

Relationships between locomotor activity, oxidative damage and life span in males and females of the linden bug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae)

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Abstract. In this study we addressed a question of whether experimental manipulations that increase life span also reduce physical activity and molecular oxidative damage. We used three phenotypes of male and female *Pyrrhocoris apterus* that survive for different lengths of time, diapausing insects, reproductive insects and insects from which the corpus allatum, the source of juvenile hormone, was surgically removed. Protein carbonyl content of the thoracic muscles was used as an index of molecular oxidative modification. Diapause or ablation of the corpus allatum (allatectomy) was associated with an extended life span of both sexes, but only those individuals that were in diapause were less active. The carbonyl content, both relative (per protein unit) and absolute (per thorax) increased with age in reproductive insects of both sexes. However, the associations between the carbonyl content and diapause and allatectomy differed in males and females. In males, the carbonyl content was not associated with either diapause or allatectomy. There was no age-related increase in the relative and absolute carbonyl levels in diapausing females, while only the increase in the relative carbonyl level was absent in allatectomized females. Overall, the results indicate that both allatectomy and diapause prolonged life span, but had different and sex-specific effects on locomotor activity and carbonyl content. Only the extension of the life span of diapausing females was correlated with both reduced locomotor activity and reduced carbonyl content.

INTRODUCTION

Early theories of ageing suggested that the longevity of an organism is inversely correlated with its mass-specific metabolic rate. This assumption is commonly referred to as the “rate of living theory” (Pearl, 1928). Contrary to this theory, there is no association between metabolic rate and life span in five species of *Drosophila* (Promislow & Haselkorn, 2002) or laboratory produced strains of *Drosophila melanogaster* (Van Voorhies et al., 2004). Similarly, within a *Drosophila* population, there is no correlation between individual metabolic rate and individual life span (Hulbert et al., 2004).

Speakman (2005) argues that the resting metabolic rate is not a good measure of total metabolism. Flight activity in insects involves a high consumption of energy (Wegener, 1996; Harrison & Roberts, 2000). Studies in which flight activity is manipulated in order to determine the role of metabolic rate in determining adult life span support Speakman’s view. For example, *D. melanogaster* (Magwere et al., 2006) and *Musca domestica* (Sohal et al., 1993; Yan & Sohal, 2000) that are allowed to fly have much shorter life spans than those prevented from flying.

Later modifications of Pearl’s (1928) theory, the “oxidative stress hypothesis”, postulate that ageing is associated with molecular damage caused by reactive oxygen species (ROS) generated by metabolic processes (Harman, 1981; Orr & Sohal, 1994; Sohal et al., 2002).

The most frequent oxidative alteration of proteins is the addition of carbonyl groups to certain amino acid residues (Levine & Stadtman, 2001). The quantity of protein carbonyl groups in different animal species and tissues increases with age (Levine & Stadtman, 2001; Linton et al., 2001). Manipulations of life span also alter the carbonyl content of tissues. In *M. domestica*, prevention of flight results in an extended life span and abolished the age-related increase in protein carbonyls (Sohal et al., 1993; Yan & Sohal, 2000). Similarly, genetic manipulations of life span in *D. melanogaster* (Orr & Sohal, 1994) and *Caenorhabditis elegans* (Adachi et al., 1998) are accompanied by the expected changes in carbonyl content. Endocrine manipulations that increase life span (defects in insulin signalling pathway or diapause) are generally accompanied by an increase in antioxidant defenses and resistance to oxidative stress (e.g. Bartke, 2001; Lin et al., 2001; Tatar et al., 2001; Holzenberger et al., 2003; Sim & Denlinger, 2008, 2009a, b), but their effects on molecular oxidative damage are mostly unknown.

In this study we used the linden bug, *Pyrrhocoris apterus* as a model to investigate the relationships between physical activity, protein oxidative damage and life span. The life span of this species can be varied experimentally by changing the photoperiod and surgical manipulation of the endocrine system (Hodkova, 2008). Short days (SD) induce reproductive diapause and pro-

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long life span. Insects kept in long days (LD) conditions are reproductive and short-lived. The effect of photoperiod on life span is partly mediated through the corpus allatum (CA), an endocrine gland producing juvenile hormone (JH). Ablation of the CA (allatectomy) prolongs the life span of LD-insects, although allatectomized insects do not live as long as insects that undergo diapause (Hodkova, 2008; Blazkova et al., 2011). Allatectomized females are non-reproductive. However, ablation of the ovary has no effect on life span of LD-females (Hodkova, 2008). Consequently, the prolongation of life span by allatectomy is not because they do not produce eggs. The difference between the life spans of allatectomized insects and those that undergo diapause is associated with the differential activity of the neurosecretory cells in the pars intercerebralis (PI) (Hodkova, 2008).

