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Chapter 5

Sexual functionality of parthenogenetic *Tetrastichus coeruleus* (Hymenoptera: Eulophidae)

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Manuscript

Wolbachia are endosymbiotic bacteria known to manipulate the reproduction of their hosts, for example by inducing parthenogenesis. In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females. In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of such traits may lead to their loss or deterioration. In addition, females may loose the ability to reproduce sexually due to ‘functional virginity mutations’ that may spread concomitantly with the *Wolbachia* infection through a population. The parasitoid wasp *Tetrastichus coeruleus* forms an ideal model to study the decay of sexual functionality, because it has both *Wolbachia*-infected populations that reproduce parthenogenetically and uninfected, sexual populations. We compared the sexual functionality of arrhenotokous (sexual) and thelytokous (parthenogenetic) *T. coeruleus* females.

Mated females had a shorter life span than virgin females, showing that either mating or sperm storage was costly. Several sexual traits of thelytokous females have degraded compared to arrhenotokous females. Arrhenotokous and thelytokous females were equally attractive to males, but thelytokous females were unreceptive to males. Furthermore, there was a clear difference in spermathecal morphology between arrhenotokous and thelytokous females. Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and spermathecal morphology of thelytokous females. However, we cannot exclude that these traits have degraded due to functional virginity mutations or accumulation of neutral mutations.

Introduction

Wolbachia are maternally inherited, intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. It has been estimated that 66% of all insect species are (partly) infected with *Wolbachia* (Hilgenboecker *et al.* 2008). To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). In haplodiploids, PI-*Wolbachia* cause diploidization of the haploid eggs by alteration of meiotic and/or mitotic processes (Stouthamer & Kazmer 1994, Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a) resulting in the production of daughters from unfertilized eggs (thelytoky).

In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females (Huigens & Stouthamer 2003). In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of costly sexual traits may lead to their loss or deterioration (Carson *et al.* 1982, Pijls *et al.* 1996, Pannebakker *et al.* 2005, Kraaijeveld *et al.* 2009). Because male traits are not expressed in parthenogenetic populations, they are likely to degenerate due to neutral mutations. Female traits, which are expressed but not used, may also be selected against when they are costly to maintain (Pijls *et al.* 1996). In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010). Virginity mutations arise during the early stages of PI-*Wolbachia* infection and affect traits in females involved in sexual reproduction, e.g. mating or egg fertilization. Accumulation of neutral mutations or selection against the maintenance of costly traits arise after a longer period of parthenogenetic reproduction and can potentially affect all traits involved in sexual reproduction, both in males and females, e.g. courtship behaviour or pheromone production.

Sexual traits may evolve to be costly when the reproductive interests of males and females are different. Sexual conflict may result in sexually antagonistic coevolution, in which males evolve adaptations that manipulate female behaviour and females evolve resistance to male manipulation (Rice 1996, Chapman *et al.* 2003, Arnqvist & Rowe 2005). In the absence of males, selection on manipulative male traits and costly female resistance traits will be absent. Adaptations evolved under sexually antagonistic coevolution are released from selection and alleles that were favoured can be replaced by others due to accumulation of random mutations (Carson *et al.* 1982, Pijls *et al.* 1996) or antagonistic pleiotropy (Pijls *et al.* 1996, Pannebakker *et* *al.* 2005). Therefore, thelytokous females may be either more receptive to mating when no males are present, which is the case in the parasitoid wasp *Leptopilina clavipes* (Kraaijeveld *et al.* 2009), or not receptive at all when a few males are still present.

