

## Lipocalin gene expression is varied in developmental stages by larval nutritional stress in *Drosophila*

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**Abstract:** Lipocalin genes *NLaz*, *GLaz*, and *Karl* are evolutionarily conserved genes in *Drosophila melanogaster*. There are studies on lipocalin gene expression differences under diverse diet conditions, but these studies have focused mainly on age-dependent expression profiles of these genes. The main aim of our study is to determine lipocalin expression in the developmental period by nutritional manipulation with an isofemale-based design. Three larval developmental periods have been researched under normal and restricted diets. We found significant differences between lines during their developmental time-related lipocalin expression. Here, we demonstrate that upregulations in the early developmental stages of lipocalin genes under stressful conditions resulted in unaffected developmental time. The possible reason for high expression is the activation of stress signal pathways in order to buffer the harmful effects of nutritional restriction. Our data showed that the early developmental period (48–72 h) is especially crucial to tolerate the dietary stress with respect to *GLaz* and *NLaz* expression. Results of this experiment have shown that the expression profiles of lipocalin genes have line-specific pathways to nutritional stress. Their expression depends on the genetic background corresponding to development time results. Our results highlight the transcriptional changes of lipocalins associated with developmental time in larvae, developed in a dietary-restricted medium.

**Key words:** Dietary stress, lipocalin gene family, *ApoD*, developmental time, *Drosophila melanogaster*

### 1. Introduction

Maintaining metabolic homeostasis is crucial for multicellular organisms (Torday and Rehan, 2012); in particular, developmental stage stability under changing environmental conditions has an important role for organisms' adaptation to the environment (Flatt and Heyland, 2011). When organisms encounter environmental stress, the characters of organisms are shaped by evolutionary adaptive mechanisms to buffer the environmental stress factors (Koehn and Bayne, 1989; Bijlsma and Loeschcke, 2005).

In nature, one of the most common environmental stresses is nutritional stress (Robbins, 1983). To imitate nature, dietary restriction has been used artificially in life history studies for almost a century. The first record of correlation between lifespan and nutrition was shown in white rats by McCay et al. (1935). Studies that followed focused on the dietary effects on different life history traits such as developmental time, lifespan, fecundity, and body size in different model organisms (Robertson, 1960;

Chippindale et al., 1993, 1997; Roth et al., 1999; Jiang et al., 2000; Lin et al., 2002; Shingleton et al., 2008; Metaxakis and Partridge, 2013; Zhu et al., 2014). These studies showed how organisms shape their life history traits under stressful conditions to buffer the harmful effects of unfavorable conditions by the regulation of stress signals (Chippindale et al., 1997; Minois, 2000; Tu and Tatar, 2003; Partridge et al., 2005; Burger et al., 2007; Doroszuk et al., 2012).

It is known that nutrition-dependent insulin signals have an important role in stress pathways (Schenk et al., 2008). One of the stress-responsive signaling pathways, c-Jun-N-terminal kinase (JNK), also known as the stress-activated protein kinase, has been identified in flies and worms (Giannakou et al., 2007; Wolf et al., 2008). Additionally, studies on lipocalin family genes showed that stress-responsive signal pathways have important roles in the regulation of lipocalin genes and metabolic adaptation of the organism to environmental challenges (Hull-Thompson et al., 2009; Pasco and Léopold, 2012;

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Ruiz et al., 2012). Lipocalin genes have diverse functions, which are protein transportation, cryptic coloration, olfaction, pheromone transport, the enzymatic synthesis of prostaglandins, and the mediation of cell homeostasis (Flower, 1996). The lipocalin gene family is evolutionarily conserved from flies to vertebrates. Neural Lazarillo (*NLaz*) and glial Lazarillo (*GLaz*), which are expressed in the *Drosophila* brain during the development of nervous system, are two homolog genes of human lipocalin gene apo-lipoprotein D (*ApoD*) (Ruiz et al., 2011). The other lipocalin gene, *Karl*, is not modified in *Drosophila*'s nervous system, unlike *NLaz* and *GLaz* (Hull-Thompson et al., 2009; Dassati et al., 2014).

