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Morphometric changes in a strain of the lineage 'Nevada', belonging to the *Brachionus plicatilis* (Rotifera) complex

Venetia Kostopoulou[1], Helen Miliou[2] & George Verriopoulos[1]

[1]Department of Zoology-Marine Biology, Faculty of Biology, National and Kapodistrian University of Athens, Athens, Greece
[2]Department of Applied Hydrobiology, Faculty of Animal Science and Aquaculture, Agricultural University of Athens, Athens, Greece

Correspondence: V Kostopoulou, Department of Zoology-Marine Biology, Faculty of Biology, National and Kapodistrian University of Athens, Panepistimiopolis, Athens 157 84, Greece. E-mail: vkostop@biol.uoa.gr

Abstract

The rotifer *Brachionus plicatilis* is an important component of aquaculture as a larval feed. Its taxonomic status has been recently re-defined as a species complex, consisting of at least 14 new species/lineages. This study deals with the lineage *Brachionus* 'Nevada', which has been shown to occur in European hatcheries. A strain of *B.* 'Nevada' was mass cultured using two commonly applied feeding regimes and analysed in terms of its morphometry. A new formula was proposed for the calculation of volume, which can be used as an index of adequacy of rotifers as feed for fish larvae. The results were related to life cycle parameters. The pre-reproductive and reproductive phases were divided into distinct size groups. Differences were also found between the two diets in morphometry and demography. Rotifers of a larger size (yeast-based diet) showed a lower growth rate and a longer reproductive period, lifespan and mean generation time compared with smaller-sized rotifers (Culture Selco®-based diet). In terms of lorica length, the present study's strain of *B.* 'Nevada' (238.5 μm) was intermediate between values reported for *Brachionus ibericus* (193.5 μm) and *B. plicatilis* sensu stricto (299 μm).

Keywords: aquaculture, cultured organisms, hatcheries, morphometry, mass culture

Introduction

The monogonont rotifer *Brachionus plicatilis* has been the subject of extensive research due to its widespread use in aquaculture (Lubzens, Zmora & Barr 2001). This rotifer is mass cultured in order to be offered as feed during the first 10–30 days after mouth opening of fish larvae. Improving the efficiency of rotifer mass cultures poses a theoretical challenge for scientists and an economical bottleneck for aquaculturists. It therefore becomes clear that this organism is of considerable commercial and scientific interest.

The research relating to this species is hampered by the fact that its taxonomy is not clear. In the last 15 years, this rotifer has been divided into different morphotypes (large and small) (Fu, Hirayama & Natsukari 1991), species (Segers 1995; Ciros-Pérez, Gómez & Serra 2001) and, most recently, into an additional array of genetically divergent lineages (Gómez, Serra, Carvalho & Lunt 2002; Suatoni, Vicario, Rice, Snell & Caccone 2006), possibly representing different species (Fontaneto, Giordani, Melone & Serra 2007 for *Brachionus manjavacas*). Accordingly, *B. plicatilis* sensu lato (s.l.) is now referred to as the *B. plicatilis* species complex, which, so far, comprises of four species (*B. plicatilis* sensu stricto (s.s.), *Brachionus ibericus*, *Brachionus rotundiformis* and *B. manjavacas*) and at least five lineages (*Brachionus* 'Nevada', *Brachionus* 'Austria', *Brachionus* 'Cayman', *Brachionus* 'Tiscar' and *Brachionus* 'Almenara'). The first two lineages have been grouped with *B. plicatilis* s.s., which represents the large morphotype (L-type), and the rest of the lineages with *B. ibericus*, which represents the medium morphotype (SM-type) (Gómez *et al.* 2002; Fontaneto *et al.* 2007). *Brachionus rotundiformis* represents the small morphotype (SS-type) (Ciros-Pérez, Gómez *et al.* 2001) (Table 1).

Morphological, ecological and physiological information on *B. plicatilis* s.l. has to be revised in view of

the new taxonomic scheme. To date, most information has been generated for the species *B. plicatilis* s.s., *B. ibericus* and *B. rotundiformis* (Ciros-Pérez, Gómez *et al.* 2001; Ciros-Pérez, Carmona & Serra 2001, 2002; Ciros-Pérez, Carmona, Lapesa & Serra 2004). In addition, a number of studies have dealt with natural populations of *B.* 'Tiscar', *B.* 'Almenara' and *B. manjavacas* (Gómez *et al.* 2002; Ortells, Gómez & Serra 2003). On the other hand, the most commonly occurring strains in European hatcheries have recently been genetically identified as belonging to *B.* 'Cayman', *B.* 'Nevada' and *B.* 'Austria', apart from *B. plicatilis* s.s. (Papakostas, Dooms, Triantafyllidis, Deloof, Kappas, Dierckens, De Wolf, Bossier, Vadstein, Kui, Sorgeloos & Abatzopoulos 2006; Dooms, Papakostas, Hoffman, Delbare, Dierckens, Triantafyllidis, De Wolf, Vadstein, Abatzopoulos, Sorgeloos & Bossier 2007). The latter lineages have been studied sporadically (*B.* 'Nevada' – Korstad, Olsen & Vadstein 1989; Øie & Olsen 1993; Olsen, Reitan & Vadstein 1993; Skjermo & Vadstein 1993; Makridis, Fjellheim, Skjermo & Vadstein 2000; Kostopoulou, Miliou, Katis & Verriopoulos 2006; Kostopoulou, Miliou, Krontira & Verriopoulos 2007, *B.* 'Austria' – Rico-Martínez & Snell 1995; Gómez & Snell 1996, *B.* 'Cayman' – Kostopoulou & Vadstein 2007).

