Aquaculture Research, 2012, **43**, 247–259 doi: 10.1111/j.1365-2109.2011.02822.x

Experimental cultivation of the Mediterranean calanoid copepods *Temora stylifera* and *Centropages typicus* in a pilot re-circulating system

Isabella Buttino[1,2], Adrianna Ianora[1], Silvia Buono[1], Valentina Vitiello[1], Maria G Malzone[1], Cira Rico[1], Antonio L Langellotti[3], Giovanni Sansone[3,4], Lorenzo Gennari[5] & Antonio Miralto[1]

[1]Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy

[2]ISPRA Istituto Superiore per la Ricerca e Protezione Ambientale, Viale Nazario Sauro, Livorno, Italy

[3]Centro Interdipartimentale di Ricerche per la Gestione delle Risorse Idrobiologiche e l'acquacoltura, CRIAcq

[4]Dipartimento delle Scienze Biologiche, Università degli Studi di Napoli 'Federico II', Naples, Italy

[5]Aquacullture consultant, Innovaqua Srl, Reggio Emilia, Italy

Correspondence: I Buttino, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy.
E-mail: isabella.buttino@isprambiente.it

Abstract

A pilot re-circulating system was used for the cultivation of two Mediterranean calanoid copepods: *Temora stylifera* and *Centropages typicus*. The system automatically concentrated the naupliar and copepodite stages. *Temora stylifera* was fed the flagellate *Rhodomonas baltica* or *Prorocentrum minimum*, whereas *C. typicus* was fed with a mixture of *R. baltica* or *P. minimum* and *Tetraselmis suecica*. Both copepods also received *Isochrysis galbana*. After 21 days, the *T. stylifera* population increased 26-fold, reaching a density of 38 000 individuals, mostly represented by nauplii (88%). The maximum density recorded was 380 ind. L^{-1}, with a production of 370 nauplii L^{-1}. On average, the egg hatching success for this copepod during the rearing period was 54%, with the highest viability in April and May (>75%). The *C. typicus* population increased more than 10-fold after 7 weeks of rearing, reaching a density of 123 000 individuals, mainly represented by nauplii (>90%). The highest naupliar production was 100 ind. L^{-1}, with a mean egg hatching success of 68%. This system may be useful to produce nauplii and copepodite stages to be used as live, alternative or complementary food for fish larvae or to provide a ready source of organisms for physiological and bioassay studies.

Keywords: copepod production, continuous rearing system, first feeding fish larvae, live diet

Introduction

Copepods represent a natural food source for marine fish larvae and, at present, both tropical and temperate species are used in aquaculture and are proposed worldwide as alternative live food for *Artemia* nauplii and/or rotifers (see Lee, O'Bryen & Marcus 2005 for a review). There are many recognized advantages in using copepods as live food for fish larvae; one of the most important is their high nutritional quality compared with commonly used *Artemia* nauplii and rotifers, in terms of protein content (44–52%), and amino acid and fatty acid composition (Båmstedt 1986; Lavens & Sorgeloos 1996; Drillet, Jepsen, Højgaard, Jørgensen & Hansen 2006). Marine copepods are naturally enriched in long-chain highly unsaturated fatty acids (HUFA), of the (n-3) series, such as eicosapentaenoic acid [20:5(n-3)] and docosahexaenoic acid [22:6(n-3)], considered to be essential for early development and rapid growth of fish, and can be further enriched with these HUFAs by manipulating their phytoplankton diet (Shields, Bell, Luizi, Gara, Bromage & Sargent 1999). Copepods are also rich in carotenoid pigments, including mono- and diesters of astaxanthin as well as unesterified astaxanthin compared with *Artemia*, which contains lower quantities of the related carotenoid canthaxanthin present only in the unesterified form (Krinsky 1965). Although both canthaxanthin and astaxanthin can be converted to

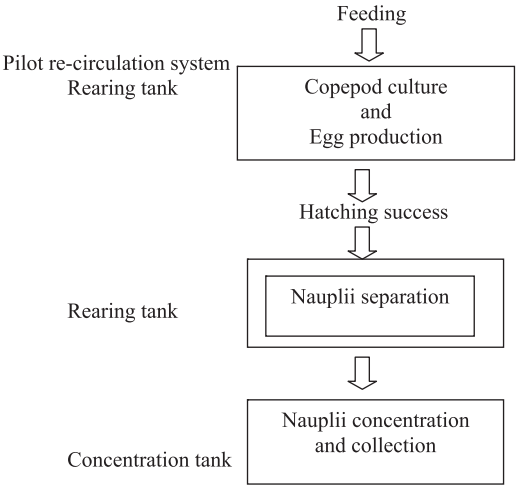
vitamin A in fish (Olson 1989), the higher quantity of total carotenoids, coupled with the apparent increased digestibility of copepods in early-developing larvae (Pederson 1984), may allow more efficient uptake and metabolism of these vitamin A precursors in fish fed copepods compared with those fed *Artemia*. Moreover, copepods contain higher levels of digestive enzymes and the early stages of many marine fish larvae, which do not have a well-developed digestive system, may benefit from the exogenous supply of enzymes from such live food organisms (Munilla-Moran, Stark & Barbour 1990; McEvoy, Naess, Bell & Lie 1998). A further advantage in the use of copepods as live fish food is the diversified sizes of larval stages, which constitute optimal prey for all larval fish developmental age groups. Moreover, the 'jerking' swimming action of most copepod nauplii and adults is believed to be an important stimulus for initiating feeding by fish larvae (Buskey 2005).

There are also negative aspects of using copepods as commercial larval fish feed, which include difficulties in rearing them at high densities (McKinnon, Duggan, Nichols, Rimmer, Semmens & Robino 2003; Lee *et al.* 2005; Støttrup 2006). Benthic harpacticoid copepods can reach densities of $40\,000\,L^{-1}$ and can be mass cultured in relatively small tanks with respect to planktonic calanoid copepods that are not easy to cultivate (see Støttrup 2006 for a review). Only recently have calanoids been successfully used as live prey to rear halibut larvae (Evjemo, Reitan & Olsen 2003), the tropical yellowtail clownfish in combination with *Artemia* and rotifers (Olivotto, Buttino, Borroni, Piccinetti, Malzone & Carnevali 2008; Olivotto, Avella, Buttino, Cutignano & Carnevali 2009), the West Australian dhufish and pink snapper larvae (Payne, Rippingale & Cleary 2001). There are several examples of subtropical extensive cultivation of copepods in ponds, in which populations can reach very high densities (see Lee *et al.* 2005), but indoor structures are much more complex to maintain, especially for temperate copepods, and remain a bottleneck for the sustainable production of copepods for commercial purposes. Zillioux (1969) was the first to propose a continuous re-circulating system, composed of two culture tanks of 100 L each, for the cultivation of the planktonic copepods *Acartia clausi* and *Acartia tonsa* at high densities. Støttrup and colleagues developed a system for the planktonic copepod *Acartia* spp. consisting of three culture units: the basis, growth and harvest tanks. Eggs produced in the basis tank were siphoned from the bottom and transferred to the growth tank. Adults were then collected and added to the basis and harvest tanks; this last tank was activated once the fish hatchery started to operate (Støttrup, Richardsen, Kirkegaard & Pihl 1986; Støttrup 2000). A similar culture method was reported by Schipp, Bosmans and Marshall (1999), who developed a controlled hatchery for the production of *Acartia* spp. with a system that easily removed undesirable organisms. An automatic system for the intensive cultivation of the estuarine tropical calanoid *Gladioferens imparipes* has also been developed by Payne and Rippingale (2001) in water volumes from 60 to 1000 L, whereby automation reduced handling and improved efficient collection of nauplii.