Lipocalin family gene expression profile differences have been identified in various nutritional stress conditions. *NLaz* transcription's relationship with oxidative stress and JNK signaling in the fat body was reported in previous studies (Thompson, 2008; Hull-Thompson et al., 2009). Recent research on *Drosophila* with high sugar diets showed that *NLaz* and *GLaz* expression is affected by a high sugar diet, but *Karl* expression is not modified with differing nutrition (Pasco and Léopold, 2012). Additionally, high expression of *NLaz* inhibits growth while increasing the organism's stress and starvation resistance (Hull-Thompson et al., 2009). For *GLaz*, the importance of gene expression for stress tolerance and lifespan was shown in former studies (Sanchez et al., 2006; Walker et al., 2006).

For holometabolous organisms, the developmental stage is a critical period for the nervous system due to environmental pressure (Rice and Barone, 2000). Developmental stage stability is highly related to organism fitness, which depends critically on growth and development. Developmental time has many different responses on other life history characters, such as short development time, decreasing sexual maturity age, and generation time, thus increasing preadult survival by shortening the developmental period, which has a high predatory risk. On the other hand, long developmental time leads to a longer growth period and bigger body size, which increases mating success (Stearns, 1992; Stearns et al., 2000). A negative correlation between dietary protein content and developmental time has been shown (Shingleton et al., 2008; Kolss et al., 2009). Restricted protein has an important role in larvae to pupae developmental time (Güler et al., 2014) due to different stress signals.

From this point of view, the main aim of our research was to identify gene expressions of *NLaz*, *GLaz*, and *Karl* during developmental time under standard and restricted food conditions. For that purpose, we designed an inbred line-based experiment to introduce genetic background effects to gene expression profiles. We compared developmental stage-related gene expressions

between the larvae of long- and short-developed inbred lines with standard and restricted diets. We investigated the relationship between lipocalin genes and larvae development under unfavorable dietary conditions for both lines. This study provides the first description of dietary effects on lipocalin transcriptional phenotypes in *D. melanogaster* larval development. Furthermore, our results identify the importance of lipocalin genes acting as a part of a stress response that adjusts developmental time in response to dietary restriction.

## 2. Materials and methods

### 2.1. *Drosophila* strains and maintenance

*D. melanogaster* populations were collected from Firtina Valley, Turkey, in 2009. One hundred isofemale lines were generated from females and all lines were maintained in standard laboratory conditions (25 °C, 12-h light and 12-h dark cycle, 55 ± 5% humidity). Fruit flies were reared in a standard sugar/yeast/agar (SY) medium (50 g/L yeast, 50 g/L sugar, 10 g/L agar, and 30 ml/L Nipagin based on the protocol of Bass et al. (2007)). All handling of flies was performed at room temperature by using CO<sub>2</sub> anesthesia. For experiments, we used a standard diet and a restricted diet, which were modified only by dilution of yeast (10 g/L).

We used an isofemale-based design, which is one of the most common methods in population genetics studies with *Drosophila* (David et al., 2005).

### 2.2. Developmental time measurement techniques

Ten isofemale lines were randomly chosen among stock colonies. For the developmental time experiment, adults were maintained in small chambers with agar and yeast paste petri dishes. After 24 h of incubation time, first-instar larvae were collected from each line. Each vial included 20 first-instar larvae and 10 vials (technical replicates) were used for the standard diet and 15 replicates for the restricted diet. The number of pupae and adults emerging from these cultures was recorded every 12 h. Larva to pupa, pupa to adult, and larva to adult developmental times were recorded for each line for the two different diet regimes.

Two lines that showed high and low developmental time differences under standard and restricted diets were selected based on their developmental time data.

