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Correlated Evolution of Morphological and Behavioral Traits in Response to Divergent Selection on Male Longevity

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Abstract

Males and females use different reproductive strategies to accrue their fitness leading to sex-specific selection, which in turn results in sexual dimorphism in many traits. However, the sexes share most of their genes and selection towards sex-specific fitness optima can be constrained resulting in intra-locus sexual conflict (ISC). The evolution of sexual dimorphism via sex-specific gene expression is predicted to be slow, suggesting that additive genetic variation can be maintained in the population as a result of ISC. Therefore, sex-specific selection on a trait under ISC is predicted to change the mean population trait value in both sexes via intersexual genetic correlation and affect sex-specific reproductive fitness in opposing directions. Earlier study used artificial divergent selection on male lifespan to show strong ISC over lifespan in the seed beetle, *Callosobruchus maculatus*, with upward-selected long-life males having low fitness compared to downward-selected short-life males and vice versa in females. In this study, I aimed to look at the correlated evolution of morphological (body size, body mass and body condition) and behavioural (mating success) traits that could affect male reproductive fitness and result in the population level changes in male reproductive fitness in response to selection on lifespan observed in the earlier study. I found that beetles from long-life selection lines were bigger and heavier than their short-life counterparts suggesting this difference can explain why females from long-life lines were more fecund. However, I did not find a significant difference in mating success between males from different selection regimes when competing for access to virgin females. The latter result suggests a possibility that short-life males might be better in mating with mated females or in post-copulatory sexual selection.

Introduction

Evolutionary trade-offs occur when investment in one trait constraints investment in another trait or traits. Trade-off between survival and reproductive effort is a fundamental constraint shaping the evolution of life-histories (Stearns 1992, Roff 2002). In sexually reproducing organisms, males and females are often faced with competition for reproductive access to one or several mates (Andersson 1994, Arnqvist and Rowe 2005). Most forms of reproductive investments are costly for the organism and directing resources to get a reproductive advantage takes a toll on organism's longevity (Williams 1957; Hamilton 1966; Partridge & Farquhar 1981; Alcock 1996; Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Prowse & Partridge 1997; Hunt *et al.* 2004; Bonduriansky & Brassil 2005; Paukku & Kotiaho 2005; Hunt *et al.* 2006).

There are several ways in which the reproductive effort can express itself. It can be morphological traits, including body size or weapons, which are beneficial for organisms that compete by fighting (Serrano-Meneses *et al.* 2007); large, colorful and otherwise conspicuous ornaments (Zahavi 1975, Andersson 1994), specialized genital properties such as anchoring spines (Johnstone & Keller 2000; Rönn & Hotzy 2011) or large ejaculate quantities (Fox *et al.* 1995; Paukku & Kotiaho 2005). But reproductive effort can also be in the form of behavioural characteristics such as higher effort in calling and/or displaying frequencies (Hunt *et al.* 2006; Sivalinghem *et al.* 2010) or aggressiveness (Andersson 1994). The expression of these traits increases mating success of their bearers, meaning that these traits evolved by the process of sexual selection (Darwin 1859, 1871; Andersson 1994).

The previously mentioned costs of reproduction are mostly connected to males, since they are often competing over access to the females (Parker *et al.* 1972, Bateson 1983,

Andersson 1994, Arnqvist and Rowe 2005, Serrano-Meneses *et al.* 2007), but for the females it would not be beneficial to express the same secondary sexual traits because unlike males, females usually do not increase their fitness linearly with increasing number of mates (Bateman 1948). Females often maximise their reproductive fitness by investing their resources in their offspring rather than in the number of mates (Bateman 1948; Parker *et al.* 1972). It can either be by putting effort on nursing their offspring to make sure they survive to adulthood (Clutton-Brock 1991), or by producing a lot of offspring in order to increase the probability that enough of them will survive (Fox 1993; Fox *et al.* 1995). Whichever the case, females will often have a higher reproductive success if they survive longer (Moya-Laraño & Fox 2006; Hotzy & Arnqvist 2009). It is therefore reasonable to predict that the life histories should differ greatly between the sexes (Partridge 1997; Hunt *et al.* 2004; Hunt *et al.* 2006; Bilde *et al.* 2009).

