Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*

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Running headline: Thermal performance curve of the metabolome
Abstract

The ability of ectotherms to respond to changes in their thermal environment through plastic mechanisms is central to their adaptive capability. However, we still lack knowledge on physiological and functional responses by which ectotherms acclimate to temperatures during development, and in particular, how physiological stress at extreme temperatures may counteract beneficial acclimation responses at benign temperatures. We exposed *Drosophila melanogaster* to ten developmental temperatures covering their entire permissible temperature range. We obtained metabolic profiles and reaction norms for several functional traits: egg-to-adult viability, developmental time, and heat and cold tolerance. Females were more heat tolerant than males, whereas no sexual dimorphism was found in cold tolerance. A group of metabolites, mainly free amino acids, had linear reaction norms. Several energy carrying molecules, as well as some sugars, showed distinct inverted u-shaped norms of reaction across the thermal range, resulting in a positive correlation between metabolite intensities and egg-to-adult viability. At extreme temperatures, low levels of these metabolites were interpreted as a response characteristic of costs of homeostatic perturbations. Our results provide novel insights into a range of metabolites reported to be central for the acclimation response, and suggest several new candidate metabolites. Low and high temperatures result in different adaptive physiological responses, but they also have commonalities likely to be a result of the failure to compensate for the physiological stress. We suggest that the regulation of metabolites that are tightly connected to the performance curve is important for the ability of ectotherms to cope with variation in temperature.

**Key-words:** NMR metabolomics, developmental acclimation, environmental stress, extreme temperatures, plastic responses, sexual dimorphism, thermal performance curve, thermal resistance.
**Introduction**

The thermal environment is central for the abundance and distribution of ectotherms and much attention has been directed towards the potential of ectotherms to adapt to current and future thermal conditions (2, 15, 16, 20, 22, 42). Predicting the future distributions of ectotherms in a warmer, but also more variable environment with more extreme thermal episodes at both ends of the thermal scale, can be addressed by studying the ability to adapt through evolutionary, behavioral or plastic responses (23). A number of recent studies suggest that some species of frogs (17), phytoplankton (24), copepods (27, 28) and insects (22) are evolutionarily constrained when it comes to coping with extreme environments due to e.g. lack of adaptive genetic variation. Thus, current and future species distributions will partly depend on migration, behavioral thermoregulation and physiological plastic responses; the latter being the focus of this study.

Acclimation is a form of physiological plastic response that organisms induce in response to changes in the environment. Laboratory studies on ectotherms investigating acclimation and the physiological changes induced during acclimation typically do so by exposing test organisms to short-term cold or hot temperatures and subsequently studying the consequences on thermal resistance and molecular phenotypes (11, 26, 39, 41, 44, 62, 64, 65) but see (7, 36). Such studies have provided novel insights into the molecular aspects of the acclimation response and have contributed to our knowledge on associations between the genotype and the phenotype. However, in their natural habitats organisms are exposed to thermal variation throughout the entire life cycle and to thermal stress for extended periods, e.g. during cold winters or hot summers (25, 50, 57), whereby molecular characteristics of long-term exposure to different thermal environments is of ecological importance (30). This includes extreme environments that may induce more physiological stress than beneficial acclimation. A linear increase in ambient
temperature may result in non-linear reaction norms at molecular levels, for example a small increase in temperature at 20°C may not be perceived in the same way as a small increase in temperature at 30°C. Molecular reaction norms across the thermal range can be investigated by quantifying a molecular phenotype such as gene expression at each temperature. This enables grouping of genes into different categories of reaction norms and quantifying levels of molecular plasticity, furthering our understanding of the underlying components of long-term thermal acclimation (7).

Metabolomics has been implemented to study consequences of age, sex and genotype in *D. melanogaster* and encompasses the power to detect biologically relevant changes in the metabolome (21). Metabolomics may thus prove to be an important tool when filling the gap between the transcriptome and the functional phenotype (47). In organisms such as springtails, fruit flies, echinoderms and fish, metabolomics has provided insight into hot and cold adult acclimation responses (39, 43, 59, 60, 70, 71). An increase in sugars, polyols and free amino acids has been related to long-term cold acclimation in both adult and larva *D. melanogaster*, as well as in winter acclimating individuals of the codling moth *Cydia pomonella* (10, 32, 52). Proline in particular, has been shown to be a central compound for the freeze tolerance of the drosophilids *D. melanogaster* and *Chymomyza costata* as well as the beetle *Alphitobius diaperinus* (33, 34). In other species such as the flesh fly, *Sarcophaga crassipalpis*, glycerol is an important polyol during cold acclimation (38, 40, 73), while this does not seem to be the case in *D. melanogaster* (29, 33). As a response to short-term cold acclimation, sugars such as trehalose and glucose have been suggested to be important (43, 64). For a comprehensive overview, and in particular more information on short-term acclimation responses, which is not the focus of this study, we refer to Purać et al. (47). In comparison, studies investigating
metabolomics of heat acclimation in insects are sparse (47) and especially metabolomics studies on long-term temperature acclimation are yet to be performed (18, 19).

Here we exposed *D. melanogaster* to ten different developmental and adult rearing temperatures spanning most of the temperature range (12 to 32°C) where this species can complete its lifecycle. We obtained detailed functional phenotypic and metabolite profiles of adult male and female flies from each thermal regime. The inclusion of extreme developmental temperatures and parallel assessments of egg-to-adult viability and developmental time, as a measure of stress perceived, allows us to separate the metabolites in two distinct groups. The “beneficial” changes in metabolites, which form part of the thermal acclimation response, and the “costly” metabolites, which we hypothesize change as a result of environmental stress leading to a departure from cellular homeostasis. By doing this we gain key ecological knowledge on the association between temperature exposure and functional and molecular phenotypes. This allows us to investigate how ectotherms perceive and plastically adapt to different thermal conditions and identify the physiological causes of the strong functional benefits and costs typically associated with thermal acclimation (35). Our goal was to provide a comprehensive analysis of the physiological background for costs and benefits associated with developmental temperature on thermal resistance. We discuss our data based on three *a priori* hypothetical types of reaction norms for the metabolome across developmental temperatures: i) No change in the metabolome across temperatures, indicating that the metabolome is unrelated to the developmental temperature, ii) Linear change in the metabolome across temperatures, indicating that the metabolome is a component of the physiological thermal response of the organism; iii) A u-shaped (or bell-shaped) change in the metabolome across temperatures, indicating a shared effect of environmental stress at extreme low (12 and 15.5°C) and high (31 and 32°C) developmental temperatures.
Materials and methods