After the selection, the developmental time experiment was repeated for the selected lines. First-instar larvae were collected from standard and restricted diet groups by using small chambers as described previously. At 48, 72, and 96 h, 5–10 larvae were picked up from the food medium and stored at –80 °C for molecular studies.

### 2.3. RNA isolation, cDNA synthesis, and real-time qPCR

RNA was isolated from 5–10 pooled larvae by homogenization in QIAzol Lysis Reagent (QIAGEN)

and treated with DNase. The RNA was quantified using a NanoDrop 2200 (Thermo Fischer Scientific). Total RNA was converted into cDNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis kit with oligo (dT) primers. For all lines, we performed two biological and two technical replicates. Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN) in a QIAGEN Rotor Gene machine with a mixture of primers according to the manufacturer's instructions. Expression of each gene relative to the housekeeping genes *α-tubulin* and *eIF-1A* was determined by the  $\Delta\Delta C_t$  method. The following PCR primers were used: *NLaz* primers, forward: 5'-CATTGACGAAGGCCAACTA-3', reverse 5'-GGATACATCGTTGTCTTCCA-3'; *GLaz* primers, forward: 5'-AGATGCCTACGGATTGGA-3', reverse 5'-TACGGCTCAAAGTCAAAGG-3'; *Karl* primers, forward: 5'-GGTTCAGTATTACGCATCCAC-3', reverse 5'-TATATGTCCTCCGTGTGCTG-3'; *α-tubulin* primers, forward: 5'-GTTTGTCAAGCCTCATAGCC-3', reverse 5'-TGGATAGAGATACATTACGCA-3'; *eIF-1A* primers, forward: 5'-TCACATTCGGGGGAACTTCG-3', reverse 5'-GTACGTCTTCAGGTTCTGGC-3'. Cycling conditions were 15 min at 95 °C (15 s 94 °C, 30 s 55 °C, 30 s 72 °C) × 45, followed by 5 min at 90 °C. Gene regions of 146 bp, 120 bp, and 169 bp were amplified respectively for *NLaz*, *GLaz*, and *Karl*.

**2.4. Analysis**

Nested analyses of variance (ANOVAs) were used to test the effects of protein concentration on larva to pupa and larva to adult developmental time. One-way ANOVA was used to test the statistical significance of the differences between larval development on standard and restricted food. All statistical analyses were done using SPSS 20.