There seems to be no causal relationship between life span and the resting metabolic rate. Basal levels of oxygen consumption are similar in diapausing, reproductive and allatectomized males of *P. apterus* (Slama, 1964a). Reproductive females of *P. apterus* consume much more oxygen than allatectomized and diapausing females (Slama, 1964b). This indicates a negative correlation between metabolic rate and life span. However, ablation of the ovary decreases oxygen consumption to the level found in allatectomized females (Slama, 1964b), and yet, this operation does not prolong life span (Hodkova, 2008).

We hypothesized that the endocrine regulation of life span in *P. apterus* might be mediated through the molecular oxidative damage caused by the metabolic processes related to physical activity. If this hypothesis is valid, then the experimental manipulations increasing life span should decrease both locomotor activity and molecular oxidative damage. The objective of the current study was to test these predictions. Life span was manipulated by changing the photoperiod and ablation of the CA. Protein carbonyl level in thoracic muscles (likely targets of oxidative damage caused by locomotion) was used as an index of molecular oxidative modification.

MATERIAL AND METHODS

Insects

Colonies of *Pyrrhocoris apterus* (L.) (Heteroptera) were reared at $26 \pm 1^\circ\text{C}$ and a diapause-preventing long-day photoperiod (LD) of 18L : 6D or diapause-promoting short-day photoperiod (SD) of 12L : 12D and supplied ad libitum with linden seeds and water. LD-insects were used in all experiments in which the corpus allatum was ablated (CA, allatectomy). The mean duration of larval development was similar under the two photoperiods (about 1 month).

Adults were collected from the stock colony within 24 h of adult ecdysis and males and females were then kept separately. Insects destined to be operated were deprived of food. Two days later, these insects were narcotized by submergence in water for 15 min and then placed in Ringer insect saline and the CA removed through an incision in the neck membrane, as previously described (Slama, 1964a). Control insects were either sham-operated (neck membrane was cut) or left intact. Results for sham-operated and intact insects were not significantly different and were combined. Insects were given food immediately

after the operation. Mortality of these insects kept individually in Petri dishes was checked every two days.

Locomotor activity

Locomotor activity was monitored by keeping individual insects in Petri dishes and using a recording device comprised of infrared beam passing horizontally through the dish to a photo-transistor. Activity was monitored in 12 min bins as the number of interruptions of the infrared light beam by moving insects and the data recorded on a computer.

Protein carbonyls

Protein carbonyl content was measured using a modification of the procedure of Levine et al. (1990), which is based on the spectrophotometric measurement of the 2,4-dinitrophenylhydrazine (DNPH) derivatives of protein carbonyls. In each sample, 10 thoraxes (without gut and fat body) were homogenized in 1 ml of 50 mM phosphate buffer (pH 7.0) with 1mM EDTA. Samples were treated with streptomycin sulfate (10%) to remove nucleic acids (Reznick & Packer, 1994) and incubated for 15 min at room temperature (RT). The homogenate was then centrifuged at 3,000 g for 10 min at 4°C . From the resulting supernatant, 10 μl aliquots were used to determine the protein content using Pierce BCA Protein Assay Kit (Genetica). Other 200 μl aliquots were treated with 800 μl of 7 mM DNPH dissolved in 2 M HCl or with 2 M HCl in the controls. Samples were then incubated for 1 h at RT, stirred every 15 min, precipitated with 1 ml of 28% trichloroacetic acid (TCA) and centrifuged at 10,000 g for 10 min at 4°C . The pellet was suspended in 1 ml of 5% TCA and centrifuged at 10,000 g for 10 min at 4°C . Finally, the pellet was washed twice with 1 ml of ethanol/ethyl acetate, 1 : 1 (vol/vol), centrifuged at 12,000 g for 10 min at 4°C and dissolved in 500 μl of 6 M guanidine hydrochloride in water. Any insoluble material was removed by centrifugation. The difference in the absorbance of the DNPH-treated and the HCl-treated samples was determined at 370 nm, and the results were expressed as nmol of carbonyl groups per g protein or per 1 thorax, using the extinction coefficient of $22.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for DNPH.

