

1 Evolution of sperm morphology in *Daphnia*

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11 Abstract

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

20 Introduction

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

34 *Daphnia* are crustaceans reproducing by cyclical parthenogenesis and as such, egg fertilization is
35 sporadic, but essential for diapause in freezing and drying habitats and for dispersal. Following periods
36 of clonal reproduction, during which females only produce genetically identical daughters, and usually
37 triggered by a change in environmental conditions, some females produce sexual eggs while others
38 produce males. During mating, generally one male, but sometimes more, attach to the female to
39 fertilize eggs which will be laid into the female brood pouch after the male(s) departed (Duneau *et al*
40 in prep.). The brood pouch is a receptacle formed by the carapace and present on the dorsal side of all
41 *Daphnia* species receiving either clonal or sexual eggs. For the latter, the cuticular structure of the
42 brood pouch changes to form a protective case which will be released upon molting, creating
43 genetically diverse egg-banks from which future populations can be established. Fertilization takes
44 most likely place in this brood pouch (Duneau *et al.* in prep.). However, although there are recordings
45 of males competing for fertilization in *Daphnia magna* (Duneau *et al.* in prep.), the extend of sperm
46 competition in this receptacle is unknown. Importantly, a water current generated by the filtering

47 apparatus oxygenates the eggs in the brood pouch (Seidl *et al.*, 2002), and it is likely that many sperm
48 may be flushed out after the male ejaculated. In this context, males are expected to evolve larger
49 ejaculates. However, assuming finite resources allocated to sperm production, this may come at the
50 cost of sperm length (Immler *et al.*, 2011), an important trait in male competition. Thus, males are
51 likely to evolve persistence traits that allow them to increase the chances of fertilization. Here we
52 investigate how sperm morphology in *Daphnia* species diverged in a phylogenetic context,
53 presumably as a response to sexual selection.

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

69 Materials and methods

70 Male *Daphnia* were either sampled from female mass cultures in the laboratory, where males
71 are naturally produced as a consequence of high density, or from females exposed to the hormone
72 methyl farnesoate (MF, 40nM final concentration) to induce male production. We induced male

73 production for *D. hyalina*, *D. zschokkei*, *D. mendotea*, *D. galeata*, *D. curvirostris* and *D. dentifera* and
74 collected naturally produced males for *D. similis*, *D. sinensis*, *D. lumholtzi*, *D. carinata*, *D. magna*, *D.*
75 *hispanica*, *D. dolichocephala*, *D. barbata*, *D. longispina*, *D. pulex* and *D. pulicaria*. Note that *D.*
76 *hyalina* and *D. zschokkei* are now synonymous of *D. longispina* and should be understood as *D.*
77 *longispina* ‘*hyalina*’ and *D. longispina* ‘*zschokei*’ (Petrušek *et al.*, 2008), hence we merged them on
78 the same branch in the cladogram.

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

94 Results

95 Sperm morphology varied greatly among *Daphnia*, ranging from about 2 µm to at least 20 µm
96 (Figure 1 and supplementary figures 1 and 2). There was a clear phylogenetic signal in sperm length
97 across *Daphnia*, but length clusters are polyphyletic. Based on recent *Daphnia* phylogenies
98 (Adamowicz *et al.*, 2009; Cornetti *et al.*, 2019) and the microscopic observations of sperm

99 morphologies, we found that an increase in sperm length evolved at least twice in *Daphnia* (Figure 1),
100 once in *Daphnia sensus lato* and once in *Ctenodaphnia*. This length variation was probably mostly due
101 to a difference in sperm compaction by an extracellular capsule. We found that clades leading to the *D.*
102 *longispina* group have lost entirely this capsule, hence leaving long filaments without protection
103 (Figure 2, Supplementary figures 1, 2 and 3).