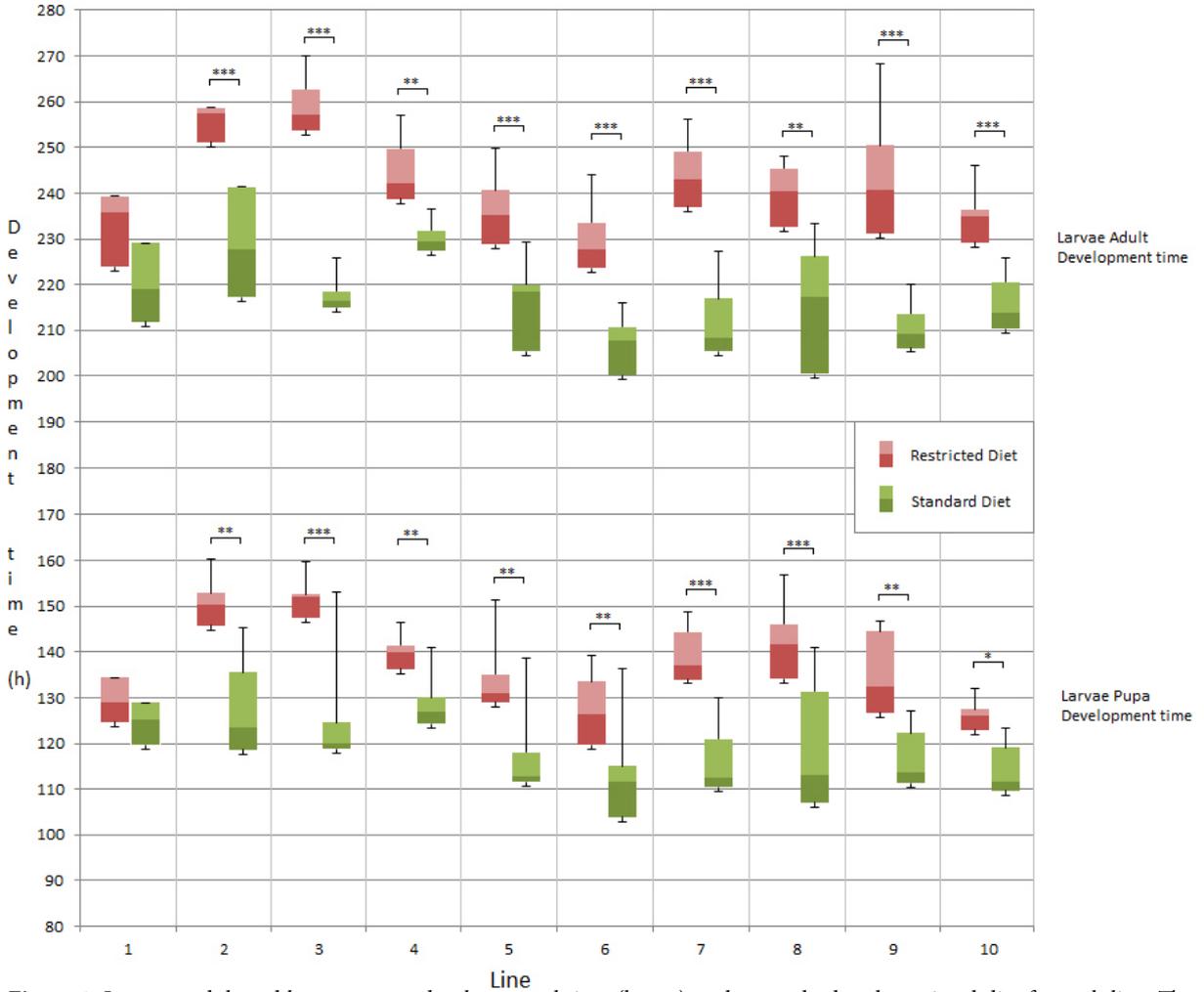
Gene expression analyses were done with SA Bioscience RT2 Profiler PCR Array Data Analysis Version 3.5 (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). This software calculated fold regulation values for each gene using the relative quantification  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001).  $\Delta C_t$  values were normalized using the mean values of two housekeeping genes: *α-tubulin* and *eIF-1A*. Student's t-test was performed with obtained data using SPSS 20.

**3. Results**

Based upon the knowledge of environmental stress signal pathways' effects on lipocalin genes expression profiles, we investigated the gene expression profiles of *GLaz*, *NLaz*, and *Karl* under standard and restricted protein conditions during developmental stages. We chose "developmental time" as a critical parameter because it is known to be affected by nutritional conditions. We focused on the expression changes in the larvae stages and investigated how much of their expression is contributed by the developmental stages. These data can explain the hypothetical relationship between developmental time and lipocalin expression, which is known to regulate stress resistance in fruit flies. To select the isofemale lines for gene expression studies, preexperiments were performed to investigate developmental time. We found variable effects of developmental time by diet restriction. The results of the two-way nested ANOVA of developmental time are presented in the Table. The line and diet effects on variation of developmental time were highly significant in both developmental stages ( $P < 0.001$ ). Genetic changes often have quantitatively variable effects on an organism's phenotype in different genetic backgrounds and in different environments. Figure 1 represents developmental

**Table.** Two-way nested ANOVAs testing for differences in mean developmental time between isofemale lines, diets, and their interactions.

