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Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases *Drosophila* longevity and stress resistance, and functionally replaces its secreted homolog NLaz

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ABSTRACT

Lazarillo (Laz) is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein first characterized in the developing nervous system of the grasshopper *Schistocerca americana*. It belongs to the Lipocalins, a functionally diverse family of mostly secreted proteins. In this work we test whether the protective capacity known for Laz homologs in flies and vertebrates (NLaz, GLaz and ApoD) is evolutionarily conserved in grasshopper Laz, and can be exerted from the plasma membrane in a cell-autonomous manner. First we demonstrate that extracellular forms of Laz have autocrine and paracrine protecting effects for oxidative stress-challenged *Drosophila* S2 cells. Then we assay the effects of overexpressing GPI-linked Laz in adult *Drosophila* and whether it rescues both known and novel phenotypes of NLaz null mutants. Local effects of GPI-linked Laz inside and outside the nervous system promote survival upon different stress forms, and extend lifespan and healthspan of the flies in a cell-type dependent manner. Outside the nervous system, expression in fat body cells but not in hemocytes results in protection. Within the nervous system, glial cell expression is more effective than neuronal expression. Laz actions are sexually dimorphic in some expression domains. Fat storage promotion and not modifications in hydrocarbon profiles or quantities explain the starvation–desiccation resistance caused by Laz over-expression. This effect is exerted when Laz is expressed ubiquitously or in dopaminergic cells, but not in hemocytes. Grasshopper Laz functionally restores the loss of NLaz, rescuing stress-sensitivity as well as premature accumulation of aging-related damage, monitored by advanced glycation end products (AGEs). However Laz does not rescue NLaz courtship behavioral defects. Finally, the presence of two new Lipocalins with predicted GPI-anchors in mosquitoes shows that the functional advantages of GPI-linkage have been commonly exploited by Lipocalins in the arthropodan lineage.

1. Introduction

Lazarillo (Laz) is a glycoprotein linked to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) tail, and first characterized in the developing nervous system of the grasshopper *Schistocerca americana* (Ganfornina et al., 1995; Sanchez et al., 1995, 2000a). Laz is expressed in a specific subset of neuroblasts, neurons and ganglion mother cells of the central nervous system (CNS). In the peripheral nervous system (PNS) Laz is detected in all sensory neurons and in a group of enteric nervous system neurons (Ganfornina et al., 1996; Sanchez et al., 1995). Its expression is neither limited to the embryonic stage, since it continues throughout adulthood, nor to the nervous system. Outside the nervous system Laz is associated mainly with the excretory system: malpighian tubules and subesophageal body. During nervous system development, Laz is involved in the growth and guidance of developing pioneer axons. Grasshopper embryos treated with a monoclonal antibody against Laz show axonal aberrant growth and misdirection (Sanchez et al., 1995). Lazarillo has been used as a marker of subsets of pioneer neurons also in other orthopterans, as in the economically important desert locust *Schistocerca gregaria* (Boyan et al., 2002, 2004; Boyan and Williams, 2004; Graf et al., 2000). However a test for grasshopper Laz functions during adulthood has not been performed yet.

Lazarillo belongs to the Lipocalin protein family, an ancient and functionally diverse family of secreted proteins that share a similar structural fold, often in the presence of low sequence similarity

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(Ganfornina et al., 2000). Their structure is defined by an eight-stranded antiparallel β-barrel that forms a binding pocket, usually hydrophobic (Flower, 1996; Flower et al., 2000; Ganfornina et al., 2006b). Molecular phylogenetic studies have shown that arthropodan Lipocalins have diversified in five main groups (Ganfornina et al., 2006a): a Laz-related clade of insect Lipocalins, a clade of Lipocalins found in Crustacea, a clade of biliproteins and related insect Lipocalins, and two large family expansions occurring in blood-feeding insects and arachnids. Grasshopper Laz has a basal position in the arthropodan Lipocalins phylogenetic tree, and the Laz-related clade is the closest relative to the chordate Lipocalin Apolipoprotein D (ApoD) (Ganfornina et al., 2000; Sanchez et al., 2003).

Lipocalins play a great number of different roles: bacteriostatic effect, retinoids transport, prostaglandins synthesis, control of reproductive behavior, and arthropod coloration among others (reviewed by Akerström et al. (2006)). The function of a given Lipocalin must be intimately related both with its hydrophobic ligands and with its site of expression. The nature of Laz physiological ligand(s) is still unknown, but a set of candidates has been studied *in vitro*. Lazarillo is able to bind retinoic acid and fatty acids (Sanchez et al., 2008).

The domain of expression of all insect Lipocalins analyzed so far commonly includes, although is not restricted to, ectodermal derivatives, with nervous system and epidermal tissues been recurrent sites of expression (reviewed by Ganfornina et al. (2006a)). Lepidopteran Lipocalins like Bilin binding protein from *Pieris brasssicae* and *Samia cynthia* or Insecticyanin from *Manduca sexta* are expressed in epidermis, and some of them have been demonstrated to play roles in cuticle coloration (Riddiford et al., 1990; Saito, 1998; Sehringer and Kayser, 2006) and prevention of oxidative damage (Schmidt and Skerra, 1994). Brain Lipocalins have been identified both in Diptera and Lepidoptera. The lepidopteran protein Hyphantrin from *Hyphantria cunea* is up-regulated in the brain in response to injury (Kim et al., 2005). Also, Bombyrin and Gallerin were isolated from pupal brain extracts of *Bombyx mori* and *Galleria mellonella* (Filippov et al., 1995; Sakai et al., 2001), but their function remains unknown.

In *Drosophila melanogaster*, two Lipocalins expressed in the nervous system are the Lazarillo homologs Neural-Lazarillo (NLaz) and Glial-Lazarillo (GLaz) (Ganfornina et al., 2000; Sanchez et al., 2003, 2000b). NLaz and GLaz have a key role in the regulation of longevity and defense against oxidative stress (Hull-Thompson et al., 2009; Ruiz et al., 2011; Sanchez et al., 2006b), and over-expressing NLaz or GLaz increases lifespan and survival against different forms of oxidative stress (Hull-Thompson et al., 2009; Walker et al., 2006).

The function of ApoD, the closest chordate Laz homolog, has also been studied extensively in diverse model organisms as well as in humans. Its expression in the brain is the most consistently up-regulated upon aging in different mammalian species (de Magalhaes et al., 2009; Loerch et al., 2008), and it is also up-regulated in many human neurodegenerative diseases (reviewed by Van Dijk et al. (2006)). Moreover, ApoD have demonstrated protective effects in the mouse brain upon oxidative stress (Bajo-Graneras et al., 2011a, 2011b; Ganfornina et al., 2008). That this protective function of ApoD is a conserved trait is supported by the results with transgenic flies expressing human ApoD. Like NLaz and GLaz, over-expressing human ApoD increases fly longevity and resistance against different stresses (Muffat et al., 2008).

So far the Lazarillo GPI-anchor is a unique feature within metazoan Lipocalins, which are mainly secreted proteins. Lipocalins linked to membranes through other mechanisms have been reported in bacteria and plants (Bishop et al., 2006; Charron and Sarhan, 2006), and indirect interactions of Lipocalins with membranes have been described for α1-acid-glicoprotein (Nishi et al., 2002), β-Lactoglobulin (Martins et al., 2008) and Tear Lipocalin (Saaren-Seppala et al., 2005). These data point to the existence of membrane receptors for Lipocalins, some of which have been already characterized, mostly for vertebrate Lipocalins (Hvidberg et al., 2005; Kawaguchi et al., 2007; Wojnar et al., 2001), but also for Insecticyanin in *M. sexta* (Kang et al., 1997).

In this work we test the protective potential of grasshopper Lazarillo in a cell culture system using the MT promoter system, and in adult fruit flies expressing grasshopper Laz with the UAS/GAL4 system. We test whether the GPI-linked Lazarillo, locally acting within and outside the nervous system, is able to promote survival upon different stress forms and extend the lifespan and healthspan of flies. Fat storage and hydrocarbon profiles are also explored as variables potentially controlled by the Lazarillo-related Lipocalins and contributing to their mechanism of regulating stress resistance and longevity. We also test whether the membrane-anchored Lazarillo is able to functionally restore the loss of NLaz by exploring stress resistance, biomarkers of aging-related damage, and behavioral phenotypes. Our purpose is to assay whether the protective capacity of NLaz, GLaz and ApoD is evolutionarily conserved in the grasshopper Lazarillo protein, and if this role can be exerted linked to the plasma membrane by a GPI-tail in a cell-autonomous manner. Finally, we found two new Lipocalins with predicted GPI-anchors in mosquitoes that point to the functional advantages of GPI-linkage as being commonly exploited by Lipocalins in the arthropodan lineage.

2. Material and methods

2.1. Cell culture

Drosophila S2 cells were maintained as semi-adherent cultures at 27 °C in Express-Five medium (Gibco) supplemented with 10% L-Glutamine, 50 U/ml Penicillin, and 50 μg/ml Streptomycin. The culture medium was replaced twice a week.

2.2. Cloning and purification of the Lazarillo protein

A fragment of the Lazarillo cDNA, translating into residues 1–192 of the precursor (Uniprot reference P49291), and thus missing the GPI signal peptide, was subcloned using the EcoRI and NotI sites into the pRmHa3 vector (Bunch et al., 1988; Sanchez et al., 2008). This system expresses the cloned sequence under the control of the inducible Drosophila metallothionein promoter, and the presence of a C-terminal His-tag sequence allows for protein purification from the culture medium.

The Laz-pRmHa3 plasmid was co-transfected with the selection vector pCoBlast (conferring blasticidin resistance) into Drosophila S2 cells using FuGENE6 (Roche) at 3:1 ratio according to the manufacturer instructions. Transfected cells were selected with 25 μg/ml blasticidin-S for 3 weeks (Invitrogen).

The recombinant protein was expressed in spinner flasks with blasticidin free medium, upon induction with 1 mM $CuSO_4$ for 5–7 days. The secreted Lazarillo protein was purified from the cell medium by immobilized metal affinity chromatography using nickel - nitrilotriacetic acid (Ni-NTA) resin (5-Prime). The Laz protein was further purified by size exclusion chromatography (Superdex prep grade 75, Amersham Biosciences) in PNEA buffer (25 mM PIPES, 150 Mm NaCl, 1 mM EDTA, 0.02% NaN_3). The purified protein was deglycosylated by treatment with peptide-N-glycosidase F (PNGase-F) from *Flavobacterium meningosepticum* (New England Biolabs) after denaturation following the protocol supplied by the manufacturer.

Bacterial recombinant Lazarillo was purified from the periplasmic space of *Escherichia coli* as previously described (Sanchez et al., 2008).

2.3. Fly generation and handling

Flies were grown in a temperature-controlled environmental incubator at 25 °C under a 12 h light–dark cycle. The stocks were routinely maintained in tubes containing our standard medium [yeast 84 g/l, sugar 84 g/l, NaCl 3.3 g/l, agar 10 g/l, wheat flour 42 g/l, apple juice 167 ml/l, propionic acid 5 ml/l; (Sanchez et al., 2006b)], or a yeast/cornmeal/agar medium [maize flour 83 g/l, agar 11 g/l, yeast 8.3 g/l, nipagine 5 g/l; (Wicker-Thomas et al., 2009)] in the case of hydrocarbon and triglyceride measurements. NLaz loss-of-function mutant (NLaz-KO) was generated in a w^{1118} background (Rong et al., 2002) and crossed with a w^{1118}-CS wild type line to generate the NW5 line used in this study (Hull-Thompson et al., 2009).

2.3.1. Generation of Lazarillo transformed Drosophila fly lines

P-element mediated transformation of w^{1118} flies was used to generate a pUASt-Laz fly line using standard procedures (BestGene Inc. USA). An EcoRI/XhoI fragment containing the full length Lazarillo cDNA (Ganfornina et al., 1995) was subcloned into the pUAST vector (FlyBase annotation FBmc0000383) to generate the UAS-Lazarillo transgene, with Upstream Activating Sequences (UAS) upstream of Lazarillo cDNA. Two different independent insertion lines of pUASt-Laz were used in our experiments, producing identical results. pUASt-Laz-8F has the insertion on X-chromosome, whereas pUASt-Laz-7F has the insertion on the third chromosome.

2.3.2. Expression of grasshopper Lazarillo under the control of GAL4 drivers

To drive the expression of the Laz transgene a set of GAL4 lines, expressing the yeast transcription factor GAL4 in different spatio-temporal patterns, were crossed with pUASt-Laz. To generate driver-only controls with minimal background differences we crossed each GAL4 line with wild-type flies (the strain w^{1118} in which the Laz transgenic lines were generated). For the hydrocarbon and triglycerides measures drivers on the third chromosome were used with the TM3 balancer. The Laz expression was achieved by crossing GAL4/TM3 flies with UAS-Laz flies. The resulting progeny bears the same genotype, excepting the third chromosome; UAS-Laz; TM3 was used as control and UAS-Laz; GAL4 was used as overexpressing Laz.

The following GAL4 drivers were used according to their main domain of expression: da-GAL4 (ubiquitous expression); $GAL4^{109(2)80}$ (expression in brain and muscle); BL7415 Repo-GAL4 and M1B-GAL4 lines (for glial expression), BL7009 Ddc-GAL4 (expression in dopaminergic and serotonergic neurons in the CNS and epidermis), BL 8699 He-GAL4 (expression in hemocytes), BL5820 31-1-GAL4 (expression in embryonic neuroblasts and neurons in the CNS and PNS), BL8816 D42-GAL4 (expression in motoneurons) and ppl-GAL4 (expression in fat body).

