The study presents a comparison of sperm traits in various species of the genus *Daphnia* plotted on a molecular phylogeny of the group (published by other authors). Two traits have been monitored: length and presence/absence of an extracellular coat (which compacts the sperm cell and possibly encloses filaments formed by the cell membrane).

I see two major problems with this manuscript that should be addressed: taxonomy and image quality.

**Taxonomy**

The authors studied sperm of 15 species of the genus *Daphnia*. However, in the abstract already, they mention a *Daphnia sensu lato* clade and a *Ctenodaphnia* clade without explaining anywhere in the manuscript the concept behind these terms.

As much as I understand (I am not a branchiopod-specialist), the genus *Daphnia* has three accepted and valid subgenera, i.e. *Daphnia* (*Ctenodaphnia*), *Daphnia* (*Australodaphnia*), and of course the nominotypical subgenus *Daphnia* (*Daphnia*), since it contains the type species of the genus *Daphnia*, i.e. *D. (D.) longispina* as pointed out by Šrámek-Hušek, Strašcraba & Brtek (1962). This seems to be covered by the zoological nomenclatural rules (see ICZN Art. 44.1), which clearly are the basis for any taxonomical, phylogenetic and evolutionary study.

If the above concept of the genus *Daphnia* and its subgenera is correct, I do not understand why the authors use the terms ‘*Daphnia sensu lato* clade’ and ‘*Ctenodaphnia* clade’. Could it be that they actually mean ‘*Daphnia sensu strictu* clade’ and ‘*Ctenodaphnia* clade’ and used these terms in the sense of/instead of subgenera? But then again, why wouldn’t they use the terms subgenera? This is very confusing, and it’s not the only confusing thing: in the course of the manuscript, the authors use the terms clade, group, sub-group, species complex, subgenus, genusX sensu strictu, genusX sensu lato without explaining any of these entities.

I suggest that the authors include a short chapter on the taxonomical status of ‘their’ species to avoid any misunderstanding and explain how they grouped the species and why.

Another problem I see is the treatment of *D. longispina*, *D. zschokkei* and *D. hyalina* in the manuscript (see my comments there). Petrusek et al. 2008 synonymized *D. zschokkei* and *D. hyalina* (and *D. rosea*, which is not part of the present study) with *D. longispina* on the basis of COI and 12S rDNA genes, retaining the name *D. longispina* because of its priority.

In their chapter Materials & Methods, the present authors explain that “*D. hyalina* and *D. zschokkei* are now synonymous of *D. longispina* and should be understood as *D. longispina ‘yalina’* and *D. longispina ‘zschokkei’* (Petrusek et al., 2008), hence we
merged them on the same branch in the cladogram.” However, Petrusek et al. (2008) did not propose such terms, they clearly synonymized the species according to the rules of the ICZN. If the authors do not agree with Petrusek et al. 2008, they should at least discuss it and explain, what they actually intend to express using the terms *D. longispina* ‘hyalina’ and *D. longispina* ‘zschokkei’, since this format is not one covered by the ICZN. Are the authors referring to morphotypes of *D. longispina*? Or do they intend to introduce subspecies (but then the quote signs don’t make sense). Given the synonymization by Petrusek et al. 2008, the authors should explain on the basis of which characters they actually identified their individuals of these ‘forms’, which they took from lab cultures.

Besides, I think that this topic could have been put much more into the focus of this study by comparing the sperm data of these three formerly separate species. Can sperm morphologies support maintaining them as separate species or support the idea of morphotypes of a single species? Would cross-fertilization tests be feasible?

Returning to taxonomy, I highly recommend that all species named in a manuscript have the (correct) species authorships attached when first mentioned. Species are scientific concepts that must be acknowledged by citing the authorship like in any other scientific concept. Besides, and particularly in groups with a complex history of nomenclature, this will avoid any misconception.

In this context, don’t miss out on Kotov 2020, Zootaxa 4803 (3): 591–599

Image quality
Given that the very purpose of this study is the investigation of sperm morphology (incl. size) it is surprising that the microscopic images are of low quality and that the maximal microscopic magnification used was 40x. With sperm of only a few µm length I would expect a 100x magnification, if not TEM anyway. Consequently, the information on morphological traits is not fully exploited and thus the results and their interpretation must remain relatively superficial.

