Aus dem Department für biomedizinische Wissenschaften
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Sperm morphology, scent marking behavior and ultrasonic vocalizations in the context of sperm competition in house mice

Diplomarbeit

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vorgelegt von

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List of abbreviations:

A-MUD	=	Automatic Mouse Ultrasound Detector
HTF	=	Human tubal fluid
IVF	=	In vitro fertilization
KLIVV	=	Konrad-Lorenz-Institut für Vergleichende Verhaltensforschung
LMM	=	Linear mixed model
SCA	=	Sperm Class Analyzer
USV	=	Ultrasonic vocalization(s)

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1. Introduction

The concept of sperm competition describes the competition between the sperm of two or more males for the fertilization of the same set of ova (PARKER 1970, 1998, Stockley 2004). It is furthermore accepted as a crucial factor influencing the selection and evolution of male reproductive anatomy, physiology and behavior among many species of mammals (Sperm Competition and Sexual Selection, 1998, Ramm et al. 2005). To gain an advantage in sexual selection, the males' mechanisms of adaption can span from different investment in mate searching strategies to timing, the frequency and duration in copulatory behavior, composition and size of the copulatory plug, the total amount of sperm per ejaculate produced and the amount of sperm ejaculated per copulation and, finally, to the adjustment of various sperm motility parameters (delBarco-Trillo et al. 2015, Firman and Simmons 2010, Klemme and Firman 2013, Ramm et al. 2005, Stockley 2004). It has not yet been investigated however, if or to what degree these beforementioned adjustments influence the sperm morphology or if the genetic quality of the individual (e.g. outbred versus inbred) also plays a role in these regulatory adaptions.

While there are comprehensible and measurable parameters of male fertility such as sperm quantity, -motility, -morphology, their susceptibility to external influence is not always clearly traceable. Putting more obvious influential variables such as climate, the individuals' genetic background, age, nutritional status, psychological stress, toxins and diseases aside, even (copulatory) behavior and social interaction in the context of courtship behavior are widely understood to be having an impact on male reproductive physiology and success.

Looking at sperm morphometrics on a microscopic level, various morphological differences in murine spermatozoa have been identified as a significantly influential factor of IVF success rates by several studies (Kawai et al. 2006). Head shape, midpiece insertion angle into the head, midpiece dimensions, sperm tail integrity (bend, location of bend), number of "loose heads" (i.e. sperm heads detached from the midpiece) and properties of the cytoplasmatic droplet are only some of the morphological criteria that have been studied and brought in conjunction with fertility (i.e. IVF success rate/percentage of fertilized oocytes) (Kawai et al. 2006, Mashiko et al. 2017). This is not

surprising, since the sperm head contains not only the genetic information and key components needed to fertilize the female oocyst, moreover its dimensions and surface structure must also be appropriately suited for the acrosome reaction to transfer the genetic information (i.e. DNA). When specifically taking a look at sperm head dimensions, clinical studies in humans have shown that larger sperm heads can be linked to infertility (Katz et al. 1986) while animal studies in stallions, bulls, lions, dogs and pigs found, that sperm head dimensions deviate between fertile and infertile/less fertile males (Casey et al. 1997, Partyka et al. 2010, Partyka et al. 2012, Verstegen et al. 2002). Similarly, sperm defects such as loose, detached sperm heads also implies an impairment of fertility and, when high in number, can lower the positive outcomes of IVFs in that the pronuclear fusion or cleavage does not occur after fertilization (Chemes et al. 1987, Chemes et al. 1999). Going back and putting sperm morphology back into the context of sperm competition, several studies show, that 1) in mammalian species in which females mate promiscuously, overall length of spermatozoa increases (Gomendio and Roldan 1991), 2) the coefficients of variation in sperm length are lower in bird species with high levels of extrapair paternity (Carballo et al. 2019) and 3) ejaculates of males experiencing a high level of sperm competition have a higher percentage of morphologically normal sperm (Gómez Montoto et al. 2011), but, besides studies proving specific genetic abnormalities result in a higher percentage of abnormal sperm in males' ejaculates (Elfateh et al. 2014) or identifying key infertility genes, responsible for morphological anomalies (Ray et al. 2017), few studies specifically compare morphometric sperm values between individuals under the influence of varying degrees of sperm competition. Similarly, there are hints, that male intrinsic quality influences spermatogenesis (Ferlin et al. 2007), but specific morphometric values have not been looked at closely and compared between individuals.

In house mice, reproductive competition and secondary sexual traits such as scent marking and vocalizing behavior, for example, can appear cryptic and incoherent at first glance, but may correlate with mate choice and ultimately reproductive success, as elaborated in the following paragraphs.

Murine scent marking with urine serves as a form of social communication; containing information about sex, reproductive status and individual identity, these scent marks are also an indicator for social dominance and represent the males` territorial boundaries (Drickamer 2001, Jemiolo et al. 1992). So not only can male mice detect possible competitors in their surroundings via revealing

olfactory urine markings, they can also determine if there is/was a possible mating partner in their vicinity; picking up the scent of a sexually mature female mouse naturally triggers behavioral patterns like the emission of ultrasonic vocalizations (USV) or “mouse song” to attract possible mating partners (Musolf et al. 2010, NYBY and al 1979). Additionally, (Thonhauser et al. 2013) could demonstrate, that scent marking increases the males’ reproductive success when females have free choice of their mating partners. So, while it is known that the amount of scent marking behavior can be an indicative factor for successful reproduction, the question if a higher expression of scent marking behavior is influenced by the level of sperm competition and, moreover, male intrinsic quality remains to be thoroughly investigated.

When presented with olfactory cues of adult, sexually active female mice (represented by soiled bedding material of female mice in this study), males produce USVs as part of their courtship behavior to attract females (Musolf et al. 2010). Studies have shown, that female mice exhibit both sexual and social partner preferences for vocalizing males, when given the choice between vocalizing and devocalized/non-vocalizing male mice (Musolf et al. 2010, Nomoto et al. 2018), suggesting quality and/or quantity of USVs playing a part in sexual selection. For the most part unfortunately, the meaning of the different syllables of mouse song (see Figure 10) and their effect/response relationship on females as well as males is not yet fully understood, despite classification efforts (Hanson and Hurley 2012). Contributing to these clarification efforts, it would be interesting to investigate if the emission of USVs, a secondary sexual trait and thereby also representing a display of the individuals male’s general fitness, is influenced by the male’s exposure to sperm competition and/or the individual’s intrinsic quality. After all, genetically higher quality males are expected to develop larger and more (elaborate) secondary sexual traits than lower quality males (Schulte-Hostedde and Schank 2009).

In the scope of this thesis, we aimed to investigate possible effects of sperm competition and genetic background on certain sperm morphology parameters and secondary sexual traits production in the form of scent marking behavior and emission of USVs in wild-derived house mice (*Mus musculus musculus*). Males of both an inbred or outbred genetic background were allocated to a “low risk” or “high risk” of sperm competition. To alter sperm competition risk, we manipulated the number of direct social interactions between males and added olfactory cues from neighboring males to a male’s territory to stage territorial intrusions. We predicted that males under a “high

risk" of sperm competition will increase sperm production and adjust sperm morphology parameters compared to "low risk" controls. We further predicted that males would increase secondary sexual traits production according to the risk of sperm competition. Additionally, we predict that inbred males produce less fertile sperm and compare worse to outbred males in regards of secondary sexual traits production.

2. Methods & Materials

2.1 Breeding facility

Breeding was conducted at the grounds of the *Konrad-Lorenz-Institut für Vergleichende Verhaltensforschung* (*KLIVV*) in rack-mounted, open “Type II long” cages with aspen wood chip bedding (*LTE E-001*, *Abedd*), red plastic mouse house and nesting material (paper tube, Nestlets by *Bioscape*) in a 12:12 hours light/dark lighting cycle. Diet consisted of dried pellets (*Type 1324, Altromin*) and tap water, both *ad libitum*, whereas the room temperature was kept at 22 °C ± 2 °C.

2.2 Animals

For the generation of animals used in this experiment, wild-caught mice (*Mus musculus musculus*) from the grounds of the Wilhelminenberg area in Vienna, Austria were coupled and represented the parent generation (F0) of all following offspring. After outbreeding three generations of these wild-caught mice (resulting in F3 generation descendants), mice with a specific genetic background were created as follows; on the one hand, an F5 “outbred” cohort was established through coupling F3 generation wild-caught mice for two generations, each time with a varying, non-relative partner. On the other hand, an F5 “inbred” cohort was established through two generations of full sibling coupling of F3 generation wild-caught mice. Weaning was performed on day 21 +/- 1 in all animals.

Despite species and breeding status, the genetic background of these wild-caught mice is naturally unclear in contrast to established laboratory mouse strains. This will contribute to the aim of the study to generate knowledge, applicable to a broader spectrum of (wild) animals since there are indicators that currently many strains of *M. musculus* used in research have impaired reproductive traits and therefore are not ideally suited as a reference for the genus *Mus* in regards of

investigating reproductive traits (e.g. sperm quality or quantity) (Beilharz 1982, Gotoh 2010, Zajitschek et al. 2009).

Finally, 36 male descendants of the F5 generation, 18 of those being “inbred” and 18 “outbred”, were then introduced to the experiment at an age of 4-5 weeks, marking the start of the experiment.

2.3 Experimental design and setup

In essence, the experimental design and conducted manipulations in the context of this thesis revolve around the question, whether the perceived risk of sperm competition, as well as the genetic quality of the individual males, have an influence on the animals’ sperm morphology and expression of secondary sexual traits. For this purpose, this thesis ultimately highlights the conduction and findings of computer-assisted sperm morphology analysis, analysis of USV recordings and image analysis of scent marking behavior, the latter two representing examples of secondary sexual traits.

