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DIPLOMARBEIT

Titel der Diplomarbeit

The Functional Significance of Genital Spines in Males of the Seed Beetle Callosobruchus maculatus

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Abstract

Male genitalia that bear spines, hooks and other conspicuous projections that cause harm to females during mating are common among animals. Yet, our knowledge about the function of such harmful genital traits is limited. In the seed beetle *Callosobruchus maculatus*, males possess genital spines that injure females during copulation. These spines aid males in sperm competition, but their exact function is unknown. Here, I explored the functional significance of the genital spines in *C. maculatus* by assessing two potential sperm competition advantages. (1) I investigated, if the genital spines increase the dispersal of accessory seminal substances throughout the females' body. (2) I explored whether the spines function as an anchor during copulation to prevent females from terminating the copulations earlier than beneficial for males. To test these hypotheses, I compared the mating performance of long and short spined males, which I generated experimentally in two complimentary ways. First, I used artificial selection to create long and short spined lines. Second, I shortened genital spines using micro laser ablation. Since copulation duration was not related to spine length, my results did not support the anchor hypothesis. However, my results showed that the dispersal of accessory seminal substances throughout the females' body increased with increasing spine length, and that long spined males achieved advantages in sperm competition. This provides the first evidence that genital spines increase male fertilization success by perforating female tissues, through which accessory seminal substances can pass more efficiently. Moreover, my results illustrate one way in which sexual selection can shape genital morphology.

1. Introduction

Male genitalia that are equipped with spines, hooks or other appendages are common among animals – especially in insects (Crudgington & Siva-Jothy 2000; Blanckenhorn et al. 2002; Stockley 2002; Arnqvist & Rowe 2005; Kamimura 2010). Such male genitalic traits can be harmful and may injure females during copulation (Merritt 1989; Crudgington & Siva-Jothy 2000; Stutt & Siva-Jothy 2001; Blanckenhorn et al. 2002; Stockley 2002; Kamimura 2007). For instance in *Drosophila melanogaster* the dorsal branches of the basal processes of the male adeagus pierce the female reproductive tract and lead to melanized scars within the females (Kamimura 2010). In bed bugs, males have even evolved specialized intromittent organs for extra-genital insemination and pierce the abdominal body wall of the females to force them into copulations, a mechanism called traumatic insemination (Stutt & Siva-Jothy 2001). In the seed beetle *Callosobruchus maculatus*, males are armed with conspicuous genital spines that injure the females' reproductive tract during copulation. Even mating a single time can leave melanized scars within the females' reproductive tract (Crudgington & Siva-Jothy 2000; Rönn et al. 2007). The internal wounds are costly to the females and can shorten their lifespan (Crudgington & Siva-Jothy 2000; Rönn et al. 2006). However, in *C. maculatus* – such as in many other cases – the specific function of the harmful genital spines is still unknown. Furthermore, it is still under discussion, how males can evolve and maintain such harmful genitalia despite the fact that they are harmful to their mates. To contribute to this discussion and to gain more insights into the potential functions of harmful genitalia, I explored the functional significance of the genital spines in *C. maculatus* in my diploma thesis. In the following sections I will first introduce the potential mechanisms that drive genital evolution (section 1.1.) and explain sexual conflict and the theories that can cause the evolution and maintenance of harmful genitalia (section 1.2.). Then I will write about the role of accessory seminal substances in sexual selection and genital evolution (section 1.3.) and mention the multiple functions of male genitalia (section 1.4.). I will finish the introduction by describing the general biology of *C. maculatus*, the potential functions of the genital spines in that species (section 1.5.) and the goal of my thesis (section 1.6.).

1.1. Rapid Genital Evolution

In species with internal fertilization genital morphology is extremely diverse and evolves rapidly. Even across closely related and morphologically very similar species genitalia can vary enormously (Hosken & Stockley 2004; Simmons et al. 2009) and in many insect taxa one actually has to inspect the genitalia to be able to identify to which species the specimen belongs to (Klotz 1970 in Gilligan & Wenzel 2008; Eberhard 1985; Simonsen 2006). There are several theories that could explain the diversity of genital morphology and rapid genital evolution. Originally, the lock and key hypothesis was proposed, where female and male genitalia were assumed to function like lock and key to avoid hybridization (Dufour 1844 in Shapiro & Porter 1989). Only the male of the same species was expected to have the right key for the females' lock, a mechanism that potentially could lead to variance in genital morphology and speciation. However, there is little evidence for the lock and key system. Instead most studies disagree with this hypothesis (Porter & Shapiro 1990; Arnqvist et al. 1997; Arnqvist 1998; Eberhard 2001; Gilligan & Wenzel 2008). Another theory, the pleiotropy hypothesis, explains the huge variation in genital morphology by selection of other traits that accidentally lead to genital modifications due to genetic correlations (Mayr 1963). Yet, this theory cannot explain why genitalia are much more variable than other morphological traits (or cannot explain why genital variance is so disproportional high). Furthermore, under the pleiotropy hypothesis selection should not act on genitalic traits. Yet, it has been shown in many species that selection acts on genitalic traits and that genital morphology affects the fitness of their bearer (Arnqvist 1998; Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; House & Simmons 2003; Takami 2003; Arnqvist & Rowe 2005; Wenninger & Averill 2006; Hotzy & Arnqvist 2009). Additionally, it is hard to imagine how harmful male genitalia could evolve and persist under pleiotropy, since such genitalic traits induce costs in females. However, pleiotropy could be partly responsible for genital evolution but cannot explain the extreme diversity of genital morphology (Hosken & Stockley 2004). More recently, sexual selection has entered the spot light of genital evolution and is getting more and more attention since the number of studies detecting correlations between male genital morphology and fertilization success is growing (Arnqvist 1998; Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; House & Simmons 2003; Takami 2003; Arnqvist & Rowe 2005; Wenninger & Averill 2006; Hotzy & Arnqvist 2009). Sexual selection mechanisms that were suggested to drive

genital evolution are (1) cryptic female choice, (2) sperm competition and (3) sexual conflict (Arnqvist 1997; Hosken & Stockley 2004), whereat it is under discussion, whether sexual conflict should be considered a force that drives genital evolution or not, since sexual conflict can also be seen as one way to generate cryptic female choice and as a product of sperm competition (Chapman et al. 2003; Arnqvist 2004; Arnqvist & Rowe 2005; Jagadeeshan & Singh 2006). Thus, starting from now, I will only refer to sperm competition and cryptic female choice when it comes to the mechanisms that drive genital evolution.

Cryptic female choice (Eberhard 1996) – which is defined as any female trait that biases male fertilization success towards certain males – can trigger male genital evolution by two mechanisms: good genes and sexy sons (Hosken & Stockley 2004). This could occur if male genitalia morphology would reflect male quality and females could use genital traits to choose males that produce attractive sons or/and inherit good genes. For example, females could potentially detect the quality of a male by his stimulation ability and choose to fertilize their eggs with the sperm of the best stimulating males (Eberhard 1985; Eberhard 2001; Hosken & Stockley 2004).

Male-male conflict – in the form of sperm competition – also plays a role in rapid genital evolution. Sperm competition arises, when the sperm of at least two males compete for fertilization of an ovum, which is very common among animals (Parker 1970; Birkhead & Møller 1998) since strict monogamy is very rare (Birkhead & Møller 1998; Arnqvist & Nilsson 2000; Griffith et al. 2008). In other words, as soon as females mate to more than one male during one reproductive period, selection will favor morphologic and physiologic traits of male genitalia that are beneficial in sperm competition. Males may outcompete rival males in many different ways. For instance they can a) transfer larger ejaculates (Gage & Baker 1991; Gage 1991; Parker 1998), b) remove the sperm of rival males (Waage 1979; von Helversen & von Helversen 1991; Tsuchiya & Hayashi 2008), c) transfer accessory seminal substances that serve in sperm competition (for accessory seminal substances see section 1.3. page 8), d) reduce female remating behavior or manipulate the reproductive behavior of the female in another way that is beneficial to the focal male (Chen et al. 1988; Chapman & Partridge 1996; Simmons 2001). Thus, sperm competition creates a variety of potential genitalic functions that may accelerate genital

evolution. Furthermore, different components of the adeagus may serve different purposes to aid different sperm competition functions (Hosken & Stockley 2004).

However, the relative importance of sperm competition and cryptic female choice in genital evolution is still unknown. In general, it is important to say that these mechanisms are not mutually exclusive and that combinations of them may lead to genital divergence in particular cases (Hosken & Stockley 2004). Furthermore, it is hard to distinguish between sperm competition and cryptic female choice, since it is often hard to determine whether a male's fertilization success is the result of the male's sperm competition ability or of cryptic female choice (Birkhead 2000; Kempenaers et al. 2000; Andersson & Simmons 2006).

1.2. Sexual Conflict and Harmful Male Traits

Sexual conflict – i.e. male-female conflict over reproduction and fertilization – is very common among animals and occurs since males and females play different roles in reproduction and maximize their fitness in different ways (Parker 1979; Arnqvist & Rowe 2005). Thus, it can be beneficial for one sex to enhance its own fitness by harming the other sex, as long as the gain in fitness outweighs the costs caused to the partner. In this way, traits that increase the fitness of one sex can be favored by sexual selection even though they are harmful and costly to the other sex (Fowler & Partridge 1989; Crudgington & Siva-Jothy 2000; Rönn et al. 2006). Sexual conflict can range from cannibalistic female spiders that consume their mates (Schneider & Lubin 1998; Schneider & Elgar 2001; Fromhage & Schneider 2005), over traumatic insemination (Stutt & Siva-Jothy 2001; Kamimura 2007; Hosken & Price 2009), to infanticidal males that kill a females' offspring to accelerate her next receptivity (Hrdy 1979; Grinnell & McComb 1996; Schneider & Lubin 1997). Thus, the variety of sexual conflict is huge. I will focus on harmful male traits that induce costs in females due to copulation, since this is the kind of sexual conflict that occurs in *C. maculatus*. During copulation, males can cause costs in females either by inflicting direct physical injuries (e.g. by harmful genital spines) or by transferring harmful accessory seminal substances (for accessory seminal substances see next section). However, it is still uncertain by which mechanisms such harmful male traits may evolve and persist (Morrow et al. 2003; Edvardsson & Tregenza

2005; Teuschl et al. 2007). Two theories could explain this – the adaptive harm hypothesis and the pleiotropic harm hypothesis (which is not to be confused with the pleiotropy hypothesis of genital evolution mentioned before). Under the adaptive harm hypothesis, Johnstone & Keller (2000) suggested that the harm itself can be beneficial. They assumed that the inflicted harm *per se* could serve males if trauma owing to mating could prohibit or decelerate females' remating behavior so that future sperm competition could be avoided or at least lowered. Furthermore, harm would be adaptive if the perceived injuries endanger the survival of the females and if this would lead to a terminal reproduction effect, i.e. the females would respond to the harm by investing more into their current reproduction (Morrow et al. 2003). Yet, many studies trying to prove the adaptive harm theory did not find the expected effects (Morrow et al. 2003; Edvardsson & Tregenza 2005; Eady et al. 2007; Teuschl et al. 2007). Instead, there is growing evidence that harmful traits, such as spiny genitalia, evolved for other reasons than causing injuries *per se* (Morrow et al. 2003; Edvardsson & Tregenza 2005; Eady et al. 2007; Y Teuschl et al. 2007; Hotzy & Arnqvist 2009). This matches the pleiotropic harm hypothesis. The pleiotropic harm hypothesis states that harmfulness of male genitalia is a negative side effect of the actual function of the harmful trait (Parker 1979; Morrow et al. 2003; Parker 2006). Pleiotropic harm, for instance, would be caused if genital armature would serve sperm competition purposes and injuries caused in females would be a by-product of that sperm competition function. In *C. maculatus* and some other insects, this seems to be the case (Kamimura 2007; Tsuchiya & Hayashi 2008; Hotzy & Arnqvist 2009).

1.3. Accessory Seminal Substances

Something else that is shaped by sexual selection and that plays an important role in sperm competition and sexual conflict are accessory seminal substances. Accessory seminal substances are peptides or proteins that are produced in the accessory reproductive glands of males (Chen 1984; Arnqvist & Rowe 2005). They are transferred into the females' body during mating and can act as neuropeptides within the females (Arnqvist & Rowe 2005). Such seminal substances may manipulate females' reproductive behavior in a way that benefits the male (Chapman et al. 1995; Gems & Riddle 1996). For example, they can enhance oviposition rate or reduce females' receptivity to subsequent

matings (Chen et al. 1988; Chapman & Partridge 1996; Simmons 2001). In many cases, this carries costs for females if the reproductive behavior of the female is modulated in a way that is suboptimal for the female. Furthermore, accessory seminal substances can actually be toxic to females (Das et al. 1980; Chapman et al. 1995; Rice 1996). But accessory seminal substances are not always used to manipulate female behavior or to serve intersexual conflict. They can also target intrasexual competition and serve in sperm competition by destroying, removing, repositioning or incapacitating stored sperm of previous mates (Radwan & Witaliński 1991; Clark et al. 1995; Chapman & Partridge 1996). Potentially, accessory seminal substances could lead to the need of additional functions of male genitalia since genital morphology could serve to increase the uptake and dispersal of accessory seminal substances within the females' body (Eberhard 1998). Hence, accessory seminal substances could provide a further engine for genital evolution.

1.4. Pre- and Postcopulatory Functions of Male Genitalia

Evolution and function of male genital structures go hand in hand. Male genital morphology is of course shaped by its functions and as indicated by the diversity of mechanisms that contribute to rapid genital evolution, male genitalia do not only serve to transfer sperm but have multiple functions. In general, the functions of male genitalia can be divided into precopulatory and postcopulatory functions. Pre copulation male genitalia may serve to gain matings (Bertin & Fairbairn 2005; Polak & Rashed 2010). This is frequently linked to sexual conflict since males and females often have different optimal mating rates (Arnqvist 1989; Arnqvist 1992) but can be linked to intrasexual competition as well (Bertin & Fairbairn 2005). Postcopulatory, male genitalia may function to increase the males' fertilization success by possessing traits that serve in sperm competition, manipulate cryptic female choice and/or aid in sexual conflict over fertilization.