104 *Ceriodaphnia*, our outgroup, have sperm of the vacuolar spermatogenesis type, like *Daphnia* species
105 and the rest of the infraorder of the *Anomopoda*. Their sperm have been described, based on electron
106 microscopy, as small (2 to 6 μm), more or less rod-shaped and strongly compacted in their capsule
107 (Figure 2A and p25-26 in Wingstrand, 1978). This information based on several *Ceriodaphnia* species
108 allowed to determine the most parsimonious ancestral *Daphnia* sperm morphology. The *Ctenodaphnia*
109 group, except for *D. dolichocephala* and *D. barbata* who had compacted and small sperm, evolved
110 non-compacted and elongated sperm, several times larger than the ancestral morphology (Figure 1 and
111 2B). A similar adaptation occurred in *Daphnia s. l.* As in (Xu *et al.*, 2015) which reported *Daphnia*
112 *pulex s. str.* sperm length, *Daphnia* from the *D. pulex* subgroup conserved the small and compacted
113 sperm morphology, while *D. curvirostris* and sister species from the *D. longispina* species complex
114 also evolved larger elongated sperm, but not as large as in *Ctenodaphnia* (Figure 1). Additionally, it
115 seems, that *Daphnia* species from the *D. longispina* species complex lost the extracellular capsule
116 compacting the sperm and have long filaments with potentially forked structures (Figure 2C). These
117 filaments can be several times the length of the sperm (not measured here) (Figure 2 and
118 Supplementary figure 3). In the same ejaculate from a single *D. longispina s. str.* male sperm can
119 have, on each side, either one long filament or many shorter ones (see Figure 2 and Supp. material). It
120 has been argued that ejaculate size increases with female receptacle length to compensate for the
121 dilution effect and that it should be accompanied by a reduction in sperm length. Considering that
122 most *Ctenodaphnia* are not much larger than *Daphnia s. l.*, it is unlikely that this hypothesis explains
123 the evolution of sperm morphology in *Daphnia*.

124 We further compared the shape of the male genital papilla across the *Daphnia* species, using drawing
125 from published keys (Benzie, 2005; Popova *et al.*, 2016) (Figure 1). Most species have no or a very

126 inconspicuous papilla, the biggest exception is the papilla structure present in *D. magna* (Figure 1).
127 This structure is also found in several species related to *D. magna*, such as *D. atkinsoni*, *D. bolivari*
128 and *D. mediterranea*, but they are not included in our study (Flössner, 2000; Benzie, 2005).

129 Discussion

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

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

150 By ejaculating in the female receptacle, males face the challenge to have their sperm flushed out, a
151 phenomenon which could be a form of cryptic female choice. To provide oxygen, the brood pouch is

152 continuously flushed with a stream of water, entering from the caudal end and leaving from the ventral
153 carapace chamber in a pulsed manner resulting from the rhythmical movements of the limbs (Seidl *et*
154 *al.*, 2002). This stream might flush out sperm. It is likely that this mechanism selects for males able to
155 produce a large number of sperm of good quality. Interspecies variations in such cryptic female choice
156 may or not vary among species, but males in each species may have different features to increase their
157 chance to be chosen. Sperm may attach and cover the inner lining of the brood pouch, so eggs would
158 not be expelled and touch the sperm as soon as they are released in the brood pouch. However, there is
159 no obvious structure supporting that freshly ejaculated sperm can attach to the inner lining. Then, they
160 may simply increase the number of sperm per ejaculate to increase the chance for few sperm to remain
161 in the brood pouch. However, as most sperm production is done early in male's life (Wuerz *et al.*,
162 2017) the total number of sperm is limited by the size of the spermiduct. By limiting the total number
163 of sperm stored, this constraint may put selection on the amount of compaction allowing to store more
164 cells in the duct. In *Daphnia*, there is an extracellular compaction process by an extracellular vacuole
165 before the mature sperm is released into the spermiduct (Wingstrand, 1978). We propose that the
166 evolutionary changes in sperm length we observed may in part be due to a change in the mechanism of
167 cell compaction before maturation.