The experiment was conducted in an air-conditioned room, tailored specifically for the requirements of this experiment. Type III cages (42,5 x 26,6 x 15,5 cm), in which the mice were kept individually, were put into the middle of 110 x 180 x 80 cm open-roof enclosures (see Figure 1). The cages were modified with an insertable, manually closable exit tube on either the far-left or far-right end of the longitudinal cage wall. Wood chip bedding (*LTE E-001*, *Abedd*), one red plastic mouse house and 2 cellulose cylinders (*Cocoon, Bioscape*) were added to the cages as bedding and enrichment respectively. Diet consisted of dried pellets (*Type 1324, Altromin*) and tap water, both *ad libitum* with the addition of sporadic treats in the form of peanut butter and apple slices placed on the lid.

To investigate if genetic quality has an influence on sperm morphology, USVs and/or scent marking behavior, “inbred” as well as “outbred” mice were distributed numerically equal over a total of 24 enclosures, with 18 enclosures containing singly housed mice and six enclosures each containing three mice housed within close proximity, after matching for subject age and body mass.

In accordance with risk of sperm competition, animals in enclosures containing singly housed mice were allocated to the “low risk” (control) group, whereas the animals in enclosures containing three singly housed mice in close proximity to on another were allocated to the “high risk” (treatment) group. These two treatment groups were created to investigate two different levels of perceived sperm competition. In this setup, “high risk” males could have audio-visual as well as visual contact at all times as to simulate an environment with neighboring territories containing male competitors.

Finally, the entirety of experimental subjects (36 mice in total) was divided by two, introducing two chronologically separated cohorts, each consisting of 18 mice. However, each cohort was matched for subject age and body mass before the introduction to the experiment and randomly allocated to the groups.

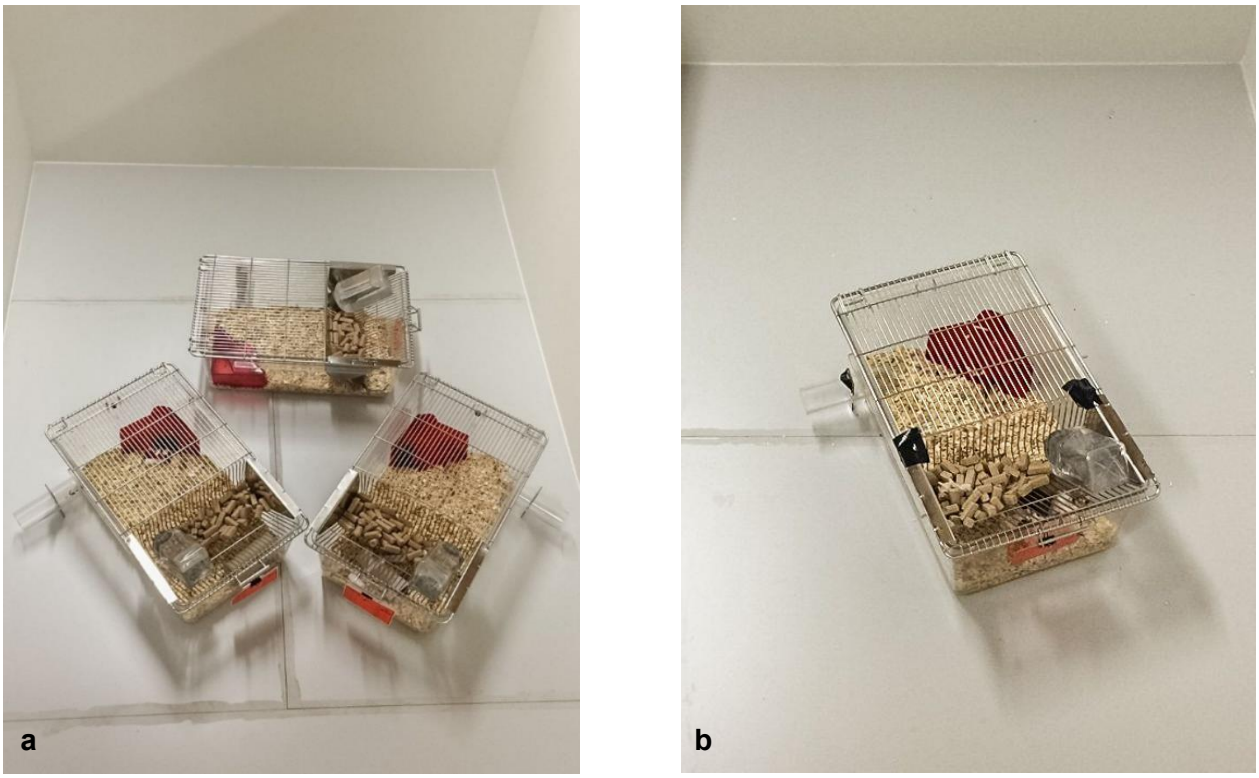

Figure 1: Photographs of **a** “high risk” enclosure containing three *type III* cages, each housing single mice, **b** “low risk” enclosure containing one cage; inserted, closable exit tube visible on the far-left side

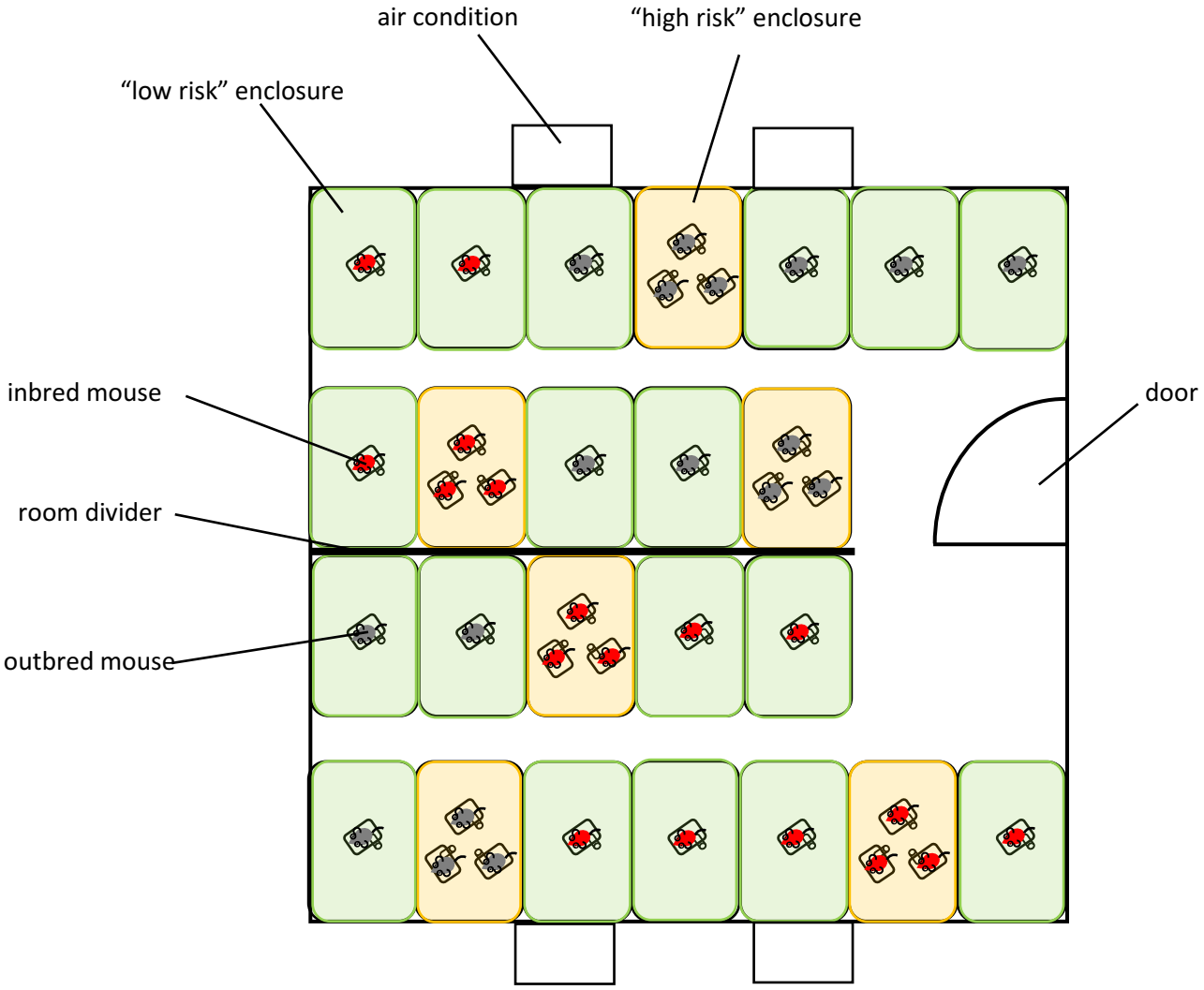

Figure 2: Experimental room setup, schematic: green enclosures represent “low risk” mice, yellow enclosures “high risk”, whereas inbred mice are colored red and outbred mice grey.

2.4 Experimental manipulations

Through strategic manipulation of the animals and their environment, the resulting fully factorial design of the study allowed to elevate the impact of genetic quality of the individual mouse as well as its perceived risk of sperm competition on sperm morphology, scent marking behavior and USV.

To facilitate the organization of the various manipulations explained below, an experimental schedule of manipulations based on a weekly routine started one day after the introduction of the animals to the experiment. While female bedding stimulation and free roaming was performed once every week, male bedding stimulation was performed twice per week. Recording of USV was conducted twice per week on all weeks except week 1 and 4. These recordings were always made after either a male bedding stimulation or during the free roaming phase. Finally, scent marked filter papers were collected on weeks 6 and 11, each time after a previously performed manipulation (male bedding stimulation, female bedding stimulation, free roaming) for three consecutive days.

