Contribution of gut content to the nutritional value of *Brachionus plicatilis* used as prey in larviculture.

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Abstract

With the aim of assessing the potential value of gut content in the rotifer *Brachionus plicatilis* during the standard procedures of enrichment and posterior residence in the larval tanks, we have examined by image analysis the changes in gut volume during the filling and subsequent evacuation process when feed is no more available. The gut filling pattern has been examined at different microalgal concentrations of *Nannochloropsis gaditana* ranging between 0.4 and 20.8×10^6 cell mL^{-1} . The rotifer gut was completely filled in 120 min and the gut volume became significantly higher in rotifers fed at higher microalgae concentrations. The gut volume accounted for up to 15% of the body volume. The rotifers evacuated quickly the gut when were filtered and placed in clean water, losing 60% of the content in the first 5 min. Contrarily, when the rotifers were rinsed carefully while maintaining the water volume, the gut was evacuated progressively and needed one hour to lose 60% of the content. Moreover, we have examined the changes in dry mass and energy. After 2 hours of gut evacuation the rotifers lost 20% of the initial dry weight, and 38 % after 24 hours of starvation. In terms of energy the rotifers lost 43% of the caloric content after 24 hour of starvation. The findings of this study confirm the importance of performing appropriate feeding protocols to supply living prey of high nutritional quality in larviculture.

Keywords: *Brachionus plicatilis*, enrichment, microalgae, gut filling pattern, gut evacuation pattern, energy content

1. Introduction

Different species and lineages of the *Brachionus plicatilis* cryptic species complex are widely used as live prey to feed marine fish larvae during the first weeks. The advantages and constraints of their use as food in aquaculture are well known (Lubzens et al., 1989; Lubzens and Zmora, 2003; Conceição et al., 2010). One of the main concerns is the nutritional quality as a prey when the rotifers are produced in mass culture. Rotifers are produced under different methods such us batch, semicontinuous and continuous cultures, as well as under high-density superintensive, water

recirculation and automatic-controlled systems (Lubzens et al., 1989; Yoshimura et al., 1997; Dhert et al., 2001; Yúfera et al., 2001; Bentley et al., 2008; Alver et al., 2010; Kostopoulou et al., 2012). The culture system may affect the body biochemical composition. In some cases, the food is almost exhausted at harvesting and the animals may present pre-starvation symptoms affecting the body dry mass and biochemical composition with the consequent poor nutritional quality as prey (Szyper, 1989; Makridis and Olsen, 1999; Kotani et al., 2009). Likewise, the use of non-expensive food sources such as baker yeast, some commercial products, and some microalgae species usually yield rotifers with nutritional deficiencies of the essential fatty acids (Watanabe et al., 1983; Ben-Amotz et al., 1987; Rainuzzo et al., 1989, 1994), vitamins and minerals (Giménez et al., 2007; Hamre et al., 2008). The potentially inappropriate nutritional quality of rotifers as food for fish larvae has been solved by re-feeding them with some microalgae species, oil emulsions, commercial microparticulated products and tailored boosters (Olsen et al., 1989; Fernández-Reiriz et al., 1993; Rodríguez et al., 1996; Palmtag et al., 2006; Demir and Diken, 2011) before being supplied to the rearing tanks.

This post-harvesting enrichment process has a double purpose. One is the incorporation of desired specific nutrients to the rotifer tissues, process that requires between 12 and 24 hours of enrichment (Rainuzzo et al., 1994; Kotani et al., 2010). The other is to use the rotifer body, and most specifically the gut, as a living capsule for transferring a given feed containing a specific compound to the fish larval gut (bioencapsulation). In this second aspect, the final quality of the ingested food depends widely on the larval feeding protocol. Gut transit time in rotifers is relatively fast and consequently the time the rotifers spend in the rearing tank before being eaten will affect the final quality as a prey (Olsen et al., 1989; Yamamoto et al., 2009). Furthermore, the survival and normal development of larvae depends not only on the adequate nutrients but also on the ingestion of the sufficient calories. Therefore, the nutritional value of rotifers depends on their biochemical composition as well as on their dry mass and caloric content (Lubzens et al., 1989).