1.5. Callosobruchus maculatus

C. maculatus are seed beetles that are pests of dried legumes (Raja et al. 2001; Tuda et al. 2006). During the last decades, they have become a model organism for sperm competition, male-female interactions, sexual conflict and sexually antagonistic

coevolution (Eady 1994a; Eady 1994b; Edvardsson & Tregenza 2005; Edvardsson 2007; Rönn et al. 2007). Females of *C. maculatus* mate multiply and store sperm of several males in their spermatheca giving rise to sperm competition. Immediately after mating, the females start cementing their eggs either directly onto the surface of host seeds or on their pods. The hatching larvae burrow into the seeds, exactly beneath the egg and develop within the seed thereby consuming it. After pupation, the mature beetles emerge from the seeds and reproduce. Adult *C. maculatus* are capital breeders and do not need any nourishment during reproduction. But if water and food are available they will forage. (Fox 1993b; Fox 1993a; Arnqvist et al. 2005)

As mentioned above, males of *C. maculatus* possess spiny genitalia and injure females during copulation, which is thought to be costly to females since multiply mating has been shown to reduce female lifespan (Crudgington & Siva-Jothy 2000). Although a correlational study showed that males with longer genital spines fertilize more eggs under sperm competition than males with shorter genital spines (Hotzy & Arnqvist 2009), the specific function of the spines is still unknown. The spines could have several potential functions. (1) They could serve as an anchor to increase copulation duration by preventing dislodgement by either females or rival males during copulation (Edvardsson & Tregenza 2005; Eady et al. 2007). (2) The spines could also increase and/or accelerate the transfer of accessory seminal substances into the haemolymph by puncturing the female reproductive tract and thereby aid the male in intrasexual competition (Lewis & Pollock 1975; Merritt 1989; Eberhard 1996; Eberhard 1998; Crudgington & Siva-Jothy 2000; Eady et al. 2007). (3) It has also been suggested that genital spines could function to directly remove sperm from rivals as they do in other species (Waage 1979; Simmons 2001), although Eady (1994) showed that this is not the case in *C. maculatus*. (4) In many animals the female immune system reacts against sperm and accessory seminal substances within the females' body (Yanagimachi & Chang 1963; Mattner 1969; McGraw et al. 2004; Fedorka & Zuk 2005). If the sperm of *C. maculatus* also has to face such immune responses, trade-offs between the immune reactions against both the male induced wounds and the sperm could decrease immune response against sperm since immune function then might focus on coping with the internal injuries. This could potentially increase sperm uptake. (5) Adaptive harm caused by the spines could either lower future sperm competition by reducing the remating probability of the females, or increase a current males' fitness by inducing a final reproduction response in females.

However, previous studies indicate that the harm due to genital spines in *C. maculatus* is not adaptive but rather pleiotropic (Edvardsson & Tregenza 2005; Hotzy & Arnqvist 2009). Moreover, I want to note that all these potential functions are not mutually exclusive and that the genital spines could of course serve multiple functions in *C. maculatus*.

1.6. The Goal of my Thesis

The aim of my diploma thesis was to answer following explicit questions:

1) Do genital spines serve in sperm competition and is the fertilization success of long spined males higher than of short spined males?
2) Do genital spines increase the dispersal of accessory seminal substances within the females' body, i.e. is the dispersal of accessory seminal substances of long spined males more effective?
3) Do genital spines serve as an anchor during copulation to prevent females from terminating copulation earlier than beneficial for the male? In other words, do long spined males copulate longer than short spined males?

To address these questions, I compared males with long and short genital spines in three main experiments:

1) I performed a sperm competition assay to test whether long spined males have a higher fertilization success than short spined males.
2) I performed a radio label experiment and traced male seminal substances of long and short spined males in different female body parts to see whether ejaculate dispersal depends upon genital spine length.
3) I compared the copulation duration of long and short spined males to see if long genital spines prolong copulation.

I obtained long and short spined males by two complimentary strategies. The first strategy was to select for genital spine length by artificial spine selection to achieve populations with either long or short genital spines. Secondly, I manipulated the genital spine length experimentally by using a micro laser ablation system to shorten spines.

So far, most studies investigating the function of genital morphology in sperm competition have been based on correlations between genital variation and male fertilization success. Such studies range from comparative studies on species level (Arnqvist 1998), over comparative studies on population level (Hotzy & Arnqvist 2009), to studies locking at the natural variation in genital morphology within populations and fertilization success (House & Simmons 2003; Wenninger & Averill 2006). However, all these studies have in common that they cannot distinguish between the effects of genital morphology on fertilization success and the effects of correlated traits on fertilization success. In my diploma thesis, I tried to solve this problem by comparing the effects of genital spine length due to artificial genital spine selection to the effects of genital spine length due to experimental genital spine ablation. If both approaches show the same results, this suggests that the effects of both spine length manipulations are due to spine length. This should minimize the risk that correlated traits are responsible for the results. Thus, in combination artificial spine selection and experimental spine ablation should be able to unveil the causal effects of the genital spines in *C. maculatus*.

2. Methods

2.1. Artificial Spine Selection

Artificial spine selection and all experiments using males from the resulting selection lines were carried out at Uppsala University, Sweden. The stock population I used to create my selection lines was *Callosobruchus maculatus* South India USA (SI USA) which is reared on mung beans (*Vigna radiata*). SI USA was originally collected in South India in 1979. The population has first been cultured in the USA for 23 years and is in Uppsala since 2002. Under the laboratory conditions we use in our lab generation time of SI USA is about three weeks. I chose the population for three practical reasons. 1) The individuals of this population are quite large and therefore easy to handle. 2) Males and females are easily distinguishable. 3) Collecting virgins is very easy and efficient since only one beetle emerges per bean (if there are several larvae within one bean only one of them survives). Using this population as the base, I selected three selection lines with long (L1, L2, L3) and three selection lines with short (S1, S2, S3) genital spines. The selection itself was done by comparing the genital spines of the males under a dissecting microscope (Leica MZ7 5). I estimated the relative spine length within a group of males and chose the third of the males possessing the longest or shortest spines to found the corresponding selection lines.

Artificial Spine Selection in the First Generation Virgin males from the stock population were randomly chosen after emergence. The males were anaesthetized with CO_2 and kept under a constant CO_2 flow for the whole selection process (max. 45 Min). To be able to compare the genital spines, the adeagus was everted using an adjustable vacuum pump connected to a tube that ended in a pipette tip. When the genitalia of a set of 12 males were fully inflated, I assessed their genital spine length and split them into half. One half contained the longer spined males and the other half contained the shorter spined males. Within these groups spine length was compared again and the four males with the longest and the four with the shortest spines were selected. Afterwards, I inflated the genitalia of the next set of 12 males and compared their genital spines in the same way. This process was repeated until 50 long spined and 50 short spined males were selected. Then, I started the two first selection lines by transferring the corresponding

selected males and 50 randomly selected virgin females from the stock population into a 1 liter glass jar and providing them with 150 g mung beans. Subsequently, I selected the males for the next two selection lines. Since it took me two to three days to select 100 males (i.e. the males for one short and one long selection line) I selected and started the six selection lines in three blocks, each two to three days apart from each other. Each block consisted of one long spined and one short spined line (first block: L1, S1; second block: L2, S2; third block: L3, S3).

Spine Selection of the Subsequent Generations In the subsequent generations, selection was basically performed as in the first generation. 12 males from the same selection line (e.g. L1) were compared in respect to their genital spine length. Again they were first split into half and then into three groups of four males with either long, short or intermediate spine length. But then only four out of the 12 males were selected. Depending on the selection line they derived from, either the four males with the shortest or the longest spines were chosen to sire the next generation. Like in the first generation, each population was founded by 50 selected virgin males and 50 virgin females. The females were randomly chosen from the same selection line as the corresponding males. All generations were selected in the same block system as in generation one. The six lines were therefore synchronized in pairs.

Artificial spine selection was performed for five generations. Males of the sixth generation were then compared in mating experiments. The selection lines were reared in 1 liter glass jars containing 150 g of mung beans and kept in climate chambers at ~30°C, ~ 55% relative humidity with a 12:12 diurnal light cycle, except for the third and fourth generation which were kept at ~26°C, ~55% relative humidity to slow down the life cycle. Selection took place at room temperature and during the selection period (two to three days) the beetles were held at room temperature to slow down their metabolism and thereby increase their lifespan. During the mating experiments, using the males of the sixth generation, beetles were kept at ~30°C and ~55% relative humidity but the matings itself were performed at room temperature.

2.2. Genital Spine Ablation - Laser Treatment

Genital spine ablation and all mating experiments carried out with males of the laser treatments were carried out at the University of Cincinnati, Ohio. As for the selection lines I used *Callosobruchus maculatus* South India USA (SI USA) for the spine ablation experiments. Beetles were sent from Lexington (Charles Fox's Lab, University of Kentucky) to Cincinnati (Michal Polak's Lab, University of Cincinnati) a couple of weeks before the experiments started. In Cincinnati they were kept in climate chambers at constant humidity with a 12:12 diurnal light cycle. To ensure the beetles would emerge during the period the laser treatment took place, the temperature was changed from ~30°C to ~20°C to slow down their metabolism and development. Shortly before the spine ablation, the beetles were put into virgin chambers and kept at the initial ~30°C to ensure enough beetles would emerge at the same time. Laser treatment and mating experiments were carried out at room temperature. Apart from that beetles were kept at ~30°C during the experimental time. To shorten the genital spines of the males I used a cutting-edge laser ablation system invented by Polak *et al.* (for more information see Polak & Rashed 2010).

I conducted four different laser treatments. In the first treatment, the strong spine ablation treatment (A), I shortened thirty ventral spines of the adeagus. In a second weaker spine ablation treatment (B) I shortened ten ventral spines. The third treatment (C) was a surgical control. Here no spines were shortened, but I hit spine-less areas of the adeagus with the laser beam ten times. In the fourth treatment (D), a second control treatment, the males were not hit by the laser beam at all. Instead, I shot the laser beam close to the adeagus ten times without hitting any tissue. To illustrate the spine ablation treatments, scanning electron microscope pictures (Figure 1, Figure 2 and Figure 3) were taken at the Department of Chemistry at the University of Cincinnati using an ESEM microscope from Phillips (XL 30 ESEM, FEI Company, Hillboro, Oregon, U.S.A.) The ESEM samples were coated with a 10 nm gold film in a vacuum desk (Denton Vacuum Desk II).

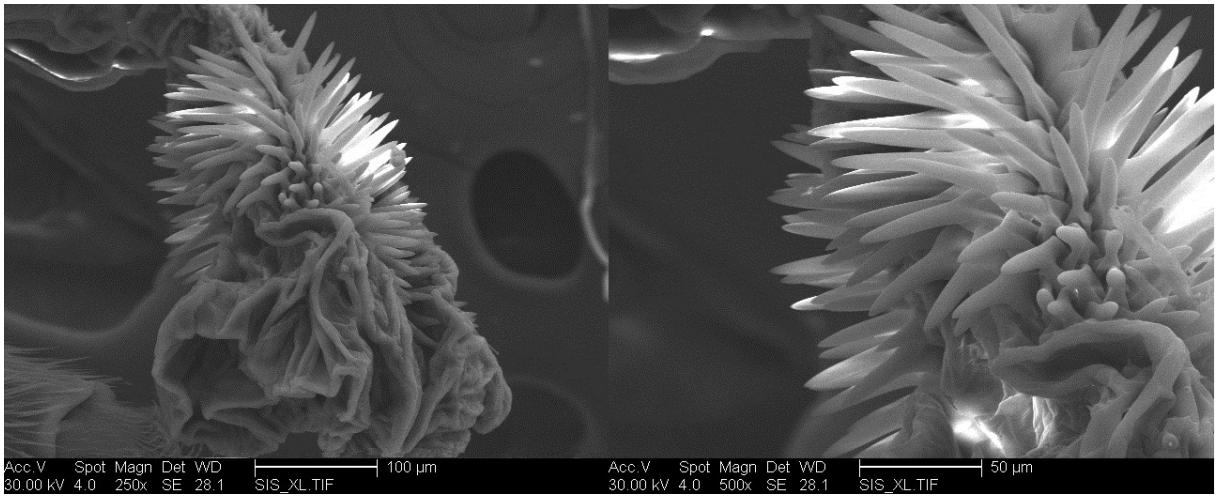

Figure 1. ESEM picture of the adeagus tip of a male of laser treatment C (i.e. the surgical control). All spines are intact since the laser only hit spine-less areas of the adeagus (no visible injuries).

