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Age- and size-dependent mating performance and fertility in a pelagic copepod, *Temora longicornis*

Mie Hylstofte Sichlau*, Thomas Kiørboe

National Institute of Aquatic Resources, Technical University of Denmark, Kavalergården, 2920 Charlottenlund, Denmark

ABSTRACT: In many species, size and age can be strong determinants of the reproductive success for both sexes. We examined age- and size-dependent reproductive performance (egg and sperm production, mating success) in a pelagic copepod, *Temora longicornis*. Compared with smaller males, larger males produced larger spermatophores containing more spermatozoa and fertilized a larger fraction of available females. Females mating with large males produced more offspring than those mating with small males. Similarly, large females had higher egg production rates as well as a higher lifetime egg production than did small females. Ageing effects were evident in this species: mortality rate increased and fertility decreased rapidly with age. The average adult longevity under optimal laboratory conditions was 30 d in both males and females, but females produced eggs for only 18 d, and males could fertilize females for only about 8 d after they matured. The strong size- and age-dependent fertility observed in this species is conducive to the development of sexual selection via mate choice for young and large partners, as has been shown in another copepod species.

KEY WORDS: Spermatophore · Spermatozoa · Egg production · Ageing · Mortality rate · DAPI · *Temora longicornis*

INTRODUCTION

Reproduction and mate choice are important determinants of the evolution and life history of all organisms (reviewed in Andersson 1994), and in many species both depend on the body size and age of the individual. Generally, larger individuals of a species produce more gametes and their mating performance typically declines with age (MacDiarmid & Butler 1999, Radwan et al. 2005, Lehmann & Lehmann 2009, Gasparini et al. 2010, Judge et al. 2010). Here we examined how reproductive performance and mortality depended on size and age in a pelagic copepod, *Temora longicornis*.

Numerous studies have shown that the variation in copepod fecundity is related to changes in food quantity and quality (Jonasdottir et al. 1995, 2009, Dam & Lopes 2003, Koski et al. 2006), salinity (Holste et al. 2009) and temperature (Ban 1994, Holste et al. 2009), but few have tested the effect of ageing on fecundity (Carlotti et al. 1997, Rodriguez-Grana et al. 2010, Ceballos & Kiørboe 2011). Ageing is the progressive decline in biological functions with advancing age and the accumulation of change in an organism over time. Ageing can have a profound negative effect on the individual fitness of some pelagic copepod species, e.g. on feeding rates, oxidative damage, egg production rate and egg hatching success (Carlotti et al. 1997, Rodriguez-Grana et al. 2010, Ceballos & Kiørboe 2011). Male ageing can have evolutionary and ecological consequences on gamete performance and fitness (see Pizzari et al. 2008). The quality of the genes that a male passes to his progeny may change with his age (Hansen & Price 1995) owing to the accumulation of deleterious mutations in the germline (Risch et al. 1987, Drost & Lee 1995,

*Email: mhsi@aqua.dtu.dk

Hurst & Ellegren 1998, Bartosch-Härlid et al. 2003, Glaser & Jabs 2004). Ageing can also affect the quantity of sperm because an increasing proportion of germ cells bear mutations as age increases, and such cells are destroyed by genetic self-guarding processes (Radwan 2003). Older males, however, have passed more episodes of selection and thus may be better adapted than the average male (Manning 1985). The breeding value (i.e. offspring performance) of older males will thus depend on specific life history characteristics (Kokko 1998, Beck & Powell 2000) and on the number of divisions a male germline undergoes after it reaches maturity (Radwan 2003). Therefore, it is not surprising that female preferences with respect to the age of the male differ among species (reviewed in Brooks & Kemp 2001). Little is known about the reproductive performance of male copepods, and less about the age dependency of male reproduction.

