How to increase productivity of the copepod *Acartia tonsa* (Dana): effects of population density and food concentration

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Abstract

In this study, we analysed the effect of population density and food concentration on the fecundity of a Mediterranean strain of *Acartia tonsa* to maximize egg production. During 4-day feeding experiments, egg hatching success and faecal pellet production were also followed. The algae *Rhinomonas reticulata* was supplied at different concentrations corresponding to 250, 500, 1000, 1500, 2000 and 3000 μg C L^{-1} day^{-1} at the following adult copepod density: 40, 80 and 160 ind. L^{-1}. Our results show a positive relationship between algal concentration and egg production under all experimental conditions confirming that the quantity of food strongly limits *A. tonsa* fecundity. Maximum egg production (57 eggs per female) was reached at the lowest density and at the maximum food concentration. Percentage of egg hatching success was not dependent on the quantity of food used. At the same food concentration, an increase in population density from 40 to 80 ind. L^{-1} induced an increase in faecal pellet production per couple which did not correspond to an increase in egg production, suggesting that higher energetic costs were shifted to swimming activity. Productivity of the *A. tonsa* Mediterranean strain is mainly limited by the quantity of food rather than by crowding conditions.

Keywords: zooplankton rearing, egg production, hatching success, algal concentration

Introduction

In the last decade, studies on copepod cultivation have increased rapidly due to the growing interest on the use of these organisms in aquaculture as alternative, live food for fish larvae (see Drillet, Frouël, Sichlau, Jepsen, Højgaard, Joarder & Hansen 2011 for a review, Buttino, Ianora, Buono, Vitiello, Malzone, Rico, Langellotti, Sansone, Gennari & Miralto 2012). In fact, evidence that copepods supply high nutritional value to larval fish growth, compared to other traditional diets is, at present, confirmed by many studies (Conceição, Van Der Meeren, Verreth, Evjen, Houlihan & Fyhn 1997; Drillet, Jørgensen, Sørensen, Ramløv & Hansen 2006; Olivotto, Buttino, Borroni, Malzone & Carnevali 2008; Olivotto, Capriotti, Buttino, Avella, Vitiello, Maradonna & Carnevali 2008; Van der Meeren, Olsen, Hamre & Fyhn 2008). There has also been a concomitant rise in the number of studies regarding the biology and reproductive physiology of some marine and estuarine world-wild distributed copepod species (Støttrup, Richardson, Kirkegaard & Pihl 1986; Buttino *et al.* 2012; Carotenuto, Esposito, Pisano, Lauritano & Perna 2012; Zhang, Wu, Pellegrini, Romano, Esposito, Ianora & Buttino 2013). As a result, copepods belonging to harpacticoids and calanoids have been highly recommended as live food for fish larvae of commercial interest (Støttrup 2006; Drillet *et al.* 2011) and specific rearing techniques

have been developed to culture large numbers of individuals as required by intensive aquaculture farms (Payne & Rippingale 2001; Payne, Rippingale & Cleary 2001; Buttino et al. 2012). In tropical areas, extensive cultivation of calanoid copepods can result in very high densities (Lee, O'Bryen & Marcus 2005); however, in temperate areas massive cultivation remains a major obstacle due to the longer life stage development and the lower productivity of temperate species with respect to tropical copepod species (Buttino et al. 2012; Carotenuto et al. 2012). In particular, calanoids from temperate regions can suffer from overcrowding rearing conditions, in terms of egg production (Miralto, Ianora, Poulet, Romano & Laabir 1996; Medina & Barata 2004; Peck & Holste 2006; Carotenuto et al. 2012), which can compromise their massive cultivation for aquaculture purposes. Protocols on how to maximize copepod production are necessary to improve copepod cultivation at a large scale.