The objective of this study is to assess the nutritional quality in terms of mass and caloric content of rotifers during the common practices of feeding larvae in the hatcheries. With this aim, we have firstly determined the gut filling pattern of rotifers

fed with different microalgal cell concentrations as well as the evacuation pattern in clean and green seawater, and secondly we have compared the dry weight and carbon and nitrogen content of overfed and starved rotifers.

2. Material and methods

Brachionus plicatilis sensu stricto, strain S-1 (Yúfera, 1982; Dooms et al., 2007) was cultured in 1L flasks at a temperature of 20 °C and a salinity of 33 ppt with gentle aeration. The microalgae *Nannochloropsis gaditana* was provided as food in all experiments.

Gut filling pattern

To determine the gut filling pattern the rotifers were first maintained under starvation conditions during 24 hours to empty the guts. The rotifers were fed with algae at six different concentrations, 0.4×10^6 , 1.2×10^6 , 2.6×10^6 , 5.2×10^6 , 10.4×10^6 and 20.8×10^6 cell ml⁻¹. The density of the rotifers was low enough to avoid that the ingestion affected the microalgal cell concentration during the experimental time. Rotifers were sampled in the six treatments at different times for a period of 240 minutes and fixed in formalin (4% v/v). Then, the body and gut volume were measured as described below, and to avoid the variability associated with body size the gut volume was presented as percentage of the total female body volume occupied by the gut (given each value as a mean of 6 to 10 individuals). The increase of gut volume was best explained by fitting an asymptotic function given by the equation:

$$V = a(1 - e^{-bT})$$

Excepting for the treatment with 0.4×10^6 cell ml⁻¹, in which the best fit was obtained with a sigmoid equation of the form:

$$V = a/(1+e^{(c-T)/b})$$

where V is the gut volume (% body volume), T is time (min), a is the asymptote, and b and c are regression parameter.

Gut evacuation pattern

To determine the evacuation rate, rotifers were first overfed with microalgae at high concentration (20.8×10^6 cell ml^{-1}) for 180 min. Then, microalgal cells were removed by filtration through a $71\mu\text{m}$ mesh sieve using two different procedures. In the first method, the rotifer culture (rotifers + medium) was poured over the filter and the collected rotifers were rinsed and transferred to a new flask with clean seawater. In the second one, clean seawater was poured over the rotifer culture placed in a container provided with a filter in order to maintain the culture volume level during the washing process. Rotifers were sampled at different times for a period of 180 min and fixed in formalin. The first sample was taken just before the filtration (considered as time 0). The gut evacuation pattern was characterized as the decrease with time of the percentage of gut volume in relation to the body volume. The best fit was given by the following exponential equation:

$$V = ae^{-bT} + ce^{-dT}$$

where V is the gut volume (% body volume), T is time (min) and a , b , c and d are the regression parameters.

Gut evacuation pattern was also determined under conditions that simulate green water rearing techniques by transferring the rotifers in a flask with 0.4×10^6 cell ml^{-1} of microalgae after filtering and rinsing the culture water.

Morphometric analyses

Determination of body and gut volume were carried out by image analysis. Between 6 and 10 rotifers of each sample were photographed under a microscope. The pictures were analyzed using the free software UTHSCSA ImageTool (University of Texas Health Science Center, San Antonio, TX, and available at <http://ddsdx.uthscsa.edu/>). Body width and length and the gut area were measured in each individual. The microalgae within the gut are easily distinguished. The females gut increases its volume progressively as the rotifer starts ingesting microalgae (Fig. 1). For the gut measurement, the criterion was to consider just the green-coloured area of the image. This area could be composed of either 1 or 2 spots, corresponding to the so-called

stomach and the intestine cavities (Kleinow et al., 1991). Rotifers after starvation may exhibit a nominal gut lumen volume of $0 \mu\text{m}^3$, this is due to the absence of microalgae, i.e. there is no coloured part in the image, as shown in figure 1A.