The microscopic images of the sperms are presented in very tiny items in an overloaded Fig. 1 and the important, bigger and more meaningful images are pushed into the supplementary material, which I do not recommend.

Other issues
The authors should generally be more precise in phrasing. Why is there no discussion of their results in the light of sperm biology of other crustaceans (rather than mammals, ascidians and nematodes)? Some conclusions drawn are not well-founded, see comments in the manuscript. Formatting of figures must be improved.
I had problems to open the dataset-file (media-1.csv). The table looks like this, when opened:

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This is not reader-friendly; it should be presented as a well-arranged table in a PDF file. I am not sure whether formatting of numbers has been compromised by perhaps me using the wrong software to open the file (?). If these long figures in the upper 19 lines are intended by the authors as they are in my screenshot, I guess they are the result of the mathematical conversion of the scale of the measuring eyepiece into µm (?). If so, they pretend a non-existent accuracy. Use not more than one decimal place.

Renate Matzke-Karasz
Evolution of sperm morphology in *Daphnia*

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Abstract

Sperm, the male most fundamental reproductive feature allowing egg fertilization, evolves under sexual selection. Two components of sperm are mainly under selection, their number and their morphology (including the associated quality). Here we study the evolution of sperm morphology in the genus *Daphnia*. Based on microscopic observations of sperm morphologies mapped on a *Daphnia* phylogeny, we found that increase in sperm length evolved at least twice in *Daphnia*, once in the *Daphnia sensu lato* clade and once in the *Ctenodaphnia* clade. Furthermore, *Daphnia s.l.* lost the ability of cell compaction by losing extracellular encapsulation and exposing large filaments. We discuss the potential reasons for such convergent evolution in sperm morphology.
Introduction

Sexual selection is a form of natural selection acting on mating and fertilization success. Hence, sperm, the most fundamental male reproductive feature allowing egg fertilization, evolve—at least in part—under such selection. Two components of sperm are mainly under selection, their number and their morphology, the latter including the associated quality. Males generally release many gametes, the ejaculate, to fertilize a few eggs. The ejaculate size is known to evolve in response to the risk of sperm competition for egg fertilization, to cryptic female choice and to female receptacle size (i.e. the dilution effect) (Roldan, 2019). In contrast, while sperm are considered as one of the most taxonomically diverse and rapidly evolving cell types (Birkhead et al., 2009; Ramm et al., 2014), the understanding of the adaptive value of sperm morphology, such as length and shape, remains largely incomplete (Lüpold & Pitnick, 2018). Sperm length does not necessarily correlate with increased swimming speed (although it is often the case, see (Tourmente et al., 2011; Rowley et al., 2019)) and structures not involved in velocity evolve most probably in response to the environment in which fertilization occurs but the details are rarely, if ever, clear (Lüpold & Pitnick, 2018).

*Daphnia* are crustaceans reproducing by cyclical parthenogenesis and as such, egg fertilization is sporadic, but essential for diapause in freezing and drying habitats and for dispersal. Following periods of clonal reproduction, during which females only produce genetically identical daughters, and usually triggered by a change in environmental conditions, some females produce sexual eggs while others produce males. During mating, generally one male, but sometimes more, attach to the female to fertilize eggs which will be laid into the female brood pouch after the male(s) departed (Duneau et al in prep.). The brood pouch is a receptacle formed by the carapace and present on the dorsal side of all *Daphnia* species receiving either clonal or sexual eggs. For the latter, the cuticular structure of the brood pouch changes to form a protective case which will be released upon molting, creating genetically diverse egg-banks from which future populations can be established. Fertilization takes most likely place in this brood pouch (Duneau et al. in prep.). However, although there are recordings of males competing for fertilization in *Daphnia magna* (Duneau et al. in prep.), the extend of sperm competition in this receptacle is unknown. Importantly, a water current generated by the filtering
apparatus oxygenates the eggs in the brood pouch (Seidl et al., 2002), and it is likely that many sperm may be flushed out after the male ejaculated. In this context, males are expected to evolve larger ejaculates. However, assuming finite resources allocated to sperm production, this may come at the cost of sperm length (Immler et al., 2011), an important trait in male competition. Thus, males are likely to evolve persistence traits that allow them to increase the chances of fertilization. Here we investigate how sperm morphology in *Daphnia* species diverged in a phylogenetic context, presumably as a response to sexual selection.

