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Novel roles for the corpus allatum hormone in the cost of sexual interactions in
the linden bug *Pyrrhocoris apterus*

RNDr. Thesis

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Annotation:

The decrease of life-span by mating is a common, yet poorly understood occurrence. Juvenile hormone (JH) is widely believed to mediate shortened life-span in insect, caused by mating. In this study we tested the role of JH in the linden bug (*Pyrrhocoris apterus*) by removal of the corpus allatum gland and thus the JH source. We then proceeded to study the lifespan of mated animals vs. virgin animals. Virgin animals without JH live longer as was expected. The effect on mated animals is however different between males and females. Surprisingly in females, JH seems to lower the cost of mating. In males, on the other hand, the cost of mating seems to be independent on JH levels. Another surprising conclusion is that the level of JH in females affects the life-span of males that mate with such females.

Declaration [in Czech]

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Novel roles for the corpus allatum hormone in the cost of sexual interactions in the linden bug *Pyrrhocoris apterus*

Úvod:

U hmyzu se běžně vyskytuje trade-off mezi délkou života a reprodukcí. Cena za reprodukci se obvykle projevuje zkrácením délky života jedince po tom, co se páří. Fyziologické mechanismy tohoto trade-off však nejsou zcela jasné. Většina dostupných studií se soustředí na samice. Některé teorie hovoří o tom, že samice investují velkou část dostupné energie do tvorby vajíček, nebo se zkrácení délky života přičítá samotnému, mnohdy traumatizujícímu, aktu páření. V této souvislosti se také často uvažuje o vlivu proteinů přídatných žláz samců. Kupříkladu protein Acp70 u *Drosophila melanogaster* zprostředkovává cenu za páření. Zkrácení délky života u samců se donedávna tolik pozornosti nevěnovalo, neboť se předpokládalo, že tvorba spermatu není nikterak náročná. U některých druhů motýlů je tomu ale právě naopak a samci svým nutričně hodnotným ejakulátem pomáhají samici pokrýt náklady na reprodukci. Jak to tak v přírodě bývá, je velmi nesnadné určit jednoho „pachatele“ zprostředkování ceny za reprodukci. Pravděpodobně se jedná o kombinaci některých předchozích jevů. (Fowler and Partridge 1989, Simons 2001, Vahed 1998, Wedell and Cook 1999)

Jako jeden z hlavních hráčů zprostředkování zkrácení délky života se často uvádí juvenilní hormon (JH) hmyzu. Negativní efekt JH na délku života byl prokázán u několika druhů hmyzu včetně plošnice *Pyrrhocoris apterus* (ruměnice pospolná, hemiptera). Vzhledem k tomu, že *P. apterus* je tradičním a zavedeným fyziologickým modelem, který díky své velikosti umožňuje reprodukovatelné chirurgické zákroky, byla tato studie provedena právě na něm. V předchozích studiích bylo dokázáno, že odstraněním žlázy corpus allatum (a tedy zdroje JH) dojde k výraznému prodloužení délky života. Vzhledem k tomu, že titr JH ovlivňuje produkci proteinů přídatných žláz a tím i jednu z možných cest zkrácení longevity, se otázka, jestli JH hraje úlohu mediátoru ceny za reprodukci, sama nabízí. (Hodková 2008, Kodrík 2000, Wigby and Chapman 2005)

Cíle práce:

Cíle přiložené práce jsou následující:

- Ověřit, že páření zkracuje délku života *P. apterus*.
- Zjistit zdali má na zkrácení délky života vliv JH samotného jedince (v tomto případě přítomnost žlázy, která ho produkuje)
- Zjistit zdali má na zkrácení délky života vliv JH sexuálního partnera

Experimenty:

Jako pokusný hmyz byla použita výše zmíněná ploštica *P. apterus* z chovů Entomologického ústavu AVČR. Pokusy byly prováděny v konstantní teplotě $26 \pm 1^\circ\text{C}$ a fotoperiodě 18h světla a 6 hodin tmy, která zabraňuje diapauze.

Pro provedení experimentu bylo nejprve nutné zjistit, zdali páření opravdu zkracuje život. Byly tedy použity panenské samice, které byly srovnávány se samicemi, které se pářily. Tento experiment byl analogicky proveden i u samců. V obou případech se potvrdilo, že páření statisticky signifikantně zkrátí délku života (Log-rank test: $p\text{-value} < 0.0001$, vizte Fig. 1 v přiloženém článku)

Další část pokusu pak spočívala ve srovnání délky života ploštic po odstranění corpus allatum (dále značeno jako CAex). Samotné odstranění CA u samic prodlouží život o 70 dní (medián z délky života 126/100 intaktních resp. CAex samic), u samců je toto prodloužení relativně menší (33 dní) nicméně i tak signifikantní ($n=70/43$; intaktní/CAex samci).

Pokusy s pářením pak naznačují, jestli je délka života krácena v závislosti na přítomnosti CA. Podle výsledků práce se zdá, že je situace rozdílná mezi samci a samicemi. U samic je cena za reprodukci větší pokud nemají CA, CA tedy snižuje negativní efekt páření u samic (detailní popis v odstavci 3.1.2 v článku). U samců je naproti tomu cena za reprodukci nezávislá na přítomnosti CA.

Zajímavé výsledky přineslo srovnání délky života v závislosti na stavu CA partnera. U samic se zdá, že přítomnost CA u partnera způsobuje cca 1/3 zkrácení longevity. Naproti tomu přítomnost CA u samice je zodpovědná za přibližně 88% zkrácení života samců (detailní popis v odstavci 3.1.3 v článku).