Source of variation	df	Mean square	F	P
<b>(a) Larva to pupa developmental time</b>				
Isofemale line	9	12,011.843	38.889	<0.001
Diet	1	213,991.971	692.819	<0.001
Isofemale line × diet	9	2515.197	8.143	<0.001
Error	2694	308.872		
<b>(b) Larva to adult developmental time</b>				
Isofemale line	9	13,500.994	34.420	<0.001
Diet	1	389,051.271	991.861	<0.001
Isofemale line × diet	9	5385.242	13.729	<0.001
Error	2532	392.244		



**Figure 1.** Larva to adult and larva to pupa developmental time (hours) under standard and restricted diet for each line. The y-axis shows the developmental time in hours. The x-axis shows the isofemale lines coded from 1 to 10, and error bars show standard errors of the means. One-way ANOVA with Bonferroni post hoc test was used for comparisons. Significant levels are indicated: \* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001.

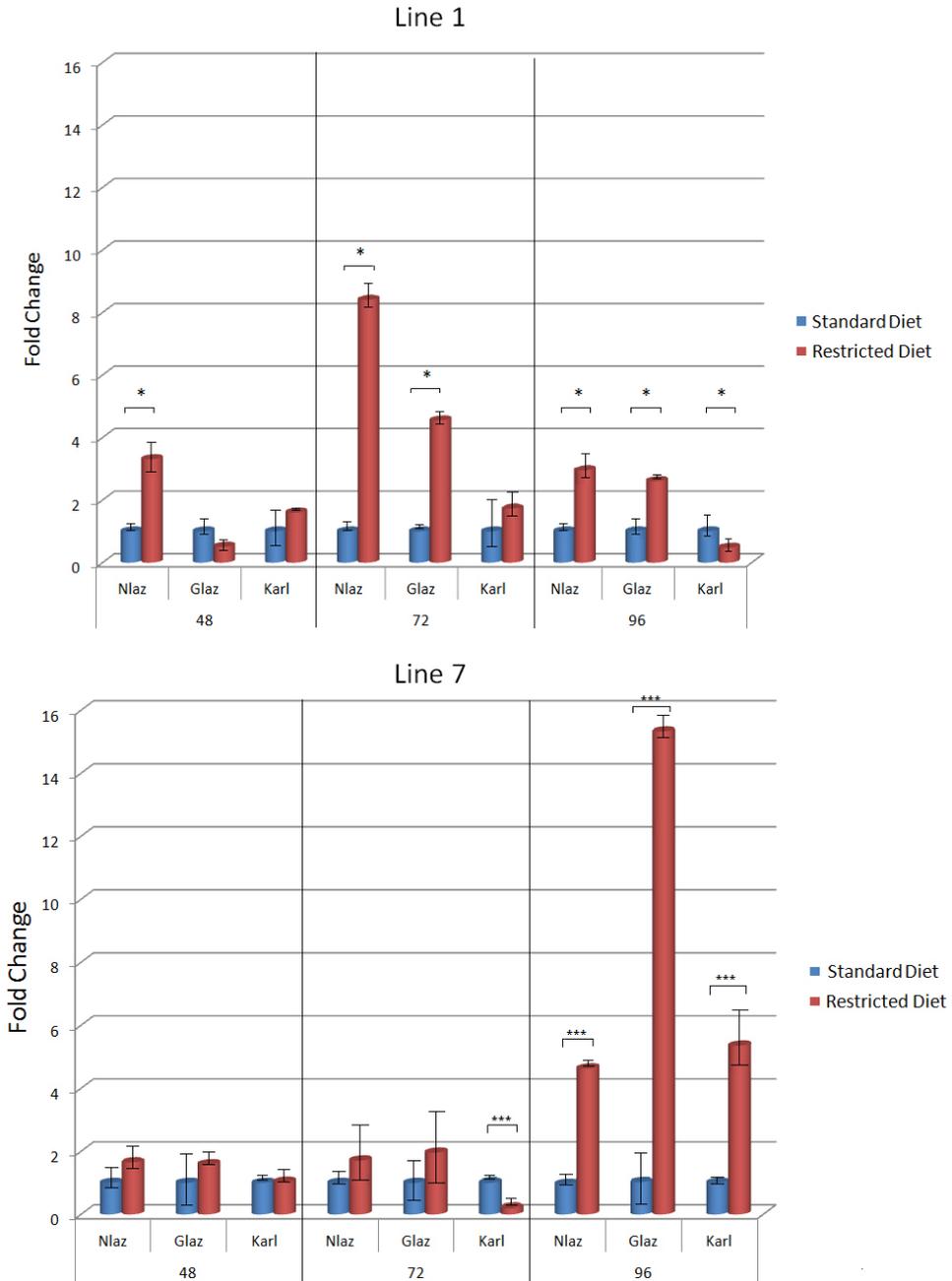
time from larva to pupa and larva to adult under two different diet conditions for ten isolines. Developmental time data showed genotype-dependent responses. One-way ANOVA with a Bonferroni post hoc comparison was performed to determine the effect of diet.

