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Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz.

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Abstract

Apolipoprotein D (ApoD), a member of the Lipocalin family, is the gene most up-regulated with age in the mammalian brain. Its expression strongly correlates with aging-associated neurodegenerative and metabolic diseases. Two homologues of ApoD expressed in the Drosophila brain, Glial Lazarillo (GLaz) and Neural Lazarillo (NLaz), are known to alter longevity in male flies. However, sex differences in the aging process have not been explored so far for these genes. Here we demonstrate that NLaz alters lifespan in both sexes, but unexpectedly the lack of GLaz influences longevity in a sex-specific way, reducing longevity in males but not in females. While NLaz has metabolic functions similar to ApoD, the regulation of GLaz expression upon aging is the closest to ApoD in the aging brain. A multivariate analysis of physiological parameters relevant to lifespan modulation uncovers both common and specialized functions for the two Lipocalins, and reveals that changes in protein homeostasis account for the observed sex-specific patterns of longevity. The response to oxidative stress and accumulation of lipid peroxides are among their common functions, while the transcriptional and behavioral response to starvation, the pattern of daily locomotor activity, storage of fat along aging, fertility, and courtship behavior differentiate NLaz from GLaz mutants. We also demonstrate that food composition is an important environmental parameter influencing stress resistance and reproductive phenotypes of both Lipocalin mutants. Since ApoD shares many properties with the common ancestor of invertebrate Lipocalins, we must benefit from this global comparison with both GLaz and NLaz to understand the complex functions of ApoD in mammalian aging and neurodegeneration.

Research highlights

GLaz and NLaz alter longevity in a sex-specific manner.
Like ApoD, GLaz increases its expression in the aged brain, but only in females.
A multivariate analysis reveals specialized and redundant Lipocalin functions.
Response to oxidative stress and control of lipid peroxidation are common functions.
NLaz and GLaz alter metabolism and reproduction in different and complementary ways.

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1. Introduction

Aging and the regulation of an organism lifespan are complex traits that are the result of multiple factors, many of which are clearly inheritable. This fact has guided the quest for genes that regulate longevity. Many genetic interventions that alter the length of life have been documented in model organisms ranging from nematodes to rodents (for recent reviews see Fontana and others 2010; Kenyon 2010). They reveal the involvement of a basic set of conserved pathways, being the nutrient sensing pathways (the insulin and insulin-like growth factor signalling (IIS) and the target of rapamycin (TOR) pathway) the best known examples (Biteau and others 2010; Broughton and Partridge 2009; Karpac and Jasper 2009; Wang and Levine 2010). These studies are leading the search for genes in humans that perform similar functions and can become candidates for pharmacological interventions to achieve a longer and healthier life. Either genetic association studies or transcriptome analysis are the main approaches to aging research in humans (Fontana and others 2010; Kenyon 2010; Passtoors and others 2008). It is striking that in a meta-genome study where the changes in brain transcriptome have been compared between mice, monkeys and humans, Apolipoprotein D (ApoD) was revealed as the most robust age-dependent up-regulated gene in the brain conserved across species (Loerch and others 2008). The same is true for a meta-array analysis using mouse, rat and human expression data in different tissues, where ApoD appears as the gene most consistently over-expressed with age (de Magalhaes and others 2009). Not surprisingly, the expression of ApoD is highly boosted by a collection of traumatic, pathological and degenerative nervous system conditions in humans as well as by cancer and age-related metabolic diseases (reviewed by Van Dijk and others 2006).

It is widely accepted that many of the genes that regulate longevity participate in defence or repair mechanisms that counteract the accumulation of damage to cell components with age. Thus, many of these genes are also thought to be important for the onset of a wide array of diseases where age is a major risk-factor. Neurodegenerative diseases are no exception. The study of ApoD and its homologous genes in model organisms should therefore prove relevant to the understanding of aging in general, and of the aging nervous system in particular.

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ApoD is a member of the Lipocalin family, small secreted lipid-binding proteins. At least three other Lipocalin genes are also expressed in the nervous system of mammals: retinol binding protein, RBP (MacDonald and others 1990), L type prostaglandin D synthase, L-PGDS (Kanekiyo and others 2007), and Lipocalin 2 (also called NGAL) (Lee and others 2009). In Drosophila, two genes are the closest homologues to ApoD: Glial Lazarillo (GLaz) and Neural Lazarillo (NLaz). They are expressed in the nervous system in a cell-type specific manner (Sanchez and others 2000). A third Drosophila Lipocalin, Karl, is not expressed in the nervous system (Hull-Thompson and others 2009). Both GLaz and NLaz are known to regulate stress resistance and lifespan in male flies (Hull-Thompson and others 2009; Sanchez and others 2006b; Walker and others 2006): loss-of-function mutants have a reduced longevity while gain-of-function manipulations result in extended lifespan. GLaz also influences fat storage (Sanchez and others 2006b) while NLaz regulates the metabolic response to stress by systemic inhibition of the IIS pathway (Hull-Thompson and others 2009). Protection against oxidative stress in the nervous system has also been demonstrated for mammalian ApoD (Ganfornina and others 2008), revealing a sufficient degree of functional conservation between ApoD and the Lazarillo genes expressed in the nervous system of Drosophila.

Single gene analyses of aging mutants often overlook pleiotropy and effects of other gene family members expressed in the same tissues. Also, in a few cases, the original interpretations derived from studies of classical longevity mutants (Lin and others 1998; Rogina and others 2000) have been challenged when other physiological, environmental (Baldal and others 2006a), or genetic (Toivonen and others 2007) factors have been taken into account. This fact highlights the need for a comprehensive understanding of the many effects of single gene mutations, as well as of the gene-environment interactions that are relevant to aging and lifespan determination.

Particularly, sex differences in the aging process are a very relevant issue that has not been explored so far for these genes. Almost all the work published on this group of Lipocalins (ApoD/Lazarillo) has been performed in males only. This work was triggered by the consistent observation that one of the Drosophila Lipocalins (GLaz) alters longevity in a sex-dependent manner in any genetic background tested: only null mutant males reduce

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their longevity, while null mutant females live as long as wild type flies. The lack of NLaz, on the other hand, shortens lifespan in both sexes. This work is at the same time an attempt to understand the origin of such a sex-specific phenotype, and an exhaustive search for the physiological processes in which these Lipocalins intervene, with the final goal of uncovering the interesting labor division structure of the two Drosophila Lipocalins homologous to ApoD.

2. Material and Methods

2.1. Fly strains and husbandry.

Flies were grown in a temperature-controlled environmental incubator at 25ºC, 60% relative humidity, under a 12h light-dark cycle.

Food recipes: (A) *"Valladolid-standard" food:* wet yeast 84 g/l, NaCl 3.3 g/l, agar 10 g/l, wheat flour 42 g/l, apple juice 167 ml/l, and propionic acid 5ml/l. (B) *"Carolina" Instant Food* (Carolina Biological Supplies). (C) *"Caltech" food:* dry yeast 15 g/l, agar 4.5 g/l, dextrose 50 g/l, sucrose 25g/l, corn meal 83 g/l, phosphoric acid 0.6 ml/l, and propionic acid 4ml/l..

GLaz and *NLaz* null mutants were generated in a w^{1118} background as previously described (Rong and others 2002; Sanchez and others 2006b). Mutations were outcrossed into a *Canton-S* wild type strain for five generations to obtain a homogeneous background with less than 5% of the original genomic background. Isogenic sister lines containing the wild type allele of *GLaz* and *NLaz* (line *G10*: $GLaz^{+/+}$, $NLaz^{+/+}$), or the mutant allele (lines *G2*: $GLaz^{-/-}$, $NLaz^{+/+}$ and *N5*: $GLaz^{+/+}$, $NLaz^{-/-}$) were selected by PCR screening for GLaz, and PCR followed by SceI restriction digest for NLaz. In a last round of outcrosses, a set of fly strains with white eyes were generated in the same way outcrossing with w^{1118}-$CS_{10.}$ The line w^{1118}-CS_{10}, a gift from Seymour Benzer (Caltech), is a 10 generation outcross of w^{1118} into the *Canton-S* background. A set of lines with white eyes were thus generated: lines *CGW* and *CNW14* as wild type isogenic controls ($GLaz^{+/+}$, $NLaz^{+/+}$), *GW* line ($GLaz^{-/-}$, $NLaz^{+/+}$) and *NW5* line ($GLaz^{+/+}$, $NLaz^{-/-}$). Most of the experiments were carried out with *G10* as wild type control and *G2* and *N5* as experimental lines unless otherwise noted.