Abychom zjistili, jestli je tento efekt výlučně vázán na opačné pohlaví, byly zároveň provedeny experimenty s homosexuálními páry. U samic se žádný vliv na délku života oproti samostatným samicím neprokázal. U samců je situace jiná. U homosexuálních párů bylo pozorováno, že se samci pokoušeli kopulovat a došlo ke zkrácení délky života o 57,9%.

Závěr:

Studie potvrdila, že jak přítomnost CA, tak i kopulace zkracuje délku života samců i samic. Rozdíl mezi silou ovlivnění délky života samců a samic může být dán rozdílnou velikostí CA. U samic je CA přibližně dvakrát větší než u samců. Způsob jakým JH ovlivňuje longevitu není přesně znám.

Nečekaným zjištěním bylo, že zkrácení délky života u samic, které se pářily, bylo větší, pokud neměly CA. Zdá se tedy, že JH není mechanismem, který by zprostředkoval cenu za reprodukci u samic *P. apterus*. Teorie o tom, že zásadní úlohu ve zkrácení života samic hrají proteiny přídatných žláz, se nepodařilo podpořit, neboť páření samic se samcem bez CA (a tedy s menším množstvím proteinů přídatných žláz) nevedlo k výraznému snížení ceny za reprodukci.

V případě samců vede páření ke snížení longevity, nezávisle na stavu CA samce. Velmi překvapivým zjištěním však bylo, že samci, kteří se pářili se samicemi bez CA, se dožili delšího věku než samci v páru s intaktní samicí. To naznačuje, že hormonální nastavení samice má vliv na longevitu samce. Jedním z vysvětlení je menší receptivita intaktních samic oproti samicím CAex. Je tedy možné, že se samci více „vyčerpali“ námluvami u CAex samice. Alternativní teorií by byla úloha feromonů samic. Další detaily a hypotézy najdete v diskusi článku. Zjištění o efektu JH samic na délku života samce je poměrně zajímavé a zasluhuje si hlubší zkoumání v budoucích projektech.

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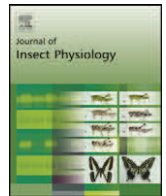
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Novel roles for the corpus allatum hormone in the cost of sexual interactions in the linden bug *Pyrrhocoris apterus*

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ABSTRACT

The cost of sexual interactions, usually expressed as a reduction of life-span, is a fundamental but poorly understood aspect of life. According to a widely accepted view, a rise in the “pro-aging” juvenile hormone (JH) might contribute to the decrease of life span caused by sexual interactions. We tested this hypothesis using the linden bug *Pyrrhocoris apterus* by removing the corpus allatum (CA), the source of JH. If JH is causally involved in the cost of sexual interactions, then the absence of CA (JH) should decrease the negative effect of sexual interactions on survival. As expected, ablating the CA significantly prolonged life-span of both virgin females and virgin males. Mated insects of both sexes lived significantly shorter than virgins. However, contrary to prediction, the decrease of life span by sexual interactions was similar in control and CA-ablated males, and was even enhanced in CA-ablated females. Another unexpected finding was that males paired with CA-ablated females lived almost as long as virgin males and significantly longer than did males paired with control females, although ablating the female CA did not cause any decrease in mating activity. On the other hand, females paired with CA-ablated males lived only slightly longer than did females paired with control males. These results highlight several important points. (1) In both genders, the negative effect of sexual interactions on insect's survival is not mediated by the insect's own CA. (2) The male CA has only minor effect on female survival, while (3) the female CA (JH) is principally responsible for the sex-induced reduction in the male survival.

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1. Introduction

Negative effects of mating on survival is of widespread occurrence, but the underlying physiological mechanisms are not well understood. In many species, male accessory gland proteins (Acps) transferred to females during mating reduce female survival, thus constituting a cost of reproduction (Arnqvist and Nilson, 2000; Gillot, 2003). In females of *Drosophila melanogaster*, Acp 70 (sex peptide) causes the mating cost (Wigby and Chapman, 2005). This peptide also stimulates biosynthesis of the principal reproductive hormone of insects, the juvenile hormone (JH) by the corpus allatum (CA) in vitro (Moshitzky et al., 1996). Mating was shown to enhance JH levels in many other species, for instance in females of the moth *Heliothis virescens* (Ramaswamy et al., 2000), males of the Caribbean fruit fly *Anastrepha suspensa* (Teal et al., 2000), males, but not females of the burying beetle *Nicrophorus orbicollis* (Scott and Panaitof, 2004; Trumbo and Robinson, 2004) or males and females of the mealworm *Tenebrio molitor* (Rolff and Siva-Jothy, 2002). A negative

effect of JH on life span was convincingly shown in grasshoppers (Pener, 1972), butterflies (Herman and Tatar, 2001) and in the linden bug *Pyrrhocoris apterus* (Hodkova, 2008), where surgical removal of the CA, the source of JH, considerably prolonged life span. Therefore, the enhanced production of JH is thought to be at the basis of elevated egg production and shorter life span after mating (Chapman et al., 1995; Rolff and Siva-Jothy, 2002; Kubli, 2003; Wigby and Chapman, 2005). However, a causal relationship between the mating-induced increase of JH levels and survival has never been experimentally tested.