Although each line had a specific pathway for its developmental time, almost all lines except the first line showed statistically longer developmental time under nutritional stress, especially from the larva to pupa period (Figure 1). This result is confirmed by previous research and literature (Onder and Yilmaz, 2009; Önder, 2010; Matzkin et al., 2011; Güler et al., 2014; Neethu et al., 2014); a negative relationship has been shown between the reachability of a protein resource and developmental time.

For gene expression studies we chose two lines out of ten to investigate developmental time in standard and

restricted dietary conditions. One of the chosen lines was the first line, which showed no statistically significant developmental time difference between the larvae raised on standard versus restricted diets (Figure 1). The second was the 7th line, which showed significant developmental time differences between standard and restricted diets, unlike Line 1 (Figure 1).

To further investigate the developmental time differences between Line 1 and Line 7, we focused on age-related expression in different stages of the development period. Figure 2 presents *NLaz*, *GLaz*, and *Karl* expressions under standard and restricted diet conditions in three different periods of the developmental stages: 48, 72, and 96 h of larval development. The relative contribution of different developmental stages in lipocalin expressions also varied with lines. Additionally, our analyses strongly



**Figure 2.** Change of *NLaz*, *GLaz*, and *Karl* gene expressions under standard and restricted diet conditions for two selected lines: Line 1 and Line 7. The y-axis shows the fold change of expression levels (mean  $\pm$  SE). The x-axis shows the genes for each analyzed developmental time period in hours. Standard diet conditions were used as the control. Error bars show standard errors of the means. Significance was determined by Student's t-test. \*\* P < 0.01; \*\*\* P < 0.001.

suggest that the difference in growing larvae on restricted diet between Line 1 and Line 7 is associated with changes in the expression of lipocalin genes. Diet affected gene expression for the lines we analyzed. Larvae of Line 1 had stable *Karl* expression levels with a slight decrease at 96 h in all investigated developmental stages. Among the other

tested molecules in Line 1 we found that transcription of the lipocalin *NLaz* was potently induced in 72-h-old larvae grown on restricted medium. Larvae raised on restricted diets showed an eightfold increase in *NLaz* expression as measured by RT-qPCR (Figure 2). Similarly, *GLaz* expression showed a fourfold increase in the same

experimental group. Additionally, at 96 h larvae raised on restricted diets showed a twofold increase in *NLaz* and *GLaz* expression in Line 1. However, in Line 7, *NLaz* and *GLaz* were only significantly upregulated at 96 h in larvae raised on the restricted diet. *GLaz* showed a 15-fold increase at this developmental time point (Figure 2).

Interestingly, the levels of lipocalins were correlated with the developmental time of the selected lines. When lipocalin expression in restricted medium did not differ from the standard medium in the 48-h and 72-h larvae, developmental time was affected in an extended way. Otherwise, if lipocalin expressions increased in early developmental stages, developmental time was minimally affected by dietary stress.