In the present study, we describe a re-circulating system to rear two different Mediterranean calanoid copepods: *Temora stylifera* (Dana, 1849) and *Centropages typicus* (Kröyer, 1849). These species are common in the Tyrrhenian (Western Mediterranean) Sea, where they reach maximum densities in autumn (*T. stylifera*) and late spring–summer (*C. typicus*) (Ribera d'Alcalà, Conversano, Corato, Licandro, Mangoni, Marino, Mazzocchi, Modigh, Montresor, Nardella, Saggiomo, Sarno & Zingone 2004). In the Gulf of Naples, both species are present throughout the year, reaching maximum densities of about 2300 ind. m^{-3} and 900 ind. m^{-3} for *C. typicus* and *T. stylifera* respectively (Di Capua & Mazzocchi 2004). Both species reproduce continuously throughout the year, but attain the highest egg numbers and egg viability in December–April for *C. typicus* and August–October for *T. stylifera* (Ianora 1998). To our knowledge, neither of the two species has ever been reported to produce resting eggs or to have over-wintering stages in the Mediterranean Sea or elsewhere. Here, we verify the amenability of these Mediterranean species for massive cultivation in large volume containers in order to obtain the year-round production of live copepods as alternative or complementary food for fish larvae or to provide a steady source of organisms for bioassay studies.

Materials and methods

The following diagram schematically shows the steps required to rear *T. stylifera* and *C. typicus* copepods, with a summary of spatio-temporal operations:



Pilot re-circulating system

For the massive cultivation of the copepods *T. stylifera* and *C. typicus*, a pilot re-circulating system (Innovaqua srl, Reggio Emilia, Italy) was used modified from the one reported by Payne and Rippingale (2001) to rear the estuarine temperate-water copepod *G. imparipes*.

The pilot system consisted of (Fig. 1):

- A 400 L water accumulation tank (A in Fig. 1);
- two 500 L cylinder–conical fibreglass rearing tanks (B in Fig. 1), connected to each other by 40 L cylindrical pre-tanks (C in Fig. 1). Each 500 L tank contained a 55 μm mesh net cylinder (d in Fig. 1) in proximity to the water outlet, to avoid animal loss during total water re-circulation. Furthermore, on the opposite side of this cylinder, another cylinder with a mesh net size of 150 or 300 μm was inserted, depending on the stage of nauplii or copepodites to be selected (e in Fig. 1). Both stages were attracted inside the cylinder by the light that was switched on using a water-immersion lamp (arrow).
- two 200 L cylinder–conical fibreglass harvesting tanks (F in Fig. 1), containing 55 μm mesh nets to concentrate nauplii and copepodites (g in Fig. 1);
- 1 bio-mechanic filter with bio-o-rings (H in Fig. 1);
- 1 skimmer (I in Fig. 1);
- 1 UV lamp (L in Fig. 1);
- 3 heater external units, one associated with the 400 L tank and two for both pre-tanks, to maintain a constant water temperature (M in Fig. 1);
- 1 Programmable Logic Control (PLC) to automatically control the re-circulation of water (separation and/or concentration of nauplii and copepodites).

The PLC programme was set up with a portable PC. The pilot rearing system was able to work in different modes:

(1) Total water re-circulation (involving 500 and 400 L tanks): In this mode, water passed from the 400 L accumulation tank (A in Fig. 1) to the pre-tank (C in Fig. 1) up to the 500 L rearing tank (B in Fig. 1) and was then discharged in the accumulation tank. Before reaching the rearing tank, water passed through the skimmer, the bio-mechanic filter and the section irradiated by the UV lamp.

(2) Partial water re-circulation (involving 500 tanks and 40 L pre-tanks): In the partial re-circulation

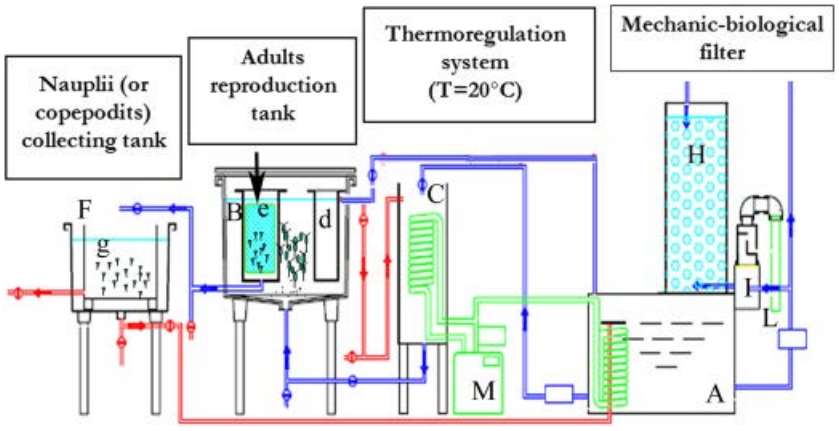


Figure 1 Schematic representation of the pilot rearing system, whereby the red line is the flux of water during the partial circulation and concentration of nauplii and the blue line is the flux in total circulation also during the separation of nauplii from the reproduction tank to the collecting tank. A, 400 L water accumulation tank; B, 500 L cylinder–conical fibreglass rearing tank; C, 40 L cylindrical pre-tank thermoregulation system; d, 55 μm mesh net filter; e, 150 or 300 μm mesh net filters (arrow in e indicates the site of the submerged lamp); F, 200 L cylinder–conical fibreglass harvesting tank containing a 55 μm mesh net filter g; H, bio-mechanical filter with bio-o-ring; I, skimmer; L, UV lamp, M, heater unit.

mode, water was conditioned by passing through the pre-tank (C in Fig. 1) and then to the rearing tank (B in Fig. 1). In this process, algae were not lost from the rearing tank but re-circulated between the two tanks. A separate circulation of the water from the 400 L tank to the UV lamp, the skimmer and the filtration system continued independently.

(3) Nauplii separation (involving 500 to 200 L tanks): This process began only if the total circulation mode was activated and consisted in the transfer of water and animals, previously photo-attracted in the region delimited by the mesh net cylinder equipped with different mesh sizes (150/300 μm) (e in Fig. 1), from the 500 L tank (B in Fig. 1) to the 200 L tank (F in Fig. 1).