2.4. Wolbachia test

The absence of infection by *Wolbachia pipiens* in 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 the PCR conditions were: 2 min 94 °C; (30 s 94 °C; 30 s 60 °C; 45 s 72 °C) × 30; 7 min 72 °C. DNA from *Wolbachia*-infected *Dirofilaria immitis* worm was used as a positive control. Primer sequences and a positive control DNA were kindly provided by Dr. F. Simon (Univ. Salamanca).

2.5. Quantitative RT-PCR

Procedures, primers, PCR parameters, and other reagents were previously described (Ruiz et al., 2011). Briefly, total RNA was extracted from 3 days old flies ($n = 25$/sample) using TRIzol (Invitrogen), and reverse transcription was performed with PrimeScript™ RT reagent Kit (Takara) according to the manufacturer instructions. PCR reactions were performed in quintuplicate for each RNA sample using the SYBR® Premix Ex Taq™ (Takara). Expression of RPL18 was use to normalized the experimental samples. Transcriptional levels were calculated following the ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

2.6. Drosophila lifespan analysis

Flies were collected within 24 h of eclosion and allowed to mate for 2–3 days. They were then separated by sex under brief CO_2

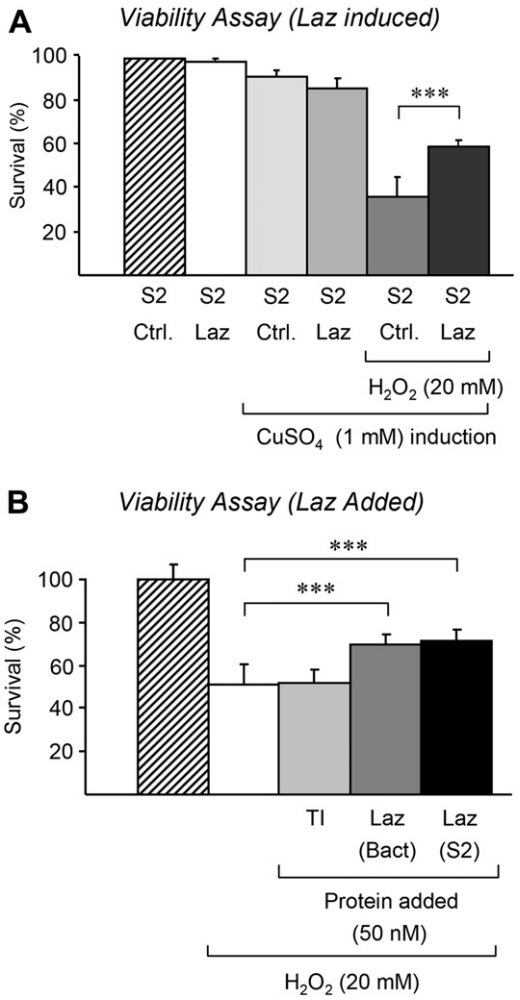

Fig. 1. Grasshopper Lazarillo promotes survival in S2 cells upon exposure to hydrogen peroxide. (A) S2 cells over-expressing Laz without its GPI tail, therefore secreted to the medium, are more resistant than wild-type cells to the oxidative stress generated by 20 mM H_2O_2. Expression is induced upon exposure to 1 mM $CuSO_4$. (B) Non-transfected S2 cells also improve their survival under oxidative stress upon addition of exogenous Lazarillo (50 nM) purified from either S2 cells culture medium or bacterial periplasm. Addition of Trypsin Inhibitor (TI, 50 nM) as a control unrelated protein does not influence cell survival. (*** Student's *t*-test, $p < 0.0005$).

anesthesia and housed in groups of 25. Every three days, flies were transferred to fresh media without anesthesia and scored for viability. Over 140 individuals were analyzed per sex, genotype and condition. The viability tests were carried out at 25 °C.

2.7. Oxidative stress, toxicity and desiccation stress assays

2.7.1. Assays in flies

Flies were collected and separated by sex as described for the longevity analysis. All tests were carried out at 25 °C. Over 60 individuals were analyzed per sex, genotype and condition. The following treatments were used:

2.7.1.1. Starvation–desiccation stress assay. Starting at 3 days of age, flies were transferred to empty plastic vials. Dead flies were scored every 2–7 h.

2.7.1.2. Hyperoxia stress assay. Adult flies (3 days old) were placed in plastic vials (20–25 flies per vial) containing our standard food and maintained in a plastic box enclosure at room temperature (25 °C) in a humidified 99.5% O_2 atmosphere. Dead flies were scored every 12 h.

2.7.1.3. Iron toxicity assay. Flies were kept on regular food until the experiment started (3 days of age), starved 3 h at 25 °C and transferred to vials with filter papers soaked in 1% glucose–15 mM $FeSO_4$ solution in water. Flies were moved everyday to new vials with fresh iron–glucose solution. Dead flies were scored every 2–7 h.

2.7.2. Assays in cell cultures

2.7.2.1. Oxidative stress assay. S2 cells were seeded at a density of 2×10^6/ml in 24-well plates, and 20 mM hydrogen peroxide (H_2O_2) was used as oxidant. When Lazarillo was generated by S2 cells, its expression was induced with 1 mM $CuSO_4$ 24 h. In other experiments, 50 nM of pure Lazarillo protein was added to the culture medium 30 min before H_2O_2 treatment. As a control protein, Trypsin Inhibitor (TI) from soybean (Sigma) was added at the same concentration. Laz and TI concentrations were determined with the Micro-BCA™ protein assay (Pierce). Two hours after treatment, cells were stained with Trypan Blue (Invitrogen) and counted in a hemocytometer. Cell viability was determined as the percentage of live cells. Each condition was assayed in triplicate, and three measurements were made in each culture well.

2.8. Climbing assay

The flies climbing ability was tested after 48 (for males), 72 and 96 h (for females) of iron treatment. Flies were placed in empty vials, and after a resting period of 10 min the animals were tapped to the bottom of the tube and the number of individuals able to climb 5 cm in 6 s was recorded. The assay was repeated three times at 1 min intervals.

2.9. Courtship behavior

Courtship and mating tests were performed during 60 min using virgin male–female pairs of 4–5 day-old flies under a Petri cover dish (40 mm diameter) used as an observation chamber. The courtship index (C.I.) represents the proportion of time a male spends actively courting the female for the first 10 min. Copulation features such as mating time, mating latency and mating performance (%) were scored for 60 min. The test was performed in the first 1–3 h of the fly daylight period.

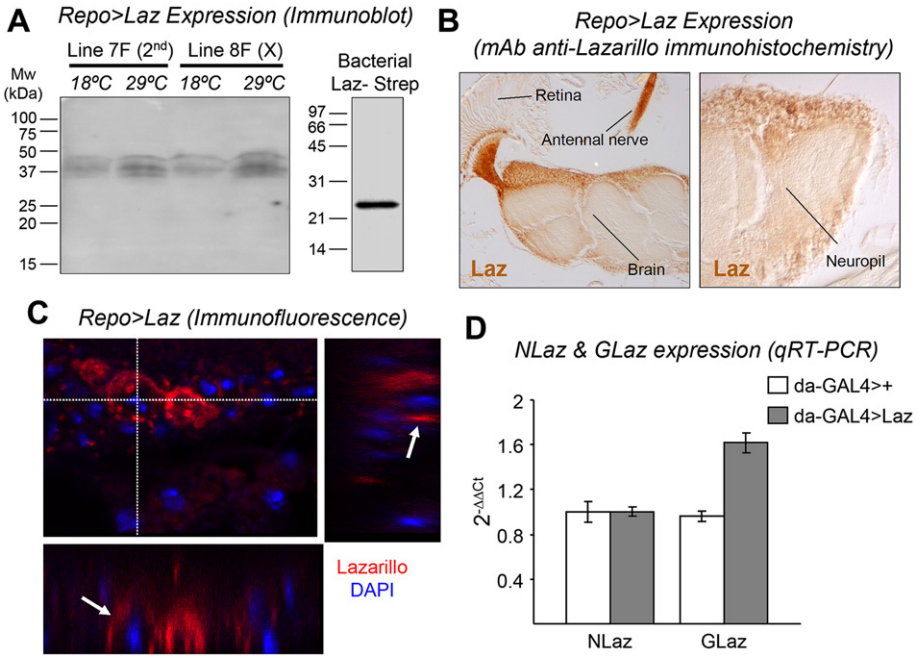

Fig. 2. Expression of grasshopper Lazarillo in transgenic flies. Influence on the expression of the *Drosophila* homologs NLaz and GLaz. (A) Immunoblot against Lazarillo with MAb 10E6 confirms its expression in flies at the protein level. Two lines representing independent insertions of the Laz gene in the *Drosophila* genome were tested (mapped to the 2nd and X chromosome). The level of expression is subject to the known temperature sensitivity of the GAL4/UAS expression system in Drosophila. Bacterially produced non-glycosylated Laz (Laz-Strep) was used as positive control and shows the difference in electrophoretic mobility with the protein produced in Drosophila. (B–C) MAb 10E6 also recognizes grasshopper Laz expressed under the control of a glial driver (Repo-GAL4). Membrane localization of the labeling is evident in confocal sections (C). (D) Quantitative PCR of NLaz and GLaz in transgenic flies over-expressing Laz compared to their driver-only controls. NLaz expression is not altered, while GLaz is slightly up-regulated when Lazarillo is present (Mann–Whitney *U* test, $p < 0.0005$).

2.10. Immunohistochemistry

Flies were fixed either with 5% glacial acetic acid, 4% formaldehyde, 85% ethanol, or 4% paraformaldehyde, and embedded in paraffin following standard procedures. Sections (5 μm) were performed with a rotary microtome (Microm), mounted in series on Polysine™ slides (Menzel-Gläser), and dried. Sections were dewaxed with xylene, and rehydrated through an ethanol series into phosphate buffered saline (PBS). Sections were then permeabilized and blocked with Tween20 (0.2% in PBS), 1% BSA and 1% normal goat serum.

The monoclonal antibody 10E6 against Lazarillo was used as primary antibody. Cy3 or HRP-conjugated goat anti-mouse IgG (Abcam) were employed as secondary antibodies. HRP development was performed with DAB (0.03%) and H_2O_2 (0.002% in 50 mM Tris, pH 8.0). For fluorescence immunohistochemistry, sections were mounted with Vectashield-DAPI (Vector Labs).

HRP immunohistochemistry sections were visualized with an Eclipse 90i (Nikon) microscope equipped with a DS-Ri1 (Nikon) digital camera. Images were acquired and processed with the NISElements BR 3.0 software (Nikon). Images from fluorescence immunohistochemistry preparations were obtained in a DMI 6000B microscope with a TCS SP5 X confocal system and a WLL laser (Leica) controlled by LAS AF software (Leica).

2.11. Immunoblots

Flies were homogenized in RIPA lysis buffer [1% NP-40, 0.1% SDS, 0.5% Na-DOC and 10% complete protease inhibitors (Roche) in PBS]. Protein extracts were separated on 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were washed (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% NaN_3) and blocked for 2 h with 0.2% gelatin, 2.5% BSA and 2.5% dry non-fat milk in the previous buffer (Ganfornina et al., 1995). Membranes were then incubated with primary antibody 10E6 (1:200 in blocking solution) for 2 h, washed again, and incubated with HRP-conjugated goat anti-mouse (1:10,000) (Dako) for 1 h at RT. Blots were developed with ECL (ImmobilonTM Western, Millipore), and the signal visualized with a digital camera (VersaDoc, BioRad).

2.12. AGE assay

Fluorescent AGEs (advanced glycation end products) were measured according to Oudes et al. (1998) with minor modifications. Flies were homogenized in 1 ml of PBS containing 10 mM EDTA. Cuticle and other debris were removed by centrifugation for 5 min at 11,000 g. Supernatants were digested by addition of 10 mg/ml trypsin (Sigma). Following incubation for 24 h at 37 °C the digested homogenates were centrifuged and filtered through a 0.22 μm sterile filter. Filtrates were diluted up to an absorbance range of 0.02–0.05 units at 365 nm. Fluorescence (excitation and emission wavelengths of 365 and 440 nm, respectively) was measured in three aliquots of each filtrate with a Shimadzu RF-5301PC spectrofluorophotometer at 25 °C.

2.13. Hydrocarbon profile and triglyceride content assays

Flies were separated by sex just after emergence and kept in groups of ten flies for 5 days at 25 °C. Each single fly was extracted with 100 μl heptane and 500 ng n-C26 (internal standard) for 5 min. Samples were injected into a Perichrom Pr200 gas chromatograph, as previously described (Wicker-Thomas et al., 2009). Hydrocarbon profiles and total amount of hydrocarbons (between 23 and 29 carbons) was calculated.

The total triglyceride content of 10 flies/sample was analyzed using the Triglyceride Liquicolor kit (Stanbio Laboratory) as described (Meunier et al., 2007). Two to four independent measures were performed for each genotype (with flies coming from independent crosses).

