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Follow-up of hatchery rotifer cultures with regard to their genetic identity

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Brachionus sp. rotifers are widely used in large numbers for larval rearing in fish industry. By practice, cultured rotifers are discriminated on the basis of their body size. Recently however, marked polymorphism has been uncovered with the use of molecular markers. Rotifers of the same size but of distinct genetic profiles have been shown to differ in environmental preferences. Thus, mass culturing practices in hatcheries should be re-considered. In this study, we have followed a number of experimental cultures from an Italian hatchery. The genetic composition of the cultures was recorded and it was revealed that a single *Brachionus* biotype was prevalent in all cultures. Results indicate that the few observed crashes occurred soon after upscaling. Diet change seems to be a possible factor leading to this outcome. In a single occasion, contamination was observed but the co-existence of different *Brachionus* types did not affect culture performance. In the future, detailed joint analyses of the genetic identity and culture preferences of the different *Brachionus* rotifers will provide useful insights into performance-related problems in hatcheries.

Key words: mass cultures, crashes, diet, contamination, genetic monitoring, 16S rRNA.

INTRODUCTION

For more than four decades (see Theilacker & McMaster, 1971) monogonont rotifers of the genus *Brachionus* have been among those planktonic organisms used with success for the rearing of numerous freshwater (Awaiss *et al*., 1992; Ludwig, 1994; Lim & Wong, 1997; Shiri Harzevili *et al*., 2003) and marine fish (Lubzens *et al*., 1989). During that time, *Brachionus* mass culturing was significantly improved (Rico-Martínez & Dodson, 1992; Dhert *et al*., 2001; Hagiwara *et al*., 2001; Park *et al*., 2001) as a result of continuous research on rotifer culture conditions (Yúfera & Pascual, 1985; Hirata *et al*., 1998; Rombaut *et al*., 1999; Gallardo *et al*., 2000; Verschuere *et al*., 2000; Suantika *et al*., 2001). Nowadays, hatcheries produce *Brachionus* rotifers at maximum possible reproduction rates and population densities, in order to meet the growing needs of the aquaculture industry (Lubzens *et al*., 2001). In spite of an apparent peak in terms of efficiency, efforts to achieve ultra-high density production are being tested (Yoshimura *et al*., 2003). Nevertheless, suppressed growth and sudden massive deaths (i.e. crashes) of rotifers are still observed in mass cultures (Comps & Menu, 1997; Cheng *et al*., 2004). It is therefore obvious that a maximum production – minimum loss pursuit is among the critical current objectives of modern hatcheries.

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Towards this direction, emphasis should be given on the genetic make-up of the rotifer cultures (i.e. strains) used in hatcheries. Hatcheries are currently using rotifer body size to distinguish their marine *Brachionus* cultures into Large (L) and Small (S) strains. This classification, originally introduced by Fu *et al.* (1991a, b) to describe two different morphotypes of *Brachionus plicatilis*, today is inaccurate. Recent molecular data have revealed that there are at least nine groups that form the *B. plicatilis* species complex (Gómez *et al.*, 2002). Therefore, the species *B. plicatilis*, as *B. plicatilis* sensu stricto (s.s.), and three biotypes ("Nevada", "Austria" and "Manjavacas"), each with possible species status, are all L rotifers. Similarly, *B. ibericus* and *B. rotundiformis* together with another three biotypes ("Cayman", "Tiscar" and "Almenara") are all S rotifers, according to the classification by Fu *et al.* (1991a, b). Further analyses even suggest the existence of more groups, up to 14, within the *B. plicatilis* species complex (Papakostas *et al.*, 2006a; Suatoni *et al.*, 2006). In addition, it has been shown that the freshwater rotifer *B. calyciflorus* also comprises a species complex (Gilbert & Walsh, 2005). All these findings suggest that strain discrimination on the basis of rotifer body size is currently unreliable.

Since cryptic speciation seems to be widespread in *Brachionus* rotifers, methods of genetic identification need to be incorporated in the rotifer culturing industry to uncover possible species interactions not yet described. Different *Brachionus* species or biotypes may have different optima with respect to culture conditions. Ortells *et al.* (2003) found that "Tiscar" and "Almenara" biotypes (both belonging to the S morphotype) have different salinity preferences. Additionally, co-existence of different *Brachionus* types (i.e. morphotypes and/or species/biotypes) in hatchery strains may result in competition and low culture performance or even crashes. Hagiwara *et al.* (1995) showed that the population growth of *B. rotundiformis* (S morphotype) and of an L type *B. plicatilis* rotifer was suppressed when cultured together.