(4) Nauplii concentration (in 200 L tanks): With this process, nauplii and copepodites collected in the 200 L tank (F in Fig. 1) were concentrated in a small volume (about 15 L) and the remaining water returned again into the 400 L tank (red line in Fig. 1 from F to A). Nauplii, collected in a beaker by opening the valve at the bottom of the tank, were placed inside the rearing system again or used in fish-feeding experiments. The concentration of nauplii and copepodites was allowed both in total or partial circulation.

Algal cultures

The dinofagellate *Prorocentrum minimum* (Pavillard) (PRO), the prymnesiophyte *Isochrysis galbana* (Parke) (ISO), the prasinophyte *Tetraselmis suecica* (Butcher) (TETRA) and the cryptophyceae *Rhodomonas baltica* (Zimmermann) (RHO) were grown in 10 L carboys filled with 0.22 μm filtered sea water at 36‰ salinity. Seawater was previously treated for 24 h with HClO, neutralized with sodium thiosulphate (v:v) and aerated for 24 h (Lavens & Sorgeloos 1996). After this treatment, Guillard F/2 medium (Sigma, Milan, Italy) was added (Guillard 1975) and monocultures were inoculated. Cultures were grown on a 24-h light cycle (5000 lux), at a mean temperature of 20 ± 1 °C, in a thermostatic chamber, aerated and maintained in the exponential growth phase by diluting with culture medium daily.

Copepod stock culture in a re-circulating system

Before starting the mass cultivation of copepods, 500 L tanks were filled with sea water filtered through a series of mesh nets (100, 10 and 1 μm) and coal cartridges. Water was left to circulate in the system for 1 week, both in total and in partial circulation. The bio-mechanical filter was incubated previously for 20 days with about 20×10^6 bacteria per gram (BIO START – Prodac International, Padova, Italy) before activating the water-circulating system. Water flow from the pre-tank to the rearing tank (partial circulation) was regulated at 0.8 L min^{-1}, while the flux in total circulation, programmed for 2 h per day, was regulated in order to substitute about 35–40% of the water each day.

Temora stylifera were fed ISO, PRO and RHO, to reach a total concentration of 3.1 mg C L^{-1} day^{-1}; *C. typicus* were fed with a mixture of ISO, TETRA and RHO or PRO, to reach a total concentration of 4.1 mg C L^{-1} day^{-1} (Table 1). These concentrations were chosen after preliminary experiments conducted in small volume tanks testing different algal combinations on the egg production rate, egg viability and per cent of cannibalized embryos (data not shown).

During total circulation, part of the algae in the rearing tank were lost, while copepods and eggs were retained by the 55 μm mesh net cylinder inserted into the rearing tank (d in Fig. 1). In March 2007, about 1500 *T. stylifera* copepods (760 *T. stylifera* adults, 250 copepodites and 450 nauplii), obtained from the II and the III generation reared in 5–10 L beakers were transferred into the 500 L rearing tank. Because of heavy infestation by harpacticoids in the second half of July and in August, a new stock of about 46 000 *T. stylifera* copepods (25 000 nauplii, 19 600 copepodites and 1500 adults) was re-introduced into the rearing tank in September.

Massive cultivation of *C. typicus* started in January 2009, when about 970 adults, 620 copepodites and 7500 nauplii, previously reared for 2 months in 10 L beakers, were inoculated into the pilot system.

Filters in the rearing tanks (55; 150 or 300 μm mesh net size, depending on the stage we wanted to collect) were removed every day and gently cleaned with fresh water, to eliminate excess algae that obstructed the nets. Temperature was maintained at 20 ± 1 and 17 ± 1 °C for *T. stylifera* and *C. typicus*, respectively, and with a 12 L:12 D photoperiod. The chemical and physical parameters (dissolved oxygen, pH, salinity and temperature) were controlled every day, while ammonia, nitrites and nitrates were controlled once a week using specific commercial kits (Tropic marin®, Wartenberg, Germany).

Feeding activity

To quantify the filtration rates in the copepod *T. stylifera*, algal concentrations were counted in the rearing tank every day, before and after total re-circulation, as

Table 1 Composition and carbon concentration of algal food supplied to *Temora stylifera* and *Centropages typicus*

	Acronyms	Species (diameter μm)	Concentration (cells ml^{-1})	mg C L^{-1} day^{-1}	References
Temora stylifera	ISO	*Isochrysis galbana* (3–5)	3.4×10^4	2.1	Renaud *et al.* (1999)
	PRO or RHO	*Prorocentrum minimum* (16)	5.5×10^3	1	Laabir *et al.* (2001)
		Rhodomonas baltica (7.53)	1.65×10^4	1	John *et al.* (2001); Patil *et al.* (2007)
Centropages typicus	ISO	*Isochrysis galbana* (3–5)	3.4×10^4	2.1	
	PRO or RHO	*Prorocentrum minimum* (16)	5.5×10^3	1	
		Rhodomonas baltica (7.53)	1.65×10^4	1	
	TETRA	*Tetraselmis suecica* (8.3)	1.25×10^4	1	Renaud *et al.* (1999)

follows: 1 L of water was collected from four different cardinal points of the tank, mixed and 15 mL fixed in 4% paraformaldehyde and left to settle. Three replicates were then counted with a Burker chamber. Fresh algal cultures were added, after total re-circulation of the water, in order to restore the initial concentrations. Percentage of feeding was calculated considering the monthly means of algal concentration 24 h after cultures were added, with respect to the initial concentration (time = 0).

Centropages typicus was fed *ad libitum* by adding algae daily at the concentrations reported above (Table 1).

Per cent egg hatching success

Egg hatching success was calculated for *T. stylifera* by siphoning the bottom of the rearing tank every 2 days. Water was filtered through a 30 μm mesh net and the filtrate was collected in a 10 L beaker to recover the embryos. Forty-eight hours after hatching, re-suspended nauplii were again placed into the rearing tank, while the remaining detritus on the bottom was discarded. *Temora stylifera* egg hatching success was calculated by collecting an aliquot of the siphoned water: the bottom was re-suspended in 100 mL seawater crystallizers. After settlement, eggs were counted on the bottom using an inverted microscope, by counting empty membranes and hatched nauplii, as described by Ianora, Poulet and Miralto (1995).

Population density and naupliar production

A census of the total population was taken almost every week as follows: 10 L of water, collected from the surface to the bottom in two different regions of the rearing tank, was filtered through a 40 μm mesh net. Adults, copepodids and nauplii were counted in triplicate and the mean value was multiplied by the volume of the tank to obtain the total number of copepods. Naupliar production was calculated as the number of individuals per 100 adults.

A preliminary test was conducted to confirm that nauplii were attracted by light into the filter inside the rearing tank: 500 mL samples were collected outside and inside the filter before switching on the light, and again after 2, 3, 9 and 12 days of continuous light. Water was filtered through a 40 μm mesh net and nauplii were counted. Furthermore, to verify the efficiency of naupliar concentration, naupliar density in the rearing tank was compared with that recorded in the 200 L concentration tank, after automatic concentration of the water.

