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Original Article

Age-related Decline of Abiotic Stress Tolerance in Young *Drosophila melanogaster* Adults

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Abstract

Stress tolerance generally declines with age as a result of functional senescence. Age-dependent alteration of stress tolerance can also occur in early adult life. In *Drosophila melanogaster*, evidence of such a decline in young adults has only been reported for thermotolerance. It is not known whether early adult life entails a general stress tolerance reduction and whether the response is peculiar to thermal traits. The present work was designed to investigate whether newly eclosed *D melanogaster* adults present a high tolerance to a range of biotic and abiotic insults. We found that tolerance to most of the abiotic stressors tested (desiccation, paraquat, hydrogen peroxide, deltamethrin, and malathion) was high in newly eclosed adults before dramatically declining over the next days of adult life. No clear age-related pattern was found for resistance to biotic stress (septic or fungal infection) and starvation. These results suggest that newly eclosed adults present a culminating level of tolerance to extrinsic stress which is likely unrelated to immune process. We argue that stress tolerance variation at very young age is likely a residual attribute from the previous life stage (ontogenetic carryover) or a feature related to the posteclosion development.

Key Words: Young age—Drosophila—Abiotic stress—Biotic stress

The notion that life span is related to the capacity to withstand stress is well known. Theories of aging posit that life span is modulated by the ability of the organism to tolerate both intrinsic and extrinsic stress (1,2). Repair and maintenance of somatic tissues appear incapable to keep pace with stress-related damages, leading to progressive decline of biological functions with age. Functional senescence describes the failure in biological systems with aging, and hence, focuses on malfunctions that progressively occur near the end of life (3). Fecundity, mobility, phototaxis, olfaction, cardiac functions are among traits that can be altered with senescence in *Drosophila melanogaster* (4). Stress tolerance traits can also decline with age, and there is ample literature dealing with this topic (1–4). In the fruit fly, studies addressing stress tolerance variations with age have generally focused on groups of flies aged of a few days versus older ones (ie, >30 days) (5). Generally, a reduction of stress resistance is reported, as a result of functional senescence (1,3,6). However, age-dependent alteration of stress tolerance can also occur in early adult life, a process which is thus likely unrelated to senescence per se. Because this early life pattern does not directly concern life-span extension or senescence, it has not been a major focus of investigation in science of aging.

To date, evidence of stress tolerance decline at young age has only been described for thermal stress. A number of studies reported

a sharp decline of heat tolerance in early life of insects including in *D melanogaster* (5,7–11). Similarly, cold tolerance has also been reported to be high soon after eclosion before decreasing dramatically at young age (12). Clearly, the ability to withstand lethal high or low temperatures is culminating in newly eclosed adults before dramatically declining over the next few days of adult life. After a few days of age, this decline either reaches a plateau or further declines progressively with aging but at a much slower rate (5,7–12). Although some features of the stress response are not directly linked to aging, there is a substantial congruence between the stress response and functional senescence, and there is strong support suggesting that the genetic basis for life span and stress resistance overlap (1). Therefore, the stress response remains a meaningful subject to be explored in aging research.

Very young flies (0- to 48-hour-old) are much less active (in terms of motor activity) and respond poorly to exogenous stimuli than older flies (13), which make young individuals particularly vulnerable to predation and environmental hazards. Therefore, a high general stress tolerance might be adaptive during this critical period. So far, it is not known whether early adult life entails a general stress tolerance or whether the response is peculiar to thermal stress. In consequence, the present work was designed to investigate whether the first days of adult life are associated with a general high and declining tolerance to a range of biotic and abiotic insults.