In this concept, detailed genetic characterizations of a large number of commercial strains have been carried out in several hatcheries worldwide and, in many cases, have provided evidence for a rather unexpected species/biotype composition of rotifer stocks (Papakostas *et al.*, 2006a, b). In the present study, we monitored and characterized genetically a number of experimental cultures from an Italian hatchery. We aimed at following the temporal genetic structure of those cultures that experienced problems or crashed. Possible biotic and abiotic parameters that affect culture performance were also investigated.

MATERIALS AND METHODS

Experimental description

Throughout this work cultures from Maricoltura di Rosignano Solvay (MRS) hatchery were studied. MRS hatchery is a modern, medium-sized hatchery located in Tuscany close to Livorno (Italy) and its main cultured species is gilthead seabream (*Sparus aurata*). In this study, rotifer production involved upscaling from stock (50 ml tubes, 19°C, 25 ppt, fresh algae: *Nannochloropsis* sp. or *Isochrysis* sp.) to starter cultures (10 L flasks). After that, rotifers were transferred to 100 L tanks where they were fed on algae and the artificial dry diet Culture Selco H (CSH) or the liquid diet (LD). CSH was based on the commercial Culture Selco Plus® and was formulated for sustaining high density rotifer batch culture whereas LD was formulated for low density rotifer batch culture. Finally, rotifers were inoculated into 1000 L tanks to start a mass culture (25°C, 25 ppt, CSH or LD). The system of batch culturing with a 3-day interval was followed.

Two strains were analyzed in this work, MRS and GBA. MRS is a local strain (i.e. isolated from the incoming seawater in MRS hatchery) which belongs to the S morphotype and has been genetically characterized as "Cayman" (Papakostas *et al.*, 2006b; Dooms *et al.*, 2007). All the studied cultures but one, started from the MRS strain. The GBA strain was provided from GreatBay Aquaculture LLC, Portsmouth, USA. It belongs to the L morphotype and has been genetically characterized as "Austria" (Papakostas *et al.*, 2006b; Dooms *et al.*, 2007). GBA was cultured in mass culture conditions once (Table 1, culture H).

For two consecutive years (2002-2003 and 2003-2004 production periods), MRS collected 139 samples from 13 cultures (from a total of 1060 batch cultures). Every sample was taken at the end of each batch culture (i.e. every 3 days) and was preserved in absolute ethanol. When a culture suffered a crash, a sample was taken as soon as this was observed. Additionally, cultures were visually monitored and performance parameters (growth, egg ratio, activity) were noted. Genetic analysis was focused on those cultures that experienced problems (bad performance, presence of flocks) or crashed.

Genetic analysis

Genetic identification of rotifers from selected cycles (for the purposes of this work) was performed through Restriction Fragment Length Polymorphism (RFLP) analysis of a 378 bp fragment of the 16S mitochondrial gene. Previous studies (Papakostas *et al*., 2005) have shown that this approach provides sufficient resolution for the purposes of this work. Furthermore, the small fragment size and the use of primers designed specifically on *Brachionus* (Papakostas *et al*., 2005) ensure high quality PCR product from individual, ethanol-preserved, rotifer DNA.

DNA extractions were performed on single rotifers (20 individuals per sample). This differs from the study of Dooms *et al*. (2007) where genetic identification of MRS samples was based on bulk extractions (see also Papakostas *et al*., 2006b). In this way, we were also able to compare and crosscheck the results of the two methods and evaluate the efficiency and sensitivity of bulk analysis.

Each *Brachionus* individual was transferred to a 0.65 ml Eppendorf tube with approximately 5 μl of absolute ethanol. To remove ethanol, samples were left for 1 h at 37°C. After that, a standard Chelex™-based DNA extraction procedure was followed (Papakostas *et al*., 2005). The PCR amplification procedure is described in Papakostas *et al*. (2005). Digestions with biotype-specific restriction endonucleases (*Apo*I for "Cayman" and *Taq*ªI for "Austria"; Papakostas *et al*., 2005) were visualized in 2.5% agarose gels (Figs 1 and 2).