Morphometric characterization of *B. plicatilis* s.l. has played a central role in the discrimination of the different morphotypes and species, which have clearly defined boundaries (Fu *et al.* 1991; Ciros-Pérez, Gómez *et al.* 2001a). However, it is not clear whether the same applies to the newly described lineages. Recent studies have shown that differences in length do exist between the lineages and the respective species (according to the classification scheme of Gómez *et al.* 2002), but it has been argued that morphometry should be used in conjunction with genetic characterization for accurate discrimination of the different lineages (Campillo, García-Roger, Martínez-Torres & Serra 2005; Kotani, Hagiwara, Snell & Serra 2005; Suatoni *et al.* 2006).

The decisive role of the genetic component in the determination of size was foreseen by earlier morphometric studies (Yúfera 1982; Serra & Miracle 1983, 1987; Snell & Carillo 1984). Looking at the effect of geographic origin, temperature, salinity or diet, they all concluded that environmental modification is restricted by the limits imposed by the rotifer genotype. It is now possible, under the new taxonomic scheme, to quantify both the genetic and the phenotypic effects on rotifer size and set the size boundaries of each lineage.

Rotifer size has important practical implications, related to the use of this organism as a larval feed in aquaculture (Lubzens *et al.* 2001). Size is a critical feature that determines the adequacy of rotifers for young larvae, because the former should not exceed the mouth opening of the latter. It therefore becomes clear that the successful cultivation of fish larvae depends, to an extent, on the morphometric characteristics of the rotifer strain used.

In this study, we present morphometric information on a strain of *B.* 'Nevada', one of the lineages grouped with *B. plicatilis* s.s. (Gómez *et al.* 2002). Owing to the role of rotifers in aquaculture, the experimental design was based on mass culturing of rotifers in aquaculture farms of the Mediterranean. Morphometric changes in *B.* 'Nevada' were determined using two different diets and related to life-cycle parameters.

Materials and methods

Experimental stock population

The strain (isolate K) used in this study was identified on the basis of mitochondrial gene COI sequencing and named according to Gómez *et al.* (2002). It belongs to *B.* 'Nevada' (GenBank accession no. AM180752). The presence of *B.* 'Nevada' has been confirmed in a number of hatcheries around Europe (Papakostas *et al.* 2006; Dooms *et al.* 2007).

The feeding regime included the microalga *Tetraselmis suecica* (LB 2286), and baker's yeast *Saccharomyces cerevisiae* or the microparticulate compound diet Culture Selco® (INVE N.V., Ghent, Belgium). *Tetraselmis suecica* was batch cultured in the medium described by Walne (1966) and modified by Laing (1991). All cultures were performed at 25 °C and 35 g L^{-1} salinity under constant illumination and aeration (Moretti, Pedini Fernandez-Criado, Cittolin & Guidastri 1999). The culture medium consisted of diluted seawater that was UV treated.

A pre-experimental low-density (~ 10 individual mL^{-1}) culture of a parthenogenetic population of *B.* 'Nevada' was maintained under these experimental conditions for several generations, using *T. suecica* as food.

Experimental procedure

Rotifer mass cultures

For the rotifer mass cultures, sampling took place in an aquaculture farm in Greece (Octapus S.A.), where this strain has been cultured for at least 7 years. Rotifers were batch-cultured in tanks (2.5 m^3 capacity)

Table 1 Mean ± [a]: SE or [b]: SD (range in parenthesis, where available) of morphometric characters in increasing order of size (*A*, lorica length; *B*, distance between spines; *C*, maximum lorica width; EL, egg length; EW, egg width; all in μm) reported in the literature for the *Brachionus plicatilis* complex