Another question is whether insect's survival may be influenced by the JH of sexual partners. This question has not yet been addressed, although it arises from available experimental data. The negative effect of Acps on female survival (Chapman et al., 1995) might depend on male JH because JH stimulates growth and protein synthesis in the male accessory glands of many insects (e.g. Chen, 1984; Yamamoto et al., 1988; Wilson et al., 2003; Parthasarathy et al., 2009) including *P. apterus* (Socha et al., 2004). Furthermore, JH participates in the regulation of pheromone production (e.g. Cusson and McNeil, 1989; Smith and Schal, 1990; Tralalon et al., 1990; Fan et al., 1999; Sréng et al., 1999; Rafaeli and Bober, 2005) and a substance(s) called dauer inducing pheromone can extend the life span of adult *Caenorhabditis elegans* (Kawano et al., 2005).

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The main aim of this study was to determine whether the CA is causally involved in the decrease of life-span caused by continuous exposure of insects to sexual partners. Hypothetically, the CA may have two principal roles in the survival cost of sexual interactions. First, the sex-induced decrease of life-span of an individual may be mediated by changes in the activity of its own CA. Second, survival may be reduced by the sexual partner's CA via its effect on the secretion of signalling substances (e.g. accessory gland peptides, pheromones). We tested these hypotheses by using males and females of *P. apterus* with surgically removed CA. If the individual's own CA mediates the cost, then the sex induced reduction of life-span should be more extensive in control (with intact CA) than in allatectomized insects. On the other hand, if the sexual partner's CA is responsible for the cost, then the reduction of life-span should be more extensive in insects exposed to allatectomized than to control partners.

2. Materials and methods

2.1. Insects

Colonies of *P. apterus* (L.) (Heteroptera) originated from insects collected in the field near Ceske Budejovice (south Bohemia). Insects were reared at 26 ± 1 °C and a diapause-preventing photoperiod of 18 h light/6 h darkness and supplied ad libitum with linden seeds and water. The mean duration of larval development was about 1 month.

2.1.1. Experiment 1: isolated virgins vs. isolated pairs

Males and females were selected separately from the colony within 24 h after adult ecdysis, and were deprived of food. Two days later, insects destined for operation were narcotized by submergence in water for 15 min and the CA was removed through the neck membrane incision under Ringer insect saline as described (Slama, 1964a,b). Control insects were either sham-operated (neck membrane was cut) or left intact. Insects were then kept in Petri dishes with linden seeds and water, either individually (virgins) or in pairs until death. Mortality, mating status and egg numbers were checked every two days. Dead sexual partners were replaced from stock insects (intact or allatectomized) or widows and widowers were combined.

Seven experimental groups were examined: virgin control males, virgin control females, virgin allatectomized males, virgin allatectomized females, control pairs (both partners with the CA), pairs with allatectomized males, pairs with allatectomized females.

2.1.2. Experiment 2: homosexual vs. heterosexual groups

Intact insects were selected from the colony within 24 h after adult ecdysis and kept in several 500 ml glass containers (cca 50 individuals per container) with continuous supply of food. Four experimental groups were examined: virgin males, virgin females, non-virgin males and females (equal numbers of males and females in container). Mortality was checked every two days.

Insect colonies used in experiments 1 and 2 were offspring of insects collected in the field in different years.

2.2. Mating activity

Earlier data based on short-term (30 min) tests indicate that the CA hormone plays no important role in the control of mating activity in *P. apterus* (Zdarek, 1966, 1968). To check whether ablating the CA influenced mating behaviour in males and females continuously exposed to one another, we recorded the mating status of individual pairs every 30 min throughout a 27 h period. Several parameters were measured: (1) temporal pattern of mating activity (changes in the number of copulating pairs), (2)

number of copulation bouts (uninterrupted mating associations) and (3) duration of a copulation bout.

2.3. Statistics

Log-rank tests were used to assess similarity of longevity between the two groups. Differences between parameters of mating activity and fecundity were analyzed by two-way analysis of variance (temporal patterns of mating proportions) or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test (numbers of copulation bouts, duration of a copulation bout, egg numbers, oviposition and post-oviposition periods). GraphPad Prism 4 software was used.

3. Results

3.1. Experiment 1 – isolated virgins vs. isolated pairs

3.1.1. Effect of the CA on survival of virgins

Ablating the CA prolonged the median life span by 70 days (103%) in virgin females (Fig. 1A). A less extensive increase of median life span by allatectomy was observed in virgin males (33 days, 37%) (Fig. 1B).