2.14. Statistical analysis

Statistical analyses were carried out with Statistica (v 5.5) software. Survival curves were analyzed using the Log-Rank Test. In maximal survival and quantitative PCR experiments the nonparametric Mann–Whitney *U* test was performed. Student's *t*-Test was

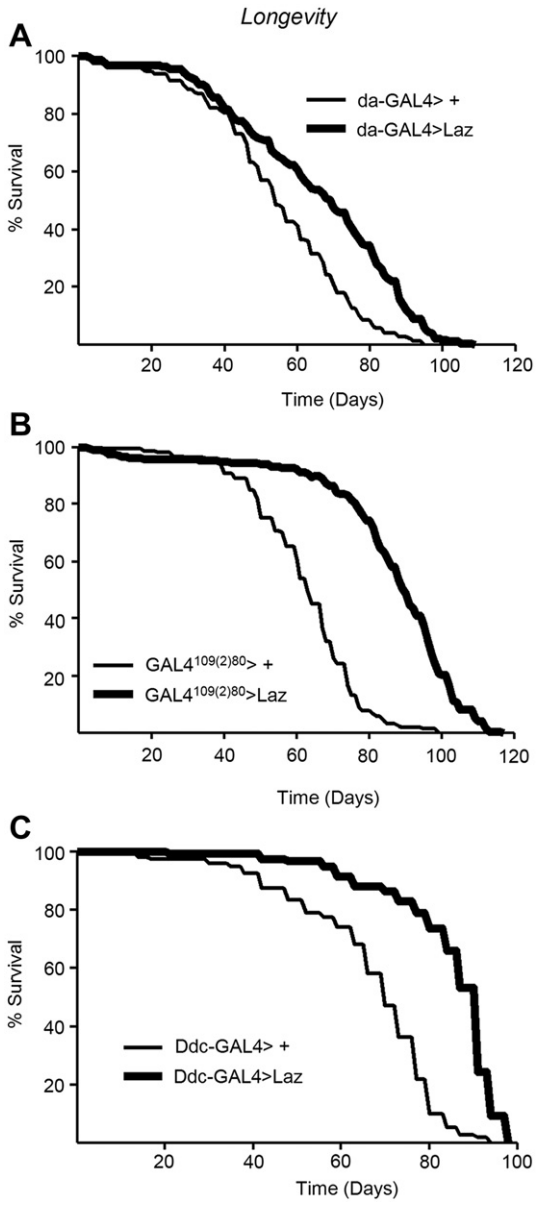

Fig. 3. The overexpression of grasshopper Lazarillo extends lifespan. (A) Drosophila lifespan increases when Laz is expressed using the ubiquitous da-GAL4 driver (da-GAL4 > Laz) compared to da-GAL4 > + control flies. Median survival time is increased by 28.3% in the Lazarillo expressing flies (Long-rank test: $p < 0.0005$). (B) Median lifespan is also increased by 43.5% when $GAL4^{109(2)80}$ drives the expression of Laz in muscles and brain (Log-rank test: $p < 0.0005$). (C) When Laz is expressed in dopaminergic and serotonergic neurons and epidermis, fly survival increases by 31.4% (Log-rank test: $p < 0.0005$). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

Table 1
Summary of the survival parameters and behavioral scores obtained with different experimental paradigms in flies of different sex and genotypes.

Background	Driver (*main site of expression*)	*N*	Sex	Experiment	Median survival or climbing success		Maximal survival	
					Sig.	% Change	Sig.	% Change
WT	Da (*Ubiquitous*)	266–418	♀	Longevity	***	**28.302**	***	**21.413**
WT		239–294	♂	Longevity	***	8.475	**	9.962
WT		93–123	♀	Desiccation	***	**25.806**	***	**24.184**
WT		105–144	♂	Desiccation	***	**23.112**	***	10.677
WT		38–69	♀	Hyperoxia	ns	−3.736	*	15.926
WT		45–63	♂	Hyperoxia	ns	0.000	ns	−0.925
WT		105–116	♀	Iron	***	17.056	***	**21.210**
WT		99–100	♂	Iron	***	**28.205**	*	5.370
WT		42–77	♀	Climbing (72 h)	***	67.532		
WT		15–40	♀	Climbing (96 h)	*	275.00		
WT		24–61	♂	Climbing (48 h)	*	16.065		
$NLaz^{NW5}$		87–118	♀	Desiccation	***	**71.678**	***	17.098
$NLaz^{NW5}$		85–119	♂	Desiccation	***	**45.038**	***	**23.149**
$NLaz^{NW5}$		68–110	♀	Iron	***	**83.167**	***	**23.811**
$NLaz^{NW5}$		62–112	♂	Iron	***	**94.130**	***	**31.214**
WT	109 (*CNS/Muscle*)	171–286	♀	Longevity	***	**43.548**	***	**40.937**
WT		136–294	♂	Longevity	***	**32.727**	***	**32.455**
WT		89–140	♀	Desiccation	***	**30.055**	***	**20.732**
WT		79–138	♂	Desiccation	ns	3.333	ns	4.409
WT		43–55	♀	Hyperoxia	ns	0.000	ns	3.577
WT		27–42	♂	Hyperoxia	***	−12.859	ns	−9.266
WT		90–105	♀	Iron	***	**24.113**	**	12.789
WT		80–97	♂	Iron	*	0.000	**	7.230
WT		24–73	♀	Climbing (72 h)	ns	−7.347		
WT		24–43	♂	Climbing (48 h)	ns	−17.395		
WT	31-1 (*Neurons*)	68–95	♀	Desiccation	*	5.849	***	**35.647**
WT		61–79	♂	Desiccation	*	14.815	***	**20.192**
WT	Ddc (*Dopaminergic and*	117–119	♀	Longevity	***	**31.479**	***	13.410
WT	*Serotonergic Neurons*)	118–121	♂	Longevity	***	**30.000**	***	11.753
WT		233–289	♀	Desiccation	***	**37.903**	***	**21.689**
WT		262–312	♂	Desiccation	ns	2.105	ns	6.748
WT		82–94	♀	Hyperoxia	***	7.154	*	6.412
WT		86–98	♂	Hyperoxia	***	**23.680**	***	**24.910**
WT		97–102	♀	Iron	*	5.282	*	7.091
WT		94–101	♂	Iron	***	−14.450	*	−3.725
WT		40–54	♀	Climbing (72 h)	ns	−1.234		
WT		23–59	♂	Climbing (48 h)	*	−45.735		
WT	D42 (*Motoneurons*)	93–100	♀	Desiccation	*	−1.990	***	−20.659
WT		80–95	♂	Desiccation	**	4.444	**	**20.815**
WT		69–86	♀	Hyperoxia	**	6.772	ns	2.785
WT		87–96	♂	Hyperoxia	ns	7.155	**	−1.509
WT		81–93	♀	Iron	ns	−14.571	*	5.864
WT		72–85	♂	Iron	ns	0.000	***	−23.170
WT		59–61	♀	Climbing (72 h)	ns	47.766		
WT		8–31	♂	Climbing (48 h)	ns	−55.288		
WT	Repo (*Glial cells*)	87–109	♀	Desiccation	***	**23.980**	***	**21.809**
WT		62–74	♂	Desiccation	**	10.484	**	**41.286**
WT		67–81	♀	Hyperoxia	*	9.282	*	7.018
WT		57–88	♂	Hyperoxia	***	−23.077	**	−9.964
WT		29–34	♀	Iron	ns	−1.29	ns	11.745
WT		13–57	♂	Iron	ns	0.000	ns	7.831
WT	M1B (*Glial cells*)	84–91	♀	Desiccation	***	**38.298**	***	**28.791**
WT		83	♂	Desiccation	**	**23.256**	**	7.483
WT		81–90	♀	Hyperoxia	***	**22.049**	***	12.899
WT		83–91	♂	Hyperoxia	***	7.618	**	7.028
WT		73–93	♀	Iron	***	**42.324**	***	**28.193**
WT		48–51	♂	Iron	ns	0.000	ns	4.141
WT	He (*Hemocytes*)	82–112	♀	Desiccation	ns	−3.468	ns	1.692
WT		81–87	♂	Desiccation	ns	0.000	*	2.688
WT	ppl (*Fat body*)	77–89	♀	Desiccation	***	**21.212**	***	**22.865**
WT		81–83	♂	Desiccation	***	18.868	***	**20.139**
WT		81–99	♀	Hyperoxia	***	13.117	**	12.258
WT		79–94	♂	Hyperoxia	***	7.877	***	**25.376**
WT		81–93	♀	Iron	***	−8.450	ns	−1.679
WT		82–94	♂	Iron	***	−25.535	*	−8.242

Values in bold indicate changes in median or maximal survival larger than 20%. Survival curves differences tested with Log-rank test. Climbing success differences tested with Student's *T*-test. $p < 0.05$ (*); $p < 0.005$ (**); $p < 0.0005$ (***).

used for the analysis of the climbing assays, cell survival, triglycerides and AGEs assays. One-way ANOVA followed by Tukey's multiple comparison post-hoc test was used for hydrocarbon profile comparisons. Kruskal–Wallis one-way ANOVA on Ranks followed by Dunn's multiple comparison post-hoc test was applied to analyze courtship experiments. Differences were considered statistically significant at $p < 0.05$ (*) $p < 0.005$ (**), and $p < 0.0005$ (***).

2.15. Sequence alignment and prediction of GPI-anchoring signal peptides

Multiple sequence alignments were performed with CLUSTAL-X2 (Larkin et al., 2007). Potential GPI cleavage sites were predicted by the big-PI Predictor (Eisenhaber et al., 1999). Hydrophobicity profiles were determined by Kyte and Doolittle's method (1982) using a window of 9 residues.

3. Results and discussion

3.1. Grasshopper Lazarillo promotes survival in S2 cells upon oxidative stress

To test whether the ability of NLaz, GLaz and ApoD to protect organisms under oxidative stress is an evolutionarily conserved function in grasshopper Laz, we used two different recombinant forms of Laz to assay their effects on S2 cell survival (Fig. 1). A secreted form of Laz was generated in *E. coli* and purified from the bacterial periplasmic space. A glycosylated and soluble form of Laz (Fig. S1), lacking the GPI tail in order to make it a secreted form, was expressed by *Drosophila* S2 cells with an inducible expression system.

We first tested the effect of expressing Laz on the survival of S2 cells exposed to oxidative stress. After 24 h of induction, S2 cells expressing soluble grasshopper Laz are more resistant to H_2O_2 (58.1% survival, Fig. 1A) than control non-transfected S2 cells (35.9% survival). The exogenous addition of purified recombinant Laz protein also promotes cell survival (Fig. 1B). Non-transfected control S2 cells show 51.4% and 51.6% survival upon H_2O_2 treatment when either no addition was performed or an unrelated protein was added to the culture. Survival increases to 71% and 69.7% respectively when Laz, either purified from S2 cells (glycosylated) or from bacteria (non-glycosylated), is added to the culture medium. A similar protective effect has been demonstrated for the mammalian Lazarillo homolog, ApoD, on primary astrocytes (Bajo-Graneras et al., 2011a), where it contributes to lower the levels of lipid peroxides and to modify the transcriptional response of cells to oxidative stress.

3.2. Expression of GPI-linked grasshopper Lazarillo in Drosophila docks the protein to the cell membrane and does not alter native Drosophila Lipocalins expression

We generated a full-length cDNA construct downstream of UAS promoter sequences in order to use the GAL4/UAS system to express the GPI-linked grasshopper Laz in Drosophila with different expression patterns according to the GAL4 driver used. Two independent single-insertion transgenic lines carrying the pUASt-Laz transgene were chosen for the study (lines 7F and 8F, mapped to the 3rd and X chromosome respectively). These transgenic lines as well as other lines used in this study were tested for the presence of *Wolbachia*, an endosymbiont of arthropods known to alter longevity related traits (Ikeya et al., 2009). No infection was detected (Fig. S2A).

Laz expression was tested by immunoblot (Fig. 2A). The protein shows the characteristic smear due to its glycosylation and an electrophoretic mobility (40 kDa) similar to the native grasshopper protein. Both transgenic lines have similar Laz expression levels. The bacterially produced non-glycosylated Laz protein is shown for comparison. Immunohistochemistry experiments (Fig. 2B–C) show the protein present in the expected cell type (glial cells in the particular case shown), and localized at the cell surface (arrows in Fig. 2C). Negative controls performed to ascertain that the monoclonal antibody 10E6 against grasshopper Laz is not cross-reacting with any Drosophila epitope are shown in Fig. S2B. These experiments confirmed that all the post-translational modifications that take place in the native grasshopper Laz, including anchoring to the cell membrane, were correctly reproduced in the transgenic flies.