Adult body length of many copepods may vary substantially with both temperature and food availability by up to a factor of 2 within a species (Berggreen et al. 1988, Dam & Peterson 1991, Arendt et al. 2005). Female fecundity typically increases with body size in both insects and crustaceans, including copepods (Honêk 1993, Aquiloni & Gherardi 2008, Ceballos & Kiørboe 2010). Spermatophore size and sperm quantity is similarly correlated with male size in insects and a single copulation by a small mature male may not provide enough sperm to fertilize all the eggs produced by a large female (McLain et al. 1990, Bissoondath & Wiklund 1996). Ceballos & Kiørboe (2010) showed that large males of *Acartia tonsa* produce larger spermatophores than do small males, and that the production of offspring in the female increases with the size of the spermatophores she receives and thus with the size of the male, probably owing to a larger content of spermatozoa in larger spermatophores. Because sperm are small and typically produced in very large numbers compared with eggs, sperm are traditionally considered an unlimited resource, but such observations suggest that sperm can be limiting. Dewsbury (1982) pointed out that sperm delivered to females in ejaculates or spermatophores, as in copepods, may be costly or slow to produce, and there is growing evidence to indicate that sperm supply can limit fertilization success and realized fecundity (Wedell et al. 2002). Spermatophore production may involve significant energetic investments for male copepods (Mauchline 1998), but the contents of sperm and semen in spermatophores is unknown in copepods. Copepod spermatophores contain various substances besides spermatozoa (Defaye et al. 2000); if such substances are nutritional, large spermatophores may increase the female's fecundity, as found in other taxa such as insects. Female copepods may therefore have an advantage in mating with large young males, because (1) it reduces her need for frequent mating and thus reduces the potential risks of mating and (2) she receives sperm of high quality and quantity, which gives her a higher reproductive success. In this study we tested the hypothesis that size and age are significant factors influencing male fecundity (mating rate and sperm production) and reproductive fitness in the pelagic copepod, *Temora longicornis.*

MATERIALS AND METHODS

Experimental copepods and general procedures

Experiments were conducted with the calanoid copepod *Temora longicornis*, which was originally obtained from the central North Sea but has been cultured in our laboratory for over a year. Cultured *T. longicornis* decrease in size during subsequent generations (Klein-Breteler & Gonzalez 1982). The culture was maintained and all experiments were conducted with food provided in excess, in darkness, at 14°C and at a salinity of 32. Temperature and salinity were within the range in which the species thrives optimally (Maps et al. 2005). *T. longicornis* is a broadcast spawner and both the female and male engage in multiple matings. The adults measure about 1 mm in cephalothorax length. Females have normally 1 to 3 attached spermatophores. In this paper, 'age' refers to age since maturation; this is closely related to absolute age because maturation age varies very little for individuals grown under standardized conditions.

Most experiments (Expts 1 to 4) were conducted with virgin adults. To make sure that the copepods were virgins, late copepodites (Stages CIV to CV) were incubated individually in 69 ml bottles. The bottles were inspected daily to obtain freshly moulted virgin adults. Copepods were incubated with food provided in excess (1000 µg C l^{-1}, phytoplankton of species *Rhodomonas salina* and *Prorocentrum minimum*). Copepods in Expt 1 were incubated in 69 ml bottles and those in Expts 2 to 4 were incubated in 630 ml screw-cap bottles. All bottles were sealed without a head space and placed on a plankton wheel that rotated at 1 rpm.

Expt 1: longevity of adult females and males

Four males or females that had matured within 24 h were placed in each experimental bottle, 75 bottles for each sex. Every second day the numbers of live copepods were counted, dead ones were removed, and new food and water was added. The experiment continued until all copepods had died. The average duration of adult life was computed as the averages of the individual death dates (with t_0 = maturation date).

Expt 2: duration of the fertile period of females, lifetime egg production and age-dependent egg production rate

One virgin female and one virgin male were placed in each experimental bottle (N = 10). After 24 h, the female was isolated and her egg production monitored daily until she did not produce eggs for 4 consecutive days. Every day the female was transferred to a new bottle, the remaining water was filtered and the eggs were counted. The experiment was repeated using a slightly modified design to ensure that the females were unlimited by mating opportunities and sperm: The virgin females (N = 10) were offered 3 males (to prevent mating and sperm limitation) and the males were replaced every 48 h throughout the experiment. Female egg production was monitored as above.

