

The impact of anaesthetic technique on survival and fertility in *Drosophila*

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Abstract. The consequences of ice and carbon dioxide anaesthetics on the survival and reproductive success of *Drosophila simulans* and *Drosophila melanogaster* were investigated after observations of high levels of mortality in *D. simulans*, possibly associated with brief chill coma. The association between brief chill coma and death is confirmed in female *D. simulans* but there is no mortality in male *D. simulans* or *D. melanogaster* of either sex. Mortality is unlikely to be associated with a strain specific cold intolerance because two geographically distinct populations of *D. simulans* were examined. In addition to the effect of ice anaesthesia, anaesthetizing newly-eclosed male *D. simulans* with CO₂ causes a reduction in fertility, which is evident 9–13 days after anaesthesia. This finding is important given that CO₂ anaesthesia is a standard technique used in *Drosophila* and other insect cultures, and may have important consequences for studies of male fertility and sperm competition.

Key words. Carbon dioxide, chill coma, *Drosophila*, fitness, insect.

Introduction

Anaesthetizing insects with ice or carbon dioxide (CO₂) is common laboratory practice (Nilson *et al.*, 2006). Amongst many functions, immobilization is often necessary for determining sex or transferring individuals from one form of housing to another. These anaesthetics are widely utilized because they are quick, reliable and easy, and typically do not pose a threat to humans (by contrast to anaesthesia by ether). However, there are some deleterious side effects associated with both techniques. For example, CO₂ anaesthesia elevates activity levels in *Drosophila melanogaster* (Nicolas & Sillans, 1989) and affects female reproductive behaviour in *D. melanogaster* by reducing sexual receptivity (Ashburner, 1989; Barron, 2000). Carbon dioxide can also cause tissue damage and mortality in newly-eclosed *Drosophila* if the cuticle has not hardened sufficiently to prevent uptake of CO₂ and subsequent rupturing of the gut (Ashburner, 1989). It is also toxic to flies infected with the sigma virus (Roberts, 1986). Anaesthesia with ice is often used as an alternative to CO₂, particularly for behavioural studies, because it has fewer and relatively minor side effects (Ashburner, 1989). However, exposure to low temperature is thought to affect

memory in some insects (Quinn & Dudai, 1976; Van Baaren *et al.*, 2005) and can trigger sperm dumping ('desperming') in female *Drosophila* (Ashburner *et al.*, 2005).

Anaesthesia with ice exploits a physiological phenomenon known as chill coma (David *et al.*, 1998) in which individuals exposed to low but nonfreezing temperatures (e.g. –1 °C) are immobilized. In *D. melanogaster*, freezing usually occurs at –18 °C (Tucic & Krunik, 1975) and adults are capable of surviving at 0 °C for substantial periods (David *et al.*, 1998; Gibert *et al.*, 2001). Provided this exposure is not long term (>15 h), *Drosophila* in chill coma are thought to recover normal activity and behaviour rapidly when they are returned to warmer temperatures. Resistance to cold stress is improved by exposing the insects to nonlethal temperatures prior to the stress (acclimation or cold-hardening) (Rako & Hoffmann, 2006). For example, exposing *D. melanogaster* adults reared at 25 °C to 12 °C for a period of time prior to cold stress lowers the temperature required to induce chill coma and reduces recovery time after chill coma (Rako & Hoffmann, 2006). This process is not reported to cause mortality in *D. melanogaster* unless the chill coma lasts more than 6 h and the flies are exposed to very low (–5 °C to –2 °C) temperatures (Rako & Hoffmann, 2006). Likewise, there are no reports of mortality associated with chill coma in *Drosophila simulans* (Gibert *et al.*, 2001).