Many insect species with female-only populations exhibit deterioration or loss of female sexual traits, as expected by theory. In the parasitoid wasp *Apoanagyrus diversicornis*, arrhenotokous and thelytokous females were equally attractive, but thelytokous females mated less often than arrhenotokous females (Pijls *et al.* 1996). Similarly, asexual *Drosophila mercatorum* females mated less often than sexual females (Carson *et al.* 1982). Thelytokous females of the parasitoid wasp *Muscidifurax uniraptor* were attractive to males, but never mated with them (Gottlieb & Zchori-Fein 2001). In contrast, thelytokous females of the parasitoid wasp *Leptopilina clavipes* mated more often than arrhenotokous females and copulations with thelytokous females lasted longer (Kraaijeveld *et al.* 2009). Differences between thelytokous and arrhenotokous females could also be found in the morphology of the sexual organs. Spermathecae are organs in which females store sperm after mating until oviposition. In the parasitoid wasp *L. clavipes*, there was a clear difference in spermathecal morphology (Kraaijeveld *et al.* 2009). Moreover, the spermathecae of thelytokous females were less capable of storing sperm than the ones of arrhenotokous females (Kraaijeveld *et al.* 2009). In the parasitoid wasp *M. uniraptor*, thelytokous females lacked a major muscle in their spermathecae, which were therefore considered to be vestigial and not capable of storing and transporting sperm (Gottlieb & Zchori-Fein 2001).

The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) forms an ideal model to study the decay of sexual functionality, because it has both arrhenotokous and thelytokous populations (chapter 2, Reumer *et al.* 2010). *T. coeruleus* is an egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. Like all Hymenoptera, *T. coeruleus* is haplodiploid. Populations from the Dutch coastal dune areas are infected with *Wolbachia* and reproduce parthenogenetically (thelytoky), while populations from the Dutch agricultural fields are not infected and reproduce sexually (arrhenotoky) (chapter 2, Reumer *et al.* 2010). The arrhenotokous and thelytokous populations diverged between 7.5 x 10^5 and 2.0 x 10^5 generations ago (chapter 3, Reumer *et al.* in prep). The *Wolbachia* infection and thelytokous reproductive mode are probably as old as this divergence. This has given all three mutation accumulation possibilities (neutral mutation accumulation, selection against the maintenance of costly traits and functional virginity mutations) time to act on traits involved in sexual reproduction in the thelytokous populations.

In this paper, we compared the sexual functionality of arrhenotokous and thelytokous *T. coeruleus* females. We predicted that several sexual traits should have degraded in thelytokous females. First, we examined whether mating is costly to females by measuring the life span of mated and virgin females. Second, we tested whether arrhenotokous and thelytokous females were equally attractive and receptive to males. Last, we studied the morphology of the spermathecae of arrhenotokous and thelytokous females.

Materials and Methods

Tetrastichus coeruleus populations

Two populations of the parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) were used in all experiments. One population was sampled from agricultural fields in Brabant (BR), The Netherlands. This population is not infected with *Wolbachia* and reproduces arrhenotokously (chapter 2, Reumer *et al.* 2010). The other population was sampled from the natural dune area Meijendel (MD), The Netherlands. This population is infected with *Wolbachia* and reproduces thelytokously (chapter 2, Reumer *et al.* 2010). More details about the sampled locations can be found in chapter 2, Reumer *et al.* (2010). In the spring and summer of 2010 individuals from both populations were obtained from the field by collecting larvae of the asparagus beetle *Crioceris asparagi*, the host of *T. coeruleus*. These larvae were reared in the lab at 20°C, light:dark 16:8, 65% relative humidity. Some of these had been parasitized by *T. coeruleus*, in which case wasps in stead of beetles would emerge from the pupae. The sex of each wasp was determined by looking under a binocular for the presence of a groove on the ventral side of the abdomen which envelopes the ovipositor. In males this groove is absent.

Cost of mating

To test whether mating is costly to females, we measured the life span of all females from the mating experiment (described below) that mated with a male. After mating, the female was transferred to a glass tube (2.5 x 8.0 cm) with a medium of agar and a foam stopper with a drop of honey. As a control we measured the life span of virgin females that never were exposed to male courtship or mating. All females were kept at 20°C, light:dark 16:8, 65% relative humidity. Female deaths were scored daily, until all females were dead.

