The effect of cytoplasmic DNA on sperm length in the seed beetle Callosobruchus maculatus (Coleoptera: Bruchidae).

Albert Larkeson Nowostawski
Abstract: The competition between sperms of different males for the fertilization of eggs should benefit the individuals with the best equipped sperm. Sperm traits, such as velocity, longevity and sperm length are regarded as particularly important in sperm competition and one would expect them to evolve adaptively. However, this notion is challenged if the trait is controlled by maternally-inherited mitochondrial (cytoplasmic) DNA, because mitochondrial genes come to an evolutionary “dead end” in males. This study used distinct lines of the seed beetle (*Callosobruchus maculatus*) with fixed nuclear and cytoplasmic lineages that gave the opportunity to assess the relative contribution of the two genomes’ effects to sperm length in *C. maculatus*. Effects of nuclear and cytoplasmic genomes were found, as well as an interaction between the genomes. The nuclear effect was apparently larger than the cytoplasmic, hence allowing a scope for the adaptive evolution of sperm length in this species. The age of mothers also had an effect on the sperm length of their sons. Moreover, a comparison with data from a sperm viability study of *C. maculatus* reveals a positive relationship between sperm length and sperm viability for the cytoplasmic lineages and a negative relationship for the nuclear lineages.

Introduction

Males are typically the sex that invests least in offspring production and this allows them to have a higher reproductive rate and hence a higher operational sex ratio (OSR) (Emlen & Oring 1977). Accordingly, this leads to an “excess” in males which results in competition over the limited resource, females. Furthermore, the competition to mate with the sex with the lower OSR (usually the female, but see e.g. Ahnesjö et al. 2001) will exert selection among the sex with the higher OSR for traits that may not always be beneficial in terms of survival, but are advantageous in terms of increased reproductive success. This “sexual” selection is thus divided into two forms; the intrasexual (i.e. male-male competition) and the intersexual (i.e. female choice) (Andersson 1994).

Sperm competition was conceptualized as a process of sexual selection by Parker (1970; 1998) and is defined as the competition between sperm of two or more males for fertilization of a given set of ova. The criteria required for sperm competition to take place are multiple mating and the coexistence of sperm from two or more males inside the female reproductive tract. The latter is especially common across insects, where females have specially adapted storage organs, spermathecae, which permits sperm mixing. Thus, insects are considered to sustain high levels of sperm competition. (Simmons 2001). The risk of being exposed to sperm competition forces accordingly the males to allocate much of their energy into trying to avoid sperm competition completely (e.g. by mate guarding or attaching mating plugs) and/or produce ejaculates that are superior to rival males’ ejaculates (e.g. by including hormones in the ejaculate that are able to manipulate the females or more competitive sperms) (reviewed in Simmons 2001).

The sperm traits; size, longevity and velocity have been regarded as especially important in sperm competition (Simmons 2001). Sperm production (i.e. sperm numbers) may also play a vital role, as has been shown in several studies where positive relationships have been found between testes size, sperm production and sperm competitive success (Parker 1998 and references therein, but see Snook 2005). Parker (1993) showed theoretically that sperm competition should exert greater selection upon sperm numbers than sperm size, but several empirical studies indicate that sperm competition is associated with variance in sperm size. They have shown that size of sperms increases with sperm competition intensity in most taxa, but decreases in fishes. (reviewed in Simmons 2001). However, lack of association between sperm competition and sperm length has also been shown, in a study across 83 mammalian species (Gage & Freckleton 2002, but see Gomendio & Roldon 1991), and similar result has been reported for the field cricket *Teleogryllus oceanicus* where no influence of sperm number or sperm length on paternity was found (Simmons *et al.* 2003). Noteworthy is that Gage and Morrow (2003) found, in the field cricket *Gryllus bimaculatus*, that males, which
produce smaller and more numerous sperms than their rivals, had higher fertilizations success under conditions of sperm competition.

The relative velocity of the sperms would intuitively be a robust measurement of fitness (the fastest sperms would be superior to their rivals in the competition to reach and fertilize most of the eggs), but no studies have so far fully addressed this issue. On the other hand, the sperm motility may play a minor role in sperm competition because much of the sperm movement inside the reproductive tract seems to be female-mediated (Arthur et al. 1998; Bloch Qazi et al. 1998 and references therein). And furthermore, it has been found in the rove beetle *Aleochara curtula* and several other species that sperm motility do not play a fundamental role in spermathecal filling (Werner et al. 2002 and references therein).