Rotifers body volume was calculated according to an ellipsoid of revolution. For each female, the width and length (μm) were obtained measuring the lorica without spines (Yúfera, 1982). Assuming the cross section in this *Brachionus* strain is circular; depth and width are equal, and the volume was calculated as follows:

$$\text{Rotifer volume } (\mu\text{m}^3) = 4/3\pi ab^2$$

where a and b are one-half of the major and minor axes (length and width) respectively. The gut cavities were considered to have a spherical shape and the volume was calculated from the measure of the area. For the calculation of the volume, the following formula was used:

$$\text{Gut volume } (\mu\text{m}^3) = 4/3(A_1\sqrt{(A_1/\pi)}) + 4/3(A_2\sqrt{(A_2/\pi)})$$

where A_1 and A_2 correspond to the area (μm^2) of the two gut cavities measured in the pictures.

Finally, as explained above since the gut volume depends widely on the rotifer size, the results are given as percentage of the whole rotifer body occupied by the gut.

Dry mass and energy content

Dry weight was determined in rotifers with their guts full of microalgae (fed 120 min with $20.8 \times 10^6 \text{ cell ml}^{-1}$), after 120 min of evacuation without microalgae and after 24 hours of starvation. Dry weight was determined by drying triplicate samples of 900-1000 individuals at 60°C . The samples were obtained from cultures gently filtered and rinsed with distilled water in order to remove saltwater and microalgae, and placed in pre-weighed glass covers. The egg/female ratio was quantified due to its positive correlation to dry weight (Yúfera and Pascual, 1989). Carbon and nitrogen content were analyzed in rotifers with their guts completely full and after 24 hours of starvation. Three 1 mg subsamples per treatment were analysed using an elemental CNHS analyser

(Thermoquest, mod. Flash 1112), using sulphanilamide as standard. Energy content was estimated using the factor of 43 J per mg of carbon (Yúfera et al., 1997).

Statistical analysis

In order to identify significant differences of rotifer gut fullness among the six treatments, the maximum experimental mean reached in a specific time of each treatment were compared. The maximum percentages of gut volume were contrasted using one-way ANOVA followed by a post-hoc Tukey's test to identify which groups were significantly different.

3. Results

The body volume of the rotifers ranged between 0.73 and $4.21 \times 10^6 \mu\text{m}^3$, the volume average was $2.05 \times 10^6 \pm 0.68 \times 10^6 \mu\text{m}^3$ (Figure 2). The maximum gut volume average obtained from rotifers with the gut completely full of microalgae, once the asymptote was attained, was $0.23 \times 10^6 \pm 0.06 \times 10^6 \mu\text{m}^3$, although gut volumes up to $0.35 \times 10^6 \mu\text{m}^3$ were observed.

The fullness of the gut during the enrichment experiment followed a saturation curve ($r > 0.963$; $P < 0.006$ in all cases), achieving its asymptote after approximately 2 hours (Figure 3; table 1). However, for the lowest cell concentration ($0.4 \times 10^6 \text{ cell ml}^{-1}$) the best fit was obtained with a sigmoid curve ($r = 0.998$; $P < 0.0001$) (Figure 3; table 1).

We found that once the gut was completely full of microalgae it occupied between 13.5-15.0% of the female volume (Table 2). In all cases the maximum gut volume occurred between 120 and 180 min after the microalgae supply, and then declined slightly at 240 min. A significant difference in the maximum gut volume was observed between concentrations higher than $5.2 \times 10^6 \text{ cell ml}^{-1}$ and concentrations of 0.4×10^6 and $1.2 \times 10^6 \text{ cell ml}^{-1}$ (Table 2).