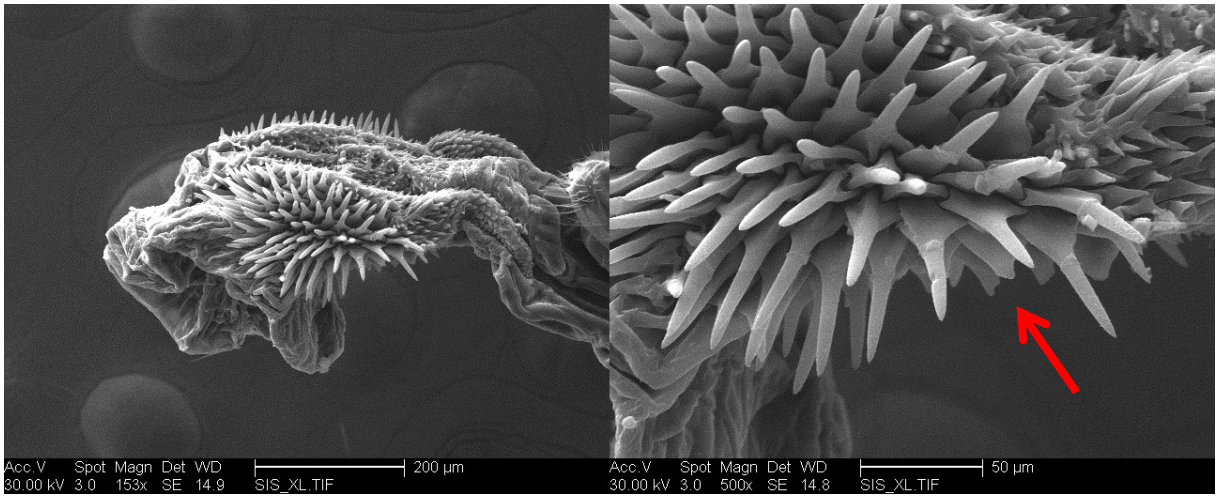

Figure 2. ESEM picture of the adeagus tip of a male of the laser treatment B (i.e. the weak spine ablation treatment in which 10 spines were cut off). The red arrow points to the area where spines were ablated.

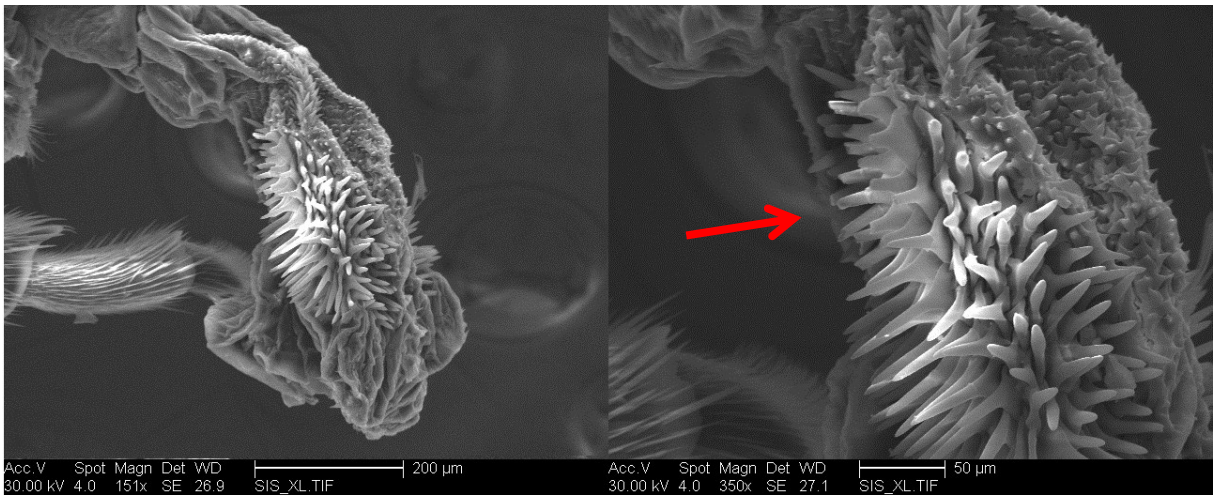

Figure 3. ESEM picture of the adeagus tip of a male of the laser treatment A (i.e. the strong spine ablation treatment in which 30 spines were cut off). The red arrow points to the area where spines were ablated.

Spine ablation process Males were randomly assigned to one of the laser treatments (A, B, C or D). Then I inflated their genitalia in the same way as for the artificial spine selection. As soon as the genitalia were fully inflated, the males were transferred to a motorized stage (Prior H117, Rocklans, MA, USA) on an inverted light microscope (Olympus, Center Valley, PA, USA), which was connected to a laser system (for more details see (Polak & Rashed 2010). I used the single shot laser modus for the spine ablation with a laser intensity of 40%. In most cases, one spine was cut per laser shot. Occasionally, two spines were cut with one laser shot if they were very close to each other. In both spine ablating treatments I shortened spines that were located on the ventral side of the adeagus. Half of the spines were cut while the male was positioned on one lateral side. Then, I turned the male to his other lateral side and cut the second half of the spines. The controls were also put on their lateral sides for the laser treatment.

2.3. Mating Experiments

The sperm competition and ejaculate dispersal experiments were performed using both males from the selection lines and males from the laser treatments. The copulation duration assay was only done with males from the selection lines.

All focal males of the selection line experiments originated from the selection lines (L1, L2, L3, S1, S2 and S3). The background males and all females derived from the nonselected SI USA stock population (I will also refer to this as the "base population"). Since the selection lines were selected in three blocks and synchronized in pairs, the mating experiments were performed in the same block system (i.e. the lines that were selected together were also used in the same experimental block). The three experimental blocks were performed three days apart from each other (first block: L1, S1; second block: L2, S2; third block: L3, S3).

In the spine ablation experiments all individuals originated from one population (SI USA). The ejaculate dispersal experiment of the spine ablation treatment was run in two blocks, conducted four days apart from each other, whereas the sperm competition experiment consisted of one block only.

2.3.1. Sperm competition Assay

For this experiment I used the following standard protocol to measure the proportion of eggs that is fertilized by the second of two males. Females were mated twice. First they were mated to a gamma irradiated, sterilized background male. They were then remated to a fertile focal male. When using the right irradiation dosage spermatozoa of sterilized males are still able to compete normally with the spermatozoa of fertile males, but eggs fertilized by the gamma irradiated males cannot hatch due to DNA damage within the spermatozoa (Ahmed et al. 1977). Thus, the proportion of eggs fertilized by the second male, i.e. P2, can be determined by counting the number of hatched versus unhatched eggs. In the P2 experiment using the males of the selection lines I started 22 to 25 replicates per line (L1 and S1: 22, L2 and S2: 25, L3 and S3: 24). In the P2 experiment using spine ablated males I started 33 replicates per laser treatment.

Sterilization of the Background Males One to three day old virgin background males were sterilized by gamma irradiation. Background males of the selection line experiment were irradiated with 80 Gy using the Cesium source of the Rudbeck laboratory in Uppsala. Background males of the laser ablation experiment were sterilized with 87.5 Gy using the Cobalt source at the Department of Nuclear & Radiological Engineering at the University of Cincinnati. I used about 80 Gy for sterilization since a previous study has shown that 80 Gy lead to complete sterilization of male *C. maculatus* but do not affect sperm competition (Ahmed et al. 1977). For irradiation, 10 to 20 males were put into one Petri dish and several Petri dishes were placed such that they received the same amount of radioactivity. After irradiation, males were separated and kept solitary in Eppendorf tubes to recover from the sterilization until the mating experiments started.

First Mating One to two days after sterilization, one to two day old virgin females were mated to one of the sterilized background males. One male and one female were introduced into a small Petri dish (Ø 3 cm) and the mating procedure was observed (this applies to all mating experiments). After copulation was completed, each female was transferred into a clean Eppendorf tube containing one (first block selection lines), three (second block and third block selection lines) or five (laser ablation experiment) mung beans as oviposition substrate. The females were kept in climate chambers until the second mating. Males of the selection lines were prepared for genital spine length

measurement after the mating. Males of the laser ablation treatments were frozen after copulation.

Second Mating Two days after their first mating, the females were remated to focal virgin males. In the selection line experiment those males derived of one of the selection lines and were randomly selected from virgin chambers one day before the mating trials. In the laser ablation experiment the focal males were prepared as follows: Three to four days before the mating trails one day old virgin males were randomly allocated to one of the four laser treatments: A: 30 spines shortened, B: 10 spines shortened, C: surgical control (spine-less area hit by the laser beam) and D: laser did not hit any tissue. The laser treatments were performed during two successive days. In total I prepared 33 males per treatment (day one: 15 males per treatment, day two: 18 males per treatment). After exposing them to the laser treatment, five (day one) or six (day two) males of the same treatment were placed into a Petri dish (Ø 3 cm) and provided with 20% sugar solution for three to four days, to recover from the treatment before the mating trials. Matings were performed like the first matings, but I placed one (spine ablation experiment) or three (selection line experiment) mung beans into the mating dish to facilitate remating of the females. When females did not remate, males (selection line experiment) or females (spine ablation experiment) were exchanged. Some of the females did not remate during the first day and were remated one day later. Those females were provided with three (selection line experiment) or five (spine ablation experiment) mung beans overnight to enhance their remating probability. In the laser ablation experiment copulation duration was recorded. Copulation start was defined as the time point when the male reached mating position (male is hanging within the female) and stopped tapping with his antennae onto the females' abdomen. Copulation was regarded as terminated as soon as the couple separated. In the selection line experiment copulation duration was not recorded, since a separate copulation duration assay was performed in this case.

Post mating procedures Successfully remated females were each transferred into a medium sized Petri dish (Ø 6cm) containing ~ 60 (selection line experiment) or ~100 (spine ablation experiment) mung beans to lay their eggs on. Mated focal males of the selection line experiment were prepared for spine measurement after the mating. The laser treatment males were returned to the climate chambers and kept solitary until they died. Once per day they were scanned to determine their lifespan. Afterwards they were

frozen for elytra length measurement. Seven days after the second mating, the remated females were removed from the Petri dishes and frozen for elytra length measurement. The beans were frozen after all eggs had hatched and the larvae had bored into the beans but before offspring emergence. The proportion of eggs fertilized by the first, sterile and second, fertile male was determined by counting the number of unhatched and hatched eggs respectively. Eggs laid between the first and second mating were counted separately to account for eggs that were laid before sperm competition occurred. As body size indicator, I measured elytra size of all mated individuals (for details of elytra size measurement see page 26). Beetles and infested beans of the laser ablation experiment were sent to Uppsala for elytra length measurement and egg counting.

2.3.2. Ejaculate Dispersal within the Females' Body

To trace seminal substances of long and short spined males within the females' body, I mated females to radio labeled males. Subsequently, I measured the proportion of radio label within different female body parts to see where the ejaculate ends up. Males were radio labeled by feeding them ^{14}C-Arginine, which seemed reasonable since ^{14}C-Arginine has been successfully used in a radio label study using *Acanthoscelides obtectus* a closely related species of *C. maculatus* (see Huignard 1983). To ensure that the ejaculate would contain a sufficient amount of ^{14}C, males were mated two times during the feeding period to get rid of the old, unlabeled ejaculate. The radio labeled males were then mated to nonvirgin females and ejaculate dispersal within these females was traced by measuring the amount of ^{14}C that ended up in bursa, spermatheca and the rest of the females' body. I started 20 replicates per selection line, plus 20 blanks in the ejaculate dispersal experiment using the selection lines and 34 replicates per laser treatment, plus 18 blanks in the ejaculate dispersal experiment using the laser treated males.

Radio Labeling of the Males First, one day old virgin males of either the selection lines or the laser treatments were mated to background females to trigger ejaculate renewal. In the selection line experiment copulation duration was recorded and used for the copulation duration experiment with intact females (see page 24). In both experiments I also started blank males that were exactly treated like the radio labeled individuals with two exceptions: 1) the blank males were not radio labeled and fed on pure 20% sugar solution, 2) The blank males of the spine ablation experiment were not exposed to the

laser procedure and the blank males of the selection line experiment derived from the base population (stock population). In the selection line experiments, males were radio labeled immediately after copulation. Each five males of the same selection line were transferred into a feeding chamber (Ø 3 cm Petri dish) containing a feeding vial filled with 340 µl of 1:1 ^{14}C-Arginine : 20% sugar solution (Arginine L-[^{14}C(U)], 250µCi, Perkin&Elmer) or pure 20% sugar solution (blanks) and kept there for 24 hours. In the spine ablation experiment males were exposed to the laser treatment between copulation and feeding period. After copulation they were randomly allocated to one of the following laser treatments: A: 30 spines shortened, B: 10 spines shortened, C: surgical control (i.e. spine-less areas hit by the laser) or the blank treatment (no laser exposure) and the corresponding treatment was conducted. In this experiment I only used one control treatment, which was the surgical control C but I did not use treatment D (i.e. laser was shot close to the adeagus but no tissue was hit by the laser). For feeding, each eight (first block: laser ablation) or nine (second block: laser ablation, and blanks) males of the same treatment were transferred into a feeding chamber containing the same ^{14}C-cocktail or blank-cocktail respectively as in the selection line experiment. The focal males and blanks of both experiments were remated to another background female one day after the first mating and afterwards returned into the feeding chamber for another day to gather more ^{14}C label. All background females were disposed after mating.

Focal Female Matings and Ejaculate Dispersal The focal females in this experiment were mated twice, which was necessary to trigger sperm competition within the females. First, they were mated to unlabeled background males when they were one day old. Three days later, they were remated to the radio labeled, focal males, except for the blank females that were mated to one of the blank males instead. After each mating females were provided with ~30 beans for oviposition. In the selection line experiment both males were weighed to the nearest 10^{-5}g before and after copulation to account for ejaculate weight and body size (Micro balance: Sartorius Genius Series ME235P-0CE, Sartorius®). In the laser ablation experiment ejaculate weight was not recorded and instead elytra length was used to indicate male body size. For the blank individuals of both experiments neither body size nor ejaculate weight were recorded. Unlabeled background males were disposed after usage in the selection line experiment, but frozen and stored for elytra length measurement in the spine ablation experiment. Radio labeled males and blank males were frozen and stored for scintillation analysis in both experiments. To ensure the

radio labeled ejaculate had time to disperse, radio labeled (and blank) females were frozen 18 hours (±10 Min) after the final copulation. 7 days later the Petri dishes containing the infested beans were frozen too and kept at –18 °C until eggs were counted. Number of eggs laid after the first and the second mating were counted separately. Beetles and infested beans of the spine ablation experiment were sent to Uppsala for elytra length measurement, egg counting, female dissection and scintillation analysis.