Expt 3: age- and size-dependent male mating performance and fertility

To estimate mating performance of males (quantified as the fraction of mating opportunities actually used) as a function of their age and size, 1 virgin male and 2 virgin females were placed in each of the 10 experimental bottles. Every 24 h the females were replaced with 2 new virgin females that had matured within 24 h. The old females were transferred individually to a new experimental bottle containing seawater and food in excess, and their nauplii production was followed daily for 10 d to determine whether the female had been fertilized and to estimate the effect of male size on the total number of nauplii that a female could produce after 1 mating. Each male was offered a total of 20 females over a period of 10 d. The size of the male was measured under a dissecting microscope.

Expt 4: size of the spermatophore

To examine the size of the spermatophore relative to the size of the male, virgin couples were incubated for 24 h (N = 115). Each female was then inspected for attached spermatophores and the water was screened for lost spermatophores by means of a dissecting microscope. The sizes of the males and of the retrieved spermatophores were measured. Images were obtained with a digital video camera (uEye, Imaging Development Systems) connected to an inverted microscope (Olympus IX71), and analyzed with the shareware Image J 1.38X. We estimated the volume of each spermatophore from its length and width assuming an ellipsoid shape.

Expt 5: sperm content in relation to spermatophore size

The final experiment was designed to estimate the total number of spermatozoa inside the spermatophores of *Temora longicornis*. We retrieved spermatophores attached to females and used either females from our lab culture (N = 30) or live females collected in the central and southern part of the North Sea in August 2010 (N = 9). To be sure that the spermatophores had not started emptying their contents, only female copepods with more than 2 attached spermatophores were used. The females have 2 genital openings, and it is believed that additional spermatophores cannot be connected to the genital openings because they are full. We modified existing DAPI protocols used for insects, bacteria and blue-green algae to stain the spermatozoa before counting (Porter & Feig 1980, LaMunyon 2001). The females were killed by freezing at −18°C. After thawing, the females were rinsed in a phosphate buffer solution (PBS, pH 7.0) and individually transferred to a microscope slide. The spermatophores not attached directly to the genital pore were separated from the female by means of fine forceps, measured, and individually placed in a 30 µl drop of PBS on a new microscope slide. The spermatophore was cut open with fine forceps to release the spermatozoa and the entire contents on the slide were transferred to a 1.5 ml centrifuge tube. The microscope slide was rinsed several times in PBS to ensure that the entire contents were transferred. The sample was diluted with PBS to 187.5 µl and centrifuged for 2 min at 2500 rpm to separate the sperm from the spermatophore. The sample was then diluted to a total volume of 200 µl with the addition of the DNA label

4,6-diamidino-2-phenylindole (DAPI; final concentration 10 µg ml^{-1}). Each sample was vortexed for 10 s and stored in the dark for at least 4 min to allow sufficient DAPI labelling. The stained sample was filtered through a 0.2 µm black polycarbonate filter and the number of spermatozoa was counted under an epifluorescence microscope (Olympus; wave length, 365 nm).

RESULTS

Longevity and age-dependent mortality

The adult longevity (mean ± SD) of virgin *Temora longicornis* was very similar between the 2 genders (females: 30.6 ± 13.4 d; males: 30.6 ± 14.6 d), as were the temporal patterns of survivorship and mortality rates (Fig. 1). The maximum individual lifespan observed among the 300 individuals of the 2 genders was 68 and 70 d for females and males, respectively. The mortality rates increased significantly with age and led to a concave shape of the log survival versus age curves (Fig. 1); hence, both male and female age and ageing become evident in the curves as elevated mortality for both genders.