Numerous studies across *Drosophila* species (David *et al.*, 1998; Goto *et al.*, 2000; Gibert *et al.*, 2001; Rako & Hoffmann, 2006) report consistent recovery from chill comas persisting for hours at a time. However, despite these findings

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and no evidence of mortality when newly-eclosed *D. simulans* are anaesthetized on ice, significant levels of mortality are observed regularly amongst >3-day-old virgin female *D. simulans* after they are subjected to short term chill coma. Typically, these flies are anaesthetized for less than 10 min and recover within minutes. Although the behaviour and activity levels of the flies appear normal after recovery, many or all are dead within 24 h. The present study investigates the relationship between ice anaesthesia and survival in *D. simulans* and *D. melanogaster*. In addition, because CO₂ anaesthesia is the main alternative to ice anaesthesia, the consequences of CO₂ anaesthesia for survival and reproductive success in *D. simulans* are also investigated.

Materials and methods

The impact of ice anaesthesia on adult survival was investigated in an Oregon-R (OreR) strain of *D. melanogaster* and two strains of *D. simulans* (*DSR* and *Michelle*). The two *D. simulans* populations originate from geographically distinct areas: *DSR* from Riverside, California, U.S.A. and *Michelle* from Tuncurry, NSW, Australia. Experiments examining the impact of CO₂ anaesthesia and ice anaesthesia on male and female reproductive success were performed on the *DSR* strain of *D. simulans* only (Fig. 1).

Eggs were harvested from stock population cages maintained at 20 °C (note that *Michelle D. simulans* stock populations are maintained at 25 °C) (LD 12 : 12 h photoperiod) by inserting a petri-dish containing a transparent red medium on which flies laid eggs for 24 h. After 24 h, the petri-dish was removed and the eggs were incubated at either 25 or 20 °C (*D. simulans* strains only) for a further 24 h until they hatched. Larvae were then picked off the medium using a metal probe and transferred to density controlled vials (5 larvae mL⁻¹) containing a standard *Drosophila* food medium (1 L of food consists of: 10 g of agar, 85 g of sugar, 60 g of oats, 20 g of yeast, 1 g of Nipagin (methyl paraben), 1 L of water), where they developed at either 25 or 20 °C until they eclosed (Fig. 1).

Newly-eclosed adults were allocated to one of four broad experimental procedures (Fig. 1), which tested the impact of: larval rearing and adult maturation temperature; the anaesthesia used to sex the flies when they were newly eclosed (ice or CO₂); and whether or not flies were acclimatized to 20 °C, on survival upon being transferred to individual vials either under ice anaesthesia or by aspiration (no anaesthetic). After adult eclosion, a general experiment procedure was employed (for sample sizes, see also Fig. 1, Table 1): adult flies were sexed within 5 h of eclosing by anaesthetizing them with either ice or CO₂, and transferred and maintained, in single sex vials at the temperature they were reared at as larvae (20 or 25 °C). After 4–8 days maturation, the impact of ice anaesthesia on survival was assayed by transferring the flies to individual vials using either ice anaesthesia or an aspirator (no anaesthetic). Adults subjected to ice anaesthesia were anaesthetized by chilling them in glass vials placed in ice (approximately 0 °C) for approximately 5 min before being transferred to individual vials containing food. The vials were returned to the temperature the flies were reared at and arranged randomly within the incubators to control for possible position effects. The flies were assayed individually for survival 18–24 h after transfer. Flies that were acclimatized prior to the survival assay were kept at 20 °C for 24 h immediately prior to being transferred to new vials either under ice anaesthesia or via aspirator. After transfer, approximately half the flies from each treatment were returned to 20 °C and the other half returned to 25 °C until they were assayed for survival. Flies were scored as either 'Alive' or 'Dead'. The flies scored as dead included some moribund flies ($n = 6$), which were capable of some movement but were grossly inactive and generally unable/unwilling to walk. These flies did not recover and were dead by the end of the day.

Male and female reproductive success

The effect of anaesthetic technique on male and female reproductive success was investigated by providing female

Fig. 1. Experimental design. We compared the impact of larval rearing and adult maturation temperature (b, d), anaesthesia (CO₂/ice) used to sex flies when newly eclosed (a, b), and prior acclimation to 20 °C (c) on survival when mature adult flies were subjected to brief chill coma during transfer to new vials. *D. simulans DSR* females were tested in parts (a) to (d) of the design. *D. simulans Michelle* females were tested in parts (b) and (d); and *D. melanogaster* females were tested in part (b) of the design only. Males of each species/strain were tested in part (b) of the design only. The impact of sexing anaesthesia (CO₂/ice) on *D. simulans DSR* male and female reproductive success was investigated using males and females from parts (a) and (b) that had been transferred to new vials by aspirator.