Species/lineage	*A*	*B*	*C*	EL	EW	Source†	Name
–	128.4 ± 5.3		78.6 ± 19.6	52.8 ± 5.0	64.2 ± 7.5	1*	Fiji (SS)
–	143.7 ± 10.1		113.7 ± 9.7	60.9 ± 6.5	65.7 ± 7.5	1*	Otoni (SS)
–	146.6 ± 12.5		90.9 ± 13.4	62.7 ± 8.5	76.8 ± 7.7	1*	Sapian (SS)
B. rotundiformis	148.5 ± 2.5[a]		120.0 ± 2.5[a]			2*	SS2 (SS)
	(131.0–165.5)		(106.0–128.5)				
–	153 ± 14[b]		120 ± 18[b]			3*	*B. rotundiformis* SS2
–	161.1 ± 13.8		98.48 ± 24.1	66.0 ± 6.6	84.0 ± 4.7	1*	Thai (SS)
B. ibericus	193.5 ± 2.5[a]		144.5 ± 2.5[a]			2*	SM2 (SM)
	(175.5–220.0)		(126.0–163.0)				
B. 'Almenara'	164–231		99–149	90		4	Salton sea
–	170 ± 9[b]		144 ± 9[b]			3*	*B. rotundiformis* KOS
–	171.7 ± 7.8		116.6 ± 7.4	65.5 ± 6.3	87.2 ± 4.7		Sap (SS/S)
–	172.6 ± 12.2		116.5 ± 19.8	71.2 ± 9.6	74.1 ± 9.7	1*	Spain (S)
	173.7 ± 10.5		98.3 ± 10.9	71.7 ± 5.4	85.1 ± 11.1		San Ton (S)
–	178 ± 17[b]		129 ± 16[b]			3*	SM2
–	188.2 ± 11.4		125.0 ± 16.9	72.5 ± 5.7	90.2 ± 9.1	1*	Tahiti (S)
	188.9 ± 14.2		109.8 ± 12.1	76.3 ± 5.1	89.8 ± 6.4		Yugo (S)
–	195.5 ± 14.3		166.3 ± 33.5	68.5 ± 8.5	86.7 ± 10.9	1*	Sing (S)
	196.0 ± 22.7		134.0 ± 14.1	76.0 ± 7.8	90.0 ± 10.2		Koshi (SS/S)
–	201.4 ± 16.2		149.6 ± 13.3	79.0 ± 7.5	97.7 ± 10.9	1*	Bani (L)
–	212 ± 14[b]		163 ± 13[b]			3*	SEI
–	212 ± 20[b]	107 ± 11[b]	172 ± 13[b]			5*	(S)
B. 'Cayman'	238 ± 36[b]					6	MRS10
B. 'Austria'	238 ± 14[b]		175 ± 11[b]			3*	*B. plicatilis* s. s. CHI
B. 'Nevada'	238.5 ± 0.6[a]	108.8 ± 0.7[a]	181.1 ± 0.6[a]	121.6 ± 0.6[a]	95.4 ± 0.2[a]	7*	K
–	247.0 ± 12.3		141.1 ± 10.1	83.0 ± 5.2	113.7 ± 8.5	1*	Nre
B. 'Nevada'	250					8,9,10	*B. plicatilis* sintef
B. manjavacas	260 ± 18[b]		192 ± 12[b]			3*	*B. plicatilis* s. s. RUS
–	262.6 ± 12.7		174.4 ± 8.9	86.2 ± 5.8	116.3 ± 3.1	1*	Vigo S (L)
B. manjavacas	266.6 ± 40.8		152.9 ± 28.2	90.2 ± 7.8	110.5 ± 12.5	1*	*B. plicatilis* s. s. RUS (L)
B. plicatilis s.s.	268 ± 30[b]					6	10–2003
–	273 ± 16[b]		207 ± 15[b]			3*	*B. plicatilis* s.s. L1
–	275 ± 24[b]	145 ± 14[b]	214 ± 17[b]			5*	(L)
B. 'Nevada'	276 ± 23[b]					6	SIN22
B. 'Austria'	286 ± 25[b]		211 ± 21[b]			3*	*B. plicatilis* s. s. AUS
B. plicatilis s.s.	299.0 ± 2.5[a]		225.5 ± 2.5[a]			2*	L1 (L)
	(274.0–341.0)		(200–269.0)				
–	300.8 ± 19.8		190.0 ± 46.4	99.2 ± 7.3	134.3 ± 30.6	1*	AT (L)
–	305.0 ± 20.2		193.4 ± 15.6	83.7 ± 6.8	120.1 ± 14.7	1*	Vigo L (L)
	328.1 ± 10.5		196.6 ± 13.0	94.7 ± 2.1	126.1 ± 3.8		No26
B. manjavacas	360.7 ± 3.6[a]	126.5 ± 1.7[a]	280.8 ± 2.8[a]			11	ATIR1, ACVF2, L5MAN
B. plicatilis s.s.	385.7 ± 2.8[a]	136.0 ± 1.3[a]	298.2 ± 1.9			11	GALL1, APET1, ACHI1 L3, ACHI2

Species/lineage: according to the classification by Gómez *et al.* (2002); specified where it has been genetically identified unless otherwise stated. Name: species and/or strain (morphotype) as given in each reference.

*Measurements correspond to 48 h old ovigerous female.

†(1) Boehm *et al.* (2000) $T = 25$, 30 °C, $S = 20$, 30‰. (2) Ciros-Pérez *et al.* (2001a) $T = 23$ °C, $S = 12$ g L^{-1}. (3) Gómez and Snell (1996) $T = 25$ °C, $S = 15$ g L^{-1}. (4) Theilacker & McMaster (1971). (5) Fu *et al.* (1991) $T = 23$ °C, $S = 20$‰ (for ease of presentation, the average of the 67 S- and L-type strains is presented in the table). (6) Kostopoulou & Vadstein (2007). (7) This study $T = 25$ °C, $S = 35$‰. (8) Øie & Olsen (1993) $T = 20$ °C, $S = 20$‰. (9) Skjermo & Vadstein (1993) $T = 20$ °C, $S = 20$‰. (10) Makridis *et al.* (2000) $T = 20$ °C, $S = 20$‰. (11) Campillo *et al.* (2005) $T = 23$ °C, $S = 12$ g L^{-1}.