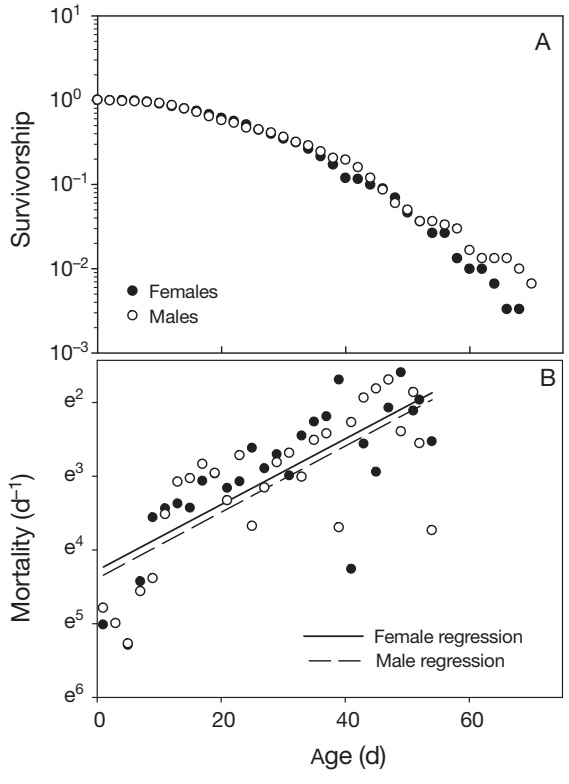

Fig. 1. *Temora longicornis*. (A) Survivorship curves for virgin females (●; N = 300) and virgin males (○; N = 300). Survivorship is defined as the fraction of individuals surviving as a function of time. (B) Instantaneous mortality rates of males and females computed for 2 d intervals as long as there were at least 10 individuals left in the cohort. Female regression: $\log_e$[mortality (d^{-1})] = −4.28 + 0.045·[age (d)] (R^2 = 0.58); male regression: $\log_e$[mortality (d^{-1})] = −4.39 + 0.045·[age (d)] (R^2 = 0.57). Both relations are statistically significant ($p < 0.001$) whereas the difference between male and female mortality patterns is not

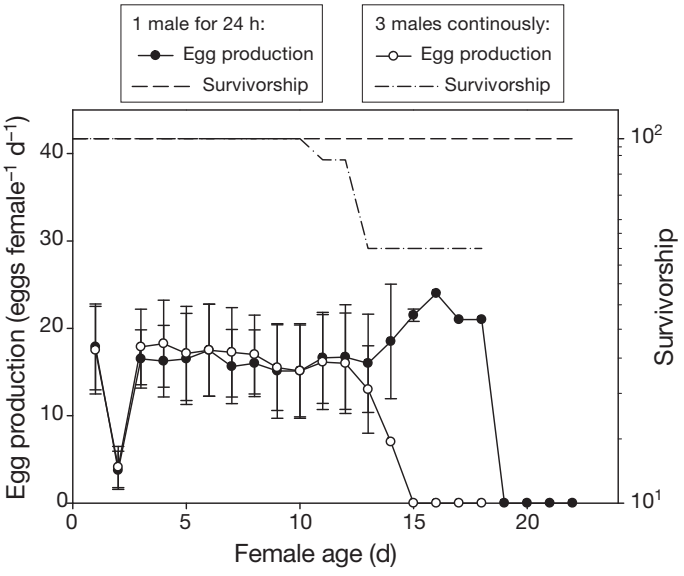

Fig. 2. *Temora longicornis*. Egg production rate (no. eggs female^{-1} d^{-1}; means ±SD) as a function of female age (d) since maturation, for females incubated with a single male for 24 h (●) and for females continuously incubated with 3 males (○) (N = 8 for each treatment). The experiment was run until the females did not produce eggs for 4 consecutive days (zero values). Only females producing eggs were included (20% of the females never produced eggs). Survivorship curves (dashed lines) during the experiment are significantly different for females with 1 male or 3 males (Mann-Whitney rank sum, p = 0.001)

Duration of the fertile period of females, and age-dependent and lifetime egg production

The fertile period of the female was short relative to the average longevity (cf. above) and varied only insignificantly between the 2 treatments, 13.9 ± 2.0 d for females incubated with a single male for 24 h and 12.3 ± 1.2 d for females incubated with males throughout the reproductive period (Fig. 2) (*t*-test, p = 0.070). The lifetime egg production was also similar between the 2 treatments (218 ± 76 vs. 191 ± 41 eggs, p = 0.388), which suggested that cessation of egg production was not due to a shortage of sperm. In both

treatments, 20% of the females did not produce any eggs during the experiment and therefore they are not included in the results. Repeated mating led to a higher mortality in females (Fig. 2).

Age-dependent male mating performance and fertility

Males mated only during the first 8 d after they matured, and the decline in mating performance had already started after 5 d (Fig. 3A). Again, this fertile period is short relative to the average longevity of males (cf. above). Also, the female's offspring production is related to the age of the male with which she had mated: young fathers sire significantly more offspring per mating than do older ones (Fig. 3B). The size of the female will also affect her offspring production (cf. below), which thus accounts for the large variance in offspring production, but all females in this experiment were virgins and of the same age (matured within 24 h). Therefore, the general decrease in offspring production is a result of the age of the male.