Automatic separation and concentration of nauplii proceeded as follows: the light installed in the mesh net cylinder in the rearing tank was switched on the previous night and, the day after, the automatic separation was activated during 2 h of total re-circulation. After nauplii had been concentrated, the remaining 15 L were collected in a beaker, filtered through a 40 μm mesh net and re-suspended in 100 mL seawater. Aliquots of 5 mL were distributed on a plexiglass plate and nauplii were counted under a Leica stereomicroscope. The total number of nauplii was obtained by multiplying the mean value by 20. Filtered sea water (1 μm mesh net) in the 400 L accumulation tank was replaced after nauplii had been concentrated.

Results

Mass cultivation of *T. stylifera*

The total number of copepods in the 500 L tank increased to 37 800 individuals (26-fold) after 21 days of rearing (Fig. 2a), and consisted mostly of nauplii (88%) (Fig. 2b). Copepodites represented $<10\%$ of the population but increased drastically to 15 000 individuals after 51 days, in mid-April. That month, copepodites represented $>37\%$ of the total population with a concomitant reduction in the number of nauplii (Fig. 2a and b). Adults increased slowly to 7000 individuals after 63 days (in May). Thereafter, the population decreased and in June, after 114 days, population numbers declined to about 200 individuals (Fig. 2a). In June–July, the population consisted only of adults (Fig. 2b).

During the first 2 weeks of September, after the reintroduction of a new stock, the *T. stylifera* adult population increased more than 17-fold with respect to the initial population (Fig. 2a), with a concomitant reduction in the number of nauplii and copepodites. After 9 days, an increase (14 times) in the naupliar population occurred and after a further 12 days, copepodite numbers increased fivefold at the beginning of October (Fig. 2a and b). Adult densities increased again after 7 days, from 13 000 to $>20\,000$. The percentage composition of the different developmental stages alternated between a dominance of nauplii, in mid-October, November and December, and a dominance of adults after a period of about 1 month (Fig. 2b). The maximum number of nauplii was recorded in December, with more than 185 000 nauplii, corresponding to 89.6% of the total population (Fig. 2b). This was the highest value recorded during the experimental period.

Egg hatching success

On average, *T stylifera* hatching success during the entire experimental period (7 months) was 54%, with the lowest values in June and mid-July (27 ±

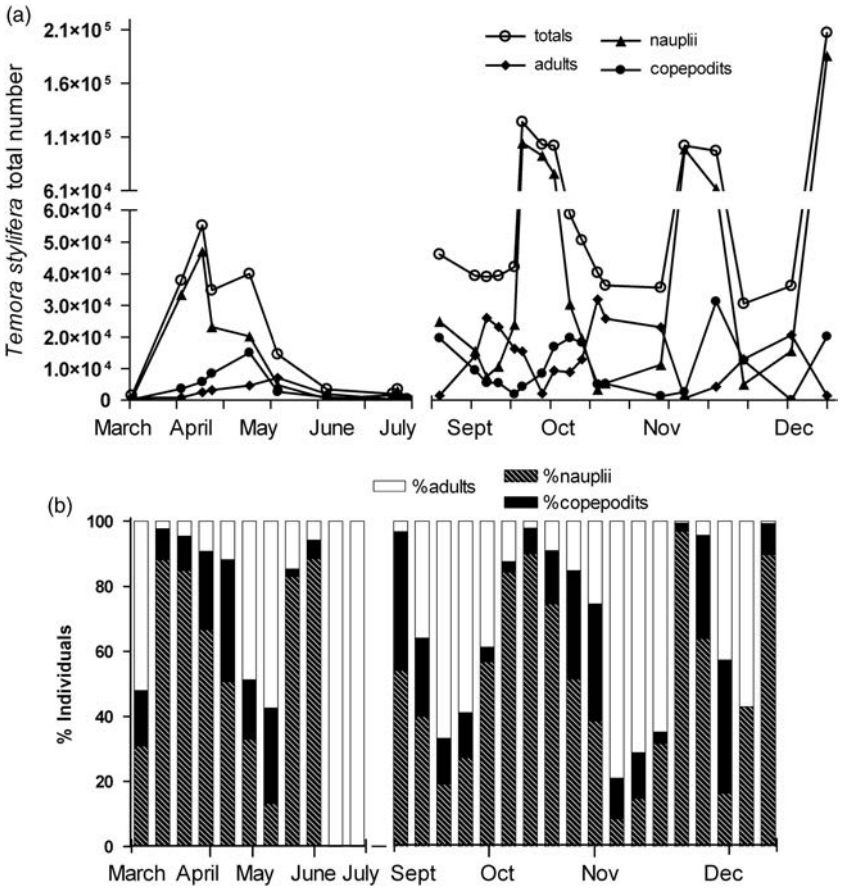


Figure 2 *Temora stylifera* population density recorded during the rearing period. (a) Total number of adults, copepodites and nauplii; (b) per cent of developmental stage composition.

29% and 18 ± 8% respectively), when the population was represented only by adults. Excluding these 2 months, per cent egg hatching success was high, with a mean of 67%. The highest hatching success was recorded in April and May, when more than 75% of the eggs hatched.

T. stylifera naupliar concentration

During 12 days of continuous light, naupliar density inside the filter increased exponentially, with a maximum of 62% recorded after 12 days. These results confirm that nauplii were photo-attracted (Fig. 3); on the contrary, their density outside the filter remained stable over time.

Figure 4 reports the per cent of nauplii concentrated in the 200 L tank, with respect to their density in the rearing tank. Naupliar density, concentrated after only one separation process, was very low compared with their density in the rearing tank (2, 7, 18 and 22% on Days 22, 31, 35 and 51 respectively). However, when the concentration process was activated twice (Fig. 4, arrows), the per cent of nauplii collected increased up to 100% of the total naupliar population calculated inside the rearing tank, suggesting that only one cycle of concentration was not sufficient to collect a representative number of nauplii.

Feeding activity

Figure 5 shows the feeding rate of *T. stylifera* copepods reared in the 500 L tank. The maximum filtration rates were obtained with RHO, with copepods filtering almost 100% of the total algae administered. Filtration rates with ISO were variable during the period considered, oscillating between 75% and 50%, with a mean value of 62.7%. Feeding rates with PRO were above 80% during the entire period.

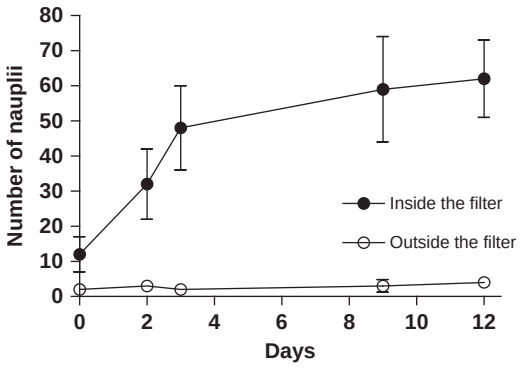


Figure 3 *Temora stylifera* nauplii density calculated inside and outside the filter (e in Fig. 1) when the submerged light was switched on (days)(± SE).