When it comes to sex-specific traits, the one major problem for organisms is that both sexes share most of the same genome. There are two types of sexual conflict that are recognised in the field. Interlocus sexual conflict occurs when a specific trait that benefits one sex decreases fitness in the other sex which results in co-evolution of antagonistic adaptations (Parker 1979; Rice 1996; Johnstone & Keller 2000; Chapman *et al.* 2003; Arnqvist and Rowe 2005). But when a shared allelic trait that is beneficial for one sex causes the other sex to be displaced from its phenotypic optimum, it causes intralocus sexual conflict (ISC) (Rice 1984; Rice & Chippindale 2001; Bonduriansky *et al.* 2008; Bonduriansky & Chenoweth 2009). A tug-of-war that can result in a situation where neither of the sexes are at their sex-specific optima (Bonduriansky and Chenoweth 2009) but instead a compromise between the two will occur. There is one way through which ISC can be resolved and that is if the expression of the gene that affects both

sexes becomes restricted to only the one sex that it benefits, resulting in sexual dimorphism (Rice & Chippindale 2001; Pischedda & Chippindale 2006; Bonduriansky & Chenoweth 2009).

There are several reasons to believe that there is large scope for ISC over lifespan and ageing (Zajitschek *et al.* 2007). The sexes represent very different environments for the expression of alleles that contribute to lifespan and ageing. Provided strong intersexual genetic correlation between the traits (Lande 1980), it is possible that sexually antagonistic selection will prevent the sexes from reaching their optimum values in life-history traits, including lifespan and demographic and reproductive ageing. Few empirical studies looked at this possibility with varying degrees of success. Thus, no ISC over ageing was found in field crickets (Zajitschek *et al.* 2007) but a more recent study on moths found evidence for ISC over lifespan (Lewis *et al.* 2011). These two studies however employed indirect quantitative genetic approach. More recently, Berg & Maklakov (in revision) used sex-specific artificial selection to show ISC over lifespan in a seed beetle *Callosobruchus maculatus*. Similar approach was utilized in the earlier study by Hunt *et al.* (2006), but in that study no support for ISC over lifespan was found. In the *C.maculatus* experiment, the authors selected directly on male lifespan in order to generate long-life and short-life lines. In the lines that were downward-selected both the males and the females lived shorter and vice versa in the upward-selected lines. This means that there is a common genetic link to longevity for both sexes and selection on male lifespan resulted in a corresponding change in female lifespan. Furthermore, short-life males had a higher relative fitness than long-life males while short-life females had a lower relative fitness than long-life females (Berg & Maklakov, in revision). These results provide strong evidence for ISC over lifespan suggesting that males live longer and the females live shorter than optimal for their reproduction in this system.

While we now know that ISC over lifespan occurs in *C.maculatus*, little is known about the mechanisms that dictate the differences in fitness between the sexes in these experimental lines. The main purpose of this study was to focus on morphological and behavioural traits that are likely to affect male reproductive success. We first wanted to assess the correlated evolution of body size, body mass and body condition in response to selection on male lifespan, because body size-related traits have been shown to affect male mating success in seed beetles (Savalli and Fox 1998; Moya-Larano *et al.* 2007; Stillwell *et al.* 2008). Secondly, we wanted to estimate the pre-copulatory mating success of males from different selection regimes. To achieve this, we tested mating success of long-life and short-life males directly competing against each other for the access to standardised background females from the source population.

Materials and Methods

The beetle populations were derived from the lab population, SI USA, from the University of Kentucky, USA. It was originally collected in south India from infested mung beans (*Vigna radiata*) in 1979 and later brought to Uppsala University from the laboratory of C. W. Fox. The beetle populations were constantly kept in 1000ml glass jars with dried beans. The jars with the beetles were, prior to the experiment, kept in climate chambers at a constant temperature of 30°C and at an air humidity of 50%. The chambers had a programmed light/dark cycle of 14 hours light and 10 hours dark. This environment closely represents the one from the beetle's natural habitat (Fox *et al.* 2003) and this is the setting that was used during the whole experiment. When a female is fertilized, she will soon start laying her eggs on beans. The larvae will hatch into the bean and start eating it from the inside while keeping the outer bean wall intact until it is ready to