Although pioneer studies have given key general descriptions to identify the main structures (Delavault & Berard, 1974; Wingstrand, 1978; Zaffagnini, 1987; Wuerz et al., 2017), only little is known about sperm morphology in *Daphnia*. All *Anomopoda*, an infraorder including *Daphnia*, have a vacuolar type of spermatogenesis (Wingstrand, 1978), *i.e.* in the testes, the spermatids are enclosed in “private” vacuoles in the nutritive cells and are exocytosed into the testicular lumen after they have decreased strongly in size and matured. After they have compacted, they are generally small, about a few microns. Sperm of *D. magna* has been more thoroughly studied with recent technology. This *Ctenodaphnia* species has larger sperm (~10 µm) encapsulated by an acellular capsule likely compacting radial arms probably to pack more sperm in the testes (Wuerz et al., 2017). The roles of the capsule and of the filaments that are only visible surrounding the cell within the vacuole with electronic microscopy (Wuerz et al., 2017) are unclear. Based on comparison with other models, it has been proposed that female secretion could dissolve the capsule and the filament could have a role in the fusion between gametes (Wuerz et al., 2017). Here, we used a robust phylogeny of the Daphnidae (Adamowicz et al., 2009; Cornetti et al., 2019) and assessed several species representing major clades within *Daphnia* to better understand the evolution of sperm morphology in this genus.

**Materials and methods**

Male *Daphnia* were either sampled from female mass cultures in the laboratory, where males are naturally produced as a consequence of high density, or from females exposed to the hormone methyl farnesoate (MF, 40nM final concentration) to induce male production. We induced male
production for *D. hyalina*, *D. zschokkei*, *D. mendotea*, *D. galeata*, *D. curvirostris* and *D. dentifera* and
collected naturally produced males for *D. similis*, *D. sinensis*, *D. lumholtzi*, *D. carinata*, *D. magna*, *D. hispanica*, *D. dolichocephala*, *D. barbata*, *D. longispina*, *D. pulex* and *D. pulicaria*. Note that *D. hyalina* and *D. zschokkei* are now synonymous of *D. longispina* and should be understood as *D. longispina ‘hyalina’* and *D. longispina ‘zschokkei’* (Petrusek et al., 2008), hence we merged them on the same branch in the cladogram.

To collect sperm, we exposed mature males to a 1 % nicotine solution ((−)-Nicotin 162.23 g/mol, from Carl Roth, Germany) to induce ejaculation as in (Duneau et al., 2012). As only mature spermatozoa are in the testicular lumen (p11 in Wingstrand, 1978; p277 in Zaffagnini, 1987), this method allowed us to describe and measure mature sperm and avoid immature ones. Presence of filaments on the sperm was recorded, but we did not measure their length. Measurements of the longest length of the sperm were performed with ImageJ (v. 1.5i) using photographs taken under phase contrast light at magnification 40x. Species with very small sperm (*D. pulex*, *D. pulicaria*, *D. dolichocephala* and *D. barbata*) we paid particularly attention that the sperm were just released from the spermiduct to reduce the possibility of degradation or to confuse them with other particles. However, it was challenging to take photographs of them, and the measurement may be less accurate than for the other species. *D. pulex* sperm length is only around 2 µm in length (Xu et al., 2015). All sperm were also observed at the moment of release from the ejaculatory opening to verify that their shape corresponds to what was observed later when they settled and were photographed. We also observed sperm morphology in sea water to confirm that osmolarity was not affecting our results. Drawing of male abdomens with the genital papilla were taken from published keys (Benzie, 2005; Popova et al., 2016).

