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Hot hypoxic flies: Whole-organism interactions between hypoxic and thermal stressors in *Drosophila melanogaster*

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Abstract

The upper critical thermal maximum (CT_{max}) of metazoans varies over a wide range, and its determinative factors, such as oxygen limitation, remain controversial. Induction of thermoprotective mechanisms after challenge by sublethal heat stress has been well documented in many organisms, including the model fly *Drosophila melanogaster*. Interestingly, however, other challenges—notably a period of anoxia—induce post-exposure thermoprotective effects in some organisms such as locusts and houseflies. Here I show, using thermolimit respirometry, that acute hypoxia during thermal stress significantly reduced the CT_{max} of *D. melanogaster*, but only below an oxygen partial pressure of about 10 kPa (39.0 ± 0.4 SE °C at 9.3 kPa vs. 36.0 ± 0.2 SE °C at 3.5 kPa). Likewise, the scope for voluntary motor activity declined sharply below 10 kPa and was essentially eliminated at 2.3 kPa. Respiratory water loss increased highly significantly below about 10 kPa. The post-CT_{max} release of a large quantity of CO_2 is shown to be independent of loss of spiracular control, but dependent at least in part on oxygen availability. The results are broadly in accord with Pörtner's oxygen limitation hypothesis, but suggest that acute oxygen limitation only becomes an important factor at partial pressures less than half of typical atmospheric levels.

Keywords: Drosophila; Heat stress; Hypoxia; Thermolimit respirometry; Multiple stressors; CT_{max}

1. Introduction

When stressors come, they may—to paraphrase King Claudius in *Hamlet*—"come not as single spies but in batallions" (Shakespeare, 1623). Because individual stressors induce a variety of protective mechanisms, and those protective mechanisms may interact and overlap additively, subtractively, or synergistically in ways that are poorly understood, the response to multiple stressors at the level of the intact, functioning organism may be complex and counterintuitive. This study addresses the interaction at the whole-organism level of two stressors, acute hypoxia and acute thermal stress, in *Drosophila melanogaster*.

Hypoxia and thermal stress are intimately related. Thermal stress elevates metabolic demands while challenging the integrity of enzyme, cytoskeletal and neural function (see Feder and Hofmann, 1999; Robertson, 2004 and references therein). Organismal responses include production of heat shock proteins (HSPs; see Parsell et al., 1993; Feder and Hofmann, 1999; Malmendal et al., 2006) and synthesis of polyol chaperones such as sorbitol (Carninci et al., 1998; Chen, 2002; Kaushik and Bhat, 2003). Hypoxia has diverse long and short term effects on insects (see review by Harrison et al., 2006). Mild hypoxia upregulates the expression of some 20 genes in *Drosophila* which have been identified and characterized (Liu et al., 2006 and references therein). Prominent among the effects of hypoxia in *Drosophila* is upregulated NO synthesis and subsequent cGMP/PKG activation (Wingrove and O'Farell, 1999) and, at least in some stages of ontogeny, increased HIF-1 expression (Ma and Haddad, 1999; see also Semenza, 2000; Lavista-Llanos et al., 2002). Other effects of hypoxia in *Drosophila* include elevated sorbitol synthesis (Chen, 2002; Chen and Haddad, 2004); interestingly, sorbitol was by then already well-known for its thermoprotective effects (Carninci et al., 1998 and references therein).

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Clearly there are molecular grounds to suspect that at least some thermal and hypoxic stress responses may interact. One particularly interesting finding in the laboratory of R. Meldrum Robertson was that exposure to anoxia increased neural thermotolerance in *Locusta migratoria* (Wu et al., 2002). In another, anoxia was found to induce cold-hardening in the housefly (Coulson and Bale, 1991). However, studies that emphasize cellular or molecular aspects of stressor responses, though highly informative, do not necessarily correlate with understanding whole-organism performance and evolutionary fitness. It is worth reminding ourselves that natural selection is the keystone of biology and that "It is the intact and functioning organism on which natural selection operates" (Bartholomew, 2005). At the whole-organism level, thermal stress elevates metabolic flux rates and decreases enzyme stability, while hypoxia challenges the delivery of sufficient oxygen to meet TCA-cycle demands. At this level of integration limited oxygen availability may challenge and ultimately overwhelm any protective mechanisms that operate singly, additively or synergestically. This was recognized by Ushakov (1964) and later by Hans-Otto Pörtner (Pörtner 2001, 2002; Pörtner et al., 2000) who maintained that in complex metazoans, thermal stress that affects fitness is not set primarily by cellular and molecular responses, but by whole-organism gas exchange limitations that cause an unsustainable transition to anaerobic metabolism. For example, at high temperatures a mismatch may occur between high oxygen demand by mitochondria and inadequate oxygen uptake and distribution by ventilation and subsequent circulation of respiratory pigments. Thus oxygen limitation, according to Pörtner, is the primary limiting factor that determines the maximum temperature an organism can sustain (CT_{max}).