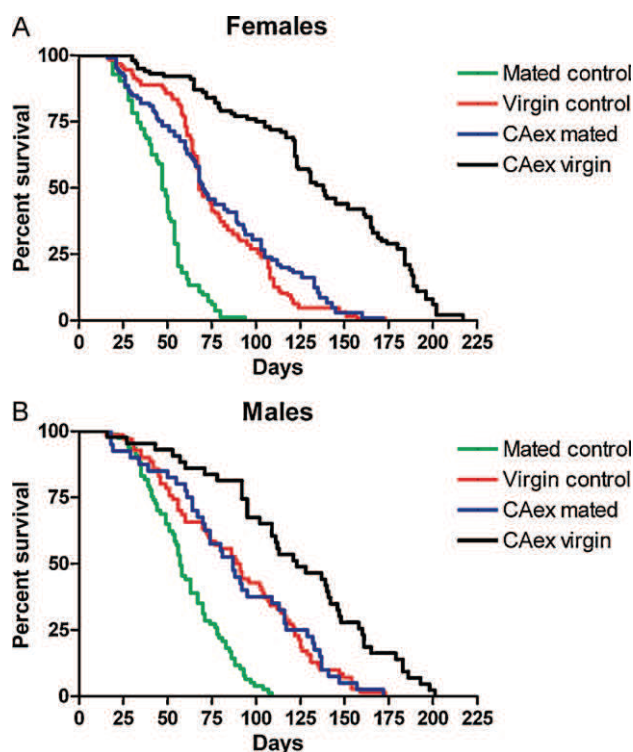


Fig. 1. Effect of the exposure to control (with the corpus allatum) sexual partners on the survival of allatectomized and control *P. apterus* females (A) and males (B). CAex – corpus allatum ablated. (A) Median life spans of females: mated control, 47 days ($n = 83$); virgin control, 68 days ($n = 126$); CAex mated, 70 days ($n = 105$); CAex virgin, 138 days ($n = 100$). Curve comparison: mated control vs. virgin control, $P < 0.0001$; mated control vs. CAex mated, $P < 0.0001$; mated control vs. CAex virgin, $P < 0.0001$; virgin control vs. CAex mated, $P = 0.4129$ (n.s.); virgin control vs. CAex virgin, $P < 0.0001$; CAex mated vs. CAex virgin, $P < 0.0001$. (B) Median life spans of males: mated control, 57 days ($n = 77$); virgin control, 90 days ($n = 70$); CAex mated, 87 days ($n = 40$); CAex virgin, 123 days ($n = 43$). Curve comparison: mated control vs. virgin control, $P < 0.0001$; mated control vs. CAex mated, $P < 0.0001$; mated control vs. CAex virgin, $P < 0.0001$; virgin control vs. CAex mated, $P = 0.9115$ (n.s.); virgin control vs. CAex virgin, $P < 0.0001$; CAex mated vs. CAex virgin, $P < 0.0001$. For all comparisons between survival curves (log-rank test), the statistical significance was confirmed by a Bonferroni correction. Insects were kept as isolated virgins or isolated pairs.

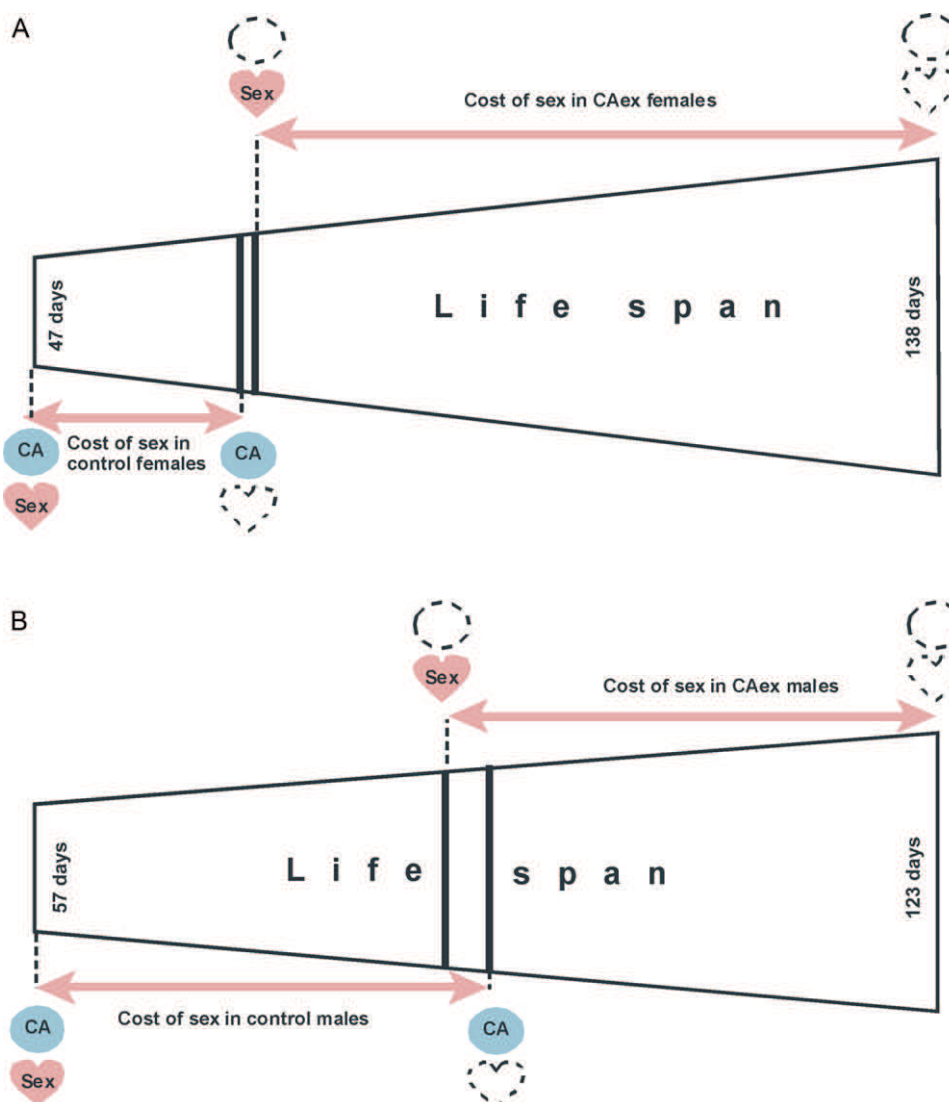


Fig. 2. Relative survival costs of sexual interactions in control and allatectomized *P. apterus* females (A) and males (B). CA – corpus allatum; CAex – CA removed. Median life spans are indicated by vertical lines. The costs of sexual interactions (horizontal lines) are expressed as proportions of the difference between maximum (both the CA and sexual partner absent) and minimum (both the CA and sexual partner present) median life spans. For other information see Fig. 1 and text.

3.1.2. Role of the insect's own CA (endogenous JH) in the survival cost of sexual interactions

Life spans of insects were compared among four groups: (a) paired insects, both sexes with the CA, (b) allatectomized insects exposed to sexual partners with the CA, (c) virgins with the CA and (d) allatectomized virgins.