come out as a full grown adult. The first beetles will start to emerge 21 days after the eggs are laid and the peak of hatching is somewhere around day 23-26. A number of different kinds of dried beans can be used for breeding *C.maculatus* (Stillwell *et al.* 2007) but we decided to use the mung bean. Firstly, it is the native larval host of *C.maculatus* and secondly, because of the small size of the mung bean, only one beetle hatches from it and it is therefore easy to generate virgin beetles by isolating single beans. The beetles can accumulate all of the resources needed for survival and reproduction during their larvae stage (Fox *et al.* 1993) and can therefore be kept aphagous, which means that they will not require any additional water or food supplies (Fox *et al.* 2006).

Selection experiment

The first part of the experiment involved divergent artificial selection on male lifespan. I used the lines previously selected upward and downward on male lifespan by E. Berg (Berg & Maklakov, in revision). Originally, there was a 30% difference in male lifespan in these lines, but after several generations without selection, this difference was reduced to 8%. The lines were kept in eight jars, as well as one jar with a population that had never been targeted for selection that we called the “base” population and this is essentially the SI USA population. Every 25 days, the beetles from the base population were moved to a new jar with new beans in order to give them opportunity to lay their eggs. Four of the populations had been targeted for downward-selection on lifespan, called the short lines, and the other four had been targeted for upward-selection on lifespan, called the long lines. In order to acquire a strong enough effect, the first selection was done on 100 pairs of beetles for every line; 800 pairs (plus five reserve pairs for every line) in total. The reason for this broad sample size is that it increased the probability of acquiring more beetles that had a longer lifespan (for the upward selections) or a shorter lifespan (for the

downward selections) than the rest of the population. 720 beans with visible “windows” (a spot on the bean that indicates that a larvae will soon be hatching) from each line were put in “virgin chambers”: aerated trays with 48 transparent and coverable wells, about 1 cm³ that can be marked on the lid in order to track the date of birth (5760 beans in total). Fertilized beans were then kept in the climate chambers. Prior to beetle hatching, 60mm petri dishes were prepared with 5.18g (+0.05g) of organic mung beans. This weight was founded on the average of six trials, in which 75 beans from a common source were counted and weighed. 840 petri dishes were prepared through carefully weighing the beans and every dish was labelled with a number 1-100 as well as a marked with the colour of population and direction of selection (long or short). Another 840 petri dishes were prepared with a visual approximate amount of 75 beans of about a full teaspoon. These were labelled identically to the first set, but with an extra mark to be able to tell the two sets apart. As the hatching peaked, one male and one female from the same line were randomly selected and put together in a Petri dish from the first set prepared with a carefully weighed amount of beans. Only males that had hatched within 48 hours were used, either hatched the same day or the day before. This time span was needed to be able to gather enough males. The females we chose mainly hatched on the day prior to pairing. The beetles were allowed to mate and lay eggs for about 30h. They were then moved to the second set of Petri dishes containing an approximate amount of 75 beans. All of the dishes from the first set with fertilized eggs were stored aside in the climate chambers. After the beetle pairs were transferred to the second set of petri dishes, all the females were moved one petri dish over. This was done within each line so that every male would be exposed to a new female. The reason for this was to ensure that male lifespan was not affected by differing female behaviour. The next day all females were moved one more time to further reduce this effect. The following day, four days

after initial pairing, all females are removed and dead males were recorded. If any males died prior to this point, they were switched for backup pairs. The males were checked daily and the date of death was recorded. Once all males had died, the results were analyzed and 720 beans with fertilized eggs from the top 15 males from each long line and bottom 15 from each short line were put in virgin chambers for continued selection on the next generation. The following generations, when the artificial selection was repeated, it was done so with 60 pairs per line instead of 100. Hereafter, when all of the males and females that would be used for the selection had been transferred to their petri dishes, the remaining beetles in the virgin chambers were transferred to new jars filled with 250g of mung beans in order to create fresh backup lines in case anything would go wrong with the experiment like knocking a full tray of petri dishes over. A protocol was created in order to avoid sibling mating during the following generations of selection. Inbreeding has proven to have an effect on male lifespan (Bilde & Maklakov 2009). The artificial selection was carried out for four generations.