Size-dependent male mating performance and fertility

Large males were superior to small males in terms of reproductive performance in 2 ways: large males both mated more frequently (Fig. 4A) and sired more offspring per successful mating than small males (Fig. 4B).

Size-dependent female reproduction

To examine whether the size of the female has an effect on total lifetime offspring production and egg production rate, data for all females were compiled. Large females had a higher total lifetime offspring production compared with small females and they also had a higher instantaneous egg production rate (Fig. 5). The size effect was substantial: a 30% increase in female body length led to a 2- to 3-fold increase in egg production, which suggests that the egg production is approximately proportional to female body volume.

Spermatophore size and sperm content

Male size and spermatophore size varied substantially, and large males produced larger spermatophores than did small males (Fig. 6A). In fact, a modest increase in male prosome length (750 to 780 µm) led to a 4-fold increase in spermatophore volume. Larger spermatophores contained more sperm cells, although sperm content was far from proportional to spermatophore volume (a 4-fold increase in volume led to an increase in sperm count from 1000 to 1300) (Fig. 6B). This modest increase in spermatozoa number with increased volume suggested that large spermatophores either have a disproportionally larger swelling capacity to create hydrostatic pressure for sperm transferral or contain disproportional amounts of additional substances that are transferred with the sperm. The average number of spermatozoa inside a

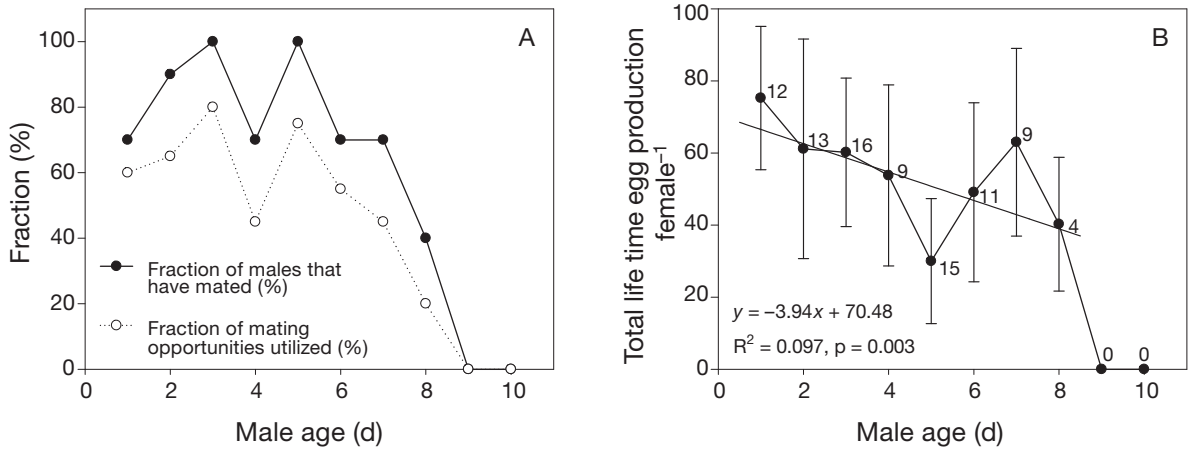

Fig. 3. *Temora longicornis*. Male mating performance and fertility. (A) The fraction of males (out of 10 males) that had mated as a function of male age since maturation (●) and the fraction of mating opportunities used (fraction of 20 females fertilized) as a function of male age (○). (B) Total life time offspring production per fertilized female ± SD as a function of male age since maturation (d). The numbers correspond to the number of fertilized females out of 20. The trend line is a linear regression that used individual values (not averages) for Days 1 to 8; the slope of this regression is statistically significant (p = 0.003) and demonstrates that young males sire more offspring per mating than do old males in the reproductive period