If the cost of sexual interactions is independent of CA, then the increase of life span produced by both virginity and allatectomy [(d) minus (a), representing the cost of both sexual interactions and CA] should be the additive benefit on life span produced by only allatectomy [(b) minus (a), representing only the cost of CA] and only virginity [(c) minus (a), representing only the cost of sexual interactions]. Otherwise, the CA may influence the cost of sexual interactions in two ways. (1) If the cost of sexual interactions is enhanced by CA, then allatectomy should reduce (or eliminate) the cost of sexual interactions and the difference in life span between (d) and (a) should be lower than the sum of [(b) minus (a)] and [(c) minus (a)]. (2) If the cost of sexual interactions is reduced by the CA, then allatectomy should enhance the cost of sexual interactions and the difference in life span between (d) and (a) should be higher than the sum of [(b) minus (a)] and [(c) minus (a)].

In females (Fig. 1A), the increase of median life span produced by both the absence of males and allatectomy [(d) minus (a)] was

91 days (193.6%), which was, by 47 days (100%), a higher benefit on life span than the sum of effects of only virginity [(c) minus (a)] (21 days, 44.7%) and only ablating the CA [(b) minus (a)] (23 days, 48.9%). These results indicate that the longevity cost of sexual interactions (the benefit of virginity) is enhanced by the absence of the CA or, conversely, the negative effect of males on female life span is reduced by the female CA.

In males (Fig. 1B), the increase of median life span produced by both the absence of females and allatectomy [(d) minus (a)] was 66 days (115.8%), which was almost exactly the additive benefit on life span produced by only virginity [(c) minus (a)] (33 days, 57.9%) and only ablating the CA [(b) minus (a)] (30 days, 52.6%). These results indicate that sexual interactions shorten male life span independently of the male CA.

Effects of the CA and sexual interactions in the regulation of life span are summarized in Fig. 2.

3.1.3. Role of the sexual partner's CA (JH) on the survival cost of sexual interactions

The median life span of control females (with CA) exposed to allatectomized males was by 7 days (14.9%) longer relative to females exposed to control males, while the benefit on median life span produced by the absence of males was 21 days (44.7%)

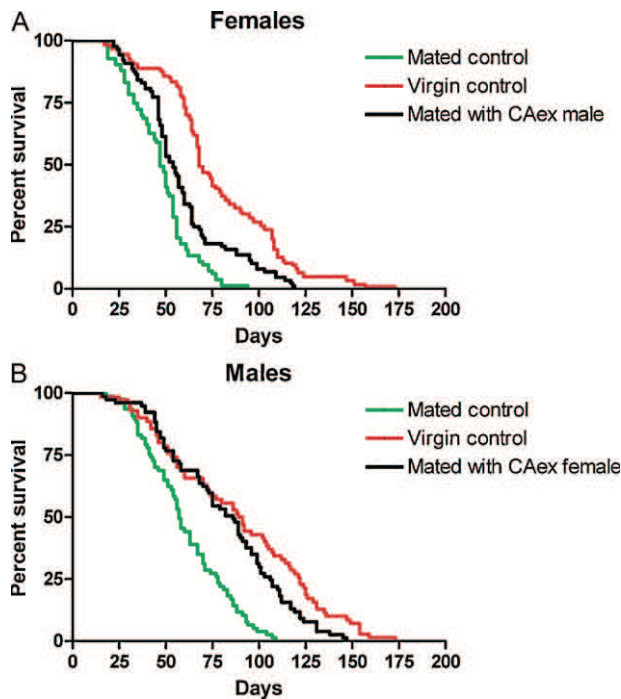


Fig. 3. Effect of the exposure to allatectomized sexual partners on the survival of control (with the corpus allatum) *P. apterus* females (A) and males (B). (A) Median life span of females mated with CAex males, 54 days ($n = 88$). Curve comparison: mated control vs. mated with CAex males, $P < 0.0003$; virgin control vs. mated with CAex males, $P < 0.0001$. (B) Median life span of males mated with CAex females, 86 days ($n = 77$). Curve comparison: mated control vs. mated with CAex males, $P < 0.0001$; virgin control vs. mated with CAex males, $P < 0.0358$. For other information see Fig. 1.

(Fig. 3A). Thus the male CA seems to be responsible for only one third of the negative effect of males on female life span.

The median life span of control males exposed to allatectomized females was by 29 days (50.9%) longer relative to males exposed to control females, which was only a slightly smaller benefit on median life span than that produced by the absence of females (33 days, 57.9%) (Fig. 3B). These results indicate that the female CA is responsible for most (87.9%) of the negative effect of females on male life span.

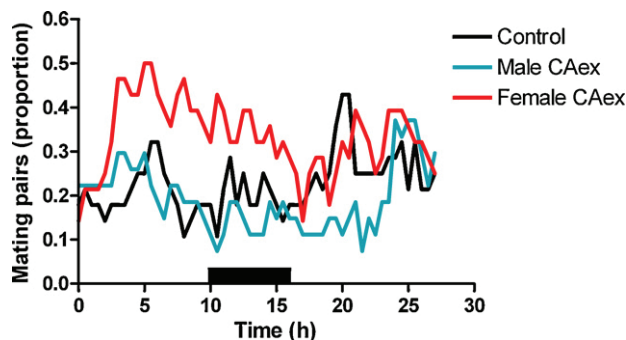


Fig. 4. Effect of allatectomy on the temporal pattern of mating proportions in *P. apterus*. Control – both partners with the corpus allatum (CA); Male CAex – male without CA; Female CAex – female without CA. Mating status was recorded every 30 min for 27 h. Time 0 = 8 h after light on. Solid column indicate the period of darkness. Two-way analysis of variance (ANOVA): The effect of time is considered not significant. The effect of allatectomy is considered extremely significant ($F = 59.94$, $DF_n = 2$, $DF_d = 108$, $P < 0.0001$); Control vs. Female CAex ($F = 56.58$, $DF_n = 1$, $DF_d = 54$, $P < 0.0001$); Control vs. Male CAex ($F = 6.85$, $DF_n = 1$, $DF_d = 54$, $P = 0.0115$). For other information see Table 1 and text.