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2.2. Wolbachia test.

The absence of infection by *Wolbachia pipiens* in all fly strains was tested by PCR of genomic DNA extracts. The following primers against the rRNA-16S gene of Wolbachia were used: 5'-GAAGATAATGACGGTACTCAC-3', 5'-GTCAGATTTGAACCAGATAGA-3' and 5'-GTCACTGATCCCACTTTA-3'. Program: 2 min 94º; (30 sec 94º, 30 sec 60º, 45 sec 72º) x15; (30 sec 94. 30 sec 52, 75 sec 72º) x15; 7 min 72º. DNA from Wolbachia-infected *Dilofilaria immitis* worms was used as positive control. Primers and a positive control DNA were kindly provided by Dr. F. Simon (Univ. Salamanca).

2.3. Lifespan analysis.

At least 100 flies of each genotype were collected within 24h of eclosion, separated by sex under brief CO_2 anaesthesia, housed in groups of 25, and maintained at 25ºC. Dead flies were counted and surviving flies were transferred to new vials with fresh food twice a week.

2.4. Oxidative stress, starvation, and desiccation resistance.

Flies collected as described for the longevity analysis were separated by sex in groups of 25 when they were 3 days old. Application of different stressors was performed as follows.

Paraquat treatment: After a period of dry starvation (3 hours) flies were transferred to vials with filter papers soaked with 1 ml of 10% sucrose-20mM paraquat (Sigma). Incubation proceeded in the absence of light and deaths were scored every 4-8 h.

Wet starvation treatment: Starting at 3 days of age, flies were transferred to vials with 1% agar in water. Dead flies were scored every 4-8 h.

Dry starvation treatment (desiccation): Starting at 3 days of age, flies were transferred to empty vials. Dead flies were scored every 4-8 h.

2.5. Body weight, fat content, and protein content.

Total wet weight, dry weight after evaporation of water, and fat-free dry weight after extraction of neutral lipids with diethyl ether, was performed as previously described (Sanchez and others 2006b). The protein content per fly was determined with the

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microBCA kit (Pierce). Three independent experiments with measurements in triplicate were performed at 3 and 30 days of age.

2.6. Lipid peroxidation levels.

A spectrophotometric assay was used to determine the concentration of free malondialdehyde (MDA-586, Bioxytech) as previously described (Sanchez and others 2006b). Three independent experiments with measurements in triplicate were performed at 3 and 30 days of age.

2.7. Population reproductive output.

Three populations of 10 males x 10 virgin females per genotype were housed in 50 ml food bottles (250ml plastic bottle). Eggs laid were counted every day after transferring parents into a new bottle, and the number of adults emerging form each bottle was counted till the culture was exhausted. Egg and adult progeny production was scored during 7 days (age of parents: 5-11 days). An estimate of fecundity and fertility per female in the population per day was calculated.

2.8. Courtship behaviour and reproduction.

Courtship and mating tests were performed during 60 min using male-female pairs of 4-5 day-old flies under a watch glass used as an observation chamber, as described previously (Ferveur and others 1995). The courtship index (C.I.) represents the proportion of time a male spends actively courting the female for 10 min. Copulation features as mating time, mating latency and mating performance (%) were scored for 60 min. Offspring numbers and sex ratio (male/female) from each mated female that yielded progeny were scored during two weeks.

2.9. Hunger driven short-term food intake behaviour.

Two groups of 20 flies per sex, previously food-deprived for 19h on 1% agar in water, were fed for 5 minutes on 4ml of 10% sucrose, 1% agar, supplemented with 0.5% food dye FD&C Blue No.1 (E-133 European Union, provided by Proquimac). After feeding, the flies were anesthetized with CO_2 and homogenized in PBS. After a short spin to pellet tissue

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debris, absorbance of the homogenate was measured at 625nm. Background absorbance was corrected with fly samples of the same genotype subjected to the same procedure without the dye. The experiment was repeated twice and performed with the white-eyed fly lines (see above) to avoid disturbances of the absorbance spectrum due to the eye pigment.

2.10. Circadian spontaneous locomotor activity.

Individual flies at 3 days of age were transferred to monitor tubes containing fresh food. Their locomotor activity was monitored using the Drosophila Activity Monitoring System (Trikinetics, Waltham, MA) with a cumulative sampling rate of 15 min, in a temperature-controlled environmental incubator at 25ºC, 60% relative humidity, under a 12h light-dark cycle. Five monitors were used, each housing 5 flies/sex/genotype and a sixth monitor recorded light, temperature and humidity. Monitoring was performed for 6 days, but analysis included data starting on day 3 (6 days of age) to avoid variations due to habituation to the housing conditions.

2.11. Climbing ability.

Flies were collected and separated by sex in groups of 10 as described for the longevity analysis. Tests were performed as previously described (Sanchez and others 2006b), after a minimum of 24 hours after anaesthesia, at 3 and 30 days of age.

2.12. qRT-PCR: GLaz and NLaz gene expression.

Flies of each genotype were collected within 24 hours of eclosion, separated by sex under brief CO_2 anaesthesia, and housed in groups of 25. They were transferred to fresh food vials twice a week until the day of processing (3, 30 and 60 days). When preceded by wet starvation, gene expression measurements were performed at 15 hours of treatment (see above). We separated heads and bodies. RNA extracted from heads should represent a good approximation to the expression in the brain, which makes most of the head tissue. RNA extracted from the body, which includes the relatively large thoracic ganglia, represents an approximation to the global expression level in whole flies.

Heads from 50 flies were cut and total RNA was extracted using TRIzol (Invitrogen). RNA concentration was measured with a Nanodrop spectrophotometer. Reverse

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transcription was done with the PrimeScript™ RT reagent Kit (Takara) according to the manufacturer instructions by using Oligo-dT primers and random hexamers. Primers of equal amplification efficiency were designed for GLaz (5'-GCGAACAATCGAAGTTTTCC-3' and 5'-ACAAGATGGCGAAGTTCTCG-3'), NLaz (5'-CGAGTACGCAGCCTATCCAT-3' and 5'-CCAGGTAGTTGGCCTTCGT-3') and the ribosomal protein L18 (RPL18, endogenous control) (5'-AGAACCGACCCCAAATCC-3' and 5'-CGACCACGATGGTAGACTCC-3'). Quintuplicate PCR reactions were performed for each RNA sample using the SYBR® *Premix Ex Taq™* (Takara) according to the manufacturer instructions in a Rotor-Gene RG-3000 thermal cycler (Corbett Research). Cycling conditions were 30 sec 95º, (5 sec 95º, 15 sec 55º, 15 sec 72º) x 35.

Melting curves were established for all conditions to check for the absence of unspecific amplifications.

RNA transcription levels were determined by the method of direct comparison of C_T values and relative quantities calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). Transcripts were normalized to RPL18 for each condition. We transform the data as Log $2^{-\Delta\Delta Ct}$ and then represent them as fold changes. In this way, up- and down-regulations are symmetrically scaled around one. Statistically significant differences of pairwise gene transcriptional changes were evaluated with a Mann-Whitney U-test, using ΔC_T of each replica (calculated by subtracting the average CT of the reference gene). The level of significance was set at $p<0.05$.

2.13. Statistics and principal component analysis.

Statistical analyses were performed with Statgraphics plus (v 5.0) software. Student's t-Test was used to assess two-sample comparisons (with the exception of qRT-PCR data, see above) with $p<0.05$ as threshold for significant changes.

To find patterns of covariation among all the physiological parameters measured, we performed a principal component analysis (PCA). The average of each parameter was arranged in two matrices (Tables S1-S2), one with 25 variables measured at 3 days of age, and another with 7 variables measured at 3 and 30 days of age. Neither longevity data nor Lipocalin expression data were included, in order to render the analysis "blind" to the genotype (the dependent variable) and lifespan (the variable we want to explain). The

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analysis was also blind to sex and age, since it is performed for 6 independent entries (2 sexes per genotype) or 12 independent entries (6 entries per age).

3. Results and Discussion

3.1. The nervous system Lipocalins GLaz and NLaz influence longevity in a sex-specific manner.

The loss of either GLaz (Sanchez and others 2006b) or NLaz (Hull-Thompson and others 2009) is known to reduce the lifespan of male flies. However, here we report that females reduce their lifespan when NLaz is absent, but their lifespan is similar to control female flies in the absence of GLaz (Fig. 1A, B). The same results are obtained before and after changing the genetic background for both mutations (from w^{1118} to *CantonS*, not shown; median survival effects stated in Fig. 1 legend). The absence of Wolbachia infection, as a potential lifespan-reducing cause, was confirmed by PCR tests (see Fig. S1 and section 2.2. in Methods).