Acclimation and rearing procedure

The laboratory population used in this study was established from the offspring of 589 mated females (five males and five females from each) caught in Denmark in 2010 (for details see Schou et al. (56)). At the time of initiating the experiment, the population had been reared in the laboratory for approximately 45 generations. Prior to the experiment, the population was maintained at 20°C at a 12:12 light : dark (L:D) photoperiod and reared on a standard Drosophila medium composed of yeast, oatmeal, sugar and agar. Parental flies used for egg production of experimental flies were density controlled during development. When the parental flies were four days of age they were transferred to bottles with the standard Drosophila medium but with a relatively high agar concentration (3%) to insure that eggs were deposited on the surface of the medium to ease removal of the eggs (55). The following day parental flies were removed from the medium (after 16 h of egg-laying) and eggs were washed off the surface of the medium and distributed into vials in groups of exactly 40 eggs. This methodology was used to ensure random grouping of eggs into vials (55). At least 20 vials with eggs were distributed to each of ten constant developmental temperatures: 12, 15.5, 18, 20, 22, 25, 27, 29.5, 31, 32°C. We expected a lower survival rate at 12, 31 and 32°C, and therefore we set up ten additional vials at these temperatures. All acclimation regimes had a 12:12 L:D photoperiod.

At the day of emergence flies were anaesthetized with CO₂, separated into sexes, mixed among vials and relocated to their respective acclimation regime. For a subset of these vials, we counted the number of emerged adults to assess the proportion of eggs developing into adult flies (egg-to-adult viability) and developmental time from the egg to the adult life stage.

Thermal tolerance assays
For each developmental temperature, male ($n = 20$) and female ($n = 20$) flies of two-three days of age were tested for their critical thermal minimum ($CT_{\text{min}}$) and their critical thermal maximum ($CT_{\text{max}}$). $CT_{\text{min}}$ and $CT_{\text{max}}$ are proposed ecologically relevant measures of cold and heat tolerance (1, 49, 57, 66). Flies were placed individually into sealed 6 mL glass vials and submerged into a glass tank containing a 20°C liquid. The transfer of flies to glass vials and experimental initiation took place at 20°C and lasted no more than 15 min. When assessing $CT_{\text{max}}$, the glass tank contained water, where the temperature was increased with a rate of 0.1°C/min. Conversely when assessing $CT_{\text{min}}$, the glass tank contained a mixture of ethylene glycol and water (1:1 v/v), and the temperature was decreased with a rate of 0.1 °C/min. The flies were continuously monitored in intervals of 2-3 min and the temperature where no movement could be induced with a flashlight and gently knocking on the vials with a stick, was noted as the upper or lower thermal limit ($CT_{\text{max}}$ and $CT_{\text{min}}$). We interpret a high $CT_{\text{max}}$ and a low $CT_{\text{min}}$ as indicating high heat and cold tolerance, respectively. A subset of the phenotypic results obtained in these assays, as well as egg-to-adult viability and developmental time, has been published elsewhere, where the focus was on changes in the proteome across those three developmental temperatures (36). This subset includes male flies developed at 12, 25 and 31 °C.

**Sample preparation for NMR**

For all developmental temperatures we prepared five replicates of 40 pooled flies per sex for NMR spectroscopy. Flies were snap frozen at three days of age and kept at -80°C. Samples were mechanically homogenized with a Kinematica, Pt 1200 (Buch & Holm A/S, Herlev, Denmark) in 1 mL of ice-cold acetonitrile (50%) for 45 s. Hereafter samples were centrifuged (10,000 g) for 10 min at 4°C and the supernatant (900 μL) was transferred to new tubes, snap frozen and stored at -80°C. The supernatant was lyophilized and stored at -80°C. Immediately before NMR measurements, samples were rehydrated in 200 mL of 50 mM phosphate buffer (pH 7.4) in
D$_2$O, and 180 mL was transferred to a 3 mm NMR tube. The buffer contained 50 mg/L of the
chemical shift reference 3-(trimethylsilyl)-propionic acid-D$_4$, sodium salt (TSP), and 50 mg/L
of sodium azide to prevent bacterial growth.

NMR experiments

NMR measurements were performed at 25°C on a Bruker Avance III HD 800 spectrometer
(Bruker Biospin, Rheinstetten, Germany), operating at a $^1$H frequency of 799.87 MHz, equipped
with a 3 mm TCI cold probe. $^1$H NMR spectra were acquired using a single-90°-pulse
experiment with a Carr-Purcell-Meiboom-Gill (CPMG) delay added, in order to attenuate broad
signals from high-molecular-weight components. The total CPMG delay was 194 ms and the
spin-echo delay was 4 ms. The water signal was suppressed by excitation sculpting, potentially
masking changes in metabolites (mostly sugar units) resonating in this region. A total of 128
transients of 32 K data points spanning a spectral width of 20 ppm were collected,
corresponding to a total experimental time of 6.5 min.

Statistical analyses of phenotypic traits

We investigated the effects of thermal regime (developmental temperature) and sex on CT$_{\text{max}}$
and CT$_{\text{min}}$ using linear models. The models had either CT$_{\text{max}}$ or CT$_{\text{min}}$ as a response variable and
consisted of the predictor parameters sex (male or female) and developmental temperature
(continuous) as well as their interaction. Both CT$_{\text{min}}$ and CT$_{\text{max}}$ models fulfilled assumptions for
parametric analyses. We performed sequential model reduction and model comparisons using F-
tests to find the minimal adequate model and to obtain $P$-values for the respective predictors.
Egg-to-adult viability was modelled using a logistic regression with developmental temperature
as the sole predictor. Developmental temperature was modelled as a quadratic effect
(continuous) as the performance curve of egg-to-adult viability across thermal regime is non-
linear (57). Several data points from the developmental temperatures 12 and 32°C were highly influential according to Cook’s statistic, but we found this to be of biological importance for the fit and therefore maintained them in the model. We detected overdispersion in the model and corrected the standard errors using a quasi-generalized linear regression (74). The change in developmental time across developmental temperatures was modeled with a Poisson generalized linear mixed model in the R-package lme4 (3). We used a mixed model with vial as a random effect to account for possible vial effects. Developmental temperature was included as a quadratic effect (continuous) to allow for the developmental time to increase at high stressful temperatures (14), while sex (male or female) was included as a factorial effect. A group of outliers caused the assumption of normality of residuals to be violated. The outliers were spread across many temperatures and in all cases represented some highly delayed flies, relative to the mean of the given developmental temperature. As the model allowed for a good parametric fit of the observed effect of temperature we chose to retain this model despite the deviation from normality. The minimal adequate model and P-values for the fixed effects of egg-to-adult viability and developmental time were obtained using sequential model reduction and by model comparisons using maximum likelihood ratio tests. All statistical analyses of functional phenotypes were performed in R (48).