Water quality

The mean oxygen, temperature and salinity values during the rearing period remained stable with time; the mean oxygen values were 5.8 ± 0.09 mg L^{-1}, temperature was maintained at an average of 20 ± 1 °C and salinity at 38.6 ± 0.13 mg L^{-1} during the entire experimental period. Nitrites, ammonia and nitrates had mean values of 0.03 ± 0.001; 0.03 ± 0.001; and 0.2 ± 0.015 mg L^{-1} respectively.

Mass cultivation of *C. typicus*

After the first week of rearing, the population increased 3.6-fold and was mainly represented by nauplii (37 000 ind.) (Fig. 6a and b). An increase in water temperature occurred accidentally at the end of January, causing high naupliar mortality, but temperature was immediately restored to 17 °C. At the end of February, the total population was 123 000 ind., corresponding to more than a 10-fold increase in the total numbers. Naupliar density declined to < 2000 ind. in March due to a daily concentration and collection of nauplii used as live prey for first-feeding clownfish larvae (Olivotto *et al.* 2008, 2009). As a consequence of the continuous collection of nauplii, their population declined in the main tank to < 4700 (Fig. 6a) and in this period adults constituted more than 88% of the total population. In April, when the collection of nauplii was stopped, the population increased again to about 17 000 ind., corresponding to 66% of the total population (Fig. 6b). In the entire 4-month period, copepodite stages remained low, reaching a maximum of 8900 ind. in February, corresponding to 22% of the total. In the other months, the contribution of copepodites was < 6% of the total population (Fig. 6b).

As for *T. stylifera*, all chemical and physical parameters remained stable during the period considered, with a mean oxygen concentration of 7.32 ± 0.3, a mean temperature of 17.1 ± 0.5 and a mean salinity of 38.4 ± 01. Nitrites, ammonia and nitrates had mean values of 0.02 ± 0.001; 0.03 ± 0.001 and 0.1 ± 0.01 mg L^{-1} respectively.

C. typicus naupliar concentration

Nauplii were collected frequently during January and February using two consecutive automatic separation

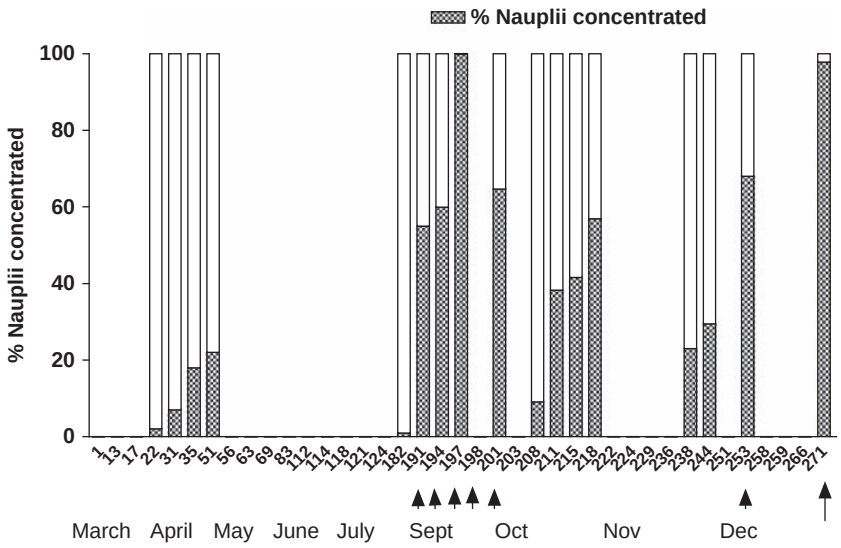


Figure 4 *Temora stylifera* per cent concentrated nauplii in the collecting tank with respect to the total naupliar population in the rearing tank, calculated after one or two (arrows) cycles of naupliar separation.

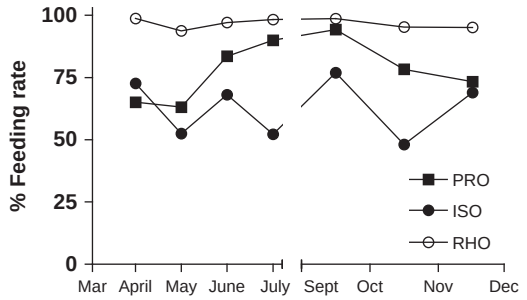


Figure 5 *Temora stylifera* per cent feeding rate in the rearing tank for the three algal diets.

processes. Figure 7 shows the per cent of nauplii concentrated with respect to the total number of nauplii in the rearing tank. The efficacy of concentration for this species was not high; moreover, the per cent of concentrated nauplii declined in March when the collection of nauplii was activated every day. In April, when the automatic separation and concentration were stopped, naupliar density increased again and the concentrated nauplii reached about 60% of the total naupliar population recorded in the rearing tank.

Discussion

The re-circulating system developed by Payne and Rippingale (2001) to rear the tropical copepod *G. imparipes* was modified in this study and used to cultivate two Mediterranean calanoid copepods: *T. stylifera* and *C. typicus*. The advantage of this system is the automation of some operations, such as the partial substitution of water or separation and/or concentration of nauplii and copepodites of a specific size. This allowed for the selection of individuals with a well-defined size to be used as live food for fish larvae (e.g. Olivotto *et al.* 2008, 2009). The automatic concentration of nauplii or copepodites considerably reduced the time required for filtration of large volumes of water, with a noteworthy gain in terms of technical support and handling. The only limitation of the system is that it targets photo-sensitive species that are attracted into the mesh net connected to the concentration tank. Both *T. stylifera* and *C. typicus* nauplii were efficiently concentrated after two consecutive separation events, while for *G. imparipens*, only one concentration event was enough to collect all nauplii present in the 500 L tank (Payne & Rippingale 2001). It is worth noting that the continuous collection of nauplii reduces the number of juveniles, as in the case of *C. typicus* in March, when nauplii were collected daily for experimental feeding of clownfish larvae (Olivotto *et al.* 2008, 2009). In the case of *G. imparipes*, the authors registered a senescent population that needed to be restocked with new adults.