Material and Methods

Fly Stocks

Experiments were performed in two different laboratories: in Paris (PAR) and Rennes (RNS). The RNS laboratory conducted experiments on a mass-bred wild *D melanogaster* line (called RNS flies) initiated in October 2011 at Plancoët (Brittany, France). Prior to the experiment, flies were maintained in 200 mL bottles at 25 ± 1°C (16L:8D) on a standard fly medium (14) consisting of deactivated brewer yeast, sucrose, and agar. To generate flies for the experiments, groups of 15 mated females were allowed to lay eggs for 6 hours in food bottles. This controlled procedure allowed larvae to develop under synchronized and uncrowded conditions at 25 ± 1°C (16L:8D). Upon emergence, virgin flies of less than 12 hours old were collected. They were sexed visually without CO_2 to avoid anesthetic stress (15) and only females were kept in food vial (30 flies/vial) that was changed every day. Virgin females aged 0- (less than 12 hours), 1-, 2-, 3-, 4-, and 5-day-old were tested for stress tolerance. The PAR laboratory used the Canton-S strain (called PAR flies). Flies were maintained in vials with standard fly medium consisting of yeast, cornmeal, and agar in a growth chamber at 25°C (12L:12D). Upon emergence, the first emerging adults were removed, and vials were left overnight for emergence. In the morning, flies of less than 12 hours old were collected and lightly anesthetized on ice to discard males. Groups of 20 females were then transferred in fresh food vials every day. Females aged from 0- (less than 12 hours) to 6- or 7-day-old were tested for stress tolerance assays.

Starvation Assays

A batch of RNS flies from each age (0- to 5-day-old) was used for determination of starvation resistance. For each age, 10 flies were put into a vial containing 2 mL of agar only at 20°C. Five replicated vials were used per age (n = 50 flies/age). After 24 hours of starvation, flies were checked four times a day (08:00, 12:00, 18:00, and 24:00) until all flies were dead. For each age, we used a control which consisted of a vial with 10 flies on standard food.

Desiccation Tolerance

To measure desiccation tolerance, 12 RNS flies from each age were individually placed in 2-mL glass vials with a plastic cap punctured to allow air circulation. These 12 vials were vertically positioned on a metal stand placed inside a desiccating sealed glass container which contained 100 g of silica gel at the bottom. Records inside the glass container (Hobo logger U12-012, Onset Computer Corporation) revealed that relative humidity was 7%–8% at 20°C. For each age, four desiccating glass containers were used (n = 48 flies/age). The vials were individually inspected every hour at 20°C, and the number of dead flies (immobile) was recorded until all flies died. For each age, a control container with 12 flies was used with a humid cotton instead of silica gel.

Tolerance to Oxidative Stress

Tolerance to two different ROS-generating agents was tested: paraquat [PQ] (cat. no. 856177, CAS Number 75365-73-0, Sigma-Aldrich) and hydrogen peroxide [H_2O_2] (33% w/v stabilized; cat. no. 141077, CAS Number 7722-84-1, PanReac AppliChem). For PQ, the first experiment consisted of exposing RNS flies of 0- to 5-day-old to 10-mM PQ administrated on a filter paper with 3% sucrose placed on top of an agar-only vial at 20°C. Five replicated vials of 20 flies were used (n = 100 flies/age). The second experiment consisted of feeding flies with 20-mM PQ on a filter paper with 3% sucrose at 25°C. Five replicated vials of 10 flies were used in this case (n = 50 flies/age). For H_2O_2, we followed exactly the same procedure: a first experiment with 1% H_2O_2 and 3% sucrose solution at 20°C (n = 100 flies/age) and a second experiment with 3% H_2O_2 and 3% sucrose solution at 25°C (n = 50 flies/age). The vials and filter papers were renewed every 2 days. For each condition, a control vial with 3% sucrose on the filter paper was used. The vials were inspected for mortality twice a day (8:00 and 18:00) for 10 consecutive days.

Exposure to Insecticides

Two different insecticides were used: malathion (CAS number CAS 121-75-5, Sigma-Aldrich) and deltamethrin (CAS Number 52918-63-5, Sigma-Aldrich). For each chemical, LD_{50} values (Lethal dose required to kill 50% of the population) were first determined in 5-day-old PAR flies to establish a specific application dose for next age-related bioassays. For malathion, flies were exposed to concentrations ranging from 0 to 2 µg/vial. For deltamethrin, flies were exposed to concentrations ranging from 0 to 63 µg/vial. The chemicals were applied into 10-mL glass vials in 250 µL of acetone. The vials were then rotated, and the acetone evaporated leaving the inside of the vial coated with the technical grade insecticide. Controls were performed as described but with acetone only. When vials were dry, flies were placed into vials. For malathion, flies were kept for 24 hours into the treated vials. The vials were plugged with cotton, and 500 µL of 1% saccharose solution was added to the plug. Survival was scored after 24 hours. For deltamethrin, flies were kept for 1 hour into the treated vial and then transferred to food vials. Each test consisted of a set of 20 flies exposed to a range of concentrations. A minimum of four replications was used per concentration. LD_{50} were calculated using logistic regression via probit analysis. Next, for the age-related bioassays, flies aged from 0- to 6-day-old for malathion or from 0- to 7-day-old for deltamethrin were exposed to the LD_{50} (malathion: 0.125 µg/vial and deltamethrin: 17 µg/vial) following the same procedure. The mortality was scored 24 hours after the exposure. For malathion, 4 to 11 replications of 20 flies were used, and for deltamethrin, 5 to 15 replications were used. For both

chemicals, a minimum of four replicated control vials were used for each age.