Statistical analysis

Log-rank (Mantel-Cox) tests were used to assess similarity of longevity between the three groups. Differences in the locomotor activity, protein carbonyl and protein levels were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test.

RESULTS

Relationship between life expectancy and locomotor activity

Diapausing SD-insects lived 2.5 times ($P < 0.0001$, females, Fig. 1A) or 1.8 times ($P < 0.0001$, males, Fig. 1B) longer than reproductive LD-insects. Ablation of the corpus allatum (CA) prolonged life span of LD-insects by 1.8 times (females, $P < 0.0001$, Fig. 1A) or 1.4 times (males, $P = 0.0006$, Fig. 1B). The results suggest that the absence of the CA (JH) contributes to the extension of life span in LD-insects, but the absence of JH alone is not responsible for the greater prolongation of life span recorded for SD-insects.

There were no significant differences in the locomotor activity of males and females (Fig. 1A, B). Ablation of the CA had no effect on locomotor activity of LD-insects. In contrast, SD-insects were 3 times less active than LD-

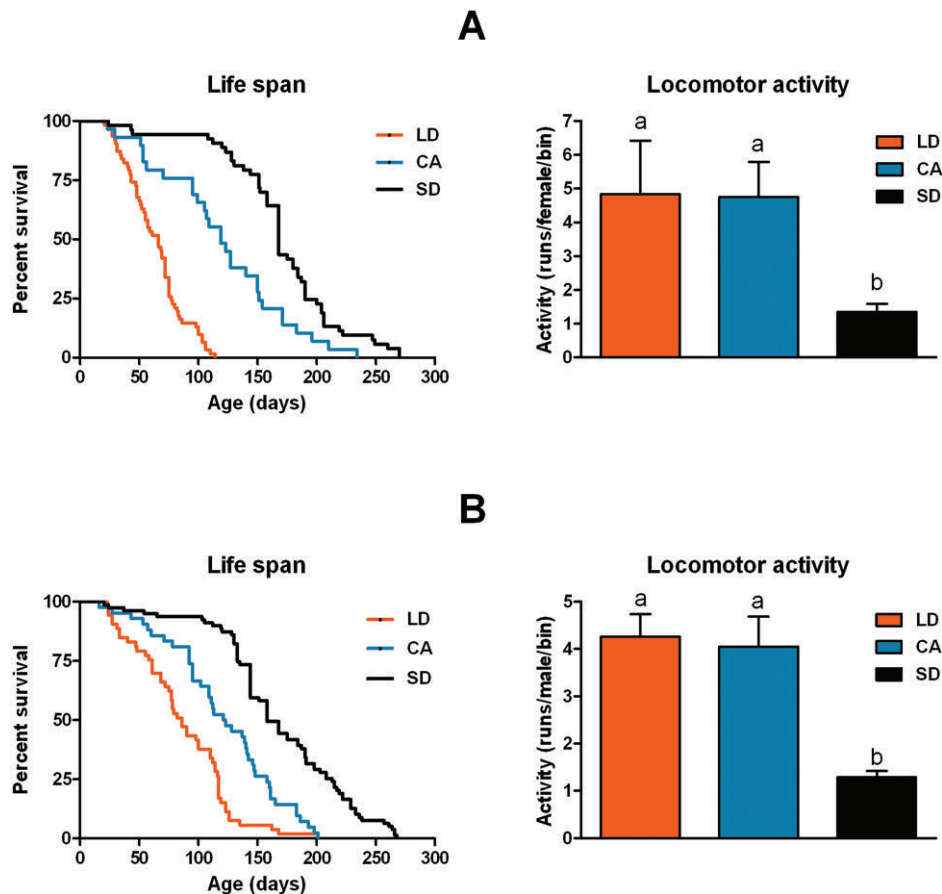


Fig. 1. Effect of photoperiod and corpus allatum ablation on life span and locomotor activity in females (A) and males (B) of *Pyrrhocoris apterus*. LD – long day (reproduction), CA – long day and allatectomy, SD – short day (diapause). Median life spans of females: LD, 66 days ($n = 62$); CA, 119 days ($n = 29$); SD, 168 days ($n = 53$). Curve comparison: LD vs. SD, $P < 0.0001$; LD vs. CA, $P < 0.0001$; SD vs. CA, $P < 0.0001$. Median life spans of males: LD, 86 days ($n = 53$); CA, 122 days ($n = 42$); SD, 158 days ($n = 79$). Curve comparison: LD vs. SD, $P < 0.0001$; LD vs. CA, $P = 0.0006$; SD vs. CA, $P < 0.0001$. Locomotor activity of females: LD, $n = 6$; CA, $n = 7$; SD, $n = 16$. $F = 8.253$, $P = 0.0017$. LD vs. CA $P > 0.05$, LD vs. SD $P < 0.01$, CA vs. SD $P < 0.01$. Locomotor activity of males: LD, $n = 6$; CA, $n = 8$; SD, $n = 14$. $F = 22.02$, $P < 0.0001$. LD vs. CA $P > 0.05$, LD vs. SD $P < 0.001$, CA vs. SD $P < 0.001$. The data for locomotor activity are the mean \pm SEM of 2-week recordings in 12 min bins. The first measurements are for 1 wk old insects. Values that are significantly different ($P < 0.01$) from each other are indicated with different letters.