Courtship and mating

To test whether arrhenotokous and thelytokous females differ in attractiveness and/or receptiveness towards males, we performed a mating experiment. Zero-to-four-day-old virgin arrhenotokous and thelytokous females were put individually in plastic tubes (1.0 x 5.0 cm), with small-mesh gauze on one side and a rubber stopper on the other side. One male from the arrhenotokous population was introduced into each tube and courtship behaviour and mating were scored. After one hour or after mating, whichever came first, the trial was stopped. Because the first element of the male courtship sequence is wing fanning, we used male wing fanning as a measure of female attractiveness. Because females may be attractive but not receptive to males, we used mating as a measure of female receptiveness. Both traits (attractiveness and receptiveness) were scored as dichotomous variables (yes/no).

Spermathecae

To examine differences in spermathecal morphology, we visualized the spermathecae of zero-to-ten-day-old virgin arrhenotokous and thelytokous females. The females were dissected in a drop of demineralized water, and the spermathecae visualized using a Nikon Labophot binocular microscope (Nikon Corporation, Tokyo, Japan) and photographed at 40x magnification. Subsequently, the length and width of the spermathecae were measured by analysing the photos using Adobe Photoshop CS4 (version 11.0; Adobe Systems, San Jose, CA, USA). To examine spermathecal shape, we used the ratio between length and width of the spermathecae for the analysis.

Statistical analysis

Statistical analyses were performed in R (version 2.12.1; R Developmental Core Team, 2010).

Survival analysis was used to test for differences in life span between mated and virgin females. Significance of the explanatory variable (mated or not) was examined in a cox proportional hazard model using a score log rank test.

Generalized linear models (glm) with a binomial error distribution were used to test for differences in male wing fanning and mating between arrhenotokous and thelytokous females. In the wing fanning model, the number of trials with males that showed wing fanning was used as the response variable and the total number of trials as the binomial denominator. In the mating model, the number of trials that ended with a mating was used as the response variable and the total number of trials as the binomial denominator. Significance of explanatory variables (female population and female age) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in deviance to a χ^2-distribution.

Analysis of covariance (ancova) was used to test for differences in the ratio between length and width (elongation) of the spermathecae between arrhenotokous and thelytokous females. Significance of explanatory variables (female population and female age) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in variance using an F-test.

Results

Cost of mating

We measured the life span of 20 females from the mating experiment that had mated with a male and that of 20 virgin females. All females were from the arrhenotokous population. Virgin females (mean survival = 30.95 days) had significantly longer life spans than females that had mated with a male (mean survival = 25.15 days) (Fig. 5.1; $Z = 3.92$, $df = 1$, $p = 0.048$).

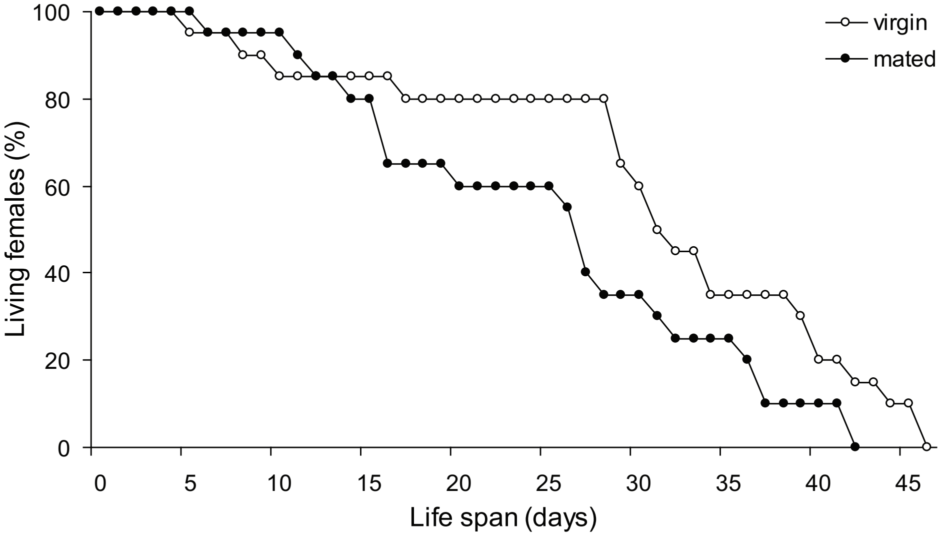

Figure 5.1: Survival probability of arrhenotokous *Tetrastichus coeruleus* females. Open circles: virgin females, solid circles: mated females.