In our system, naupliar production of *T. stylifera* reached a maximum of 370 ind. L^{-1} while for *C. typicus* the highest naupliar production was 100 ind. L^{-1}. These values were much lower than those reported for the tropical species *G. imparipes*

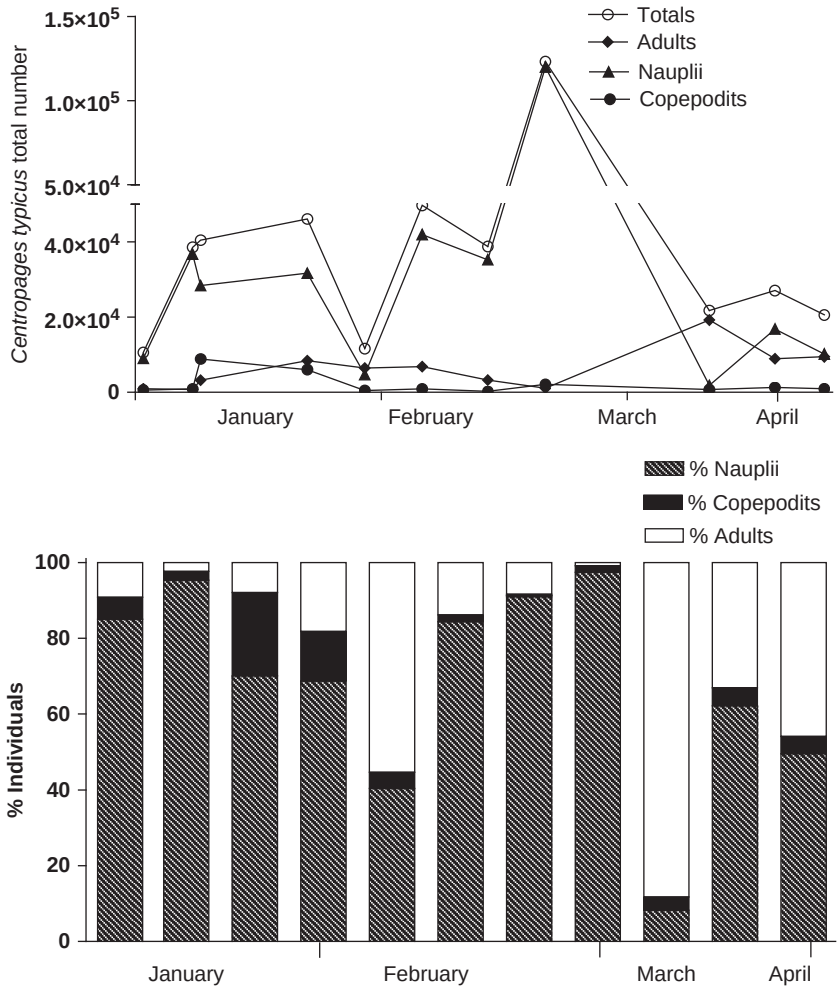


Figure 6 *Centropages typicus* population density recorded during the rearing period. (a) Total number of adults, copepodites and nauplii; (b) per cent developmental stage composition.

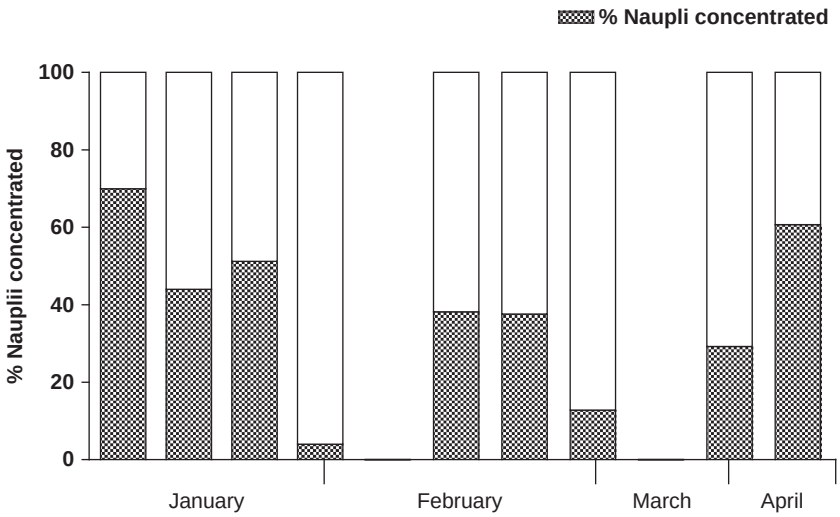


Figure 7 *Centropages typicus* per cent concentrated nauplii in the collecting tank with respect to the total naupliar population in the rearing tank, calculated after two consecutive cycles of naupliar separation.

(1000 ind. L^{-1}) (Rippingale & Payne 2001) or for other cultivated copepod species such as *A. tonsa* (2000 ind. L^{-1}) (Støttrup 2006). This may largely depend on the amenability of certain copepods for mass cultivation. For example, *G. imparipes* adults attach to the wall of the tank using fine hair sensillae on the surface of the prosome, thereby minimizing collisions between individuals (Rippingale & Payne 2001). By contrast, *T. stylifera* and *C. typicus* are free-swimmers and may be sensitive to overcrowding, which potentially reduces feeding and egg production and can lead to cannibalism of eggs as reported for *C. typicus* (Miralto, Ianora, Poulet, Romano & Laabir 1996). *Temora stylifera* and *C. typicus* also have longer development times: 19 days for both *T. stylifera* (Carotenuto, Ianora, Buttino, Romano & Miralto 2002) and *C. typicus* (Carlotti, Bonnet & Halsband-Lenk 2007) compared with the very short generation times of 11 and 9 days for *G. imparipes* (Payne & Rippingale 2001) and *A. tonsa* (Leandro, Tiselius & Queiroga 2006) respectively. On the other hand, in laboratory experiments, *T. stylifera* and *C. typicus* are much more productive than *G. imparipes*, with 120 and 25 eggs $female^{-1} day^{-1}$, respectively (Payne *et al.* 2001; Carotenuto, Ianora, Di Pinto & Miralto 2006), or *A. tonsa* (laboratory cultivation = 26–37 eggs $female^{-1} day^{-1}$) (Jepsen, Andersen, Holm, Jørgensen, Højgaard & Hansen 2007). Hence, their potential for aquaculture purposes is worth exploring.

In our system, the mean naupliar production (number of nauplii/100 adults) was almost 60% from March to June and 50% from September to December, with a peak of 97%. These results are not comparable to those of short-term laboratory studies testing different diets in small containers (100 mL crystallizing dishes), which considered only egg hatching success. For example, higher hatching success was reported for the same species by Buttino, Ianora, Buono, Vitello, Sansone and Miralto (2009) using the same flagellates *R. baltica* (93% hatched eggs) and *P. minimum* (92% hatched eggs), but these data did not consider mortality occurring during the development of different naupliar stages.

In the case of *C. typicus*, 100% hatching success over a 10-day period in small containers was obtained by Miralto, Ianora and Poulet (1995) with a diet of the flagellate *Lingulodinium* (formerly *Gonyaulax*) *polyedra*. In our cultivation tanks, we used a combination of *R. baltica*, *P. minimum* and *I. galbana* and obtained a peak of 97 nauplii per 100 adults in March. The mean value calculated during the entire experimental period was 68 nauplii per 100 adults.

For both species, naupliar production increased when the adult population decreased and, vice versa, similar to what has been observed in the field by Ianora and Buttino (1990) for *C. typicus* and *A. clausi*. In our study, egg hatching success for *T. stylifera* was the lowest when the population was represented only by adults. Environmental factors, such as the quantity or the quality of available food, may be responsible for such oscillations at sea. However, as the quantity and quality of food were constant in our system, we suggest that a mechanism of population density control may be acting on adults by modifying their reproductive rate. Alternatively, both species may have produced resting eggs even if such eggs have not yet been observed for these species in the Mediterranean Sea. Hence, we cannot exclude that oscillations in high and low naupliar production could have been due to different egg hatching times.