NMR data and analyses

The spectra were processed using iNMR (http://www.inmr.net). An exponential line-broadening of 0.5 Hz was applied to the free-induction decay prior to Fourier transformation. All spectra were referenced to the TSP signal at -0.017 ppm, automatically phased and baseline corrected. The spectra were aligned using icoshift (54). The region around the residual water signal (4.85-4.67 ppm) was removed in order for the water signal not to interfere with the analysis. The high- and low-field ends of the spectrum, where no signals except the reference signal from TSP
appear, were also removed (i.e., leaving data between 9.5 and 0.5 ppm). The spectra were normalized to total intensity in order to suppress separation based on variations in amount of sample. Metabolite assignments were done based on chemical shifts only, using earlier assignments and spectral databases previously described (13, 39, 45), and comparison with *Drosophila* metabolites identified by mass spectrometry (8).

All multivariate analyses were carried out on Pareto scaled data (12) using the SIMCA13 software (Umetrics, Malmö, Sweden). Principal component analysis (PCA) was performed to assess the overall temperature and sex dependence of the metabolome. To further investigate the differentiation in the metabolome between sexes across developmental temperatures we performed a PCA based on the difference between individual female sample spectra and the median male spectrum at each temperature as well as the difference between median female spectrum and individual male sample spectra at each temperature. In contrast to all the other analyses in this study the intensities were not centered here, and therefore the deviation from zero in the scores corresponds to the sex difference. The change in differentiation across developmental temperatures was modelled in a linear model with developmental temperature as a cubic term. The inclusion of developmental temperature as a cubic term was necessary to fulfill assumptions of parametric analyses.

Although a PCA is highly informative when analyzing the difference in metabolite profiles across the thermal acclimation gradient, additional variation can be extracted from the metabolite profiles by separating the different combinations of sex and temperature regimes using orthogonal projection to latent structures discriminant analysis (O2PLS-DA) (67). Hierarchical cluster analysis (HCA) of the Euclidean distance between the O2PLS-DA scores for each combination of developmental temperature and sex was carried out using Ward’s
method in R (48), to enable visualization of the differentiation in a dendrogram. The O2PLS-DA models were validated by cross validation. Randomly chosen groups of samples were left out to predict group membership for the excluded samples, until predicted values had been obtained for all samples.

To investigate the predictability of the male and female metabolomes from the two life history traits egg-to-adult viability and developmental time, as well as from the developmental temperatures, we used OPLS models (68, 69). We modeled the dependence of the metabolome on the developmental temperatures using two approaches based on our prior hypotheses: i) a linear change with developmental temperature, tested by correlating intensities with developmental temperatures \textit{(linear prior)} and ii) a u-shaped (or bell-shaped) change in intensities across developmental temperatures, tested by modeling a fit of the intensities on a categorical variable with two states, intermediate (18-29.5°C) and extreme (12, 15.5, 31 and 32°C) developmental temperatures \textit{(u-shape prior)}. This categorical variable is a simple representation of metabolite changes that are dependent on the deviation from intermediate non-stressful developmental temperatures rather than the absolute temperature value. The OPLS models were validated by cross validation. All samples for each combination of sex and temperature were left out one at a time, so that the predicted parameter for that combination of sex and temperature was only based on the relationship between that parameter and the metabolite concentration for the other samples.

To avoid restricting the analyses to our prior hypotheses on the relationship with developmental temperature, we also used the results from the PCA described above to infer the overall patterns of metabolite change across developmental temperatures. We first rotated the principal
components in order to find the component that showed the most linear response to the
developmental temperatures, which then allowed us to identify the shape of the component that
explained as much of the remaining variation as possible. For both male and female flies, the
first rotated component was rather linear (linear component), while the second rotated
component was u-shaped (u-shape component).

Given that the rotated components from the PCA, as well as our prior hypotheses, dictate the
potential for a linear or a u-shaped reaction norm of individual metabolites across
developmental temperatures, we aimed at identifying which specific metabolites conformed to
the two reaction norms. First, we correlated individual metabolite intensities with the linear
component and u-shape component from the rotated PCA. To investigate whether the same
pattern was observed when using our prior hypotheses, we correlated the individual metabolite
intensities with OPLS component scores of the overall metabolome in both the linear prior
model and the u-shape prior model. The same type of correlation was performed on the OPLS
component scores of the overall metabolome on egg-to-adult viability and developmental time.
All correlations were performed on each sex separately, except for egg-to-adult viability.
Finally, we also tested which metabolites correlated with the overall differences in the
metabolome between sexes. The difference between sexes was determined as the first
component in a PCA of the spectral differences between male and female samples (described
earlier). The significant correlations were calculated using peak correlations within the
integration range used to assign the metabolite (Table 1) and verified by looking at the
correlation of other characteristic chemical shifts from that metabolite (Table 1). Significant
spectral correlations were identified by applying sequential Bonferroni correction (P < 0.05) for
an assumed total number of 100 metabolites. The correlations were performed in MATLAB
(The MathWorks, Natick, 2015).
Results

Thermal resistance, egg-to-adult viability, and developmental time

We observed a linear relationship between developmental temperature and thermal tolerances (Fig. 1A-B; Table 2). Cold tolerance, measured as CT$_{\text{min}}$, was approximately 8°C lower in flies acclimated at 12°C compared to flies acclimated at 32°C, with no effect of sex (Fig. 1B; Table 2). In comparison, CT$_{\text{max}}$ (heat tolerance) showed a very similar pattern, but with a total increase of only 2°C in flies acclimated at 32°C compared to flies acclimated at 12°C and overall females were more heat tolerant than males (Fig. 1A; Table 2). The sex differentiation in CT$_{\text{max}}$ was highest at low developmental temperatures, and decreased with increasing developmental temperatures (Fig. 1A; Table 2). Egg-to-adult viability assessed across developmental temperatures had a quadratic shape with approximately 40 to 50% survival at the extremes (12 and 32°C) and with approximately 60 to 80% survival at the intermediate temperatures (Fig. 1C; Table 2). Developmental time decreased from approximately 49 days at 12°C to approximately 7.5 days at 29.5°C (Fig. 1D). There was no interaction between sex and temperature for developmental time, but males did develop significantly slower than females (Fig. 1D; Table 2).