Courtship and mating

In total, 96 individual females were tested: 50 arrhenotokous females and 46 thelytokous females. Males showed wing fanning in 31 of the 50 trials (62%) with arrhenotokous females and in 30 of the 46 trials (65%) with thelytokous females. There were no significant differences in wing fanning frequency between female populations, female age or their interaction (Fig. 5.2; overall: $\Delta_{deviance} = 3.92$, $df = 3$, $p = 0.27$; interaction: $\Delta_{deviance} = 0.0001$, $df = 1$, $p = 0.99$; female population: $\Delta_{deviance} = 0.11$, $df = 1$, $p = 0.74$; female age: $\Delta_{deviance} = 3.82$, $df = 1$, $p = 0.051$).

Twenty of the 50 trials (40%) with arrhenotokous females ended with a mating, while none of the thelytokous females mated with a male. All of the matings were preceded by male wing fanning behaviour. Arrhenotokous and thelytokous females differed significantly in mating frequency (Fig. 5.2; overall: $\Delta_{deviance} = 31.23$, $df = 3$, $p < 0.0001$; female population: $\Delta_{deviance} = 30.95$, $df = 1$, $p < 0.0001$). There were no significant effects of female age or the interaction between female age and female population on mating frequency (interaction: $\Delta_{deviance} < 0.0001$, $df = 1$, $p = 1.00$; female age: $\Delta_{deviance} = 0.22$, $df = 1$, $p = 0.64$).

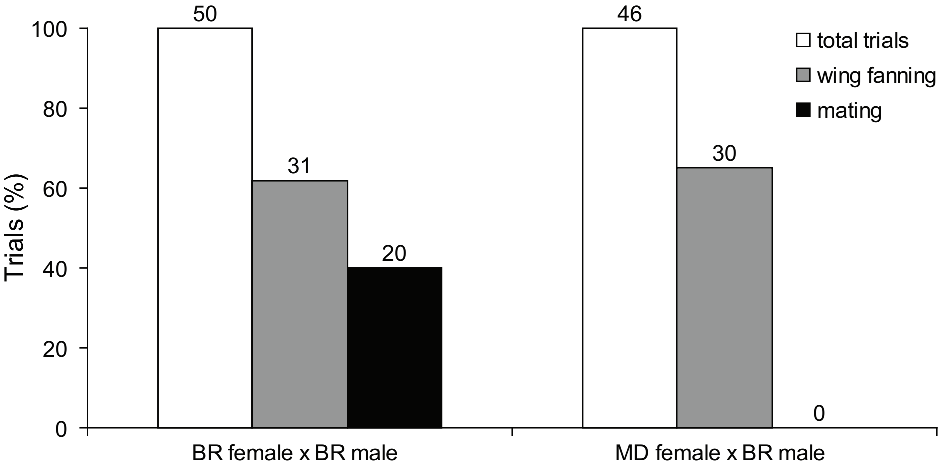

Figure 5.2: Percentage of trials in which arrhenotokous (BR) *Tetrastichus coeruleus* males exhibited wing fanning behaviour and percentage of trials that ended with a mating, for arrhenotokous (BR) and thelytokous (MD) *T. coeruleus* females. White bars: total number of trials, grey bars: percentage of trials in which males showed wing fanning behaviour, black bars: percentage of trials that ended with a mating. Number of trials are indicated above the bars.

Spermathecae

In total, we visualized and measured the spermathecae of 55 females: 30 from arrhenotokous females and 25 from thelytokous females. All spermathecae from arrhenotokous females were circular with a smooth surface (Fig. 5.3A). Five of the spermathecae from thelytokous females were similar to those from arrhenotokous females, while the other 20 spermathecae were more elongated and often had bulges (Fig. 5.3B-D). The spermathecae of arrhenotokous and thelytokous females differed significantly in the ratio between length and width of the spermathecae (overall: $F_{3,51} = 7.70$, $p = 0.0002$; female population: $F_{1,53} = 23.46$, $p < 0.0001$). There were no significant effects of female age or the interaction between female age and female population on the ratio between length and width of the spermathecae (interaction: $F_{1,51} = 0.14$, $p = 0.71$; female age: $F_{1,53} = 0.83$, $p = 0.37$).