These observations led us to search for causal links that might explain how GLaz regulates longevity differently in males and females, and to find the fine structure of functional relationships between the two nervous system Drosophila Lipocalins.

3.2. Male and female flies differ in their temporal and spatial regulation of Lipocalin genes expression.

Since both Lipocalins are expressed within and outside the nervous system (Hull-Thompson and others 2009; Sanchez and others 2000; Sanchez and others 2006b), we investigated how much of their expression is contributed by the brain in young flies. We focused our study on the expression changes in the brain, either through aging or upon stress. These data can then guide the comparisons between the fly Lipocalins and the mammalian homologue, ApoD, known to be the most up-regulated gene in the aged brain.

Brains of male flies have stable expression levels of both Lipocalins throughout life; NLaz expression being higher than that of GLaz at all ages explored (Fig. 1C, triangles). In contrast, the brains of females show interesting and opposite expression changes with age: GLaz increases and NLaz decreases with aging (Fig. 1C, circles). Curiously, it is the sum of

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the two Lipocalins what is kept roughly constant throughout aging in females. The increase of GLaz expression with age in female brains echoes the pattern followed by ApoD in mice, macaques, and humans (Loerch and others 2008).

The fact that NLaz expression in females shows an opposite pattern, suggests that different regulatory elements are responsible for the temporal control of each Lipocalin, and that GLaz gene regulation must have commonalities with the age-dependent networks controlling ApoD expression in the mammalian CNS. Also, these gene-regulation networks differ with sex for both Drosophila Lipocalin genes.

Interestingly, the level of Lipocalins in the brain of young flies correlate with their longevity reduction when each Lipocalin is missing. Note that GLaz expression is the lowest in young wild type females, and its absence does not alter longevity. These data support the idea that early adulthood represents a critical period for Lipocalin function, affecting parameters that will result in lifespan alterations later on.

The relative contribution of head and body in Lipocalin expression also varies with sex (Fig. 1D). In young females, GLaz has the highest ratio body/head. In males, the expression of both Lipocalins is higher in the brain than in the body, with NLaz again showing higher expression levels than GLaz.

3.3. Starvation triggers a differential expression of Lipocalins in the brain.

The males of both mutants had been already described as starvation sensitive (Hull-Thompson and others 2009; Sanchez and others 2006b), and NLaz expression is known to be induced by starvation (Hull-Thompson and others 2009). However, our previous works used different regimes of starvation (wet or dry starvation) and were performed only in males. Here we analyze in detail how brain expression of the two Lipocalins is altered upon food deprivation (without water deprivation, to avoid a more complex stress).

Expression of NLaz and GLaz in the fly head is differentially induced upon food deprivation in wild type flies (Fig. 2A,B). Interestingly, no induction of NLaz is observed in the head tissue where its expression is constitutively high. These data are compatible with NLaz working in the control of systemic metabolism and with the fat body tissue being a source of NLaz under stress conditions (as we had previously shown using a dry

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starvation paradigm) (Hull-Thompson and others 2009). In contrast, GLaz is highly induced in the head upon starvation, suggesting that GLaz might be involved in a nervous system regulatory loop triggered by nutrient deprivation. These results show the existence of differential domains of action of the two Lipocalins.

3.4. A multivariate analysis of the physiological parameters that contribute to lifespan modulation by Lipocalins.

Given that longevity is the result of an intricate puzzle of interactions between genetic components and physiological and environmental variables, we need to uncover the full complexity of effects of a single mutation in order to learn how each gene alters the network of causal links underlying lifespan regulation.

With that in mind, we have undertaken an analysis of multiple parameters and their dependence on NLaz and GLaz expression. We have screened variables including metabolic (levels of various metabolites, stress-nutrition relationships), behavioral (reproduction, food intake, locomotor activity), as well as stress resistance, and analyzed them by principal component analysis (PCA). PCA has been successfully used in a multitrait study by Baldal and others (2006b) pertaining starvation resistance and aging evolution, and by Andersen and others (2005), in a study uncovering how heat stress and age induced maternal effects influence physiological parameters in the offspring. This type of analysis extracts features of the parameters variation as a set of new variables (the principal components, PCs) that are uncorrelated with one another and successively account for maximal amounts of variation. Our analysis was performed with 25 variables at 3d of age (Fig. 3A-B; Fig. S2A-B), and a subset of 7 variables mainly related to metabolic parameters at 30d of age (Fig. 3C, Fig. S2C-D). Values obtained for all variables are listed in supplementary tables (Tables S1-S2). The PCA has uncovered sex, genotype, and age-specific patterns.

Some life history parameters clearly separate genotypes. The two principal components explaining 69% of the variation in the sample studied at 3d of age separate wild type flies from either mutant, and in turn segregate each mutant into opposite corners of the multivariate space (Fig. 3A). This pattern reflects the existence of specialized functions

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separating Lipocalin mutants from each other (component 1), as well as common functions separating both mutants from the wild type (component 2). For example, lipid peroxidation and relative fat content contribute to the separation of wild type from either mutant (black labels in Fig. 3B). Variables in the domain of reproduction, locomotor activity, food intake and metabolism (relative water content, starvation resistance) separate GLaz from NLaz mutants (red and green labels in Fig. 3B).

Other life history parameters contribute to sex or age differences regardless of genotype (Fig. S2). Metabolic parameters (wet weight, protein content or lipid peroxidation), response to stress (starvation, desiccation or paraquat resistance) and diurnal *vs.* nocturnal locomotor activity are important variables in the separation of sexes in all genotypes (Fig. S2A-B). In the analysis of variables at two ages (Subset 2) it is interesting that only females are segregated by age in the component 1 by 2 plot (explaining 63% of de variance). Metabolic parameters (relative water content in opposition to relative fat) and climbing ability have the most weight for the separation of young female flies from old female flies in all genotypes (Fig. S2C-D).

3.5. Sex-specific modulation of longevity by GLaz is dependent on metabolic parameters related to protein homeostasis.

Studying the multivariate space, we searched for components that would cause GLaz mutant females to cluster with WT females, and would therefore explain the observed longevity curves. Interestingly, the stronger association of *GLaz*$^{-/-}$ females with wild type flies arises in the PCA analysis performed for the subset of 7 variables measured at both 3 and 30d of age (Fig. 3C). The association only occurs for young GLaz mutant females, further supporting the idea that the function of Lipocalins in early adulthood is crucial for longevity determination. Young wild type females, with their low levels of GLaz expression, show a combination of parameters similar to that of *GLaz*$^{-/-}$ young females. Old GLaz mutant females, however, are displaced to the opposite corner of the component 2 by 3 plane of the multivariate space (arrows in Fig. 3C). The variables with more weight in the "mutant GLaz females – wild type" association are the protein content and relative fat free content (with a negative weight, red labels) and the wet weight and relative water content

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(with a positive weight, green labels). Variables related to lipids (relative fat content or lipid peroxidation levels) do not contribute to this association.

Our analysis indicates that the patterns of metabolism, especially during early adulthood, contribute importantly to the final outcome in longevity, resulting in a shorter lifespan in NLaz mutants and in GLaz mutant males, but a similar lifespan in GLaz mutant females and wild type flies. Some of the parameters included in our PCA analysis are further described and discussed hereafter.

3.6. Energy intake and homeostasis are altered through aging in the absence of Lipocalins.

The loss-of-function mutants of both Lipocalins show pleiotropic effects at different levels of energy management (intake-balance-expenditure, Fig. 4A) that contribute to their segregation in the multivariate space (Fig. 3A-B). Particularly, the PCA analysis shows that energy management differences between genotypes are part of the functional specialization between both Lipocalins, and also contribute to the unaltered lifespan in females lacking GLaz (Fig. 3C).

In addition, the behavioral response to starvation is increased in young *NLaz*$^{-/-}$ flies, but not in *GLaz*$^{-/-}$ flies (Fig. 4B). We have previously described that overall weight in males is increased in the absence of NLaz (Hull-Thompson and others 2009), and decreased in the absence of GLaz (Sanchez and others 2006b). Here we show that *NLaz*$^{-/-}$ flies start life with a different body size and increase their fat content with age, while *GLaz*$^{-/-}$ mutants maintain a low fat content throughout life in both sexes (Fig. 4C). These results are in line with the food intake increase observed in NLaz mutant flies (Fig. 4B).