NMR metabolomics: Overall effects of developmental temperature and sex

Examples of metabolite NMR spectra of male flies acclimated at three different temperatures are presented in Fig. 2. In order to characterize the overall metabolite response to developmental temperatures in male and female flies, respectively, metabolite NMR spectra were analyzed by principal component analysis (PCA) (Fig. 3A-B). Visual inspection of the PCA score plots reveals large variations in principal components 1 and 2 (PC1 and PC2) due to developmental temperatures for both sexes, but only segments of these are linear, and neither PC1 nor PC2 are linearly correlated with developmental temperature (Fig. 3A-B). The overall grouping of samples can be described as circular. Thus, samples from flies developed at similar temperatures
appear close to each other (Fig. 3A-B). But there is also a notable similarity between female flies reared at the maximum developmental temperature (32°C) and female flies reared at the minimum developmental temperature (12°C) (Fig. 3B), and this congruence was also apparent (though not to the same extent) in the male flies (Fig. 3A). In order to make some general conclusions on the overall metabolite change across developmental temperatures, we rotated the scores from the sex specific PCAs such that the first rotated component showed as high a correlation as possible with developmental temperature (*linear component*) (Fig. 3C-D). For both sexes, removal of the linear variation resulted in the next component having a distinct u-shape (*u-shape component*) (Fig. 3E-F), indicating a dependence on the deviation from the intermediate temperature. The individual metabolites underlying these linear and u-shaped reaction norms of the overall metabolome are investigated below.

We also performed a PCA on the spectral differences between male and female samples to investigate how the differentiation between sexes varies with developmental temperature. PC1 represents the majority of the sexual differentiation (Fig. 4) and showed a significant curved reaction norm across developmental temperatures (Female replica minus median male: $F_{(3,42)} = 181.84, P < 0.001$; median female minus male replica: $F_{(3,45)} = 140.92, P < 0.001$). Inspection of the resulting 95% confidence intervals revealed that the lowest differentiation between sexes (low scores at PC1) occurs at low and high developmental temperatures (Fig. 4). Conversely, there is large differentiation at benign temperatures.

O2PLS-DA in combination with HCA was used to illustrate the overall variation between flies at different temperatures and sexes in a dendrogram (Fig. 5). The dendrogram shows that the effect of developmental temperature on the metabolome is stronger than the effect of sex only at
the lowest temperatures (12–15.5°C) (Fig. 5). At 17–20°C males and females form separate
groups, but within the same cluster. Interestingly, flies reared at intermediate to high
temperatures (22–32°C) form separate clusters for males and females, in which females cluster
with flies reared at lower temperatures, and males form a cluster on their own (Fig. 5).

NMR metabolomics: Predicting phenotypes

In order to assess the ability to predict the developmental temperature, extreme temperatures,
developmental time and egg-to-adult viability from metabolite data, we performed OPLS
modeling. Egg-to-adult viability was evaluated with metabolite changes merged for males and
females as no sex-specific egg-to-adult viability estimates were obtained. As shown in Table 3
all models were significant based on the total predictability, $Q^2$ ($Q^2 \geq 0.5$ is considered
significant), providing statistical support for a set of metabolites with linear reaction norms
across developmental temperatures, as well as a set of metabolites with inverted u-shaped
reaction norms across developmental temperatures. Both for males and females the
developmental temperature can be accurately predicted ($Q^2 = 0.90$ and $Q^2 = 0.84$, respectively)
using a three-component linear model (linear prior) (Fig. 6). In comparison, the discrimination
between extreme temperatures and those of the intermediate temperatures (u-shape prior) was
not as accurate, although statistical significant. Finally, developmental time and egg-to-adult
viability could also be predicted from the metabolome, but again not with the same accuracy as
the developmental temperature.

NMR metabolomics: Individual metabolites

The individual metabolite variation forming the basis for the linear and u-shaped reaction norms
are shown in Fig. 7. Overall, metabolites that correlate with the linear prior are largely identical
with the metabolites correlating with the linear component, while metabolites that correlate with
u-shape prior also correlate with u-shape component. Metabolites correlating with the linear terms were dominated by a decrease in amino acids with increasing developmental temperature. Ten metabolites, arginine, histidine, isoleucine, leucine, methionine sulfoxide, phenylalanine, proline, tryptophan, tyrosine, and fatty acid, decreased linearly in both males and females, while only phosphocholine increased. Additionally, male flies showed an increase in nicotinamide ribotide, maltose, acetate and the galactoside 1-O-(4-O-(2-aminoethyl phosphate)-β-D-galactopyranosyl)-glycerol, and decreases in 3-hydroxykyneurenine and glutamate. In females, the only additional linear effect was an increase in glutamine. The u-shaped terms were dominated by changes in energy carrying molecules that were decreased at extreme temperatures (showing the reverse behavior of the scores displayed in Figs 3D and 3F). Five metabolites showed this inverted u-shape response in both males and females: NAD+, NADP+, AMP, mannose and β–alanine. Males showed additional changes along the same axis in glucose, the galactoside and hydroxyisovalerate, and in the opposite direction in isoleucine and valine. Females showed additional changes in maltose, glutamate, glutamine and proline. No metabolite showed significant changes in opposite directions in males and females.

The individual metabolite changes that correlated with developmental time and viability are also shown in Fig. 7. The metabolites that correlated with the linear terms also correlate with developmental time, except for a reversal of the sign of the correlation coefficient (Fig. 7). Egg-to-adult viability shared a high number of metabolites with the u-shaped terms, but also here with a reversal of the sign of the correlation coefficient (Fig. 7).
Discussion

How organisms perceive and adapt to changes in temperature is central for our understanding of species ecology, species distributions and ecological networks. Numerous studies have used metabolomics to investigate associations between temperature and metabolite composition in ectotherms (10, 19, 32, 37, 39, 45). Here we investigated functional phenotypes and the metabolome in female and male flies from the developmental thermal range that *D. melanogaster* can tolerate, enabling estimation of reaction norms of groups of metabolites. The resulting relationship between metabolites and the functional phenotypes allows for an improved understanding of how organisms respond and adapt to cope with thermal stress via eco-physiological interactions.