Table 1
Effect of allatectomy on mating activity in *Pyrhocoris apterus*.

Insect pairs	<i>n</i>	Copulating pairs (%) ^a	Number of copulation bouts ^b	Duration of copulation bout (h) ^b
Normal male	28	71.4	3.1 ± 0.4 ^a	2.7 ± 0.3 ^a
Normal female				
Normal male	28	67.9	3.0 ± 0.3 ^a	4.5 ± 0.4 ^b
Allatectomized female				
Allatectomized male	27	70.4	2.7 ± 0.3 ^a	2.8 ± 0.3 ^a
Normal female				

Two-week old insects were monitored every 30 min during 27 h.

^a Pairs observed to mate at least once within 27 h.

^b Means ± SEM are given. Values with different letters are significantly different at $P < 0.01$ – 0.001 (ANOVA, Tukey's Multiple Comparison Test).

3.1.4. Mating activity

Means of 54 recordings of proportions of mating pairs (every 30 min. during 27 h) were not significantly different between control pairs (both partners with CA, 22.6 ± 0.01%) and pairs of a control female and allatectomized male (19.2 ± 0.02%), but pairs of a control male and allatectomized female showed a significantly higher mean proportion of mating pairs (33.4 ± 0.01%) than pairs of the other two groups (one-way analysis of variance, $P < 0.001$) (Fig. 4). Two-way analysis of variance confirmed the significant increase of mating activity caused by ablating the female CA ($P < 0.0001$), but also indicated a slight, but significant decrease of mating activity caused by ablating the male CA ($P < 0.05$) (Fig. 4). Proportions of mating pairs (checked every second day at arbitrarily chosen hours within photophase) did not show any clear tendency to decrease or increase (data not shown). This observation is consistent with *Zdarek's (1970)* finding that the mating activity of *P. apterus* does not essentially change throughout the insect's life.

Proportions of pairs observed to mate at least once (67.9–71.4%) and numbers of copulation bouts (2.7–3.1) recorded within 27 h were similar in the three groups, but copulation duration was significantly longer in pairs with an allatectomized female (4.5 h) compared to other two groups (2.7–2.8 h) (Table 1). Differences in the temporal patterns of mating proportions between pairs with an allatectomized female and the other two groups were evidently attributable to differences in the copulation duration. The results indicate that absence of the CA (lack of JH) has no significant effect on the mating activity of males, but females with CA ablation show shorter re-mating intervals (higher receptivity) relative to control females.

3.1.5. Egg production in virgin and mated females

Egg production was monitored in a fraction of females from Fig. 1A and Fig. 3A. Three experimental groups were examined: virgin control, mated control (paired with control male), mated with allatectomized male (Table 2). There was no significant difference in the rate of egg production (eggs/oviposition period) between virgin females and females mated with control males or between females mated with control males and females mated with allatectomized males. Total number of eggs was significantly higher in virgins than in females mated with control males. This difference is attributable to a significantly longer oviposition period in virgin females. Allatectomized females did not produce eggs.

3.2. Experiment 2 – homosexual vs. heterosexual groups

To discriminate between effects of sex-dependent and sex-independent interactions on life span, survival was compared between insects kept in homosexual or heterosexual groups.

The median life span of females kept without males (114 days) was longer than the median life span of females kept with males

Table 2
Egg production in females with intact corpus allatum.

Females	<i>n</i>	Eggs (total)	Eggs/oviposition period	Oviposition period (days)	Post-oviposition period (days)	Life span (days) ^a
Virgin control	75	433.0 ± 18.0 ^a	7.4 ± 0.2 ^a	59.9 ± 2.4 ^a	20.7 ± 2.5 ^a	80.2 ± 3.5 ^a (70.0)
Mated control	56	312.0 ± 21.8 ^b	7.8 ± 0.3 ^{ac}	41.6 ± 2.1 ^b	8.2 ± 0.9 ^b	49.7 ± 2.2 ^b (50.0)
Mated with males CAex	58	396.7 ± 21.0 ^a	8.6 ± 0.3 ^{bc}	46.1 ± 2.5 ^b	13.4 ± 1.9 ^b	59.6 ± 3.4 ^b (54.0)

Means ± SEM are given. Values with different letters are significantly different at $P < 0.05$ – 0.001 (ANOVA, Tukey's Multiple Comparison Test).

^a Numbers in parenthesis are median life spans.