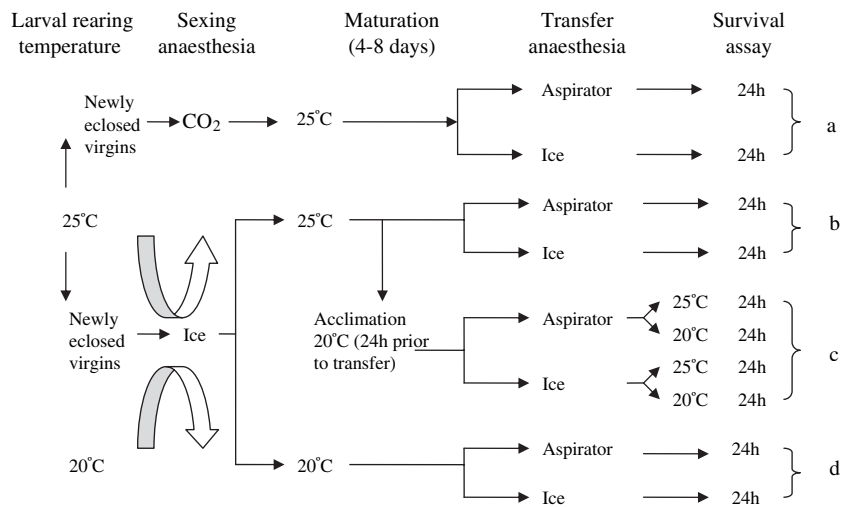


Table 1. The number of replicates (*n*) for each species/strain in each experiment treatment and the percentage of flies that survived.

Species/strain	Sex	Anaesthesia when newly eclosed	Rearing temperature (°C)	Transfer method	<i>n</i>	% survival	
<i>Drosophila simulans</i> DSR	♂	Ice	25	Ice	10	100	
				Aspirator	10	100	
	♀	Ice	25	Ice	50	4	
				Aspirator	50	100	
		CO ₂	25	Ice	50	4	
				Aspirator	50	100	
		Acclimation a) 25 b) 20 c) 25	Ice	9	100		
			Aspirator	4	100		
	Acclimation a) 25 b) 20 c) 20	Ice	13	69			
		Aspirator	3	100			
	<i>Drosophila simulans</i> Michelle	♂	Ice	25	Ice	10	90
					Aspirator	10	100
♀		Ice	25	Ice	19	21	
				Aspirator	10	100	
		Ice	20	Ice	17	53	
				Aspirator	5	100	
<i>Drosophila melanogaster</i>		♂	Ice	25	Ice	10	100
					Aspirator	10	100
		♀	Ice	25	Ice	10	100
					Aspirator	10	100

Treatments where *Drosophila simulans* were acclimated to 20 °C are marked with 'Acclimation'.

The three temperatures refer to (a) larval rearing temperature, (b) temperature flies were acclimated to for 24 h prior to experiment and (c) the temperature flies recovered at after the experiment.

D. simulans DSR with a single male in a four-treatment design that balanced the anaesthetic technique (ice or CO₂) both males and females were subjected to when they were newly eclosed. Each female was kept with a male in the same vial and allowed to lay eggs for 5 days before both the male and female were removed. The vials were then maintained at 25 °C for 8 days, which allowed most of the larvae to complete their development and eclose, and ensured that the vials only contained offspring from the original pair. The number of offspring produced by each pair was counted as a measure of their reproductive success.

Statistical analysis

The frequency of survival/death with respect to anaesthesia technique was investigated using chi-square analyses. The offspring production data were normally distributed and were analysed using a general linear model. The terms included as fixed factors in the model were female anaesthesia technique when newly eclosed (ice/CO₂), male anaesthesia technique when newly eclosed (ice/CO₂), and an interaction between these terms. Terms were removed after stepwise deletion, with interactions removed before main effects and with least significant terms removed first. There was no significant interaction between male and female anaesthetic technique. All data were analysed in SPSS version 14 (SPSS Inc., Chicago, Illinois).