for 4 days, under conditions characteristic of Mediterranean hatcheries (25 °C, 35 g L^{-1}, 24 h:0 h light: dark cycle). Two routinely used feeding regimes were chosen: baker's yeast *S. cerevisiae* (treatment 1MC, three replicates) and Culture Selco® (treatment 2MC, three replicates). *Tetraselmis suecica* was added in order to improve the nutritional value of yeast in the first case (Hirayama & Watanabe 1973), and the quality of the culture medium in the second (Dhert, Rombaut, Suantika & Sorgeloos 2001). Nevertheless, the phytoplankton levels used were near (1.5×10^4 cells mL^{-1} in treatment 1MC) or below (0.2×10^4 cells mL^{-1} in treatment 2MC) 'green water' levels (Reitan, Rainuzzo, Øie & Olsen 1993; Dhert *et al.* 2001) and not high enough to satiate rotifers (Hirayama & Ogawa 1972). The levels of yeast and Culture Selco® corresponded to the recommended levels for mass culture production (Moretti *et al.* 1999; Suantika, Dhert, Nurhudah & Sorgeloos 2000) and were therefore considered to be satiating. According to the above and as shown by previous experiments (Kostopoulou *et al.* 2006), baker's yeast and Culture Selco® were considered to be the main food sources of the two feeding regimes. Baker's yeast and Culture Selco® show differences in their dry weight composition (Table 2), which result in concomitant differences in their energy content. Based on the energy conversion factors given by Beukema & De Bruin (1979), baker's yeast and Culture Selco® have an energy content of 15.8 and 18.2 respectively. A more analytical description of the experimental design can be found in Kostopoulou *et al.* (2006) as Experiment one.

Rotifer samples (10 mL) were collected twice daily at 8:00 and 16:00 hours for morphometry measurements and were preserved in 4% buffered formaldehyde solution.

Life-table analysis

The two feeding regimes of the rotifer mass cultures were examined in life-table experiments (*S. cerevisiae* and *T. suecica* – treatment 1LT, Culture Selco® and *T. suecica* – treatment 2LT). Rotifers of known age (F_2 generation of the pre-experimental culture) were cultured individually in a 24-well culture plate (3424 Mark II), which was immersed in a water bath to avoid evaporation. The culture volume was 1 mL. Rotifers were examined at 8 h intervals (08:00, 16:00 and 24:00 hours). Every day (between 10:00 and 12:00 hours), the culture medium was renewed. The offspring were counted and removed. The experiment was terminated when the last rotifer died.

Data acquisition and analysis

Rotifer mass cultures

Three characters of the lorica of each individual were measured, according to Fu *et al.* (1991): lorica length (*A*), distance between spines (*B*) and maximum lorica width (*C*). These were determined using a Zeiss Axiolab microscope (× 10) (Carl Zeiss Jena, Germany), with a Sony digital Hyper Had camera attached to it. An image analysis system (Image Pro-Plus v 3.01, Media Cybernetics LP, Silver Spring, MD) was used. For higher accuracy in the calculation of volume (*V*), all measurements (*A*, *B* and *C*) were incorporated, and not just length (*A*) as suggested by Ruttner-Kolisko (1977). Accordingly, the upper half of the body (from *C* to *B*) was approximated to a cylinder and the other half (*C* to the rear end) to a half-sphere (Fig. 1). The following formula was used:

$$V = \pi/12C^3 + \pi/16(C+B)^2(A - C/2)$$

The length (EL) and width (EW) of the eggs were also measured. Their volume (EV) was approximated to that of an ellipsoid of revolution (Yúfera 1987)

$$\mathrm{EV} = \pi/6\,\mathrm{EL}\,\mathrm{EW}^2$$

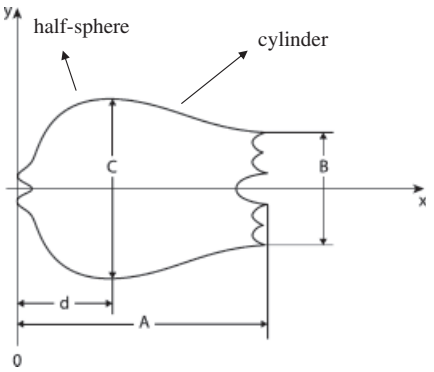
The relative egg volume (REV) was calculated on the basis of the above estimates:

$$\mathrm{REV} = \mathrm{EV}/V$$

Table 2 Literature values of protein, lipid and carbohydrate content (% dry weight) of the main diet types used in this study

	Proteins	Lipids	Carbohydrates	Source
Saccharomyces cerevisiae	53.2	1.0	41.3	Ben-Amotz, Fishler and Schneller (1987)
	30.65	3.4		Dendrinos and Thorpe (1987)
	30.5 ± 2.6	7.7 ± 2.1	46.2 ± 4.2	Frolov, Pankov, Geradze, Pankova and Spektorova (1991)
Culture Selco®	35	15		Suantika *et al.* (2000)
		18		Dhert *et al.* (2001)
	36.8	16.8	33.8	http://www.inve.com
	45	15	30	http://www.aquaculture.ugent.be

Values appear as mean ± SE.



Volume of half sphere: $V_{sphere/2} = 2/3\ \pi\ d\ (C/2)^2 = 1/6\ \pi\ d\ C^2$

Volume of cylinder:

- mean radius of cylinder: $r = (C/2 + B/2)/2 = 1/4\ (C + B)$
- volume of cylinder: $V_{cylinder} = \pi\ r^2\ (A - d) = \pi/16\ (C + B)^2\ (A - d)$

Total volume: $V = V_{sphere/2} + V_{cylinder}$

$V = 1/6\ \pi\ d\ C^2 + \pi/16\ (C + B)^2\ (A - d)$

$\downarrow\ d = C/2$

$V = \pi/12\ C^3 + \pi/16\ (C + B)^2\ (A - C/2)$

Figure 1 Calculations that yielded the formula for rotifer body volume.