Energy storage (in the form of fat storage) is clearly altered in opposite directions by the two Lipocalins. However, only male and not female GLaz mutants shorten their lifespan. On the other hand, NLaz mutants show both increased food intake (this work) and increased IIS activity (Hull-Thompson and others 2009). In order to understand the relevance of food storage and food intake in lifespan determination we need to know how Lipocalins influence energy expenditure. We have analyzed locomotor activity patterns and reproduction as the major forms of energy output.

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3.7. Lipocalins influence the patterns of daily energy expenditure in locomotor activity.

The patterns of daily spontaneous locomotor activity also differ with genotype and sex (Fig. 5), showing complementary patterns. Overall activity is higher in *GLaz*$^{-/-}$ males (Fig. 5A,C), which are more active than wild type flies especially at night (Fig. 5D). *NLaz*$^{-/-}$ females are overall less active (Fig. 5B,C), but the difference is mostly due to diurnal activity (see Table 1).

Shortened resting periods at night have been reported to correlate with aging (reviewed by Grotewiel and others 2005). Our data show that the highest sex difference within a given genotype is the large increase in nocturnal activity of *GLaz*$^{-/-}$ males (Fig. 5D) compared to mutant females of the same genotype. Interestingly, *GLaz*$^{-/-}$ female activity is more similar to the wild type pattern (Fig. 5 and Table 1). One could argue that this effect will contribute to an accelerated senescence of *GLaz*$^{-/-}$ males and therefore a shortening of lifespan occurring only in males of this genotype. However, the lower nocturnal activity observed in males *NLaz*$^{-/-}$ is not accompanied by an increase in lifespan. Our data support the idea that the amount of activity *per se* does not correlate with longevity, in agreement with Koh and others (2006), who report sleep fragmentation rather than total sleep time as the relevant parameter influencing longevity.

3.8. Reproductive output and courtship behavior is differentially altered in each Lipocalin mutant.

Reproductive parameters separate GLaz from NLaz mutants in the multivariate space analyzed at 3d of age (Fig. 3B). A lower fecundity is observed in both mutants (Fig. 6A), but fertility of *NLaz*$^{-/-}$ mutants does not differ from wild type (Fig. 6B). An estimation of developmental success, or "egg quality" (% of eggs reaching adulthood) is higher in *NLaz*$^{-/-}$ mutants and lower in *GLaz*$^{-/-}$ mutants compared to wild type flies (Fig. 6C). We can therefore discard the existence of major fertilization or developmental problems at least in NLaz mutants. However, given that GLaz mRNA is detected very early in embryogenesis (0-2 hours embryos, Sanchez and others 2000), the *GLaz*$^{-/-}$ eggs that do not reach adulthood

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might have developmental problems. Alternatively, they can be unfertilized eggs. We therefore tested whether mating is hampered in the absence of Lipocalins.

Mating behavior is indeed altered in both *GLaz*$^{-/-}$ and *NLaz*$^{-/-}$ flies. When paired with wild type females, males of both mutant genotypes are less involved in courting (Fig. 6D), and a lower percent of pairs actually mate (Table 2). Wild type males paired with mutant females (Fig. 6E) also display a deficient courtship, and therefore the alteration of behavior is dependent on both sexes. When *NLaz*$^{-/-}$ males and females pair, the courtship index is even lower (Fig. 6E), and mating success is strikingly low (Table 2), which would account for the low number of eggs per female observed in population experiments (Fig. 6A). On the contrary, pairs composed of two GLaz mutant flies are more effective in triggering the courtship behavior than pairs of *GLaz*$^{-/-}$ with wild type flies. This result would be compatible with pheromone signaling being off-set in GLaz mutants, both at the emission and reception levels. Interestingly, the GLaz or NLaz mutants who successfully mate show longer mating latencies (Table 2), but this effect is cancelled when mutant males are paired with wild type females.

The altered mating behaviors could modify the final reproductive output of the population. However, the fertility of successfully mated mutants does not differ from wild type values (Fig. S3A). Thus, we can conclude that in experiments performed in fly populations (Fig. 6B) the *GLaz*$^{-/-}$ eggs that do not reach adulthood are mostly unfertilized eggs, while the fertilized ones reach adulthood at a normal rate, discarding major developmental problems also for *GLaz*$^{-/-}$ mutants.

In addition, we analyzed the lifespan of virgin females, which results in a pattern identical to the one observed for mated females: similar in wild type and *GLaz*$^{-/-}$ and shortened in *NLaz*$^{-/-}$ mutants (Fig. S3B). These virgin females do lay eggs, although all of them are unfertilized. The putative differential constraint to lifespan is not therefore dependent on the energy spent by the female in producing eggs proper.

Even though reproduction has been shown to correlate negatively with lifespan in many cases (Baldal and others 2006a; Tatar and others 2003; Toivonen and Partridge 2009), instances of genetic interventions that alter longevity and reproductive output independently have also being documented (Dillin and others 2002; Giannakou and others

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2007) (Grandison and others 2009a; Partridge and others 2005), challenging the existence of a direct causal relationship between reproduction and lifespan. Lipocalin mutants support the latest view, since the sex differences observed in longevity can not be fully explained by a net reduction in the energy spent in the production of eggs. The control of courtship behaviors are part of the pleiotropic functions of the two nervous system Lipocalins clearly deserving future investigation.

3.9. Environment-genotype interactions: Food composition influence on Lipocalins phenotypes.

Food composition is an important environmental parameter that influences stress resistance and reproductive output. We have performed a nutritional analysis of three common fly food recipes including the standard one used in our laboratory, and the one used by collaborators that have also worked on NLaz or GLaz function (Fig. 7A, see Methods). Each recipe differs in the amount of yeast and the source of sugar. However, a clear pattern emerges. For wild type flies, the higher the proportion Net Fat / Total Carbohydrates (Fig. 7B), the higher the fertility (Fig. 7C) and the lower the oxidative stress resistance (Fig. 7D-F).

Characterizing this interaction in the fly strains used in our analysis has been important, since the GLaz mutation effects on reproduction are significantly higher in diets with high or medium Fat/Carbohydrate ratio ("Valladolid" and "Carolina" foods, Fig. 7C), while sensitivity to stress observed in both mutants is only observed in diets with medium to low Fat/Carbohydrate ratio ("Carolina" and "Caltech" foods, Fig. 7D-F shows the results for NLaz mutants).

Previous studies analyzing diet effects on lifespan (Grandison and others 2009b; Skorupa and others 2008) have been performed with different wild type strains. Our set of experiments emphasizes the importance of controlling diet when characterizing the effects of single genes influencing lifespan (see also Baldal and others 2006a). The effects of Lipocalin genotype in both reproductive output and stress resistance are best observed when flies are fed on our standard "Valladolid food", which contains a medium Fat/Carbohydrate proportion.

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It is interesting to note that the largest differences in the stress response upon changes in diet are observed in the wild type flies. Without NLaz, the differences between feeding regimes are reduced. These results should not be surprising giving the role of NLaz in the control of metabolism in response to environmental stress (Hull-Thompson and others 2009). In the absence of NLaz, the IIS pathway is activated and flies become less sensitive to diet modifications. On the contrary, when IIS activity is genetically reduced (Grandison and others 2009a), lifespan extension becomes insensitive to nutrient supplemented diets.

3.10. ¿How do brain Drosophila Lipocalins help us to understand the aging process?

Several general ideas relevant to aging and lifespan determination are supported by our study.

3.10.1. Early adulthood is a crucial period for lifespan determination.

Experiments where the IIS pathway was manipulated at different time points in life, both in *C. elegans* (Dillin and others 2002) and Drosophila (Giannakou and others 2007), have shown that in order for this pathway to influence lifespan, it needs to operate exclusively during adulthood, with such an influence decreasing at older ages. Two lines of evidence suggest that a brain Lipocalin critical period also exists early in adulthood for their influence on lifespan: (1) their temporal expression pattern in wild type flies, and (2) the clustering of GLaz mutant females with wild type flies only at young ages in the multivariate space of metabolic parameters.

This time period, critical for both IIS and Lipocalin function, is coincident with the reproductive period of the organism (both in worms and flies), and could be interpreted in the light of the disposable soma theory of aging (Kirkwood 1977), where natural selection forces are predicted to decline sharply once the organism is no longer engaged in passing its genes to the next generation (Kirkwood 2008). Lipocalin function would be under selection in early adulthood only, and subsequent consequences of Lipocalin expression on the aging brain or body would be exaptations of this early adulthood selected functions (Gould and Vrba 1982).

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3.10.2. The sex-specific lifespan modulation by Lipocalins is better explained by metabolic than by reproduction phenotypes.