insects (females $P < 0.01$, males $P < 0.001$). The results indicate that the absence of JH is not responsible for the negative effect of diapause on locomotor activity.

Effect of age on protein carbonyl content

Reproductive insects

The carbonyl content was determined from 0–1 weeks up to 6–7 weeks of age, when 20–25% of the population had died. In both females (Fig. 2A) and males (Fig. 2B) the carbonyl content increased with age. The relative carbonyl level (per g protein) increased by 71% ($P < 0.01$) in females and 72% ($P < 0.01$) in males between 0–1 and 6–7 weeks. The absolute carbonyl (per thorax) level increased by 71% ($P < 0.01$) in females and 81% ($P < 0.001$) in males between 0–1 and 6–7 weeks.

Diapausing insects

In females (Fig. 2A), there were no significant age-dependent changes in either the relative or absolute carbonyl levels up to 7–8 weeks of age. In males (Fig. 2B), the relative carbonyl level increased by 58% ($P < 0.05$)

and 69% ($P < 0.01$) between 0–1 and 6–7 or 7–8 weeks, respectively. The absolute carbonyl level increased by 53% ($P < 0.05$) between 0–1 and 5–6 weeks. Thus, the age-related increase in carbonyl content did not occur in diapausing females.

Relationship between life span and carbonyl content

The carbonyl contents of thoraxes of short-lived reproductive insects and long-lived diapausing or allatectomized insects, all aged between 6–7 weeks, were compared.

Reproductive vs. diapausing insects

The relative (per g protein) and absolute (per thorax) carbonyl contents were, respectively, 53% ($P < 0.05$) and 48% ($P < 0.01$) higher in reproductive than diapausing females (Fig. 2A). In contrast, reproductive males had only a slightly higher relative (21%) and absolute (16%) carbonyl contents than diapausing males and these differences were not significant (Fig. 2B). In both sexes, protein levels were not significantly different in reproductive and diapausing insects (Fig. 2A, B).