168 Female cryptic choice may select for higher number hence smaller sperm, but sperm of different males
169 may also compete within the receptacle imposing an additional selection pressure on sperm
170 morphology. Such sexual selection on sperm morphology through male-male competition will
171 increase as males are more numerous at a given time and regularly mate at the same time with a
172 female. The change in sperm size as a result of sperm competition is a commonly seen evolutionary
173 pattern (see for an example Vielle *et al.* (2016), including extreme examples like giant *Drosophila*
174 sperm (Lüpold & Pitnick, 2018). However, it is difficult to assess the intensity of sperm competition
175 based solely on sperm size. In *Caenorhabditis elegans*, experimentally enhanced sperm competition
176 leads to the evolution of larger sperm (LaMunyon & Ward, 2002). However, in *Drosophila*
177 *melanogaster*, the competitive advantage was present only in females with relatively long sperm
178 storage organs (Miller & Pitnick, 2003). When sperm are non-motile, the outcome of sperm

179 competition generally resembles a lottery in which having more tickets than your competitor increases
180 your chances to win, males are thus expected to invest in sperm production. Assuming finite resources
181 allocated to sperm production, this may then come at the cost of sperm size (Immler *et al.*, 2011).
182 When sperm competition is high, the total number of sperm may be maximized balancing the size of
183 the sperm *per se* and its compaction. However, when sperm competition is low, males may invest less
184 in sperm size and there may be little benefit from expending energy in mechanisms insuring constant
185 sperm length (Bauer & Breed, 2006; Immler *et al.*, 2008). However, because it often correlates with
186 velocity, increase in sperm size can also be selected if sperm are motile at some point of the
187 fertilization process. It is difficult to assure that *Daphnia* sperm are always non motile. Sperm in the
188 brood pouch are probably not motile but we observed them on a microscope slide and an *in vivo*
189 assessment would be necessary to ascertain sperm behavior (Lüpold & Pitnick, 2018). *Daphnia* males
190 ejaculate into the brood pouch filled with water (the same used for our observation); it is therefore
191 likely that the absence of motility observed in water on a microscope slide reflects what is happening
192 in the brood pouch. However, it has been shown that fluid surrounding eggs of externally fertilized
193 species induced modification to sperm motility (Yoshida *et al.*, 2013). In such scenario, the capsule
194 might break, the sperm would attach to the oocyte, crawl to an eventual specific fusion site and larger
195 sperm with filaments may be advantageous in the race to this site.

196 Our phylogenetic analysis of sperm morphology revealed a monophyletic clade (i.e. *D. longispina*
197 species complex) with sperm exposing filaments of diverse shape and length. In *Daphnia magna*, it
198 seems that filaments exist and are inside the extracellular vacuole (Wuerz *et al.*, 2017), but they are
199 difficult to resolve even with electron microscopy and therefore our knowledge about their
200 ultrastructure is limited. Nevertheless, the vacuole is expected to break before the sperm cell fuse with
201 the oocyte and filaments to be exposed. *Daphnia* species from the *D. longispina* species complex
202 evolved non-compacted sperm and the apparent loss of the capsule exposes especially long, sometimes
203 numerous, filaments. Those exaggerated long structures are unlikely to be flagella and not used to
204 move towards the eggs as they are extremely flexible and can be forked. But, as sperm features are
205 expected to be adaptations to their specific fertilization environment (Pitnick *et al.*, 2009), filaments

206 almost certainly have a role in fertilization. If we cannot not exclude that they have a role in
207 attachment to the brood pouch to avoid being flushed out with the water flow generated by the female,
208 it is very likely that they have a role in the fusion with the oocyte and potentially with crawling at its
209 surface to reach a fusion site and win the eventual sperm competition. It may be that with reduced
210 selection for compaction, readiness to fuse with the oocyte may be favored by the exposure of those
211 long filaments.