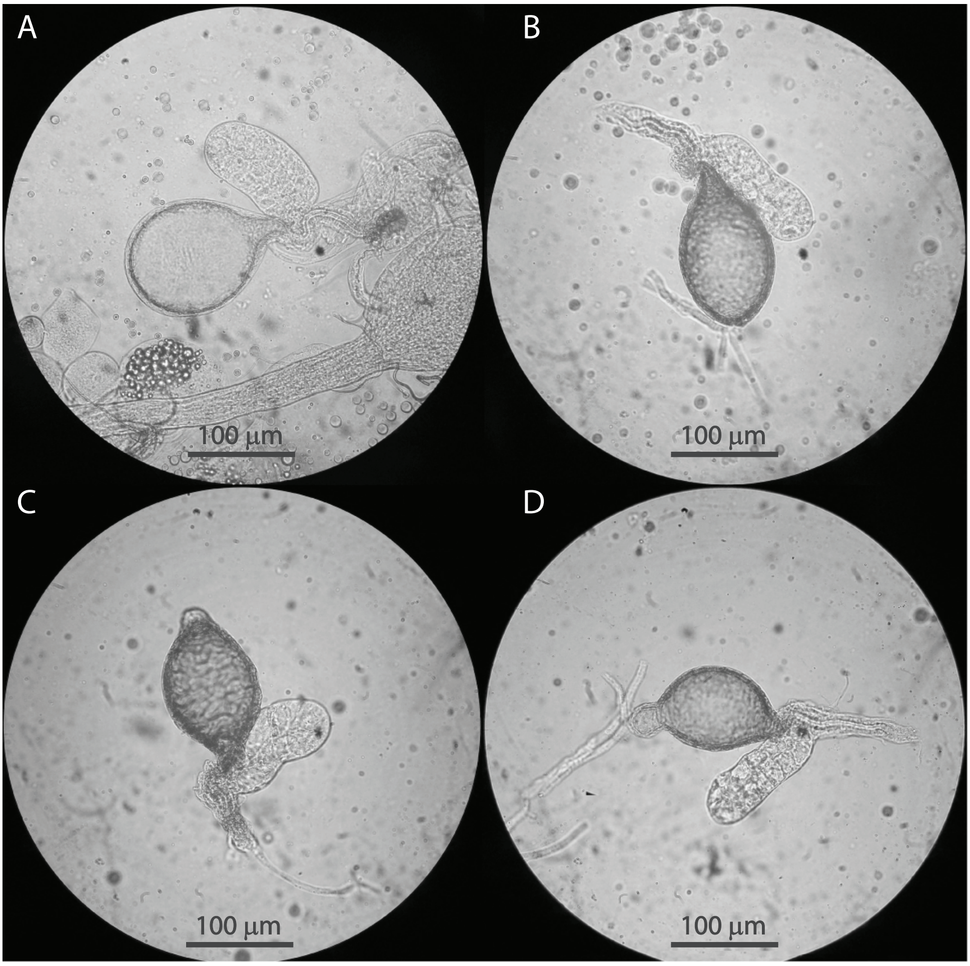

Figure 5.3: Spermathecae of arrhenotokous (A) and thelytokous (B-D) *Tetrastichus coeruleus* females. The spermatheca in B was slightly more elongated than the one in A. The spermathecae in C and D exhibited small and large, respectively, bulges as seen in many spermathecae of thelytokous females, but never in arrhenotokous females.