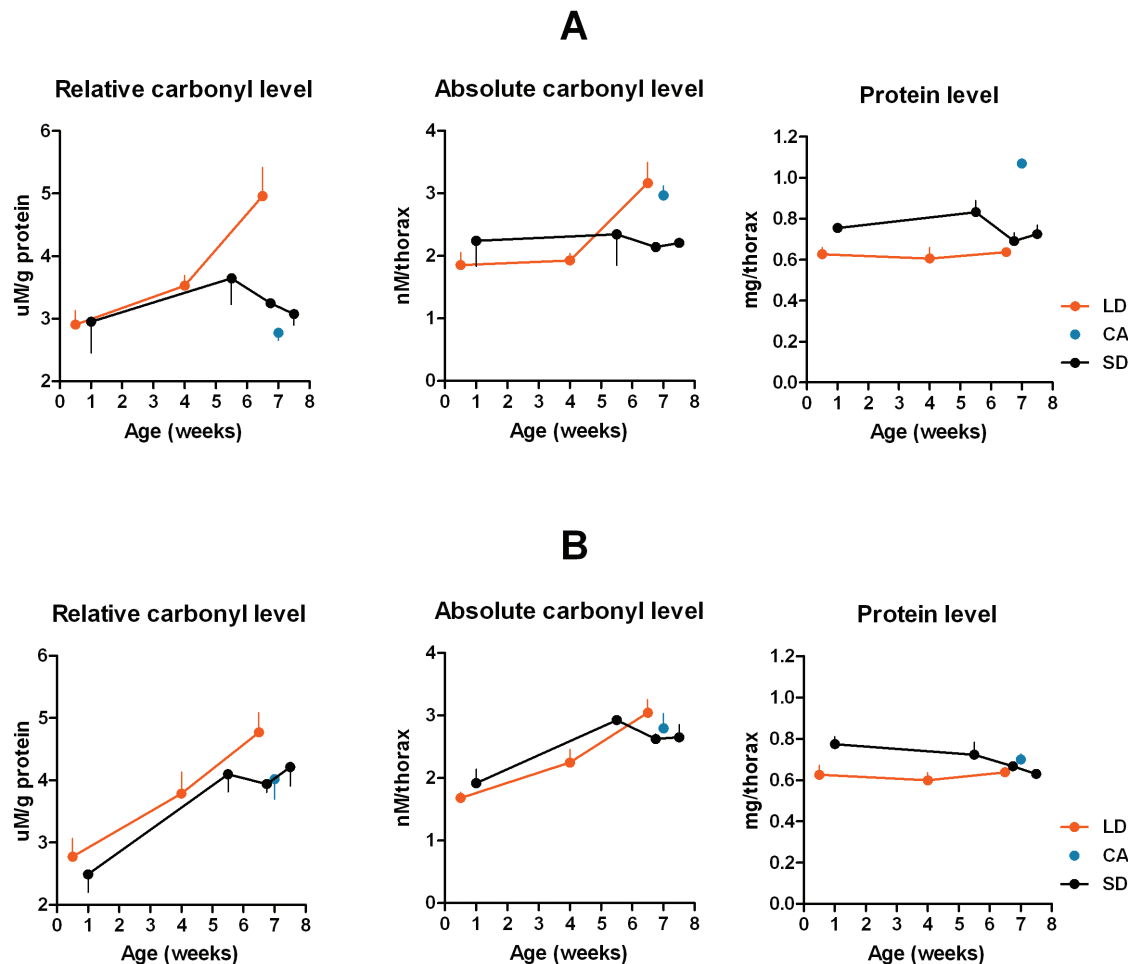


Fig. 2. Effect of photoperiod and corpus allatum ablation on levels of protein carbonyls and proteins in thoraxes of females (A) and males (B) of *Pyrrhocoris apterus*. LD – long day (reproduction), CA – long day and allatectomy, SD – short day (diapause). Each point represents the mean \pm SEM of determinations for $n = 3-5$ samples, with 8–10 thoraxes per sample. Differences between ages: (A) Relative carbonyl level: LD – $F = 12.26$, $P = 0.020$. 0–1 wk vs. 6–7 wk, $P < 0.01$; 3–5 wk vs. 6–7 wk, $P < 0.05$. SD – $F = 0.7861$, $P = 0.5946$. Absolute carbonyl level: LD – $F = 11.77$, $P = 0.0018$. 0–1 wk vs. 6–7 wk, $P < 0.01$; 3–5 wk vs. 6–7 wk, $P < 0.01$. SD – $F = 0.06246$, $P = 0.9785$. Protein level: LD – $F = 0.1408$, $P = 0.8700$. SD – $F = 1.903$, $P = 0.1931$. (B) Relative carbonyl level: LD – $F = 9.748$, $P = 0.0031$. 0–1 wk vs. 6–7 wk, $P < 0.01$. SD – $F = 9.138$, $P = 0.0033$. 0–1 wk vs. 5–6 wk, $P < 0.05$; 0–1 wk vs. 6–7 wk, $P < 0.05$; 0–1 wk vs. 7–8 wk, $P < 0.01$. Absolute carbonyl level: LD – $F = 5.975$, $P = 0.0069$. 0–1 wk vs. 6–7 wk, $P < 0.001$; 3–5 wk vs. 6–7 wk, $P < 0.05$. SD – $F = 5.789$, $P = 0.0147$. 0–1 wk vs. 5–6 wk, $P < 0.05$. Protein level: LD – $F = 0.3122$, $P = 0.7376$. SD – $F = 3.510$, $P = 0.0571$. Comparison of values for insects aged between 6 and 7 weeks: (A) Relative carbonyl level: $F = 9.628$, $P < 0.0134$. LD vs. CA $P < 0.05$, LD vs. SD $P < 0.05$, CA vs. SD $P > 0.05$. Absolute carbonyl level: $F = 30.8$, $P = 0.0015$. LD vs. CA $P > 0.05$, LD vs. SD $P < 0.01$, CA vs. SD $P < 0.01$. Protein level: $F = 75.51$, $P < 0.0001$. LD vs. CA $P < 0.001$, LD vs. SD $P > 0.05$, CA vs. SD $P < 0.001$. (B) Relative carbonyl level: $F = 2.442$, $P = 0.1487$. Absolute carbonyl level: $F = 1.307$, $P = 0.3227$. Protein level: $F = 4.337$, $P = 0.0530$.