Tolerance to Septic Bacterial Infections

To assess the tolerance to biotic stress, we exposed PAR flies to a septic jab of bacteria (suspension of *Escherichia coli* and *Micrococcus luteus*). Cultures of *E coli* and *M luteus* were grown in Lysogeny broth medium (LB) for 24 hours under shaking at 29°C. An equal mixture of concentrated pellet of the bacterial cultures was prepared after centrifugation of the mixture and measurement by optical density at 600 nm (OD = 200). Flies of 0- to 6-day-old were lightly anesthetized on ice. Septic injuries were induced by pricking the thorax of the flies with a tungsten needle previously dipped into the concentrated bacterial pellet. Controls were performed with sterile pricking. For each age, four to six replicated vials, each consisting of 20 infected flies, were used. After pricking, the flies were transferred into food vials changed every 2 days and maintained at 29°C in a growth chamber during the experiment. Mortality was scored after 120 hours. Because a certain level of mortality could result from needle pricking, the mortality rate (M) in the treatment was corrected against that in the control for each age according to Abbott's correction:

Tolerance to Fungal Infections

Natural infections by entomopathogenic fungi were realized using *Beauveria bassiana*. The spores, kept at −80°C in 20% glycerol, were incubated at 25°C and darkness in 90-mm petri dishes filled with growing medium: 500 mg peptone (Fluka, 77199), 10 g malt extract (Fluka, 70167), 10 g glucose, and 7.5 g agar in 500 mL of distilled water (pH adjusted to 6.5). After sporulation, five sets of 10 RNS flies were infected for each age (n = 50 flies/age). On the day of infection, flies were slightly anesthetized with CO_2 and then transferred to a petri dish containing the sporulating *B bassiana*. Flies were hand-shacked for 1 minute until they were covered with spores and then transferred back to food vials. For each age (0- to 5-day-old), a set of 10 control flies was transferred to a petri dish that contained only agar. Flies were then maintained at 25°C in food vials that were changed every 2 days. Mortality was scored twice a day (08:00 and 18:00) until all the flies died.

Statistical Analyses

Temporal measures of survival were used to compute survival curves which were compared among ages with log-rank tests, together with a log-rank test for trend (to detect a consistent trend with increasing age). These tests were performed in Prism V 5.01 (GraphPad Software Inc., 2007). Because post hoc analyses are not available and are not reliable with log-rank tests, additional tests such as General Linear Model (GLM) are required to determine where the differences lie among groups (16). Thus, mean time to death values (in desiccation, starvation, oxidative, and fungal infection assays) were compared by GLM with Gamma error (inverse link), as Gamma distribution is appropriate to model time-to-event data. For insecticide exposure, the response was a survival rate after 24 hours. To test the effects of age on these data, we used a GLM with binomial error (logit link) because a binomial distribution is appropriate to model binary data or percentages. Tukey's tests were performed following GLMs with "glht" function in the "multcomp" package. For septic bacterial infection, mortality was compared among ages using Kruskal–Wallis test and followed by post hoc Dunn tests in the "dunn.test" package. All these analyses were conducted using the statistical software "R 3.0.3."

Results

Abiotic Stressors

Tolerance to starvation was affected by the age of young flies (Figure 1). Temporal survival curves were distinct (χ^2 = 67.39, df = 5, p < .001), but there was no trend with increasing age (χ^2 = 0.11, df = 1, p = .750). Mean time to death under starvation varied according to age (F = 22.86, df_N = 5, df_D = 294, p < .001). Multiple comparisons showed that 1- and 5-day-old flies died the most rapidly, whereas 3-day-old flies survived the longest, confirming the lack of consistent pattern related to age. No mortality was found in controls.