Once the microalgae had been removed from the culture medium, the gut volume decreased quickly. In the case of rinsing directly over a filter, a sharp drop was noticed during the first few minutes from 13.5-15.0 to 6.0-7.0% of the body volume. Afterwards, the gut maintained this volume during the following two hours after

filtration, but after 24 hours became completely empty. In the case of adding a microalgae concentration of 0.4×10^6 cell ml^{-1} to the rotifer culture once it has been filtrated, no significant differences in the emptiness process were found. Contrarily, when the filtration was carried out as gently as possible maintaining the rotifers submerged during the process, the gut volume decreased progressively at a lower rate (Figure 4).

The importance of this short-term enrichment was evaluated in terms of weight and energy content as well (Table 3). Dry weight was obtained in a population with egg/female ratio of 0.39 that after 24 hours of starvation declined to 0.17 egg/female. The rotifers experienced a dry weight decreased of 128 ng (19.71% of body weight) after 2 hours of starvation, and of 254 ng (38.22% of body weight) after 24 hours of starvation. Both carbon and nitrogen content decreased during 24 hours of starvation but this drop was more moderate for nitrogen and consequently C/N ratio decreased from 4.96 to 4.72. The energy content of the rotifer biomass declined from 19.78 to 18.39 $\text{J} \cdot \text{mg}^{-1} \text{DW}$ representing a loss of 42.65 % in each individual (Table 3).

4. Discussion

Body size distribution of a population of *Brachionus* spp is affected by different environmental factors such as feeding conditions and temperature, but it is mainly defined by the lineage and strain (Yúfera 1982; Snell and Carrillo 1984; Yúfera, 2001; Kostopoulou et al., 2009). In this study the morphometric measurements and the extrapolated body volume of the *B. plicatilis* strain S-1 agree with previous measurements carried out since this strain was isolated in late seventies (Yúfera 1982; Yúfera et al., 1989).

In addition to the effect of the body size which is related to the age of individuals (Kostopoulou et al., 2009), the gut volume varied widely depending on its microalgae content, that is, on the time passed since the start of re-feeding and on the time spent without microalgae in the water after the enrichment process.

The filling process occurred relatively fast. In our *B. plicatilis* strain, the gut was almost

full in nearly an hour from the start of the enrichment, reaching the 80% of the total gut volume, and in two hours the rotifers exhibited the gut completely full. In our study the gut volume when is full reaches values up to 230 pL (15% of the rotifer body volume), which is a notable part of the prey unit ingested by the fish larvae. Kleinow et al. (1991) estimated the gut volume of other *B. plicatilis* strain (200 μm lorica length) in 60-120 pL. Baer et al. (2008) working with the lineage *Brachionus* “Cayman” (168 μm lorica length) fed on latex beads found much lower values (18 pL; 1.18% of body volume). Besides the different methodology and environmental conditions, this difference can be due to a lower ingestion of the inert beads compared to microalgae as well as to an underestimation of the ingested volume because the space between the rigid beads was not taken into account as stated by the authors.

Interestingly, the gut volume after the enrichment depends on the microalgal concentration. The maximum gut fullness was achieved above a concentration threshold of $5.2 \times 10^6 \text{ cell ml}^{-1}$. There was no significant variation in the gut fullness for the higher tested food concentrations. Alternatively, concentrations lower than $5.2 \times 10^6 \text{ cell ml}^{-1}$ resulted in significant lower gut fullness, and therefore the rotifers would not contribute to the larvae diet with all their potential caloric content. In this study the rotifer density was low enough to prevent variation in the cells concentration, but if the enrichment is performed at high rotifer densities the cell concentration may fall below this threshold. In fact, these results reflect to some extent the ingestion behaviour observed for this species. The Ingestion rate of *B. plicatilis* as other zooplankters depends on the algal concentration following a saturation pattern (Yúfera and Pascual, 1985; Montagnes et al., 2001; Hotos, 2003; Yúfera, 2007).