In this article, we analyse the effect of crowding and food concentration on the fecundity of the calanoid copepod *Acartia tonsa*. This species has largely been studied for aquaculture purposes (Støttrup 2006; Drillet, Goetze, Jepsen, Højgaard & Hansen 2008; Drillet, Jepsen, Højgaard, Jørgensen & Hansen 2008; Drillet et al. 2011) or for ecotoxicological applications (Bielmyer, Grosell & Brix 2006; Gorbi, Invidia, Savorelli, Faraponova, Giacco, Cigar, Buttino, Leoni, Prato, Lacchetti & Sei 2012; Jarvis, Miller, Lenihan & Bielmyer 2013). Moreover, the effect of food quality and quantity on *A. tonsa* reproduction is well documented (Støttrup & Jensen 1990; Ismar, Hansen & Sommer 2008; Zhang et al. 2013), but to our knowledge few data are available on the effects due to overcrowding (Medina & Barata 2004; Jepsen, Andersen, Holm, Jørgensen, Højgaard & Hansen 2007). Here, we report on how crowding and different food concentrations can influence egg production rate, hatching success and faecal pellet production in *A. tonsa*, in a small-scale basis incubation, to define the best rearing conditions for this species to maximize productivity.

Materials and methods

Algal culture

The cryptophyta *Rhinomonas reticulata* (CCAP 995/2; from the culture collection of the Scottish Association for Marine Science SAMS, Obam, UK) was used as food for the copepod *Acartia tonsa* in addition to *Isochrysis galbana* (CCMP 1323; from the culture collection of the Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow, AR, USA) and *Rhodomonas baltica* (CCAP 979/9; from the culture collection of the Scottish Association for Marine Science SAMS). At the ISPRA laboratory in Livorno (Italy), *R. reticulata* was cultured in a temperature-controlled room at 20°C, using 500 mL flasks filled with 0.22 μm filtered seawater (FSW) enriched with f/2 medium (Guillard 1975) at 30 g L^{-1} salinity, on a 14 h light:10 h dark cycle (5000 lux). Filtered seawater for medium was previously treated with HClO (0.04% v:v) for 24 h and then with sodium thiosulphate 12.5% (v:v) for further 24 h. Sea water was aerated 24 h (Lavens & Sorgeloos 1996) to remove chloride residues.

Carbon contents of the algae were converted from cell volumes according to the formula reported by Strathmann (1967).

Copepod culture

Acartia tonsa has been reared through multiple generations for the last 5 years at the ISPRA laboratory in Livorno (Italy). Adults are reared in a 50 L aquarium containing 20 L of 0.22 μm FSW at 30 g L^{-1} salinity and are fed mixture of the algae *I. galbana*, *R. reticulata* and *R. baltica* at a final concentration >300 μg C L^{-1} per day. The aquarium is maintained at 20°C with a 14 h L:10 D photoperiod.

Incubation experiments

Healthy mature females and males, with intact appendages, no visible defects and that were actively swimming, were randomly sorted from the aquarium and isolated for the tests as follows: 1, 2 and 4 couples, corresponding to 40, 80 and 160 ind. L^{-1}, were distributed in crystallizers containing 50 mL 0.22 μm mesh net FSW. Age of the adults was not known. Copepods were not acclimated before starting the different incubation protocols and eggs produced on the first day corresponded to copepod fecundity in the aquarium.

Eight crystallizers and four replicates were set up for each treatment. The algae *R. reticulata* was supplied during the exponential growth phase, at

the following final concentrations: 0.45×10^4; 0.9×10^4; 1.8×10^4; 2.7×10^4; 3.6×10^4 and 5.4×10^4 cells mL^{-1} corresponding to 250, 500, 1000, 1500, 2000 and 3000 µg C L^{-1} day^{-1} respectively. After 24 h, animals were transferred to new crystallizers containing fresh media and algae, and eggs and faecal pellets (fp) were counted under an inverted Olympus microscope. Egg hatching success was determined 48 h later by adding 0.2 mL Lugol solution and counting nauplii settled on the bottom of crystallizers. The experiment lasted 4 days to test the effects of food concentration and adult stocking density on the reproduction of the copepod *A. tonsa*.

Data analysis

Egg production per female, fp per couple and the percentage of egg hatching success were compared using student's *t*-test and One-way ANOVA to analyse any significant differences among treatments. When significant differences ($P < 0.05$) were found, a Tukey's multiple comparison test was used to determine specific differences among treatments. All statistical analyses were conducted using the GraphPad Prism Program. Data are presented in the graphs as mean ± standard error (SE).

Results

Egg production

At the lowest food concentration of 250 µg C L^{-1}, mean egg production rate was <10 eggs per female for all 4 days, independent of the number of couples incubated (Fig. 1a). On average, the number of eggs produced ranged from 5.83 to 5.94 eggs female^{-1}, for two- and four-couple incubation experiments, with values similar to those obtained in one-couple incubation experiments (7.67 eggs female^{-1}).