Female Dissection Dissection was performed under a dissecting microscope (Leica MZ7 5) using micro forceps. Focal females were three times rinsed in 70% ethanol to get rid of all outer radio label due to copulation (the same procedure applies to the blank females). The washing dish was rinsed after each female. Females were dissected on a small square of gelatin (1.2 cm^2) in a Petri dish lid (Ø 12cm). The gelatin square was placed on a drop of water (10µl) to fix it. The rinsed female was placed on the gelatin and one elytron was removed using micro forceps. Then, elytra size was measured twice using a dial calipers (SMIEC, 0.02mm exact). The measured elytron was placed into a fresh Eppendorf tube. Thorax and abdomen of the female were separated and thorax and head were also transferred into the Eppendorf tube. Subsequently, a drop of water (10µl) was dripped on the gelatin for further dissection. I removed the bursa copulatrix and the spermatheca and transferred each of them into a new Eppendorf tube. The rest of the females' body and the gelatin (which already sucked in all the haemolymph etc.) were placed into the first Eppendorf tube, already containing the elytra, thorax and head of the female. This lead to 3 Eppendorf tubes per female containing: (1) rest of the female's body, (2) bursa, (3) spermatheca. The Eppendorf tubes were stored at -18 °C until they were prepared for scintillation analysis. Dissecting instruments were rinsed in 70% ethanol after each female.

Preparation for Scintillation Analysis The frozen radio labeled males were three times rinsed in 70% ethanol to wash off all outer label. Then, they were put into a new Eppendorf tube and crushed using a steel pestle. Afterwards, they were frozen again until tissue solubilizing. The steel pestle was cleaned after each usage. Two days before scintillation analysis, the samples (radio labeled and blank males and radio labeled and blank female body parts) were defrosted and tissue solubilizer was added. I used two different tissue solubilizers. In the selection line experiment I used TS-2 (Koch-Light Research Laboratories Ltd, England), in the spine ablation experiment I used Solvable

(PerkinElmer, USA). The amounts of tissue solubilizer I added to the different scintillation samples were the same in both experiments: 1) focal males: 100 µl, 2) rest of the female body: 200 µl 3) bursa copulatrix 50 µl, 4) spermatheca: 50 µl. After adding the tissue solubilizer, all samples were centrifuged for 1 Min at 10.7x1000 rpm to ensure that the tissues were actually covered by the tissue solubilizer. 24 hours later the scintillation cocktail was added. In both experiments each sample was supplied with 1.5 ml scintillation cocktail (Optiphase 'Hisafe' 2, PerkinElmer, USA).

Scintillation Analysis 25 hours after adding the scintillation cocktail, the samples were analyzed with a Liquid Scintillation Analyzer (Packard, TRI-CARB 2100TR) to measure the radioactivity of the different samples. Samples were analyzed twice, for ten minutes each. Scintillation analysis was performed in blocks that were consistent with the experimental blocks (selection line experiment: three blocks; spine ablation experiment: two blocks). Each block contained between 6 and 9 blanks in addition to the labeled samples to measure the background radioactivity.

Preparation of the Data for Statistical Analysis First, I took an average of the readings of the two scintillation cycles for the CPM (counts per minutes) data for all types of samples and removed some misreads. If there were two readings, the mean of both readings was used. If there was only one reasonable reading (e.g. due to a misread or missing reading), this one was used. I compensated for the background by subtracting the mean reading of the corresponding blanks from the mean of the sample readings. This led to the "true" ^{14}C-label for all observations that could be used in the statistical tests. The ^{14}C-label in the spermatheca was quite low and when subtracting the blank readings from the spermatheca readings some of the values became negative. Thus, I added a constant of 2.3 to all spermathecal readings to get rid of negative values. Then, I calculated three new variables: 1) total signal in females (i.e. sum of bursa and rest of female's body), 2) proportion of ^{14}C in the rest of female's body (i.e. ratio of rest of female's body to total signal) and 3) proportion of ^{14}C in spermatheca (i.e. ratio of spermatheca to total signal).

2.3.3. Copulation Duration Assay

In this assay I measured the copulation duration of couples with females who had intact hind legs and were thus able to terminate matings and females who could not terminate matings due to hind leg ablation. I mated these females to males of the different selection lines and analyzed the difference between the female treatments in respect to the genital spine length of the males. In contrast to the other mating experiments, this experiment was only carried out using males of the selection lines. Per selection line, I mated 20 males to females with intact hind legs, and 20 males to females with ablated hind legs.

Female Treatments Focal females in this experiment were randomly selected from the base population and were allocated to one of two following treatments: 1) intact treatment and 2) ablated treatment. In the latter treatment the females were anesthetized under a steady CO_2 flow and the hind legs were ablated using micro scissors. I cut the hind legs in the middle section of the femur such that the females could not reach the male with their hind legs during copulation. Therefore these females could not kick off the male to terminate copulation. Hind leg ablation was performed about 30 Minutes before the mating experiments started. In the intact treatment females had intact hind legs and were thus able to terminate matings.

Mating experiments One to two days old virgin females of both female treatments were mated to one day old virgin males of the different selection lines. Copulation length was documented as follows: Copulation start was defined as the time point when the male reached mating position (male is hanging within the female) and stopped tapping with his antennae onto the females' abdomen. Copulation was regarded as terminated as soon as the couple separated. Before and after mating, males were weighed to the nearest 10^{-5} g to account for body size and ejaculate weight (Micro balance: Sartorius Genius Series ME235P-0CE, Sartorius®). After usage, females were frozen and stored for elytra size measurement. Males, who had mated to intact females, were radio labeled after the mating and used in the ejaculate dispersal experiment (see page 20). Males, who had mated to females with ablated hind legs, were prepared for genital spine measurement.

2.4. Spine Length of the Selection Lines

To check if the genital spine length of the selection lines actually differed across the selection lines, spine length was measured of all males used in the sperm competition experiment carried out with the selection lines and the males who mated to ablated females in the copulation duration experiment. This lead to approximate 40 (36 to 40) replicates per selection line, and 111 replicates of the base population.

Inflation and Fixation of Male Genitalia Preparation for the spine length measurement took place a couple of hours after the males were used in the different mating experiments. The genitalia were inflated in the same manner as for the artificial spine selection (see page 13). As soon as the genitalia were fully inflated, the males were placed into boiling water for 20 seconds. This led to primary fixation of the genitalia due to coagulation of the proteins. Then, each male was transferred into a small Eppendorf tube containing 60µl of Bouin's Solution for final fixation. The males were stored in Bouin's solution at room temperature for about 3 weeks.

Spine Length Measurement Males were washed in 70% ethanol and transferred into an Eppendorf tube containing 70% ethanol. Several Eppendorf tubes turned out to be not completely airtight and dried out. In some of these cases the genital spines were covered by residue crystals, which were removed using a needle. Within the next three days, genital spines were measured as follows: The genitalia were cut off with micro scissors, put in a small, flattened drop of glycerol on a microscope slide and placed under a dissecting microscope (Leica MZ8). Each adeagus was oriented in two different positions to measure the length of both the lateral and the ventral spines. In each position, the 5 longest spines were measured using a CAD digitizing tablet (SummaSketch III, Summagraphics) connected to the dissecting microscope. Spine length was measured as the distance between tip and base of the spine. All measurements were performed at the same magnification and the genitalia were always oriented in the same manner.

2.5. Body Size Measurement

Elytra length was measured of all individuals used in the mating experiments (spine ablation and selection line experiments) except for: 1) the males of the ejaculate dispersal experiment carried out with the selection lines 2) the males of the copulation duration experiment using the selection lines. In these both cases body weight was used as body size. Males were weighed two times in immediate succession to the nearest 10^{-5}g and the mean of the measurements was taken (Micro balance: Sartorius Genius Series ME235P-0CE, Sartorius®). Elytra size of the focal females of the ejaculate dispersal experiments was measured twice using a dial calipers (SMIEC, 0.02mm exact). Elytra size of all other individuals was measured using a CAD digitizing tablet (SummaSketch III, Summagraphics) connected to a dissecting microscope (Leica MZ8). Each individual was placed on a small piece of blue tack and the elytra were oriented horizontal under the microscope. Both elytra were measured twice and the mean of the measurements was taken. All measurements were performed at the same magnification.

2.6. Statistical Analysis

Statistical analyses were performed using SYSTAT 11 (Wilkinson 2004) and GenStat v.10.0 (Payne et al. 2007).

Continuous response data was analyzed in Systat11 by running analyses of variance (ANOVA), analyses of covariance (ANCOVA) and regressions. This applies to the selection line data as well as to the spine ablation data. In each model, all possible factors and covariates that could affect the model were tested. Factors and covariates that did not contribute to the fit of the model were removed from the model. A few outliers (i.e. studentized residual >3) were also removed from the analyses. The distribution of the residuals of the response variable of all general linear models was tested for normality using the Kolmogorov-Smirnov-Test. In cases with non-normally distributed residuals, transformation of the data was performed to meet the assumptions of normally distributed errors. In some cases the model was run using the original data although the Kolmogorov-Smirnov-Test was significant, because visual inspection of the residuals showed that they

were nicely distributed (symmetrical and dome-shaped) and could be treated like normally distributed.

Ratio response data was analyzed in Genstat running analyses of deviance (ANDEVA). This applied to the P2 data of both, the selection line experiment and the spine ablation experiment. In both cases, I modeled the number of hatched eggs laid after the second mating in a generalized linear model with binomial errors, using a logit link function and an empirically derived dispersion parameter. The total number of eggs laid after the second mating was used as the binomial denominator. As I did in the general linear models, I tested those factors and covariates that could have contributed to the fit of the models, and factors and covariates that did not contribute to the fit of the models were removed from the final models.

For the selection line data I additionally ran two sample T-tests to detect differences between the long and short spined selection lines. The means that were used for the two sample T-test were generated by running the corresponding general linear model of the response variable without the effect of selection, saving the residuals and calculating the mean residual per selection line.

3. Results

3.1. Selection Lines

3.1.0. Definitions of Some Variables

In all selection line data "block" refers to the block system that was used for the artificial spine selection. At the same time this matches to the experimental blocks of each mating experiments since the experiments were performed using the same block system. Each block contained one long and one short spined selection line (i.e. L1 and S1, L2 and S2 or L3 and S3). The factor "group" is used in connection with the data of the genital spine length of the selection lines. It refers to males that were used in two different mating experiments (the first group of males was used in the sperm competition experiment, whereas the second group of males used in the copulation duration experiment). I distinguish between these males since the spine length of them was not measured at the same time but consecutively.

3.1.1. Spine Length and Artificial Spine Selection

To test whether the long spined lines differed in spine length from the short spined males I ran two ANCOVAs for lateral and ventral spine length respectively (Table 1 and Table 2).

Differences in Lateral Spine Length Selection had definitely a very large effect on lateral spine length (Table 1). Furthermore selection had worked into the right direction with all long spined lines having longer lateral spines than the short spined lines (Figure 4). Interestingly the lateral spine length of males of the different groups differed significantly too (Table 1). Males of the different blocks also differed in lateral spine length (Table 1). The interactions between block and group and block and selection had a large impact on lateral spine length too, although selection definitely had the biggest effect on lateral spine length (Table 1). Male body size, the interaction between selection and group and the interaction between block, selection and group were not significant (Table 1).

Table 1. ANCOVA of lateral spine length of the selection lines

Source	SS	DF	F-ratio	P-value
Selection	0.014	1	34.945	< 0.001
Block	0.006	2	7.743	0.001
Group	0.010	1	23.295	< 0.001
Block*Selection	0.004	2	4.715	0.010
Block*Group	0.007	2	8.613	< 0.001
Selection*Group	0.001	1	3.015	0.084
Block*Selection*Group	0.000	2	0.569	0.567
Elytra length	0.000	1	0.088	0.767
Error	0.089	216		

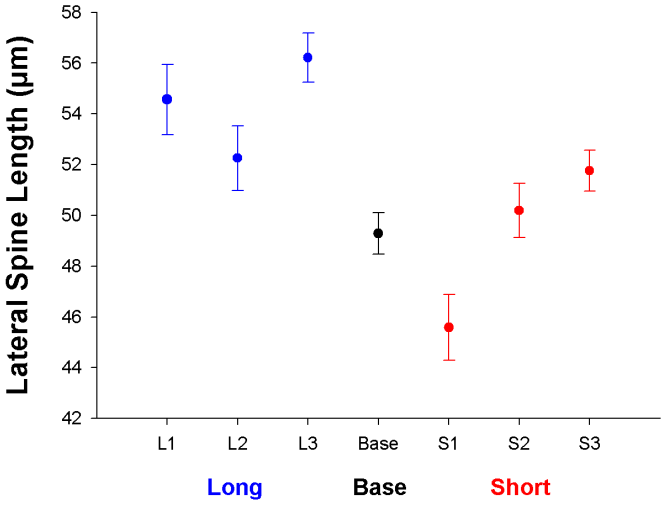

Figure 4. Means and standard errors of lateral spine length (in µm) of the six selection lines and the base population, after five generations of selecting the genital spine length of the selection lines.

Differences in Ventral Spine Length I ran the same model for the ventral spine length and selection also had a large effect on ventral spine length (Table 2) at which all long spined lines had significant longer ventral spines than the short spined lines (Figure 5). The second factor that contributed to the variation in ventral spine length was block (Table 2). All other tested factors, covariates and interactions did not have a significant effect on the length of the ventral genital spines (Table 2).