Further indications of sperm longevity and size playing important roles in sperm competition have been shown. Bernasconi et al. (2002) provided several explanations why the female reproductive tract is toxic for sperms, and hence one should expect relative high longevity being awarded in this sperm-hostile environment. Moreover, the sperm size and the morphology of the female reproductive tract have been found to co-evolve in several species, both across invertebrates (e.g. Dybas & Dybas 1981; Pitnick et al. 1995; Morrow & Gage 2000) and birds (Briskie & Montgomery 1992). This coevolution, or “evolutionary chase”, would be similar to the one Holland and Rice (1998) describes as “sexual selection chase-away”, where, for example, male genitalia are evolving as a response to female morphology and/or behaviour. This process is more likely to be more promoted across polyandrous species than monoandrous species (see Arnqvist 1998).

The mitochondria are the power stations of the cell, supplying the cell with most of its energy through oxidative phosphorylation. These organelles are located in the cytoplasm of cells and contain their own genome (Nass & Nass 1963). Much of the mitochondrial genes, however, have throughout evolution been transferred into the nucleus (Martin & Herrmann 1998). Many of the essential functions for the mitochondria are nowadays controlled by nuclear DNA (nDNA). For example, the formation of the mitochondrial membranes is a process exclusively controlled by nuclear genes, whereas oxidative phosphorylation is controlled by both nDNA and mitochondrial DNA (mtDNA) (see Attardi & Schatz 1988).

The mtDNA codes only for a very small fraction of the total number of genes in the cell. In humans for example the mtDNA holds 13 protein-coding genes (Gray et al. 1999) whereas it is as many as 20 000 – 25 000 in the nDNA (IHGSC 2004). Moreover, different cells types contain different number of copies of mitochondria, ranging between 1 000 and 100 000 copies in human diploid cells (Chinnery & Schon 2003). The oocyte contains approximately 100 000 copies while the sperm contains less than a hundred (Chinnery & Schon 2003; St John et al. 2005), all located along its midpiece and/or tail. Another very important and significant distinction between the mtDNA and nDNA is the mode of transmission to the offspring. The nuclear genome is inherited biparentally across sexually reproducing species while the mitochondrial genome is strictly maternal inherited (e.g. Giles et al. 1980). Studies by, among others, Gyllensten et al. (1991) have found some, but extremely low, levels of paternal leakage of mtDNA to the offspring in some species, but its impact on the organism’s phenotype is negligible.

A consequence of the maternal inheritance of mtDNA is that selection on mitochondrial genes can only occur in females. Males are, hence, evolutionary “dead ends” for mitochondrial genes and this has some intriguing effects. Phenotypic variation in male-specific fitness traits that are encoded by mtDNA cannot be transmitted to the offspring. Accordingly, mtDNA mutations, that are advantageous for males, are not necessary maintained in the population if they are not advantageous for females. The antagonistic coevolution between sexes, where a genotype have high fitness in one sex but low fitness in the other (see e.g. Rand et al. 2001; Arnqvist & Rowe 2002), could, theoretically, lead to that
deleterious mtDNA mutations in males can be maintained in a population, if the mutation is beneficial, neutral or slightly deleterious in females (Frank & Hurst 1996). If the mitochondria genome encodes for some sperm traits, the consequences could be that these traits would be constrained in their ability to evolve adaptively.

A functional mtDNA is believed to be essential to withhold high sperm quality and reproductive success and mutations in the mitochondrial genome may consequently have severe effects on the individuals fitness (reviewed in St John et al. 2005). It has been found that the sperm motility phenotype, in humans, is conditioned by mtDNA haplogroup, i.e. different mitochondria haplotypes result in different motility phenotypes (Ruiz-Pesini et al. 2000). Similarly, a study by Dowling et al. (2006) found that the relative proportion of viable (living) sperms, taken from the seminal vesicles of male seed beetles, *Callosobruchus maculatus*, was influenced by cytoplasmic (presumably mitochondrial) genes. Moreover, the amount of mitochondria in the sperm is likely to covary with the level of sperm competition. This has been shown in a comparative study across primate species by Anderson & Dixson (2002), where the volume of the midpiece (the part of the sperm in mammals which contain the mitochondria) across different species was positively correlated with residual testis size and, hence, was likely to affect sperm competition (but see Snook 2005). Accordingly, there are strong indications of mitochondria being crucial for sperm functions and traits. Taking this and the strict maternal inheritance of mtDNA into consideration, it gives rise to a major unresolved question in evolutionary biology; Is the potential for the adaptive evolution of sperm traits constrained by the underlying genetic architecture?