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

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298 Figure legends

299 **Figure 1:** Evolution of sperm and genital papilla morphologies in Daphniidae. Phylogeny of
300 Daphniidea is modified from Cornetti et al. 2019 and Adamowicz et al. 2009. Purple, pink and green
301 represent respectively the sperm that are small, large with capsule or large with filaments exposed.
302 Drawings represent the genital papilla of the males (from Benzie 2005 and Popova et al. 2016) and
303 indicate the atypical exaggerated structure of *D. magna*. Scale bar represent 0,1mm. Photographs show
304 an example of sperm for each species. The graph represents the difference in sperm length among
305 males. The mean sperm length was calculated with 2 to 3 ejaculates and is intended to provide a rough
306 sense of the traits.

307 **Figure 2:** Examples of Daphnia sperm morphologies. A- *Ceriodaphnia laticauda*, ancestral small and
308 encapsulated elongated sperm (purple color in figure 1). Photo from (Wingstrand, 1978). B- *D.*
309 *magna*, large and encapsulated elongated sperm of the groups (salmon color in figure 1); C and D - *D.*
310 *longispina*, two examples of typical sperm non encapsulated and elongated sperm with filaments
311 (green color in figure 1).

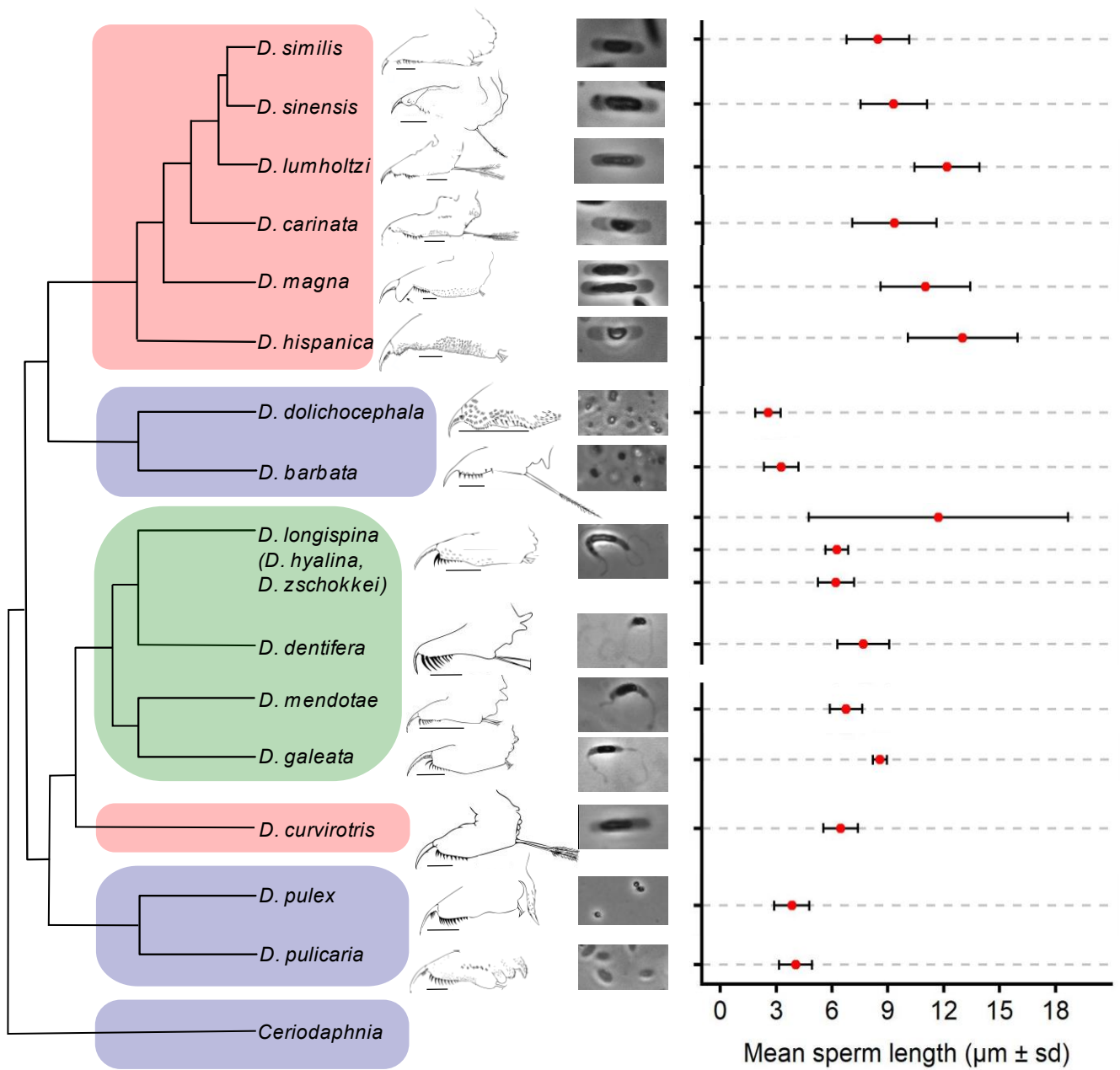


Figure 1: Evolution of sperm and genital papilla morphologies in Daphniidae. Phylogeny of Daphniidea is modified from Cornetti et al. 2019 and Adamowicz et al. 2009. Purple, pink and green represent respectively the sperm that are small, large with capsule or large with filaments exposed. Drawings represent 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.

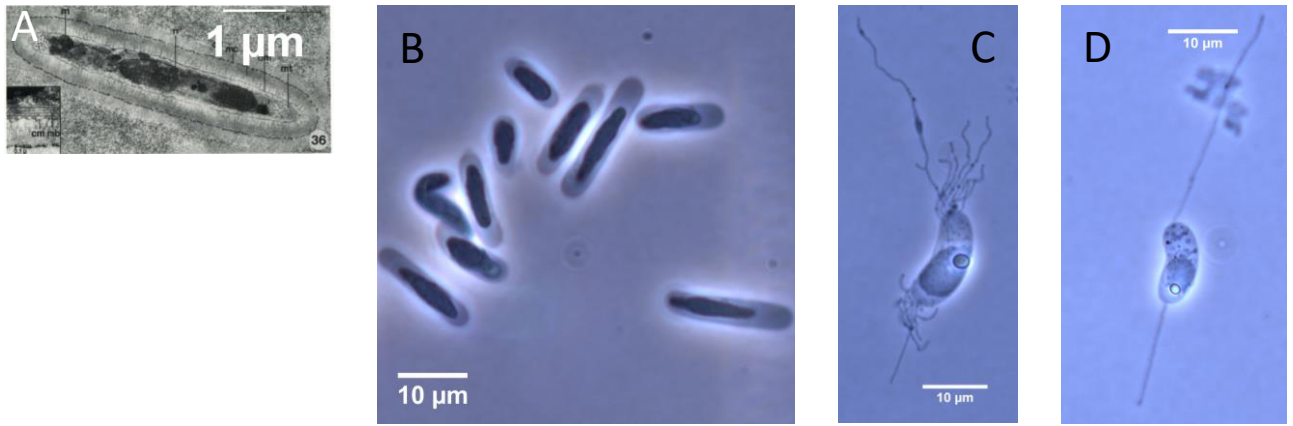


Figure 2: Examples of *Daphnia* sperm morphologies. A- *Ceriodaphnia laticauda*, ancestral small and encapsulated elongated sperm (purple color in figure 1). Photo from (Wingstrand, 1978). B- *D. magna*, large and encapsulated elongated sperm of the groups (salmon color in figure 1); C and D - *D. longispina*, two examples of typical sperm non encapsulated and elongated sperm with filaments (green color in figure 1).