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The rapid cold hardening response of *Drosophila melanogaster*: Complex regulation across different levels of biological organization

Johannes Overgaard [a,*], Jesper Givskov Sørensen [b], Emmanuelle Com [c], Hervé Colinet [d]

[a] Zoophysiology, Department of Biosciences, Aarhus University, C.F. Møllers Alle 3, Building 1131, DK-8000 Aarhus C, Denmark
[b] Genetics, Ecology & Evolution, Department of Biosciences, Aarhus University, Ny Munkegade 114-116, Building 1540, DK-8000 Aarhus C, Denmark
[c] Proteomics Core Facility Biogenouest, INSERM U1085 IRSET, Campus de Beaulieu, Université de Rennes 1, 263 Avenue du Général Leclerc CS 2407, 35042 Rennes Cedex, France
[d] Université de Rennes 1, UMR CNRS 6553 Ecobio, 263 Avenue du Général Leclerc CS 74205, 35042 Rennes Cedex, France

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ABSTRACT

Rapid cold hardening (RCH) is a form of thermal acclimation that allows ectotherms to fine-tune their physiological state to match rapid changes in thermal environment. Despite progress in recent years, there is still a considerable uncertainty regarding the physiological basis of RCH in insects. Here we investigated the physiological response of adult *Drosophila melanogaster* to a gradual reduction of temperature from 25 to 0 °C followed by 1 h at 0 °C. As expected, this RCH treatment promoted cold tolerance, and so we hypothesized that this change could be detected at the proteomic level. Using 2D-DIGE, we found that only a few proteins significantly changed in abundance, and of these, we identified a set of four proteins of particular interest. These were identified as two different variants of glycogen phosphorylase (GlyP) of which three spots were up-regulated and another was down regulated. In subsequent experiments, we quantified upstream events by measuring the *GlyP* mRNA amount, but we found no marked effect of RCH. We also examined downstream events by measuring GlyP activity and the level of free sugars. We found no effect of RCH on GlyP activity. On the other hand, screening of whole animal sugar contents revealed a small increase in glucose levels following RCH while trehalose content was unaltered. This study highlights a complex regulation of GlyP in relation to RCH where we found associations between the cold tolerance, the protein abundance and the metabolite concentrations but no changes in mRNA expression and enzyme activity. These data stress the necessity of combining the hypothesis-generating power of an 'Omics' approach with subsequent targeted validations across several levels of the biological organization. We discuss reasons why different biological linked levels do not necessarily change stoichiometrically.

1. Introduction

The rapid cold hardening response (RCH) in insects represents a fast acclimatory response thought to play a role for insects' ability to respond to natural diurnal temperature variation (Koveos, 2001; Kelty, 2007; Overgaard and Sørensen, 2008; Lee and Denlinger, 2010). At the organism level RCH is known to improve survival to acute cold stress, reduce negative effects of cold exposures on activity and reproduction and decrease the temperature of chill coma (Lee et al., 1987; Kelty and Lee, 1999; Shreve et al., 2004; Overgaard et al., 2007; Lee and Denlinger, 2010; Teets and Denlinger, 2013). The mechanisms underlying this response have therefore been widely studied in insects, including *Drosophila melanogaster* (Lee and Denlinger, 2010; Teets and Denlinger, 2013). As a broad generalization studies of RCH have found that the physiological transitions underlying RCH have clear similarities to those found during the more profound physiological modification that occur during seasonal cold acclimation in insects (Teets and Denlinger, 2013). Thus RCH has been associated with changes in compatible osmolytes (Chen et al., 1987; Michaud and Denlinger, 2007; Overgaard et al., 2007) but see (MacMillan et al., 2009), alterations of membrane composition or fluidity (Overgaard et al., 2005, 2006; Lee et al., 2006; Michaud and Denlinger, 2006), expression of heat shock proteins (Goto et al., 1998; Kelty and Lee, 2001; Nielsen et al., 2005; Li and Denlinger, 2008), improved ability to maintain and recover ion and metabolic homeostasis (Overgaard et al., 2007; Armstrong et al., 2012; Teets et al., 2012; Teets and Denlinger, 2013; Findsen et al., 2013) and prevention of cold-induced apoptosis (Yi et al., 2007). The physiological responses associated with RCH are, however, often small and there

* Corresponding author. Address: University of Aarhus, C.F. Møllers Allé, Building 1131, 8000 Århus C, Denmark. Tel.: +45 8942 2648; fax: +45 8942 2586.
E-mail address: biojo@biology.au.dk (J. Overgaard).

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is still a considerable uncertainty and debate regarding the generality and importance of the physiological modifications mentioned above (Teets and Denlinger, 2013).