Table 2. ANCOVA of ventral spine length of the selection lines

Source	SS	DF	F-ratio	P-value
Selection	0.021	1	47.974	< 0.001
Block	0.005	2	5.829	0.003
Group	0.000	1	0.037	0.848
Block*Selection	0.002	2	2.168	0.117
Block*Group	0.000	2	0.001	0.999
Selection*Group	0.001	1	1.699	0.194
Block*Selection*Group	0.000	2	0.228	0.796
Elytra length	0.000	1	0.324	0.570
Error	0.096	216		

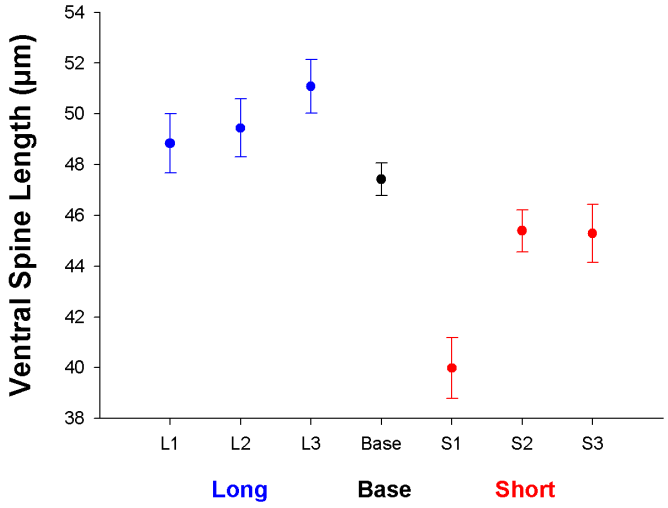

Figure 5. Means and standard errors of ventral spine length (in µm) of the six selection lines and the base population, after five generations of selecting the genital spine length of the selection lines.

Two two sample T-tests using the mean residuals per selection line for lateral and ventral spine length respectively showed the same results as the ANCOVAs. Both, the lateral spines and the ventral spines, differed significantly due to selection (Two sample T-test: lateral spines: N = 6, df = 4, t = 3.787, p = 0.019; two sample T-test: ventral spines: N = 6, df = 4, t = 6.466, p = 0.003).

All these results allow the conclusion that selection has been efficient since both lateral and ventral spine length have evolved in the predicted direction in all selection lines. Furthermore, I ran an ANOVA for male body size (elytra length) and an ANCOVA for male ejaculate size to see whether selection had affected those traits as well. But neither body size (see Table 3) nor ejaculate size (ANCOVA: $F_{(1,213)} = 0.169$, p = 0.681, Table 13

page 40) were affected by the selection. When looking at the male body size only males of the different blocks differed significantly in body size (Table 3). All other tested factors did not contribute to the variance in body size (Table 3). This suggests that the selection lines only differed in spine length due to selection and that I had not accidentally selected for other correlated traits while selecting on genital spine length.

Table 3. ANOVA of male body size (i.e. elytra length) of the selection lines

Source	SS	DF	F-ratio	P-value
Block	0.032	2	4.423	0.013
Selection	0.001	1	0.238	0.626
Group	0.004	1	1.212	0.272
Block*Selection	0.008	2	1.145	0.320
Block*Group	0.017	2	2.296	0.103
Selection*Group	0.000	1	0.050	0.823
Block*Selection*Group	0.002	2	0.215	0.807
Error	0.793	217		

Strength of the Artificial Spine Selection Across the three blocks, the mean lateral spine length of the long versus short line differed by 0.24 – 1.05 SD's of the base population. For the ventral spine length the means differed by 0.60 – 1.32 SD's of the base population. Expressed in the proportion of mean spine length of the base population, the corresponding numbers were 0.04-0.18 and 0.09-0.19. Thus, the genital spine length responded strongly to the selection process.

Table 4. Within block response of genital spine length to selection process after 5 generations of artificial spine selection Difference in genital spine length within the blocks is given as (a) the difference between the means in µm (i.e. mean of long spined line minus mean of short spined line, (b) the difference of means of the long minus the short spined lines in proportion of the SD of the base population (i.e. difference between the means/SD of base) and (c) the difference of the means of the long and short spined lines in proportion of the mean of the base population (i.e. difference between the means/mean of base).

	Diff. between means (in µm)		Diff. in prop. of SD of the base population		Diff. in prop. of mean of the base population	
	lateral	ventral	lateral	ventral	lateral	ventral
Block 1	8.984	8.851	1.051	1.318	0.182	0.187
Block 2	2.067	4.052	0.242	0.603	0.042	0.085
Block 3	4.456	5.792	0.521	0.862	0.090	0.122

Another way the strength of the response to the artificial spine selection can be expressed, is by calculating the heritability in all six lines as deviations from the base line values. The heritability of a selected trait is: $h2 = 2R/S$ (Falconer & Mackay 1996), whereat h2 is the heritability of the selected trait, R is the response to selection in SD's per generation

and S is the selection intensity in SD's. Since I selected 30% of the males the corresponding intensity of selection was 1.1 in each line (Falconer & Mackay 1996). Using the above formula I calculated the heritability of lateral and ventral spine length in each line. The mean heritability for ventral and lateral spines across all selection lines was 0.185 (min = 0.077, max = 0.402), which is quite low for a morphological trait. However, it is important to note that this estimate of overall h2 is a conservative estimate, since the selection process was assumed to be perfect – which it was probably not. Therefore, the intensity of selection used in the calculations was exaggerated and the true heritability should be somewhat higher. Anyway, the results show that genital spine length is a heritable trait.

3.1.2. Sperm competition Assay

Some females that did not remate and the corresponding couples were excluded from the data set (remaining replicates per selection line: L1: 16, S1: 18, L2: 19, S2: 20, L3: 20, S3: 19).

Sterilization Only 6 out of 2279 eggs laid in between the matings (i.e. fertilized by the irradiated males) hatched. Hatching rate of eggs fertilized by sterilized males was thus only 0.3%.

P2 across the Selection Lines To test differences in P2 between the selection lines I ran an ANDEVA of the number of hatched eggs that were laid after the 2nd mating with binomial errors, using the total number of eggs after 2nd mating as the binomial denominator, a logit link function and an empirically derived dispersion parameter. According to the ANDEVA, the proportion of hatched P2 eggs was marginally non-significantly affected by selection (Table 5). The blocks did not differ in the proportion of hatched P2 eggs (Table 5), but time between mating had an effect on P2 (Table 5). I tested the effect of all possible covariates, factors and interactions. But none of them contributed to the fit of the model. Thus, they were removed from the model (female size, sterile male size and focal male size: all $p > 0.1$; female age and focal male age: both $p > 0.2$; interaction between block and selection: $p > 0.9$).

Table 5. ANDEVA of the number of hatched P2 eggs with the total number of eggs laid after the second mating as the binomial denominator

Source	SS	DF	F-ratio	P-value
Block	35.52	2	0.83	0.438
Selection	151.17	1	3.55	0.062
Time between matings	171.17	1	4.01	0.048
Residual	42.63	105		
Change			2.31	0.063

Although the effect of selection was marginally non-significant according to the ANDEVA, looking at the mean P2 per population showed that all long spined lines had a higher fertilization success than the short spined lines (Figure 6). Furthermore, when I compared the mean residuals of the P2 per selection line with a two sample T-test, the selection lines did differ significantly in their P2 values due to selection (Two sample T-test: N = 6, df = 4, t = 5.916, p = 0.004). Thus, according to the T-test, selection for long spines has definitely resulted in a higher fertilization success.

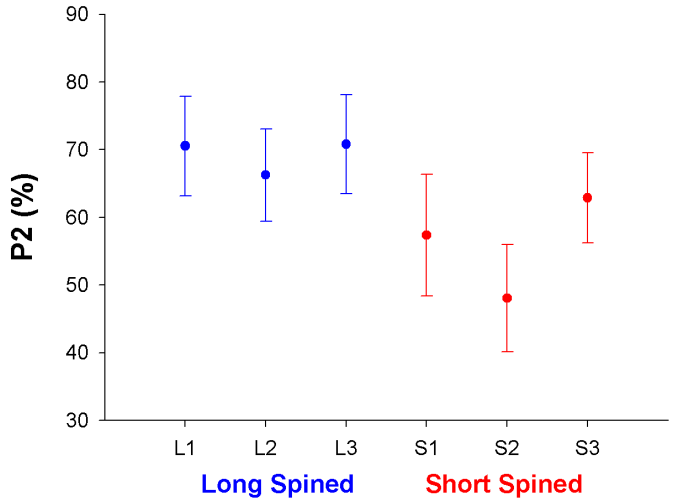

Figure 6. Means and standard errors of P2 across the six selection lines, after five generations of genital spine selection. P2 is shown as the percentage of eggs fertilized by the males of the selection lines under sperm competition with a sterilized background male.

Gonadotropic Effect of Genital Spine Length To test if spine length had a gonadotropic effect on females, and that is that males of the long spined selection lines induced females to lay more eggs, I ran an ANCOVA for the total (hatched plus unhatched) number of eggs laid after the second mating. Since the original residuals were not normally

distributed, square transformed data was used to run this model. Selection did not have any effect on the number of eggs a female laid after the second mating (see Table 6). Thus a gonadotropic effect is unlikely. Covariates that affected the number of eggs laid after the second mating were the number of eggs laid between the matings and female size (Table 6). All other tested factors and interactions were not significant (Table 6).

Table 6. ANCOVA of the number of eggs laid after the second mating

Source	SS	DF	F-ratio	P-value
Selection	2248628.162	1	0.368	0.546
Block	5922210.306	2	0.484	0.618
Eggs between matings	1.29130E+08	1	21.106	< 0.001
Female size	2.64402E+07	1	4.322	0.040
Block*Selection	1.42731E+07	2	1.166	0.316
Error	6.24048E+08	102		

3.1.3. Ejaculate Dispersal within the Female's Body

Some females had to be excluded from the data set since a) they did not remate (each one of S1 and of S3), b) their bursa copulatrix was accidentally ruptured during dissection (each one of S1, L2 and L3). Furthermore, some females had to be excluded because of misreads of the scintillation analyzer. Remaining replicates per selection line were: L1: 18, S1: 15, L2: 17, S2: 18, L3: 19, S3: 18.

Total ^{14}C-Signal within Females To test which covariates had an impact on the amount of ^{14}C-label within the radio labeled females, I first ran a multiple regression for the total ^{14}C-signal of the radio labeled females. Since the residuals of the original data of the total radio label in females were not normally distributed, square root transformed data was used in the analysis. It turned out that the total amount of radio label in females was very strongly dependent up on the amount of radio label in the males (Table 7). Additionally, focal male body size affected the total amount of radio label in females and female size potentially could have had an effect too (Table 7). Interestingly, the total signal in females was lower when the focal male and/or focal female were larger. All the other covariates – both males' ejaculate sizes, base male size, eggs laid between the matings and eggs laid after the second mating – did not contribute to the variation of the total ^{14}C-signal in the females (Table 7).

Table 7. Multiple regression of total ^{14}C-signal in females Total signal is given as ^{14}C counts per minute. Multiple R: 0.903, Squared multiple R: 0.816, Adjusted squared multiple R: 0.800

Source	β (coefficient)	Std Error	t	P (2 tailed)
Constant	121.347	35.705	3.399	0.001
Base male ejaculate	-0.156	0.162	-0.961	0.339
Base male body size	-0.008	0.029	-0.268	0.789
Focal male ejaculate	0.115	0.158	0.727	0.469
Focal male body size	-0.052	0.025	-2.107	0.038
Focal female body size	-25.647	13.966	-1.836	0.070
Eggs between matings	0.094	0.105	0.896	0.373
Eggs after 2nd mating	-0.149	0.181	-0.823	0.412
Male signal	0.001	< 0.001	19.120	< 0.001

Since the amount of radio label in males had a very large impact on the total amount of radio label in females (see Table 1) and both signals were strongly, positively correlated ($R = 0.924$, $N = 102$) the male ^{14}C-signal could not be used as a covariate in the models for subsequent analysis.

Amount of ^{14}C-label that Left the Bursa Copulatrix I ran an ANCOVA of the ^{14}C-label that dispersed into the rest of the females' body while keeping the ^{14}C-label within the bursa copulatrix constant, to test whether the selection lines differed in the proportion of ^{14}C that dispersed from the females' bursa copulatrix into the rest of the females' body. Selection, block and focal female body size all had a significant effect on the amount of ^{14}C that left the bursa copulatrix and dispersed throughout the female's body, but most variation was caused by selection (Table 8). The interaction between selection and block did not have an effect on ejaculate dispersal (Table 8). None of the other covariates (base male ejaculate size and body weight, focal male body and ejaculate size, final mating time, number of eggs laid before and after the second mating, as well as total number of eggs) contributed to the fit of the model (for all of them $p > 0.1$). Thus, they were excluded from the model.

Table 8. ANCOVA of the ^{14}C-signal as counts per minutes in the rest of the female's body

Source	SS	DF	F-ratio	P-value
Signal in bursa	3.86129E+08	1	571.241	< 0.001
Block	7756773.228	2	5.738	0.004
Selection	4207216.545	1	6.224	0.014
Focal female body size	2708028.484	1	4.006	0.048
Block* Selection	1636580.697	2	1.211	0.303
Error	6.28631E+07	93		

A two sample T-test across the selection lines using the mean residual per lines showed the same picture: the amount of radiolabel in the rest of the females' body was significantly different between females mated to long and females mated to short spined males (Two sample T-test: N= 6, df = 4, t = 3.127, p = 0.035). As predicted, the proportion of ^{14}C in the rest of the females' body was higher across the long spined lines (Figure 7).

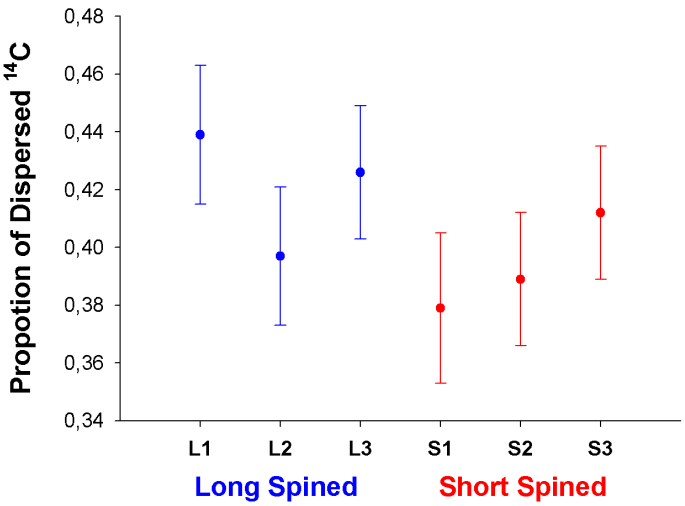

Figure 7. Proportion of ^{14}C that left the bursa copulatrix and dispersed throughout the females' bodies. Shown are the means and SE of the dispersed radio label in females that were mated to radio labeled males of the different selection lines after 5 generations of genital spine length selection.