#### 4. Discussion

The main objective of our study was to identify the lipocalin gene expression profiles in larval development under two different diet conditions. We used two isofemale inbred lines selected according to their developmental time on standard and restricted diets. The selected lines' larvae were collected at three different development points, 48, 72, and 96 h, and analyzed for lipocalin gene expressions for the two diets. The results of the lines' developmental time and lipocalin gene expression contribute to the understanding of the relationships between diet stresses, lipocalin genes, and developmental time. This is an important result of this study because most previous studies mainly focused on lipocalin gene expression changes in adult flies.

We found that the gene expression of *NLaz* for Line 1 increased significantly under stressful conditions for each developmental stage. Line 7, which showed significantly high developmental time differences between restricted and standard diet conditions, also showed similar profiles for *NLaz* gene expression at 48 and 72 h under standard (control) and restricted conditions. Flies lacking in *NLaz* have decreased energy storage and show a decreased resistance to starvation; however, overexpression of *NLaz* results in an increase of starvation resistance (Rong et al., 2002). In agreement, Hull-Thompson et al. (2009) showed that loss of *NLaz* function reduces stress resistance and lifespan in flies. Accordingly, *NLaz* is transcriptionally regulated by JNK signaling and is required for JNK-mediated stress and starvation tolerance. Reduced activity of the insulin/insulin-like growth factor signaling (IIS) pathway increased stress tolerance and prolonged lifespan (Kapahi et al., 2004), consistent with phenotypes occurring in the overexpression of *NLaz* (Hull-Thompson et al., 2009). We demonstrated that expression of the *NLaz* gene was higher in restricted groups than standard in all analyzed developmental time groups and that it increased with the developmental period in Line 1 while showing a sharp increase at 72 h. Differently, in Line 7 *NLaz* remained

at a similar level to that of the control at the beginning at the developmental period and showing a sharp increase at 96 h. These results follow the previous findings from the phenotypical level, where higher expression levels of *NLaz* were found in relation to increased stress tolerance (Rong et al., 2002; Hull-Thompson et al., 2009). JNK signaling is activated in flies as a result of dietary restriction (Clancy et al., 2002). However, JNK signaling influences IIS signaling indirectly through the action of lipocalin signals (Hull-Thompson et al., 2009). These findings showed that *NLaz* expressed in early developmental stages was characterized by the same developmental time in the two dietary conditions as compared with those in late-expressed developmental stages, with extended developmental time in a dietary-restricted medium. The comparisons of the developmental stage-dependent expression between the lines showed divergent expression patterns between the lines. We can speculate that increasing expression of the *NLaz* gene occurring in the early developmental period can be characterized by increased dietary stress tolerance in larval developmental time.

For *GLaz* expression in Line 1, an increase at 72 and 96 h but not at 48 h was detected as a probable reason for late development stage genetic expression. Line 1 is the line that showed no significant developmental time differences between restricted and standard diet conditions; in other words, dietary stress did not affect developmental time in Line 1. *GLaz* overexpression resulted in a longer lifespan and an increase in resistance to starvation and hyperoxia in adult flies (Walker et al., 2006). In contrast, *GLaz*-null mutant flies showed a decrease in mean lifespan and lower resistance to starvation (Sanchez et al., 2006). It is known that *GLaz* expression is increased with a high sugar diet (Pasco and Leopold, 2012) and stress like hyperoxia, high temperatures, and paraquat exposure (Muffat et al., 2008). From these points of view, it can be speculated that restricted yeast in the diet leads to a stress response at the *GLaz* expression level. The overexpression of *GLaz* and *NLaz* together protects the larvae from the detrimental effects of diet restriction on developmental duration. In the analysis of Line 7, like *NLaz*, the expression of *GLaz* between standard (control) and restricted groups only differed significantly in the late stages of larval development at 96 h. This analysis indicated that the developmental hour-related upregulation of the lipocalins under dietary stress conditions can be involved in the developmental length. As a result of upregulation of *NLaz* and *GLaz*, it is likely that individuals could buffer the effects of nutritional stress. This result is quite similar to the previous study of Thompson (2008), which indicated that *NLaz* acts downstream of JNK to promote starvation and stress and tolerance-maintained metabolic homeostasis. With respect to increasing gene expressions of *NLaz* and *GLaz*, organisms become more resistant to dietary stress. The

relationship between the *NLaz* and *GLaz* genes and stress resistance has been indicated by previous studies (Sanchez et al., 2006; Walker et al., 2006; Thompson, 2008; Hull-Thompson et al., 2009).