Tolerance to desiccation was markedly affected by young age (Figure 2). Survival curves were distinct among ages (χ^2 = 111.10, df = 5, p < .001) and there was a significant trend with increasing age (χ^2 = 94.11, df = 1, p < .001). Mean time to death under desiccating conditions varied according to age (F = 41.10, df_N = 5, df_D = 282, p < .001). A clearcut reduction of desiccation tolerance was noted with newly eclosed flies (0-day-old) surviving the longest, 1- to 3-day-old flies showing intermediate response, and the 4- and 5-day-old flies being the least tolerant to desiccation. No mortality was found in controls.

Survival of flies exposed to ROS-generating agents was also affected by young age (Figure 3). For the lowest PQ concentrations (10 mM of PQ; Figure 3, top), we found that temporal survival curves were markedly divergent among ages (χ^2 = 153.30, df = 5, p < .001) and that there was a trend with age (χ^2 = 147.40, df = 1, p < .001). For very young flies (0- and 1-day-old), the curves were right-censored

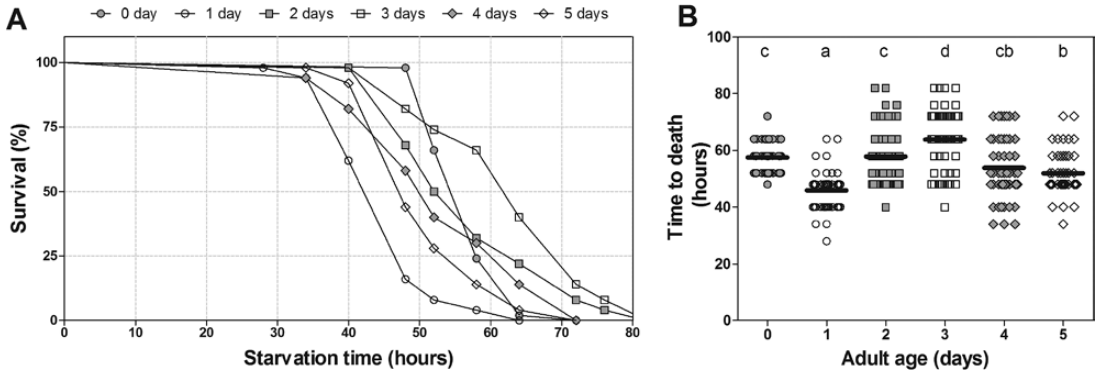

Figure 1. (**A**) Temporal survival curves of flies submitted to starvation in the six age groups tested (0–5 days). (**B**) Scatter plot showing the time to death values. The horizontal black lines indicate the mean for each age. Different letters indicate significant difference.

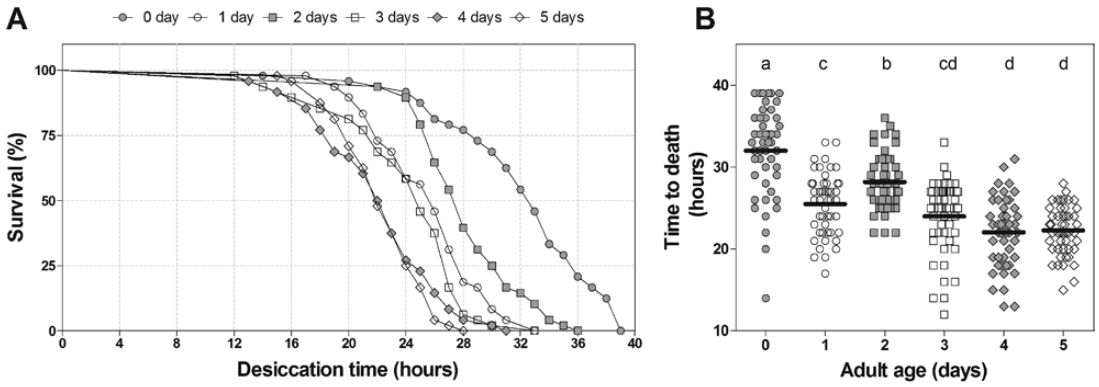

Figure 2. (**A**) Temporal survival curves of flies submitted to desiccation in the six age groups tested (0–5 days). (**B**) Scatter plot showing the time to death values. The horizontal black lines indicate the mean for each age. Different letters indicate significant difference.