From each sample of the rotifer mass cultures, the dimensions of at least 50 randomly selected individuals were measured. Each individual was classified according to the following scheme. The life cycle of the rotifer was divided into pre-reproductive, reproductive and post-reproductive phases. The first two phases were further divided into a number of life-history stages, which differed in the overall shape and size. The pre-reproductive phase included neonates (same shape as the egg) and young (same shape as the non-ovigerous female but smaller in size). The reproductive phase comprised of non-ovigerous females, females with a single egg and females with multiple eggs. Finally, the post-reproductive phase consisted of post-reproductive females, which can be discriminated due to the degenerate digestive organs and gonads and general swelling of the body (Carmona, Serra & Miracle 1989; Ricci & Fascio 1995). In order to quantify changes in lorica shape in the aforementioned stages, the ratio of *C*/*A* was also calculated. No sexual females or males were observed; experimental populations were all parthenogenetic.

Life-table analysis

From the life-table analysis, the duration of the following life-cycle phases was obtained: lifespan, juvenile period (time to first egg), reproductive phase, post-reproductive phase, egg development time and time interval between subsequent egg layings. The growth rate was calculated as follows:

$$r = \ln \mathrm{Ro}/T$$

where Ro is the total number of offspring per female and T is the mean generation time, given by

$$T = \Sigma(x\ Ux)/\Sigma Ux$$

where x is the age of the female at egg hatching and Ux is the number of offspring at age x (Birch 1948; Andrewartha & Birch 1954; Laughlin 1965).

Statistical analysis

Normal distribution and homogeneity of variance were assessed before analysis. The morphometric data were analysed using the non-parametric tests of Mann–Whitney for two-sample comparisons and Kruskal–Wallis for multiple sample comparisons. With respect to the life-table analysis, comparisons between and within treatments were tested using analysis of variance. Comparison of means was conducted with Fisher's LSD multiple-range test. Differences were considered to be significant at $P < 0.05$. All analyses were performed using STATGRAPHICS, v.5. The measurements taken at the beginning of the batch cultures were not included in the statistical tests. Mean values ± standard error (SE) are presented.

Results

Rotifer mass cultures

Individuals in the various life-history stages of treatment 1MC were generally larger in size, compared with treatment 2MC (Table 3): the difference in the distance between spines (*B*) was clearly demonstrated for all stages, followed by maximum lorica width (*C*) and then by lorica length (*A*), which differed in only two life-history stages. When all measurements were incorporated into the calculation of volume (*V*), significant differences were observed in all life-history stages except one. Within each treatment, a gradual increase in size was evident, from the pre-reproductive to the reproductive phase, slowing thereafter during the post-reproductive phase. The maximum size was attained by females bearing multiple eggs. Neonates had a significantly lower *C*/*A* ratio than the rest of the life-history stages ($P < 0.001$). Young individuals showed similar *C*/*A* ratios as non-ovigerous females, whereas maximum *C*/*A* values

Table 3 Mean values ± SE of lorica length (*A*, μm), distance between spines (*B*, μm), maximum lorica width (*C*, μm) and volume (*V*, 10^6 μm^3) in the different life history stages of the populations of treatments 1MC and 2MC

	n		*A*			*B*			*C*			*V*		
Life history stages	**1MC**	**2MC**	**1MC**	**2MC**	P_A	**1MC**	**2MC**	P_B	**1MC**	**2MC**	P_C	**1MC**	**2MC**	P_V
Pre-reproductive phase														
Neonate	24	51	146.5 ± 1.5^a	137.6 ± 1.2^a	***	66.8 ± 0.9^a	56.2 ± 1.0^a	***	100.6 ± 1.3^a	95.4 ± 0.8^a	***	0.80 ± 0.02^a	0.64 ± 0.01^a	***
Young	193	359	178.8 ± 1.0^b	178.0 ± 0.7^b	*NS*	84.5 ± 0.8^b	80.7 ± 0.6^b	***	132.3 ± 1.0^b	134.5 ± 0.6^b	*	1.72 ± 0.03^b	1.68 ± 0.02^b	*ns*
Reproductive phase														
Non-ovigerous	756	478	219.4 ± 0.6^c	221.7 ± 0.7^c	*NS*	103.7 ± 0.4^c	97.4 ± 0.5^c	***	166.3 ± 0.5^c	165.4 ± 0.6^c	*ns*	3.21 ± 0.03^c	3.11 ± 0.03^c	*
Female with single egg	134	252	238.9 ± 0.9^d	238.2 ± 0.7^d	*NS*	112.9 ± 1.0^d	106.6 ± 0.8^d	***	182.3 ± 1.1^d	180.4 ± 0.6^d	*	4.15 ± 0.05^d	3.95 ± 0.03^d	***
Female with multiple eggs	11	61	249.0 ± 2.3^e	247.2 ± 1.7^e	*NS*	125.9 ± 2.9^e	117.3 ± 0.9^e	**	199.3 ± 1.7^e	194.3 ± 0.7^e	*	5.21 ± 0.11^e	4.79 ± 0.05^e	**
Post-reproductive phase														
Post reproductive	18	34	248.5 ± 4.4^e	234.4 ± 2.7^{de}	**	123.1 ± 2.9^e	109.1 ± 2.0^{de}	*	190.9 ± 3.6^{de}	181.9 ± 2.2^d	*	4.83 ± 0.20^d	4.00 ± 0.12^d	**
P			***	***		***	***		***	***		***	***	

Means within a column having a different letter in superscript are significantly different. n: sample size.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
NS: non significant.

were attained by females with multiple eggs ($P < 0.001$).