Body mass, body size and body condition

The first thing we measured was body mass for each line, a sample of ten males and ten females was randomly picked out from every population. Only beetles that had hatched the same day were chosen so that they would not have lost too much of their allocated resources. They were then weighed individually and the lines and sexes were compared to each other (Fig3). The second thing we measured was the elytra length of the beetles. These are the chitin shields covering their hindwings (which in turn are used for flight). The elytra length was measured using a stereoscope and computerized coordinate system. The mean of elytra measurements was then analyzed together with the beetle mass in a regression plot.

Behavioural Study

In this part of the study, we wanted to test the long line versus the short line in a competitive mating situation. The experiment was carried out in a climate room, in which the temperature was 30°C and the humidity was 50%, same as in the climate chambers. Ten virgin males from every short line were individually put together with one of ten virgin males from every long line in 35mm petri dishes together with a virgin female from the SI USA base population, for a total of 160 different trials. In order to tell the males apart, one of them was marked with water based non-toxic face paint that was allowed to dry for a few minutes. Face paint has been used in previous experiments with *C.maculatus* (Maklakov and Bonduriansky 2009) and it has not been detected that it affects the male's ability to behave naturally. However, in order to account for possible effect by the paint, for every ten trials between a short and a long line, the short line male was marked in five trials and the long line male was marked the other five trials.

Sometimes a female would not be receptive to the males mating attempts. In such cases the female was switched in for a new one after 5-10 minutes. To analyse the effect of the selection on male mating success, we used a general linear mixed model (GLMM) with binomial error distribution and logit link function. Here, the response variable was defined as the mating order (which beetle mated first) with 1 if short-life males mated first and 0 if long-life males mated first. We controlled for the effect of marking by including it as a fixed effect in the model.

Population was fitted as two selection-specific random effects. Thus, if the mean of the marking effect would deviate from 50%, it would indicate significant effect of the selection on mating order. In order to obtain error bars around this estimate, we performed a parametric bootstrap following Gelman and Hills (2007).

Results

By the time of the behavioural study (F4), the mean difference in lifespan between the lines was at approximately 40%.

In all the generations there was a significant difference between the lines (Fig 1). A fit model analysis in JMP with replicate set as a random effect, generation F0 had $p=0.046$, generation F1 had $p<0.0001$, generation F2 had $p=0.0037$ and generation F4 had $p=0.0015$.

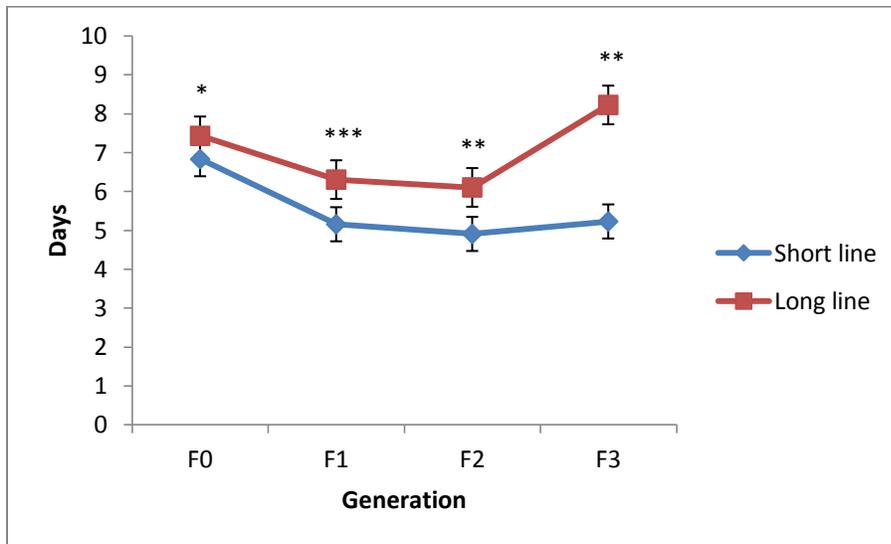


Figure 1. Mean male lifespan \pm standard errors in days for every generation of the selection experiment.

Beetle body mass analysis showed a significant difference between the lines $p=0.0077$ as well as between sexes $p<0.0001$ but not line*sex (crossed) $p=0.1183$ (Fig 2), (Table 1).