**Results**

Sperm morphology varied greatly among *Daphnia*, ranging from about 2 µm to at least 20 µm (Figure 1 and supplementary figures 1 and 2). There was a clear phylogenetic signal in sperm length across *Daphnia*, but length clusters are polyphyletic. Based on recent *Daphnia* phylogenies (Adamowicz et al., 2009; Cornetti et al., 2019) and the microscopic observations of sperm
mophologies, we found that an increase in sperm length evolved at least twice in *Daphnia* (Figure 1), once in *Daphnia sensu lato* and once in *Ctenodaphnia*. This length variation was probably mostly due to a difference in sperm compaction by an extracellular capsule. We found that clades leading to the *D. longispina* group have lost entirely this capsule, hence leaving long filaments without protection (Figure 1, Supplementary figures 1, 2 and 3).

*Ceriodaphnia*, our outgroup, have sperm of the vacuolar spermatogenesis type, like *Daphnia* species and the rest of the infraorder of the *Anomopoda*. Their sperm have been described, based on electron microscopy, as small (2 to 6 µm), more or less rod-shaped and strongly compacted in their capsule (Figure 2A and p25-26 in Wingstrand, 1978). This information based on several *Ceriodaphnia* species allowed to determine the most parsimonious ancestral *Daphnia* sperm morphology. The *Ctenodaphnia* group, except for *D. dolichocephala* and *D. barbata* who had compacted and small sperm, evolved non-compacted and elongated sperm, several times larger than the ancestral morphology (Figure 1 and 2B). A similar adaptation occurred in *Daphnia s. l.* As in (Xu *et al.*, 2015) which reported *Daphnia pulex s. str.* sperm length, *Daphnia* from the *D. pulex* subgroup conserved the small and compacted sperm morphology, while *D. curvirostris* and sister species from the *D. longispina* species complex also evolved larger elongated sperm, but not as large as in *Ctenodaphnia* (Figure 1). Additionally, it seems, that *Daphnia* species from the *D. longispina* species complex lost the extracellular capsule compacting the sperm and have long filaments with potentially forked structures (Figure 2C). These filaments can be several times the length of the sperm (not measured here) (Figure 2 and Supplementary figure 3). In the same ejaculate from a single *D. longispina s. str.* male sperm can have, on each side, either one long filament or many shorter ones (see Figure 2 and Supp. material). It has been argued that ejaculate size increases with female receptacle length to compensate for the dilution effect and that it should be accompanied by a reduction in sperm length. Considering that most *Ctenodaphnia* are not much larger than *Daphnia s. l.*, it is unlikely that this hypothesis explains the evolution of sperm morphology in *Daphnia*.

We further compared the shape of the male genital papilla across the *Daphnia* species, using drawings from published keys (Benzie, 2005; Popova *et al.*, 2016) (Figure 1). Most species have no or a very
inconspicuous papilla, the biggest exception is the papilla structure present in *D. magna* (Figure 1).

This structure is also found in several species related to *D. magna*, such as *D. atkinsoni*, *D. bolivari* and *D. Mediterranea*, but they are not included in our study (Flössner, 2000; Benzie, 2005).

**Discussion**

Our assessment of sperm morphology uncovered clearly structured phylogenetic variation in sperm length and in the presence or absence of long filaments. The reason for this variation is unclear but may be in part explained with the strength and intensity of post-copulatory sexual selection. Strength of sexual selection is a function of how often male ejaculates compete for fertilization, in particular direct sperm competition and sperm competition through cryptic female choice.

The frequency of sex in *Daphnia* depends on the species and of the environment, being eventually under local adaptation (Roulin et al., 2013). In unstable and short-lived habitats, such as small rockpools or ponds in unstable or strongly seasonal environments, such as deserts and arctic sites, few asexual generations occur before diapause recommences. In stable environments, such as large lakes and ponds in temperate mild climatic regions, many asexual generations may occur before the next sexual generation comes, if it ever comes in a mother’s lifetime. Traditionally, the later type received more attention by *Daphnia* researchers, leading to the wrong impression that sexual reproduction, and thus the occurrence of males, is generally rare. Therefore, it is difficult to estimate the intensity and frequency of sexual selection in the system, especially for each species. In certain populations, males can be periodically abundant, and several males can be found copulating at the same time with a female (Duneau et al, in prep). It is not clear whether fertilization would occur in the brood pouch or in the oviduct. However, the fact that most genital papilla are inconspicuous suggest that fertilization is realized in the brood pouch as the access to the oviduct seems complicated. Thus, cryptic female choice and male-male competition via sperm competition, both likely to shape the evolution of sperm morphology, may occur in this female receptacle.