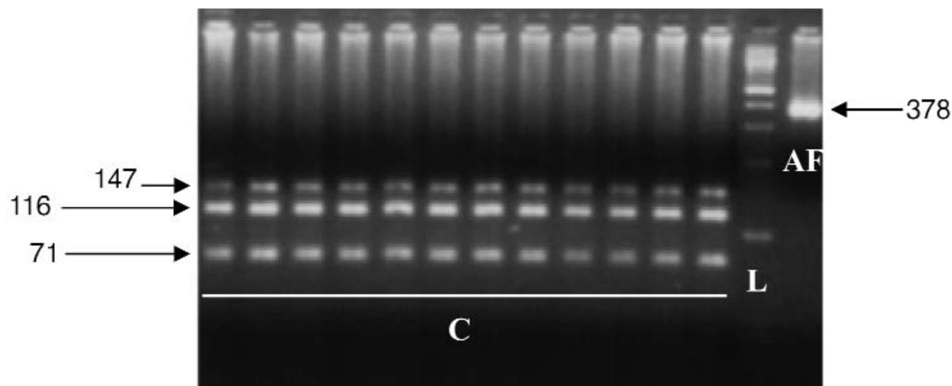

FIG. 1. Example of digestion with the restriction endonuclease *Apo*I in samples of the MRS culture F. All samples produced the pattern 147, 116, 71, 25*, 19* bp specific to the "Cayman" biotype (Papakostas *et al*., 2005). C: "Cayman" biotype; L: 100 bp DNA ladder; AF: the amplified fragment of 378 bp. * Fragment not shown because of the small size.

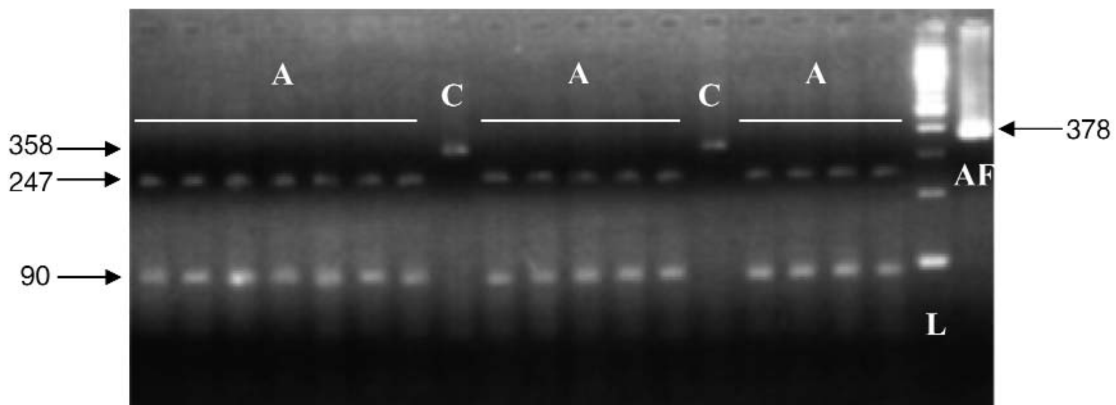

FIG. 2. Example of digestion with the restriction endonuclease *Taq*ªI in samples from culture H (cycle 8). C: "Cayman" biotype (358, 20* bp); A: "Austria" biotype (247, 90, 21*, 20* bp) (Papakostas *et al*., 2005); L: 100 bp DNA ladder; AF: the amplified fragment of 378 bp. * Fragment not shown because of the small size.