Discussion

Sexual traits may evolve to be costly when the reproductive interests of males and females are different. We therefore examined whether mating is costly to *T. coeruleus* females. This was only tested for arrhenotokous females, because thelytokous females never mated with males. The results showed that mated females had a shorter life span than virgin females. Next, we compared the sexual functionality of arrhenotokous and thelytokous *T. coeruleus* females. The results showed that arrhenotokous and thelytokous females were equally attractive to males, but that thelytokous females never mated with males. Furthermore, there was a clear difference between spermathecae from arrhenotokous and thelytokous females. Arrhenotokous females had circular spermathecae with a smooth surface, whereas the spermathecae of thelytokous females were more elongated and often had bulges.

Thelytokous *T. coeruleus* females were equally attractive to males as arrhenotokous females. Therefore, male courtship does not seem to be costly to females, as is the case in the field cricket *Gryllus bimaculatus* and the parasitoid wasp *Leptopilina clavipes* (Bateman *et al.* 2006, Reumer *et al.* 2007, respectively).

Similar to many other insects, mating was costly to *T. coeruleus* females. Many male insects are known to manipulate females through proteins that they transfer to females during mating along with the seminal fluid (Chapman *et al.* 1995, Wigby & Chapman 2005, South & Lewis 2011). Such manipulative male proteins may cause a reduction in female receptivity, an increase in female fecundity and the removal of sperm of previous mates, but it can also have substantial side-effects, such as mating-induced reduction in female life span (e.g. Fowler & Partridge 1989, Chapman *et al.* 1995, Rice 1996, Garcia-Gonzalez & Simmons 2010, South & Lewis 2011, Xu & Wang 2011). Females are expected to evolve counter-adaptations to reduce the harmful effects of male manipulation (Chapman *et al.* 2003, Arnqvist & Rowe 2005). However, when no males are present in a population, which is the case in most PI-*Wolbachia*-infected populations (Huigens & Stouthamer 2003), female adaptations against male manipulation may be released from such selection. Therefore, thelytokous females may be more receptive to mating when they have evolved without males for a long period of time. This seems to be the case in the parasitoid wasp *L. clavipes*, where thelytokous females mated more frequently with males than arrhenotokous females and copulations lasted longer (Kraaijeveld *et al.* 2009), although in this species mating does not result in a shortened female life span (Reumer *et al.* 2007). However, when males occur at low frequency in a thelytokous population, females may still be selected to avoid the cost of mating, while they no longer need to mate to fertilize their eggs. In such cases, thelytokous females may be less receptive or not receptive at all to mating. In agreement with this, males are found in low frequencies in thelytokous populations of *T. coeruleus* (chapter 2, Reumer *et al.* 2010) and thelytokous *T. coeruleus* females never mated with males. Although thelytokous *T. coeruleus* females never mated in the lab, Reumer *et al.*

(chapter 3, in prep) found clues for occasional matings between males and thelytokous females in the field. Because of these sporadic matings, females may still be exposed to the cost of mating and adaptations of females against male manipulations keep evolving. Alternatively, the unreceptiveness of thelytokous females may be due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010).

However, the longevity cost may also have been due to a cost of sperm storage. If this is true, then there would be selection against traits involved in sperm storage. Arrhenotokous females need to be able to store and transport sperm in order to produce female offspring. Thelytokous females however, do not need sperm to reproduce and traits involved in sperm storage may be released from selection in order to maintain them. In agreement with this, the spermathecae of arrhenotokous and thelytokous *T. coeruleus* females differed in their morphology. All spermathecae of arrhenotokous females were circular with a smooth surface, whereas the spermathecae of thelytokous females were more elongated, often had bulges and differed between individuals. This suggests that thelytokous females no longer use their spermathecae, which have become vestigial. In addition, the unreceptiveness of thelytokous females may be a response to the possible cost of sperm storage in stead of a cost of copulation. Alternatively, the spermathecae may have degraded due to neutral mutation accumulation, because these organs are no longer used by thelytokous females.

In conclusion, we observed a longevity cost of mating or sperm storage in *T. coeruleus* females. Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and spermathecal morphology of thelytokous females. However, we cannot exclude that these traits have degraded due to functional virginity mutations or accumulation of neutral mutations.

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