Functional phenotypes

We propose that the functional phenotypes support the interpretation of the metabolomic results, whereby their detail and generality across species deserves some attention. The life-history data (egg-to-adult viability and developmental time) are in line with previous studies (14, 42, 57), and provides detailed information on consequences of developmental temperatures on fitness components. Our finding that upper thermal limits are less plastic than lower thermal limits is also in line with previous findings (22, 61). Thus studies investigating reaction norms of thermal limits in thirteen species of *Drosophila* (58), as well as studies on fish (31) and lizards (9) lead to the same conclusions. We also note that the linear increase of $CT_{\text{min}}$ with increasing temperatures is a characteristic shared across *Drosophila* species, while the slope of the linear increase of $CT_{\text{max}}$ is highly species specific (58). This linearity suggests that the physiological mechanisms involved in the acclimation are continuously adjusted, instead of an “edge” effect, in which a temperature threshold facilitates a given physiological mechanism, promoting a large change in thermal tolerance within a small temperature range. It is well known that males
develop slower than females across the entire thermal range (46). Less well established are the
coloristics of the sexual dimorphism that may exist in the reaction norms of upper and lower
thermal limits. We here show that the sexual dimorphism in CT\textsubscript{min} is very small, if present at all.
Conversely, females had consistently higher CT\textsubscript{max} than males, and with some indication that
this difference was largest at the lowest temperatures.

The metabolome and the relationship with functional phenotypes
As for the functional phenotypes, we also found large effects of the developmental temperature
on the metabolome. The majority of these effects were directional across developmental
temperatures, in accordance with these metabolites being a component of the physiological
thermal response of the organism (hypothesis ii). This group of metabolites mainly consisted of
amino acids that increased with decreasing developmental temperatures, a pattern which was
consistent across sexes (Figs. 3 and 7). The increase in free amino acids at lower temperatures is
in accordance with earlier studies on \textit{D. melanogaster} and \textit{Cydia pomonella} (10, 32, 52),
including proline, which has been shown to be important for cold acclimation in several insect
species (33, 34). We did not observe any consistent change in alanine, which has been related to
heat and cold shock responses (39, 43), but also seems to be less important for \textit{Drosophila}
species than in other insects (47). Glutamate and glutamine have been related to the heat shock
response in \textit{D. melanogaster} (39), but we did not find a consistent pattern for these metabolites,
and it is therefore likely that their regulation is unrelated to long-term heat acclimation.
Phosphocholine stood out as the only metabolite increasing at higher developmental
temperatures in both sexes, whereas the galactoside, which exclusively occurs in males (45),
also increased at higher temperatures (Fig. 7). Phosphocholine has not been connected to
thermal acclimation before, and given the strong correlation we show here, this makes it an
interesting new candidate metabolite involved in thermal acclimation. Phosphocholine is an
intermediate of phosphatidylcholine, a class of phospholipids and a well-known component of cell membranes. The increase of phosphocholine at higher temperatures is likely a result of restructuring of biological membranes as a part of thermal acclimation, but studies directed towards this metabolite are needed for further hypothesis development. $CT_{\text{min}}$ and $CT_{\text{max}}$ both correlated linearly with developmental temperature, making a correlation between these tolerance traits and the metabolome redundant in terms of identifying metabolites directly related to thermal resistance. The metabolite data presented characterize the constitutive metabolome, and does not reflect the changes that occur up until the point of critical temperature determination during the thermal tolerance assays. Nevertheless, it can be concluded that these metabolites are part of a large set of physiological mechanisms enabling insects to adapt plastically to diurnally and seasonally changing thermal environments. It also shows that the inherent structures of the metabolite changes and their temperature dependence share strong similarities with the temperature dependence of the functional phenotypes.

An increase in sugars with decreasing developmental temperatures has been reported in several studies and there is a consensus that these are important for long-term (10, 32) as well as short-term cold acclimation (43, 64). Central sugars from the above studies, such as fructose and trehalose, were not identified in the current study, but we found both mannose and to a lesser extent glucose to decrease at both extreme low and high developmental temperatures (Fig. 7). This pattern is unexpected, but confirms earlier suggestions of sugars to be related to a thermal stress response (10), and thus not only connected to cold acclimation but to thermal stress in general. Mannose and glucose were part of a group of metabolites showing an inverse u-shaped norm of reaction across temperatures (Figs. 3 and 7). These metabolites were also detected when we identified metabolites related to the non-linear norm of reaction of egg-to-adult viability (Figs. 1 and 7). Consistently across sexes, this group of metabolites, which is primarily related
to energy metabolism (NAD+, NADP+ and AMP), was depleted at the lowest and highest
temperatures. We interpret this group, together with the depleted sugars, as a set of “cost”
metabolites, which characterize a departure from cellular homeostasis, and thus challenges the
acclimation of the organism at the edges of the investigated environmental gradient (hypothesis
iii). These molecules are potential candidates for molecular estimators of cellular thermal stress
or stress in general, and constitute an example of a commonality between high and low
temperature stress. Such similarities indicate that apart from the commonly observed low and
high temperature specific responses (4, 36), shared cellular challenges during cold and heat
stress also exist. Indeed, results from selection experiments show how flies selected for
increased heat resistance are both more heat and cold resistant (5) while a short term heat shock
prior to cold shock can increase cold resistance (6).

Sex effects on the metabolome

In general, the effect of sex on the metabolome was stronger than the effect of developmental
temperature, except at low rearing temperatures (Fig. 5). This was supported by the PCA on
metabolomic sex differences, which provides evidence for a peak in sex differentiation at
intermediate temperatures, intermediate differentiation at high temperatures and low
differentiation at low temperatures (Fig. 4, see Fig. 7 for individual metabolites). The decrease
in differentiation between males and females at extreme temperatures is not consistent with
observations on thermal limits, and thus likely driven by the common metabolomic response to
stress at extreme temperatures. Strong sex effects on the metabolome have previously been
identified (21). We provide evidence that supports this conclusion, but also show that the
metabolomic differences between males and females are highly dependent on developmental
temperature and thus environment specific (Fig. 4). Male and female specific acclimation
responses have previously been observed in ectotherms (21, 63). Such dimorphism can lead to
different selection intensities in males and females exposed to stressful temperatures and ultimately lead to sex-specific life-history evolution as the degree of plasticity can impact on the strength of selection (51, 72). Our data pinpoint traits and physiological mechanisms, which in variable thermal environments, are likely to be under different selection pressures in males and females.