Hence, over the duration of eleven weeks, for each cohort the following experimental manipulations were conducted:

- Female bedding stimulation
- Male bedding stimulation
- Free roaming of “high risk” mice
- Recording of USV
- Scent mark collection
- Feces collection

2.5 Procedures/manipulations in detail

The various “bedding stimulations” performed in this experiment can be regarded as a simulation of indirect encounters of two or more mice through olfactory cues. Since olfaction plays a crucial role in social and sexual interaction in mice (Yang and Crawley 2009), the bedding stimulation procedure should be a sufficient trigger for a realistic and adequate reaction in the male mice of this study.

- Female bedding stimulation

A fistful of wood chip bedding material was taken out of a pooled sample of cage bedding of wild-derived, sexually mature female mice and placed centrally on the lid of the male’s cage. It was ensured, that males were always exposed to the bedding of unfamiliar, novel females. This should, according to the Coolidge effect (Carr et al. 1970, Dewsbury 1981), maximize the male’s sexual interest in the female and therewith potentially also elicit male competitive behavior (Cunningham et al. 2013, Oakeshott 1974).

- Male bedding stimulation in “high risk” males

A fistful of wood chip bedding material was taken out of a pooled sample of cage bedding of all three mice of the respective enclosure and placed centrally on each lid of the males` cages to simulate territorial intrusion of male competitors indirectly through olfactory cues.

- Male bedding stimulation in “low risk” males

As a measure of good scientific practice, a fistful of wood chip bedding material was taken out of the male’s own cage and then placed centrally on the lid of said male’s cage. This should represent a standardized, controlled experimental conduction and equal treatment throughout all groups to minimize confounding factors such as manipulation effects.

- Free roaming

As a means of controlled engagement, this “free roaming” procedure simulates a direct confrontation of competing males without the risk of severe injuries. In “high risk” groups, each male, one at a time, is granted 30 minutes of free roaming in the whole enclosure. The exit tube of the modified Type III cage is opened and the respective male can exit and enter its own cage at any time during the entire 30 minutes. During this time, “high risk” animals could freely engage in direct contact with other males through the metal grid cage lid. After the procedure, all surfaces in the enclosures as well as the animals themselves were visually checked for possible injuries (i.e. blood stains, abnormal behavior etc.). Similarly, singly housed, “low risk” males were also granted free roaming inside their respective enclosures. Of course, without any competitors present in the enclosure, these “low risk” males had no possibility of direct interaction with other males.

- USV recording

For the examination of USV behavior, Microphones (*USG Electret Ultrasound Microphone, Avisoft Bioacoustics/Knowles FG*), capable of recording sound in the ultrasonic range, were installed approximately 50 cm centrally above the cage in “low risk” enclosures (center of “high risk” enclosures respectively) hanging from a framework installed on top of the enclosures. Running through an A/D-converter (*UltraSoundGate 416Hb, Avisoft Bioacoustics*), which was connected to a *Windows 7* laptop, recording was processed with *RECORDER USGH (Avisoft-RECORDER 4.2)* software with a sampling rate of 300 kHz and 16-bit format. For optimized sensitivity and sound capturing, microphones were placed inside a plastic parabola. Each recording lasted for 25 minutes, resulting in five 5-minute-long *.wav* files. Prior to each recording event, the males experienced either 1) a female bedding stimulation, 2) a male bedding stimulation or 3) free roaming.

- Scent mark collection

After an odor stimulation with either female or male bedding material, 5 x 5 cm filter paper squares were dropped centrally onto the cage floor through the lid and collected after a time period of 18 hours. Males could interact freely with the filter paper during this time period. Afterwards, the collected filter papers were photographed under UV light and then stored into a -18 °C freezer until further usage.

- Feces collection

Although not directly relevant for this thesis, but an important part of the study in general, feces samples were collected to measure fecal corticosterone and testosterone metabolites as a means of determining if or how these values influence sperm production.

All manipulations were consistently performed during the dark phase of the lighting cycle under red light to avoid any additional disturbance of the animals. Also, every manipulation was conducted at its previously determined, specific time of day to minimize confounding factors such as diurnal fluctuations of hormone levels (Touma et al. 2004) and general activity (e.g. sleeping/awake).

2.6 Sperm sampling for morphology analyses

Sperm samples for morphology analysis were acquired through *post mortem* dissection of both epididymides. After dissection, the epididymides were immediately put into a 1000 µl drop of human tubal fluid (HTF) medium, submerged under a closing layer of mineral oil and incubated at 37 °C in 5 % CO_2 enriched air for 10 minutes. This ensures all motile sperm to swim freely and spread out in the drop of HTF medium. Two 10 µl aliquots of each HTF/sperm solution sample were then pipetted onto two duplicate sample slides and gently smeared across the slide, trying to avoid morphological damage through the application of too much force, as well as the formation of unwanted flag patterns at the distant end of the smear. The resulting smear was stained via full submersion in *SpermBlue* (Microptic S.L.) staining solution for 90 seconds, followed by a gentle submersion in distilled water for 4 seconds to prevent over-staining, placing the slide in a 80-90° angle to let superficial drops of liquid drain off by gravity and, finally, drying the smear on a 37 °C heating plate until no excess moisture was visible.

2.7 Sperm morphology analysis

The morphological attributes of mouse spermatozoa analyzed in this thesis include sperm head morphometrics (note the characteristic, physiological head shape of rodent spermatozoa with the distinctive hook shape at the distal part of the head, opposite of the midpiece joint; see Figures 3, 7) such as head ARC, -width and -elongation (Elongation = (ARC-Width) / (ARC+Width)), alongside midpiece width in µm and total number of loose heads counted in a previously marked-off area of the sample slide (see Figures 4, 5).

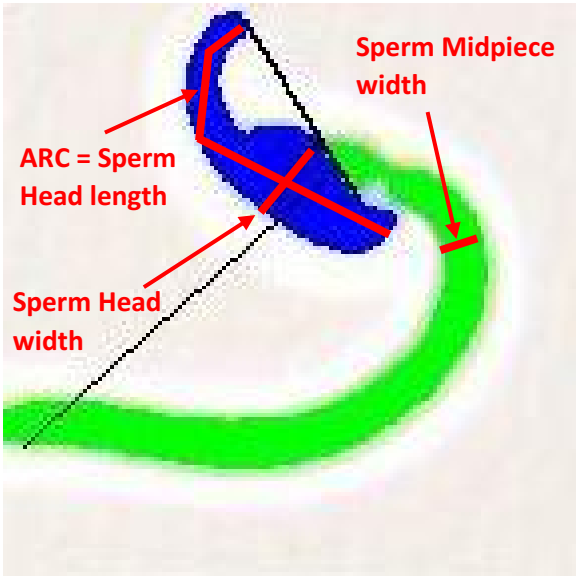

Figure 3: Digital image as captured in SCA for computer-assisted sperm morphology analysis partially showing a physiologically shaped mouse spermatozoon. By default, the software renders the measured head area in blue and the midpiece area in green.

Therefore, the readily prepared and fully dried smear on the sample slide was placed under a *Nikon Eclipse E200* bright field microscope with 10x magnification eyepiece, 60x magnification lens (resulting in a total magnification of 600x) and blue filter, connected via a *Basler acA780-75gc CCD GigE* camera to a *Windows 10* PC running *Microptic Sperm Class Analyzer (SCA) 6.10.1 Research Edition* software for morphological sperm analysis. Using the *Morphology* module of the *SCA* software, between 50-60 unique spermatozoa in 47-58 individual fields were captured and digitally measured. To avoid false duplication of captured spermatozoa, the slide was moved strictly in an undeviating, meandering pattern.

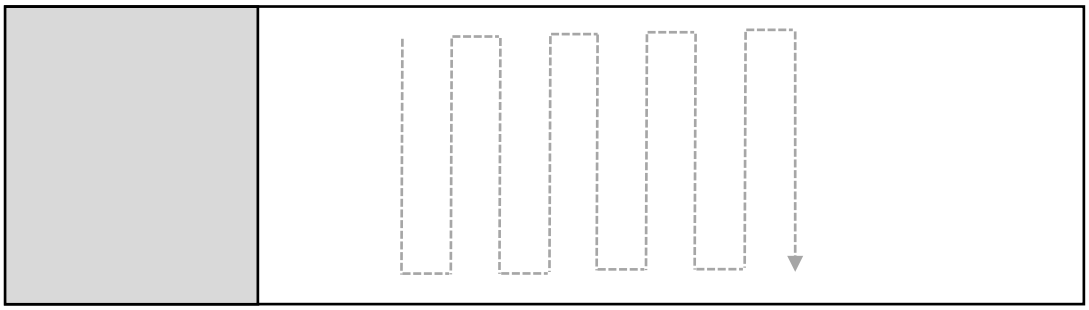

Figure 4: Sketch of a sample slide and the meandering viewing pattern, which was used for SCA sperm morphology analyses. The grey area on the left side represents the matte labeling surface

Additionally, the number of loose heads on each sample slide was counted by manually scanning the area of [one vertical sample slide length] x [the diameter of the viewing field in the microscope at a magnification of 600x] (see Figure 6). For better comparability between samples, all measurements started from a previously determined starting point located at a distance of 15mm from the edge of the matte labeling surface.

Finally, the counted number of loose sperm heads was divided by the total sperm number of the respective drawn sample to obtain a “loose head ratio” for a fairer comparison of values.