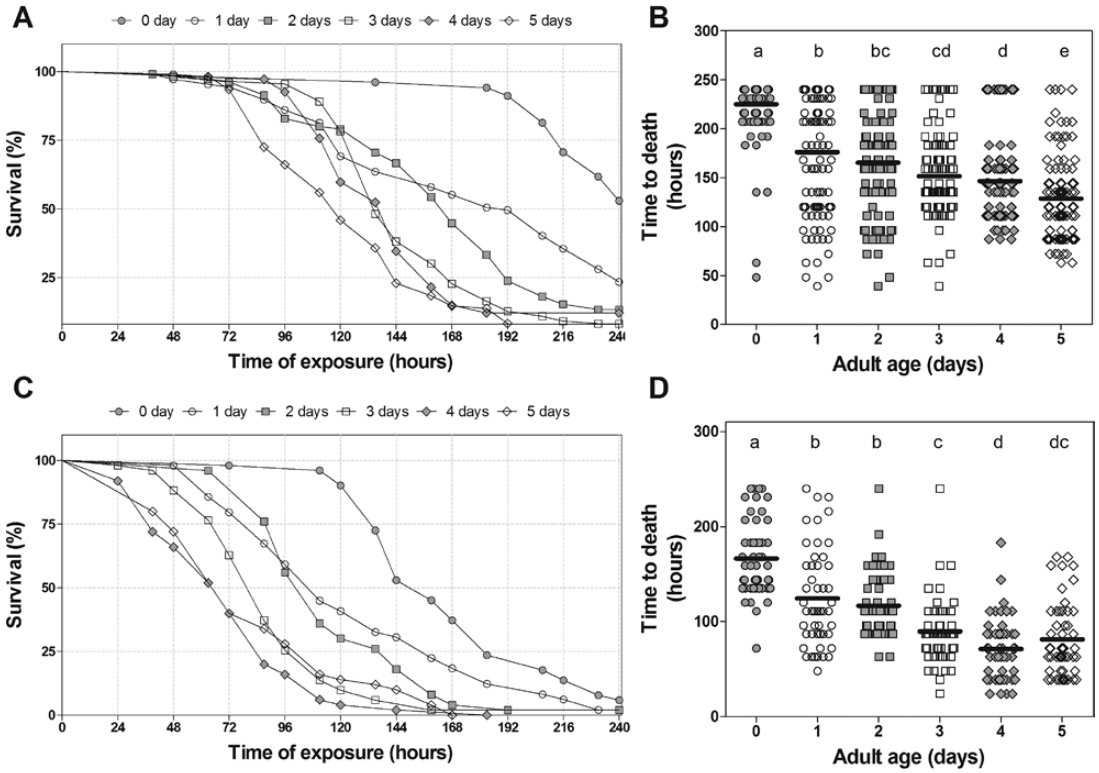

Figure 3. Temporal survival curves of flies submitted to oxidative stress in the six age groups tested (0–5 days) using 10-mM PQ (**A**) or 20-mM PQ (**C**). Scatter plots showing the time to death values for 10-mM PQ (**B**) or 20-mM PQ (**D**). The horizontal black lines indicate the mean for each age. Different letters indicate significant difference.

after 10 days, whereas older fly groups had reached 100% mortality. Mean time to death was significantly affected by age ($F = 56.64$, $df_N = 5$, $df_D = 594$, $p < .001$). For the second assay (20 mM of PQ; Figure 3, bottom), temporal survival curves were also affected by age ($\chi^2 = 90.90$, $df = 5$, $p < .001$) and a trend with age was confirmed ($\chi^2 = 79.43$, $df = 1$, $p < .001$). Mean time to death was affected by age ($F = 40.06$, $df_N = 5$, $df_D = 294$, $p < .001$). For both PQ concentrations, multiple comparisons showed that newly eclosed flies (0-day-old) survived longest to oxidative stress whereas 5-day-old flies survived the shortest, and intermediate ages showed intermediate responses. For both assays, mortality in controls was insignificant (<10%).