Eggs were significantly larger in terms of size (Table 4) and relative egg volume (REV – mean standard ± error; single eggs: 1MC = 15.5 ± 0.2%, 2MC = 14.2 ± 0.2%, $P < 0.001$; multiple eggs: 1MC = 11.3 ± 0.3%, 2MC = 10.5 ± 0.1%, $P < 0.05$) in treatment 1MC, compared with treatment 2MC. Single eggs were also significantly larger with respect to length (EL), volume (EV) and REV ($P < 0.001$), than multiple eggs, irrespective of the treatment.

Life-table analysis

The growth rate (*r*) was significantly higher ($P < 0.001$) in treatment 2LT, compared with 1LT. The lifespan ($P < 0.05$), duration of reproduction ($P < 0.001$), post-reproductive period ($P < 0.001$) and mean generation time ($P < 0.001$) were significantly longer in treatment 1LT, than in 2LT. The total number of offspring per female (Ro), juvenile period and egg development time did not differ between treatments (Table 5). In treatment 2LT, the time interval between subsequent egg-layings was significantly shorter until the deposition of the fourth egg, similar between the fourth and fifth laying and significantly longer from the fifth to the eighth laying, compared with 1LT (Table 6).

Discussion

This study shows variations in the morphometric characters of a *B.* 'Nevada' strain between as well as within experimental treatments. The population was categorized into life-history stages, which corresponded to distinct size groups, with the exception of the post-reproductive phase. There was an increase in all morphometric variables from the pre-reproductive to the reproductive phase in both treatments. The maximum increase in lorica length (*A*) from neonate to full size (ovigerous female with multiple eggs) was 70–80% and within the range of previously reported values (Yúfera 1982; Snell & Carillo 1984; Korstad *et al.* 1989; Yúfera, Pascual & Olivares 2005). Within the reproductive phase, the presence and number of eggs positively influenced rotifer size. This could be attributed to the accumulation of yolk, which is needed for the formation of eggs. Hence, there is an increase in size up to the attainment of reproductive maturity and a fluctuation

Table 4 Mean values ± SE of length (EL, μm), width (EW, μm) and volume (EV, 10^6 μm^3) of parthenogenetic eggs carried by females (single: one egg at a time; multiple: more than one eggs at the same time) in the populations of treatments 1MC and 2MC

Number of eggs carried by female	*n*		EL			EW			EV		
	1MC	2MC	1MC	2MC	P_{EL}	1MC	2MC	P_{EW}	1MC	2MC	P_{EV}
Single egg	134	252	124.8 ± 0.8	119.8 ± 0.7	***	98.5 ± 0.4	93.7 ± 0.3	***	0.64 ± 0.01	0.55 ± 0.01	***
Multiple egg	22	122	108.5 ± 1.2	104.3 ± 0.5	**	99.9 ± 0.7	95.4 ± 0.4	***	0.57 ± 0.01	0.50 ± 0.01	***
P			***	***		*NS*	***		***	***	

n: sample size.
$^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.
NS: non significant.

Table 5 Mean ± SE of the main life-table parameters of the rotifer

	Treatment 1LT	Treatment 2LT	*P*
Growth rate (*r*, ind. day^{-1})	0.27 ± 0.02	0.31 ± 0.01	***
Total number of offspring (Ro)	5.60 ± 0.55	5.27 ± 0.42	*NS*
Lifespan (h)	285.0 ± 14.5	237.0 ± 17.2	*
Juvenile period (h)	44.5 ± 1.5	44.6 ± 3.2	*NS*
Duration of reproduction (h)	216.8 ± 16.6	164.0 ± 18.2	***
Post-reproductive period (h)	17.7 ± 3.9	8.9 ± 1.5	***
Mean generation time (h)	147.9 ± 7.5	123.9 ± 6.0	***
Egg development time (h)	22.7 ± 0.4	22.2 ± 0.6	*NS*

$^*P<0.05$, $^{***}P<0.001$.
NS: non significant.
$n = 24$.

Table 6 Interval egg laying (mean ± SE, in hours) in the two treatments

Interval egg laying	Treatment 1LT	Treatment 2LT	*P*
1st–2nd	20.1 ± 1.3	18.2 ± 1.4	***
2nd–3rd	27.9 ± 2.4	22.7 ± 1.7	***
3rd–4rth	41.7 ± 4.1	25.2 ± 3.3	***
4th–5th	47.8 ± 2.7	42.1 ± 6.4	*NS*
5th–6th	37.1 ± 2.3	48.0 ± 5.0	*
6th–7th	37.6 ± 2.7	61.9 ± 3.7	***
7th–8th	34.1 ± 3.1	72.2 ± 0.0	*
8th–9th	48.1 ± 15.6	–	*NS*

$^*P<0.05$, $^{***}P<0.001$.
NS: non significant.
$n = 24$.

thereafter during the reproductive phase, in close connection to egg production.

Further examination of lorica shape (*C*/*A*) revealed that neonates had a more oblong shape (lowest *C*/*A*), compared with young individuals, which were rounder and similar to the rest of the life-history stages. Thus, neonates can be discriminated from young based on shape and size, and young can be discriminated from the rest based on size. In addition, the high *C*/*A* values of post-reproductive females are in agreement with the swelling of the body, as reported by Carmona *et al.* (1989) and Ricci & Fascio (1995).