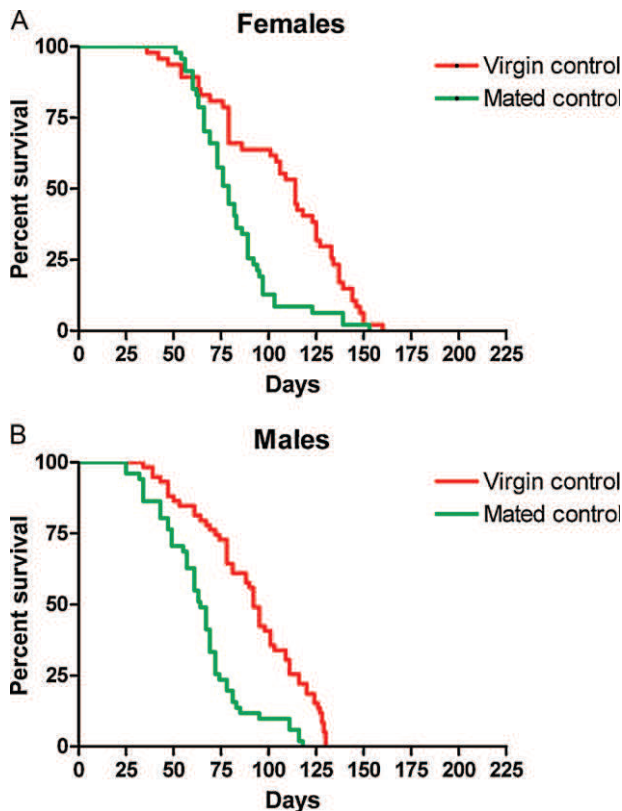


Fig. 5. Survival of heterosexual and homosexual groups of females (A) and males (B). Median life spans of females: homosexual group, 114 days ($n = 47$), heterosexual group, 79 days ($n = 47$); $P = 0.0001$. Median life spans of males: homosexual group, 92 days ($n = 59$); heterosexual group, 64 days ($n = 51$); $P < 0.0001$. Differences between survival curves were analyzed by log-rank test.

(68 days) (Fig. 5A). The absolute life spans of females originated from the colony used in this experiment were longer than life spans of females originated from the colony used in experiment 1 (Fig. 1A). However, the relative benefits of virginity on life spans were similar, 44.3% and 44.7%, respectively. These results indicate that female survival is not influenced by homosexual interactions.

The median life span of males kept without females (92 days) was by 43.8% longer than the median life span of males kept with females (64 days), suggesting an important negative effect of heterosexual interactions on male life span (Fig. 5B). In experiment 1, isolated virgin males lived 57.9% longer than males in heterosexual pairs (Fig. 1B). A slightly higher benefit of virginity on life span of isolated males compared to males kept in homosexual groups indicates a slight negative effect of homosexual interactions on male survival. In the absence of females, males were observed to court other males.

4. Discussion

We show that the CA (JH) as well as sexual interactions significantly reduce life span in both males and females of *P. apterus*. The most important outcome of our study is the disclosure

of novel and unexpected roles of the CA on longevity costs as a function of sexual interactions.

4.1. CA and survival of virgins

Consistent with earlier results (Hodkova, 2008), ablating the CA remarkably prolonged life span of virgin females of *P. apterus* (Fig. 1A). In addition we showed that a less extensive, but significant increase of life span was produced by ablating the CA in males (Fig. 1B). A higher survival cost of the CA in females relative to males might be related to the CA volume, which is nearly two times larger in females than in males (Hodkova and Socha, 2006; Socha and Hodkova, 2006).

Although it is clear that JH plays a role in the control of longevity, the way it does so remains unknown. Metabolic rate and egg production (both stimulated by JH in females) seem not to be important in determining longevity, because oxygen consumption is similar in CA-ablated and ovary-ablated females of *P. apterus* (Slama, 1964a), while ovary-ablation has no effect on life span in either intact or allatectomized females (Hodkova, 2008). Furthermore, allatectomy has no effect on oxygen consumption in males of *P. apterus* (Slama, 1964b).

A tissue that may potentially link CA-signalling and the physiological processes related to aging is the fat body. In females of many insects, including *P. apterus*, JH promotes vitellogenin synthesis in the fat body (Socha et al., 1991; Wyatt, 1997; Soller et al., 1999). Interestingly, antiDAF-16 of *C. elegans* (the homolog of dFOXO) represses vitellogenin mRNA, and the knockout of vitellogenin genes prolongs life span (Murphy et al., 2003). However, the absence of vitellogenins cannot explain life span extension by the CA ablation in males of *P. apterus*, because they do not produce vitellogenins (Socha et al., 1991). Life span of *D. melanogaster* is extended by transgenic over-expression of the insulin signalling responsive transcription factor encoded by *dfoxo* in the fat body (Ginnakou et al., 2004; Hwangbo et al., 2004). Defining interactions between JH and FOXO will be the subject for the next phase of our study of the endocrine regulation of aging in males and females of *P. apterus*.

4.2. CA and survival of females exposed to males

As expected, continuous exposure to males significantly reduced the life span of control females. Relative benefit of virginity on life span was similar in isolated virgins vs. females kept in isolated pairs (44.7%, Fig. 1A) and in females kept in homosexual vs. heterosexual groups (44.3%, Fig. 5A), although the absolute longevity were different (females originated from different laboratory colonies). An unexpected finding was that the reduction of life span (50% survival) caused by sexual interactions was more extensive in allatectomized than in control females (Fig. 2). A possibility that the presence of males stimulates the production of JH in intact females cannot be excluded. However, the benefit of allatectomy on life span was higher in virgin than in non-virgin females (Fig. 2), indicating that the negative impact of the CA on female survival was rather enhanced by virginity. Interestingly, mating as well as *D. melanogaster* sex peptide inhibit JH biosynthesis in the Mediterranean fruit fly (*Ceratitis capitata*) CA

in vitro (Moshitzky et al., 2003). These results contrast with the stimulatory effect of *D. melanogaster* sex peptide on the CA of *D. melanogaster* and a moth *Helicoverpa armigera*, where JH production by CA in vitro increases after mating in these and other species (Moshitzky et al., 1996; Shu et al., 1998; Fan et al., 1999; Holbrook et al., 2000). In any case, our results provide direct evidence that enhanced activity of the CA, including JH production, is not the likely mechanism by which sexual interactions shorten the life span in females of *P. apterus*. On the contrary, the cost of sexual interactions (the difference between survivals of virgins and non-virgins) was enhanced by the absence of the CA (Fig. 2).