The aim of this study was to examine if cytoplasmic genes affect variation in sperm length in the seed beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae). The beetle is a polygamous species with sperm mixing inside the female reproductive tract, and hence intense sperm competition (see e.g. Eady 1994; 1995). The sperm that was being measured was taken from 25 artificially breed (cytonuclear introgression) lines, in which each line is characterized by orthogonal combinations of distinct nuclear genome and mitochondrial (cytoplasmic) lineages. By comparing the variation in sperm length between the lines, it was possible to assess if this sperm trait is controlled by nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) or an interaction between them both. The results will elucidate if and how the sperm length phenotype in this species is able to evolve adaptively. An additional dimension to the study was added by including both old and young mothers of the focal males to determine whether there are maternal effects on sperm length.

**Methods**

*C. maculatus* is a widespread pest of stored legumes. The life cycle of the beetle, in laboratory conditions, is approximately 3 weeks long, and the female oviposits for about six days on the surface of seeds (Dowling et al. 2006). The larvae hatch and burrow themselves into the seed, where they develop. When fully developed, individuals emerge from the seeds as adults that are immediately capable of reproducing.

The beetles used in this study were derived from five distinct outbreed *C. maculatus* wild-type populations. These stock populations are Brazil (BR), California (CA), Lossa (LO), Oyo (OY) and Yemen (YE). The obtaining and maintenance of these populations, previous to this study, is described in Dowling et al. (2006). The 25 different “introgression” lines used in this study were created by using back-crossing to introgress the five cytoplasmic lineages so that each was expressed in each of the five nuclear lineages (Table 1). The beetles have, previous to this study, been treated with antibiotics, in order to eliminate any maternally inherited cytoplasmic bacteria, such as *Wolbachia* (see Dowling et al. 2006). Accordingly, the
cytoplasm of these beetles are not contaminated with DNA from other bacterial organisms (known to inhabit the cytoplasm) and can be presumed to only contain mitochondrial DNA.

Table 1. The 25 "mt introgression" lines, constituted by five cytoplasmic haplotypes (mtDNA) and five nuclear backgrounds (nDNA).

<table>
<thead>
<tr>
<th>mtDNA</th>
<th>BR</th>
<th>CA</th>
<th>LO</th>
<th>OY</th>
<th>YE</th>
</tr>
</thead>
<tbody>
<tr>
<td>nDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR</td>
<td>BR x BR</td>
<td>BR x CA</td>
<td>BR x LO</td>
<td>BR x OY</td>
<td>BR x YE</td>
</tr>
<tr>
<td>CA</td>
<td>CA x BR</td>
<td>CA x CA</td>
<td>CA x LO</td>
<td>CA x OY</td>
<td>CA x YE</td>
</tr>
<tr>
<td>LO</td>
<td>LO x BR</td>
<td>LO x CA</td>
<td>LO x LO</td>
<td>LO x OY</td>
<td>LO x YE</td>
</tr>
<tr>
<td>OY</td>
<td>OY x BR</td>
<td>OY x CA</td>
<td>OY x LO</td>
<td>OY x OY</td>
<td>OY x YE</td>
</tr>
<tr>
<td>YE</td>
<td>YE x BR</td>
<td>YE x CA</td>
<td>YE x LO</td>
<td>YE x OY</td>
<td>YE x YE</td>
</tr>
</tbody>
</table>

For the assay, 25 large petri dishes with each 100 grams of black eyed beans were arranged in each block. The beetles (of approximately equal sex ratio) were added to each dish in such numbers that the average density per bean would not exceed four eggs. The dishes were then kept in an incubator at constant temperature (30ºC) and humidity (50%). To test the maternal effect on sperm length, the 25 cytonuclear lines were duplicated so that one replicate was propagated by young mothers (newly hatched mothers collected at day 24 of the lifecycle) and the other replicate by old mothers (collected at day 31 of the life cycle). Approximately 17 days into the beetles’ life cycle the beans were isolated from each other and put into so called “virgin chambers”. The first adults started to emerge at day 20 and the assay for each block was always conducted for a maximum of five days. Virgin males and females were collected each morning at around 0800h for that day’s assay. Each evening at around 2000h all hatched beetles in the virgin chambers were discarded, hence guaranteeing that the beetles collected the following morning would not be older than 12 hours.