The evacuation of the gut content is also quick and follows an exponential decay curve. It has been reported that the concentration and harvest of rotifers should be performed under water; otherwise the animals could be damaged (Dhert, 1996). We found as well that the rotifer culture filtration generates a sudden decrease of the gut volume, however when the microalgae removal is carried out in a gently way, with submerged filters, the gut volume decrease occurs progressively. Therefore, the protocol followed before rotifers are provided to the larvae deeply influences their quality as live feed.

The gut evacuation curve is not significantly different from that obtained when 0.4×10^6

cell ml⁻¹ of *Nannochloropsis* is added to the culture. The use of the green water has been proven to have a positive correlation with growth and survival of the fish larvae when added along with the rotifers to the tanks (Reitan et al., 1993). However, according to our results, it doesn't contribute much to gut volume, at least during the first two hours, and at so low concentration as tested in the present experiment that simulates the concentration in rearing tanks. In fact, the gut volume after 15 min of evacuation is the same as the maximum obtained when we fed the rotifers with 0.4 x 10⁶ cell ml⁻¹ and therefore it is not possible to detect any effect.

We found that the time necessary for an optimal short-term enrichment ranges between 120 and 180 minutes. A longer enrichment period, as shown in figure 3, leads to a decrease in gut volume probably due to a rebound effect. Baer et al. (2008) also found the same phenomenon, that may be the consequence of the start of evacuation after a given gut residence time.

The dry weigh of a single individual in this strain of *B. plicatilis* ranges approximately between 200 and 900 ng depending on temperature and fecundity status (Yúfera, 2007). Average values in mass culture and holding 0.15-0.60 egg/female ranges between 400 and 700 ng that represents a reasonable figure of the type of prey item supplied to the fish larvae. We found that the gut content after the post-harvesting refeeding may account for the 38% of the mass of a single female. Besides, we found that 20% of the rotifer weight is lost in two hours in the rearing tanks or even in only few minutes when the rotifers are transferred to the tanks without enough care. Furthermore, a prolonged starvation, as can be the case of uneaten rotifers spending the night in the rearing tanks, may lead to a loss of 43% of the total calories ingested by fish larvae when they eat a rotifer.

Under routine conditions a single individual of this strain ingests around 350 cell min⁻¹ of *N. gaditana* when fed at a concentration of 20 x 10⁶ cell min⁻¹ (Yúfera, 2007). This means, taking the average dry weight of a single female in a population growing at 20°C and 0.40 egg/female to be 627 ng (Yúfera et al., 1997) and the average dry weight of a single cell of *N. gaditana* to be 3.3 pg (Yúfera and Pascual, 1985), that each rotifer would ingest approximately 137 ng, that is, 22.11% its own body weight in algal food in

the two hours required to fill completely the gut (assuming that this moment is just before the evacuation starts). These calculations are close to the results found here.

This experiment has been done with the microalgae *N. gaditana* which is commonly used in larviculture. Some changes in the demographic parameters and in the ingestion and evacuation pattern may occur when fed on other fresh microalgae species/strains (Yúfera et al., 1983; Sayegh et al., 2007) or commercial diets and enriching formula (Dhert et al., 2001; Naz, 2008, Kotani et al., 2009) also usual in the rotifers culture and enrichment. Likewise, some variations may occur in other *Brachionus* strains with different body size (Yúfera and Pascual, 1985; Hotos, 2003) or changing the environmental conditions (Lebedeva and Orlenko, 1995; Montagnes et al., 2001; Ferreira et al., 2009). In spite to these expected variations, the present study confirm the importance of maintaining the gut content as a relevant contribution of the nutritional quality of the rotifers offered as food for fish and crustacean larvae.