The present findings imply that downstream consequences of JH effects, such as the synthesis of vitellogenins by fat body or eggs production are not causally related to the sex-dependent decrease of female life span, because these activities are absent in allatectomized females (Socha et al., 1991). This conclusion is also supported by observations that the rate of egg production is similar in mated and virgin females and the lifetime production of eggs was even higher in the latter (Table 2). Consequently, the difference in longevity between non-virgin and virgin females must be due to the costly effects of sexual interactions as such.

A cost of mating that is independent of the rate of egg production was also identified in other insect species. For example mated females have a shortened life span even if the cost of egg production is excluded in *D. melanogaster* (Fowler and Partridge, 1989; Chapman et al., 1995), *C. capitata* (Chapman et al., 1998) or the seed beetle *Callosobruchus chinensis* (Yanagi and Myiatake, 2003). In these species, manipulations of egg production by genetic interventions, X-radiation or the availability of oviposition sites revealed a separate cost of egg production, in addition to the cost of mating (Chapman et al., 1998; Sgro and Partridge, 1999; Yanagi and Myiatake, 2003). In *P. apterus*, an additional (mating-independent) cost of egg production seems unlikely, because ablating the ovary does not prolong female life span (Hodkova, 2008), but additional costs of vitellogenin synthesis and/or other reproduction related processes have not been excluded.

In *D. melanogaster*, the negative effect of mating on female longevity is caused by peptides made in male accessory glands (Acps) (Chapman et al., 1995; Wigby and Chapman, 2005). The accessory glands of allatectomized males of *P. apterus* are involuted and contain about four times lower amount of proteins than the accessory glands of control males (Socha et al., 2004). Consequently, it was likely that females exposed to allatectomized males received lower amounts of Acps, because the allatectomy of males did not enhance their mating activity (Table 1, Fig. 4). Therefore, we expected a strong negative effect of the male CA on female life span. In contrast to our expectation, females exposed to allatectomized males lived only slightly longer than females exposed to control males (Fig. 3A). These results suggest that the negative effect of males on female life span is principally independent of the male CA, leaving thus the effect of Acps on female survival open to question. Either the cost of mating depends on factors other than Acps or a low amount of Acps produced in the absence of CA is sufficient to reduce female life span. Several possible causes of a negative effect of mating on female life span have recently been discussed (De Loof, 2011). Alternatively, sexual interactions without mating may be responsible for the survival cost. Further studies should unravel the relative importance of mating and mating-independent effects of males on female survival.

Another question for future research concerns the role of Acps for reproductive success. Females do not benefit from the male CA through increased egg production (Table 2). In contrast, a critical role for JH in the regulation of male reproduction, especially through the expression of Acps, was suggested in *Tenebrio castaneum* (Parthasarathy et al., 2009).

4.3. CA and survival of males exposed to females

Continuous exposure to females significantly reduced the median life span of control males (Figs. 1B and 5B), and this effect was independent of the male's own CA (endogenous JH) (Fig. 2). Given that the growth of accessory gland size is impaired in allatectomized males (Socha et al., 2004), our results indicate that the production of accessory fluid is not important in determining longevity of mated males of *P. apterus*. In contrast, the extra production of accessory gland products were thought to be responsible for much of the cost of reduced longevity associated with high mating rates in males of the stalk-eyed flies *Cyrtodiopsis dalmani* (Pomiankowski et al., 2005).

The most striking effect in our study was that males continuously exposed to allatectomized females had significantly longer life span than males exposed to control females (Fig. 3B), although numbers of copulation bouts were similar and the copulation duration was even longer in the former (Table 1). Hence, it is not the mating itself that mediates the negative effect of the female CA on male longevity. On the other hand, the shorter genital associations (lower female receptivity) caused by the female CA allow males to spend longer time courting and that may be ultimately responsible for the cost of male survival associated with the female CA. Notably, the main cost of sexual interactions arises from courtship e.g. in males of *D. melanogaster* (Cordts and Partridge, 1996) or *Ceratitis capitata* (Papadopoulos et al., 2010).

An alternative or additional mechanism responsible for the effect of the female CA on life span of males might involve pheromones. In *C. elegans*, sugar-like chemicals (acarosides) at very low concentrations act as a sex pheromone that attract males to hermaphrodites and the same group of compounds at higher concentrations trigger young worms to enter the long-lived dauer stage (Srinivastan et al., 2008). The dauer pheromone may also increase the life span of adult worms (Kawano et al., 2005). Interestingly, exogenously applied JH analogue, fenoxycarb, suppressed sex-pheromone production and receptivity in adult females of a moth *H. armigera* (Rafaeli and Bober, 2005), but allatectomy prevented pheromone production and calling, e.g. in a moth *Pseudaletia unipuncta* (Cusson and McNeil, 1989) or a cockroach *Supella longipalpa* (Smith and Schal, 1990). Although the regulation of pheromone production by JH appears to be widespread among insects, a potential role for pheromones in connecting sexual activity with longevity has not yet been examined. In any case, the effect of a female reproductive hormone on the male life span observed in our study is presently unique and deserves further attention.

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