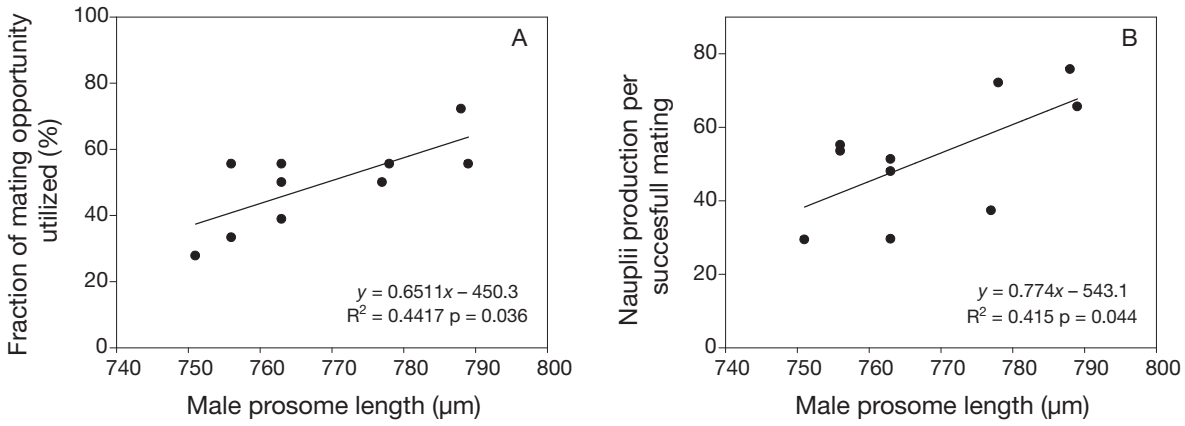

Fig. 4. *Temora longicornis*. (A) Fraction of mating opportunities that males had used (%) as a function of male size. (B) Nauplii production per successful mating as a function of the father's size. The experiment lasted 8 d and each male (N = 10) was offered 2 new virgin females every day. Same experimental individuals as in Fig. 3

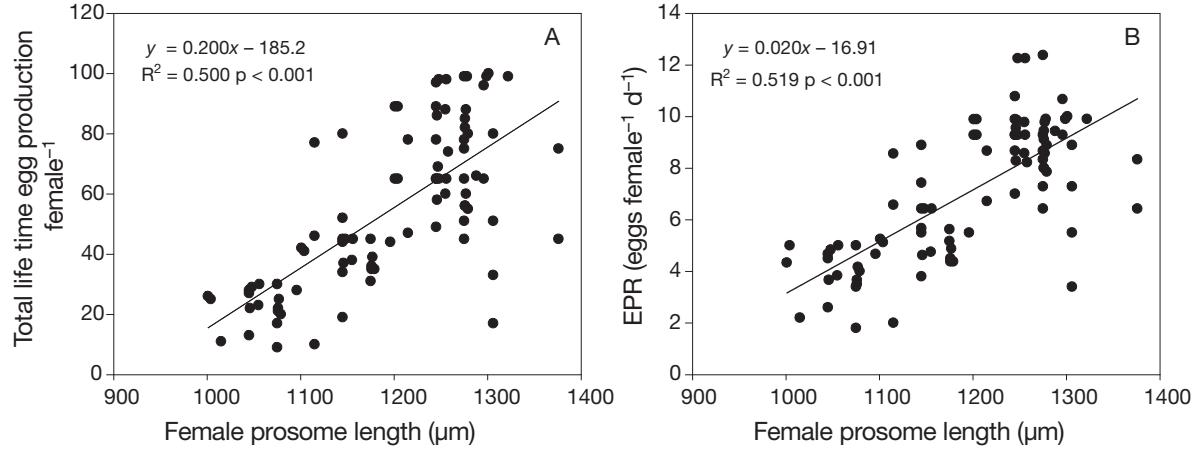

Fig. 5. *Temora longicornis*. (A) Lifetime egg production as a function of the female prosome size (N = 89). (B) Egg production rate (EPR) as a function of female prosome length. The egg production was followed for 10 d