In accordance to the disposable soma theory, our first working hypothesis was that GLaz mutant females maintain a lifespan similar to the wild type because they would be able to allocate more resources to maintenance and less to reproduction. We then confirmed that GLaz mutant females reproduce less and survive more. However, since NLaz mutant females reduce both fecundity and lifespan, we must conclude that survival and reproduction are in this particular case uncoupled.

Curiously, the PCA analysis shows that it is the alteration of metabolic parameters related to the protein content of the fly what mainly accounts for the observed sex differences in longevity. The final protein content and the fat free dry weight of the organism depends of protein synthesis, which has a strong evolutionary conservation among the genetic modifiers of aging (Smith and others 2008).

NLaz is also related to the control of metabolism. When NLaz is expressed in the fat body, it is able to repress IIS activity in systemic target tissues. Also, the loss of NLaz increases signaling through IIS (Hull-Thompson and others 2009). The experiments in this work predict that, in addition, NLaz must be a negative regulator of food intake. NLaz is thus expected to fulfill a role similar to Leptin in the nervous system, inhibiting food intake and promoting energy expenditure. Loss of NLaz mimics therefore the Leptin deficiency state in mouse models of obesity (Ingalls and others 1950; Maffei and others 1995). Curiously, mice where the neural/brain specific insulin receptor is knocked out, exhibit increased food intake and mild adiposity (Bruning and others 2000), just as the NLaz mutant flies. A direct interaction of ApoD with the cytoplasmic portion of the Leptin receptor has been suggested (Liu and others 2001), although it is still questionable how a secreted Lipocalin can access the cytoplasmic side of the plasma membrane for such an interaction to occur.

In contrast with NLaz, GLaz is not regulated by JNK in the peripheral tissues (Hull-Thompson and others 2009). Here we demonstrate that it is instead induced by food deprivation in the brain, where no NLaz transcriptional response is detected. This pattern of

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differential transcriptional response in the brain further supports the existence of specialized functional domains for these two Lipocalins.

3.11. Two brain Lipocalins in Drosophila: a labor division strategy in longevity regulation.

Our work highlights that the two Drosophila Lipocalins expressed in the nervous system have both functional redundancies and specializations. The response to oxidative stress and accumulation of lipid peroxides are among their common functions, while the transcriptional and behavioral response to starvation, the pattern of daily locomotor activity, storage of fat along aging, fertility, and courtship behavior differentiate NLaz from GLaz mutants. This framework is guiding our current research, as more details need to be elucidated. However, we can already start questioning: how many of these shared or unique functions are conserved in the mammalian homologues also expressed in the brain?

The basal position of ApoD in the phylogenetic tree of vertebrate Lipocalins (Ganfornina and others 2000; Sanchez and others 2006a) suggests that ApoD shares many properties with the common ancestor of invertebrate Lipocalins. However, we have to take into account that neither GLaz nor NLaz is a true orthologue of ApoD. Our molecular phylogenetic analyses strongly suggest that the Drosophila Lipocalins originated from an independent duplication event, taking place within the invertebrate lineage. Subsequently, the resulting genes have diverged both in their protein coding sequence and their regulatory sequences.

Since ApoD in the adult mammalian brain is expressed in glial cells, one might be tempted to directly conclude that GLaz is the closest Drosophila homologue. Furthermore, the expression data we report in this work strongly suggest that GLaz regulation through aging is most similar to the robust increase of mammalian ApoD in the aged brain (de Magalhaes and others 2009; Loerch and others 2008). Interestingly, ApoD is most similar to GLaz in protein sequence, but to NLaz in the intron-exon structure of the gene (Sanchez and others 2003). We have also data showing that ApoD is up-regulated by oxidative stress in astrocytes, and that this induction is mediated through the JNK pathway (Bajo-Grañeras, Ganfornina and Sanchez, unpublished observations), which is comparable to the NLaz

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JNK-mediated induction by stress in Drosophila (Hull-Thompson and others 2009). Thus, if we want to extrapolate the Drosophila data to learn about the functions of ApoD in mammalian aging and neurodegeneration we must benefit from a global comparison with both GLaz and NLaz, as the one reported here.

To understand the multigenic control of aging, we need to take into account that a layer of complexity is added due to the fact that each gene has pleiotropic effects, and each one has differing degrees of specialization or redundancy with members of the same gene family. This fact represents a daunting complication for the task of predicting the actions of putative anti-aging or anti-neurodegeneration drugs. However, complexity should not keep us from investigating till we get a comprehensive understanding of the aging process.

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Authors' contributions and approval of manuscript

MR performed the food intake and courtship behaviour experiments, contributed to carry out the longevity curves and the molecular biology analyses and participated in the design of the study, the statistical analysis, and in writing the manuscript. AA performed and analyzed courtship behaviour experiments and contributed to the draft of the manuscript. IC carried out the circadian activity experiments. MDG and DS contributed equally by designing the study, performing all other experiments (stress responses, molecular biology experiments, metabolism and biochemical analyses), the statistical analysis, and writing the manuscript. All authors participated in the critical interpretation of data and read and approved the final manuscript.

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Figure Legends

Figure 1. Lipocalin loss-of-function mutants show sex-specific patterns in longevity that can be related to their different spatiotemporal patterns of expression.
(A) Male lifespan determination of *GLaz*$^{-/-}$ (G), *NLaz*$^{-/-}$ (N) compared to isogenic wild type flies (G10 line). Median survival time is reduced by 15.5% in the *GLaz* mutant ($p<10^{-5}$), and by 22.5% in the *NLaz* mutant ($p<10^{-5}$). ***(B)*** Lifespan is also reduced in *NLaz* females (30.8% reduction, $p<10^{-5}$), but not in GLaz mutant females (p=0.034). Reductions in median survival in the *w1118* genetic background (not shown) were 27.8 and 34.3% for *GLaz* and *NLaz* mutant males, and 37.2% for *NLaz* mutant females. Log-rank test was used for statistical analysis. N=114-213/genotype. ***(C)*** Age effect in NLaz and GLaz transcription in wild type fly heads assayed by qRT-PCR. ***(D)*** Transcription levels of NLaz and GLaz in wild type body RNA extracts. The RPL18 gene is used as endogenous control. The expression level of GLaz in 3 day-old male heads is used as calibrator (Cal.) for both graphs. Symmetrical Fold Change was chosen for data representation for equal display of down-regulations and up-regulations. Statistical differences assayed by Mann-Whitney U-test. * p<0.05 ** p<0.01.

Figure 2. Differential expression of Lipocalins in the brain upon starvation.
(A) Changes in expression of GLaz and NLaz in the head of wild type male flies at 3 days of age in control conditions and after 15 hours of wet starvation treatment. ***(B)*** Changes in expression of both genes in the same conditions as in A, but in females. qRT-PCR using RPL18 as endogenous control and the value of GLaz in 3d wild type male heads as calibrator (Cal.) for both graphs. Symmetrical Fold Change was chosen for data representation for equal display of down-regulations and up-regulations. Statistical differences assayed by Mann-Whitney U-test. ** p<0.01.

Figure 3. Principal Component Analysis of variables excluding Lipocalin gene expression and lifespan parameters.
(A-B) Scatterplot and biplot of the two principal components explaining 69% of the variation in the sample of 25 variables measured in 3 day-old flies (Subset 1). The two-dimensional space defined by these components separate wild type flies from either mutant,

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and sends each mutant to a separate corner of the multivariate space reflecting functional specialization. Acronyms for variables with small weight in the components are omitted in the biplot. ***(C)*** Biplot of components 2 by 3 extracted from subset 2 (7 variables measured in 3 day- and 30 day-old flies). Young *GLaz*$^{-/-}$ females are closely related to wild type females (green arrow), while the old *GLaz*$^{-/-}$ females are projected in the opposite corner of the two dimensional space (red arrow). Symbols: black = wild type, red = *NLaz*$^{-/-}$, green = *GLaz*$^{-/-}$, circles = females, triangles = males, filled symbols = 3 day-old flies, open symbols = 30 day-old flies. Acronyms: Climb: climbing ability; Des: dessication resistance; DevS: developmental success; DL: diurnal locomotor activity; F: fecundity; Fert: fertility; FInt: food intake; LipP: lipid peroxidation; NL: nocturnal locomotor activity; PQ: paraquat resistance; Prot: protein content; RFat: relative fat content; RFatFr: relative fat free weight; RW: relative water content; SSL: sunset locomotor activity; St: starvation resistance; TL: total locomotor activity; WW: wet weight.