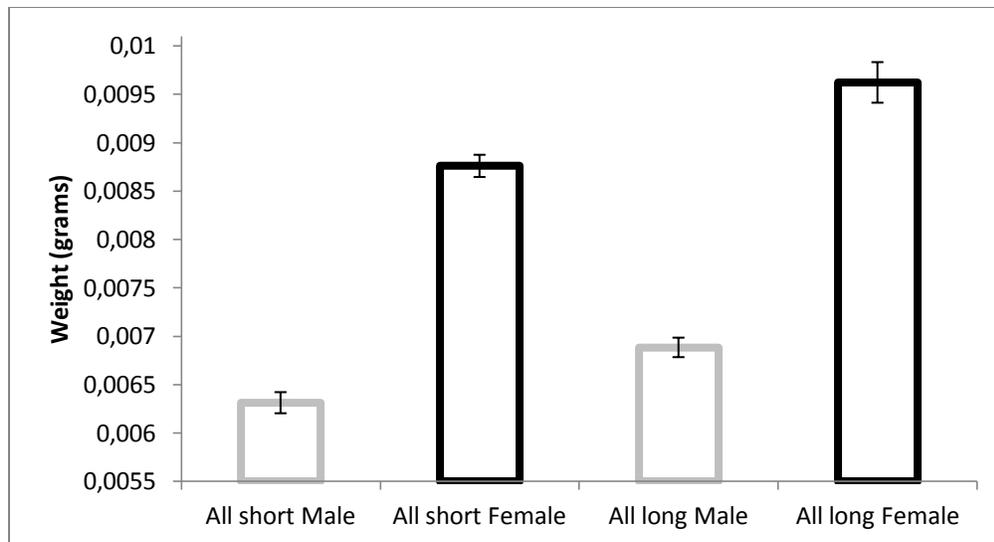


Figure 2. Mean male and female body mass \pm standard errors in short-life and long-life selection lines.

Table 1. Linear mixed model of responses to beetle body mass. The fixed effect parameters; Sex, Selection and Sex crossed with selection (Sex*Selection) are represented with degrees of freedom (Df), F-value and P-value. The random effect parameters; Population nested with Selection (Pop.[Selection]) and Population crossed with Sex nested with Selection (Pop.*Sex[Selection]) are represented with Variance Components (Var. Comp.) and Confidence Intervals (CI) 95% Lower – 95% Upper.

Parameter	Df	F/Var. Comp.	P/CI
Sex	1, 6	1054.19	<.0001
Selection	1, 6	15.45	0.0077
Sex*Selection	1, 6	3.32	0.1183
Pop.[Selection]		5.38×10^{-8}	$-2.29 \times 10^{-8} - 1.31 \times 10^{-7}$
Pop.*Sex[Selection]		-2.26×10^{-8}	$-4.28 \times 10^{-8} - 1.76 \times 10^{-8}$

Beetle size analysis showed a significant difference between the lines $p=0.0013$ as well as between sexes $p<0.0001$ but not line*sex (crossed) $p=0.8413$ (Fig 3) (Table 2).