By ejaculating in the female receptacle, males face the challenge to have their sperm flushed out, a phenomenon which could be a form of cryptic female choice. To provide oxygen, the brood pouch is
continuously flushed with a stream of water, entering from the caudal end and leaving from the ventral
carapace chamber in a pulsed manner resulting from the rhythmical movements of the limbs (Seidl et
al., 2002). This stream might flush out sperm. It is likely that this mechanism selects for males able to
produce a large number of sperm of good quality. Interspecies variations in such cryptic female choice
may or not vary among species, but males in each species may have different features to increase their
chance to be chosen. Sperm may attach and cover the inner lining of the brood pouch, so eggs would
not be expelled and touch the sperm as soon as they are released in the brood pouch. However, there is
no obvious structure supporting that freshly ejaculated sperm can attach to the inner lining. Then, they
may simply increase the number of sperm per ejaculate to increase the chance for few sperm to remain
in the brood pouch. However, as most sperm production is done early in male’s life (Wuerz et al.,
2017) the total number of sperm is limited by the size of the spermiduct. By limiting the total number
of sperm stored, this constraint may put selection on the amount of compaction allowing to store more
cells in the duct. In Daphnia, there is an extracellular compaction process by an extracellular vacuole
before the mature sperm is released into the spermiduct (Wingstrand, 1978). We propose that the
evolutionary changes in sperm length we observed may in part be due to a change in the mechanism of
cell compaction before maturation.

Female cryptic choice may select for higher number hence smaller sperm, but sperm of different males
may also compete within the receptacle imposing an additional selection pressure on sperm
morphology. Such sexual selection on sperm morphology through male-male competition will
increase as males are more numerous at a given time and regularly mate at the same time with a
female. The change in sperm size as a result of sperm competition is a commonly seen evolutionary
pattern (see for example Vielle et al. (2016), including extreme examples like giant Drosophila
sperm (Lüpold & Pitnick, 2018). However, it is difficult to assess the intensity of sperm competition
based solely on sperm size. In Caenorhabditis elegans, experimentally enhanced sperm competition
leads to the evolution of larger sperm (LaMunyon & Ward, 2002). However, in Drosophila
melanogaster, the competitive advantage was present only in females with relatively long sperm
storage organs (Miller & Pitnick, 2003). When sperm are non-motile, the outcome of sperm
competition generally resembles a lottery in which having more tickets than your competitor increases your chances to win. Males are thus expected to invest in sperm production. Assuming finite resources allocated to sperm production, this may then come at the cost of sperm size (Immler et al., 2011).

When sperm competition is high, the total number of sperm may be maximized balancing the size of the sperm per se and its compaction. However, when sperm competition is low, males may invest less in sperm size and there may be little benefit from expending energy in mechanisms insuring constant sperm length (Bauer & Breed, 2006; Immler et al., 2008). However, because it often correlates with velocity, increase in sperm size can also be selected if sperm are motile at some point of the fertilization process. It is difficult to assure that Daphnia sperm are always non motile. Sperm in the brood pouch are probably not motile but we observed them on a microscope slide and an in vivo assessment would be necessary to ascertain sperm behavior (Lüpold & Pitnick, 2018). Daphnia males ejaculate into the brood pouch filled with water (the same used for our observation); it is therefore likely that the absence of motility observed in water on a microscope slide reflects what is happening in the brood pouch. However, it has been shown that fluid surrounding eggs of externally fertilized species induced modification to sperm motility (Yoshida et al., 2013). In such scenario, the capsule might break, the sperm would attach to the oocyte, crawl to an eventual specific fusion site and larger sperm with filaments be advantageous in the race to this site.