When the food concentration was doubled, mean egg production rate, calculated over 4 days, doubled in one-couple incubation experiments, from 7.6 to 16.3 eggs female^{-1} (Fig. 1b). A similar increase was observed in experiments with two couples. In experiments with four couples, egg production increased 1.5-fold, on average from 5.9 to 8.8 eggs female^{-1}, and this value was statistically lower than those recorded in one-couple incubation experiments.

A fourfold increase in algal concentration (1000 µg C L^{-1}) induced almost a fourfold increment in mean egg production rates in one-couple and two-couple incubation experiments (Fig. 1c). In four-couple experiments, egg production doubled with respect to the lower food concentration and remained statistically lower than values calculated for one-couple experiments.

At 1500 µg C L^{-1}, egg production was similar to values obtained with 1000 µg C L^{-1} for all three groups (Fig. 1d) with a mean production of 30.7 and 19.5 eggs female^{-1} for one- and two-couple experiments. Fecundity of four-couple remained statistically lower than those recorded in one-couple experiments, with a mean 10 eggs female^{-1} calculated over 4 days.

An increase in algal concentrations to 2000 and 3000 µg C L^{-1} induced the highest egg production in one-couple experiments with 50.6 eggs female^{-1}, and 57.3 eggs female^{-1} respectively, calculated after 4 days of feeding (Fig. 1e and f). In two-couple experiments, a maximum of 43.7 eggs female^{-1} was recorded on day 4 with 3000 µg C L^{-1}.

In four-couple experiments fed 2000 µg C L^{-1}, the number of eggs produced was only 15 eggs female^{-1} after 4 days and with a mean value that remained significantly lower than that recorded in one-couple experiments. Only at the highest food concentration (3000 µg C L^{-1}) did egg production increase more than 3.5 times in four-couple incubation experiments, with respect to the lowest food concentration; this value was not significantly different from that recorded in one- and two-couple experiments (Fig. 1f).

Hatching success

Egg hatching success remained high (mean >85%) for all treatments with a reduced variability recorded at the highest algal concentrations (from 1500 to 3000 µg C L^{-1}). Interestingly, somewhat lower hatching success was observed in one-couple experiments at food concentration from 250 (mean 83.8% ± 6.1 SE) to 500 µg C L^{-1} (83.3% ± 6.6 SE), even if these differences were not significant. Possibly egg viability depended on male fertility and was higher when there were more males in containers.

Faecal pellet production

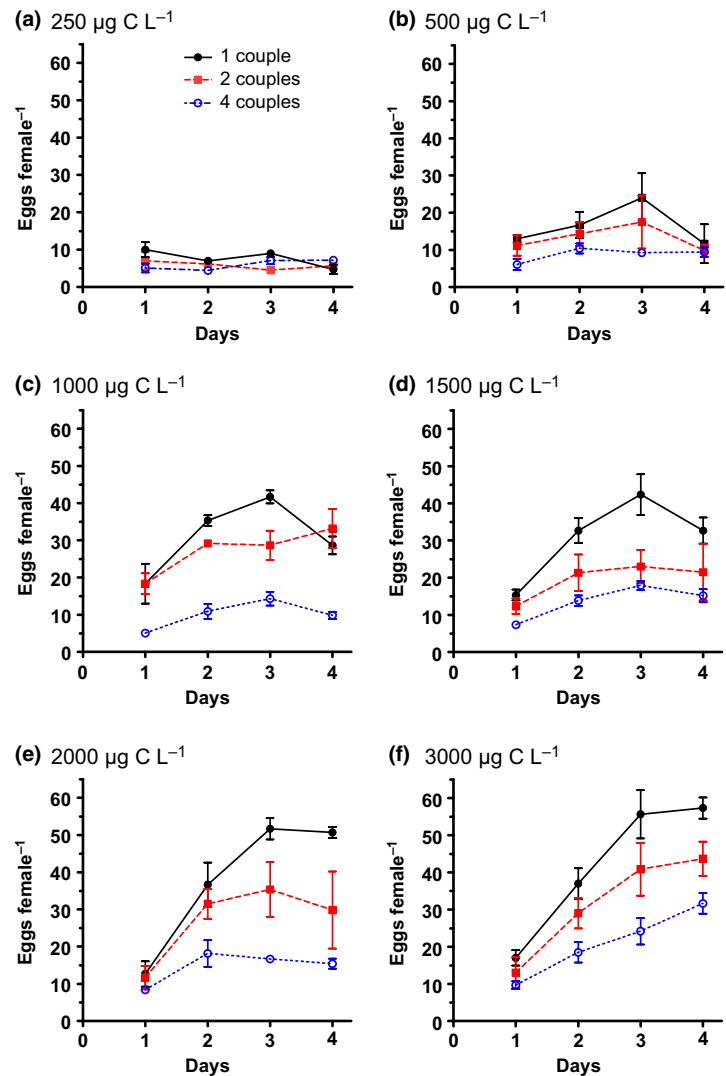At the lowest food concentration, the number of fp was almost 55 per couple on average in