^{14}C-Label in the Spermatheca Two facts strongly suggest that the variation in spermathecal readings reflect contamination. 1) There was no effect on the signal strength due to the male signal, whereas the total signal in the female was highly dependent on the male signal (see Table 7 and Table 9). 2) The label in the spermatheca was strongly dependent on the label in the rest of the female's body while it was less dependent on the label in the bursa copulatrix (Table 9). Therefore, I excluded the spermathecal data from the any further analysis.

Table 9. ANCOVA of ^{14}C-signal as counts per minutes in the spermatheca

Source	SS	DF	F-ratio	P-value
Selection	0.244	1	0.061	0.806
Block	30.772	2	3.845	0.026
Focal male body size	11.140	1	2.783	0.099
Focal female body size	5.739	1	1.434	0.235
Focal male ejaculate size	4.260	1	1.065	0.305
Signal male	2.871	1	0.717	0.400
Signal in rest of female	27.447	1	6.858	0.011
Signal in bursa	12.540	1	3.133	0.081
Block*Selection	0.699	2	0.087	0.916
Error	316.161	79		

3.1.4. Copulation Duration Assay

One couple did not mate and was excluded from the data set. Some males transferred no or a suspicious low amount of ejaculate and the corresponding matings were also removed from the data set since those pairs most probably did not copulate successfully. Remaining replicates of the intact female treatment per selection line: L1: 18, S1: 19, L2: 20, S2: 19, L3: 20, S3: 19. Remaining replicates of the ablated female treatment per selection line: L1: 18, S1: 18, L2: 19, S2: 18, L3: 19, S3: 19.

Copulation Duration and Spine Length First, I ran an ANOVA for copulation duration to test whether the female treatment affected copulation duration, and to assess the interaction between female treatment and selection. Both, female treatment and the interaction between selection and female treatment had an impact on mating duration (Table 10). Whereat copulation duration was most dependent on female treatments, i.e. copulation duration was much longer for ablated females than for intact females (Figure 8). Selection itself also had an effect on copulation duration but only when females had ablated hind legs (see Table 10, Table 11 and Table 12). The interaction between female treatment and block had a significant effect on copulation duration, while block itself and the interactions between selection and block did not contribute to the variance in mating duration (Table 10). The same applied to the interaction between selection, female treatment and block (Table 10).

Table 10. ANOVA of copulation duration of all female treatments

Source	SS	DF	F-ratio	P-value
Selection	47.214	1	4.334	0.039
Female treatment	2076.428	1	190.586	< 0.001
Block	8.800	2	0.404	0.668
Selection*Female treatment	65.152	1	5.980	0.015
Selection*Block	58.945	2	2.705	0.069
Female treatment*Block	148.547	2	6.817	0.001
Selection*Female treatment*Block	1.599	2	0.073	0.929
Error	2331.525	214		

Additionally, I ran an ANCOVA for the copulation duration of each female treatment to see whether selection had an effect on copulation duration when females were able or not able to terminate the copulation. Since the original residuals of the copulation duration for the intact female treatment were not normally distributed, ln (natural logarithm) transformed data was used to run the ANCOVA for the intact female treatment. The residuals of copulation duration of the ablated female treatment were normally distributed and thus the original data could be used.

When analyzing the copulation duration of ablated females, selection had a big impact on copulation duration and ejaculate weight also affected copulation duration (Table 11). The effects of block and the interaction between block and selection on copulation duration were not significant (Table 11). Since male body size (ANCOVA: $F_{1,103} = 0.002$, $p = 0.966$), female body size (ANCOVA: $F_{1,99} = 0.349$, $p = 0.556$) and female age at mating (ANCOVA: $F_{1,98} = 0.938$, $p = 0.335$) did not contribute to the fit of the model they were excluded from the analysis. In contrast to the ablated female treatment, selection had no effect on copulation duration when females were intact (Table 12). All variance in copulation duration of intact females was mainly due to differences between the blocks, but the interaction between block and selection and female age at mating also affected copulation duration of intact females (Table 12). Female body size (ANCOVA: $F_{1,103} = 0.012$, $p = 0.914$), male body size (ANCOVA: $F_{1,103} < 0.001$, $p = 0.996$) and ejaculate size (ANCOVA: $F_{1,103} = 0.563$, $p = 0.455$) did not contribute to the model and were excluded from the model.

Table 11. ANCOVA of copulation duration of ablated females

Source	SS	DF	F-ratio	P-value
Selection	107.485	1	5.732	0.018
Block	104.497	2	2.786	0.066
Ejaculate weight	71.303	1	3.802	0.054
Block*Selection	28.740	2	0.766	0.467
Error	1950.325	104		

Table 12. ANCOVA table of copulation duration for intact females Since the original residuals of the mating were not normally distributed ln (natural logarithm) transformed data was used in the analysis.

Source	SS	DF	F-ratio	P-value
Selection	0.000	1	< 0.001	> 0.999
Block	1.336	2	16.900	< 0.001
Female age	0.160	1	4.050	0.047
Selection*Block	0.291	2	3.678	0.029
Error	4.231	107		

The results of a two sample T-test for ablated females and intact females respectively showed the same picture. When females could not terminate the mating (ablated female treatment), copulation duration differed significantly between the long and short spined lines (Two sample T-test: N = 6, df = 4, t = 3.905, p = 0.017) and long spined males mated for a longer time. But, when females were intact and thus able to terminate the mating, copulation duration did not differ between long and short spined males (Two sample T-test: N = 6, df = 4, t = -0.300, p = 0.779).

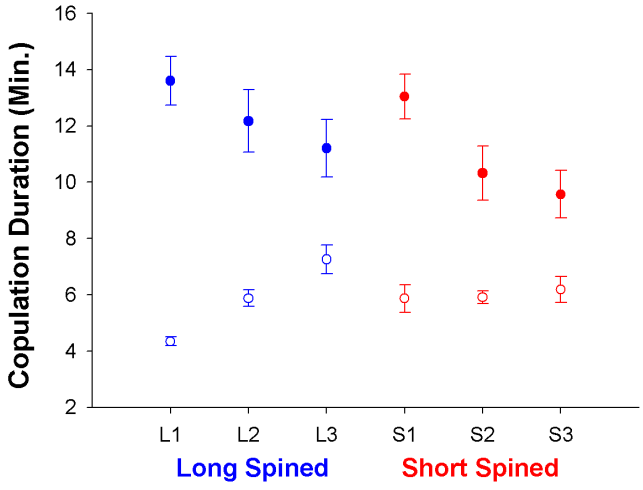

Figure 8. Means and standard errors of the copulation duration across the selection lines after five generations of genital spine selection. Hollow circles are used for the intact female treatment (i.e. females with intact hind legs), whereas filled circles are used for the ablated female treatment (i.e. females with ablated hind legs).

Copulation Duration and Ejaculate Weight To assess whether selection indirectly affected the ejaculate size of the selection lines and to test if ejaculate size was affected by male body size and female treatment I ran an ANCOVA of the ejaculate weight of the selection lines. Ejaculate size did not differ due to selection (Table 13). As mentioned before, this indicates that I was not indirectly selecting for differences in ejaculate size when selecting for male genital spine length. Ejaculate weight was significantly higher when females were ablated then when females were intact (Table 13). Furthermore, male body size affected ejaculate size significantly (Table 13) with larger males transferring larger ejaculates (see Figure 9). Copulation duration almost had an effect on ejaculate size (Table 13). The interaction between female treatment and block was also on the border to affect ejaculate size significantly (Table 13). Everything else (block, the interaction between selection and female treatment, the interaction between selection and block and the interaction between selection, female treatment and block) did not have an effect on ejaculate weight (Table 13).

Table 13. ANCOVA of ejaculate weight

Source	SS	DF	F-ratio	P-value
Selection	0.927	1	0.018	0.893
Female treatment	200.748	1	3.951	0.048
Block	22.792	2	0.224	0.799
Selection*Female treatment	19.455	1	0.383	0.537
Selection*Block	195.020	2	1.919	0.149
Female treatment*Block	271.669	2	2.273	0.071
Selection*Female treatment*Block	9.757	2	0.096	0.908
Male weight	587.799	1	11.568	0.001
Copulation duration	192.916	1	3.797	0.053
Error	10772.304	212		

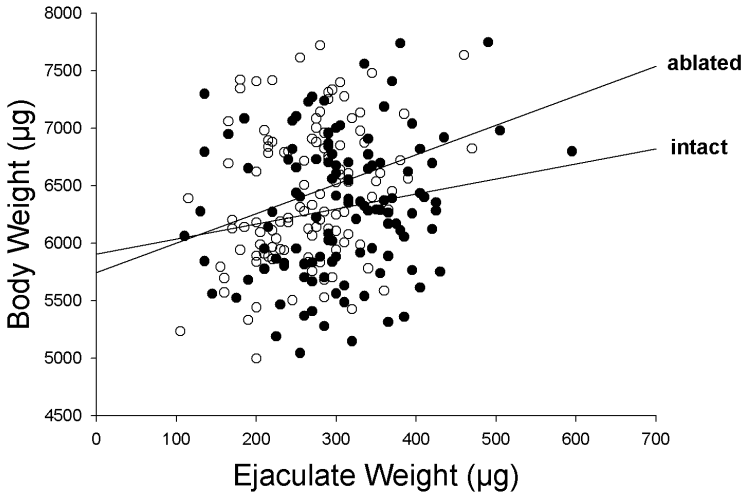

Figure 9. Correlation between male body weight and ejaculate weight of males mated to females with intact or ablated hind legs. Male body weight and ejaculate weight are shown in µg. Data points of males that mated with ablated females are filled, data points of males that mated with intact females are hollow.

3.2. Genital Spine Ablation – Laser Treatment

3.2.1. Differences between the Laser Treatments

I ran several ANOVAs to test if males of the laser treatments responded differently to the laser treatments before running the actual models for the results of the mating experiments. For most tested variables, like copulation duration, life span, number of eggs that females laid after the second mating and male body size, there was no difference between males of the different laser treatments (see Table 14). But the surgical control males of the ejaculate dispersal experiment ingested significantly more ^{14}C than the spine ablation treatments (see Table 14 and Figure 10). The last model was run with square root transformed data since the original residuals of the ingested ^{14}C amount (i.e. radio label of the males) were not normally distributed. Furthermore, the second control treatment (treatment D, where no tissue was hit by the laser and which was only used in the P2 experiment) appeared deviant. Although the effects were not significant, the D treatment had the lowest mating duration, the lowest life span, and females mated to these males laid the fewest eggs after the 2nd mating. This was not due to an incidental difference in male body size, since the D treatment males were not smaller than the males of the other treatments (Table 14).

Table 14. ANOVAs assessing for differences between all laser treatments

Response variable	Source	SS	DF	F-ratio	P-value
Copulation duration	laser treatment	7.089	3	0.226	0.878
	error	980.728	94		
Life span	laser treatment	41.348	3	1.578	0.200
	error	838.292	96		
Male body size	laser treatment	2.709	3	0.900	0.444
	error	96.291	96		
Eggs laid after 2nd mating	laser treatment	267.484	3	0.308	0.819
	error	27783.906	96		
Ingested amount of ^{14}C-label (square root transformed male ^{14}C-signal)	laser treatment	95297.785	2	4.272	0.017
	block	18708.620	1	1.677	0.199
	male size	14572.745	1	1.306	0.256
	block*treatment	85352.255	2	3.826	0.026
	error	925802.442	83		

Since the laser treatment definitely influenced the feeding behavior of the males, i.e. males of the surgical control treatment ingested significantly more radiolabel than the spine ablation treatments, it could be problematic to retain the surgical control treatment in the analysis. Similarly, the second control treatment (treatment D) also showed signs of undesirable effects. For that reason, I decided to run all analyses of the spine ablation experiment twice. Once including the two control treatments and once excluding both control treatments from the analyses.

To check if the laser treatments in which genital spines were actually ablated (i.e. treatment A and B) differed in their degree of injury, I ran the same tests as above but including treatment A and B only. The spine ablation treatments neither differed in mating duration, life span, the number of eggs laid after second mating, body size nor in the amount of ^{14}C they ingested (Table 15). Thus, the only difference between them was the number of spines that were ablated.

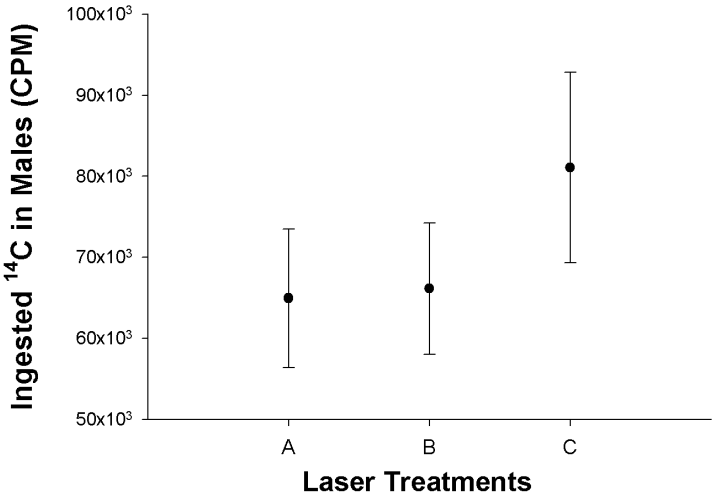

Figure 10. The amount of ^{14}C in counts per minute that was ingested by males of the different laser treatments. Shown are the means and standard errors for each treatment. In treatment A thirty genital spines were ablated. In treatment B ten genital spines were ablated. In treatment C no spines were cut by the laser but instead spine-less areas of the adeagus were hit by the laser beam 10 times.