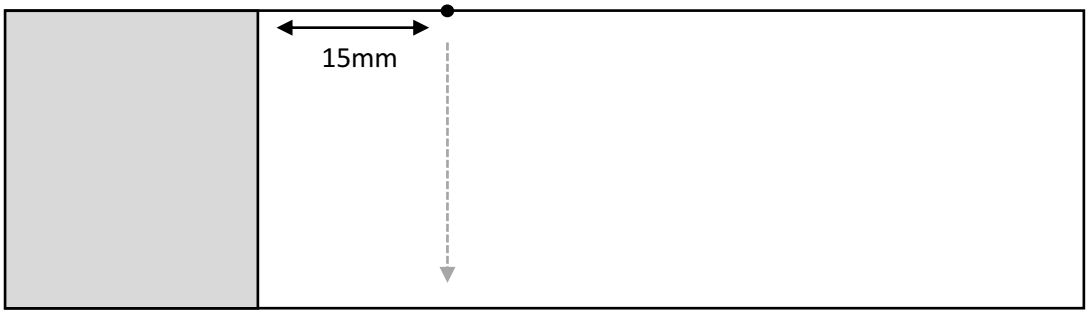

Figure 5: Sketch of a sample slide with starting point for quantitative loose head analysis, 15 mm off the matte labeling surface (grey area).

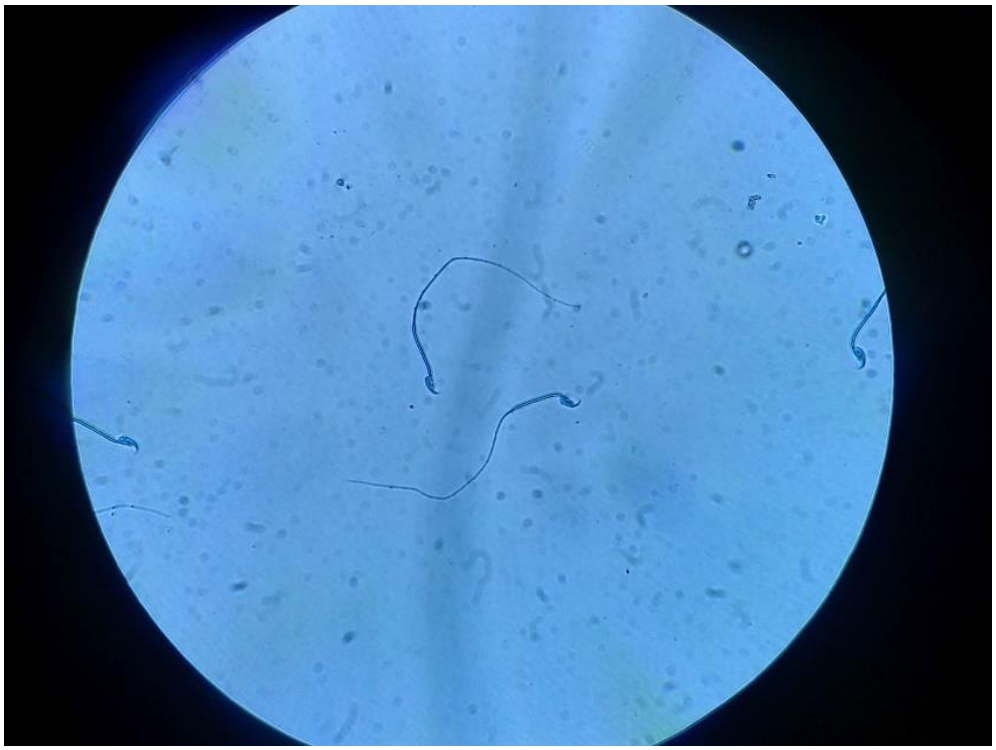

Figure 6: Photograph of the viewing field in the microscope with the inserted blue filter at 600x magnification, taken with a cellphone camera through the ocular of the microscope. Multiple physiologically shaped spermatozoa are visible either wholly or partially.

Figure 7: Digital images as captured in SCA for computer-assisted sperm morphology analysis. **a**: Image containing a single spermatozoon with clear view on a physiologically shaped hook, visible on the distal head portion. **b**: Analysis mask as automatically applied by the SCA program, showing midpiece (green), head (blue), midpiece insertion angle (black lines) and the area in which to take these measurements (red box).

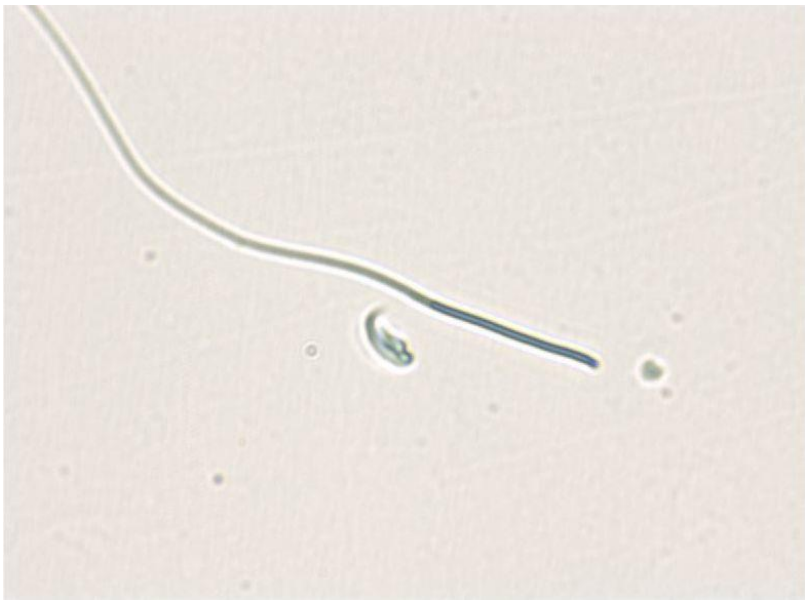

Figure 8: Digital image as captured in SCA for computer-assisted sperm morphology analysis showing a loose head. The head of the spermatozoon is clearly separated from the midpiece.

2.8 Analysis of collected scent marks

For analysis of murine scent marking behavior, front and back sides of each of the collected filter papers were photographed under UV light to visualize any fluorescent urine stains as bright, blue colored areas in contrast to the darker blue appearing, unstained filter paper (Desjardins et al. 1973). The resulting *.jpg* files were then used for further analysis using *FIJI ImageJ 1.51 n*. After importing each picture, the picture was first cropped to the edges of the filter paper to remove any excess empty areas. Then, after manually setting a detection sensitivity threshold for every picture to ensure a measurement as exact as possible to the edges of the stained area(s), a manually programmed macro script was run to measure and count the stained area(s) of the filter paper.

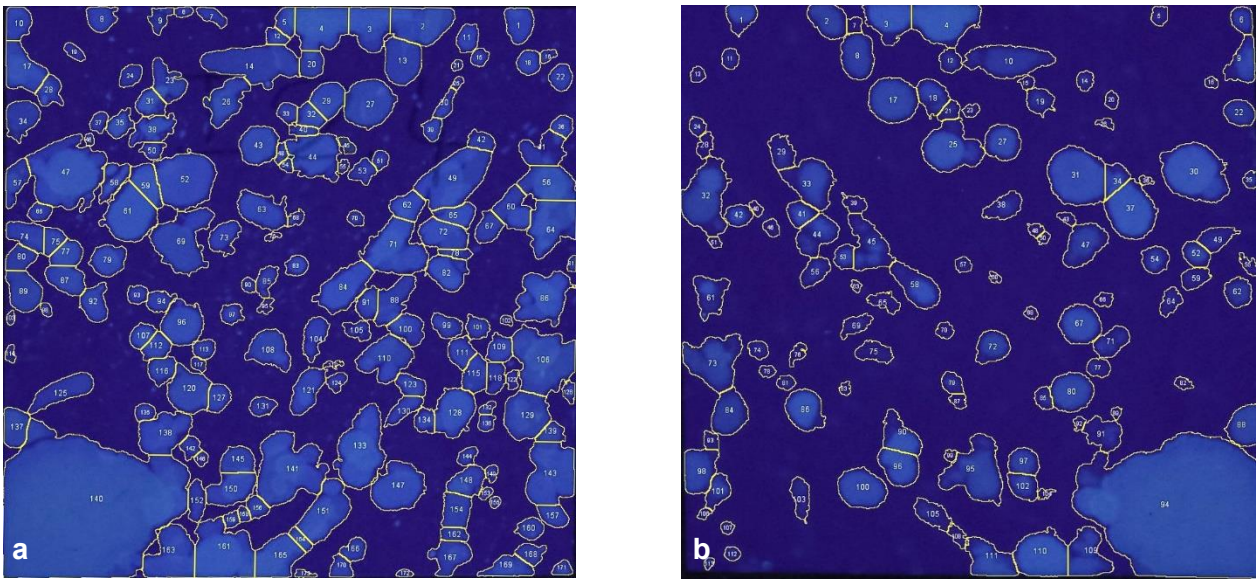

Figure 9: Digital photograph under UV light showing a filter paper with urine stains (bright blue in color, surrounded by yellow edges) on the front (**a**) and back (**b**) of the same filter paper. Using manually generated scripts for *FIJI ImageJ 1.51 n*, these stains can be numbered in ascending order and then measured in area (cm^2).

For a fairer comparison between the total stains counted on each filter paper, a calculated score was created. Using the formula $\frac{(Stain\ count\ front)^2+(Stain\ count\ back)^2}{500}$, smaller urine stains that usually show up only on one side of the filter paper (as a result of scent marking behavior) were attributed a higher score than the larger urine stains. This should reduce the number of false positives in the form of these larger urine stains that soaked through the filter paper and appeared on both sides of the filter paper.