Figure 1 *Acartia tonsa* egg production per female ($\pm$ SE) per day calculated at different densities and fed the algae *Rhinomonas reticulata* at 250 µg carbon L^{-1} (a); 500 µg carbon L^{-1} (b); 1000 µg carbon L^{-1} (c); 1500 µg carbon L^{-1} (d); 2000 µg carbon L^{-1} (e) and 3000 µg carbon L^{-1} (f).

one-couple experiments whereas it increased to more than 100 fp couple^{-1} in two- and four-couple experiments at the same food concentration (Fig. 2). This increment was statistically different with respect to one-couple experiments. At all other food concentrations, there was a strong increase in fp production in one-couple experiments with increasing food concentrations up to 2000 µg C L^{-1}. However, there was no evident increase in fp production in both two-couple and four-couple experiments even if food concentration increased suggesting that food is a limiting factor at these concentrations.

A synthesis of the results is shown in Figure 3 which reports the relationship between increasing egg and fp production with respect to increasing food concentrations. A direct proportional increase

in egg production with respect to food concentration is evident for both one- and two-couple incubation experiments up to 1000 µg C L^{-1} (Fig. 3a). A further increase in the food concentration did not enhance egg production proportionally. When food concentration increased 12-fold, there was only a fivefold increase in egg production. At highest adult density (four couples), egg production increased slowly and a doubling in egg production was recorded only with an eightfold food increase.

Fp produced by one couple increased almost fivefold with an eightfold food increase, and a further increase in food concentration did not induce any increase in fp production (Fig. 3b). In two-couple experiments, fp production barely increased and at 2000 and 3000 µg C L^{-1} food concentrations fp increased only twofold. At the highest
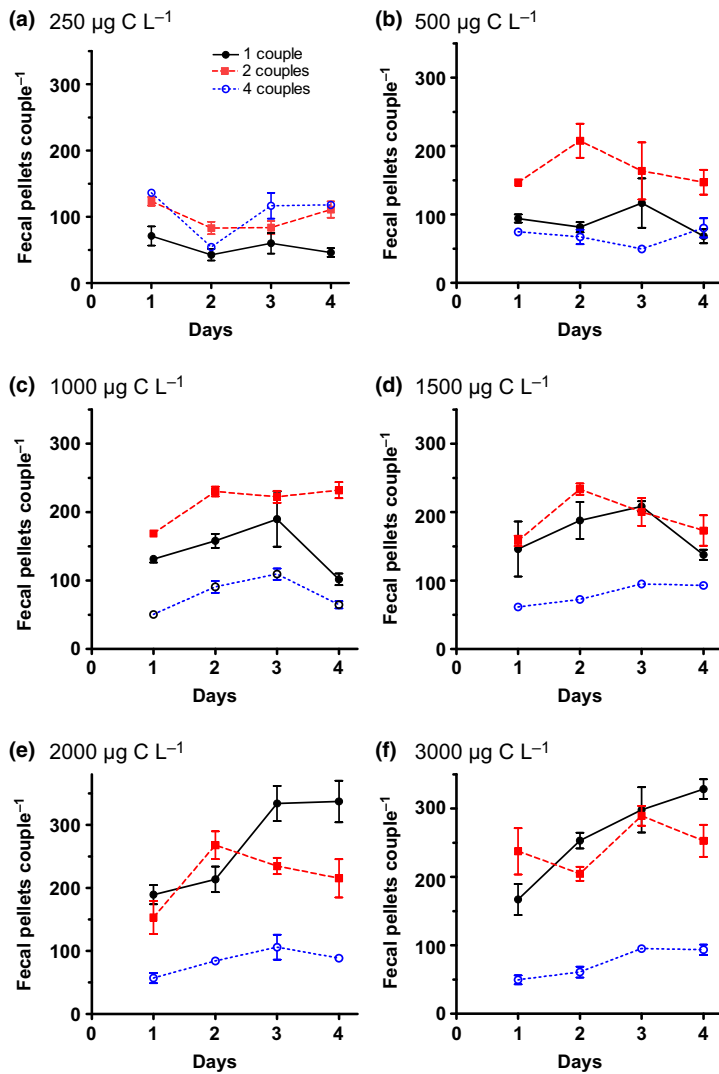
Figure 2 *Acartia tonsa* fecal pellet production per couple ($\pm$ SE) per day calculated at different densities and fed the algae Rhinomonas reticulata at 250 μg carbon L^{-1} (a); 500 μg carbon L^{-1} (b); 1000 μg carbon L^{-1} (c); 1500 μg carbon L^{-1} (d); 2000 μg carbon L^{-1} (e) and 3000 μg carbon L^{-1} (f).