Reproductive vs. allatectomized insects

Reproductive females had 79% ($P < 0.05$) higher relative carbonyl content than allatectomized females. On the other hand, the absolute carbonyl content was not significantly different in reproductive and allatectomized females. The lower relative carbonyl content is attributable to the higher (68%, $P < 0.001$) level of proteins in allatectomized than in reproductive females (Fig. 2A). Carbonyl and protein levels in allatectomized males were similar to those in diapause males and were not significantly different from the values for reproductive males (Fig. 2B).

DISCUSSION

We tested the hypothesis that the extension in life span that results from the experimental manipulation of endocrine signals may be mediated through a decrease in the molecular oxidative damage associated with physical activity. Predictions of the hypothesis were not unambiguously confirmed by the present results.

Prediction #1: Manipulations that extend life span should be associated with reduced locomotor activity

Both diapausing SD-insects and allatectomized LD-insects lived longer than reproductive LD-insects (Fig. 1). In accordance with prediction #1, diapausing insects were considerably less active than reproductive insects. In con-

trast, allatectomy did not reduce locomotor activity in LD-insects (Fig. 1). This result contradicts prediction #1. However, allatectomy increased life span to a lesser extent than diapause (Fig. 1), suggesting that the absence of JH was not the only cause of the long life span of insects that undergo diapause.

A previous study indicates that the pars intercerebralis (PI) of the brain is responsible for the difference in the life spans of diapausing and allatectomized females (Hodkova, 2008). Ablation of the PI increases the life span of allatectomized LD-females of *P. apterus* (Hodkova, 2008) and this operation also decreases locomotor activity to that characteristic of diapausing females (Hodkova, unpubl. data). Accordingly, the extension of life span associated with the simultaneous absence of CA (JH) and reduced PI-signalling (in diapausing insects) is accompanied by a decrease in locomotor activity, while the absence of JH alone (in allatectomized insects) prolongs life span without affecting locomotor activity.

Prediction #2: Manipulations that extend life span should be associated with a reduction in molecular oxidative damage

A correlation between increased oxidative damage and age and a correlation between manipulations that increase life span and a reduction in oxidative damage and/or increase in resistance to oxidative stress are the most frequent outcomes of the research that support the oxidative stress hypothesis (Perez et al., 2009). Consistent with these findings, protein carbonyls in thoracic muscles accumulated with age in the reproductive males and females of *P. apterus* (Fig. 2). However, alterations in carbonyl content caused by diapause or allatectomy that prolonged life span differed between sexes.

In females of *P. apterus*, both diapause and allatectomy abolish the age-related increase in the relative carbonyl level (Fig. 2A). The relative carbonyl content measured at about 7 weeks of age was significantly higher in reproductive females than in diapausing and allatectomized females. Using the median life span as the end point (1.0), at 7 weeks of age reproductive females reached 74% of their physiological age (life expectancy), whereas diapausing and allatectomized females reached only 29 and 41% of their life expectancy, respectively. Thus, the increase in the relative carbonyl content was associated with the physiological ageing of females, in accordance with prediction #2 and with the oxidative stress hypothesis.