Since we intended to assay the effects of overexpressing Laz in wild type flies, we quantified the expression of the native

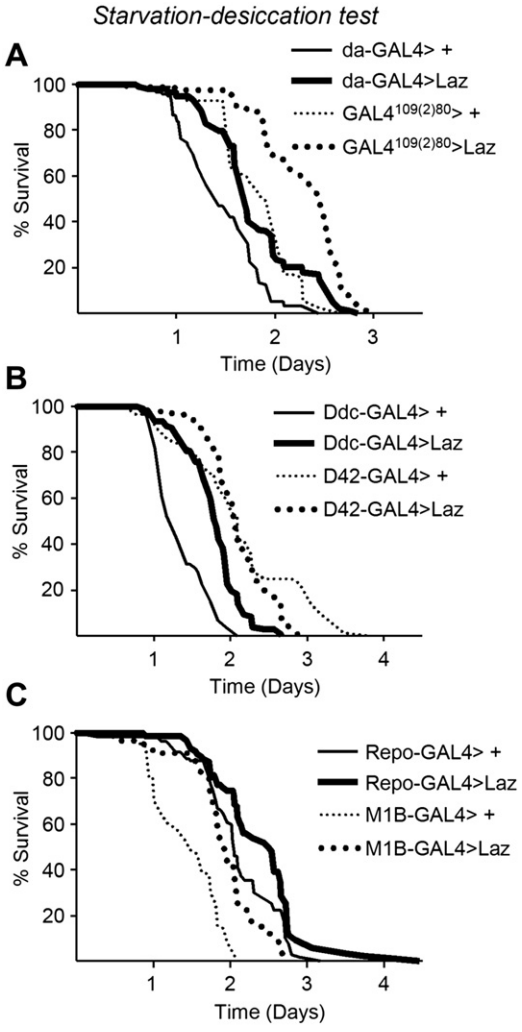

Fig. 4. Lazarillo protects flies against starvation–desiccation stress. (A) Resistance against starvation–desiccation increases when Lazarillo is expressed in flies using da-GAL4 or GAL4$^{109(2)80}$ drivers. Median survival increases by 25.8 and 30% respectively (Log-rank test: $p < 0.0005$ in both cases). (B) Median survival is increased by 37.9% (Log-rank test: $p < 0.0005$) when Lazarillo expression is driven by Ddc-GAL4. However, using a motoneuron driver (D42-GAL4) no changes in median-survival upon starvation–desiccation are observed. (C) Expression in glial cells (Repo-GAL4 or M1B-GAL4 drivers) also promotes survival upon starvation–desiccation (survival increases 24% and 38.3% respectively; Log-rank test: $p < 0.0005$ in both cases). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

homologous Drosophila genes (GLaz and NLaz) under these circumstances. NLaz gene expression was unaltered (Fig. 2D), while GLaz gene was slightly (1.6-fold) up-regulated in flies over-expressing Laz ubiquitously. Increases of 7–10 fold in GLaz ubiquitous expression have been reported to produce mild longevity extensions (Walker et al., 2006). Therefore, though we cannot discard a contribution of the mildly altered expression of GLaz upon transgenic Laz over-expression, other results presented and discussed below cast doubt about the functional significance of these minor changes.

3.3. Membrane bound grasshopper Lazarillo increases lifespan with both ubiquitous and nervous system-enriched expression

Laz overexpression produces a significant increase in fly longevity. Median survival increases in female flies by 28%, 43.5% and 31.4% when Laz is expressed either ubiquitously (da-GAL4 driver, Fig. 3A), in the nervous and muscular system ($GAL4^{109(2)80}$ driver, Fig. 3B), or in the dopaminergic–serotonergic neurons and epidermis (Ddc-GAL4 driver, Fig. 3C). Effects in male flies (Table 1) are less prominent when Laz is expressed ubiquitously (8.5%), but very similar when expressed under the control of $GAL4^{109(2)80}$ or Ddc-GAL4 drivers (32.7% and 30% respectively). Similar results were obtained with both Laz transgenic lines. The survival curves shown in Fig. 3 are the average of data obtained independently in cohorts from both lines. Survival curves obtained independently for each UAS:Laz line are shown in Fig. S3. Table 1 summarizes the results of all tests performed with male and female flies.

The magnitude of the effects obtained with Laz overexpression contrasts lifespan extension obtained in transgenic male flies producing GLaz with similar expression patterns: 0–21% in ubiquitous patterns (actin or Hsp70 drivers), 23–29% when driven by $GAL4^{109(2)80}$, and no effect when expressed pan neuronally (elav driver) or in motor neurons (D42 driver) (Walker et al., 2006). Thus, grasshopper Laz is able to extend lifespan of flies more efficiently than the native GLaz, especially when the expression domain included neurons ($GAL4^{109(2)80}$ and Ddc-GAL4 drivers). So far, no analysis of NLaz effects on longevity have been performed with neuronally enriched patterns. On the other hand, ubiquitous overexpression of NLaz with the da-GAL4 driver produces a 42–50% increase in median longevity of male flies (Hull-Thompson et al., 2009), a larger effect than that obtained with Laz.

The Ddc-GAL4 line used drives the expression of UAS-transgenes with the dopa decarboxylase promoter, the enzyme controlling the synthesis of dopamine (DA). DA is not only a neurotransmitter in insect CNS, but has also a role in the sclerotization of pigmented cuticle. The Ddc gene is expressed in late embryogenesis, when cuticle hardening is taking place (Wang et al., 1996), and in the epidermis of third instar larvae (Scholnick et al., 1983). Also, DA levels peak within the first hours after pupa eclosion (Neckameyer and Weinstein, 2005). DA contributes to adult cuticle hardening in this period, but also regulates cuticular sex-specific hydrocarbon biosynthesis in female flies (Marican et al., 2004; Wicker-Thomas and Hamann, 2008). Curiously, the later effect is dependent on DA production in the brain, and not on local production of DA by Ddc activity in the epidermis. In addition, Ddc expression can be induced in the larval and adult epidermis upon local infection, as part of the immune response (Davis et al., 2008). Ddc basal expression in adult epidermis is small compared to the expression in the CNS domain (Davis et al., 2008). In view of these data the large extension of lifespan caused by Laz expressed under the control of the Ddc driver (Fig. 3C) has probably an important contribution of the neuronal domain of expression (dopaminergic and serotonergic neurons in the CNS), where Ddc is expressed throughout life. However, below we describe sex-dependent effects of Laz on stress resistance when expressed

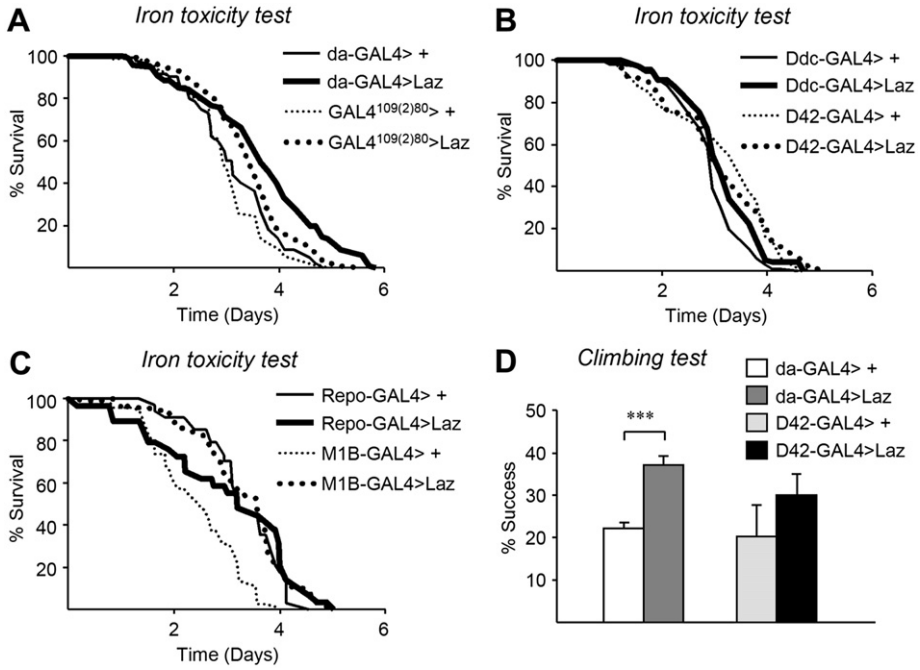

Fig. 5. Expression of Lazarillo improves survival and locomotor behavior of flies exposed to iron. (A) Wide range expression of Lazarillo (da-GAL4 > Laz or $GAL4^{109(2)80}$ > Laz) increase median survival upon $FeSO_4$ treatment by 17 and 24.1% (Log-rank test $p < 0.0005$). (B) A small increase in median lifespan (5.3%; Log-rank test: $p < 0.05$) is observed when Lazarillo is expressed by dopaminergic and serotonergic neurons and the epidermis of flies exposed to $FeSO_4$. In contrast, D42-GAL4 > Laz flies show no change in median survival. (C) Expression in glial cells with the repo-GAL4 driver does not modify fly resistance to iron intoxication. However, M1B-GAL4 > Laz flies significantly increase their survival 42.3% (Log-rank test: $p < 0.0005$). (D) Climbing ability was assayed after 72 h of iron exposure in ubiquitous and motoneuron overexpressor flies. Only ubiquitous expression of grasshopper Laz improves fly climbing ability after iron exposure (Student's *T*-test: $p < 0.0005$). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

under the control of Ddc-GAL4. The putative contribution of Laz epidermal expression will be discussed.

3.4. Lazarillo protects flies against different forms of stress in a cell-type dependent manner within the nervous system

Laz increases survival of flies upon starvation–desiccation stress when expressed with ubiquitous or wide range patterns (23.1–30% median survival increase, Fig. 4A and Table 1). Protective effects are obtained when Laz is expressed by dopaminergic–serotonergic neurons and epidermis (Ddc-GAL4 driver, Fig. 4B), or by a subset of embryonic neuroblasts and neurons in the CNS and PNS (31-1-GAL4 enhancer trap insertion; Fig. S4). However, no protection is obtained when Laz is driven to motoneurons by D42-GAL4 (Fig. 4B). These effects are, moreover, sex-dependent (see below). In contrast, glial cell expression of grasshopper Laz consistently protects both male and female flies from starvation–desiccation stress with survival extensions ranging 10.4–38.3% (Fig. 4C and Table 1).

When flies are subjected to oxidative stress (exposure to 15 mM $FeSO_4$, Fig. 5), Laz is able to extend their survival when expressed with the glial driver M1B (Fig. 5C), the Ddc driver (Fig. 5B), the nervous and muscular systems driver ($GAL4^{109(2)80}$, Fig. 5A) or the ubiquitous driver (da-GAL4). These protective effects are therefore also cell type-dependent and could be sensitive to variations in timing or magnitude of overexpression.

We also assayed whether Laz expression either ubiquitously or in neuron-enriched patterns would modify the locomotor performance of Fe^{2+}-intoxicated flies. Generic overexpression of Laz (da-GAL4 driver) improves climbing performance after 72 h of iron treatment (Fig. 5D and Table 1) and the protecting effect is maintained up to 93 h of treatment (Table 1). However, expression of Laz with D42 or Ddc drivers shows no significant effect (Fig. 5D and Table 1). Thus, the ubiquitously-expressed grasshopper Laz is not only able to extend the flies' lifespan under normal or stress conditions, but also to improve their healthspan.

Finally, when oxidative stress is provoked by hyperoxia, neuronal or glial enriched expression of Laz is beneficial for flies (Fig. 6 and Table 1) with significant effects ranging from a mild 6.8% to a substantial 23.7% increase in survival. However, no statistically significant survival extension was obtained with a wide expression of Laz (Fig. 6A).

3.5. Local effects of GPI-linked Lazarillo outside the nervous system promote survival under starvation and oxidative stress

The expression domains of *Drosophila* GLaz and NLaz and grasshopper Laz include cells outside the nervous system. GLaz is expressed in mesodermal cells, gut epithelium, and salivary glands primordia during embryogenesis (Sanchez et al., 2000b), and in adult hemocytes (Sanchez et al., 2006b). NLaz is expressed in developing mesodermal cells and sessile cells in the gut lumen (Sanchez et al., 2000b), and in the developing and adult fat body (Hull-Thompson et al., 2009; Sanchez et al., 2000b). Finally grasshopper Laz is expressed in cells at the tip of the Malpighian tubules, nephrocytes of the subesophageal body, and in a group of mesodermal cells that might be the precursors of sessile hemocytes, nephrocytes, or fat body cells (Sanchez et al., 1995). Thus, systemic effects of Lipocalins might be part of their longevity-regulation and stress-protection functions.

Interestingly, when expressed in hemocytes, grasshopper Laz has no effect in starvation–desiccation resistance (Fig. S4B). However, flies expressing Laz in the fat body increase their resistance to both starvation–desiccation and hyperoxia stress (Fig. S5A–B and Table 1). Iron exposure appears to be deleterious for flies overexpressing Laz in fat-body (Fig. S5C).

Expression of NLaz in the fat body is triggered by the JNK signaling pathway and promotes resistance to starvation and oxidative stress (Hull-Thompson et al., 2009). Since native NLaz is a secreted protein, we have proposed NLaz as an important component of the systemic control of metabolism necessary under stress conditions. In support of that idea, expression of NLaz in pericardial cells or hemocytes also protect against a starvation–desiccation stress (Hull-Thompson et al., 2009). On the other hand, GLaz is not expressed by fat body cells, but controls fat storage in these cells (Sanchez et al., 2006b), probably acting at a distance once secreted to the hemolymph by hemocytes. Our results show that the GPI-linked grasshopper Laz protects against stress when expressed in fat body cells (Fig. S5 and Table 1), but not in hemocytes (Fig. S4B and Table 1), suggesting that not only systemic effects, but also local effects of these Lipocalins in the fat body tissue are important for stress resistance.