adult density, fp production did not increase at any food concentration supplied, confirming that food is a limiting factor in this experimental condition.

Discussion

Fecundity of calanoid copepods has been generally shown to be directly affected by high population density due to overcrowding or competition for available food (Miralto *et al.* 1996; Peck & Holste 2006). Recently, due to the growing interest to produce copepods as live food for fish larvae, researchers have devoted much attention on how to maximize copepod production. Medina and Barata (2004) evaluated the efficiency of a static-renewal culture system for *A. tonsa* to be used in

ecotoxicology tests. The authors achieved a copepod density up to 2000 ind. L^{-1}; however, at this concentration, egg production and copepod development rates were reduced (0.4–8 eggs female^{-1}) when compared to the lower density of 500 ind. L^{-1} (3–32 eggs female^{-1} at 500 ind. L^{-1}) over a 26-day period. The authors suggested that lower production at high population density could be due to the quantity and quality of food supplied (total carbon concentration ranging from 378 to a maximum of 945 μg C L^{-1} for each algae *R. reticulata* and *I. galbana*). Our results show that at a very low algal concentration (250 μg C L^{-1}) egg production was always low and was independent of the population density (Fig. 1a). On the contrary, at increasing food concentrations, egg production increased after 2/3 days, and this is much more evident at the lowest
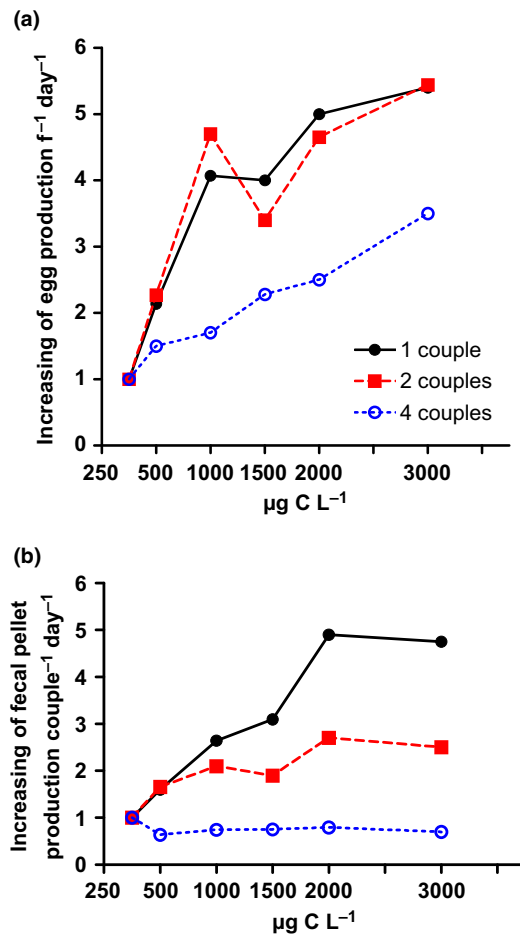
Figure 3 Relationship between food concentration (μg carbon L^{-1} *Rhinomonas reticulata*) and egg production per female per day (a) or fecal pellet production (b), calculated for different numbers of couples.

population density condition. This could be due to food limitation in the main culture tank, where, in fact, food was supplied to a concentration >300 μg C L^{-1}. Our results indicated that this food concentration is not sufficient to sustain high egg production (see also Zhang *et al.* 2013).