Figure 4. Energy intake and homeostasis are altered in Lipocalin mutants.
(A) Variables exploring the flow of energy in Lipocalin mutants. ***(B)*** Behavioral response to starvation is increased in *NLaz*$^{-/-}$ but not in *GLaz*$^{-/-}$ mutants. ***(C)*** Age and genotype effect in relative fat content. *GLaz*$^{-/-}$ mutants have a constitutive reduction in their neutral fat stores while fat increases with age in *NLaz*$^{-/-}$ mutants. Values normalized with respect to wild type young males. Statistical differences assayed by Student's T-test. * $p<0.05$; ** $p<0.01$.

Figure 5. Lipocalin mutants show sex-specific alterations in their circadian locomotor activity.
(A-B) Average of three 24h periods of locomotor activity in wild type, *GLaz*$^{-/-}$ and *NLaz*$^{-/-}$ males (A) and females (B). ***(C)*** Average total locomotor activity is higher in *GLaz*$^{-/-}$ males and lower in *NLaz*$^{-/-}$ females . ***(D)*** Locomotor activity during the dark phase. *GLaz*$^{-/-}$ males are much more active at night than the wild type, while *NLaz*$^{-/-}$ males reduce their activity. N=25/sex/genotype. Statistical differences assayed by Student's T-test. * $p<0.05$; ** $p<0.01$.

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Figure 6. Reproductive output is modulated differently by GLaz and NLaz.
(A-B) The absence of both Lipocalins reduces fecundity (A) but only *GLaz*$^{-/-}$ mutant has a lower fertility (B). Three populations of 10 males x 10 females where scored for egg and adult progeny production during 7 days (from 5-11 days of age). The average per day is represented. ***(C)*** The percent of eggs reaching adulthood was calculated from the experiments shown in A-B. ***(D-E)*** Courtship behavior is altered in *GLaz*$^{-/-}$ and *NLaz*$^{-/-}$ mutants. Courtship index is shown for pairs of each genotype with wild type females (D) and pairs of Lipocalin mutant females with wild type or mutant males (E). Other parameters of the courtship behavior and mating success are shown in Table 2. N= 34-52 pairs per class. Statistical differences assayed by Student's T-test. * $p<0.05$; ** $p<0.01$.

Figure 7. Food composition influences reproductive output and stress resistance.
(A) Variables exploring the flow of energy upon different nutritional regimes in Lipocalin mutants. ***(B)*** Nutrient analysis (performed by Aquimisa, Salamanca, Spain) of the three food recipes used in this work yielded the proportion of Net Fat vs. Total Carbohydrates as the most salient difference (represented normalized with respect to values in Valladolid food recipe). ***(C)*** Reproductive output (fertility) of each genotype when flies are fed in the three types of food. Three populations of 10 males x 10 females per diet condition where scored for progeny production during 2 days (from 5-6 days of age). The average per day is represented. ***(D-F)*** Survival curves under paraquat treatment of wild type and *NLaz*$^{-/-}$ adults born and raised in each type of food. A similar pattern is observed for *GLaz*$^{-/-}$ mutants (not shown). Log-rank test was used for statistical analysis. N=45-75/genotype/sex. Significant reduction in survival was observed only in the Valladolid and Caltech foods ($p<10^{-4}$).

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Table Legends

Table 1. Patterns of daily locomotor activity in Lipocalin mutants.

Average of three 24h periods of locomotor activity in wild type, *GLaz*$^{-/-}$ and *NLaz*$^{-/-}$ flies. Sunrise and sunset are defined as the period of the light switch ±1h. N=25/sex/genotype. Bold lettering indicates statistical differences assayed by Student's T-test. [a] Different from WT ($p<0.05$). [b] Different from WT ($p<0.01$)

Table 2. Courtship behavior is altered in Lipocalin mutants.

Courtship behavior parameters obtained from experiments shown in Fig. 6D-E. Average ± Standard Error is represented. Bold lettering indicates statistical differences assayed by Student's T-test ($p<0.05$): [a] Different from WT-M x WT-F pairs, [b] Different from mutant-M x mutant-F pairs.

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Legends for Supplementary Information

Figure S1. PCR test for Wolbachia infection.

PCR of genomic DNA extracts from all wild type and mutant strains used in this work. Primers against the rRNA-16S gene of Wolbachia endosymbiont are used. DNA from *Dilofilaria immitis* worms infected with Wolbachia is used as positive control. A plasmid containing the cDNA of α-1-microglobulin (a1m, also a Lipocalin) or no DNA are used as negative controls.

Figure S2. Principal components separating sex and age.

(A-B) Scatterplot and biplot of components 2 by 3 extracted from the sample of 25 variables measured in 3 day-old flies (Subset 1). The two-dimensional space defined by these components separate males from females of the three genotypes. ***(C-D)*** Scatterplot and biplot of the two principal components explaining 63% of the variation in the sample of 7 variables measured in 3 day- and 30 day-old flies (Subset 2). These components are also able to separate samples according to sex. In addition, they separate females according to age. Symbols: black = wild type, red = *NLaz*$^{-/-}$, green = *GLaz*$^{-/-}$, circles = females, triangles = males, filled symbols = 3 day-old flies, open symbols = 30 day-old flies. Acronyms for the variables are listed in Table S1 and S2.

Figure S3. Reproduction – Lifespan relationship.

(A) Fertility of successfully mated mutant pairs does not differ from wild type when scoring the offspring of mated couples from courtship behavior analysis (Fig. 6D-E). N= 34-52 pairs. Statistical differences assayed by Student's T-test ($p<0.05$): [a] Different from WT-M x WT-F pairs. ***(B)*** Lifespan of virgin females is reduced in *NLaz*$^{-/-}$ mutants ($p<10^{-5}$), but is similar to wild type in *GLaz*$^{-/-}$ mutants. Log-rank test was used for statistical analysis. N=97-120/genotype.

Table S1. Matrix for Principal Component Analysis. Subset 1.

Averages of the 25 variables measured in 3 day-old flies. Transcription of GLaz or NLaz and longevity parameters are not included.

Table S2. Matrix for Principal Component Analysis. Subset 2.

Averages of the 7 variables measured in 3 day-old and 30 day-old flies. Transcription of GLaz or NLaz and longevity parameters are not included.

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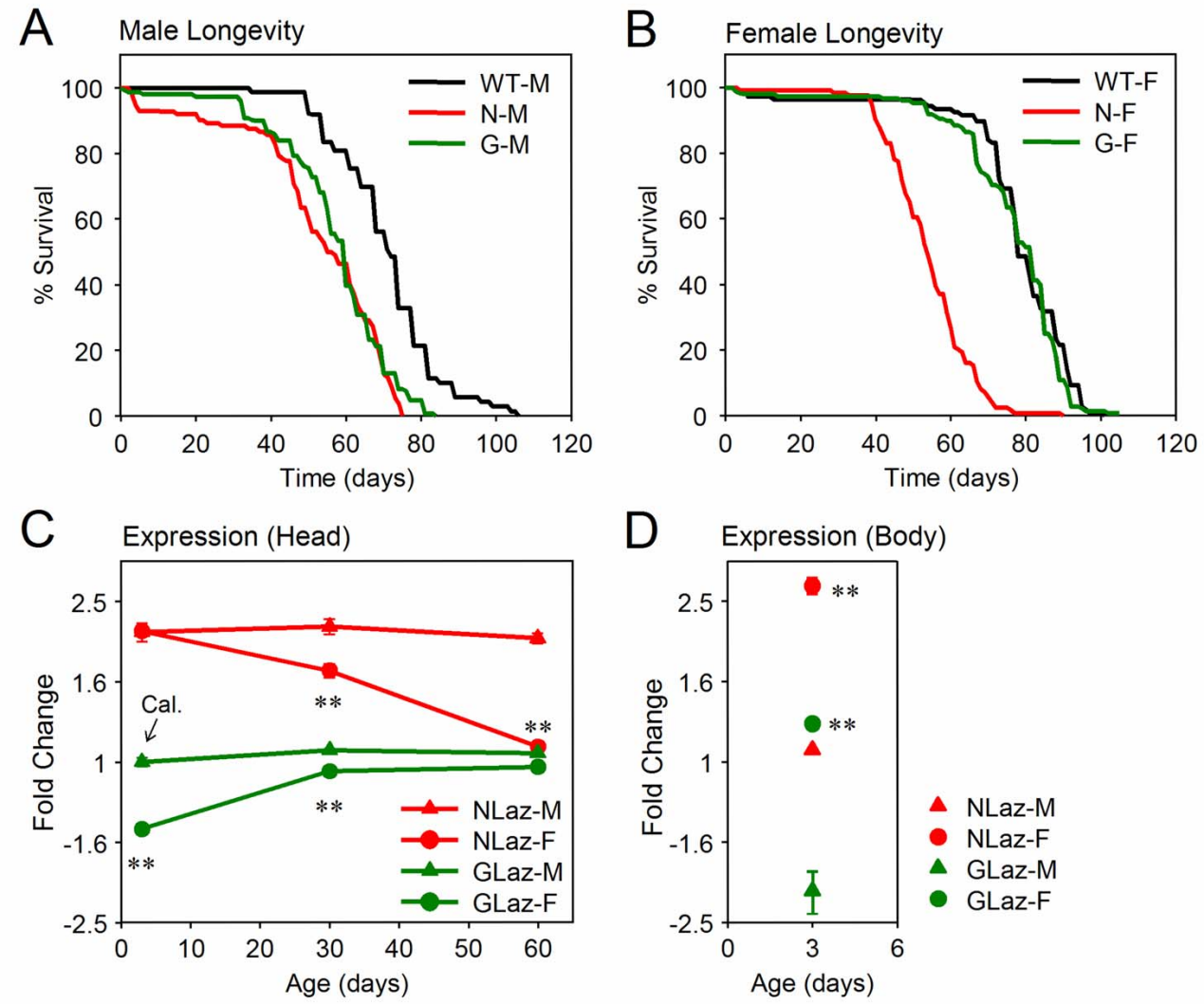