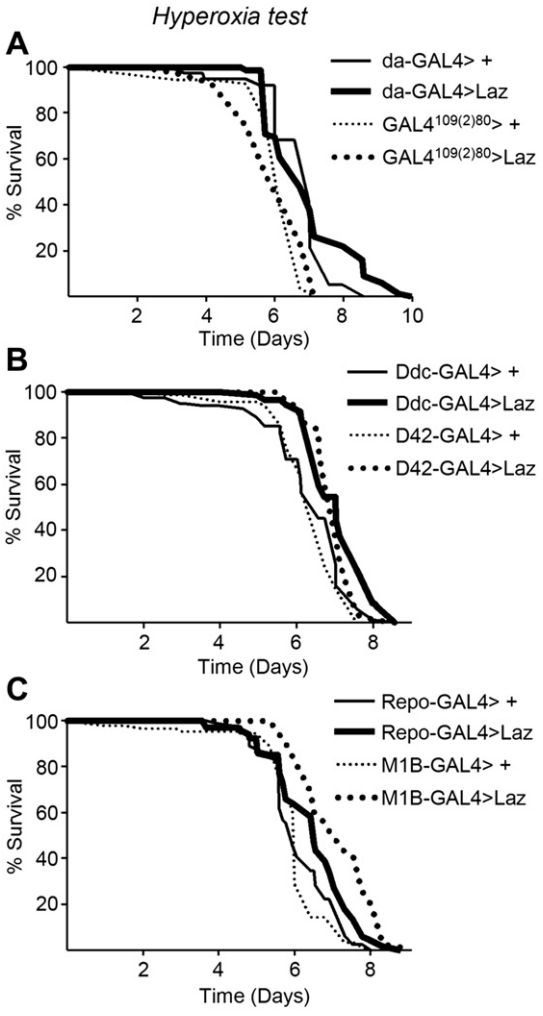

Fig. 6. Grasshopper Laz protects against hyperoxia in a cell-type dependent manner. (A) Wide range expression of Lazarillo (da-GAL4 > Laz or $GAL4^{109(2)80}$ > Laz) does not modify median survival upon hyperoxia. (B) The expression of Laz in dopaminergic and serotonergic neurons and epidermis (Ddc-GAL4 > Laz) or in motoneurons (D42-GAL4 > Laz) increases slightly their median survival: 7.1 and 6.8% (Log-rank test: $p < 0.0005$ and $p < 0.005$ respectively). (C) When Lazarillo is expressed in glial cells using M1B-GAL4 driver, it produces a significant survival expansion in female flies exposed to hyperoxia: 22% increase in median survival (Log-rank test: $p < 0.0005$). Conversely, repo-GAL4>Laz flies show a milder increase (9.3%) in median-survival (Log-rank test: $p < 0.05$). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

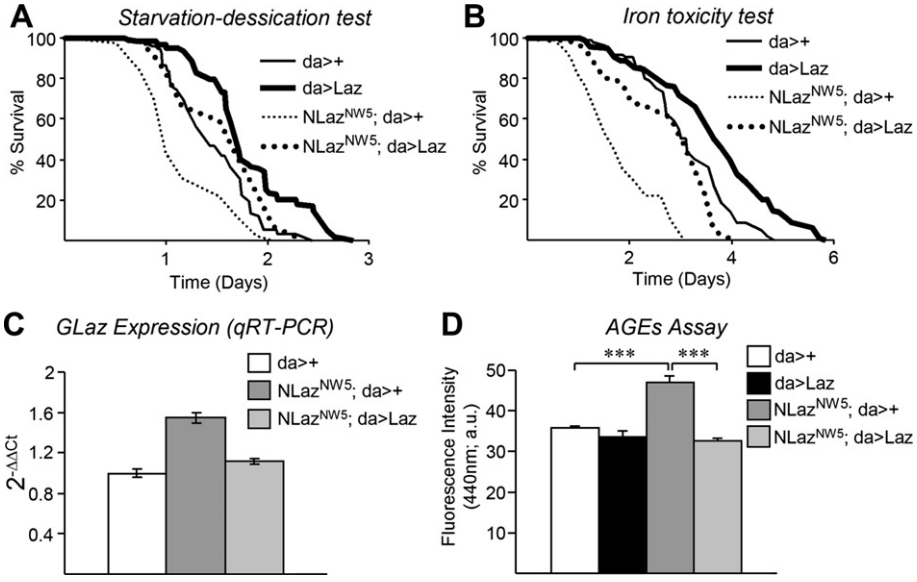

Fig. 7. Grasshopper Lazarillo expression rescues NLaz null mutant flies from stress-sensitivity and accelerated-aging phenotypes. (A, B) Homozygous NLaz^{NW5} flies are more sensitive to starvation–desiccation and iron exposure than control flies. Ubiquitous expression of grasshopper Laz returns NLaz null mutant flies to or above control levels. Median survival increases by 71.7% (NLaz ^{NW5}; da-GAL4 > + *versus* NLaz ^{NW5}; da-GAL4 > Laz) in the starvation–desiccation test and 83.2% in the iron toxicity assay (Log-rank test: $p < 0.0005$ in both cases). Female data is shown. The complete data for both sexes is shown in Table 1. (C) GLaz transcript levels slightly increase in homozygous NLaz^{NW5} flies (da-GAL4 > + *versus* NLaz ^{NW5}; da-GAL4 > +). Overexpression of grasshopper Laz returns GLaz expression back to control levels (NLaz ^{NW5};da-GAL4 > + *versus* NLaz^{NW5};da-GAL4 > Laz flies). (D) Advanced glycation end products (AGEs), which normally accumulate upon aging, are basally increased in NLaz null mutant young female flies reflecting an accelerated aging process. Grasshopper Laz expression rescues this phenotype completely (Student's *t*-test, $p < 0.0005$ in both cases).

3.6. Lazarillo protects flies against different forms of stress in a sex dependent manner

Using loss-of-function mutants we have previously shown that (i) lifespan extension by GLaz is sex dependent, (ii) NLaz and GLaz expression change in opposite directions (only in females) upon aging, and (iii) the relative contribution of head and body in Lipocalin expression also varies with sex (Ruiz et al., 2011). A multivariate analysis uncovered that differences in metabolic parameters, particularly protein and relative water content, account for the observed sex-specific patterns of longevity.

Upon different types of stress, grasshopper Laz also shows sex-dependent effects. When expressed by the Ddc driver, resistance to starvation–desiccation stress is promoted only in females (Fig. S6A), resistance to hyperoxia is promoted in both sexes (Fig. S6B), and resistance to iron exposure is slightly enhanced in females and decreased in males (Fig. S6 C). Some of these differences could be explained by functional differences of Laz in the Ddc cuticular domain of expression. Since epidermal DA production is triggered in stress situations (Neckameyer and Weinstein, 2005; Sekine et al., 2011) and Ddc functions downstream of the stress-responsive JNK signaling cascade upon a septic cuticle injury (Davis et al., 2008), a contribution to epidermal Laz expression might not be negligible under the stress situations used in our experiments. DA production is particularly important in females, where it controls the last biochemical steps toward the production of female-specific long-chain hydrocarbons with pheromonal functions (Wicker-Thomas and Hamann, 2008). Although a quantitative profile of Ddc expression in the epidermis has not been reported yet, our data are compatible with a significant expression of membrane-bound Laz expression in female epidermis (driven by the Ddc promoter) as a putative cause for the sex-specific effects in starvation–desiccation resistance.

Similarly, females expressing Laz in the nervous system and muscles (109-GAL4 driver) show a larger resistance to starvation–desiccation than males, but neuronal Laz (31-1 or D42 drivers) shows larger protective effects in males than in females (Table 1).

Interestingly, overexpression of grasshopper Laz extends lifespan in both males and females (Fig. 3 and Table 1).

3.7. Grasshopper Lazarillo rescues stress-sensitivity and premature AGEs accumulation in NLaz null mutant flies

At least two arthropodan Lipocalins are predicted to be the minimal ancestral set of Lipocalins (Sanchez et al., 2006a). However, awaiting full genome sequencing, no other grasshopper Lipocalins have been reported so far. Among the Drosophila Lipocalins, NLaz and grasshopper Laz show many shared properties, in addition to their neuronal expression domain. They both have higher % similarity in protein sequence (Ganfornina et al., 2000) and intron–exon structure similarity index (Sanchez et al., 2003) than the Laz–GLaz pair. Also NLaz, but not GLaz, bears an extended C-terminal segment similar in size to the GPI-anchoring signal of Laz (Ganfornina et al., 2006a) that could represent a vestigial NLaz tail (Fig. 9).

We therefore set to examine whether the ancient Laz is able to rescue the phenotypes caused by the loss of NLaz. The NLaz loss-of-function mutation is known to reduce longevity, and to increase the flies sensitivity to oxidative and starvation–desiccation stress (Hull-Thompson et al., 2009; Ruiz et al., 2011). Ubiquitous expression of the GPI-linked grasshopper Laz (da-GAL4 driver) is in fact able to restore stress resistance to or above control levels, both under starvation–desiccation stress and iron induced oxidative stress (Fig. 7A–B). Median survival increases by 71.7% and 83.2% respectively with respect to control flies.

A slight increase in GLaz mRNA expression (1.6 fold increase) is observed when NLaz is absent (Fig. 7C). As it has been described for the vertebrate homologue ApoD in the mouse (Bajo-Graneras et al., 2011a, 2011b; Ganfornina et al., 2008; Ganfornina et al. 2010), without additional stress the absence of NLaz might cause an imbalance in tissue homeostasis, generating a basal level of stress. This in turn might cause the observed slight GLaz up-regulation.

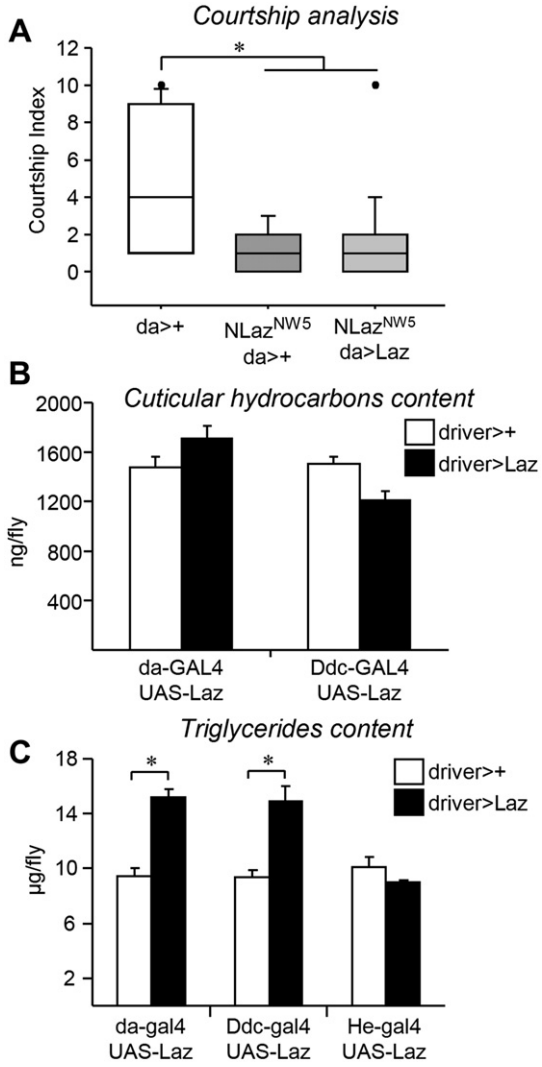

Fig. 8. Ubiquitous expression of Lazarillo promotes fat storage, but does not alter phenotypes related to pheromonal signaling. (A) Homozygous NLaz^{NW5} flies show a decreased courtship index and expression of Lazarillo under the control of da-GAL4 does not rescue this phenotype. (B) Total amounts of cuticular hydrocarbons are not altered by Laz expression neither ubiquitously nor when expressed in a nervous system enriched pattern (Ddc-GAL4 > Laz). (C) Triglyceride contents increase in flies overexpressing Laz with da-GAL4 or Ddc-GAL4 drivers. Laz has no effect on fat storage when expressed only in hemocytes (He-GAL4 > Laz). (* Student's *T*-test: $p < 0.05$). Female data is shown in all panels. The complete data for both sexes is shown in Table 2.

Since both proteins are secreted within and outside the nervous system, though from different cell types, they might be functionally compensating for each other. However, GLaz expression is equivalent to control levels when membrane-linked grasshopper Laz is expressed with the da-GAL4 driver. Therefore, the rescue of NLaz null mutation by grasshopper Laz cannot be ascribed to functional compensation by GLaz. These GLaz expression changes, together with those observed when grasshopper Laz is expressed in wild type flies (Fig. 2D), are indicative of the existence of stochastic small variations in the expression levels for GLaz. Whether they represent biologically relevant variations would need further study.

During normal aging, reducing sugars react nonenzymatically with proteins or nucleic acids forming advanced glycation end-products (AGEs) that accumulate within cells. A 40% increase in AGEs has been reported in *Drosophila* between 10 and 75 days of age (Oudes et al., 1998). Fluorescent AGEs are reliable biomarkers of aging-related damage. They track the effects of factors that condition mortality rates (e.g., temperature in the case of ectothermic animals like *Drosophila*), but are irreversible and thus not affected by other potentially reversing factors like dietary restriction (Jacobson et al., 2010). If the lack of NLaz does impose a basal stress in the tissues, we should expect an accelerated aging occurring in the null mutant flies. This hypothesis is confirmed, since young 3–5 day old NLaz mutant flies exhibit 31.6% more fluorescent AGEs than control flies (Fig. 7D). Interestingly, the ubiquitous expression of grasshopper Laz fully rescues the acceleration of aging in mutant NLaz flies (Fig. 7D). The beneficial action of grasshopper Laz therefore must prevent age-related damage to occur.