We did not acclimate copepods as we considered the first incubation day corresponding to the productivity in the main culture tank. This species rapidly adapts to changing food conditions and is very efficient in transforming ingested materials into eggs (Kiørboe, Møhlenberg & Hamburger 1985). This has allowed us to confirm that the fast increase in egg production, recorded after the second day, was due to increasing food concentrations independent from the population density (Fig. 1b–f).

We incubated copepods in small volumes and extrapolated results to a much bigger cultivation practice. At present, we do not know how other factors, such as swimming behaviour, limited mating choice or food competition affect *A. tonsa* fecundity. It is known that population densities modify copepod behaviour and consequently food uptake in other species (Kiørboe 2008; Dur, Souissi, Schmitt, Michalec, Mahjoub & Hwang 2011), as for example in *Eurytemora affinis* at a density of 160 ind. L^{-1}, independent of the volume. Miralto *et al.* (1996) found a depressed fecundity in *Centropages typicus* at densities as high as 1000 ind. mL^{-1} and attributed this to physical stress due to increasing number of collisions among individuals. This could be further investigated for *A. tonsa* species.

Buttino *et al.* (2012) suggested that overcrowding effects were responsible for reduced egg production rate observed when high population density was reached in a pilot experimental system for two Mediterranean copepod species *Temora stylifera* and *Centropages typicus*. Also Peck and Holste (2006) found that egg production rate diminished at high densities of *A. tonsa*. The authors fed copepods with a concentration of *Rhinomonas* spp. >50,000 cells mL^{-1} (corresponding to about 3000 μg C L^{-1}) and recorded a maximum egg production >40 eggs female^{-1} when adult stocking density was 65 ind. L^{-1}. On the contrary, Jepsen *et al.* (2007) who tested the effect of different stocking densities on mortality rate, egg production and hatching success of the same copepod *A. tonsa*, found no differences in egg production and hatching success for densities ranging between 100 and 600 ind. L^{-1}. Authors reared copepods with *Rhodomonas salina* at a carbon concentration of about 950 μg C L^{-1} and reported egg production rates from 37.3 to 22.5 eggs female^{-1} at 100 and 600 ind. L^{-1} respectively (Table 1). Genetic differences among the tested strains, one Baltic and the other Mediterranean, could possibly explain these differing results.

It is also well known that both food quality and quantity influence egg production and egg viability in *A. tonsa*; Zhang *et al.* (2013) reported that a monoalgal diet of *R. reticulata* at a concentration of 500 μg C L^{-1} supported the highest egg production rate in *A. tonsa* reared in 50-ml crystallizers (corresponding to 40 ind. L^{-1}) with a mean egg production of 24.4 eggs female^{-1} day^{-1}. *I. galbana* was considered the worst food which

Table 1 *Acartia tonsa* results of egg production and percentage of egg hatching success reported in different studies at various rearing conditions ($\pm$ SD except where specified)

Food type	Algal concentration	Adult density ind. L^{-1}	Eggs f^{-1} day^{-1}	% hatching success	References
Rhodomonas baltica	500 µg C L^{-1} (4.0 × 10^3 cells mL^{-1})	40	21.9 $\pm$ 5.9	78.2 $\pm$ 13.4	Zhang *et al.* (2013)
Rhinomonas reticulata	500 µg C L^{-1} (9.0 × 10^3 cells mL^{-1})	40	24.4 $\pm$ 7	76.0 $\pm$ 9.4	
Isochrysis galbana	500 µg C L^{-1} (3.8 × 10^4 cells mL^{-1})	40	12.8 $\pm$ 5	78.2 $\pm$ 13.4	
Rhodomonas salina	>950 µg C L^{-1} (>2.0 × 10^4 cells mL^{-1})	63.2 $\pm$ 13.1 120.8 $\pm$ 18.2	37.3 $\pm$ 13.9 31.3 $\pm$ 11.4	90.7 $\pm$ 4.7 86.7 $\pm$ 9.4	Jepsen *et al.* (2007)
Rhodomonas spp.	≥5.0 × 10^4 cells mL^{-1}	65	50.9	48.4 $\pm$ 8 (SE)	Peck and Holste (2006)
Isochrysis galbana + *Rhinomonas reticulata*	>945 µg C L^{-1} (>5.0 × 10^4 cells mL^{-1})	<500 <1000 <2000	3–32 2–23 0.4–8	>80	Medina and Barata (2004)
Rhinomonas reticulata	3000 µg C L^{-1} (5.4 × 10^4 cells mL^{-1}) 1000 µg C L^{-1} (1.8 × 10^4 cells mL^{-1})	40 40 160	41.7 $\pm$ 9.45 31 $\pm$ 4.98 10 $\pm$ 1.91	>80	Present study