Results

Survival

All flies anaesthetized on ice during the transfer to individual vials recovered within 1 min of transfer and displayed normal activity levels. However, within a short period (< 24 h), there was a highly significant effect of anaesthetic method on *D. simulans* DSR female survivorship ($\chi^2 = 184.62$, d.f. = 2, $P < 0.0001$; Table 1). All females transferred to new vials with an aspirator (no anaesthetic) survived, but 96% of females that were anaesthetized on ice during the transfer process died. The anaesthesia method used to sex newly-eclosed females (ice or CO₂) had no effect on their survival after allocation to individual vials (Table 1). Likewise, 79.0% of *D. simulans* Michelle females died after brief chill coma ($\chi^2 = 16.35$, d.f. = 2, $P < 0.0001$; Table 1). No females died if they were transferred to vials using an aspirator and, with the exception of one male *D. simulans* Michelle, there was no mortality amongst male *D. simulans* or in *D. melanogaster* of either sex associated with ice anaesthesia (Table 1). The high levels of mortality reported above were all observed in flies reared at 25 °C. However, a lower rearing temperature (20 °C) improved the survival of flies subjected to ice anaesthesia. All DSR *D. simulans* females reared at 20 °C survived ice anaesthesia. However Michelle *D. simulans* still exhibited mortality after anaesthesia with ice: 47.1% females died after brief chill coma despite being reared at 20 °C, whereas no females died after being transferred with

an aspirator ($\chi^2 = 3.657$, d.f. = 1, $P = 0.054$; Table 1). The statistical power of the previous test is limited by the small number of replicates ($n = 5$) of females transferred with an aspirator. Acclimatizing *D. simulans* DSR females reared at 25–20 °C for 24 h prior to anaesthesia with ice reduced female mortality to 18.2% (Table 1). All deaths (four out of 22) were found in females that were returned to 20 °C after acclimation and anaesthesia.

Male and female reproductive success

The method by which *D. simulans* DSR females were anaesthetized when newly eclosed (ice or CO₂) had no effect on the number of offspring they produced ($P = 0.974$). However, the method by which males were anaesthetized when newly eclosed did have a significant effect on offspring production (Table 2). Males collected under CO₂ anaesthesia produced approximately 42 ± 5.2 offspring (mean \pm SE) whereas males collected under ice anaesthesia produced 61 ± 3.8 offspring. Males collected under CO₂ anaesthesia therefore produced on average 31.4% fewer offspring than males collected under ice anaesthesia.

Discussion

Survival

The present study reports high levels of female mortality associated with brief chill coma or cold shock in *D. simulans* but no effect of cold shock on survival in *D. melanogaster*. High mortality in *D. simulans* is unlikely to be strain-specific because it has been replicated in flies originating from two geographically and therefore presumably genetically distinct populations. Although CO₂ anaesthesia increases chill coma recovery time in *D. melanogaster* (Nilson *et al.*, 2006), *D. simulans* mortality is not associated with the anaesthesia technique (CO₂ or ice) that flies were subjected to when newly eclosed. This is not surprising given the long interval between anaesthetics in this experiment.

It is unclear why female *D. simulans* are prone to death after brief chill coma. Sex and age is known to affect cold resistance in *D. melanogaster*, although the reports are con-