TABLE 1. Follow-up of the 13 studied MRS cultures (numbers of individuals genetically characterized are in parentheses)

	Culture	Cycle	Feed type	Genetic identity	Remarks
2002-2003	A1*	0	*Isochrysis* sp.	"Cayman"	
		1 to 3	LD	(20 individuals	
		4a to 8a	LD	from each cycle)	
		4b to 8b	CSH		
	B1	0	*Isochrysis* sp.	"Cayman" (20)	
		1 & 2	LD	"Cayman" (20 & 20)	
		3	CSH	"Cayman" (20)	Crash
	C1	0	*Isochrysis* sp.	"Cayman" (20)	
		1 & 2	LD	–	
		3	LD	"Cayman" (20)	
		4 & 5	CSH	–	
		6	CSH	"Cayman" (20)	Bad performance
	D1	0	*Isochrysis* sp.	"Cayman" (20)	
		1 to 5	LD	–	
		6		"Cayman" (20)	
		7 to 10		–	
		11		"Cayman" (20)	
	E1	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1	LD	"Cayman" (20)	Crash
2003-2004	A2	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1 to 3	CSH	–	
		4		"Cayman" (20)	Presence of flocks – culture discarded
	B2	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1 to 9	CSH	–	
		10		"Cayman" (20)	
		11 to 25		–	
		26		"Cayman" (20)	
	C2	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1	CSH	"Cayman" (20)	
		2		"Cayman" (20)	Crash
	D2	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1 to 7	CSH	–	
		8		"Cayman" (20)	
	E2	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1 to 5	CSH	–	
		6		"Cayman" (20)	
	F	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1 to 4	CSH	–	
		5		"Cayman" (20)	
		6 to 14		–	
		15		"Cayman" (20)	
		16 to 28		–	
		29		"Cayman" (20)	
	G	0	*Nannochloropsis* sp.	"Cayman" (20)	
		1	CSH	"Cayman" (20)	Crash

TABLE 1. continued

Culture		Cycle	Feed type	Genetic identity	Remarks
2003-2004	H	0	*Nannochloropsis* sp.	"Austria" (20)	
		1 to 4		–	
		5		"Austria" (20)	
		6 & 7		–	
		8		"Austria" (18) & "Cayman" (2)	
		9	CSH	–	
		10		"Austria" (13) & "Cayman" (7)	
		11		–	
		12		"Austria" (8) & "Cayman" (12)	
		13 to 15		–	
		16		"Austria" (5) & "Cayman" (15)	

– : genetic analysis not performed
* : after cycle 3, cultures were separated into a and b series differing in feed type

RESULTS

In total, 13 experimental cultures were genetically analyzed (Table 1). Among those, four crashed (B1, E1, C2 and G), two were interrupted (C1 and A2), six performed well and were used as controls (in the sense that they differed from other cultures that crashed or were interrupted), and one (culture H) was examined since it was the only culture reared from the GBA strain. Cultures that crashed lasted no more than four cycles. Culture B1 crashed in cycle 3, when diet was changed from LD to CSH. Cultures E1, G and C2 crashed soon after upscaling. Cultures C1 and A2 were interrupted because of bad performance (poor growth and presence of flocks, respectively). The six non-problematic cultures performed well for long periods: culture F reached 29 cycles and culture B2 was interrupted after completing 26 cycles. Culture H (GBA strain) was maintained for 16 cycles (Table 1). Contamination was suspected after the first few cycles as gauged by the observation of S type rotifers.

A total of 1000 rotifers from 50 cycles of all studied cultures were genetically identified (Table 1). From each selected cycle, 20 individuals were analyzed. All MRS strain cultures were identified as "Cayman" (Fig. 1). Culture H on the other hand, was identified as "Austria" (GBA strain) for the first five cycles (see Table 1). Suspected contamination was verified at cycle 8 since two (out of 20) rotifers were found to be "Cayman". Interestingly, analysis of the subsequent cycles showed that the "Cayman" biotype (presumably from MRS strain), became more abundant and eventually dominated the culture (Table 1, Figs 2 and 3). Figure 3 illustrates the course of culture H. It can be seen that "Cayman" increased steadily from 10% at cycle 8 to 75% at cycle 16, at which point the culture H was interrupted. During that time, no crash or bad performance was observed in culture H.