3.8. Courtship behavior and cuticular sex pheromones are not altered by Lazarillo expression

NLaz not only modulates longevity and regulates the metabolic response to stress (Hull-Thompson et al., 2009; Ruiz et al., 2011), but also participates in fly mating behavior (Ruiz et al., 2011). Loss of NLaz leads to deficiencies in courtship that are dependent on functional alterations in both sexes, since both wild type males paired with mutant females and mutant males paired with wild type females are less involved in courting. The courtship index decreases to even lower levels when both flies are NLaz null mutants (Ruiz et al., 2011). Here we show that a ubiquitous overexpression of Laz is not able to rescue the courtship defects produced by the lack of NLaz (Fig. 8A), indicating that Lazarillo is not able to substitute NLaz in all of its functions.

We have proposed that courtship defects in NLaz mutants might be due to its role as a transporter of pheromones either at the emission or reception level. As mentioned above, the main sex-pheromones in insects are cuticular long-chain unsaturated hydrocarbons (HC) synthesized from fatty acid precursors in epidermal cells. They, together with visual and acoustic cues, play an important role in male courtship behavior (Wicker-Thomas et al., 2009). Interestingly, HC are also important factors modulating survival upon desiccation. A causal association between HC composition, their resulting global melting point and desiccation survival has been well documented (Foley and Telonis-Scott, 2011; Rouault et al., 2004).

We assayed the profile of HC in Laz overexpressing flies, comparing global expression (da-GAL4 driver) with nervous system enriched expression (Ddc-GAL4 driver). In no case the expression of Laz produced major changes in the profile of HC in males or females (Tables S1 and S2). The total amount of HC (between 23 and 29 carbons), calculated for da, and Ddc-driven expression of Laz, also showed no significant differences (Fig. 8B and Table 2).

3.9. Neutral fat stores are promoted by Lazarillo

Since Laz does not change the profiles or total amounts of HC, they cannot account for its ability to increase starvation–desiccation resistance in flies. We therefore tested whether greater metabolic stores, particularly fat stores, could be the mechanism by which flies can better resist the lack of nutrients and water in our starvation–desiccation test.

A significant increase in total triglycerides content is actually observed when grasshopper Laz is expressed ubiquitously (da-GAL4 driver) or in dopaminergic cells (Ddc-GAL4 driver) both in males and females (Fig. 8C and Table 2). However, the effect is not attained when Laz is expressed in hemocytes (He-GAL4 driver).

Opposite to Laz overexpression, NLaz and GLaz null mutant adults exhibit reduced triglycerides stores and are sensitive to diverse forms of starvation (Hull-Thompson et al., 2009; Sanchez et al., 2006b). Also, the fat body tissue is markedly reduced in GLaz mutants (Sanchez et al., 2006b). Our results support a model in which the control of fat storage by Lipocalins is a conserved

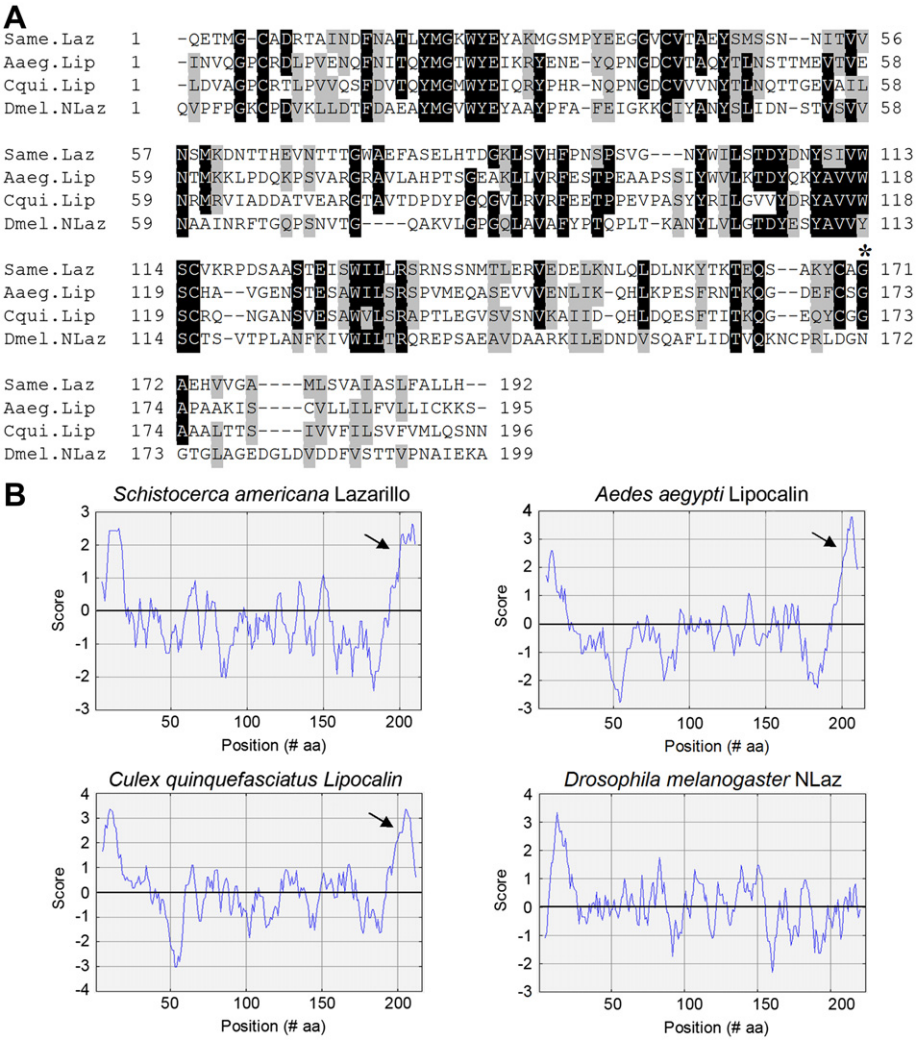

Fig. 9. GPI-linkage is a shared character in Orthoptera and Diptera Lipocalins. (A) Multiple sequence alignment of Lazarillo (Same.Laz), NLaz (Dmel.NLaz) and the two predicted GPI-linked Lipocalins from the mosquitoes *Aedes aegypti* (Aaeg.Lip) and *Culex quinquefasciatus* (Cqui.Lip). Sequences were aligned with CLUSTAL-X2. Black and gray boxes show residue identities and similarities respectively. Potential GPI cleavage sites for Aaeg.Lip and Cqui.Lip were predicted by the big-PI Predictor software and are highlighted with an asterisk. They align with the experimentally demonstrated GPI-signal peptide cleavage site of Same.Laz. (B) Hydrophobicity plot of the Lipocalin protein sequences determined by Kyte and Doolittle's method using a window of 9 residues. The hydrophobic domains of the GPI-anchoring signal are indicated by arrows.

ancestral function, promoting both starvation and desiccation resistance.

3.10. Tissue specificity and range of action of Lazarillo related Lipocalins: contributions to the control of the aging process

The ubiquitous expression of grasshopper Laz, anchored to cell membranes, is able to accomplish many functions. In wild type background it promotes lifespan extension, decreases starvation–desiccation sensitivity, and improves survival and locomotor activity upon iron treatment. In addition it decreases AGEs formation in the accelerated-aging NLaz mutant background, and rescues its sensitivity to starvation–desiccation and oxidative stress.

Some of these functions are also possible when grasshopper Laz is expressed by subsets of cells, within or outside the nervous

Table 2
Total hydrocarbon and triglyceride content in Laz over-expressing vs. control flies.

	Control males	Laz males	Control females	Laz females
Hydrocarbons (ng/fly)				
da-GAL4 x UAS-Laz	1474 ± 203	1207 ± 71	1471 ± 90	1704 ± 104
Ddc-GAL4 x UAS-Laz	1289 ± 66	1186 ± 65	1499 ± 60	1207 ± 73
Triglycerides (μg/fly)				
da-GAL4 x UAS-Laz	7.04 ± 0.25	**10.77 ± 0.3**[a]	**9.48 ± 0.8**[b]	**14.9 ± 0.3**[a]
Ddc-GAL4 x UAS-Laz	7.03 ± 0.23	**8.06 ± 0.49**[a]	**9.41 ± 0.44**[b]	**14.91 ± 1.09**[a]
He-GAL4 x UAS-Laz	7.04 ± 0.20	6.62 ± 0.25	10.12 ± 0.70	9.00 ± 0.13

Average ± SEM is represented. Values in bold and shaded in gray indicate significant differences assayed by Student's *T*-test.
[a] Difference between Laz overexpression and driver-only control ($p < 0.05$).
[b] Difference between females and males of the same genotype ($p < 0.05$).

system. We have reasoned above that the sex-independent longevity extension obtained with the Ddc-GAL4 driver could be mostly dependent on the neuronal expression, while the female-specific resistance to starvation–desiccation stress is better explained by the epidermal expression of Laz. On the other hand, the results obtained with restricted patterns of expression (e.g. hemocytes or fat body) clearly show that Laz can perform its protection against starvation–desiccation when locally expressed in the fat body and not in hemocytes, which contrasts with the long-range actions of the secreted GLaz or NLaz homologs (Hull-Thompson et al., 2009; Sanchez et al., 2006b). We have shown that secreted forms of grasshopper Laz are also able to protect insect S2 cells in culture when exposed to oxidative stress, both in an autocrine and a paracrine manner. Thus, Lazarillo has the potential of acting both, from the extracellular space or linked to the cell membrane. Interactions of the secreted GLaz and NLaz with cell membranes upon which they exert their protective functions are predicted as a result.

The restricted neuronal expression of the 31-1-GAL4 driver (similar to the embryonic expression pattern of grasshopper Laz) consistently, though moderately, improves survival upon starvation–desiccation in a sex-independent manner. Glial restricted expression of Laz does cause large survival increases upon different forms of stress, reflecting the wider range of protective functions exerted by glial cells. As we have shown for mouse ApoD (Bajo-Graneras et al., 2011a), protecting glial cells might represent a beneficial strategy to maintain the nervous system homeostasis and ultimately improve the whole organism survival. Our results with grasshopper Laz are in agreement with the protecting effects of glial-derived ApoD on oxidation-challenged dopaminergic neurons in the mouse (Bajo-Graneras et al., 2011a), as well as global effects on survival upon systemic oxidative stress (Ganfornina et al., 2008).

3.11. Ancestral vs. derived functions of the Lazarillo-related Lipocalins. Is the GPI-linked grasshopper Lazarillo an evolutionary rarity?

We have shown that grasshopper Laz is able to perform protective functions when expressed in a variety of patterns. Moreover, a ubiquitous expression of Laz rescues NLaz null mutants from stress sensitivity and accelerated aging phenotypes. However, functional equivalence between grasshopper Laz and Drosophila NLaz is not all-inclusive since deficient courtship phenotypes of NLaz null mutants are not rescued. A divergent evolution of hydrophobic ligand binding specificities could account for the differences between grasshopper Laz and NLaz.

These results strongly support the hypothesis that some functions (e.g., lipid storage control, protection against oxidative stress, and oxidative age-related damage), all of them impacting on life-span regulation, represent the ancestral suite of functions for this group of Lipocalins. The Lazarillo-related Lipocalins include ApoD, which also extends lifespan and protects against oxidative stress in the fly (Muffat et al., 2008). Other functions, such as the specific signaling required for proper courtship, might be part of the autapomorphic characters evolved independently in different lineages, like the NLaz-dependent courtship behavior in Drosophila, or the axillary odorant binding by human ApoD (Zeng et al., 1996).

The features currently present in the extant Drosophila Lipocalins might be the result of a gene duplication occurring before the divergence of Orthoptera and Diptera. As mentioned above this would predict the existence of a GLaz-like gene in grasshoppers, but it also predicts that more Lipocalins derived from a GPI-linked ancestor might exist in insects. Thanks to the genome sequencing of diverse insect species, we were able to identify two Laz-like mosquito Lipocalins with an extended C-terminal segment that is not only similar in size to the GPI-anchoring signal of Laz, but also has the characteristic hydrophobicity profile and predicted cleavage site (Fig. 9). This finding strongly supports the hypothesis of an ancestral GPI-signal peptide as the origin of the vestigial NLaz C-terminal tail, and that insects have exploited the functional possibilities provided by GPI-linked Lipocalins in more lineages than previously thought.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ibmb.2012.07.005.