flicting. For example, in some studies, young females (6–7 days old) are thought to be relatively resistant to cold stress compared with males of the same age and older females (Norry & Loeschcke, 2002; Rako & Hoffmann, 2006), whereas, in other studies, young (2–8 day old) females are found to be less resistant than males when exposed to temperatures between –2 °C and –8 °C (Jensen *et al.*, 2007; see also Sejerkilde *et al.*, 2003). The present study shows that young (4–8 day old) virgin *D. simulans* females are strongly susceptible to cold stress (at 0 °C) compared with males and newly-eclosed females, but there is no effect of cold stress on female *D. melanogaster*. It is possible that sex related differences in thermal tolerance are only evident at lower temperatures (i.e. below 0 °C) in *D. melanogaster*. The decrease in *D. melanogaster* female cold shock tolerance is suggested to be related to the onset of sexual maturity (Jensen *et al.*, 2007). This could also be true for *D. simulans* and may be further related to their egg load. In the present study, all *D. simulans* (and *D. melanogaster*) females were virgin and had a high egg load, which may affect thermal tolerance. The study by Jensen *et al.* (2007) reports the greatest difference in cold tolerance between male and female *D. melanogaster* when they are 2 days old, which is before they reach sexual maturity (Pitnick *et al.*, 1995) and therefore before they lay eggs, although females can have a high egg load at that age (personal observation.). Interestingly, *D. melanogaster* female cold tolerance is similar to that of males when they are 8 days old (Jensen *et al.*, 2007). Because the males and females were enclosed together, females of this age should have a reduced egg load owing to active reproduction/egg laying. Although, the mechanisms of chilling injury are poorly understood in general (Sinclair & Roberts, 2005), some progress has been made in cockroaches, indicating a role for ion homeostasis in chilling injury and mortality (Košťál *et al.*, 2004). In *D. melanogaster*, it is likely that phospholipid fatty acid composition in cell membranes changes in response to chilling so as to preserve membrane function (Overgaard *et al.*, 2005). Additionally, in *D. melanogaster*, recovery from cold shock is associated with up-regulation of expression of the *Frost* (*Fst*) gene (Goto, 2001; Qin *et al.*, 2005). Unfortunately, however, the role of these factors in determining *D. simulans* cold tolerance remains unclear.

Rearing temperature and acclimation have an important influence on survival and recovery from chill coma in *D. melanogaster*

Table 2. General linear model of offspring production in *Drosophila simulans* DSR with respect to male anaesthesia method (ice or CO₂) and female anaesthesia method (ice or CO₂) when newly eclosed.

Source	Type III sum of squares	d.f.	Mean square	F	Significance
Corrected model	4430.496*	1	4430.496	8.547	0.005
Intercept	127550.006	1	127550.006	246.052	0.000
Male anaesthetic method	4430.496	1	4430.496	8.547	0.005
Error	24364.198	47	518.387		
Total	155429.000	49			
Corrected total	28794.694	48			

Neither the interaction between male and female anaesthesia technique, nor female anaesthesia technique had a significant effect on offspring production and these factors were removed from the model.

* $R^2 = 0.154$ (adjusted $R^2 = 0.136$).

(Lee *et al.*, 1987; Goto, 2001; Sinclair & Roberts, 2005; Rako & Hoffmann, 2006). In support of this, female *D. simulans* DSR reared at 20°C exhibit no mortality after chill coma, and females acclimatized to 20°C for 24h prior to chill coma show reduced mortality. By contrast, almost 50% of female *Michelle* *D. simulans* die despite being reared at 20°C. One potentially important difference between *Michelle* and DSR *D. simulans* is the stock population rearing temperatures. The stock populations of *Michelle* are kept at 25°C whereas all other stocks used in this experiment are maintained at 20°C. If chill coma tolerance is costly, it may be selected against under permanently warm conditions (Gibert *et al.*, 2001). It is therefore possible that laboratory conditions have created different selective environments that could explain the differences observed between the two strains of *D. simulans* used in the present study.

Reproductive success

The present study also investigates the consequences of the anaesthesia technique used to sex flies when they are newly eclosed for female and male reproductive success. There is no effect of ice or CO₂ anaesthesia on female fecundity. However, significant reductions in the fertility of male *D. simulans* associated with CO₂ anaesthesia when males were newly eclosed are found. How CO₂ reduces fertility is unclear but a similar effect is found in another fly. Moloo & Kutuza (1975) report that CO₂ appears to reduce the insemination capability of male Tsetse flies (*Glossina fuscipes fuscipes*). *Drosophila* are thought to be particularly susceptible to CO₂ prior to their wings unfurling (Ashburner, 1989). However, because they are