Our phylogenetic analysis of sperm morphology revealed a monophyletic clade (i.e., D. longispina species complex) with sperm exposing filaments of diverse shape and length. In Daphnia magna, it seems that filaments exist and are inside the extracellular vacuole (Wuerz et al., 2017), but they are difficult to resolve even with electron microscopy and therefore our knowledge about their ultrastructure is limited. Nevertheless, the vacuole is expected to break before the sperm cell fuse with the oocyte and filaments to be exposed. Daphnia species from the D. longispina species complex evolved non-compacted sperm and the apparent loss of the capsule exposes especially long, sometimes numerous, filaments. Those exaggerated long structures are unlikely to be flagella and not used to move towards the eggs as they are extremely flexible and can be forked. But, as sperm features are expected to be adaptations to their specific fertilization environment (Pitnick et al., 2009), filaments
almost certainly have a role in fertilization. If we cannot exclude that they have a role in attachment to the brood pouch to avoid being flushed out with the water flow generated by the female, it is very likely that they have a role in the fusion with the oocyte and potentially with crawling at its surface to reach a fusion site and win the eventual sperm competition. It may be that with reduced selection for compaction, readiness to fuse with the oocyte may be favored by the exposure of those long filaments.

As it is often the case when studying the evolution of sperm morphology, it is difficult to clearly identify the role of sperm features. However, it is certainly evolving driven by the intensity of sexual selection in the system. Daphnia is a cyclical parthenogenetic species, where periods of sexual reproduction are interspersed with period of asexual reproduction. Cyclical parthenogenetic species, like Daphnia, aphids, and rotifers, alternate sexual and asexual reproduction. The number of asexual generations in between two sexual reproductive events may be highly variable, but because the less frequent sexual reproduction is, the lower the average intensity of selection. Thus, one can wonder how the variation in intensity of sexual selection among cyclical parthenogenetic species influences the evolution in sperm morphology. Here, the missing information on the sexual process in Daphnia species makes it difficult to assess the relative role of cryptic female choice and sperm competition but both are likely to play a role in the convergent evolution or maybe, even more interestingly, the conserved evolution of sperm length in Daphnia. Altogether, our results support the idea that sexual selection plays a role in cyclical parthenogenetic invertebrate species and encourage further investigations.

Acknowledgements

Jürgen Hottinger for help in the laboratory, Jeremias Brand, Vitor Faria, Lukas Schärer and Axel Wiberg for discussions about the study. DD was supported by the French Laboratory of Excellence project ‘TULIP’ (ANR-10-LABX-41; ANR-11-IDEX-0002–02). MM was supported by an FWF Austrian Science Fund stand-alone project (P29667-B25), DE was supported by a grant from the Swiss National Science Foundation.
References


Figure legends

**Figure 1:** Evolution of sperm and genital papilla morphologies in Daphniidae. Phylogeny of Daphniidea is modified from Cornetti et al. 2019 and Adamowicz et al. 2009. Purple, pink and green represent respectively the sperm that are small, large with capsule or large with filaments exposed. Drawings represent the genital papilla of the males (from Benzie 2005 and Popova et al. 2016) and indicate the atypical exaggerated structure of *D. magna*. Scale bar represent 0.1mm. Photographs show an example of sperm for each species. The graph represents the difference in sperm length among males. The mean sperm length was calculated with 2 to 3 ejaculates and is intended to provide a rough sense of the traits.

**Figure 2:** Examples of *Daphnia* sperm morphologies. A- *Ceriodaphnia laticauda*, ancestral small and encapsulated elongated sperm (purple color in figure 1). Photo from (Wingstrand, 1978). B- *D. magna*, large and encapsulated elongated sperm of the groups (salmon color in figure 1); C and D - *D. longispina*, two examples of typical sperm non encapsulated and elongated sperm with filaments (green color in figure 1).
Figure 1: Evolution of sperm and genital papilla morphologies in Daphniidae. Phylogeny of Daphniidea is modified from Cornetti et al. 2019 and Adamowicz et al. 2009. Purple, pink and green represent respectively the sperm that are small, large with capsule or large with filaments exposed. Drawings represent the genital papilla of the males (from Benzie 2005 and Popova et al. 2016) and indicate the atypical exaggerated structure of *D. magna*. Scale bar represent 0.1mm. Photographs show an example of sperm for each species. The graph represents the difference in sperm length among males. The mean sperm length was calculated with 2 to 3 ejaculates and is intended to provide a rough sense of the traits.
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Supplementary figure 1: Illustration of *Daphnia* sperm morphology by species.
Supplementary figure 2: Illustration of Daphnia sperm morphology by species.
Supplementary figure 3: Examples of *Daphnia longispina* sperm morphologies.