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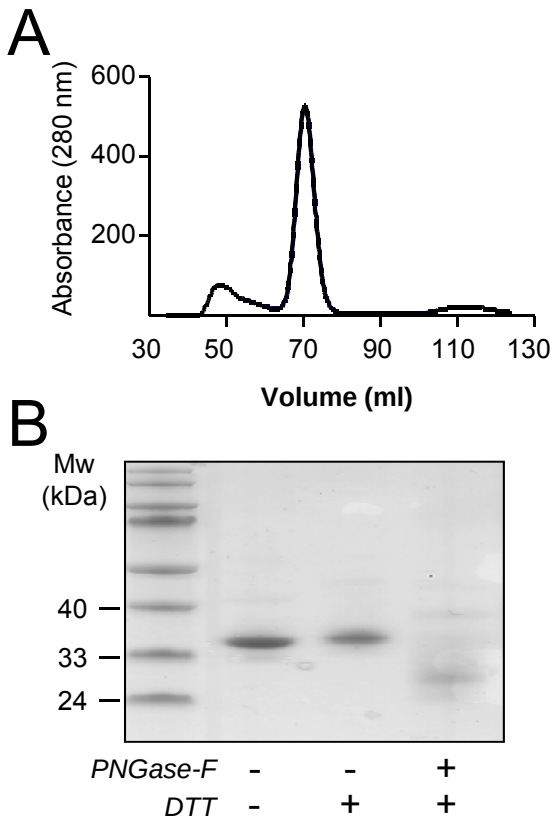
Figure S1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400
200
Absorbance (280 nm)
30
50
70
90
110
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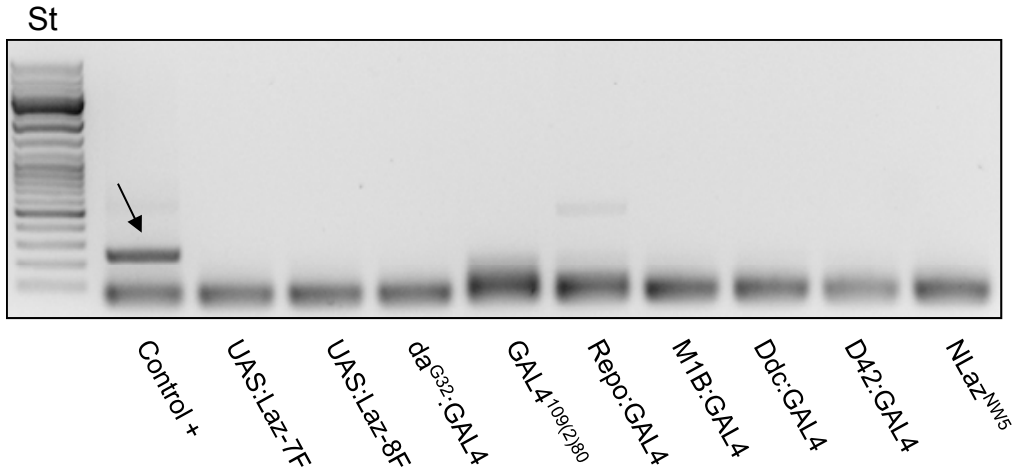
Volume (ml)
B
Mw
(kDa)
40
33
24
PNGase-F - - +
DTT - + +

Figure S2


St
Control +
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UAS:Laz-8F
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NLazNW5

Figure S3

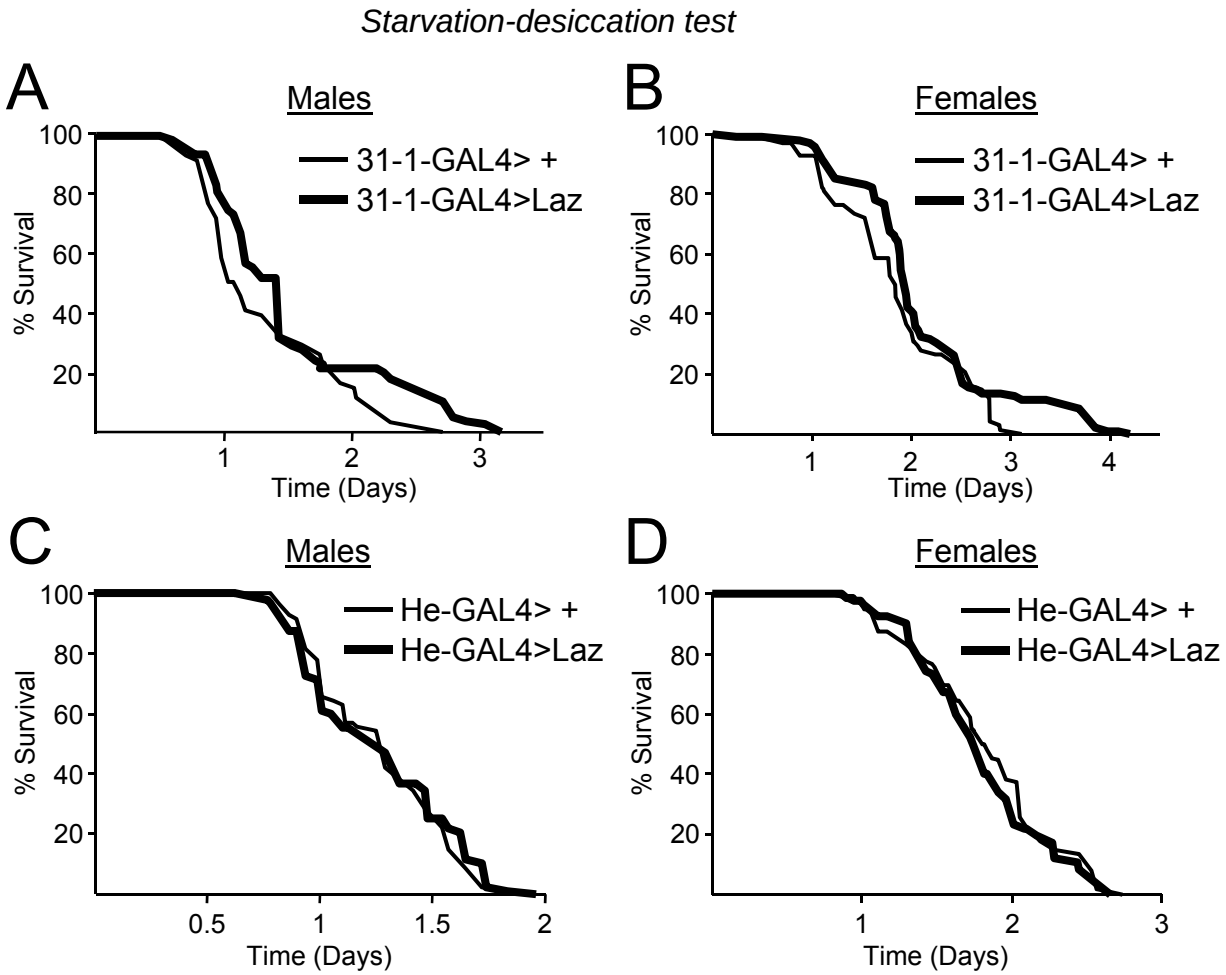
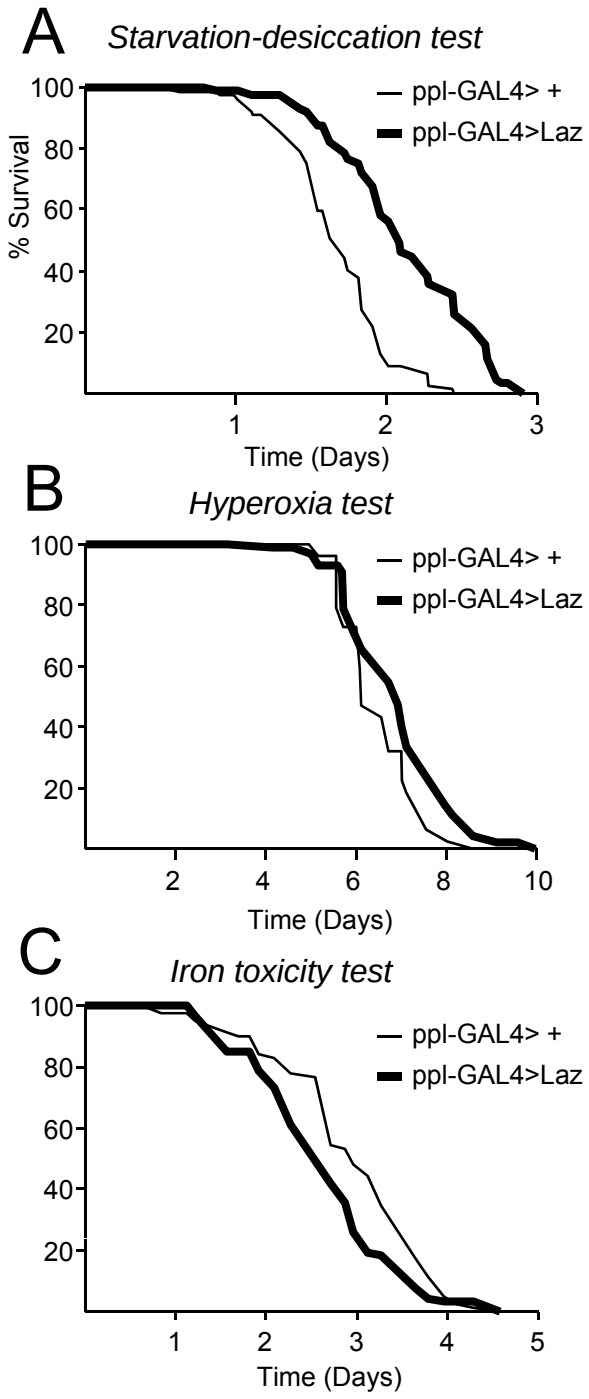

Starvation-desiccation test
A
Males
31-1-GAL4> +
31-1-GAL4>Laz
% Survival
Time (Days)
B
Females
31-1-GAL4> +
31-1-GAL4>Laz
% Survival
Time (Days)
C
Males
He-GAL4> +
He-GAL4>Laz
% Survival
Time (Days)
D
Females
He-GAL4> +
He-GAL4>Laz
% Survival
Time (Days)

Figure S4


A
Starvation-desiccation test
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ppl-GAL4>Laz
% Survival
100
80
60
40
20
1
2
3
Time (Days)
B
Hyperoxia test
ppl-GAL4> +
ppl-GAL4>Laz
100
80
60
40
20
2
4
6
8
10
Time (Days)
C
Iron toxicity test
ppl-GAL4> +
ppl-GAL4>Laz
100
80
60
40
20
1
2
3
4
5
Time (Days)

Figure S5

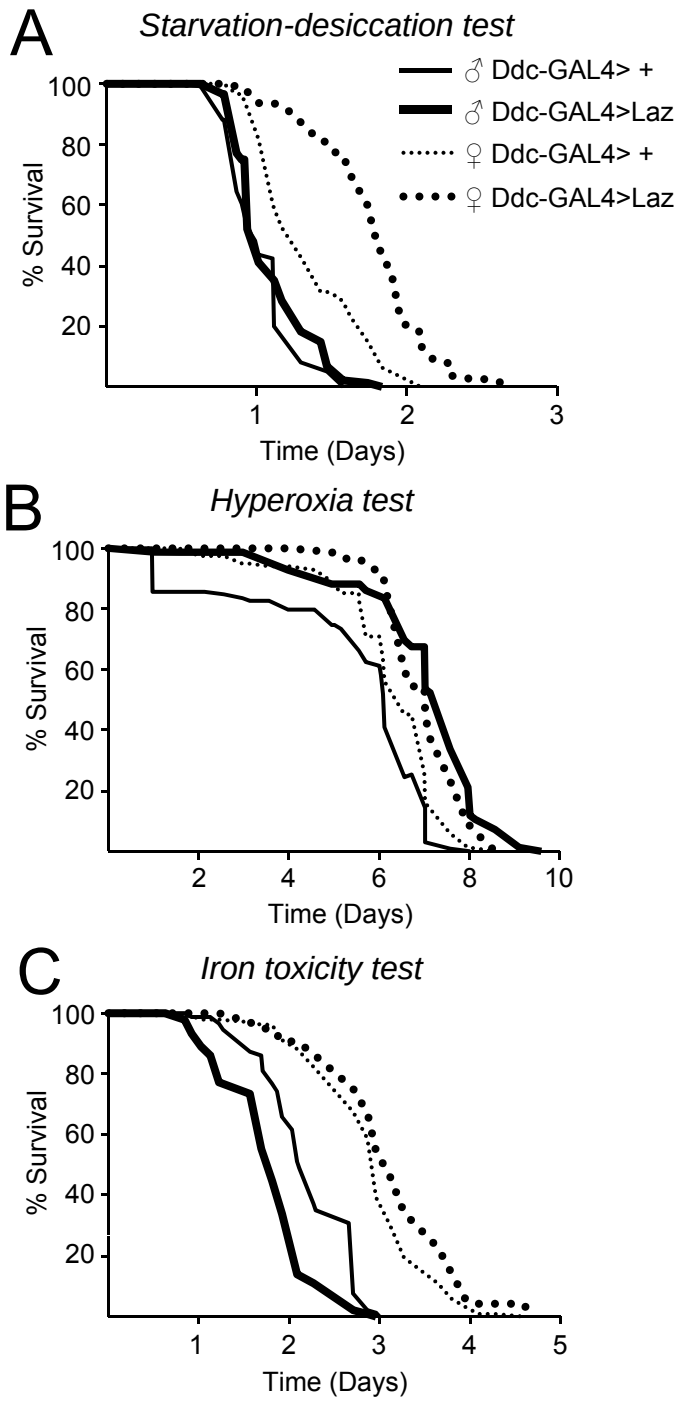

A
Starvation-desiccation test
♂ Ddc-GAL4> +
♂ Ddc-GAL4>Laz
♀ Ddc-GAL4> +
♀ Ddc-GAL4>Laz
% Survival
Time (Days)
B
Hyperoxia test
% Survival
Time (Days)
C
Iron toxicity test
% Survival
Time (Days)

Table S1. Analysis of differences between hydrocarbon profiles in male flies.