2.9 Analysis of USV Recordings

A great part of the analysis of USV recordings consisted of identification and classification of different syllables of mouse song. While different types of syllables can clearly be differentiated and classified in regards of frequency, duration, amplitude, shape and repetitions (see Figure 10), little is known about their true meaning, albeit male mice appear to vocalize exclusively for females (Musolf et al. 2010, Portfors 2007). However, several studies suggest male USVs being part of murine courtship behavior to attract females and therewith also playing a role intra-sexual competition (Musolf et al. 2010, Nomoto et al. 2018, Sales 1972).

For the Analysis of the recorded segments, the *Windows* program *STx v4.3.5 (9286)* (*Acoustics Research Institute*, Vienna, Austria) and the Automatic Mouse Ultrasound Detector (*A-MUD) 3.2* (improved by Zala et al. 2017) were used. The 5 x 5-minute-long *.wav* files per animal per recording event were processed using a semi-automatic method; first, USVs were automatically detected by *A-MUD*. Second, the detected segments were manually checked and classified into 15 different syllable types (see Figure 10), also regarding frequency, amplitude and total amount of each respective USV type. Spectograms for manual annotation were generated using the transcription function in *STx* with a frame of 4ms, a range of 50 dB (floor at -80 dB) and an overlap of 75 % in a Hamming window.

In addition to the automatic detection *post-recording*, all recording sessions were manually supervised and any segments, in which USVs were apparently captured, marked for the facilitation of later analysis.

Example	Label	Syllable type	Definition
(< 5ms)	us	ultra short	syllables below 91kHz, that are shorter than 5ms (short dots)
(<10 ms)	s	short	syllables below 91kHz, that are shorter than 10ms
	f	flat	syllables below 91kHz, with less than 5kHz frequency modulation
	d	down	syllables below 91kHz, that decreases in frequency for more than 5kHz
	up	up	syllables below 91kHz, that increases in frequency for more than 5kHz
	u	u-shaped	syllables below 91kHz, that first decreases, then increases in frequency for more than 5kHz each
	ui	u-shaped inverted	syllables below 91kHz, that first increases, then decreases in frequency for more than 5kHz each
	c	complex	syllables below 91kHz, that contain two or more directional changes in frequency and more than 5kHz modulation of frequency
	c2	complex 2	syllables below 91kHz, consisting of two elements that are separated by one frequency jump but without time separation
	c3	complex 3	syllables below 91kHz, consisting of three elements that are separated by two frequency jumps but without time separation
	c4	complex 4	syllables below 91kHz, consisting of four elements that are separated by three frequency jumps but without time separation
	c5	complex 5	syllables below 91kHz, consisting of five or more elements that are separated by four or more frequency jumps but without time separation
	h	harmonic	syllables below 91kHz, that have an harmonic element
(< 91 kHz)	uh	ultra high	all syllables above 91kHz
	uc	unclassified	syllables that cannot be classified according to any other of the 14 categories due to background noise or not defined spectrographic features (shape) that can be measured.

Figure 10: List of different USV syllable types expressed by mice. The various shapes in the “Example” column represent the visible amplitude of the vocalization in the spectrogram, only sufficiently visible and audible to humans in slowed-down sound files.

Recording segments apparently void of any significant signs of USV were randomly selected (n=24) and analyzed for USV. As expected though, in a total of 24 randomly selected 5-minute-long sequences, only one USV of the Type “s” was found in one sequence. It can therefore be assumed that, besides the selected recordings that have been checked for USV during the “live” recording, the remainder of the non-marked recordings contain no significant amount of USV for further analysis.

2.10 Statistics

Statistical analysis was performed using *Microsoft Excel 2016* and *SPSS 26* by *IBM*, while graphs were created using *Graph Pad Prism 8.2.1.441*. The data sets were processed in a linear mixed model (=LMM) in *SPSS*, with either (loose head ratio) or (midpiece width in µm) or (head ARC in µm) or (head width in µm) or (head elongation) as the dependent variable, (treatment), (genetic status), the (interaction between treatment and genetic status) as fixed factors, (body mass at the end of the experiment) or (sperm number) as fixed covariate and (replicate) as a nested term within the (group) as a random factor for the sperm morphology analyses. Similarly for the scent marking analyses, a LMM with either the (scent mark count score) or (total area marked in %) as dependent variables, (treatment), (genetic status), the (interaction between treatment and genetic status) and (stimulation) as fixed factors and (body mass at the end of the experiment) or (sperm number) as fixed covariate and (replicate) as a nested term within the (group) as a random factor was processed. A model selection was then performed for every model, while the residuals were tested for normal distribution using a Shapiro-Wilk test.

3. Results

3.1 Sperm morphology analysis

When investigating the loose head ratios, we found no significant difference between the “high risk” versus “low risk” of sperm competition treatment groups (LMM: $F_{1,33} = 1.903$, p=1.177). Interestingly though, we found a marginally non-significant difference between outbred and inbred mice (LMM: $F_{1,34} = 3.887$, p=0.057), as inbred mice showed an overall higher loose head ratio than outbred mice (see Figure 11). Additionally, we found no significant interaction of treatment and genetic status (LMM: $F_{1,32} = 0.081$, p=0.778).

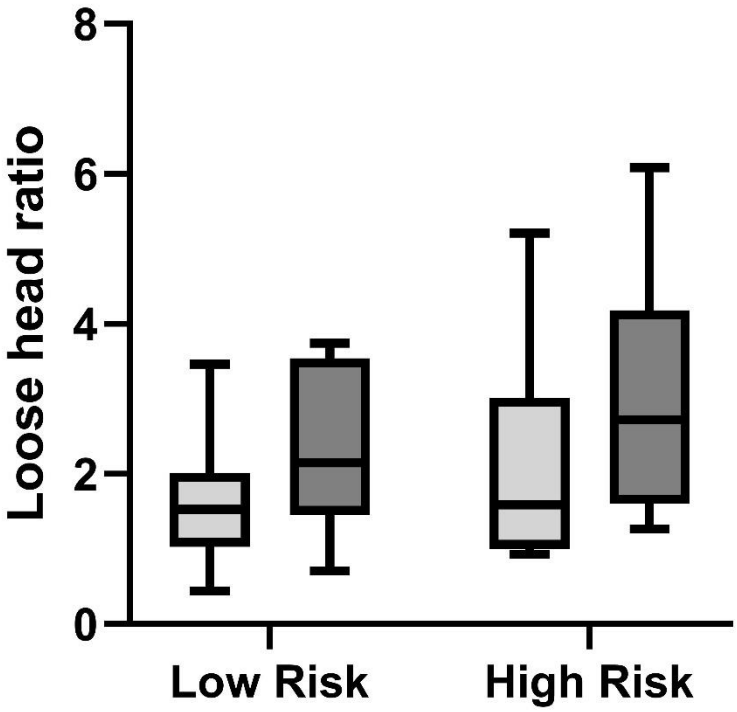

Figure 11: Comparison of the loose head ratio of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (light grey) or inbred (dark grey)

We found no significant differences in midpiece widths between the different treatments (LMM: $F_{1,27} = 0.051$, p=0.823) or the different genetic statuses (LMM: $F_{1,4} = 0.180$, p=0.693) (see Figure 12). Equally, we found no significant interaction of treatment and genetic status (LMM: $F_{1,27} = 1.239$, p=0.275).

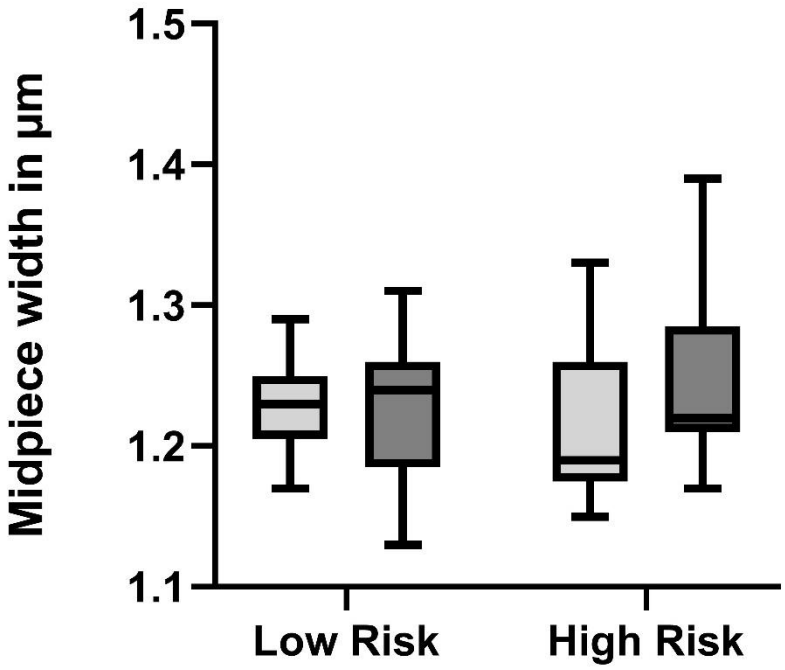

Figure 12: Comparison of the sperm midpiece width of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (light grey) or inbred (dark grey)

We found no differences in sperm head morphometric parameters of “low risk” versus “high risk”, or outbred versus inbred mice: sperm head ARC was comparable between genetic statuses (LMM: $F_{1,3} = 0.436$, p=0.558) (see Figure 13), treatments (LMM: $F_{1,27} = 0.209$, p=0.651) and the interaction of treatment and genetic status had no effect (LMM: $F_{1,26} = 0.164$, p=0.688).