At the beginning of each day, the virgin males and females of each line were collected and stored separated, on a temperature-controlled (30ºC) bench. In each sample a male was mated with a female from the same introgression line. The reason for this procedure was that the sperm are mainly in bundles inside the male (Damian Dowling pers. comm.) and the fluid(s) inside the female reproductive induces the sperm to detach (see also Elofsson 2005), thus making them easier to measure. After approximately 90 minutes, when the level of sperms in the female spermatheca was at its highest (Eady 1994), the females were dissected under a stereoscope and the spermatheca was removed using Watchmaker’s forceps. The sperms in the spermatheca were squeezed out in a 5 µl drop of saline buffer solution on a microscope glass slide. The saline buffer solution used was DPBS buffer pH 7.4 (Werner et al. 2002). A cover slip was then gently pressed over the drop, so that the sperms, across all samples, were subsequently measured on the same plane. Eight random photos were taken of each sample using a Hamamatsu OrcaIIIm camera, a Leica DMRX/E light microscope with 400x magnification and the computer software program Openlab v. 3.0.9. The experiment was conducted in three blocks, each separated in time by one generation.

Ten males in each lineage, randomly scattered over the three blocks, were used, making in total the assay comprise of over 2 000 pictures (25 lineages x 8 males/lineage x 10 photos/male). The pictures were then imported to the computer software program ImageJ v. 1.34 where the sperm’s length was measured. It was suggested that the influence of nDNA and mtDNA could perhaps vary between different parts of the sperm, and therefore the measurement of each sperm was divided into three measurements: whole length, head length and tail length. The whole length was measured once and the head length measured three times, due to its short length and consequently the higher risk of measurement errors. The tail length was obtained by subtracting the average of the head length from the whole length. In order to assess repeatability of the sperm length measure, approximately 5% of the pictures
were measured twice, with the second measurement taking place at least two days after the first measurement, blind to the length of the first measure.

The statistical analysis was done in SYSTAT v. 11 and SAS v. 9.1 using Analysis of Variance (ANOVA) in which all terms were included as fixed effects with the exception of Block, which was included as a random effect. The a priori significance criterion was 0.05.

Results

Mean lengths across all sperms was for whole length 170.34 µm (n = 2008, S.E. = 0.17), for tail length 153.52 µm (n = 2008, S.E. = 0.16) and for head length 16.82 µm (n = 2008, S.E. = 0.022). The repeatability of the sperm length measurements was calculated using the equation provided in Lessells & Boag (1987). The measures were highly repeatable for both head length (R = 0.71, n = 105, p < 0.001) and whole length (R = 0.999, n = 105, p < 0.001). Due to the reason that tail length was not measured directly no repeatability calculation was done on this length measurement.

The nDNA had a strong significant effect on whole length (F$_{4, 222}$ = 18.30, p < 0.001), head length (F$_{4, 222}$ = 15.94, p < 0.001) and tail length (F$_{4, 222}$ = 16.42, p < 0.001) of the sperm. The cytoplasmic effect of the three length measurements was statistically significant for whole length (F$_{4, 222}$ = 3.40, p = 0.01) and tail length (F$_{4, 222}$ = 3.09, p = 0.017), but not significant for head length (F$_{4, 222}$ = 2.10, p = 0.082). The interaction between nDNA and the cytoplasmic DNA was significant for tail length (F$_{16, 222}$ = 1.72, p = 0.044) and nearly significant for whole length (F$_{16, 222}$ = 1.65, p = 0.057), whereas it was non significant for head length (F$_{16, 222}$ = 0.74, p = 0.75). (Figure 1). The sperm lengths (tail and whole lengths) of some cytoplasmic lineages were longer when expressed in some nuclear backgrounds, but shorter when expressed in others.