induced the lowest egg production rate (<13 eggs female^{-1} day^{-1}) at the same adult density and carbon concentration.

In this study, we confirmed that *R. reticulata* is a good food for *A. tonsa*; however, a very high number of eggs (>57 eggs female^{-1} day^{-1}) was produced at our maximum food concentration tested (3000 µg C L^{-1}) and only at the lowest adult density. To our knowledge, this is the highest value reported until now under these feeding conditions. Peck and Holste (2006) recorded a similar egg production rate of 50.9 eggs female^{-1} day^{-1} for the Baltic population of *A. tonsa* reared at a nominal adult density of 65 ind. L^{-1}. On average, our results are also similar to those from Jepsen *et al.* (2007) at the same food concentration, but only for one-couple experiments (corresponding to 40 ind. L^{-1}) with a mean production of 31 eggs. However, at the highest density, corresponding to 160 ind. L^{-1}, mean egg production rate was significantly lower (10 eggs female^{-1} on average). At the concentrations used in our experiments a positive relationship between algal concentration and egg production is evident in all experiments confirming that the quantity of food strongly limits *A. tonsa* fecundity.

Similar to our results, in all of the cited studies percentage of egg hatching success was always higher than 85%, independent of stocking density, except for the results obtained by Peck and Holste (2006) which reported value <50% (Table 1). Higher variability in egg hatching success was

found in our experiments at the lowest food concentrations suggesting that this could be due to a food limiting effect. It is well known that *A. tonsa* produces three different type of eggs, which differ in the hatching times (Madhupratap, Nehring & Lenz 1996; Marcus 1996): subitaneous eggs, which hatch in 24–48 h, quiescent eggs that may forego hatching in unfavourable conditions and hatch when environmental conditions are restored, and resting or diapauses eggs which have an obligatory refractory phase that may span from a few months to several years. In our experiments, *A. tonsa* Mediterranean strain seems to produce only subitaneous eggs under these rearing conditions.

An interesting finding that has emerged from this study is that at the same food concentration, an increase in population density from 40 to 80 ind. L^{-1} induces an increase in fp production per couple. Similar effects have been reported for the harpacticoid copepod *Tisbe holothuriae* (Gaudy & Guerin 1982; Sibly, Williams & Jones 2000). For this species, crowding is also known to reduce reproductive output and larval viability (Brand 1985). In our experiments, at increasing density (160 ind. L^{-1}), fp production remained under 84 fp couple^{-1} on average, at food concentrations from 500 to 3000 µg C L^{-1}. Only at a very low food concentration (250 µg C L^{-1}) did fp increase to >106 fp couple^{-1}, probably because in this condition food, rather than space, becomes a strong limiting factor. High faecal pellet production

reduces water quality in a mass cultivation system, with consequent risks for uncontrolled growth of pathogenic microorganisms. In our study, information on fp provided an estimate of energetic input, in terms of food supplied that was converted into egg production and conditions when food represented a limiting factor for copepod reproduction (Fig. 3b). In fact, although an increase in grazing activity from 80 to 40 ind. L^{-1} was evident at the highest food concentrations, this did not lead to an evident increase in egg production, suggesting that higher energetic costs were spent in swimming activity or in somatic growth at lowest population density.

Our results show that productivity of our *A. tonsa* Mediterranean strain seems mainly limited by the quantity of food rather than by crowding conditions and that high egg production rates can be obtained by increasing food concentrations to values higher than 2000 μg C L^{-1}. However, if the collection of eggs is a priority, it is convenient to maintain lower population density than those reached in recent studies (2000 ind. L^{-1}), preferring many small tanks instead of a few larger containers, with evident advantages in terms of handling and management.

Acknowledgments

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