**Perspectives and Significance**

The presented NMR data provide novel insights into the underlying physiology of the large effects of developmental temperature observed in the functional phenotypes. We found developmental temperature to be a strong predictor of the metabolome, however this association was sex-dependent, especially at higher developmental temperatures. Evidence that the metabolome is a sensitive indicator of physiological state and biological age in *D. melanogaster* has previously been reported (21, 53). We show here that the metabolomic fingerprint also accurately predicts developmental temperatures by a mixture of “beneficial” metabolites related to the functional phenotypes cold and heat tolerance and “cost” metabolites with signatures associated with the fitness phenotype egg-to-adult viability through energy carrying molecules.
Acknowledgements

We are grateful for the technical assistance offered by Doth Andersen and Morten Bjerring, and to Johannes Overgaard for comments on the manuscript. This research was funded by the Graduate School of Science and Technology at Aarhus University to MFS, and by the Danish Natural Science Research Council with a frame grant to VL and a Sapere aude stipend to TNK (DFF – 4002-00036) and by the Department of Biomedical Sciences at the University of Copenhagen to AM.
References


37. **Kristensen TN, Overgaard J, Hoffmann AA, Nielsen NC, Malmendal A.** Inconsistent effects of developmental temperature acclimation on low-temperature performance and


45. Pedersen KS, Kristensen TN, Loeschcke V, Petersen BO, Duus J, Nielsen NC, Malmendal A. Metabolomic signatures of inbreeding at benign and stressful


58. Schou MF, Mouridsen MB, Sørensen JG, Loeschcke V. Linear reaction norms of thermal limits in *Drosophila*: predictable plasticity in cold but not in heat tolerance.


Figure 1. Functional phenotypes across developmental temperatures. Average thermal tolerance limits, CT\textsubscript{max} (A) and CT\textsubscript{min} (B), as well as egg-to-adult viability (C) and developmental time (D) across thermal regimes. Flies developed at ten different thermal regimes from egg to adult and remained there until their thermal tolerance limits were assessed at two-three days of age. Both CT\textsubscript{max} and CT\textsubscript{min} were linearly and positively correlated with developmental temperature, whereby heat tolerance was increased in flies that developed at higher temperatures while cold tolerance was increased in flies developed at lower temperatures. Egg-to-adult viability had a quadratic norm of reaction as flies developed at temperatures below 15°C and above 27°C had a decreased survival. We did not register sex specific egg-to-adult viability, and therefore use diamonds as points in display (C). Developmental time decreased with increasing temperatures. Minimal adequate models from the statistical analyses are plotted with the shaded area representing the 95% confidence interval. Parametric bootstrapping was used to obtain the confidence interval of the fitted line for developmental time, which was modeled with a mixed model. As we found no effect of sex in CT\textsubscript{min} only one line representing the pooled data from both sexes has been plotted. Error bars are standard errors of the mean.

Figure 2 \textsuperscript{1}H NMR spectra of \textit{D. melanogaster} metabolites. NMR spectra of male flies after acclimation at 12, 22 and 32°C (M12, M22 and M32, respectively). The displayed spectra show the median intensities of all spectra in that temperature regime. Spectra were acquired with a CPMG delay of 194 ms at 25°C and sample pH was 7.4. *The full name of the galactoside is 1-O-(4-O-(2-aminoethyl phosphate)-β-D-galactopyranosyl)-glycerol.

Figure 3. PCA on the overall metabolite variation across temperatures and sexes. (A) male and (B) female flies. Note that the axes in A and B do not necessarily represent the same metabolite changes. Below each PCA plot we have displayed rotated scores for males (C-D) and
females (E-F). The rotations were performed such that the first rotated component correlated as much as possible with the developmental temperature \((\text{linear component})\) and the next component explained as much as possible of the remaining variation \((\text{u-shape component})\). These scores represent the norm of reaction for the change in concentration across developmental temperatures for groups of correlated metabolites. The correlation of the rotated scores with different metabolites is presented in Fig. 7.

Figure 4. Sex differentiation in the metabolome across developmental temperatures. PC1 from a PCA based on the difference between individual female sample spectra and the median male spectrum at each temperature as well as the difference between median female spectrum and individual male sample spectra at each temperature. Higher scores in PC1 indicate a stronger differentiation between male and female samples, as the metabolite intensities was not centered. For comparison, the total score length across all averaged PCs (square roots of sum of squares) are also plotted for each developmental temperature. Minimal adequate models from the statistical analyses are plotted with the shaded area representing the 95% confidence interval.

Figure 5. Similarities between combinations of developmental temperatures and sex. The grouping at the dendrogram illustrates circumstances where the effect of sex on the metabolome was stronger than the effect of developmental temperature (22-32°C), and \textit{vice versa} (12-20°C). The dendrogram was derived by hierarchical cluster analysis of O2PLS-DA scores. The length of the vertical axis is a measure of the dissimilarities between clusters/groups of treatments. Males are displayed with triangles while females are displayed with circles. In the tip labels F/M indicate female/male, and the following number indicates the developmental temperature.

Figure 6. Prediction of developmental temperature from the metabolome. OPLS model of temperature regime based on male (filled triangles) and female (open circles) flies. The
predicted values are calculated using cross-validation with all measurements for one condition left out from the model calculation when predicting the temperature regime for that condition. The predictabilities of the models ($Q^2$) are 0.90 and 0.84 for males and females, respectively (Table 3). Positions were calculated as medians. Error bars represent one standard error ($n \leq 5$).