Similarly, we found no effect of treatment, genetic quality or the interaction of both on sperm head width (LMM: $F_{1,31} = 0.488$, p=0.490), (LMM: $F_{1,31} = 0.592$, p=0.448), (LMM: $F_{1,31} = 0.179$, p=0.675) (see Figure 14) or sperm head elongation (LMM: $F_{1,31} = 0.002$, p=0.962), (LMM: $F_{1,31} = 0.563$, p=0.459), (LMM: $F_{1,31} = 0.059$, p=0.809) (see Figure 15).

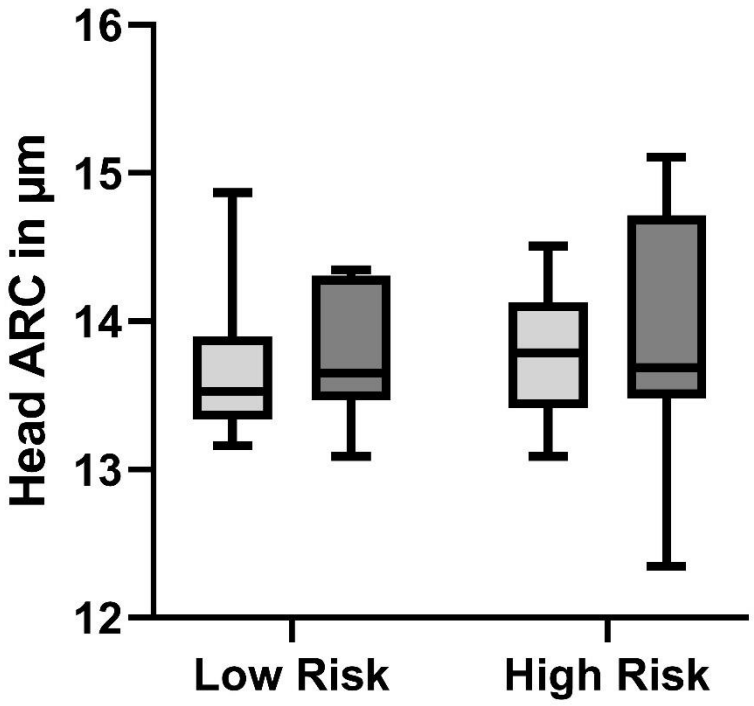

Figure 13: Comparison of the sperm head ARC of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (light grey) or inbred (dark grey)

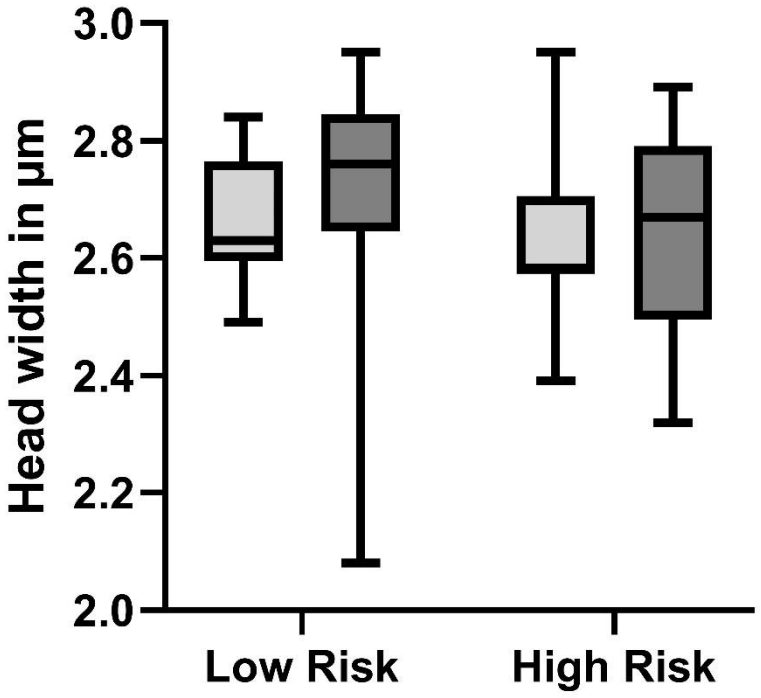

Figure 14: Comparison of the sperm head width of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (light grey) or inbred (dark grey)

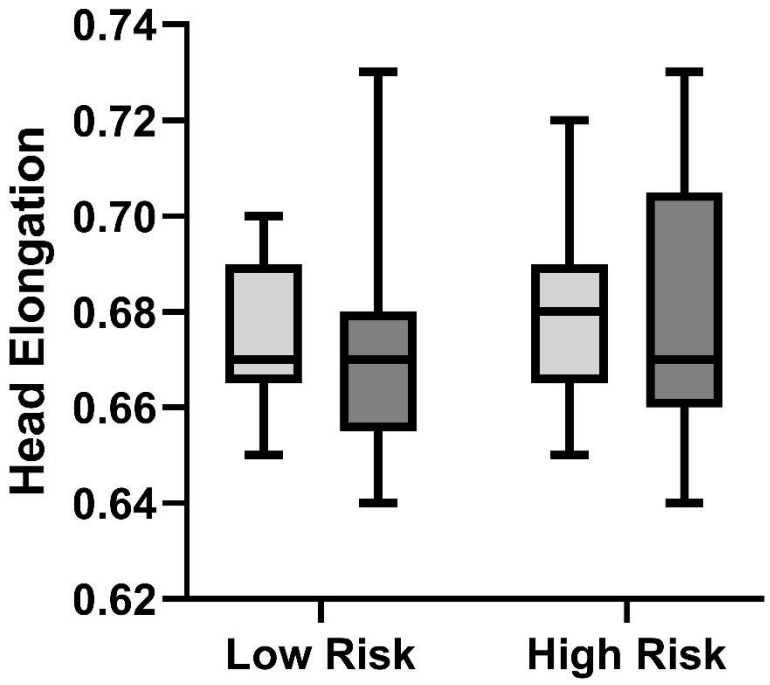

Figure 15: Comparison of the sperm head elongation of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (light grey) or inbred (dark grey)

3.2 Scent mark analysis

During comparison of the stain count scores, we found that males under the influence of a “high risk” treatment had significantly higher stain count scores than males exposed to a “low risk” treatment (LMM: $F_{1,60} = 4.110$, p=0.047) and that female bedding is a significantly stronger stimulant to elicit scent marking behavior in mice than male bedding (LMM: $F_{1,60} = 6.054$, p=0.017) (see Figure 16). There was, however, no difference between inbred versus outbred males (LMM: $F_{1,4} = 1.277$, p=0.323) or the interaction of treatment and genetic status (LMM: $F_{1,147} = 1.388$, p=0.240).

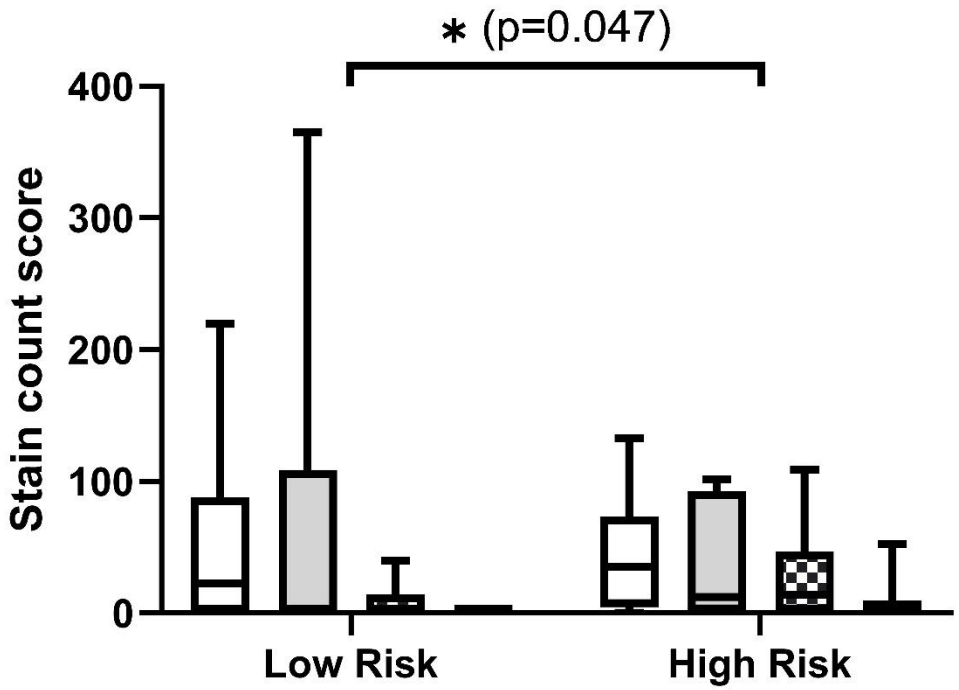

Figure 16: Comparison of the stain count score of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (white under female bedding stimulation, light grey under male bedding stimulation) or inbred (checkered under female bedding stimulation, diagonally striped under male bedding stimulation)

Looking at the percentages of total marked areas, we found no effect of our treatment (LMM: $F_{1,64}$ = 2.632, p=0.110), manipulation of genetic statuses (LMM: $F_{1,4}$ = 0.499, p=0.519) or interaction of treatment and genetic status (LMM: $F_{1,207}$ = 0.594, p=0.442). Again, we found a significant effect of the type of bedding stimulation on male scent marking behavior (LMM: $F_{1,65}$ = 6.450, p=0.013), as males that had been stimulated with female bedding marked significantly more than those stimulated with male bedding (see Figure 17).