Table 15. ANOVAs assessing for differences between treatment A and B (i.e. laser treatments in which genital spines were ablated)

Response variable	Source	SS	DF	F-ratio	P-value
Copulation duration	laser treatment	0.370	1	0.037	0.847
	error	463.935	47		
Life span	laser treatment	11.640	1	1.141	0.291
	error	479.625	47		
Male body size	laser treatment	2.005	1	2.362	0.131
	error	39.894	47		
Eggs laid after 2nd mating	laser treatment	1.661	1	0.006	0.941
	error	14037.318	47		
Ingested amount of ^{14}C-label	laser treatment	3.12240E+07	1	0.016	0.900
	block	1.43447E+10	1	7.286	0.009
	male size	2.50696E+09	1	1.273	0.264
	block*treatment	3.02728E+07	1	0.015	0.902
	error	1.12221E+11	57		

3.2.2. Sperm competition Assay

Some of the laser ablation males died before they could mate (during the recovery time and in the mating chambers) and were excluded from the analyses below (A: 6, B: 2, C: 3, D: 8). Females who did not remate and the corresponding males were also excluded from the analyses (A: 2, B: 5, C: 2, D: 2). Remaining replicates per treatment: A: 25, B: 26, C: 28, D: 23.

Sterilization Only 10 out 2737 eggs laid in between the matings (i.e. fertilized by the irradiated males) hatched. Thus, the hatching rate of eggs sired by sterilized males was only 0.4%.

Analysis of P2 I ran two ANDEVAs of the number of hatched eggs that were laid after 2nd mating one with and one without control treatments. I used binomial errors, using the total number of eggs after 2nd mating as the binomial denominator, a logit link function and an empirically derived dispersion parameter. Independent of whether the control treatments were included (Table 16) or not (Table 17), males of the different laser treatments did not differ in their fertilization success. Although the trend was in the predicted direction when the analysis was restricted to the laser treatments in which genital spines were actually ablated, i.e. P2 was lower for males that had more spines removed (see treatment A and B in Figure 11). When all treatments were included, the eggs between matings had an effect on fertilization success (Table 16). This effect was not seen when control treatments were excluded from the analysis (Table 17).

Table 16. ANDEVA of P2 of the males of the laser ablation experiment with all laser treatments included

Source	Mean deviance	DF	Deviance ratio	P-value
Laser treatment	8.37	3	0.81	0.490
Eggs between matings	46.60	1	4.52	0.036
Residual	10.31	95		
Change			1.74	0.148

Table 17. ANDEVA of P2 of the males of the laser ablation experiment without control treatments

Source	Mean deviance	DF	Deviance ratio	P-value
Laser treatment	0.00	1	< 0.001	0.984
Eggs between matings	26.68	1	2.43	0.126
Residual	10.99	46		
Change			1.21	0.306

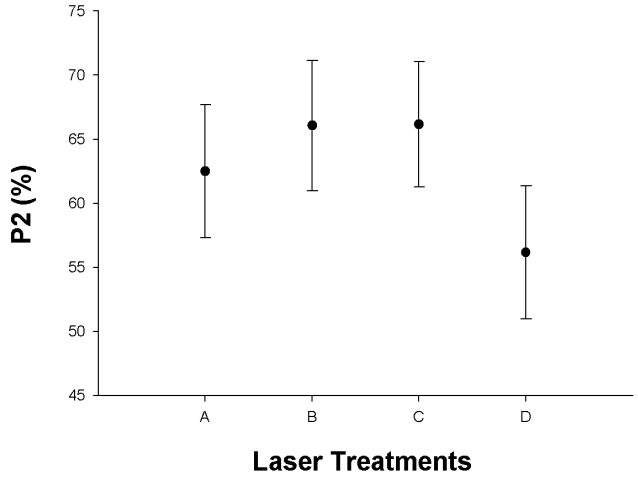

Figure 11. **Fertilization success (P2 in %) of the males of the laser treatments.** The graph shows the means and standard errors of the percentage of eggs that was fertilized by males of the different laser treatments in competition with a sterilized background male. In treatment A thirty genital spines were ablated. In treatment B ten genital spines were ablated. Treatment C served as a surgical control at which spine-less areas of the adeagus were hit with the laser beam 10 times. In treatment D the laser beam was shot 10 times besides the adeagus without hitting any tissue.

3.2.3. Ejaculate Dispersal within the Female's Body

The scintillation cocktail and the tissue solubilizer I used in this experiment (Solvable) did not completely merge when high amounts of tissue solubilizer were used. Samples containing 200 µl tissue solubilizer (rest of the female's body) stayed turbid. All the other samples (focal male, bursa copulatrix and spermatheca) cleared up after shaking them and stayed clear for the whole scintillation analysis. But, since the turbidity of all samples containing the rest of the females' bodies was constant for all samples, I decided to count them normally with the scintillation analyzer. Some females had to be excluded from the data set since a) their bursa copulatrix was accidentally ruptured during dissection, b) they did not remate or c) the scintillation readings were striking low or missing (total amount of removed females per laser treatment: A: 6, B: 8, C: 6). Remaining replicates per laser treatment: A: 28, B: 26, C: 28.

Proportion of ^{14}C-label in the rest of the females' body As for the data from the selection line experiment, I ran ANCOVAs of the ^{14}C-label that dispersed into the rest of the females' body while keeping the ^{14}C-label within the bursa copulatrix constant, to test whether the laser treatments differed in the proportion of ^{14}C that dispersed from the females' bursa copulatrix into the rest of the females' body. When all laser treatments were included into the model, the original residuals of the amount of ^{14}C in the rest of the females' body were not normally distributed. Thus, square root transformed data was used for the analysis. Furthermore two cases with student < 3 were excluded from the analysis. When the control treatment was excluded, the original data could be used since the residuals were normally distributed then. Both, when the control treatment was included (Table 18) and excluded (Table 19) from the analysis, the laser treatments did not significantly differ in the proportion of ^{14}C that left the bursa and dispersed into the rest of the female's body. But in both cases focal female size contributed significantly to the variance of the proportion of ^{14}C in the rest of the females' body (see Table 18 and Table 19), whereat more radiolabel dispersed throughout the body of large females (Pearson Correlation: R = 0,279, N = 82, Least Square Regression: t = 2.601, N = 82). When the control was included, the number of eggs a female laid significantly affected the proportion of ^{14}C that dispersed into the rest of the females' body (Table 18). Interestingly, the more eggs females laid the more radio label entered the haemolymph or vice versa (Pearson Correlation: R = 0.278, N = 82, Least Square Regression: t = 2.591, N

= 82). This effect was much less important and not significant when the control was excluded from the analysis (Table 19). All other tested factors and interactions did not have an effect on the radio label in the rest of the females' body (see Table 18 and Table 19).

But even though the laser treatments did not significantly differ in their ^{14}C-disperal, the trend pointed into the predicted direction when the control treatment was excluded, i.e. less radio label dispersed throughout females that mated to males with more spines ablated (Figure 12).

Table 18. ANCOVA of the amount of ^{14}C-label that left the bursa copulatrix and dispersed within the female's body including the control treatment

Source	SS	DF	F-ratio	P-value
Laser treatment	704.552	2	1.702	0.190
Block	138.047	1	0.667	0.417
Signal in bursa	31311.323	1	151.310	< 0.001
Focal female size	1564.084	1	7.558	0.008
Eggs total	1076.091	1	5.200	0.026
Laser treatment*Block	548.670	2	1.326	0.272
Error	14899.363	72		

Table 19. ANCOVA of the amount of ^{14}C-label that left the bursa copulatrix and dispersed within the female's body without the control treatment

Source	SS	DF	F-ratio	P-value
Laser treatment	2124050.831	1	1.255	0.268
Block	354774.107	1	0.210	0.649
Label in bursa	1.37055E+08	1	80.998	< 0.001
Female size	7822650.100	1	4.623	0.037
Eggs total	5186777.064	1	3.065	0.087
Block*Laser treatment	3802461.294	1	2.247	0.141
Error	7.95281E+07	47		

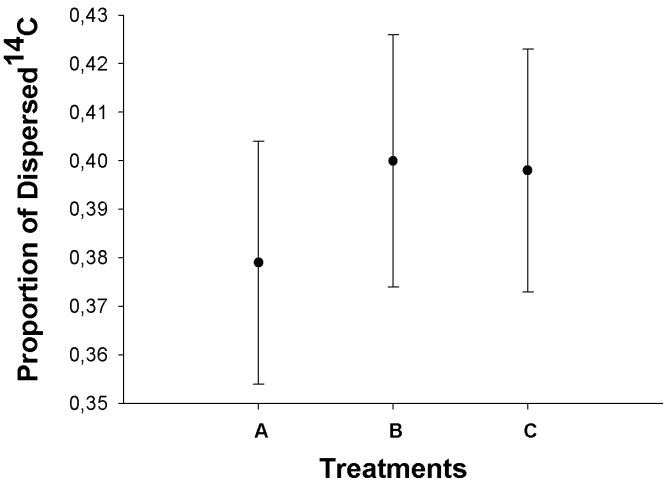

Figure 12. The proportion of ^{14}C that left the bursa copulatrix and dispersed into the rest of the females' bodies. The graph shows the means and standard errors of the dispersed radio label of females mated to radio labeled males of the different laser treatments. In treatment A thirty genital spines of the males were ablated. In treatment B only ten genital spines of the males were ablated. Treatment C served as a surgical control at which spine-less areas of the adeagus were ten times hit with the laser beam.

^{14}C-Label in the Spermatheca Since I used the same method to determine the amount of ^{14}C-label in the spermatheca as in the experiment using the selection lines, I assume that the variation in the spermathecal readings of the laser ablation experiment also reflects contamination rather than anything else. Therefore, I excluded the spermathecal data from the analyses.

4. Discussion

First, I will discuss which hypotheses of the functional significance of genital spines are supported or not supported by my results (sections 4.1. and 4.2.). I will then look at my results in respect to the evolution of genital morphology and the evolution of harmful male traits (section 4.3. and 4.4.). Finally, I will discuss artificial spine selection and experimental spine ablation as methods to explore the function of genital spines (section 4.5.). Since some of the experimental spine ablation treatments I conducted appeared unsuccessful and are therefore hard to interpret, I will mainly discuss the results of the selection line experiments. Thus, if not otherwise mentioned, all interpretations will refer to the results of the selection line experiments.

4.1. Genital Spines, Ejaculate Dispersal and Male Fertilization Success

My results strongly indicate that the genital spines of male *C. maculatus* serve in sperm competition by puncturing the female reproductive tract to increase the ejaculate dispersal throughout the females' body. Males of the long spined selection lines fertilized more eggs in the sperm competition assay than males of the short spined selection lines, which indicates that spine length is linked to male fertilization success and that males with longer spines fertilize more eggs under sperm competition. This result is in agreement with the results of a previous comparative study in *C. maculatus*, where populations with longer genital spines had a higher fertilization success, when mated to females of a background population, than populations with shorter genital spines (Hotzy & Arnqvist 2009). Both studies indicate that genital spines serve to increase male fertilization success and that male fertilization success is linked to genital spine length. Furthermore, and this has not been shown before, my results demonstrate that ejaculate passage throughout the females' body is higher when females mate to long spined males. As far as I know, this is the first experimental evidence for the hypothesis that genital spines may serve to increase the passage of accessory seminal substances into the females' haemolymph, although it has been suggested in different insects (Lewis & Pollock 1975; Merritt 1989; Eberhard 1996; Eberhard 1998; Crudgington & Siva-Jothy 2000). For instance, Lewis & Pollock (1975) and Eberhard (1998) assumed that genital spines in the blowfly *Lucilia*

sericata serve this function. They based this suggestion on the fact that the spiny adeagus of *Lucilia sericata* has three openings, one terminal sperm exit and two lateral exits for accessory seminal substances, and that most of the spines are located next to the lateral apertures. A similar genital morphology is found in *Lucilia cuprina* and was also suggested to improve the uptake of accessory seminal substances (Merritt 1989). Furthermore, Crudgington & Siva-Jothy (2000) suggested that the genital spines of *C. maculatus* might serve the same function and pierce the reproductive tract to increase the passage of accessory seminal substances into the female haemolymph. This suggestion is now confirmed by my results. In addition, Crudgington & Siva-Jothy (2000) suggested that the increased passage of accessory seminal substances could lead to an increased oviposition rate due to accessory seminal substances. This seemed likely, since accessory seminal substances have a gonadotropic effect in many insect species (Pickford et al. 1969; Das et al. 1980; Chen 1984; Chen et al. 1988; Gillott 2003; Arnqvist & Rowe 2005). Furthermore, a recent study showed that oviposition is in fact induced by male-derived extracts in *C. maculatus* (Yamane & Miyatake 2010a). Yet, I did not find any evidence for a gonadotropic effect in *C. maculatus* due to spine length. Females mated to males of the long spined lines did not lay more eggs after mating than females mated to males of the short spined lines, despite the fact that more radiolabeled ejaculate entered the haemolymph of females mated to long spined males. It is interesting that I did not find a gonadotropic effect due to spine length, especially since Yamane & Miyatake (2010a) detected accessory seminal substances that induced oviposition in *C. maculatus*. However, a possible explanation for this could be that only a certain amount of the seminal substance is needed to trigger oviposition and that increasing amounts of the corresponding seminal substance do not further increase oviposition rate after a certain threshold is reached. In this case, maybe both the long and the short spined males already reached this level of stimulation and thus no difference in oviposition rate was detectable in my study. Hence, it would be interesting to test the effect of different amounts of the male derived substances on oviposition rate in *C. maculatus*, to see if this assumption can be confirmed. Since I did not detect any difference in the gonadotropic response due to spine length, the differences in male fertilization success I detected cannot be explained by a gonadotropic effect. However, accessory seminal substances do not have to modulate male fertilization success by increasing the oviposition rate. It is known that they influence male fertilization success in many other ways. For instance, accessory seminal substances of the acarid mite *Caloglyphus berlesei* may reposition rival sperm into the

distal end of the spermatheca so that its usage for fertilization is unlikely or reduced (Radwan & Witaliński 1991). In Drosophila accessory seminal substances influence sperm displacement (i.e. sperm removal from spermatheca by the female) and thereby affect male fertilization success (Clark et al. 1995). Sperm displacement has been reported in *C. maculatus* (Eady 1994b) and is likely to be affected by accessory seminal substances. Moreover, it has also been shown that male derived extracts inhibit female mating receptivity in *C. maculatus* (Yamane et al. 2008; Yamane & Miyatake 2010b). In the current study I only measured differences in P2 between long and short spined males and did not investigate the effects of spine length on female mating receptivity. Thus, the effect of spine length on male fertilization success I measured was not due to differences in female mating receptivity. However, since I could show that ejaculate dispersal increases with spine length in *C. maculatus,* spine length could potentially influence the strength of the inhibiting effects on female mating receptivity detected by Yamane et al. (2008). This means that long spined males could potentially more efficiently inhibit female remating behavior. Thus, it would be interesting to measure the effects of spine length on female mating receptivity. Since the increased fertilization success of long spined males can neither be explained by increased oviposition nor by a decreased remating probability of the females, it very is likely that either additional accessory seminal substances – which have not been detected in *C. maculatus* so far – or, additional functions of the detected accessory seminal substances influenced the P2 I measured in my experiments. However, further investigations are needed, to detect which accessory seminal substances influence male fertilization success in *C. maculatus* and which particular functions they serve.