Survival of flies exposed to H_2O_2 was affected by young age (Figure 4). For the lowest H_2O_2 concentrations (1%; Figure 4, top), temporal survival curves differed among ages ($\chi^2 = 181.20$, $df = 5$, $p < .001$), and there was a significant trend with age ($\chi^2 = 153.70$, $df = 1$, $p < .001$). Only the older groups (4- and 5-day-old) reached 100% mortality after 10 days. Mean time to death was affected by age ($F = 61.81$, $df_N = 5$, $df_D = 594$, $p < .001$). For the second assay using 3% of H_2O_2 (Figure 4, bottom), temporal survival curves were also affected by age ($\chi^2 = 44.07$, $df = 5$, $p < .001$), and a trend with age was confirmed ($\chi^2 = 20.71$, $df = 1$, $p < .001$). Mean time to death was affected by age ($F = 15.39$, $df_N = 5$, $df_D = 294$, $p < .001$). For both H_2O_2 concentrations, multiple comparisons showed that 0-day-old flies survived longest to oxidative stress whereas 5-day-old flies survived the shortest, and intermediate ages showed intermediate responses. For both assays, mortality in controls was insignificant (<10%).

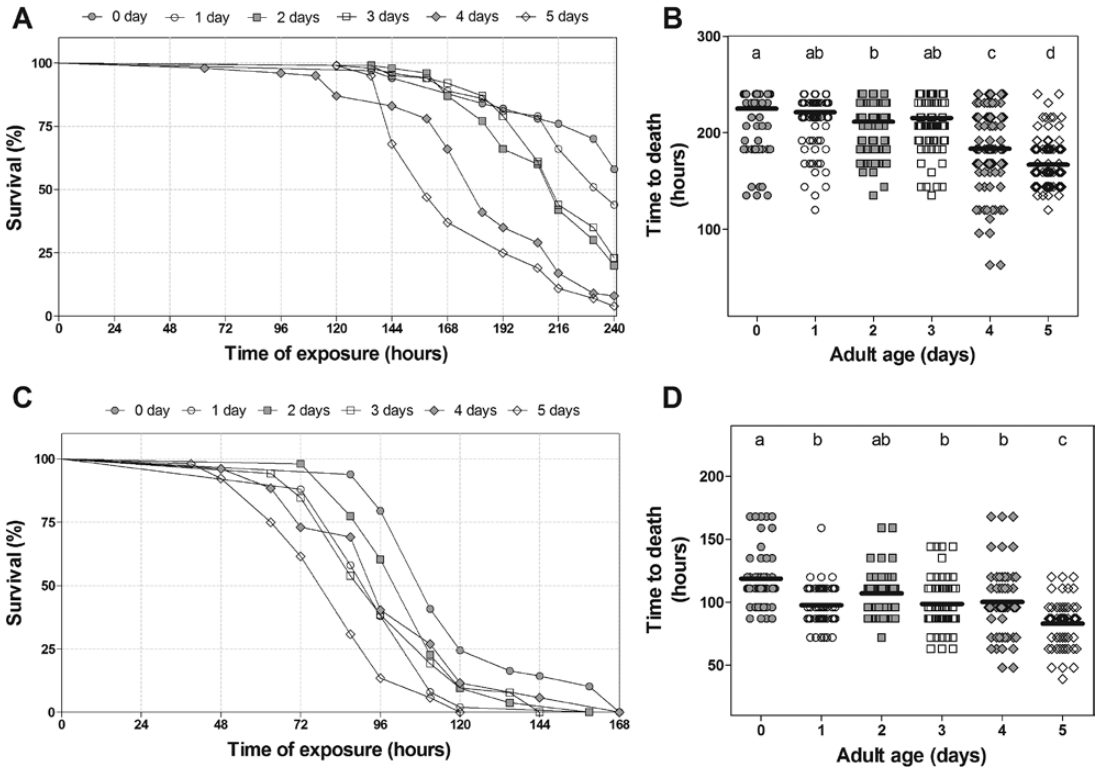

Figure 4. Temporal survival curves of flies submitted to oxidative stress in the six age groups tested (0–5 days) using 1% H_2O_2 (**A**) or 3% H_2O_2 (**C**). Scatter plots showing the time to death values for 1% H_2O_2 (**B**) or 3% H_2O_2 (**D**). The horizontal black lines indicate the mean for each age. Different letters indicate significant difference.