The calculation of volume using our formula yielded higher values than those of previous studies, most of which have applied Ruttner-Kolisko's (1977) formula (Epp & Lewis Jr 1979; Yúfera 1987; Guisande & Mazuelos 1991; Galindo, Guisande & Toja 1993; Walz, Sarma & Benker 1995; Kirk 1997). Pourriot & Rougier (1991) have also modified Ruttner-Kolisko's (1977) formula, although still using lorica length as the only determinant, and obtained higher estimates that agree with our values. It is suggested that, because Ruttner-Kolisko's (1977) formula refers to *Brachionus* at the genus level, whereas Pourriot & Rougier's (1991) and this study's formulae refer to *B. plicatilis* species complex, they should be used preferentially. It is suggested that the latter two formulae represent a more accurate description of *B. plicatilis* shape.

According to the present results, most of the growth (50–60%) occurs before the beginning of the reproductive phase. This is in agreement with previous studies, where growth deceleration was correlated to the extrusion of the first egg (Snell & Carillo 1984; Carmona *et al.* 1989; Ricci & Fascio 1995). The resources directed towards reproduction reduce those available for growth and maintenance and for this reason these two processes cannot take place simultaneously. The latter has been shown to occur in many different organisms (Snell & King 1977; Mole & Zera 1993; Landwer 1994; Jokela & Mutikainen 1995). It should be noted that, in this study, the onset of reproduction did not differ between treatments in

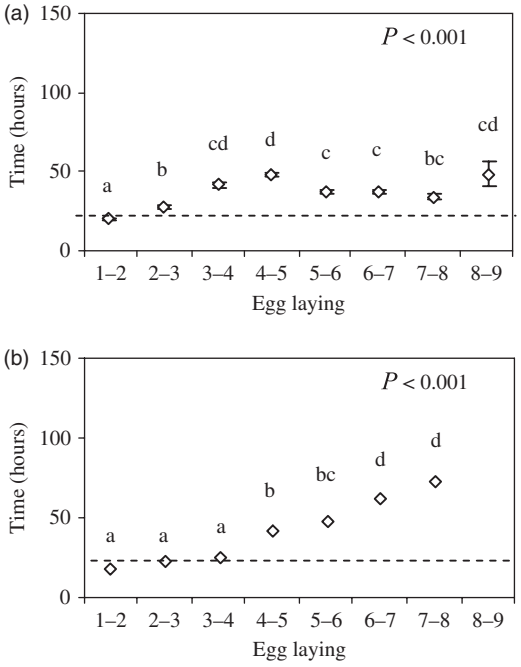


Figure 2 Time interval (in hours, mean ± standard error) between subsequent egg layings in increasing order: (a) 1LT and (b) 2LT. Letters designate significant differences. The discontinuous line represents egg development time.

terms of size (see young, Table 3) and duration (see juvenile period, Table 5). It is suggested that for reproduction to begin, a fixed threshold is probably required (Roff 2000), in terms of size and duration, the latter two being possibly interrelated.

The formation of single and multiple eggs depends on the duration of egg development time in relation to the time interval between subsequent egg layings. Multiple eggs arise when interval egg laying is less than egg development time and vice versa (Fig. 2). In this study, egg development time was similar between treatments (Table 5), whereas interval egg laying differed (Table 6). The faster deposition of the first four eggs observed in treatment 2LT can explain the higher abundance of females with multiple eggs reported earlier (Kostopoulou *et al.* 2006). Despite the above-mentioned differences, the appearance of females with multiple eggs was more pronounced at the beginning of the reproductive phase in both treatments (Fig. 2, Table 6). Single eggs were larger than multiple eggs in both treatments. The same has been shown in *Daphnia*, where offspring size decreases with clutch size (McKee & Ebert 1996). This difference in size could be linked to the frequency of egg laying; faster deposition of eggs (see Fig. 2, Table 6) results in eggs of smaller size (see Table 4), as also supported by previous studies (DeMeester 1995; Stelzer 2005).

Between-treatment variation consisted of rotifers in treatment 1MC (fed mainly on yeast) being overall larger in volume compared with those in treatment 2MC (fed mainly on Culture Selco®). The difference in size was more important for *B* (ranging between 4.7% and 19%), followed by *C* (0.5–5.4%) and much less for *A* (0.3–6%). In previous studies, it has been shown that lorica length is largely genetically determined (Snell & Carillo 1984; Serra & Miracle 1987). Environmental modification of *A* is possible, but to a lesser extent: compared with the present study, wider fluctuations in *A* have been reported previously (5–15%), due to the type of diet (Yúfera 1982; Snell & Carillo 1984; Korstad *et al.* 1989), food availability (Temprano, Moreno, Carmona & Serra 1994; Hansen, Wernberg-Møller & Wittrup 1997), culture conditions (Suantika, Dhert, Sweetman, O'Brien & Sorgeloos 2003) or hormone addition (Gallardo, Hagiwara, Tomita, Soyano & Snell 1997). This could be attributed to the wider range of conditions tested in the aforementioned studies.

Lorica length (*A*) was similar between treatments in most life-cycle phases (young and reproductive females), despite the respective differences in volume (Table 3). According to the present study, lorica length appears to be the most conservative character to environmental perturbation, and therefore more suitable to study the size limits of species/lineages. Width measurements (*B*, *C*) and volume are more sensitive and can be used preferentially to detect phenotypic variability. Comparison with other strains is required in order to reinforce the above finding.