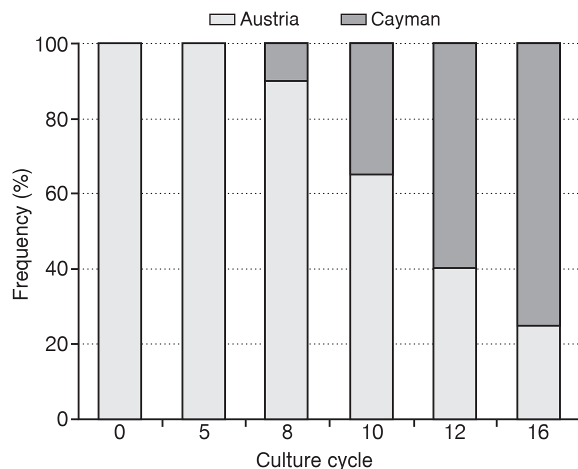

FIG. 3. Genetic follow-up of culture H. A total of 20 rotifers were studied in each cycle (numbers of individuals from each biotype are shown in Table 1, culture H).

DISCUSSION

This study is the first attempt to monitor *Brachionus* mass cultures by taking into account the genetic make-up of the used strains. Many researchers have investigated the different problems encountered in *Brachionus* mass cultures (e.g. Hirayama, 1987; Hagiwara *et al.*, 1995). In general, both abiotic, such as ammonia levels (Yu & Hirayama, 1986), and biotic

parameters like microbial interactions (Reguera, 1984; Cheng *et al*., 1997) are known to cause crashes. In this work, numerous rotifers from a series of experimental cultures performed in MRS were genetically characterized. Results revealed no evidence for a correlation between the genetic background of a culture and its performance.

The genetic identification of samples from the studied MRS strain cultures showed that a single *Brachionus* biotype existed, namely the “Cayman”. Our results are in agreement (regarding biotype composition) with those of Dooms *et al*. (2007) who developed a bulk-based DGGE methodology for genetic screening of rotifer strains. Despite the fact that numerous cycles were monitored and analyzed in our study, no genetic variation was noticed between high- and low-performing cultures, before or immediately after a crash. More interesting was the course of culture H that started with “Austria” rotifers (L type) and ended with “Cayman” (S type) dominance. It has been shown that *Brachionus* species with smaller body size reach higher population densities in cultures (Fernández-Araiza *et al*., 2005). The outcome of culture H though could also be attributed to the culturing conditions (25 °C, 25 ppt) that seem to favour “Cayman”. There are many indications to propose that L type rotifers prefer lower temperatures in contrast to S type rotifers. Fielder *et al*. (2000) noticed that S type rotifer *B. rotundiformis* was more tolerant than L type *B. plicatilis* at temperatures higher than 23 °C. Ortells *et al*. (2003) found L type *B. plicatilis* in low temperatures and high oxygen levels, whereas S type rotifers (*B. ibericus*, “Tiscar”, “Almenara” and *B. rotundiformis*) were associated with higher temperatures. The pattern of gradual increase of “Cayman” (10%, 35%, 60%, 75%; see Table 1 and Fig. 3) to its final dominance over “Austria” in culture H, lends further support to the idea that favourable conditions permitted such a biotype replacement. In other words, an initial contamination event in culture H was carried over and aggravated over the course of subsequent cycles. Alternatively, contamination may have been persistent and repetitive, however, in that case we would not obtain a monotonically increasing trend for “Cayman”.

The dominance of “Cayman” over “Austria” explains to some extent previous findings (Papakostas *et al*., 2006b) where most of the used strains in hatcheries were found to be monocultures (i.e. composed of a single *Brachionus* type). Rotifers at mass culture level are grown under stable conditions. Fixed environmental conditions are expected to favour certain *Brachionus* types and lead to monocultures. The impact (positive, negative or neutral) of this possible genetic impoverishment on the efficiency of a culture needs however, further investigation. So far, there are not enough data to suggest that mixed cultures (i.e. cultures of more than one *Brachionus* types) perform better or worse than monocultures. Although it has been shown that *Brachionus* rotifers have higher growth rates in monocultures than in mixes (Hagiwara *et al*., 1995; Fernández-Araiza *et al*., 2005), this does not necessarily determine the outcome in mass culturing scale. Besides, although culture H was not a mixed culture from the beginning, it experienced no problems in this study. It seems that more experiments are needed towards this direction before safe conclusions are drawn.