4.2. Genital Spines and Copulation Duration

It has also been suggested that the genital spines in *C. maculatus* serve as an anchor during copulation to prolong copulation and to prevent either the female or rival males to terminate the copulation too soon (Simmons 2001; Morrow et al. 2003; Edvardsson & Tregenza 2005; Rönn et al. 2007). Yet, I did not find any effect of spine length on copulation duration when females were able to terminate matings. Hence, my results do not support the suggestion that genital spine length is linked to copulation duration and thus do not support the anchor hypothesis. However, copulation duration was affected by

spine length when females could not terminate the copulation, with long spined males mating for a longer time than short spined males. This could indicate two things: First, copulation duration could be prolonged by long spines if it takes long spined males longer to either position the adeagus within the female or to loosen themselves from the female. Second, the optimal copulation duration could differ between long and short spined males, if long spined males also transfer larger ejaculates. Yet, ejaculate size did not differ between long and short spined males and thus it is unlikely that their optimal copulation duration differs. Therefore, it is more likely that long spined males face mechanical difficulties, either when freeing them from the female at the end of the copulation, or when positioning the adeagus within the female at the beginning of the copulation. However, my data indicate that the spines do not prevent females from terminating the matings earlier than beneficial for the male. In addition, a comparative study did not find an effect of rival males on copulation duration of long and short spined *C. maculatus* populations, which suggests that moreover the spines do not serve to hinder rival males to dislodge the copulating male from the female (Rönn et al. unpublished data). Thus, although I cannot exclude that the spines might have evolved as an anchor, there is no evidence that an anchor ability of the spines leads to the maintenance of long genital spines, since the anchor ability of the spines does not differ within the natural variance of genital spine length of the populations today.

4.3. Genital Evolution and Sexual Selection

Three main hypotheses have been invoked to explain the remarkable rapid evolution of genitalia: 1) the lock and key hypothesis, 2) the pleiotropy hypothesis and 3) sexual selection hypothesis. 1) Under the lock and key hypothesis male and female genitalia are assumed to serve as a species specific lock and key system to avoid hybridization, and thus genital variance has to be extremely high to ensure that the lock and key system works (Dufour 1844 in Shapiro & Porter 1989). 2) According to the pleiotropy hypothesis the rapid evolution of genital morphology can be explained by selection on non-genitalic traits that are genetically correlated with genitalic traits. As mentioned in the introduction most empirical evidence contradicts both the lock and key hypothesis and the pleiotropy hypothesis and supports the connection between sexual selection and genital evolution instead. My results also provide further evidence that genital evolution is driven by sexual

selection and that the genital spines in *C. maculatus* evolve via sexual selection. This is in agreement with many studies showing the importance of sexual selection in rapid genital evolution and genital divergence (Arnqvist 1998; Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; House & Simmons 2003; Takami 2003; Bertin & Fairbairn 2005; House & Simmons 2005; Wenninger & Averill 2006; Hotzy & Arnqvist 2009; Polak & Rashed 2010). Under the sexual selection hypothesis, sperm competition and cryptic female choice are assumed to shape genital evolution and to fuel genital divergence (Hosken & Stockley 2004). In the current study, however, I could not distinguish, whether sperm competition, cryptic female choice or both of those mechanisms were responsible for the increased fertilization success (measured as P2) of long spined males, since the method I used to measure male fertilization success does not allow such conclusions. However, the increased ejaculate dispersal in long spined males suggests, that the spines are a product of sperm competition rather than cryptic female choice. Female sperm choice due to male genital spine length would be possible in *C. maculatus* if male spine length is correlated to male quality. Tadler (1999) demonstrated cryptic female choice in the seed bug Lygaeus simulans, where females modulate the males' fertilization success depending on the genitalic traits of the males. Thus, it would be interesting to conduct experiments that show whether the selection on genital spine length in *C. maculatus* is due to sperm competition, cryptic female choice or both of those mechanisms.

4.4. Pleiotropic vs. Adaptive Harm of the Genital Spines

As mentioned in the introduction, two theories are used to explain harmful male traits that are costly to females such as the genital spines in *C. maculatus*: a) the adaptive harm hypothesis and b) the pleiotropic harm hypothesis. Male induced harm in females is assumed to be adaptive if the harm itself is beneficial for the male. For instance, harm could prohibit or decelerate females' remating behavior and decrease the chance of future sperm competition if the females respond to the harm by investing more into their current reproduction (Johnstone & Keller 2000; Morrow et al. 2003). In contrast, harm is assumed to be pleiotropic if the induced harm is a by-product of another function of the harmful trait (Parker 1979; Morrow et al. 2003; Parker 2006). My study suggests strongly that the inflicted harm in *C. maculatus* is pleiotropic. My results show that the injuries in

females caused by the spines increase the dispersal of seminal fluid throughout the females' body and that this is linked to male fertilization success. This suggests that the spines serve to increase ejaculate dispersal within the female and that the harm is a side-effect of that function rather than that the injuries are induced to harm the female *per se*. Furthermore, assuming that the inflicted harm increases with spine length, oviposition rate was not linked to the strength of harm in this study, since females did not lay more eggs when they were mated to long spined and thus more harmful males. This indicates that the oviposition rate was not dependent on the strength of the harm as expected under the adaptive harm hypothesis. Thus, adaptive harm is even more unlikely. Hence, my findings provide more evidence for the pleiotropic harm hypothesis and are in concert with previous studies, which have indicated that the harm in *C. maculatus* is pleiotropic rather than adaptive (Morrow et al. 2003; Edvardsson & Tregenza 2005; Hotzy & Arnqvist 2009; Gay et al. 2010).

4.5. Artificial Spine Selection and Experimental Spine Ablation

Artificial spine selection worked well and turned out to be a good method to generate males with different genital spine lengths. In all six selection lines, spine length responded in the predicted direction to the selection process. Furthermore, spine length showed significant heritability. In contrast, the genital spine ablation using the laser edge-ablation system did not work satisfying, since the control treatments appeared deviant and the spine ablation treatments did not differ significantly from each other. However, I want to point out that these problems were most likely the result of choosing imperfect spine ablation treatments and control treatments and that laser surgery has been used successfully when investigating the function of male genitalic traits in *Drosophila bipectinata Duda* (Polak & Rashed 2010). Looking at the average 230 spines possessed on the adeagus of *C. maculatus*, ablating 10 (i.e. ~ 4%) and 30 (i.e. ~ 13%) spines was probably not enough to see a significant difference in P2 or ejaculate dispersal between the different treatments. Furthermore, the control treatments I choose turned out to be problematic, since the amount of injury was probably different between the control treatments and the spine ablation treatments. This is indicated by the fact that the control males ingested significantly more radio label than the spine ablated males. If this was due to more severe, or less severe injuries caused by the laser treatment is hard to say, but this

difference made the control treatments non-informative. Nevertheless, I think it would be worth to repeat the laser surgery experiment conducting stronger spine ablation treatments and a better control treatment instead, especially since the results of the spine ablation indicate that the expected treatment effects were potentially there, but probably too weak to be detected. In both, the P2 experiment and the ejaculate dispersal experiment, the trends pointed into the predicted directions when the control treatments were excluded from analysis (i.e. P2 and ejaculate dispersal were both lower for males with more spines removed). By removing more spines it could be possible to detect differences between males with more and less genital spines removed. Furthermore, the weak spine ablation treatment I conducted (only 10 genital spines removed) might be a good control treatment for future experiments. A spine ablation experiment with more reasonable treatments could thus provide a more direct picture of the functional significance of the genital spines in *C. maculatus*.

4.6. Conclusion

My results strongly suggest that the genital spines serve to increase the males' fertilization success by increasing the passage of seminal fluid into the female haemolymph. This provides further evidence for sexual selection as an engine that drives genital evolution and for the link between genital morphology and male fertilization success. Moreover, my results suggest that the harm inflicted in females due to the genital spines is of pleiotropic rather than of adaptive nature. In addition, I did not find an effect of genital spine length on copulation duration in *C. maculatus* and thus my data does not support the anchor hypothesis.

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Zusammenfassung

Genitalstacheln, -haken und dergleichen, die Weibchen während der Kopulation verletzen, sind bei Männchen vieler Tierarten zu finden. Die Funktion solcher Genitalstrukturen und die Kräfte, die zu ihrer Evolution und Beibehaltung führen, werden seit Jahrzenten diskutiert. Der Adeagus von *Callosobruchus maculatus* (Coleoptera: Bruchidae) beispielsweise ist mit Stacheln versehen, die den weiblichen Geschlechtstrakt durchbohren und Narben hinterlassen. Eine komparative Studie hat gezeigt, dass Männchen mit längeren Genitalstacheln unter Spermienkonkurrenz höhere Befruchtungserfolge erzielen als kurzstachelige Männchen. Auf welche Weise die Stacheln den Befruchtungserfolg erhöhen, blieb allerdings ungeklärt. Im Rahmen meiner Diplomarbeit habe ich zwei Hypothesen über die Funktion der Genitalstacheln untersucht, die Perforierungshypothese und die Ankerhypothese.

Die Perforierungshypothese besagt, dass die Genitalstacheln dazu dienen, den weiblichen Geschlechtstrakt zu perforieren, damit mehr Ejakulat der Männchen in die Hämolymphe der Weibchen gelangt. Das ist für die Männchen vorteilhaft, da sich im Ejakulat Substanzen befinden, die das weibliche Verhalten zugunsten der Männchen manipulieren. Obwohl angenommen wird, dass Genitalstacheln bei mehreren Insekten diese Funktion haben, ist diese Diplomarbeit meines Wissens die erste Studie, in der diese Hypothese überprüft wird. Laut der zweiten Hypothese – der Ankerhypothese – dienen die Genitalstacheln als Anker während der Kopulation, um zu verhindern, dass die Weibchen die Kopulation früher beenden, als es für die Männchen optimal ist. Dieser Hypothese zufolge ist zu erwarten, dass langstachelige Männchen länger kopulieren als kurzstachelige Männchen, da sich längere Stacheln effektiver verankern sollten.

Um die Perforierungshypothese zu prüfen, wurden lang- und kurzstachelige Männchen mit ^{14}C markiert und untersucht, ob sich das Ejakulat von langstacheligen Männchen besser im weiblichen Körper ausbreitet. Die Ankerhypothese wurde getestet, indem die Kopulationsdauer von Paaren verglichen wurde, bei denen die Männchen entweder lange oder kurze Genitalstacheln hatten. Zusätzlich wurde überprüft, ob langstachelige Männchen unter Spermienkonkurrenz einen höheren Befruchtungserfolg haben, da in beiden Hypothesen davon ausgegangen wird, dass das so ist. Die lang- und

kurzstacheligen Männchen, die in den Versuchen verwendet wurden, wurden mit zwei sich ergänzende Methoden erzeugt. Zum einen wurden Selektionslinien mit langen und kurzen Stacheln gezüchtet, zum anderen wurden Genitalstacheln mit einem Mikrolaser gekürzt, um Männchen mit unterschiedlicher Stachellänge zu erzeugen.

Langstachelige Männchen kopulierten nicht länger als kurzstachelige Männchen, daher sprechen die Resultate gegen die Ankerhypothese. Des Weiteren zeigte es sich, dass sich mehr Ejakulat von langstacheligen als von kurzstacheligen Männchen im Körper des Weibchens ausbreitete und dass langstachelige Männchen unter Spermienkonkurrenz einen höheren Prozentsatz von Eizellen befruchteten als kurzstachelige Männchen. Diese Resultate sprechen dafür, dass die Genitalstacheln von *C. maculatus* die Ausbreitung des Ejakulats im weiblichen Körper fördern, und dass dies langstacheligen Männchen Befruchtungsvorteile verschafft. Die Studie illustriert darüber hinaus, wie sexuelle Selektion – in Form von Spermienkonkurrenz – die Morphologie von männlichen Genitalien beeinflussen kann.

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