Our results indicate that high mortality occurred during the CI–CII stages in *T. stylifera* (data not shown). Copepodite stages never reached more than 40% of the total population and we estimated a loss of 60–90% from nauplii to copepodites 12 days after the peak in the total number of nauplii. The causes for high CI–CII mortality are unknown but may be related to suboptimal food conditions during growth. A diet that combines *R. baltica* with *I. galbana* has been shown to be appropriate in providing desirable fatty acid and protein composition for the culturing of *A. tonsa* (Kleppel, Hazzard & Burkart 2005), but may not be sufficient to maximize production for all copepod species. Moreover, if we calculate the growth rate as from the time of the inoculum to when it reaches the maximum adult density, our results indicate that *C. typicus* grows much faster (50 days) compared with *T. stylifera* (4 momths). This may imply that the diet was suboptimal for *T. stylifera* as both species have the same development time (19 days reported in the literature) or that *C. typicus* is more fecund, producing double the number of eggs as *T. stylifera* with a higher hatching success. As copepod species probably differ in nutritional requirements, future research efforts to improve production in *T. stylifera* should focus on testing other flagellate diets and the possible use of nutritional supplements provided in microencapsulated form. Moreover, feeding rates calculated for *T. stylifera* were based on the 'clearing rate' theory. However, some algae may have sedimented to the bottom of the rearing tank, consequently causing us to overestimate the feeding rates.

In the case of *C. typicus*, preliminary experiments in 10 L beakers testing four different monoalgal diets (PRO, RHO, ISO and TETRA) in eight different combinations (data not shown) indicated that a mixture of PRO and TETRA yielded the best production in terms of number of eggs and per cent egg hatching success. ISO alone was not ingested, as in the case of *T. stylifera*, and a very high per cent of cannibalized eggs was found at the bottom of the beaker (data not shown). It has been demonstrated that nauplii of *T. stylifera* and *C. typicus* have different swimming behavioural patterns, with consequences on food encounter rates and predation pressure (Paffenhöfer, Strickler, Lewis & Richman 1996; Titelman & Kiørboe 2003). This should further be taken into consideration when designing experimental feeding experiments with studies.

Similar to *T. stylifera*, *C. typicus* also showed an alternating pattern of high- and low-density production of nauplii, copepodites and adults with a periodicity of about 30 days. The per cent of copepodites was always very low compared with adults and nauplii, indicating that mortality was highest in these stages.

In conclusion, our culturing system needs to be optimized to obtain higher naupliar production and lower copepodite mortality, possibly by integrating the present diet with other foods. For example, *C. typicus* has been shown to have an omnivorous and/or a carnivorous feeding behaviour both in nature and in the laboratory (Calbet, Carlotti & Gaudy 2007), and may not be amenable to long-term culturing on pure algal diets. Our results show that this culturing system has the potential to rear these ecologically important calanoid species through multiple generations, albeit at much lower concentrations than other cultured copepod species. The system also offers the possibility of obtaining large quantities of *T. stylifera* and *C. typicus* throughout the year, which may find applications in genetic and eco-toxicology studies.

Acknowledgments

Special thanks are due to Francesco Esposito and Mario Di Pinto for their support with algal cultures, Alfonso Toscano, Paola Cirino and Davide Caramiello for their helpful suggestions during the set-up of the pilot system and to Francesca Rinna for her contribution during the experimental phase of her master thesis.

Funding: This work was supported by the Regione Campania (project DGR no 889-30/06/2006 to IB).

References

Båmstedt U. (1986) Chemical composition and energy content. In: *The Biological Chemistry of Marine Copepods* (ed. by E.D.S. Corner & S.C.M. O'Hara), pp. 1–58. Oxford University Press, New York, NY, USA.

Buskey E.J. (2005) Behavioral characteristics of copepods that affect their suitability as food for larval fishes. In: *Copepods in Aquaculture* (ed. by C.-S. Lee, P.J. O'Bryen & N.H. Marcus), pp. 91–106. Blackwell Publishing, IA, USA.

Buttino I., Ianora A., Buono S., Vitello V., Sansone G. & Miralto A. (2009) Are monoalgal diets inferior to plurialgal diets to maximize cultivation of the calanoid copepod *Temora stylifera*? *Marine Biology* **156**, 1171–1182.

Calbet A., Carlotti F. & Gaudy R. (2007) The feeding ecology of the copepod *Centropages typicus* (Kröyer). *Progress in Oceanography* **72**, 137–150.

Carlotti F., Bonnet D. & Halsband-Lenk C. (2007) Development and growth rates of *Centropages typicus*. *Progress in Oceanography* **72**, 164–194.

Carotenuto Y., Ianora A., Buttino I., Romano G. & Miralto A. (2002) Is postembryonic development in the copepod *Temora stylifera* negatively affected by diatom diets? *Journal of Experimental Marine Biology and Ecology* **276**, 49–66.

Carotenuto Y., Ianora A., Di Pinto M., Sarno D. & Miralto A. (2006) Annual cycle of early developmental stage survival and recruitment in the copepods *Temora stylifera* and *Centropages typicus*. *Marine Ecology Progress Series* **314**, 227–238.

Di Capua I. & Mazzocchi M.G. (2004) Population structure of the copepods *Centropages typicus* and *Temora stylifera* in different environmental conditions. *ICES Journal of Marine Science* **61**, 632–644.

Drillet G., Jepsen P.M., Højgaard J.K., Jørgensen N.O.G & Hansen B.W. (2006) Strain-specific vital rates in four *Acartia tonsa* cultures II: life history traits and biochemical contents of eggs and adults. *Aquaculture* **279**, 47–54.

Evjemo J.O., Reitan K.I & Olsen Y. (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L) with special emphasis on the nutritional value. *Aquaculture* **227**, 191–210.

Guillard R.R.L. (1975) Culture of phytoplankton for feeding marine invertebrates. In: *Culture of Marine Animals* (ed. by W.L. Smith & M.H. Chanley), pp. 26–60. Plenum Press, New York, NY, USA.

Ianora A. (1998) Copepod life history traits in subtemperate regions. *Journal of Marine Systems* **15**, 337–349.

Ianora A. & Buttino I. (1990) Seasonal cycles in population abundances and egg production rates in the planktonic copepods *Centropages typicus* and *Acartia clausi*. *Journal of Plankton Research* **12**, 473–481.

Ianora A., Poulet S.A. & Miralto A. (1995) A comparative study of the inhibitory effect of diatoms on the reproduc-

tive biology of the copepod *Temora stylifera*. *Marine Biology* **121**, 533–539.

Jepsen P.M., Andersen N., Holm T., Jørgensen A.T., Højgaard J.K. & Hansen B.W. (2007) Effects of adult stocking density on egg production and viability in cultures of the calanoid copepod *Acartia tonsa* (Dana). *Aquaculture Research* **38**, 764–772.