“Cayman” monocultures have been found to be common in hatcheries, and among S type rotifers “Cayman” is the most prevalent (Papakostas *et al*., 2006b). A total of six strains from the MRS hatchery, under different labels, were also found to be “Cayman” (Papakostas *et al*., 2006b). Since “Cayman” is an S type rotifer, it might be favoured by hatchery practices. As Lubzens *et al*. (2001) reported, there is an increasing need of small size rotifers for newly cultured marine species and part of this may be fulfilled by small-sized strains. Furthermore, exchange of strains with good performance characteristics or no crashes is frequently practiced by hatcheries. Favourable culture conditions and contamination phenomena may comprise alternative routes for the widespread presence of the “Cayman” biotype in hatcheries. However, to further evaluate the dominance of “Cayman”, the specific genetic make-up of the exchanged strains combined with monitoring practices should be addressed. On the other hand, great care should be taken by hatcheries when L type *Brachionus* culturing is desired. In this work, it has been shown that L rotifers could be susceptible when contaminated by “Cayman” and a possible way to maintain strain composition would be the application of appropriate culture conditions. According to what was already discussed, lower temperatures should favour L rotifers.

In this study, most of the observed problems and crashes coincided mainly with changes in the culture conditions. Cultured rotifers need time to adapt to novel conditions. Rapid changes in temperature or salinity have negative impacts on rotifer experimental cultures (Fielder *et al*., 2000). In the same way, abrupt

diet changes might cause similar shocks to rotifers under mass culture conditions. Diet is known to be a key factor in rotifer culturing (Yúfera *et al*., 1993) and the growth rate of *Brachionus* rotifers may vary substantially under different diets (Peña-Aguado *et al*., 2005). In this work, all four MRS cultures that ended to a crash did not last more than four cycles. This occurred soon after culture inoculation (cycle 0) when rotifers were transferred from 100 L to 1000 L tanks and algae were substituted with artificial medium. Changing all these parameters might be the reason that led cultures B1, E1, C2 and G to crash. Furthermore, culture C1 did not perform well and this was noticed soon after a diet change from LD to CSH. However, there is a degree of variability in our observations. For example, many other cultures that did not end to a crash were inoculated in the same way. Likewise, culture A1 (cycles 1 to 8b) had its diet changed from LD to CSH at cycle 4 without problems. Therefore, it seems that change of culture conditions *per se* is not necessarily detrimental. It is possible that longer acclimatization periods will help rotifer adaptation and improve culture efficiency (Lubzens *et al*., 1995). However, in a hatchery there are always many other reasons that can cause problems to cultures. For example in the present study, culture A2 was discarded because of flocks (aggregates of dead algal cells, rotifer faeces and feeding rests). Flocks are difficult to remove through filtration and tend to remain in the rotifer culture during the subsequent cycles. They may reduce air circulation and act as substrates for the development of infectious agents. As a result, culture performance is below optimum and crashes are frequently triggered.

In hatcheries, rotifers are exposed to conditions far different than those in their natural environment (see Lubzens *et al*., 2001). It is, therefore, possible that mass cultures could become unstable. Strain adaptation prior to inoculation is suggested to be an influential factor for the stability of mass cultures. The recent molecular description of the *B. plicatilis* group (Gómez *et al*., 2002) is expected to increase the resolution of modern studies. Highly evaluated and well-standardized methods of genetic analysis (Papakostas *et al*., 2006a, b; Dooms *et al*., 2007) can facilitate the fast screening of numerous samples from hatcheries. Thus, hatchery practices can now be re-examined and correlated with *Brachionus* species and types previously ignored.

CONCLUSIONS

In this work, genetic identification revealed that all the MRS strain cultures, irrespective of their performance, are monocultures. Culturing conditions seem to favour the "Cayman" biotype. In one occasion (culture H), the L starter strain (GBA, "Austria") was outperformed by "Cayman" under the studied hatchery conditions. It is suggested that culturing conditions might play an important role in the *Brachionus* species composition of the hatchery strains. In view of these findings, the actual benefits of strain exchange among hatcheries need to be re-evaluated. It is recommended that together with the optimal culture parameters, the genetic profile of the exchanged strains should be determined as well. Finally, the few performance-related problems were observed soon after upscaling and were linked to diet change. It is suggested that an extension of the time that rotifers are co-fed on algae and mass culturing diets (CSH or LD) should improve culture performance. Further experimentation is however needed towards this direction.

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