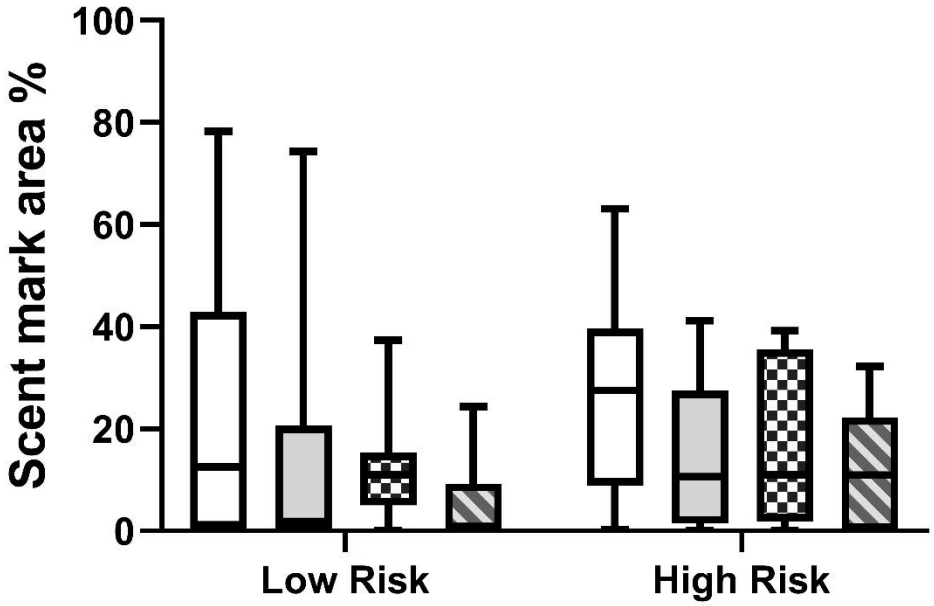

Figure 17: Comparison of the percentage of marked area on the filter papers of males that were kept under a “high risk” versus “low risk” of sperm competition and that were either outbred (white under female bedding stimulation, light grey under male bedding stimulation) or inbred (checkered under female bedding stimulation, diagonally striped under male bedding stimulation)

3.3 USV analysis

The recording and collection of USV proved to be more difficult than anticipated, since very few mice did actually emit meaningful USV during the recording sessions. Only 13 of the 216 recorded 25-minute long sequences contained actual mouse song syllables. Of these 13 sequences, four animals (38.4, 13.6, 28.2 and 18.5) have repeatedly sung, resulting in a total of 9 actively singing individuals out of 36.

Among these singing mice, some trends can be determined; 1) most males react the strongest (e.g. emitting USV) during the female bedding stimulation, 2) more USV were emitted during the second recording event, 3) the three highest emitting USV males are all outbred, “low risk” treatment individuals, singing during the female bedding stimulation.

Animal ID	Recording	Genetic Status	Treatment	Stimulation	#USV	uc	us	s	f	d	up	u	ui	c	c2	c3	c4	c5	h	*uh*
O-oo38.4	1st	Outbred	Low Risk	bedding	28	0	0	4	5	0	13	1	0	0	5	0	0	0	0	0
O-oo13.6	1st	Outbred	Low Risk	bedding	5	0	0	0	1	0	1	0	2	0	0	1	0	0	0	0
O-ii2.7	1st	Inbred	Low Risk	bedding	34	0	10	16	0	2	3	0	0	0	3	0	0	0	0	0
O-oo1.7	1st	Outbred	High Risk	bedding	66	0	0	0	3	1	28	4	5	0	24	1	0	0	0	1
O-ii9.2	1st	Inbred	High Risk	bedding	55	0	0	6	2	1	40	0	2	2	2	0	0	0	0	0
O-oo38.4	2nd	Outbred	Low Risk	bedding	484	0	6	63	37	6	327	19	12	1	13	0	0	0	0	1
O-oo13.6	2nd	Outbred	Low Risk	bedding	2833	4	35	88	119	206	604	226	600	297	390	242	19	0	1	6
O-oo20.6	2nd	Outbred	Low Risk	bedding	1266	0	22	146	325	159	361	13	196	12	30	1	0	1	0	2
O-oo13.7	2nd	Outbred	High Risk	bedding	87	0	1	4	9	3	36	2	4	0	15	13	0	0	0	2
O-ii18.5	2nd	Inbred	High Risk	bedding	80	0	32	21	9	3	5	0	3	1	6	0	0	0	0	1
O-ii28.2	2nd	Inbred	High Risk	bedding	143	0	82	45	6	0	4	0	0	0	5	1	0	0	0	1
O-ii18.5	2nd	Inbred	High Risk	bedding	75	0	26	26	3	2	14	0	2	0	2	0	0	0	0	0
O-ii28.2	2nd	Inbred	High Risk	bedding	57	0	12	25	7	2	9	0	1	0	0	1	0	0	0	0

Figure 18: Table containing data extracted from the USV recording sessions. Age of the animals was 6 weeks on the 1st recording, 14 weeks on the 2nd. "#USV" represents the total amount of all USV counted in the respective 25-minute long sequence.

4. Discussion

The results of the sperm morphology analysis could not confirm our hypothesized increase in morphological sperm quality parameters (Loose head ratio, sperm midpiece width, sperm head width, sperm head ARC and sperm head elongation) with increased risk of sperm competition. In fact, when it comes specifically to murine sperm morphology, (Firman et al. 2011) demonstrated in a comparative study, that sperm competition does not affect sperm hook morphology. This is interesting, since several other reproductive traits such as sperm motility, sperm number and testes size can indeed diverge between monogamous and polygamous (e.g. under the influence of sperm competition) populations of different taxa/species bred under a selective regime (Firman and Simmons 2010, Hosken and Ward 2001, Simmons and García-González 2008). Similarly, manipulating the genetic quality of the males did, at best, only have a marginal effect on sperm morphology, as inbred males showed a slightly higher number of loose sperm heads per ejaculate than outbred males. This finding could be explained by the inbreeding depression this group experienced, since inbreeding can lower semen quality and overall reproductive performance (van Eldik et al. 2006). Furthermore, (Gotoh 2010) indicated that, by inbreeding mouse strains, sperm head abnormalities can be enhanced. However, this does not explain why other morphological sperm parameters did not decline with inbreeding. Whether sperm morphology can ultimately be influenced by sperm competition or genetic status still remains to be an interesting question; could the intensity/duration of experienced sperm competition by the individual or the number of generations of breeding possibly be the deciding factor to elicit an effect? Admittedly, the duration of our study exceeded the 34.5 days it takes house mice to undergo a full cycle of spermatogenesis (OAKBERG 1956), but our study exposed F5-generation mice to sperm competition over the course of several weeks, not years or even generations, in which evolutionary adaptions on morphological sperm traits could possibly occur. This also begs the question to what degree morphological sperm traits are genetically constraint or embedded in the genome. Compared to sperm motility and total sperm number, both sperm traits that have been proven to be susceptible to evolutionary change under the influence of selective breeding of 12 generations of house mice experiencing varying degrees of sperm competition (Firman and Simmons 2011), sperm morphology could be more difficult to alter in a shorter-term experimental study design. After all however,

the evolution of sperm morphology in the context of sperm competition received little attention so far in the scientific community (Pizzari and Parker 2009). Regarding the age of the animals at the beginning of this study, it is also interesting to discuss whether the prepubescent age of the animals at the introduction of the experiment (4 – 5 weeks of age at introduction) interfered with a thorough investigation of sexual behavior and biology. Physiologically, house mice can be sexually mature at an age of 6 - 8 weeks (The Laboratory Mouse, Second Edition, 2012, Hedrich 2012). This creates a possible discrepancy of 1 – 4 weeks, meaning that we could have expected the male mice in our study to be sexually mature in a time frame ranging from as early as week 2 of 11 of our experiment to as late as week 5 of 11 of our experiment. When investigating sexual behavior and sperm competition, this could naturally pose a problem since these prepubescent males do not necessarily respond to sexual cues or engage in sperm competition (both behaviors naturally starting with the onset of puberty). Nevertheless, the question whether it is ultimately advantageous or disadvantageous to introduce prepubescent males to this kind of experimental design is difficult to answer and remains.

The results of the scent marking analysis varied from the sperm morphology analysis in that we found a significant treatment effect on the scent mark count score and a highly significant effect of the bedding stimulation on both the scent marking score and the total marked area of the filter paper. This contrasts the findings of a previous study by (Ramm and Stockley 2008), in which they demonstrated in an experiment over 22 weeks with similar social manipulations, that the average levels of scent marking did not differ significantly between “low” versus “high risk” of sperm competition treatments. The social experience of these “low risk” males however also consisted of sporadic simulation of territorial intrusion by other males (e.g. olfactory cues), compared to no intruders in “low risk” males in our study. This fact significantly hinders comparability between the two studies but could be taken into consideration when planning future behavioral experiments incorporating different levels of sperm competition. Furthermore, in our study the scent marking response to female bedding was far stronger compared to the response to male bedding and “high risk” males produced more scent marks than “low risk males”, insinuating a potential prioritization of investment in courtship behavior over aggressive, competitive behavior. However, the genetic background did not show a significant effect on scent marking behavior on either measured parameter. The fact, that we can see stronger treatment responses in scent marking behavior than

in sperm morphology could be owed to the much more spontaneously adaptable nature of behavioral patterns compared to the adaption of physiological or anatomical traits such as sperm morphology. Whereas, on the topic of female versus male bedding stimulations, the results spark the question if there is a preference in the attraction of possible female mating partners versus the assertion of dominance over possible male competitors. Making certain predictions in regards of animal behavior is not easy and requires well planned experimentation and analysis, so further investigations are necessary to prove any assumptions.