The difference in size between treatments 1MC and 2MC can be related to life-history differences. The rotifers of treatment 2LT produced the first four eggs at a faster rate, compared with those of treatment 1LT. The total number of offspring was similar between the two treatments, but the reproductive period lasted longer in the case of treatment 1LT. This means that the rotifers of treatment 1LT had more time for the production of the same number of offspring, compared with those of treatment 2LT (also demonstrated by the growth rate of the two treatments). According to the above, the time spent in the production of each offspring of treatment 1LT can explain their larger size. The inverse relationship between the rate of offspring production (i.e. growth rate) and its resulting size, as well as the positive relationship between the total duration of the reproductive phase

and offspring size is also supported by previous studies (Duncan 1989; Korstad *et al.* 1989; Stelzer 2005). According to the results of this study, the production of the first few offspring (four in this study) appears to play a decisive role in rotifer size and dynamics.

The larger size of rotifers of treatment 1MC points towards a higher energy content per individual (Epp & Lewis Jr 1979). However, the diet of the latter treatment is of a lower energy compared with the diet of treatment 2MC. Previous studies on cladocerans (Gliwicz & Guisande 1992; Boersma 1995; McKee & Ebert 1996) and rotifers (Guisande & Mazuelos 1991; Galindo *et al.* 1993; Green 1998) have shown that, over a range of middle to high levels of food supply (like the ones used here), individuals respond by producing offspring of increasing size with decreasing food availability. In the present study, it is the energy content that seems to play the decisive role. According to the literature (Gliwicz & Guisande 1992; Kirk 1997), this increased investment in individual offspring (also demonstrated in REV differences between treatments) concomitantly increases the offspring's ability to survive periods of food scarcity, thus conferring higher starvation resistance. Large offspring will eventually develop into large females, which will in turn produce large eggs (Yúfera 1987; Pourriot & Rougier 1991; Temprano *et al.* 1994; Walz *et al.* 1995; Green 1998).

Such morphological responses to food availability have been considered to be adaptive, given that a trade-off between the size and the number of offspring exists (Winkler & Wallin 1987; Yampolsky & Scheiner 1996). Examples of such trade-offs can be found in *Daphnia* (Gliwicz & Guisande 1992; Boersma 1995; McKee & Ebert 1996), copepods (Auel 2004) and other species of rotifers (Guisande & Mazuelos 1991; Galindo *et al.* 1993). In our case, there was no difference in the total number of offspring, but rather in the rate of their production (offspring per unit of time). Therefore, the trade-off in this study can be found between the size and the number of offspring in the unit of time. The observed responses can be attributed to the diet offered, and specifically to the type of dry food. In particular, the above differences could be associated with the energy content of baker's yeast and Culture Selco®.

The strain of *B.* 'Nevada' used in this study ('K', see Table 1) is similar in length to strain 'sintef', but smaller by 16% than strain 'SIN22', all belonging to the same lineage. In comparison with the species, strain 'K' is larger by 23% in lorica length compared with *B. ibericus* and smaller by 25% than *B. plicatilis* s.s. Overall, the grouping of the newly defined lineages in terms of size (Table 1) follows their phylogenetic position (Gómez *et al.* 2002) – with the exception of *B.* 'Cayman' – although the size limits of each lineage are not clearly defined. Therefore, the view by Campillo *et al.* (2005), Kotani *et al.* (2005) and Suatoni *et al.* (2006) that morphometry does not represent an accurate criterium for the discrimination of species/lineages holds true, unless more studies resolve the size boundaries of the species/lineages within the complex. In addition, the morphotype, as defined in previous studies not taking into account the new taxonomic scheme, should be viewed with caution.

In this study, it was shown that the main morphometric changes in strain 'K' of *B.* 'Nevada' were due to growth. The increase in size during growth is not evenly distributed: reproduction marks a deceleration in growth. Diet also plays a role, but of smaller importance. Batch cultures of rotifers fed on the two feeding regimes of the present study showed differences in the (%) abundance of life-history stages (Kostopoulou *et al.* 2006). According to the present study, the aforementioned differences could be related to respective differences in size. Rotifer size, in particular volume, is important from an aquacultural point of view, because it could be related to biomass (Ruttner-Kolisko 1977; Pauli 1989). The proposed formula of volume appears to be the most sensitive index of morphometric differentiation, because it incorporates all morphometric parameters and not just lorica length, as proposed by earlier studies (Ruttner-Kolisko 1977; Pourriot & Rougier 1991). Therefore, the formula of volume of the present study can be used to estimate total rotifer biomass in culture, which can be translated into the amount of food offered to fish larvae. Rotifer volume may represent a more significant measure of adequacy of rotifers as feed for fish larvae than rotifer numbers, as also proposed by others (Yoshimura, Usuki, Yoshimatsu, Kitajima & Hagiwara 1997), considering that rotifer cultures with equal numbers of rotifers may show differences in biomass, due to differences in the (%) abundance of life-history stages. In order to obtain the peak of rotifer production, at least for *B.* 'Nevada', one should aim for the beginning of the reproductive phase (45 h, Table 5), when egg production is highest and rotifers attain their maximum size. This has been shown for batch-cultured *B.* 'Nevada' fed lipid-rich diets (Culture Selco®) (Kostopoulou *et al.* 2006). It is suggested that shortening the duration of batch cultures from 4 days to 2–3 days will result in higher production rates.

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