John M.A., St, Clemmesen C., Lund T. & Köster T. (2001) Diatom production in the marine environment: implications for larval fish growth and condition. *ICES Journal of Marine Science* **58**, 1106–1113.

Kleppel G.S., Hazzard S.E. & Burkart C.A. (2005) Maximizing the nutritional values of copepods in aquaculture: managed versus balanced nutrition. In: *Copepods in Aquaculture* (ed. by C.-S. Lee, P.J. O'Bryen & N.H. Marcus), pp. 49–60. Blackwell Publishing, IA, USA.

Krinsky N.I. (1965) The carotenoids of the brine shrimp, *Artemia* salina. *Comparative Biochemistry and Physiology* **16**, 181–187.

Laabir M., Buttino I., Ianora A., Kattner G., Poulet S.A., Romano G., Carotenuto Y. & Miralto A. (2001) Effect of specific dinoflagellate and diatom diets on gamete ultrastructure and fatty acid profiles of the copepod. *Temora stylifera*. *Marine Biology* **138**, 1241–1250.

Lavens P. & Sorgeloos P. (1996) *Manual on the production and use of live food for aquaculture*. FAO Fisheries Technical Paper, 361, Rome, Italy.

Leandro S.M., Tiselius P. & Queiroga H. (2006) Growth and development of nauplii and copepodites of the estuarine copepod *Acartia tonsa* from southern Europe (Ria de Aveiro, Portugal) under saturating food conditions. *Marine Biology* **150**, 121–129.

Lee C.-S., O'Bryen P.J. & Marcus N.H. ed. by (2005) *Copepods in Aquaculture*, 1st edn. Blackwell Publishing, IA, USA.

McEvoy L.A., Naess T., Bell J.G. & Lie Ø. (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglussus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. *Aquaculture* **163**, 237–250.

McKinnon A.D., Duggan S., Nichols P.D., Rimmer M.A., Semmens G. & Robino B. (2003) The potential of tropical paracalanoid copepods as live feeds in aquaculture. *Aquaculture* **223**, 89–106.

Miralto A., Ianora A. & Poulet S.A. (1995) Food type induces different reproductive responses in the copepod *Centropages typicus*. *Journal of Planktonic Research* **17**, 1521–1534.

Miralto A., Ianora A., Poulet S.A., Romano G. & Laabir M. (1996) Is fecundity modified by crowding in the copepod *Centropages typicus*? *Journal of Planktonic Research* **18**, 1033–1040.

Munilla-Moran R., Stark J.R. & Barbour A. (1990) The role of exogenous enzymes in digestion in cultured turbot larvae (*Scophtalmus maximus* L.). *Aquaculture* **88**, 337–350.

Olivotto I., Buttino I., Borroni M., Piccinetti C.C., Malzone M.G. & Carnevali O. (2008) The use of the Mediterranean calanoid copepod *Centropages typicus* in Yellowtail clownfish (*Amphiprion clarkii*) larviculture. *Aquaculture* **284**, 211–216.

Olivotto I., Avella M.A., Buttino I., Cutignano A. & Carnevali O. (2009) Calanoid copepod administration improve yellow tail clownfish (*Amphiprion clarkii*) larviculture: biochemical and molecular implications. *AACL Bioflux2* **3**, 355–367.

Olson J.A. (1989) Provitamin A function of carotenoids: the conversion of β-carotene into vitamin. *American Journal of Nutrition* **119**, 105–108.

Paffenhöfer G.A., Strickler J.R., Lewis K.D. & Richman S. (1996) Motion behavior of early copepodid stages of marine and planktonic copepods. *Journal of Plankton Research* **18**, 1699–1171.

Patil V., Källqvist T., Olsen E., Vogt G. & Gislerød H.R. (2007) Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquaculture International* **15**, 1–9.

Payne M.F. & Rippingale R.J. (2001) Intensive cultivation of the copepod calanoid *Gladioferens imparipes*. *Aquaculture* **201**, 329–342.

Payne M.F., Rippingale R.J. & Cleary J.J. (2001) Cultured copepods as food for West Australian dhufish (*Glaucosoma hebraicum*) and pink snapper (*Pagrus auratus*) larvae. *Aquaculture* **194**, 137–150.

Pederson B.H. (1984) The intestinal evacuation rate of larval herring (*Clupea harengus* L) predating on wild zooplankton. *Dana* **3**, 21–30.

Renaud S.M., Thinh L.V. & Parry L.D. (1999) The gross chemical composition and fatty acid composition of 18 species of tropical australian microalgae for possible use in mariculture. *Aquaculture* **170**, 147–159.

Ribera d'Alcalà M., Conversano F., Corato F., Licandro P., Mangoni O., Marino D., Mazzocchi M.G., Modigh M., Montresor M., Nardella M., Saggiomo V., Sarno D. & Zingone A. (2004) Seasonal patterns in plankton communities in a pluriannual time series at a coastal Mediterranean site (Gulf of Naples): an attempt to discern recurrences and trends. *Scientia Marina* **68**, 65–83.

Rippingale R.J. & Payne M.F. (2001) *Intensive Cultivation of a Calanoid Copepod Gladioferens imparipes: A Guide to Procedures, ISBN 1 74067 070 1*. Department of Environmental Biology. Curtin University of Technology, Perth, Australia, 60pp.

Schipp G.R., Bosmans J.M.P. & Marshall A.J. (1999) A method for hatchery culture of tropical calanoid copepods, *Acartia* spp. *Aquaculture* **174**, 81–88.

Shields R.J., Bell J.G., Luizi F.S., Gara B., Bromage N.R. & Sargent J.R. (1999) Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: relation to dietary essential fatty acids. *Journal of Nutrition* **129**, 1186–1194.

Støttrup J.G. (2000) The elusive copepods: their production and suitability in marine aquaculture. *Aquaculture Research* **31**, 703–711.

Støttrup J.G. (2006) Review on the status and progress in rearing copepods for marine larviculture. Advantages

and disadvantages. Among Calanoid, Harpacticoid and Cyclopoid copepods. In: *Vances en Nutriciün Acuícola VIII. VIII Simposium Internacional de Nutriciün Acuícola, ISBN 970-694-333-5*, Universidad Autünoma de Nuevo Leün, Monterrey, Nuevo Leün, México, 15–17 Noviembre. (ed. by Suârez L.E. Cruz, D.R. Marie, M.T. Salazar, López M.G. Nieto, Cavadoz D.A. Villarreal, Cruz A.C. Puello & A.G. Ortega.), pp. 62–83. Monterrey, Mexico.

Støttrup J.G., Richardsen K., Kirkegaard E. & Pihl N.J. (1986) The cultivation of *Acartia tonsa* Dana for use as live food source for marine fish larvae. *Aquaculture* **53**, 87–96.

Titelman J. & Kiørboe T. (2003) Motility of copepod nauplii and implications for food encounter. *Marine Ecology Progress Series* **247**, 123–135.

Zillioux E.J. (1969) A continuous re-circulating culture system for planktonic copepods. *Marine Biology* **4**, 215–218.