Looking at the results of the USV analysis, we can only make very limited assumptions due to the relatively small sample size. Among the vocalizing males, we can see a stronger reaction to female than to male bedding during bedding stimulation, most USVs being emitted by “low risk” outbred males during the second of two recording events. As mentioned above in the discussion of scent mark analysis, this could also be a hint to a potential prioritization of investment in courtship behavior over aggressive, competitive behavior and possibly interpret these USV more as a means to attract females and improve courtship success than to vocally fend off competitors. After all, the low response rate to male bedding could be expected, since (Portfors 2007) could show, that male mice predominantly vocalize for females. The overall low response rate of USVs, however, could be in line with the findings of (Portfors and Perkel 2014, Sewell 1972), which indicate that the majority of USVs are emitted when the animals are in close proximity to each other. Additionally, young, prepubescent age and lack of courtship experience due to a rather isolated sexual maturation also have to be taken into consideration as causes for the low amount of emitted USVs. As indicated by Ramm and Stockley 2009, the presented olfactory cues (e.g. soiled bedding of sexually mature female mice) could also be not a strong or frequent enough stimulant to elicit the presumed effects investigated in this study. Ultimately, although some animals responded strongly, most animals showed little to no reaction to treatment and/or stimulation, creating a lot of outlier data in the statistical analysis. This, of course, complicates the analysis of the results and further reduces the effective sample size(s).

Arguably, the animals in the “high risk” treatment group of this study, were also granted almost direct contact to other males through their cage lids. When looking at the lack of significant differences between the treatments in the results though, it begs the question whether direct contact is 1) a stronger or weaker cue to trigger adaptive strategies than olfactory cues, 2) the time episodes, the animals were granted this level of contact was adequate or 3) if maybe a similar level of contact to sexually mature females would have elicited the presumed effect.

Other unexpected, influential factors can be found in the scent mark analysis using the square filter papers, which have been placed into the center of the cages and were open to free interaction by the animals. This resulted in filter papers being gnawed on, thus reducing the measurable area (see Figure 19, additional material) or some even entirely displaced into locations, where it has been almost impossible for the respective animal to reach it to mark with urine. In one instance, for example, the animal placed the filter paper between the cage wall and the wall of the plastic mouse house, leaving practically no room for interaction and therefore resulting in an unmarked filter paper. In addition to that, a total of three animals have been found dead for no apparent reason on the following morning after no-manipulations dates during routine health checks. These individuals had to be replaced by similar “back-up” mice, thus introducing them newly into the running experiment and therewith also introducing a non-reproducible variable. Room temperature also proved to be difficult in this experimental setting during the summer of 2017 in Vienna. With outside temperatures regularly exceeding 30 °C, even the newly installed air conditioning units at times struggled to keep the temperatures below 28 °C inside the experimental room. And although the thermoneutral zone of the house mouse is currently still debated to be around 30 °C (Speakman and Keijer 2012), temperature consistency alone could possibly be another influential factor in this experimental setup.

In conclusion, the gathered results could only prove a partially significant difference between the “low risk” and “high risk” treatment groups, as sperm morphology parameters (Loose head ratio, sperm midpiece width, sperm head width, sperm head ARC and sperm head elongation), total area marked on filter paper with scent marks or USV emissions were not affected by the males’ experienced level of sperm competition, while “high risk” males showed a higher scent marking score. We could also find a significantly higher scent marking response on female bedding stimulations compared to male bedding stimulations, whereas the genetic status of the individual had no significant effect on any of the measured parameters (except a marginally non-significant difference between genetic statuses in loose head ratios). It could be suggested to investigate these measured parameters over a longer time period, spanning multiple generations. Such future studies could possibly elicit stronger effects and elucidate, if or to what degree sperm competition influences sperm morphology and the production of secondary sexual traits, when experienced over a longer time.

5. Summary

The concept of sperm competition describes the competition between the sperm of two or more males for fertilization of the same set of ova and represents an underlying theory for the arms race in the field of sexual conflict. To gain an advantage over competitors, male mice develop adaptation strategies that lead to the evolution of sexual traits linked to reproductive success during sexual selection. Sperm morphology, scent marking behavior and the emission of USVs are among those traits and are possibly susceptible to change under the influence of external factors. In this study, we conducted an experiment on male house mice of varying genetic background (either inbred or outbred controls) and perceived risk of sperm competition ("low risk" versus "high risk") and assessed the effects on morphometric sperm parameters (Loose head ratio, midpiece width, head width, head ARC and head elongation), expression of scent marking behavior and emission of USV. The results hinted, that genetic status could have an influence on the number of abnormal sperm per ejaculate, but furthermore showed, that there is no significant difference between the two treatment groups or genetic statuses when looking at all the other measured morphometric parameters. Scent marking behavior analysis revealed a significantly higher response rate to female bedding stimulation compared to male bedding stimulation and partially higher scent marking expression in "high risk" treatment mice, while the small sample size of the USV data prohibited decisive assumptions. Future, possibly long-term studies are required to test, if and to what degree sperm competition influences male sperm morphology and the production of secondary sexual traits.

6. Zusammenfassung

Das Prinzip der “sperm competition“ beschreibt den Wettbewerb zwischen den Spermien zweier oder mehr männlicher Individuen um die Befruchtung der Eizellen eines weiblichen Individuums und stellt eine Grundlegende Theorie im Wettrüsten auf dem Gebiet des sexuellen Konflikts dar. Um sich einen Vorteil gegenüber Mitbewerbern zu verschaffen, entwickeln männliche Mäuse Anpassungsstrategien, die zur Evolution sexueller Merkmaler führen, die mit dem Reproduktionserfolg während der sexuellen Selektion im Zusammenhang stehen. Spermien Morphologie, Markierungsverhalten und die Emission von Lautäußerungen im Ultraschall-Bereich (USV) zählen zu diesen Merkmalen und sind möglicherweise dem Äußeren Einfluss unterlegen. In dieser Studie haben wir männliche Mäuse unterschiedlicher genetischer Qualität (Inzucht oder Auszucht) im Rahmen eines Experiments unterschiedlichen Graden an „sperm competition“ ausgesetzt, um Veränderungen verschiedener morphologischer Spermienparameter (Kopflose Spermien/Ejakulat, Breite des Spermienmittelstücks, Spermienkopf -breite, -länge und -elongation) beziehungsweise im Markierungs- und Gesangverhalten zu untersuchen. Die Ergebnisse zeigten, dass die genetische Qualität der Männchen einen Einfluss auf die Anzahl an abnormalen Spermien haben könnte und darüber hinaus, dass es keinen signifikanten Behandlungs-Effekt gibt oder die genetische Qualität einen Einfluss hat im Bezug auf die restlichen gemessenen Spermienparameter. Außerdem konnten wir zeigen, dass sich die Reaktionen der männlicher Mäuse im Markierungsverhalten auf weibliche Geruchsnoten signifikant von Reaktionen auf männliche Gerüche unterscheiden und es eine teilweise stärkere Reaktion in den Behandlungsgruppen gibt, die einem höheren Maß an „sperm competition“ ausgesetzt waren. Obwohl die generierten Ergebnisse im Kontext dieser Arbeit keinen soliden Beweis liefern konnten für einen deutlichen Unterschied zwischen den untersuchten Behandlungen, bleiben noch einige Fragen offen, die weitere Forschung auf diesem Gebiet erfordern für deren Aufklärung.

7. References

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8. Appendix

8.1 List of figures

Figure 19: Digital photograph under UV light showing a filter paper with urine stains (bright blue in color, surrounded by yellow edges). on the front (**a**) and back (**b**) of the same filter paper. Red circles indicate the gnawed off material at the edges (black areas)

8.2 Additional material

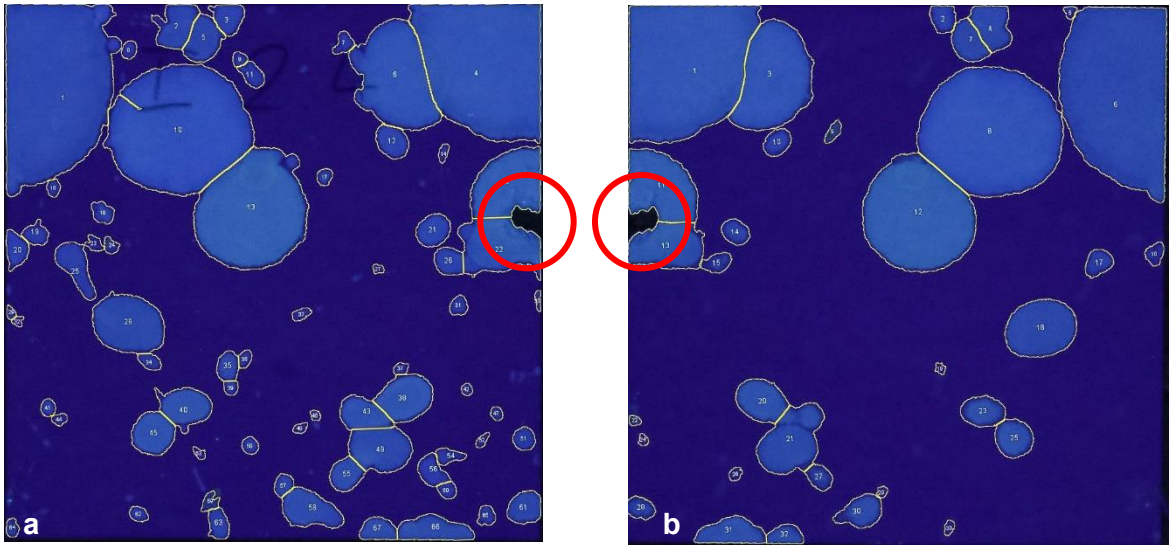

Figure 19: Digital photograph under UV light showing a filter paper with urine stains (bright blue in color, surrounded by yellow edges). on the front (**a**) and back (**b**) of the same filter paper. Red circles indicate the gnawed off material at the edges (black areas)

9. Acknowledgements

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