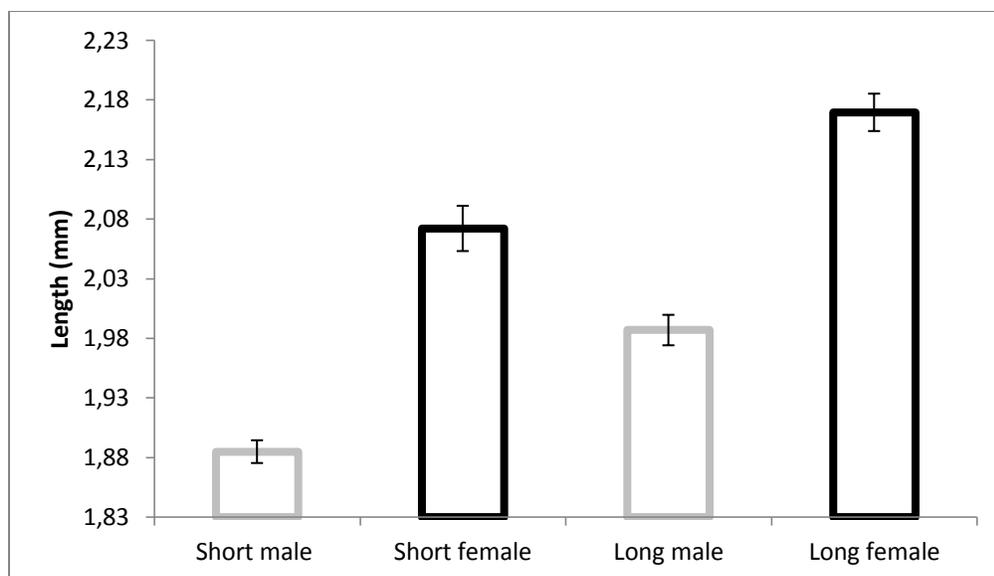


Figure 3. Mean male and female body size \pm standard errors in short-life and long-life selection lines.

Table 2. Linear mixed model of responses to beetle body size. The fixed effect parameters; Sex, Selection and Sex crossed with selection (Sex*Selection) are represented with degrees of freedom (Df), F-value and P-value. The random effect parameters; Population nested with Selection (Pop.[Selection]) and Population crossed with Sex nested with Selection (Pop.*Sex[Selection]) are represented with Variance Components (Var. Comp.) and Confidence Intervals (CI) 95% Lower – 95% Upper.

Parameter	Df	F/Var. Comp.	P/CI
Sex	1, 6	278.15	<.0001
Selection	1, 6	32.33	0.0013
Sex*Selection	1, 6	0.04	0.8413
Pop.[Selection]		3.69×10^{-4}	$-3.8 \times 10^{-4} - 1.12 \times 10^{-3}$
Pop.*Sex[Selection]		1.02×10^{-4}	$-4.62 \times 10^{-4} - 6.66 \times 10^{-4}$

Beetle condition analysis of responses to body mass showed significance in selection $p=0.0014$, body size $p<0.0001$, selection*body size (crossed) $p=0.038$ and sex*selection*body size (crossed) $p<0.0001$ (Table 3), (Fig 4) and (Fig 5).

Table 3. Linear mixed model of beetle condition with response to body mass including body size as a covariate. The fixed effect parameters; Sex, Selection, Sex crossed with selection (Sex*Selection), Body size, Sex crossed with Body size (Sex*Body size), Selection crossed with Body size (Selection*Body size) and Sex crossed with selection and body size (Sex*Selection*Body size) are represented with degrees of freedom (Df), F-value and P-value. The random effect parameters; Population nested with Selection (Pop.[Selection]), Population crossed with Sex nested with Selection (Pop.*Sex[Selection]), Population crossed with Body size nested with selection (Pop.*Body size[Selection]) and Population crossed with sex and body size nested with selection (Pop.*Sex*Body size[Selection]) are represented with Variance Components (Var. Comp.) and Confidence Intervals (CI) 95% Lower – 95% Upper.

Parameter	Df	F/Var. Comp.	P/CI
Sex	1, 1	0	1.0
Selection	1, 5.59	33.99	0.0014
Sex*Selection	1, 1	0	1.0
Body size	1, 12.32	581.26	<0.0001
Sex*Body size	1, 147.3	0.52	0.47
Selection*Body size	1, 12.32	5.4	0.038
Sex*Selection*Body size	1, 147.3	18.89	<0.0001
Pop.[Selection]		5.81×10^{-8}	$-2.7 \times 10^{-8} - 1.43 \times 10^{-7}$
Pop.*Sex[Selection]		-2.3×10^{-8}	$-3.61 \times 10^{-8} - -9.95 \times 10^{-9}$
Pop.*Body size[Selection]		-4.55×10^{-7}	$-2.1 \times 10^{-6} - 1.19 \times 10^{-6}$
Pop.*Sex*Body size[Selection]		3.22×10^{-6}	$2.44 \times 10^{-6} - 3.99 \times 10^{-6}$