sexed before or after the peak eclosion period, and wings unfurl relatively quickly, most, if not all, of the males in the present study have unfurled wings and hard cuticles when they are exposed to CO₂. Carbon dioxide is known to affect sexual receptivity or copulation latency in female *D. melanogaster* (Ashburner, 1989; Barron, 2000) and longevity and fecundity in young adult flies (Perron *et al.*, 1972). Carbon dioxide is also suggested to affect sperm movement to the spermatheca within females of the moth *Utetheisa ornatrix* (LaMunyon & Eisner, 1993) and the dung fly *Scatophaga stercoraria* (Hellriegel & Bernasconi, 2000), and sperm storage in female *Tribolium castaneum* (Fedina & Lewis, 2004). This effect has been attributed to CO₂ inhibiting female muscular movement, but an alternative explanation is that it may directly affect sperm viability. Although it is unclear why male *D. simulans* exposed to CO₂ suffer reduced fertility, this finding has important implications for fertility studies in *Drosophila* in particular, and insects in general, because CO₂ is used frequently in experimental work. The use of CO₂ may cause underestimates of male fertility and, amongst other things, affect the dynamics of sperm competition.

We reveal the dramatic effects of ice anaesthesia on female survivorship and of CO₂ anaesthesia on male fertility in *D. simulans*. These findings call for additional work to investigate the underlying physiological mechanisms of anaesthesia in *Drosophila* and highlight the need for careful investigation from researchers to ensure that anaesthesia does not affect the outcome of their studies. To help *Drosophila* researchers assess anaesthesia techniques, an overview of the known side-effects of CO₂ and ice anaesthesia for *D. melanogaster* and *D. simulans* is presented in Table 3. Although we advocate the avoidance of

Table 3. Summary of the side effects of CO₂ and ice anaesthesia in *Drosophila melanogaster* and *Drosophila simulans*.

Anaesthesia	Side effects	<i>Drosophila melanogaster</i>	<i>Drosophila simulans</i>	References
CO ₂	Gut rupture before cuticle hardened	✓	✓	Ashburner (1989)
	Increased activity levels/locomotor activity	✓	?	Vandijken <i>et al.</i> (1977), Nicolas & Sillans (1989)
	Reduced sexual receptivity	✓	?	Ashburner (1989), Barron (2000)
	Toxic to flies with sigma virus	✓	?	Roberts (1986)
	Exposure as pupae increases development time and decreases weight at eclosion	✓	?	Kaiser (1995)
	Reduces longevity and fecundity	✓	?	Perron <i>et al.</i> (1972)
	Increases chill coma recovery time (if chill coma occurs within 90 min of anoxia)	✓	?	Nilson <i>et al.</i> (2006)
Ice	Males have decreased fertility	?	✓	Present study
	Short chill coma at 0 °C results in death of mature virgin females	×	✓	Present study
	Females less cold tolerant than males at temperatures below zero	✓	?	Sejerkilde <i>et al.</i> (2003), Jensen <i>et al.</i> (2007)
	Desperming/sperm dumping	✓	?	Ashburner <i>et al.</i> (2005)
	Memory loss	✓	?	Quinn & Dudai (1976)
	Recovery time affected by sex, age, rearing temperature and length of anaesthesia	✓	✓	David <i>et al.</i> (1998), Gibert <i>et al.</i> (2001), Rako & Hoffmann (2006)
	Tropical populations more cold sensitive	✓	✓	Gibert <i>et al.</i> (2001)

either CO₂ or ice anaesthesia whenever possible, alternative techniques facilitating the handling of flies, such as aspiration, may also affect fly behaviour. For example, physical shocks to flies that can occur during aspiration increase copulation latency (Barron, 2000). If researchers require the use of ice anaesthesia in their experiments, we suggest that flies are acclimatized to lower temperatures (e.g. 10–20 °C) for at least 24 h prior to entering chill coma. In addition, flies should be allowed 24 h recovery after CO₂ or ice anaesthesia to ensure any side effects of anaesthesia do not affect the outcome of studies. Finally, CO₂ should not be used as an anaesthetic in any study of male fertility or sperm competition without demonstrating that it has no effect on male fertility in the study species.

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