Pörtner's oxygen limitation hypothesis was mostly formulated with marine animals in mind, but the situation for terrestrial animals, and especially tracheate arthropods, is more complex. Insects use a highly efficient tracheal system for direct O_2 delivery and are capable of staggeringly high metabolic flux rates during activities such as flight (Joos et al., 1997 and references therein). It would therefore appear that oxygen limitation *per se* is unlikely to affect CT_{max} (see discussion in Chown, 2001; Klok et al., 2004). Using a newly developed technique for multimodal, low-variance CT_{max} determination, i.e. thermolimit respirometry or TLR (Lighton and Turner, 2004), Klok et al. (2004) undertook the first experimental test of Pörtner's oxygen limitation hypothesis and concluded that in the insect they examined (a tenebrionid beetle, *Gonocephalum simplex*), hypoxia did not affect CT_{max}. In the other animal in that study, the isopod *Armadillidium vulgare*, a slight effect of hypoxia on CT_{max} was observed, which the authors concluded was an effect of that terrestrial expatriate species' less efficient gas exchange capabilities (primarily gills and respiratory pigment vs. tracheae), in accordance with Pörtner's oxygen limitation hypothesis. They concluded that because studies had shown that membrane damage probably sets the CT_{max} in insects (Denlinger and Yocum, 1998), "such determinants of CT_{max} at the cellular level *in insects* would make the CT_{max} independent of oxygen availability" (Klok et al., 2004, emphasis added).

Although most insects are generally thought to be highly tolerant of hypoxia (Chown, 2001; Klok and Chown, 2003; Klok et al., 2004 and references therein; Schmitz and Harrison, 2004), all is not necessarily as it seems. Indeed, some insects such as dragonflies are—surprisingly—oxygen-limited even in normoxia, and are able to significantly increase their metabolic flux rates during flight if they are exposed to $pO_2 > 21$ kPa (Harrison and Lighton, 1998). This observation is particularly interesting in light of the long episodes of atmospheric hyperoxia during which extensive radiation of insect taxa, including episodes of gigantism, occurred (Graham et al., 1995; Dudley, 2000). As detailed in a recent review by Harrison et al. (2006), even small insects such as *D. melanogaster*, which one would expect to be little challenged by hypoxia in view of their small size and limited diffusion distances, do in fact display a suite of ontogenetic responses to even fairly mild hypoxia. Among these are reductions in body size and survival and increases in development time, particularly at higher temperatures (Frazier et al., 2001; Peck and Maddrell, 2005); behavioral changes (Dillon and Frazier, 2006); and significant changes in tracheal morphology (Henry and Harrison, 2004).

Thus hypoxic effects in *Drosophila* become significant at levels not far below normobaric normoxia. Yet, in the only study that has explicitly tested the CT_{max} of insects as a function of hypoxia (Klok et al., 2004), no correlation between these variables was found. Could hypoxic and thermal protective mechanisms be interacting in insects? Were Klok et al. (2004) dealing with an unusually well-aerated insect? Plainly, more data are needed, and *D. melanogaster*, with its sequenced genome, well-characterized genetic lines, and known ontogenetic sensitivity to hypoxia, is a good candidate for further investigation into the interaction of thermal and hypoxic stressors.

2. Methods

2.1. Animals

D. melanogaster, strain Oregon-R wild type, were reared at moderate density at $25 \pm 2\,^{\circ}C$ at 12:12 L/D cycle. Adult flies were released, and the flies that enclosed over the subsequent two days were collected in narrow vials prepared with feeding medium, 10–20 flies per vial, and used 5–10 days later for the experiments described here.

2.2. Thermolimit respirometry

The procedures we used are slightly modified from Lighton and Turner (2004). Briefly, we enclosed individual flies in a leak-proof chamber, 6 mm i.d., bored through a