The age of the focal males' mothers had a strong significant effect on whole sperm length (F$_{1, 224}$ = 15.94, p < 0.001), tail length (F$_{1, 224}$ = 14.20, p < 0.001) and head length (F$_{1, 224}$ = 7.48, p = 0.007). “Old” mothers had sons with longer sperm compared to “young” mothers (Figure 2). There was no effect of block in any of the statistical analyses.

An assay has recently been conducted on the cytoplasmic and nuclear effect on sperm viability in C. maculatus (Dowling et al. 2006). Using the same 25 introgression lines that have been used in this study and following similar procedures Dowling et al. (2006) examined the relative number of living and dead sperms from samples of dissected male seminal vesicles. Because of the similarity in experimental design between the sperm viability assay and this sperm length assay a comparison of the two datasets was made in order to find any association. The average whole sperm length per cytoplasmic lineage tended to be positively

---

**Figure 1.** Interaction plot of nuclear and cytoplasmic (cyto) effect on sperm whole length.

**Figure 2.** Maternal age effect on sperm length (adjusted least squares means of GLM ± 1 standard error). Sample size are n = 125 for young and n = 126 for old.
related to average sperm viability ($r^2 = 0.76$, $n = 5$, $p = 0.055$) and the nuclear lineage showed a tendency towards a negative relationship ($r^2 = 0.73$, $n = 5$, $p = 0.063$) (Figure 3).

![Figure 3. Relationship between mean sperm length and mean sperm viability across the five cytoplasmic lineages (left graph). Relationship between mean sperm length and mean sperm viability across the five nuclear lineages (right graph).](image_url)

**Discussion**

This study found that the variation of sperm length across the 25 introgression lines was a result of both nuclear and cytoplasmic effects. Furthermore, there was a significant interaction between nuclear and cytoplasmic lineages on sperm tail length and a tendency towards a significant interaction on whole length. The finding of an intergenomic interaction confirms findings by others (e.g. Rand *et al.* 2001; Ballard & James 2003; Zeyl *et al.* 2005; Dowling *et al.* 2006) of interaction between nDNA and the cytoplasm (presumably the mtDNA) in components that are presumed to be associated with fitness. This study is one of the first to show that sperm quality is influenced by the natural variation in cytoplasmic genes and the interaction between cytoplasmic and nuclear genes.

If cytoplasmic genes are primarily responsible for determining the sperm length phenotype, then this would constrain the possibility for this trait to evolve adaptively. It means that these length-associated genes will not be transmitted upon the fertilization of the egg and, hence, there can be no selection for or against these genes. However, the nuclear effect on sperm length is apparently stronger than the cytoplasmic effect (based on a comparison of the F values gained from the relevant ANOVAs) and thus there is a scope for the adaptive evolution of sperm length in this species. Notably, the results of a recent study suggested that sperm length is inherited down the maternal line in the field cricket (*Gryllus bimaculatus*) (Morrow & Gage 2001). This could point towards that the sperm trait, length, being partly encoded by genes that are either on the X-chromosome (see also Ward 2000) or, as found in this study, by genes in the cytoplasm (presumably mitochondrial genes).

The absence of an intergenomic interaction and cytoplasmic effect on the sperm head length indicates that the head of the sperm is probably merely a transport capsule for the nDNA and possibly not affected by differential energetic outputs associated with different mitochondrial haplotypes. The sperm tail length, on the other hand, was affected by cytoplasmic genes and this may point to an important energy demand for this part of the sperm. Since some studies indicate that sperm production is energetically costly (e.g. Dewsbury 1982; Olsson *et al.* 1997) it is plausible that the mitochondrial haplotype will have a pivotal influence before and/or during spermatogenesis in the germ line (reviewed in Moore & Reijo-Pera 2000). The mitochondria is, furthermore, believed to power the sperm motility (e.g. Perotti 1973; Bão *et al.* 1992) (with the exception for some insect species [reviewed in Baccetti & Afzelius 1976; Werner 1999]), and this could link sperm motility with sperm...
length if there is an association between length and velocity in this species (see Cardullo & Baltz 1991; Gomendio & Roldan 1991).