Fig. 1

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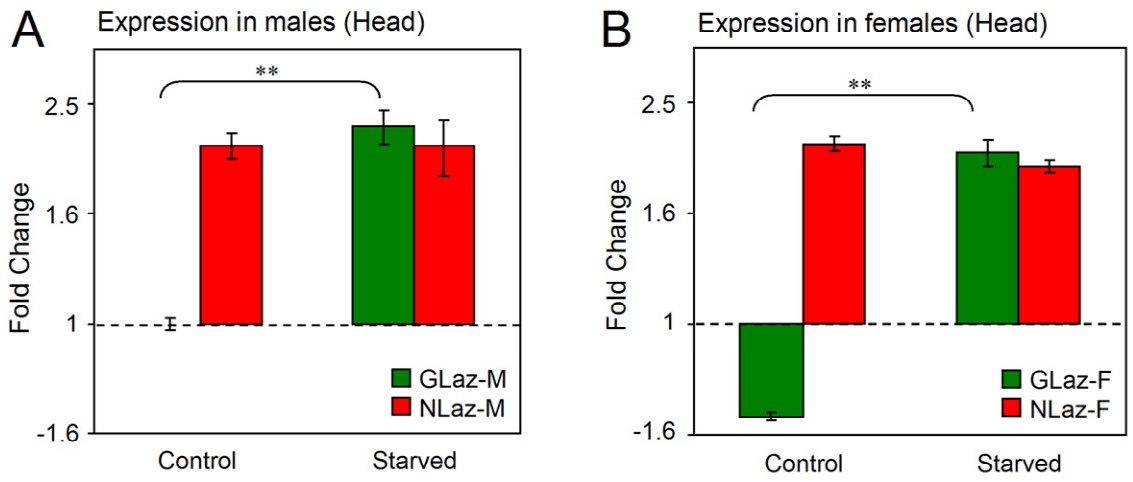

Fig. 2

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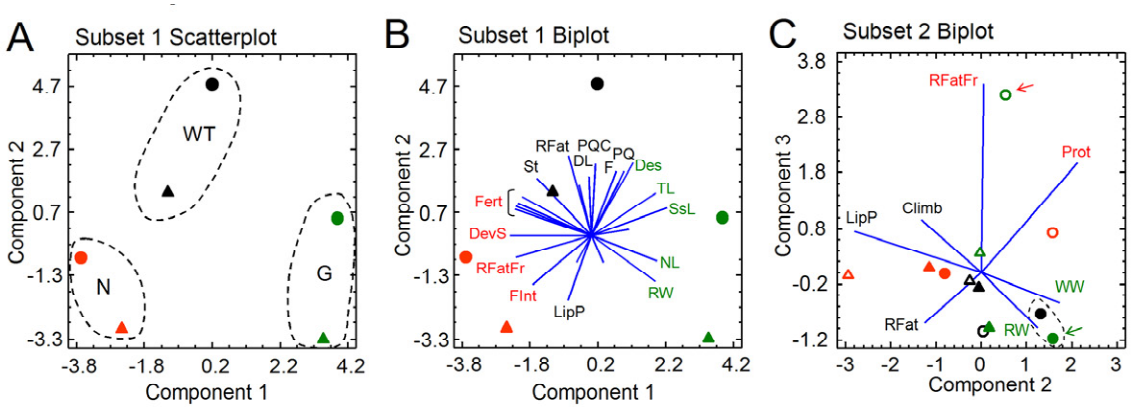

Fig. 3

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Fig. 4

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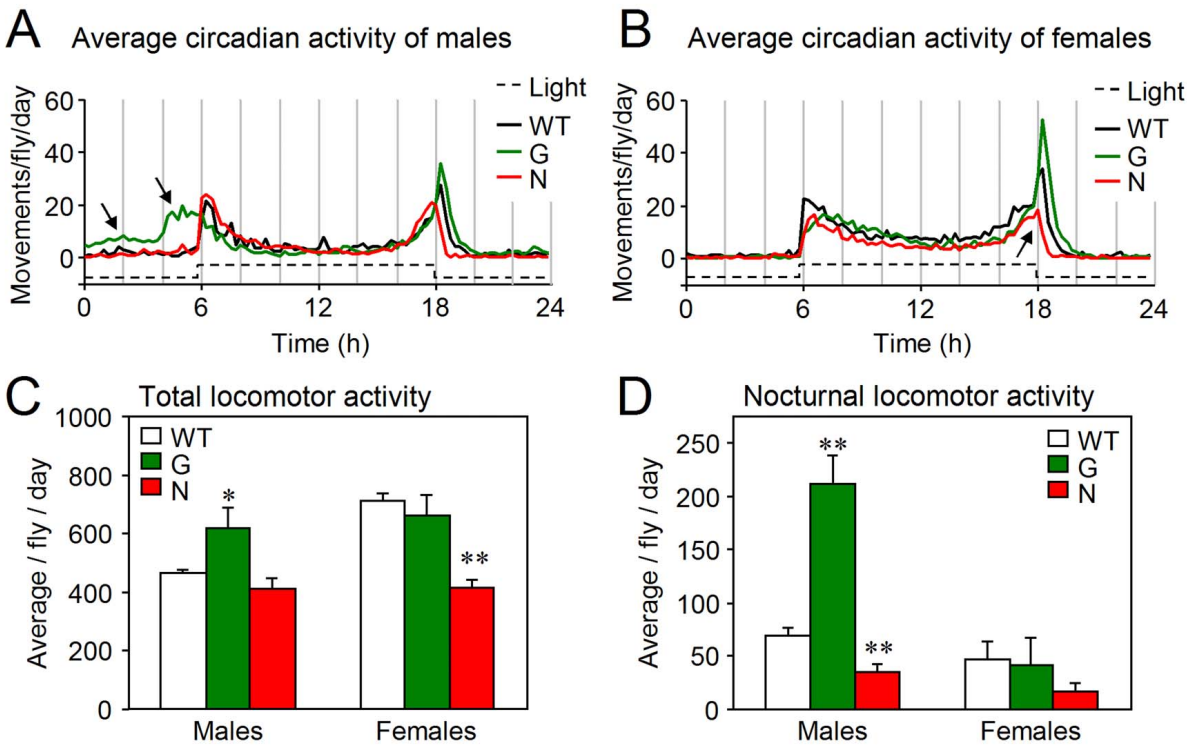