Unlike *NLaz* and *GLaz*, *Karl* gene expression showed no significant increase during development in Line 1; on the contrary, *Karl* was downregulated at 96 h of the development period in Line 1. These results correspond with Pasco and Léopold's study (2012), which showed that the expression of *Karl* is not modified by changing the sugar component. Previous work indicated that a P element insertion in the *Karl* gene leads to the extension of fly development time to about 100 h (Mensch et al., 2008). Therefore, *Karl* is a candidate gene that is associated with developmental time but transcriptional data about the study are not available. In Line 7, *Karl*'s expression decreased in the restricted group at 72 h of the development period. However, at 96 h high expression was shown under stressful conditions rather than the standard conditions for all lipocalin genes. A possible reason for the overexpression could be the pupation stage effects on metabolic homeostasis. Expression data show that *Karl* expression is very low in the developmental stages (Chintapalli et al., 2007); however, our results show that nutrient manipulation can lead to overexpression of *Karl*, dependent on the genetic background in developmental stages. An opinion is that the *Karl* gene modulates insulin signaling like *NLaz* (Hull-Thomson et al., 2009).

Our results have indicated major differences between Line 1 and Line 7 at the phenotypical and molecular levels. This is an interesting finding as the analyses performed for both lines and two diets indicate lipocalin genes as an important group in expression changes during the larval development in a distinct way.

High expression at late developmental time has no significant correlation with developmental time unaffected by dietary restriction. The late developmental period might be too late to tolerate nutritional stress effects for developmental time for the possible reason of the coming pupation stage. Our overexpression of lipocalins shortly before pupation may prolong the time taken to reach the critical size required for pupation. Despite this speculation, considering the relationship between stress tolerance and lipocalins in *Drosophila*, it can be suggested that early larval

developmental periods are more important for genetic buffering to dietary stress. Generally, there is no specific expression pathway for time-dependent developmental periods, unlike age-specific expression. Despite Ruiz et al. (2011) showing age-specific *NLaz* and *GLaz* expression for each sex in *D. melanogaster*, it is hard to identify developmental stage-specific expression. On the other hand, *GLaz* and *NLaz* show low levels of larval expression and are highly expressed in pupae and adult flies (Sanchez et al., 2000; Maynard, 2010). Likewise, *Karl*'s expression is very low during developmental stages (Chintapalli et al., 2007). Contrarily, our results do not support these findings. The tested lines show lipocalin expression at different time points of larval developmental stages by dietary stress. Moreover, the study by Mensch et al. (2008) suggested that the mutated *Karl* gene with P element insertion as a candidate affects developmental time. Our results highlight the large potential of the relationship between lipocalin genes and developmental time.

It is possible that lipocalin expression was buffered in the optimum diet composition in comparison to the lipocalins expressed in the yeast-restricted poor diet. However, differences of lipocalin expressions in both lines indicated that the genetic background of inbred lines has strongly influenced the transcription phenotype.

The main goal of this study was to identify the expression profile of lipocalin genes in larval developmental periods under nutritional stress; it needs to be noted that the effects of developmental time can be related to lipocalin genes. We identified *NLaz*, *GLaz*, and *Karl* expressions during development under two different diet conditions. This framework needs to be investigated in more detail based on tissue and molecular pathways to understand the nutritional effects of lipocalin gene expressions during the developmental stage.

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