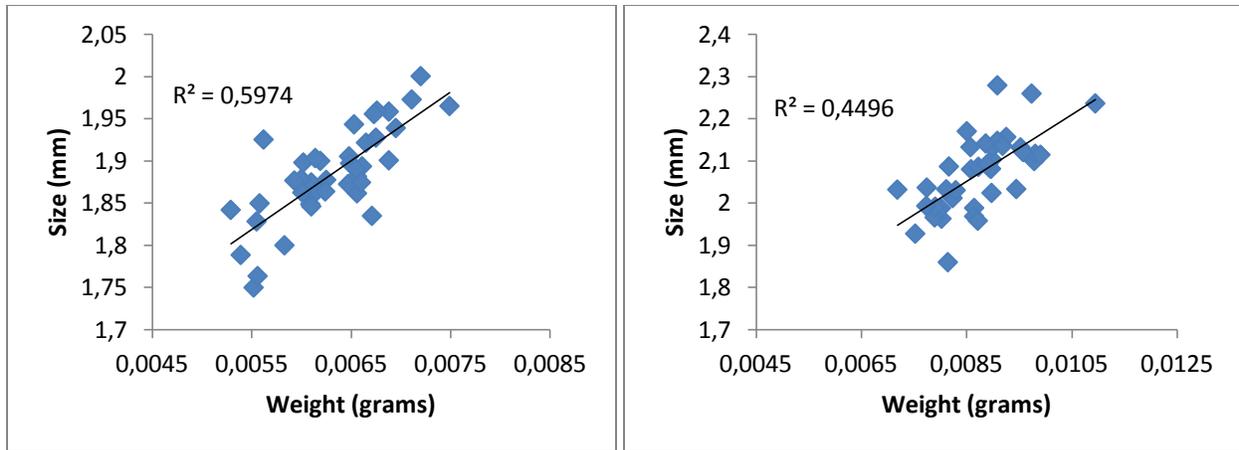


Figure 4. Beetle size versus mass for short-life males (left) and females (right) with a regression line and R^2 -value.

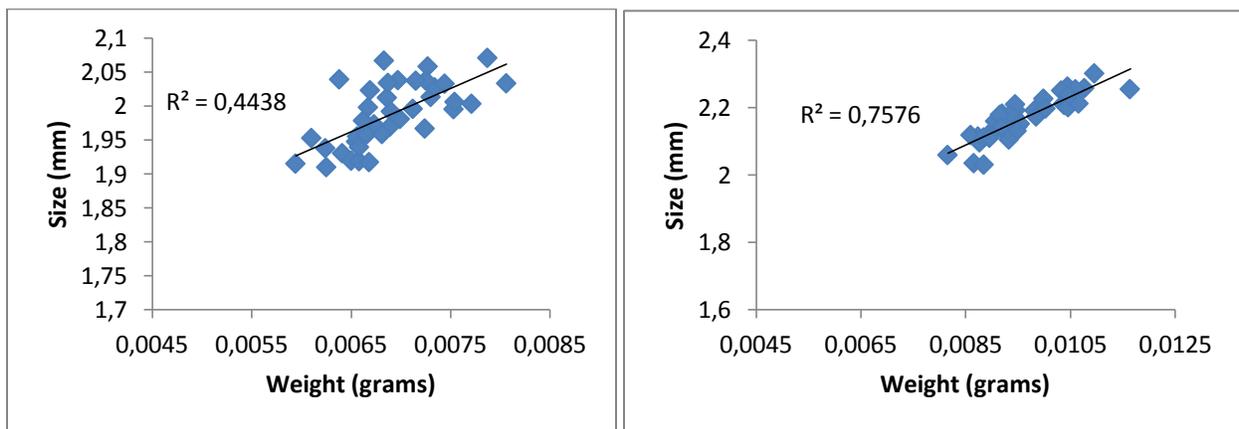


Figure 5. Beetle size versus mass for long-life males (left) and females (right) with a regression line and R^2 -value.

There was a significant effect of marking on mating order but no significant effect of the selection (the proportion of short-life males being first: estimate = $0.578 \pm$ parametric bootstrap quantiles $0.494 - 0.654$). This result indicates a trend towards higher mating success of short-life males, because the lower quantile is very close to 0.5. Larger sample size might be necessary to show the significance.