In addition to the more hypothesis driven studies of RCH, several studies have utilized 'Omics' technologies to uncover putative genes or metabolites associated with RCH (Qin et al., 2005; Michaud and Denlinger, 2007; Overgaard et al., 2007; Teets et al., 2012; Vesala et al., 2012). Surprisingly these studies have revealed that only few or even sometimes no genes are affected by RCH treatments compared for example to the responses associated with rapid heat hardening (Sørensen et al., 2005) or with gradual cold acclimation (Vesala et al., 2012). Overall these results suggest that elements downstream the transcriptional machinery might be sufficient to carry out RCH. It is becoming increasingly clear that the relationship across different levels of biological organizations cannot be assumed to be linear, i.e. that a transcriptional change leads to a comparable translational changes leading to a comparable change in protein activity, etc. (Feder and Walser, 2005; Suarez and Moyes, 2012; Malmendal et al., 2013). Thus little is still known of the relation between transcriptional, translational and organism responses in relation to insect RCH response. For example, except from a study of Li and Denlinger (2008) that focused on brain proteins, no study has investigated changes at the proteomic level following RCH. It is an interesting observation that some previous studies have found transcriptional regulation (Qin et al., 2005) while others did not (Vesala et al., 2012; Teets et al., 2012). Even more puzzling is the observation that RCH can take place in *D. melanogaster* in the absence of protein synthesis since a clear RCH response was found in flies where the translational machinery was blocked by cycloheximide (Misener et al., 2001).

Together, these incongruent observations prompted us to assess proteomic response to RCH and to further examine whether up- and downstream events are correlated across different levels of biological organization. The aim of the present study was to decipher the underpinnings of RCH through investigation across different levels of biological organization using proteomics as an explorative starting point. Here we used 2D-DIGE proteomics to identify putative changes in protein profile following RCH treatments in *D. melanogaster*. We further examined if the changes found at the protein level were consistent with observations across four levels of biological organization. Thus here we describe for the first time in one study the consequences of RCH at the level of mRNA, protein abundance, protein activity, protein product and whole organismal performance.

2. Materials and methods

2.1. Origin and maintenance of experimental flies

A mass bred laboratory population of *D. melanogaster* was used in the experiment. The population was created by mixing flies from >500 isofemale lines collected in October 2010 at Karensminde fruit farm at the Danish peninsula of Jutland (flies were kindly shared by Mads Fristrup Schou and Volker Loeschcke). After establishment of a mass bred population the flies were maintained on a standard oatmeal-sugar-yeast-agar *Drosophila* medium under low to moderately high larval density conditions at 25 °C, relative humidity (RH) of 50% and 12 h light/12 h dark cycles. Flies used for experiments also developed and lived under these conditions.

Adult flies from the mass bred population were transferred to bottles with yeasted media in order to stimulate egg production (~500 flies on 35 ml media). Flies from these bottles were placed on spoons with media for egg laying (~10 pairs/spoon) and 14–20 h later eggs were collected in batches of 40 eggs that were transferred to fresh food vials with 7 ml fly food. Vials were placed at constant 25 °C for development and emerging flies were then collected and transferred to fresh bottles at 25 °C until sexual maturation. 2–3 days after emergence flies were sexed under CO_2 anesthesia and saved in fresh food vials with a density of 25 flies/vial. Flies were placed on fresh media every second day during the subsequent 5 days before onset of experiments and sampling.

2.2. Experimental protocol

The purpose of the present study was to investigate putative physiological mechanisms underlying RCH. Here we used a large-scale proteomic assay to reveal proteins modified during the RCH treatment but importantly we also examined the up- and downstream relations of such proteins at other levels of biological organization (ranging from transcriptional regulation to whole organism performance). To achieve this we compared a group of flies exposed to a RCH treatment with an untreated control group. Changes found at the proteomic level were subsequently related to transcriptional activity using qPCR and in other experimental series we measured the enzymatic activity and product from a candidate protein to explore the possible downstream events of the proteomic modifications.

2.3. Cold hardening treatment and assessment of thermal tolerance

Rapid cold hardening was induced using the protocol described in Overgaard et al. (2005). Flies were gradually ramped down from 25 to 0 °C at a rate of 0.1 °C min^{-1} followed by 1 h at 0 °C. To assess the thermal tolerance of the flies, we exposed untreated controls and rapid cold hardened flies acutely to 1 h at −6 °C in pre-cooled water bath. For both treatment groups we used ten vials each with ten female flies which were transferred to empty glass vials before being acutely cold shocked. Vials were provided with a moist stopper to ensure high humidity. After cold exposure flies were transferred to fresh food vials and survival was evaluated 20 h later from the flies' ability to move any body part.

2.4. Sample preparation

Samples for use in subsequent biochemical, proteomic and transcriptional analysis were taken immediately after the RCH treatment and a similar amount was sampled directly from the constant 25 °C cabinet (to avoid confounding effects of starvation and desiccation). 300 flies were sampled from each treatment (14 vials of 25 flies from each treatment). These samples were transferred directly to liquid nitrogen and placed at −80 °C until analysis (in some cases 25 flies were adequate for several assays and each replicate was split accordingly).

In an additional follow-up experiment we reared flies under similar conditions and exposed them to the same acclimation treatments (RCH and Control). These flies were also sampled immediately after treatment, but here we also sampled flies 2 and 6 h after the RCH treatment (and a similar control for time was taken from 25 °C group). For each time point we sampled flies for 5 replicates for determination of gene expression, sugar contents (glucose and trehalose) and glycogen phosphorylase (GlyP) activity (12 vials of 25 flies). These samples were also snap frozen in liquid nitrogen and saved at −80 °C until later investigations.

2.5. 2D-DIGE proteomics

For both phenotypes (control at 25 °C and RCH), four biological replicates, each consisting of a pool of 25 females, were used for proteomics. The protein extraction procedure was performed as previously described in Colinet et al. (2013). Total protein concentration was determined using the Bradford Protein Assay Kit