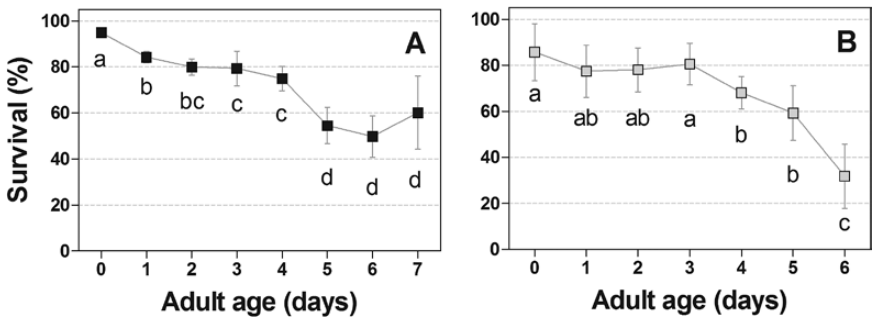

Figure 5. Mean survival (±*SE*) of flies submitted to insecticide, deltamethrin (**A**) or malathion (**B**), in the eight age groups tested (0–7 days). Different letters indicate significant difference.

Survival of flies exposed to the two insecticides is presented in Figure 5. For deltamethrin (Figure 5, left), survival was significantly affected by age (χ^2 = 332.51, *df* = 7, *p* < .001). Multiple comparisons indicated that survival was the highest in young flies (ie, 0- and 1-day-old) and the lowest in 5- to 7-day-old flies, and intermediate in 2- to 4-day-old flies. Mortality was insignificant in controls (<5%). For malathion (Figure 5, right), survival was also affected by age (χ^2 = 63.22, *df* = 6, *p* < .001). Multiple comparisons indicated the highest survival in young flies and the lowest survival in older flies. Mortality was insignificant in controls (<5%).

Biotic Stressors

Mortality of flies submitted to bacterial infection is presented in Supplementary Figure 1. Mortality in control flies was not negligible with values sometime superior to 15%. Mortality in controls was slightly affected by age (χ^2 = 13.58, *df* = 6, *p* = .03). Mortality of flies that received a needle prick with bacteria was different according the age (χ^2 = 19, *df* = 6, *p* = .004). However, there was no evident age-related pattern. The Abbott's corrected mortality varied with age (χ^2 = 19.82, *df* = 6, *p* = .002), but no clear age-related pattern was found.

Survival of flies submitted to fungal infection is presented in Supplementary Figure 2. Temporal survival curves were marginally different (χ^2 = 10.25, *df* = 5, *p* = .068), and there was no trend with increasing age (χ^2 = 3.37, *df* = 1, *p* = .06). Mean time to death slightly differed with age (*F* = 2.86, df_N = 5, df_D = 294, *p* = .015), and multiple comparisons only found a difference between 1- and 3-day-old flies. No mortality was found in controls.

Discussion

Previous studies have reported that the ability to withstand lethal high or low temperatures is culminating in newly eclosed adult insects before

dramatically declining over the next few days of adult age (5,7–12). Many facets of the stress response are generic because stress is monitored at the cellular level based on macromolecular damage without regard to the type of stress that is inflicted (17,18) Therefore, we speculated that the high level of thermal tolerance observed in newly eclosed adults could be part of a generic stress tolerance syndrome. The present work was thus designed to investigate whether newly eclosed adults present a high and declining tolerance to a range of biotic and abiotic insults. We found that young flies displayed a high resistance to most of the abiotic stressors tested (desiccation, PQ, H_2O_2, deltamethrin, and malathion), but no clear age-related pattern was found for starvation and biotic stress (bacterial or fungal infection). Hence, it seems that stress tolerance in early adult life is not peculiar to thermal stress but is part of a nonspecific abiotic stress tolerance response.

So far, the mechanisms underlying the variation of stress tolerance at young age are unknown, but a marked reduction in the induced expression of the heat shock proteins (Hsps) was found to accompany the decline of heat (5,11) and cold tolerance (12). Because Hsps are part of a generic cellular stress response (18), they may confer tolerance to a large array of stress including chemicals (19). Here, we found that newly eclosed adults were consistently more tolerant to xenobiotics. Tolerance to PQ and H_2O_2 require an efficient antioxidative defense system which includes a whole set of enzymes such as superoxide dismutase, catalase, and glutathione-S-transferase (GST) (20). Resistance and detoxification of insecticides such as pyrethroids and organophosphates also require a high activity of detoxifying enzymes (21). For instance, the activity of the GST system correlates with resistance to deltamethrin (22) and malathion (23). In addition, transgenic-induced overexpression of GST provides multiresistance to UV, heat, PQ, and H_2O_2 (24). As for Hsps, several studies have reported an ontogenetic pattern of GST activity, peaking in pupae before declining during the first days of adult stage (25–27). Thus, a high expression of various proteins involved in generic stress protection mechanisms (such as Hsps or ROS scavengers, 18) in pupae and newly eclosed adults may partly underlie this age-related pattern.