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Figure captions

Fig. 1. Examples of images taken of *Brachionus plicatilis* used for the experiments. The gut content is outlined in yellow. A) After 24 hours of starvation. B) After 15 minutes feeding on 20.8×10^6 cell ml^{-1} of microalgae. C) After 30 minutes feeding on 20.8×10^6 cell ml^{-1} of microalgae.

Fig. 2. Frequency histogram of the body volume (μm^3) of all the rotifers used in this study.

Fig. 3. Gut filling pattern in *Brachionus plicatilis* fed on six different microalgae concentrations. All values are means, $n=6-10$.

Fig. 4. Gut evacuation pattern in *Brachionus plicatilis*. The red line was obtained after

rinsing the rotifer culture in a submerged filter causing the least stress as possible. The green and blue lines correspond to a drastic microalgae removal protocol. The blue one was obtained after placing the rotifers in clean seawater. The green one corresponds to the evacuation in a culture with 0.4×10^6 cells·ml⁻¹. Mean $\pm$ S.D.

Table 1. Regression parameters of the curves describing the filling and evacuation patterns.

<i>Filling</i>					
Algae concentration (cell ml ⁻¹)	a	b	c		R
0.4 x 10 ⁶	6.1057	13.0356	64.3676		0.9976
1.2 x 10 ⁶	6.9127	0.0467			0.9634
2.6 x 10 ⁶	9.2225	0.0478			0.9710
5.2 x 10 ⁶	13.7317	0.0358			0.9756
10.4 x 10 ⁶	12.8481	0.0472			0.9733
20.8 x 10 ⁶	12.8627	0.0523			0.9722
<i>Evacuation</i>					
Filtering procedure	a	b	c	d	R
Submerged in clean water	3.6301	0.6073	11.0027	0.0098	0.9864
Non-submerged	6.9995	0.2220	5.4759	1.4447·10 ⁻¹¹	0.9356
Non-submerged + microalgae	7.5058	3.1430	6.1711	0.0017	0.9083

Table 2. Maximum volume (percentage of body volume) occupied by the gut of *B. plicatilis* at different cell concentrations. Letters indicate significant differences within treatments. Values expressed as mean $\pm$ S.D. Tukey's test ($F_{(5,35)}$, $p < 0,0001$).

Cell conc. (cell.ml ⁻¹)	0.4·10 ⁶	1.2·10 ⁶	2.6·10 ⁶	5.2·10 ⁶	10.4·10 ⁶	20.8·10 ⁶
Maximum volume (%)	6.44 $\pm$ 1.59 a	7.25 $\pm$ 4.42 a	11.55 $\pm$ 3.70 ab	14.97 $\pm$ 3.48 c	13.58 $\pm$ 1.67 bc	14.28 $\pm$ 2.87 bc

Table 3. Dry weight (DW), carbon content (%C), nitrogen content (%N), C:N ratio per female and energy content ($\text{J}\cdot\text{mg}^{-1}\text{DW}$ and mJ ind^{-1}) as well as percentage of dry mass and energy lost under starvation in females with the gut completely full of microalgae, after 2 hours of starvation and after 24 hours of starvation. Egg:female ratio is also included for comparison. Values are expressed as mean $\pm$ S.D.

	Gut full	Starved for 2 h	Starved for 24 h
DW (ng)	663.3 ± 109.5	532.5 ± 15.2	409.7 ± 82.5
Egg/female	0.39	0.43	0.17
%C	46.01 ± 0.13	-	42.78 ± 0.96
%N	9.27 ± 0.04	-	9.07 ± 0.17
C/N	4.96 ± 0.02	-	4.72 ± 0.08
$\text{J}\cdot\text{mg}^{-1}\text{DW}$	19.78 ± 0.06	-	18.39 ± 0.42
mJ ind^{-1}	13.12 ± 1.18	-	7.52 ± 0.48
lost DW (%)	-	19.71	38.22
lost energy (%)	-	-	42.65

Figure 1

Figure 2

Figure 3

Figure 4