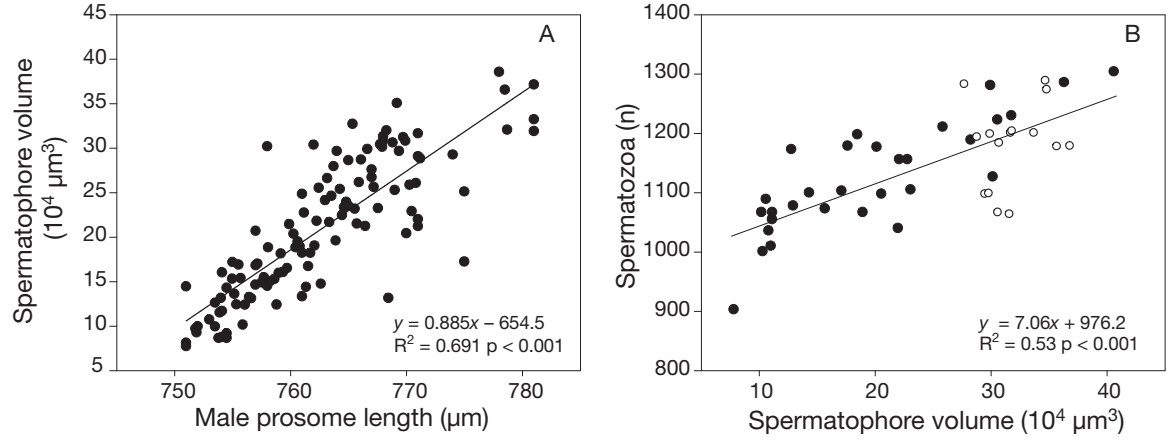

Fig. 6. *Temora longicornis*. (A) Spermatophore volume (10^4 µm^3) as a function of male size (µm) (N = 115). (B) Number of spermatozoa as a function of the spermatophore volume (10^4 µm^3) in a laboratory culture (●) (N = 30) and in field samples (○) (N = 15)

single spermatophore was 1126 ± 92, which corresponds to about 5 times the number of eggs that a female can produce after a mating event.

DISCUSSION

Our results showed 3 clear findings. (1) Ageing effects were evident. Mortality rate increased with age, and fertility decreased rapidly with age. This latter effect was stronger in males than females. Also, in both genders, the reproductive period was significantly shorter than the average longevity. (2) Several aspects of reproductive performance increased with size in both males and females: large females produced more offspring than small ones, and large males mated more often, produced larger spermatophores containing more sperm cells and sired more offspring. (3) Repeated mating was not only potentially advantageous (e.g. in terms of higher genetic variability) for females, but could also have had disadvantages as it led to higher mortality. While the absolute rates and durations reported in this study applied to our laboratory culture and conditions of the experiments, we believe that the patterns apply generally to this species.

Young males of *Temora longicornis* have a higher mating success and a higher reproductive success compared with old males. Whether this is due to females preferring young males, young males performing better (better sperm quality and quantity) or young males being more efficient in capturing and mating with females is unknown. In theory, female preference for males of particular ages is thought to be maintained largely through the benefits accrued by choosy females (Manning 1985, Hansen & Price 1995, Kokko 1998, Beck & Powell 2000, Proulx et al. 2002). One possible explanation for the elevated offspring production in young males is declining sperm number in old males, or increasing damage to the DNA or the spermatozoan cell membrane as seen in other species (Vishwanath & Shannon 1997, Irvine et al. 2000). Theory predicts that sperm quality declines with age owing to the accumulation of de novo mutations in the germline cells (Hansen & Price 1995, 1999). Females mating with old males may therefore suffer reduced fertility. Sperm age may be independent of male age; successful males may replenish their sperm frequently and so have better fertilization success than less successful competitors regardless of age (Siva-Jothy 2000). The old males in this experiment were not virgins and were offered new females daily. Therefore, the possibility is low that their sperm is old, and the observed decline in male success with age is probably an effect of male age. Direct trade-offs between fitness components and age-specific differences in survival may reduce the fertility of older males and instead promote the evolution of female preference for young males and those of intermediate age (Hansen & Price 1995, 1999, Kokko 1997, Beck & Powell 2000, Beck et al. 2002). Males in particular, but also females, can survive beyond their reproductive time period (30.6 versus 8 or 15 d). Such post-reproductive life may be an evolutionary adaptation to low mate-encounter rates if the reproductive performance is maintained in the absence of matings, as shown in another pelagic copepod species (Ceballos & Kiørboe 2011).