Mechanisms determining the low relative carbonyl levels in thoracic muscles differed in diapausing and allatectomized females. In diapausing females, neither the absolute carbonyl level nor total proteins increased with age (Fig. 2A). Either the oxidative damage of proteins did not occur or the oxidized proteins were replaced by new proteins. In allatectomized females, on the other hand, the increase in the absolute carbonyl content was not suppressed, but was accompanied by an increase in the amount of total proteins (Fig. 2A). These additional proteins might counterbalance the negative effect of the enhanced absolute carbonyl level on muscle function. It is

possible that the difference in the absolute carbonyl levels between diapausing and allatectomized females is related to different PI-signalling (Hodkova, 2008) and different locomotor activity (Fig. 1A). In *D. melanogaster*, genetic ablation of the insulin-like producing cells in the PI resulted in an extension of life span and increased resistance to oxidative stress (Broughton et al., 2005), but effects of this treatment on carbonyl levels or locomotor activity were not measured. Hypothetically, reduced PI-signalling in diapausing females of *P. apterus* (Hodkova, 2008) might be responsible for a decrease in locomotor activity and, consequently, the maintenance of a low absolute carbonyl level. Physical activity has a positive effect on protein carbonylation in *M. domestica* (Sohal et al., 1993; Yan & Sohal, 2000). This hypothesis, however, is not applicable to diapausing males (see below).

JH accelerates flight muscle histolysis and stimulates ovarian maturation in many insects (cited in Lorenz, 2007), including the flightless *P. apterus*, which display non-functional wing polymorphism (Socha & Sula, 2006, 2008). The histolysis of the flight muscles can provide additional substrates for the growing oocytes (e.g. Mole & Zera, 1993; Tanaka, 1993; Stjernholm et al., 2005). Conversely, the absence of ovarian development associated with high locomotor activity might be responsible for a reallocation of resources to thoracic muscles in allatectomized females of *P. apterus*.

In approximately 7 week old males of *P. apterus*, the carbonyl content (both relative and absolute) of the thoracic muscles was similar in all three longevity groups (Fig. 2B). At this age, reproductive males were 57% of their physiological age, whereas diapausing and allatectomized males were 31 and 40% of their physiological age, respectively. Thus, the increase in carbonyl content was more associated with the chronological rather than the physiological age. These results contradict prediction #2.

The lack of a correlation between carbonyl levels and life span may imply one of two possibilities. (1) Analysis of carbonyl content in whole tissue homogenates did not reveal differences in carbonylation of specific enzymes. Selective age-related protein carbonylation occurs in the matrix of the flight-muscle mitochondria of *D. melanogaster* (Das et al., 2001) and different tissues of rats (Goto et al., 1999). (2) Carbonyl levels in thoracic muscles are not reliable markers of ageing in *P. apterus*. The increase in lipid peroxidation, accumulation of oxidative damage to DNA or alterations in membrane fatty acid profiles represent alternative mechanisms resulting in the association between oxidative damage in organisms and decrease in life span (Agarwal & Sohal, 1994; Magwere et al., 2006; Kregel & Zhang, 2007). In *C. elegans*, the abundance of short-chain saturated fatty acids (C14:0, C15:0, C16:0) increases at the expense of long-chain classes (C18:0, C20:0, C22:0) and susceptibility to oxidation decreases in the longest-lived mutants (Shmookler Reis et al., 2011). Consistent with these finding, the induction of diapause or allatectomy in *P. apterus* females resulted in an accumulation of phospholipids

with C16:0/C18:2 acyl chains at the expense of C18:0/C18:2 molecular species (Hodkova et al., 2002).

A recent study of the molecular regulation of diapause in *Culex pipiens* indicates that antioxidant enzymes are important in promoting the survival of diapausing females (Sim & Denlinger, 2011). It is possible that sexual differences in protein carbonylation recorded in the thoracic muscles of diapausing *P. apterus* (Fig. 2) are related to a higher antioxidant defense in females than in males. This explanation is supported by the finding that the carbonyl content in the brain microsomal fraction of *P. apterus* adults injected with paraquat (a strong elicitor of oxidative stress) is higher in males than females (Krishnan et al., 2007).

CONCLUSION

The results show that the effects of endocrine interventions, either suppression of JH-signalling (CA ablation) or suppression of both JH- and PI-signalling (diapause), on life span, locomotor activity and protein carbonylation differ between the sexes and also between interventions. Only the extension of the life span of diapausing females was correlated with both reduced locomotor activity and reduced accumulation of protein carbonyls during ageing, in accordance with the predictions of the hypothesis tested. Further work on identifying the molecular pathways that result in the oxidative damage of specific proteins and other macromolecules in different tissues is essential for the next phase of the study of the relationship between the stress associated with oxidation and ageing.

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