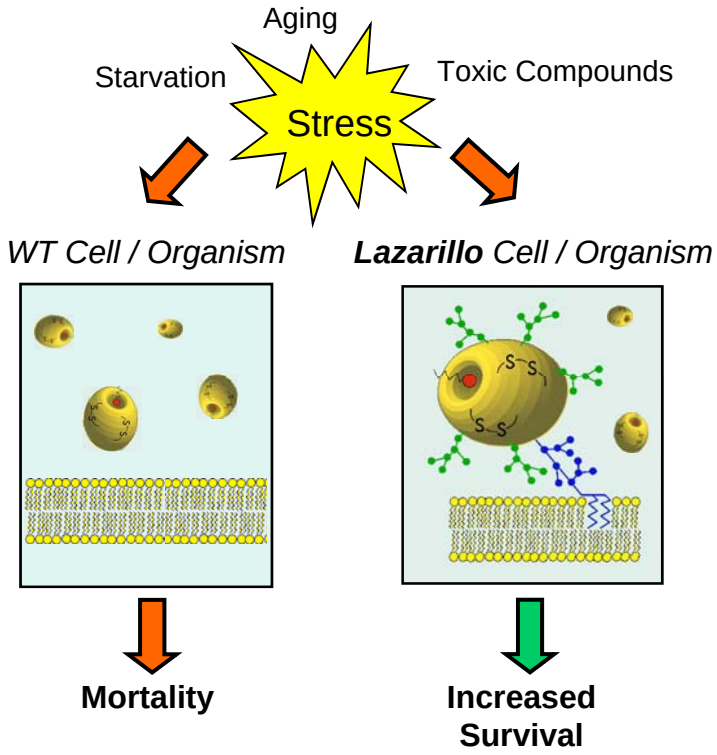
HC	*da-GAL4>+*	*da-GAL4>Laz*	F	P	*Ddc-GAL4>+*	*Ddc-GAL4>Laz*	F	P	*elav-GAL4>+*	*elav-GAL4>Laz*	F	P
Total HC	1346 ±2 7	1298 ± 82	0.31	0.59	1289 ± 66	1185 ± 64	1.26	0.28	2819 ± 396	2792 ± 143	0.04	0.54
2-Me-C22	0.28 ± 0.04	0.27 ± 0.03	0.08	0.78	0.32 ±0.02	0.26 ± 0.03	2.27	0.15	0.18 ± 0.03	0.16 ± 0.03	0.56	0.01
9-C23:1	4.89 ± 0.12	4.55 ± 0.20	2.09	0.17	4.13 ± 0.10	3.99 ± 0.13	0.76	0.40	2.87 ± 0.13	3.17 ± 0.11	0.81	0.95
7-C23:1	45.24 ± 0.74	**40.51 ± 1.07***	13.14	0.001	42.71 ± 0.57	**38.42 ± 0.59***	27.16	0.001	37.06 ± 1.25	40.31 ± 0.57	0.54	0.92
5-C23:1	2.90 ± 0.07	**2.18 ± 0.11***	32.55	0.001	3.03 ± 0.09	**2.69 ± 0.05***	12.02	0.002	2.21 ± 0.09	2.25 ± 0.06	0.66	0.97
C23	15.32 ± 0.42	**20.62 ± 0.35***	94.43	0.001	15.73 ± 0.28	**19.44 ± 0.35***	68.23	0.001	15.64 ± 0.50	16.17 ± 0.36	0.52	0.18
2-Me-C24	3.19 ± 0.19	3.63 ± 0.09	4.59	0.05	3.47 ± 0.20	4.20 ± 0.16	9.47	0.006	2.85 ± 0.24	2.97 ± 0.06	0.22	0.69
9-C25:1	3.75 ± 0.10	3.71 ± 0.08	0.07	0.79	2.77 ± 0.17	2.54 ± 0.11	1.41	0.25	3.52 ± 0.39	3.50 ± 0.16	0.00	0.27
7-C25:1	9.46 ± 0.17	7.40 ± 0.27	41.55	4.58	9.18 ± 0.24	**7.32 ± 0.21***	34.25	0.001	12.88 ± 0.84	11.89 ± 0.49	0.02	0.80
5-C25:1	0.12 ± 0.04	0.11 ± 0.04	0.04	0.84	0.07 ± 0.02	0.12 ± 0.03	1.85	0.19	0.12 ± 0.03	0.08 ± 0.02	0.01	0.54
C25	3.03 ± 0.17	**4.03 ± 0.17***	17.18	0.001	2.92 ± 0.13	**4.19 ± 0.26***	18.49	0.001	3.17 ± 0.27	3.16 ± 0.21	0.01	0.07
2-Me-C26	6.00 ± 0.32	6.83 ± 0.49	2.03	0.17	6.93 ±0.20	**8.24 ± 0.45***	7.11	0.02	8.92 ± 0.52	8.49 ± 0.34	0.90	0.34
C27	1.31 ±0.13	1.83 ± 0.24	3.61	0.07	1.92 ± 0.10	2.30 ± 0.22	2.36	0.14	1.90 ± 0.23	1.68 ± 0.17	0.01	0.001
2-Me-C28	4.17 ± 0.15	3.89 ± 0.77	0.13	0.72	5.42 ± 0.21	5.23 ± 0.26	0.32	0.58	7.07 ± 0.41	5.51 ± 0.24	0.00	0.13
C29	0.35 ± 0.03	0.44 ± 0.12	0.55	0.47	0.63 ± 0.07	**0.83 ± 0.06***	5.63	0.02	0.71 ± 0.09	0.48 ± 0.05	0.00	0.13

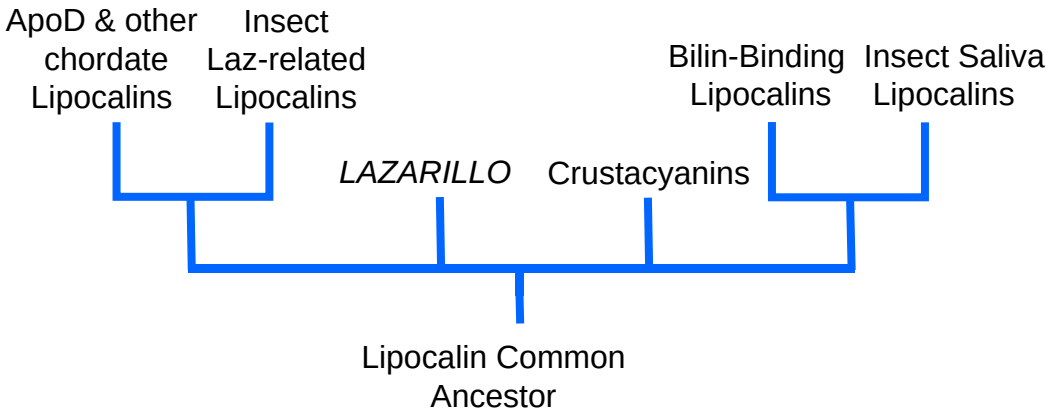
Total amount of hydrocarbons (HC) in ng/fly are stated in the first raw. All other data shown are the mean percentages (± SEM) of cuticular hydrocarbons produced by individual males from each genotype (5 day old flies, n=10 per genotype). HC identities are given in the first column. HC composition is listed as the carbon chain length followed by the number of double bonds. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison post-hoc test. F and P values are listed for each comparison. Values in bold and highlighted with asterisks indicate significant HC variations between Laz over-expression and its driver-only control.

Table S2. Analysis of differences between the HC profiles of females.

HC	***da-GAL4>+***	***da-GAL4>Laz***	F	P	***Ddc-GAL4>+***	***Ddc-GAL4>Laz***	F	P	***elav-GAL4>+***	***elav-GAL4>Laz***	F	P
Total HC	1434 ± 88	1477 ± 80	0.02	0.30	1499 ± 60	1207 ± 73	0.13	0.72	3280 ± 236	2863 ± 139	0.80	0.34
2-Me-C22	0.54 ± 0.08	0.57 ± 0.03	0.96	0.55	0.64 ± 0.05	0.79 ± 0.07	0.15	0.70	0.57 ± 0.05	0.68 ± 0.05	0.71	0.42
9-C23:1	0.20 ± 0.04	0.26 ± 0.02	0.02	0.26	0.36 ± 0.08	0.46 ± 0.04	1.75	0.20	0.17 ± 0.03	0.20 ± 0.01	0.21	0.32
7-C23:1	1.29 ± 0.08	1.75 ± 0.19	0.24	0.90	**2.26 ± 0.41***	**2.44 ± 0.25***	5.32	0.03	1.83 ± 0.28	1.49 ± 0.07	0.00	0.22
5-C23:1	0.06 ± 0.02	0.04 ± 0.02	0.16	0.001	0.16 ± 0.04	0.10 ± 0.03	0.31	0.59	0.05 ± 0.02	0.08 ± 0.02	0.11	0.11
C23	9.05 ± 0.31	10.22 ± 0.41	0.35	0.51	**9.91 ± 0.42***	**11.44 ± 0.23***	5.23	0.03	9.38 ± 0.23	8.90 ± 0.15	0.62	0.01
7,11-C25 :2	1.29 ± 0.11	2.34 ± 0.14	0.42	0.29	**1.21 ± 0.12***	**1.69 ± 0.10***	35.21	0.001	1.43 ± 0.06	1.55 ± 0.06	0.55	0.50
2-Me-C24	1.57 ± 0.17	1.41 ± 0.09	0.33	0.47	2.18 ± 0.11	2.78 ± 0.14	0.66	0.43	1.46 ± 0.21	2.02 ± 0.23	0.03	0.66
9-C25:1	4.34 ± 0.26	5.02 ± 0.34	0.01	0.03	4.51 ± 0.10	4.88 ± 0.17	2.60	0.12	3.39 ± 0.32	4.16 ± 0.29	0.02	0.11
7-C25:1	1.88 ± 0.15	2.30 ± 0.27	0.89	0.02	1.58 ± 0.07	1.82 ± 0.05	1.82	0.19	1.82 ± 0.12	1.69 ± 0.27	0.00	0.001
5-C25:1	0.08 ± 0.02	0.09 ± 0.03	0.62	0.01	0.09 ± 0.03	0.12 ± 0.02	0.06	0.81	0.08 ± 0.02	0.08 ± 0.02	0.61	0.69
C25	7.86 ± 0.26	10.37 ± 0.31	0.60	0.44	**7.16 ± 0.26***	**7.87 ± 0.14***	39.31	0.001	8.91 ± 0.28	8.79 ± 0.44	0.29	0.49
7,11-C27 :2	11.53 ± 0.65	15.00 ± 0.25	0.10	0.93	**11.53 ± 0.37***	**12.70 ± 0.29***	24.83	0.001	13.99 ± 0.49	12.62 ± 0.45	0.32	0.88
2-Me-C26	19.90 ± 0.94	19.59 ± 0.42	0.36	0.17	22.82 ± 0.58	25.28 ± 0.90	0.09	0.77	20.61 ± 1.32	25.18 ± 1.01	0.26	0.03
9-C27:1	5.19 ± 0.30	4.60 ± 0.30	0.71	0.65	3.95 ± 0.15	2.70 ± 0.11	1.92	0.18	4.13 ± 0.19	3.71 ± 0.18	0.47	0.56
7-C27:1	2.48 ± 0.22	2.03 ± 0.11	0.16	0.67	1.29 ± 0.05	1.09 ± 0.08	3.38	0.08	1.94 ± 0.13	1.35 ± 0.07	0.59	0.65
C27	7.43 ± 0.47	5.60 ± 0.27	0.63	0.30	6.43 ± 0.24	5.25 ± 0.09	11.30	0.003	7.40 ± 0.38	6.47 ± 0.33	0.87	0.19
7,11-C29 :2	7.27 ± 0.36	5.74 ± 0.21	0.28	0.73	7.99 ± 0.35	7.04 ± 0.30	13.62	0.002	7.25 ± 0.28	6.46 ± 0.22	0.28	0.29
2-Me-C28	14.40 ± 0.64	10.62 ± 0.50	0.08	0.29	**13.27 ± 0.54***	**9.28 ± 0.29***	21.81	0.001	13.41 ± 0.54	12.40 ± 0.64	0.99	0.32
9-C29:1	0.93 ± 0.26	0.57 ± 0.12	0.12	0.75	0.12 ± 0.04	0	1.58	0.22	0.19 ± 0.05	0.02 ± 0.01	0.11	0.002
7-C29:1	0.30 ± 0.10	0.06 ± 0.02	0.12	0.08	0.07 ± 0.02	0.03 ± 0.03	5.81	0.03	0.16 ± 0.05	0.03 ± 0.01	0.06	0.007
C29	1.27 ± 0.16	0.68 ± 0.11	0.89	0.90	1.24 ± 0.09	1.04 ± 0.10	8.96	0.008	1.02 ± 0.08	1.02 ± 0.05	0.08	0.56

Total amount of hydrocarbons (HC) in ng/fly are stated in the first raw. All other data shown are the mean percentages (± SEM) of cuticular hydrocarbons produced by individual females from each genotype (5 day old flies, n=10 per genotype). HC identities are given in the first column. HC composition is listed as the carbon chain length followed by the number of double bonds. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison post-hoc test. F and P values are listed for each comparison. Values in bold and highlighted with asterisks indicate significant HC variations between Laz over-expression and its driver-only control.


Aging
Starvation
Toxic Compounds
Stress
WT Cell / Organism
Lazarillo Cell / Organism
Mortality
Increased Survival


ApoD & other chordate Lipocalins
Insect Laz-related Lipocalins
LAZARILLO
Crustacyanins
Bilin-Binding Lipocalins
Insect Saliva Lipocalins
Lipocalin Common Ancestor