Size is a heritable trait in copepods (McLaren 1976, McLaren & Corkett 1978) and females that choose large males may therefore sire larger sons and daughters that likewise sire more offspring (Weatherhead & Robertson 1979). Morphologically, body size is positively correlated with reproductive organ size and total number of gametes, and it is well documented in comparisons between species that egg production rates in copepods, like in many other organisms, increase with size (Kiørboe & Hirst 2008). Comparisons within species are rare and the relationship observed here for *Temora longicornis* is much more pronounced than comparisons between species, where numerical egg production increases with body mass (or volume) to a power of 0.2 (Kiørboe & Hirst 2008). The size dependency of reproductive performance in male copepods has previously been examined in only one other species, *Acartia tonsa* (Ceballos & Kiørboe 2010), and is in accordance with our observation in *T. longicornis* that large males produce larger spermatophores and sire more offspring per mating than do small males. We showed that this is related to a higher content of sperm cells in the spermatophores.

Multiple mating (polyandry) in female insects is reported to increase fecundity and egg viability across a range of taxa (Arnqvist & Nilsson 2000). In copepods, polyandry is often observed both in laboratory and field populations (many attached spermatophores), and it is typically assumed that copepods belonging to the superfamily Centropagoidea, such as *Temora longicornis*, need to mate several times during their life because they lack dedicated sperm storage organs (spermathecae). This is, for example, the case in a sibling species, *T. stylifera* (Barthélémy et al. 1998). However, a female of *T. longicornis* receives sufficient sperm in 1 mating to fertilize all the eggs she produces in her life. In our

study, the lifetime egg production was 203 ± 62 eggs and the number of sperm per spermatophore ranged from 900 to 1300. This means that the female only use 15 to 22% of the sperm in 1 spermatophore to fertilize all of her eggs. Consistent with this, we find that multiple matings in *T. longicornis* do not increase the reproductive output in the females. We did not examine whether the offspring from one or several mating opportunities had different fitness levels, e.g. in terms of higher survival, and whether multiple mating secures higher genetic variation or allows for cryptic mate choice leading to fitter offspring. It is unclear whether cryptic mate choice is at all feasible in copepods because the genital opening may be blocked by the spermatophore or in some species by a cement plug, but there is molecular evidence in one such species, *Lepeophtheirus salmonis*, that a batch of eggs may in fact be sired by several fathers (Todd et al. 2005). However, there is a mortality penalty related to matings; repeated mating leads to a higher mortality in females (Fig. 2), probably owing to elevated energy consumption or damage or injury by the male when mating. This has also been demonstrated for another copepod species (*Oithona davisae*; Ceballos & Kiørboe 2011) and in several insect species (Campbell 2005, Wenninger & Hall 2008); thus, any advantages of multiple matings have to be traded off against the mortality penalty. In some species of insects, the seminal fluid contains substances that increase the male's relative paternity at the expense of female fitness (Chapman et al. 1995, Simmons 2001, Gillott 2003), which explains the elevated mortality of females that mate multiple times (Arnqvist & Nilsson 2000). The volume of the spermatophores and the volume of the total number of spermatozoa leave plenty of space in the spermatophores for seminal fluids.

Titelman et al. (2007) reviewed indirect evidence to suggest that mating in pelagic copepods was nonrandom and the result of mate choice. The strong age- and size-dependency of the reproductive output in both males and females and the mortality penalty to mating, in at least the females, are conducive to the development of sexual selection through mate choosiness. Necessary conditions for the development of choosiness are a high mate encounter rate, a cost to mating (in terms of elevated mortality or lost future mating opportunities) and, finally, that mates are of different quality and being choosy implies a fitness benefit (Kokko & Monaghan 2001, Shuster 2007). We have shown for *Temora longicornis* that mating with large, young partners implies higher offspring production in both males and females, and have provided evidence (higher mating success of young, large males) that is consistent with mate choosiness. Our study, together with recent observations of sexual selection through mate choosiness in other species of copepods (Ceballos & Kiørboe 2010), adds further evidence that sexual selection is an important determinant of the behavioral ecology and population dynamics of pelagic copepods.

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