₂ and CO₂ treatments differ significantly where marked with *. N₂ and CO₂ treatments differ from the control where marked with † or §, respectively. See text for details of statistics.