It should be pointed out that the relationship between fitness and sperm size is still somewhat unclear and several hypotheses have been put forward on how sperm length may contribute to success in sperm competition (reviewed in Simmons 2001). For example, as mentioned in the introduction, there could be a positive relationship between sperm length and sperm velocity and/or longevity. Moreover, longer sperm might be better at plugging storage organ(s) and/or resisting sperm displacement, compared to shorter sperms. No studies have so far showed a relationship between sperm longevity and sperm length (Simmons 2001). Additionally, the only studies showing a positive correlation between sperm size and velocity have dealt with amoeboid sperms (Radwan 1996; LaMunyon & Ward 1998), where the sperm progresses by “cell crawling” (see Stossel 1994). Nonetheless, increased length would most likely mean a higher energy output for the sperm (reviewed in Parker 1998), and there seems to be no obvious reason for a male to allocate energy in producing longer sperms if it will not provide him with any fitness advantages.

An effect of age of mothers on sperm length was found in this study, and the effect was apparent in all three length measurements. One straightforward explanation for this result would be that the condition of mothers is passed on to their sons. Older mothers tend to be smaller and in poorer condition and accordingly producing sons with shorter sperm. However, it was found that older mothers produced sons with the longest sperm, and this was counter the expectation. Moreover, there might not be any association between the condition of the male and sperm length. Although Gage (1994) has found a positive relationship between male body size and sperm size across butterflies, no relationship was found across mammals (Gage 1998). In addition, a minor assay (n = 29) in connection with this study compared male weight with sperm size and found no correlation (data not shown). Therefore, it seems unlikely that in C. maculatus the sons’ condition, determined by maternal effects, will have an effect on sperm length.

The most plausible explanation is instead probably that the larval density (i.e. egg density per bean) varied consistently between the two maternal age treatments. Although the density was held at the maximum of four eggs per bean, larval density was never quantified. Hence, it is possible that there were small but consistent differences between the treatments. A larval density influence on sperm length is nevertheless a very interesting observation. However, further empirical experimentation is required in order to fully understand the nature of these environmental effects on sperm length. It should also be pointed out that longer sperm do not necessary need to be an indication of higher fitness (see Gage and Morrow 2003), and it could well be that in C. maculatus small/short sperms are the most beneficial for males to produce. However, as discussed above, if longer sperm are more energetically costly, then they probably also confer a fitness advantage.

The check for association between viability and sperm length provided some interesting results. Mean sperm lengths for a given cytoplasmic lineage tended to be positively correlated to mean viability, whereas in the nuclear lineage the opposite trend was observed, with a tendency towards a negative correlation. Although the trends were not statistically significant (the low number of lineages used meant that it was very difficult to achieve statistical significance), sperm length was nonetheless strikingly related to sperm viability (76% and 73% for the cytoplasmic and nuclear lineages respectively).

These opposite relationships between the cytoplasmic and nuclear lineages are confusing, and probably difficult to interpret within the scope of this study. It would be tempting to assign this as a very straight-forward evolutionary antagonistic coevolution in sperms, leading to sub-optimal traits (see Birkhead et al. 2005). This would mean that the two genomes are in conflict with each other and when one genome codes for the longest length the other codes for
the shortest, and vice versa. However, this kind of association is not found (Figure 3) and instead there seems to be little consistency in what effect the sperm length has on viability in the cytoplasmic or the nuclear lineage. It is therefore not easy to draw any advanced conclusions from these results.

In conclusion, my main focus of this study has been to investigate whether cytoplasmic DNA effects sperm length. The results suggest that mitochondria genes play a role in the formation of sperm length, especially the tail, but the nuclear background has also a major influence, both by itself and in interaction with the cytoplasmic lineage. This indicates that the ability for the sperm to evolve its length adaptively may be somewhat constrained, but not totally excluded. The further findings of this study (the effect of age of mothers on sperm length and the relationship between sperm viability and sperm length), illustrate the diversity of factors affecting sperm traits and the complexity that surrounds them.

Acknowledgements

I thank my supervisors Damian Dowling and Göran Arnqvist for giving me the opportunity to work with this very intriguing topic, and for the support and assistance I received from them throughout the degree project. Furthermore, I thank Ted Morrow for helpful discussions of sperm morphology and sperm competition and Stefan Gunnarsson for technical assistance when using the microscope.

Reference