Pupae are immobile and freshly eclosed fruit flies (0- to 48-hour-old) and have a very limited mobility (13), and hence, they cannot escape from environmental stress through behavioral avoidance, which likely make them more susceptible to unfavorable environmental conditions, including xenobiotics or temperature (5,26). Moreover, it might be important to protect the sensitive biosynthetic machinery during metamorphosis. In fact, transcripts related to stress response are invariably associated with metamorphosis in all investigated phyla, presumably as part of a general protective mechanism (28). Therefore, the high stress tolerance of young adults might be an "ontogenetic carry over" resulting from previous immobile and metamorphosing stage. This hypothesis is supported by the observation that total RNA declines substantially at young age, exhibiting a dramatic drop in the first days of adult life (29). Such decline was found to be unrelated to senescence but rather reflected the transition from larvae to adult. The overall gene expression is very high during metamorphosis, and the nucleic acids subsequently become nonfunctional and drastically decline at young age (29). A similar early-age sharp decline in protein synthesis pathway has been reported (30). Therefore, it is evident that many aspects of young adult's physiology are still affected by the striking events that occurred during the holometabolous transformation from larvae to adult, and the early-age stress tolerance might be a byproduct of these events. Finally, in addition to a possible ontogenetic carry over (a passive phenomenon), it should be underlined that the development is not fully completed upon adult emergence. Several developmental processes still occur during the first hours/days of adult stage (an active phenomenon). For instance, the central nervous system continues to develop after eclosion (13,31). Newly enclosed adults also have to attain reproductive maturity, within about 2 days after the eclosion (32). Therefore, it is most likely that stress tolerance variation at young age is an attribute related to the previous life stage (ontogenetic carry over) and/or to the posteclosion development rather than to the effect of aging per se.

We found that the tolerance to desiccation and xenobiotics is culminating in newly eclosed adults before dramatically declining over the next few days. However, we could not find any distinct age-related pattern for tolerance to starvation and bacterial and fungal infection. Even if stress responses share common elements, a specificity still arises because the types of lesions and macromolecular damages vary with the type of stress (18). Increased desiccation tolerance does not necessarily imply tolerance to starvation. In fact, selection for increased desiccation tolerance does not affect starvation tolerance (33), which suggests that both processes are mechanistically independent. Metabolic network analysis also supports the notion that physiological responses to desiccation and starvation are somewhat specific (34). The absence of age-related pattern on tolerance to biotic insults suggests that the high level of abiotic stress tolerance observed in newly eclosed adults is unrelated to the immune functions which require cellular and humoral responses. Immunosenescence is ubiquitous and a typical consequence of aging (35). However, with respect to theories of aging, one could expect no or little decline of stress tolerance traits at young age because physiological performances are likely not declining precisely from the onset of adult age. Bacterial infection was performed with injection, and this resulted in some weak mortality in controls. This method has some limitations as it bypasses the first natural step of infection (36), thus, alternative methods, such as administration through food, should be further evaluated. Finally, two different strains were used. Both strains showed a similar pattern, that is, newly eclosed adults presented a high tolerance to abiotic stress. Further experiments could test whether the responses observed in these strains can be generalized to a larger range of strains and genotypes.

Ontogeny and age are important sources of variation of stress tolerance. For ontogeny, the most documented source of variation results from difference among life stages. Concerning the age, a reduction in stress tolerance is typically reported because of senescence. Here, we show that within the adult stage, deep phenotypic variations are detected at young age, and these are obviously unrelated to senescence. It is likely that this within-life-stage variation is linked to a passive or an active developmental (maturational) process at young adult age. Such variation may confound age-related studies if unaccounted for. Many Drosophila studies use young adults (less than 1-week-old) as experimental reference. It is therefore implicitly considered that during the first week of adult life, all aspects of the organism's biology remain relatively stable, but this assumption is clearly not warranted (13,31). Particular care should be taken in comparative aging studies that include very young adults as these may show atypical phenotypic responses that are unrelated to age.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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