**Figure 7. Metabolite changes correlated to temperature regime and survival characteristics.** We identified reaction norms of individual metabolites across developmental temperatures using two hypotheses derived from rotated components of sex-specific PCA’s of the metabolome as well as two prior hypotheses. We calculated the correlation coefficient (R) between individual metabolite intensities and the *linear component*, which describes the PCA component rotated to show the highest correlation with developmental temperature (Fig. 3C-D), and the *u-shape component*, which was obtained as the second component after removing the linear variation (Fig. 3E-F). Reaction norms following our prior hypotheses were tested by correlating individual metabolite intensities with developmental temperature (*linear prior*), and with a categorical variable discriminating the two highest and lowest temperatures (12, 15.5, 31 and 32°C) from the intermediate temperatures (*u-shape prior*). The tests of these prior hypotheses, as well as the correlations to phenotypic traits developmental time and egg-to-adult viability, were calculated as the correlation coefficient (R) with OPLS model scores for the respective parameters. Egg-to-adult viability was evaluated for males and females together as there were no sex-specific egg-to-adult viability estimates. *Sex diff. F-M* is the first component in a PCA of the spectral differences between male and female samples (Fig. 4), whereby significant correlations with this parameter indicate that the given metabolite contributes to the sexual dimorphism observed in the metabolome. Significant correlations with the *linear component* and/or the *linear prior* shows evidence of an approximate linear norm of reaction of the given metabolite across developmental temperatures, with a negative correlation coefficient representing a decrease in concentration with increasing temperatures and *vice versa.*
Conversely, a significant correlation with the *u-shape component* and/or the *u-shape prior*, illustrates that the change in concentration from intermediate temperature to high and low temperatures is in the same direction. Here a negative correlation coefficient represents a decrease in concentration at extreme temperatures (bell-shaped reaction norm), while a positive correlation coefficient represents an increase in concentration at extreme temperatures (u-shaped reaction norm). Significant spectral correlations were identified by applying sequential Bonferroni correction (P < 0.05) for an assumed total number of 100 metabolites. Only significant correlations are presented. Note that although this procedure minimizes the false positives, it does nothing to limit the false negatives. *The full name of the galactoside is 1-O-(4-O-(2-aminoethyl phosphate)-β-D-galactopyranosyl)-glycerol.*
Table 1. $^1$H NMR chemical shifts and integration range used for correlations. The identity of the metabolites was verified using all the listed chemical shifts. The signal intensities used for the correlations presented in Fig. 7 were calculated as the total intensity within the integration range. Note that some of the signals used to estimate intensities of different metabolites overlap with other signals.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chemical Shifts (ppm)</th>
<th>Integration Range (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD+</td>
<td>9.32, 9.13, 8.82, 8.42, 6.08</td>
<td>9.146, 9.128</td>
</tr>
<tr>
<td>NADP+</td>
<td>9.28, 9.09, 8.82, 8.40, 8.13</td>
<td>8.832, 8.810</td>
</tr>
<tr>
<td>AMP</td>
<td>8.60, 8.25, 6.13</td>
<td>8.609, 8.584</td>
</tr>
<tr>
<td>glucose</td>
<td>5.21</td>
<td>5.213, 5.207</td>
</tr>
<tr>
<td>maltose</td>
<td>5.40, 5.22</td>
<td>5.406, 5.398</td>
</tr>
<tr>
<td>mannose</td>
<td>5.17</td>
<td>5.174, 5.169</td>
</tr>
<tr>
<td>galactoside*</td>
<td>4.46, 4.18, 3.93, 3.76, 3.61</td>
<td>4.456, 4.448</td>
</tr>
<tr>
<td>fatty acid</td>
<td>5.31, 1.28, 0.89</td>
<td>5.325, 5.288</td>
</tr>
<tr>
<td>3-hydroxykynurenine</td>
<td>7.44, 6.89, 6.69</td>
<td>6.704, 6.677</td>
</tr>
<tr>
<td>acetate</td>
<td>1.93</td>
<td>1.933, 1.930</td>
</tr>
<tr>
<td>alanine</td>
<td>1.47</td>
<td>1.474, 1.460</td>
</tr>
<tr>
<td>beta-alanine</td>
<td>3.16, 3.54</td>
<td>2.552, 2.530</td>
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<tr>
<td>arginine</td>
<td>1.91, 1.73</td>
<td>1.742, 1.723</td>
</tr>
<tr>
<td>asparagine</td>
<td>2.93, 2.83</td>
<td>2.930, 2.916</td>
</tr>
<tr>
<td>aspartate</td>
<td>2.80, 2.65</td>
<td>2.796, 2.785</td>
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<td>glutamate</td>
<td>2.34, 2.12, 2.05</td>
<td>2.360, 2.320</td>
</tr>
<tr>
<td>glutamine</td>
<td>2.44, 2.12</td>
<td>2.460, 2.420</td>
</tr>
<tr>
<td>histidine</td>
<td>7.78, 7.05</td>
<td>7.055, 7.043</td>
</tr>
<tr>
<td>hydroxyisovalerate</td>
<td>2.34, 1.24</td>
<td>1.248, 1.239</td>
</tr>
<tr>
<td>isoleucine</td>
<td>1.00, 0.92</td>
<td>1.007, 0.989</td>
</tr>
<tr>
<td>lactate</td>
<td>4.10, 1.31</td>
<td>1.321, 1.306</td>
</tr>
<tr>
<td>leucine</td>
<td>1.73, 0.95</td>
<td>0.962, 0.930</td>
</tr>
<tr>
<td>methionine sulfoxide</td>
<td>3.88, 3.01, 2.74, 2.32</td>
<td>2.739, 2.734</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>7.41, 7.31</td>
<td>7.424, 7.401</td>
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<tr>
<td>phosphocholine</td>
<td>4.15, 3.58, 3.21</td>
<td>4.163, 4.138</td>
</tr>
<tr>
<td>proline</td>
<td>2.12, 2.01</td>
<td>2.039, 1.995</td>
</tr>
<tr>
<td>tryptophan</td>
<td>7.72, 7.52, 7.31</td>
<td>7.729, 7.711</td>
</tr>
<tr>
<td>tyrosine</td>
<td>7.18, 6.89</td>
<td>7.194, 7.169</td>
</tr>
<tr>
<td>valine</td>
<td>1.03, 0.98</td>
<td>0.987, 0.970</td>
</tr>
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</table>
Table 2. Statistics of functional phenotypes. Results from the statistical analysis of the change in cold tolerance (CT_{min}), heat tolerance (CT_{max}), egg-to-adult viability and developmental time as a function of developmental temperature. We used sequential model reduction to find the minimal adequate model, such that model reductions were halted if predictors were part of a significant interaction. Effect sizes from the minimal adequate model are presented together with test statistics and significance level for the tested parameters. CT_{min} and CT_{max} were analyzed with linear models and P-values were obtained with F-tests. Egg-to-adult viability and developmental time were both analyzed with generalized linear models with temperature as a quadratic term in which the significance of fixed effects was assessed with likelihood ratio tests. All model coefficients given are for males (except for egg-to-adult viability), and are thereby the deviation from the females. The second-degree term is given in parentheses. **P < 0.01; ***P < 0.001.
<table>
<thead>
<tr>
<th>Trait</th>
<th>Parameter</th>
<th>Estimate</th>
<th>s.e.</th>
<th>$F_{(d.f.)}$ / $\chi^2_{(d.f.)}$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$CT_{\text{min}}$</td>
<td>Intercept</td>
<td>-4.578</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Developmental temperature</td>
<td>0.412</td>
<td>0.004</td>
<td>9852.20 $(1,434)$</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>-</td>
<td>-</td>
<td>0.19 $(1,434)$</td>
<td>0.662</td>
</tr>
<tr>
<td></td>
<td>Sex*Developmental temperature</td>
<td>-</td>
<td>-</td>
<td>1.73 $(1,433)$</td>
<td>0.189</td>
</tr>
<tr>
<td>$CT_{\text{max}}$</td>
<td>Intercept</td>
<td>37.601</td>
<td>0.130</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Developmental temperature</td>
<td>0.123</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>-0.692</td>
<td>0.184</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sex*Developmental temperature</td>
<td>0.019</td>
<td>0.008</td>
<td>6.79 $(1,349)$</td>
<td>0.009**</td>
</tr>
<tr>
<td>Egg-to-adult viability</td>
<td>Intercept</td>
<td>0.66</td>
<td>0.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Developmental temperature</td>
<td>-0.81 (-4.85)</td>
<td>0.50 (0.53)</td>
<td>212.53 $(2)$</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Developmental time</td>
<td>Intercept</td>
<td>2.55</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Developmental temperature</td>
<td>-26.56 (9.61)</td>
<td>0.28 (0.25)</td>
<td>501.24 $(2)$</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>0.03</td>
<td>0.01</td>
<td>9.67 $(1)$</td>
<td>0.002**</td>
</tr>
<tr>
<td></td>
<td>Sex*Developmental temperature</td>
<td>-</td>
<td>-</td>
<td>0.24 $(2)$</td>
<td>0.930</td>
</tr>
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</table>
Table 3. OPLS model statistics for parameter prediction from metabolite data.