Fig. 5

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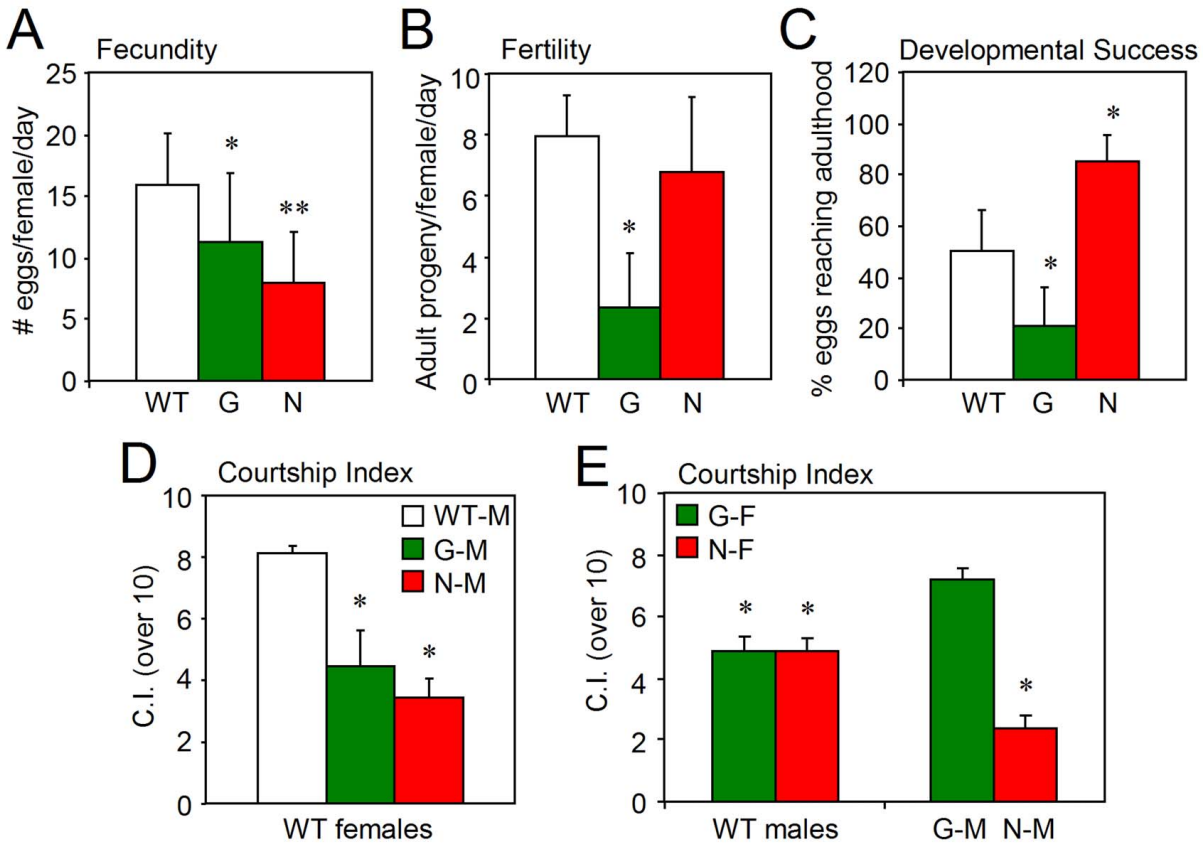

Fig. 6

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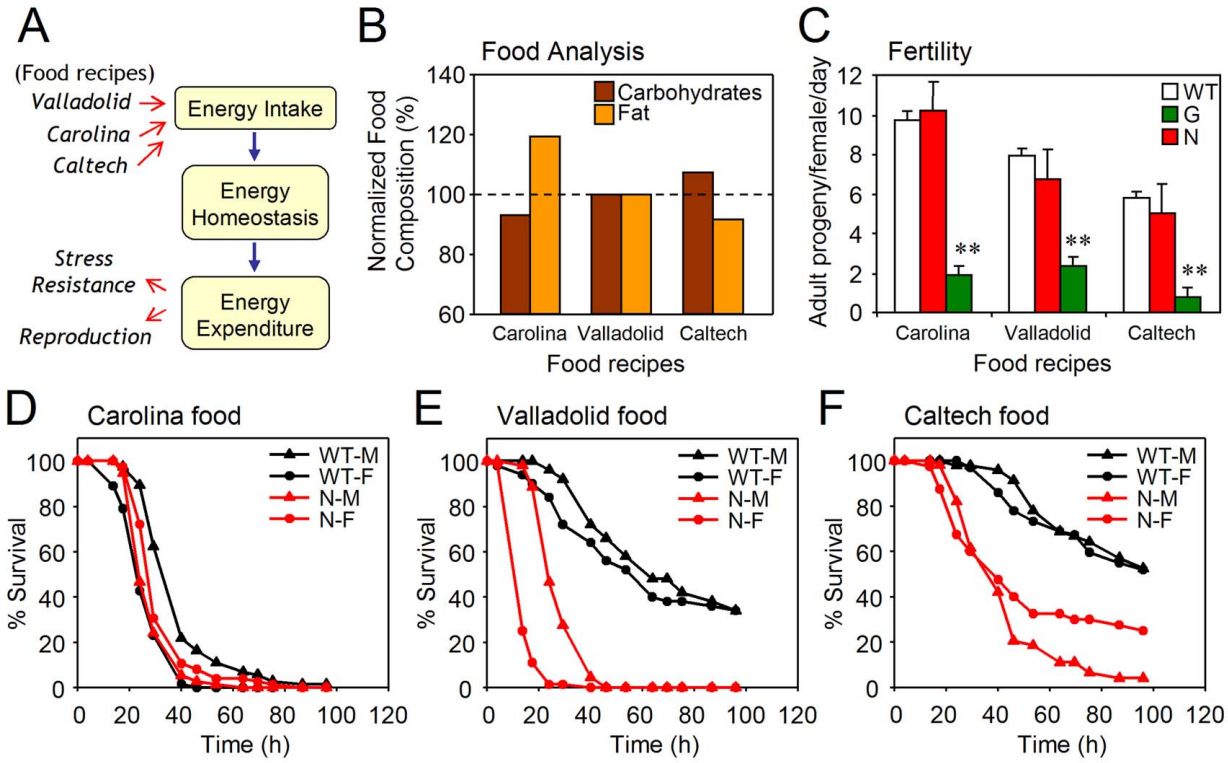

Fig. 7

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Table 1. Patterns of daily locomotor activity in Lipocalin mutants

Genotype/Sex	Total Activity	Diurnal Activity	Nocturnal Activity	Sunrise Activity	Sunset Activity
WT males	463.1 ± 14.2	203.69 ± 9.9	69.23 ± 7.4	76.21 ± 11.6	113.97 ± 18.6
GLaz$^{-/-}$ males	**615.22 ± 52.3[a]**	**137.19 ± 31.8[a]**	**212.07 ± 36.1[b]**	**116.64 ± 18.4[a]**	149.33 ± 17.8
NLaz$^{-/-}$ males	412.03 ± 35.1	188.89 ± 35.7	**37.74 ± 7.7[b]**	**99.03 ± 3.0[a]**	89.37 ± 4.2
WT females	710.05 ± 27.5	416.43 ± 17.0	46.16 ± 17.3	93.85 ± 5.4	153.61 ± 23.7
GLaz$^{-/-}$ females	660.17 ± 74.7	**361.89 ± 29.4[a]**	41.79 ± 25.6	**53.12 ± 1.7[b]**	203.37 ± 21.9
NLaz$^{-/-}$ females	**415.19 ± 28.7[b]**	**264.43 ± 15.4[b]**	17.19 ± 8.0	**53.98 ± 3.7[b]**	**59.59 ± 2.9[b]**

[a] different from WT ($p<0.05$)
[b] different from WT ($p<0.01$)

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Table 2. Courtship Behavior

Pair Genotypes	Courtship index	Mating time	Mating latency	% mating	Fraction mating
WT-M x WT-F	8.12 ± 0.27	22.15 ± 0.54	13.04 ± 1.37	74.0%	(37/50)
N-M x N-F	**2.38 ± 0.40[a]**	22.0 ± 0.0	**26.0 ± 11[a]**	**4.4%**	**(2/45)**
N-M x WT-F	**3.45 ± 0.64[a]**	21.15 ± 0.97	16.08 ± 3.77	**37.1%**	**(13/35)**
WT-M x N-F	**4.86 ± 0.45[a,b]**	24.13 ± 0.99	**23.88 ± 5.13[a]**	**22.9%**	**(8/35)**
G-M x G-F	7.18 ± 0.40	**19.73 ± 0.57[a]**	**31.62 ± 2.41[a]**	**52.0%**	**(26/50)**
G-M x WT-F	**4.46 ± 1.16[a,b]**	21.0 ± 1.21	15.17 ± 1.80	**55.8%**	**(19/34)**
WT-M x G-F	**4.88 ± 0.48[a,b]**	24.25 ± 1.0	**31.56 ± 4.77[a]**	**30.8%**	**(16/52)**

[a] different from WT-M x WT-F ($p<0.05$)

[b] different from mutant-M x mutant-F ($p<0.05$)

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