Discussion

By resuming the artificial selection on these lines, we managed to surpass the mean difference, which was previously achieved in five generations (Berg and Maklakov, in revision), in only four generations. It is interesting to notice that in both cases, our mean lifespan curves closely follow the same pattern (Berg & Maklakov, in revision). During the first three generations of selection all life spans were going down, even in the long lines. But by the fourth generation, long line lifespan takes off upwards, while the short line lifespan does so only slightly. It takes longer time before any effect can be seen in upwards selection and this is consistent with earlier attempts at artificial selection on lifespan (Hunt *et al.* 2006), suggesting that there is a greater heritability for shorter life than longer.

Looking at the morphological differences between the lines, the short line beetles were significantly lighter and smaller compared to the long line beetles. It is possible that one of the main reasons for the difference in longevity between the lines is that the short lived beetles have a limit in how much resources they can allocate to somatic maintenance. Seed beetles in this population are maintained as capital breeders that acquire all of their resources at larval stage and smaller size means less overall resources. But when we look at the condition analysis, another trait comes into light. The analysis shows that females in long lines and males in short lines have steeper slope of regression of body mass on body size. This result suggests that selection on condition may potentially be stronger on females in long lines and males in short lines and that large long-life females and short-life males allocate disproportionately larger amount of resources to body mass. It makes sense because long-life females can either survive for longer or produce more eggs. But why do short-life males allocate more resources to maintain relatively large body mass? We cannot know for certain at this point, but perhaps this indicates that large

short-life males have more resources to allocate to their ejaculates (which can be as large as 10% of total body mass of *C. maculatus* males). If so, short-life males can be more successful in sperm competition. This is an interesting line for future research.

While the effect of body size on mating success in beetles remains contradictory, it has earlier been discovered that *C. maculatus* female lifetime reproductive success increases if they mate with larger males (Fox *et al.* 1995b), mainly because a larger male produces larger volumes of ejaculate fluids that serve as a nourishment for females (Moya-Laraño & Fox 2006; Hotzy & Arnqvist 2009). Nonetheless, larger males do not necessarily enjoy a higher reproductive success and there are a lot of examples of sexual size dimorphism across the insect taxa that are biased towards small males (Blackenhorn 2000). Moreover, small male beetles have been shown to be more successful in scramble competition (Moya-Laraño *et al.* 2007).

The mating success experiment showed no significant effect for quicker mating in short line males but it does indicate a trend, significance was reached when using a one-tailed test in the analysis. This suggests that even though success in scramble competition for virgin females does not seem to be the main reason behind the higher reproductive success in short line males, it should not be completely ignored as it might still have a potential effect.

There are of course a number of other factors linked to reproductive fitness, which could make a difference in reproductive success between long line and short-life males. For example, genital spines function by anchoring the male to the insides of the female and may allow the male to copulate longer and transfer more sperm before being kicked off by the female (Rönn *et al.* 2007; Rönn & Hotzy 2011). Perhaps there are differences in how efficient these genital spines function between short-lived and long-lived males, copulation duration is by itself an important

factor in reproduction (Edvardsson & Canal 2006) as proteins in the male's ejaculate may affect male success in sperm competition (Chapman *et al.* 1995, Arnqvist and Rowe 2005)

This study followed the experimental procedures of previous work conducted with this species. This will decrease the appearance of inconsistent results when comparing similar studies to each other (Zwaan 1999). If this experimental consistency will be kept at a common level by many researchers, it would mean that studies would become much more comparable to previous results and will also be of more use for further studies in the same field of research.

To summarize, I successfully selected on male lifespan to produce lines with 40% difference in this trait. The effect size increased steadily across three generations of selection, although significance did not increase due to increased variance between the experimental populations within the selection treatments. There was no significant relationship between short lifespan and higher mating success with virgin females in males but short-life males were significantly smaller and lighter than long-life males. Long-life females were significantly larger and heavier than short-life females. However, compared to their body size, large short-life males invested relatively more in body mass compared to long-life males while large long-life females invested relatively more into their body mass compared to short-life females. These results tentatively suggest stronger indirect selection on condition in short-life males and long-life females. While there is still no answer as to how short-life males achieve higher reproductive fitness, this study suggests some promising future directions. More research is necessary to investigate the roles of male mating success with mated females and post-copulatory sexual selection (sperm competition) in generating high reproductive success of short-life males. Finally, physiological traits such as locomotory activity, metabolic rate and metabolic efficiency should be investigated.

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