The capability of the *D. melanogaster* metabolome to predict the environmental parameter developmental temperature was tested using OPLS models. The test was done both with developmental temperature as a continuous parameter (*linear prior*), as well as a categorical parameter that discriminates the two highest and lowest temperatures (12, 15.5, 31 and 32°C) from the intermediate temperatures (*u-shape prior*). We also assessed the predictability of developmental time and egg-to-adult viability. All parameters except egg-to-adult viability were predicted separately for each sex.

<table>
<thead>
<tr>
<th>Predicted parameter</th>
<th>Metabolome</th>
<th>A</th>
<th>N</th>
<th>$R^2$</th>
<th>$Q^2$</th>
</tr>
</thead>
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<td><strong>Linear prior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>1+2</td>
<td>46</td>
<td>0.58</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1+2</td>
<td>50</td>
<td>0.46</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td><strong>U-shape prior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1+1</td>
<td>46</td>
<td>0.48</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1+1</td>
<td>50</td>
<td>0.33</td>
<td>0.73</td>
<td></td>
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<tr>
<td>Developmental time</td>
<td>Male</td>
<td>1+2</td>
<td>46</td>
<td>0.58</td>
<td>0.71</td>
</tr>
<tr>
<td>Female</td>
<td>1+3</td>
<td>50</td>
<td>0.64</td>
<td>0.60</td>
<td></td>
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<tr>
<td>Egg-to-adult viability</td>
<td>Both</td>
<td>1+2</td>
<td>96</td>
<td>0.51</td>
<td>0.61</td>
</tr>
</tbody>
</table>

1 *A* describes the number of model components where the first number accounts for the predictive component(s) correlating with the predicted variable, and the second the orthogonal component(s).

2 *N* describes the number of observations included in the model.

3 $R^2$ describes how much of the total metabolite variation that is explained by the model.

4 $Q^2$ represents the predictability of the total model and is related to the statistical validity of the model. $Q^2 \geq 0.5$ is considered significant and is bold in the table. $Q^2$ was calculated using cross-validation with all measurements for one condition left out at a time.
M12
1H chemical shift (ppm)
10 9.5 9 8.5 8 7.5 7 6.5 6 5.5 5 4.5 4 3.5 3 2.5 2 1.5 1 0.5
maltose
glucose/maltose
valine/tyrosine
proline
phosphocholine
methionine sulfoxide
leucine
lactate
isoleucine/histidine
glutamine
glutamate
aspartate/asparagine
alanine/acetate
fatty acid
galactoside
mannose
AMP
NAD+

M22

M32

1H chemical shift (ppm)
10 9.5 9 8.5 8 7.5 7 6.5 6 5.5 5 4.5 4 3.5 3 2.5 2 1.5 1 0.5
maltose
fatty acid
mannose
Residual water
galactoside
phosphocholine
β-alanine
serine
glutamine
acetate
arginine
alanine
fatty acid
valine
isoleucine/leucine
fatty acid
Actual developmental temperature (°C)

Predicted developmental temperature (°C)

Females

Males
<table>
<thead>
<tr>
<th></th>
<th>Linear component (M)</th>
<th>Linear component (F)</th>
<th>U−shape component (M)</th>
<th>U−shape component (F)</th>
<th>Linear prior (M)</th>
<th>Linear prior (F)</th>
<th>U−shape prior (M)</th>
<th>U−shape prior (F)</th>
<th>Developmental time (M)</th>
<th>Developmental time (F)</th>
<th>Egg−to−adult viability</th>
<th>Sex diff. F−M</th>
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<tr>
<td>nicotinamide ribotide</td>
<td>0.76</td>
<td>0.82</td>
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<td></td>
<td>0.55</td>
<td>-0.69</td>
<td>-0.69</td>
<td>-0.88</td>
<td>-0.41</td>
<td>0.65</td>
<td>0.61</td>
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<tr>
<td>NAD+</td>
<td>-0.78</td>
<td>-0.8</td>
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<td>0.61</td>
<td>0.61</td>
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<td>NADP+</td>
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<td>-0.75</td>
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<td></td>
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<tr>
<td>AMP</td>
<td>-0.55</td>
<td>-0.73</td>
<td>-0.53</td>
<td>-0.87</td>
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<tr>
<td>glucose</td>
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<td>-0.57</td>
<td>-0.6</td>
<td>0.46</td>
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<td>maltose</td>
<td>0.65</td>
<td>-0.84</td>
<td>0.76</td>
<td>-0.84</td>
<td>-0.56</td>
<td>-0.73</td>
<td>0.84</td>
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<td>mannose</td>
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<td>-0.84</td>
<td>-0.76</td>
<td>-0.84</td>
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<td></td>
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<td>0.94</td>
<td>0.84</td>
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<tr>
<td>fatty acid</td>
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<td>-0.6</td>
<td>-0.71</td>
<td>-0.61</td>
<td>0.68</td>
<td>0.84</td>
<td>0.76</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3−hydroxykynurenine</td>
<td>-0.68</td>
<td>-0.62</td>
<td>0.6</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>acetate</td>
<td>0.79</td>
<td>0.75</td>
<td>-0.63</td>
<td>-0.83</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>alanine</td>
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<td>-0.78</td>
<td>-0.71</td>
<td>-0.72</td>
<td>-0.58</td>
<td>-0.61</td>
<td>0.72</td>
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