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Fatty acid composition of *Turbatrix aceti* and its use in feeding regimes of *Coregonus maraena* (Bloch, 1779): is it really a suitable alternative to *Artemia* nauplii?

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Summary

By incorporating the free-swimming nematode *Turbatrix aceti* into early feeding regimes of the European whitefish *Coregonus maraena*, the suitability of this nematode species was investigated as an alternative to *Artemia* nauplii. During a 14-day feeding trial in a total of 25 aquaria each 1.7 L (each treatment n = 5, 255 larvae/tank) *T. aceti* was used either as the sole live food or in combination with *Artemia* nauplii or microdiet to determine the effect of *T. aceti* on growth performance and survival rate of *C. maraena*. By analysing the fatty acid composition of *T. aceti* prior to and after enrichment with INVE spresso® it was investigated whether the amount of n3-polyunsaturated fatty acids (n3-PUFA) in *T. aceti* could be further enhanced. Supplementation of *Artemia* nauplii with *T. aceti* increased growth significantly within the first 5 days of rearing in comparison to the non-supplemented food treatments (14.39 ± 0.15 mm compared to 13.44 ± 0.18 mm; mean ± SE). However, growth and survival of juvenile *C. maraena* on nematode-supplemented *Artemia* nauplii did not differ significantly from non-supplemented *Artemia* nauplii at the end of the 14-day rearing period (15.22 ± 0.15 mm compared to 14.86 ± 0.24 mm). All feeding treatments containing *Artemia* nauplii showed significantly higher growth and lower mortality at the end of the experiment in comparison to diets containing only the microdiet or *T. aceti* or a combination thereof. The overall low performance of *T. aceti* alone can most likely be explained by an insufficient capacity of *C. maraena* to digest this nematode species efficiently. Enrichment with INVE spresso® successfully increased the proportion of DHA in the *T. aceti* tissue. The results reveal that *T. aceti* cannot be considered a full alternative to *Artemia* nauplii, at least not in the rearing of *C. maraena*, but might be a useful vector of essential fatty acids within the early rearing period of this and potentially other fish species when provided as live food along with *Artemia* nauplii.

Introduction

Live food organisms in aquaculture are currently limited to relatively few species, most prominently the brine shrimp *Artemia salina,* and rotifers such as *Brachionus* sp. (Lavens and Sorgeloos, 1996). *Artemia* nauplii account for approximately 40% of the total amount of live food in aquaculture and are particularly convenient in hatchery operations as they can be stored over long time periods and are readily available when needed (Lavens and Sorgeloos, 2000). Due to the rapidly growing demand of hatcheries for live food organisms, the search for non-*Artemia* alternatives is increasing (Sorgeloos et al., 2001).

Several nematodes were suggested recently as cost-effective alternatives to fish- and crustacean larvae (Brüggemann, 2012; Weber and Traunsburger, 2014). Nematodes have been shown to be especially successful when used in combination with inert artificial diets as co-feeding regimes (Kahan, 1980). Particularly the use of the free-living nematode *Panagrellus redivivus* has been shown to result in high survival and growth of shrimp larvae and carp species, similar to conventional live food organisms such as *Artemia* nauplii (Rottmann et al., 1991; Kumlu et al., 1998; Focken et al., 2006; Brüggemann, 2012). Other free-living nematode species, such as *Caenorhabditis elegans*, *C. briggsae* and *T. aceti*, have been used in the culture of adult fish (Hofsten et al., 1983), but their suitability as live food to rear fish larvae has been poorly studied (Brüggemann, 2012). In particular, the free swimming nematode *Turbatrix aceti*, used for years as fish feed in ornamental fish culture (Hofsten et al., 1983), must be considered as an interesting candidate for feeding of larval fish. *T. aceti* is easy to culture in high densities and with a low risk of contamination by pathogenic microorganisms due to the natural antibiotic properties of the growth medium (Buck et al., in press). Contrary to many other free-living nematode species, *T. aceti* is able to swim actively in the water column (Brüggemann, 2012), which can increase its availability to pelagic fish larvae and limit the amount of live food necessary for feeding. More importantly, *T. aceti* possess the ability to produce polyunsaturated fatty acids (PUFA) *de novo* (Rothstein and Götz, 1968) and therefore might be suitable to overcome nutritional deficiencies commonly found in *Artemia* nauplii (Lavens et al., 1989). Several authors have demonstrated the value of n3-PUFA enrichment strategies: docosahexaenoic acid

(DHA) and eicosapentaenoic acid (EPA) in particular have been shown to enhance growth and survival of various fish larvae during the production process (Gapasin et al., 1998; Noori et al., 2011b).

Accordingly, growth, survival and general condition of the European whitefish *Coregonus maraena* have shown significant improvement on artificial diets containing more than 0.5% n-3 PUFA (Watanabe et al., 1989). The goal of this project was to investigate the fatty acid composition of *T. aceti* before and after enrichment with commercial PUFA solution and to evaluate the general suitability of this nematode species as a solitary live food or in co-feeding regimes during the early rearing period of *C. maraena*.

Materials and methods

Culture of *Turbatrix aceti* and fatty acid supplementation

The nematode *T. aceti* was grown according to Buck et al. (in press) on a 50 : 50 solution of apple cider and water added to 25 g L^{-1} peptone at approximately 20°C under gentle aeration in two 5-L plastic bottles at xenic conditions. Prior to feeding, *Artemia* nauplii and nematodes were harvested and their respective densities (ind. ml^{-1}) assessed three times under a stereomicroscope and a counting chamber in 0.1 ml water to calculate the daily feeding treatments. For PUFA enrichment, harvested nematodes were transferred into a 50 ml PE tube filled with tap water and subsequently enriched with Selco S.presso® (INVE-Aquaculture) according to the producer's instructions: 1 g L^{-1} of the emulsion was added to the nematodes at T_0 and T_{12} and harvested after 24 h. The nematodes were then rinsed through a 20 μm gauze and stored frozen at −80°C for subsequent fatty acid analyses. Simultaneously, non-enriched nematodes, Minipro™ and *Artemia* were frozen at −80°C until analysis.

Experimental design

Larvae of *C. maraena* were obtained at 2 days post hatch (dph) from a Schleswig-Holstein fish hatchery (Fischbrutanstalt Altmühlendorf, Schleswig-Holstein, Germany) and transferred in oxygen filled plastic bags to the Centre for Aquaculture Research (Zentrum für Aquakulturforschung- ZAF) in Bremerhaven, Germany.

After arrival the larvae were transferred into independent static 1.7 L aquaria (five replicates per treatment) with a stocking density of 150 larvae L^{-1}. Larvae were reared at 17°C under natural photoperiod conditions (11 h : 13 h light : dark regime) and continuously aerated. Prior to the experiment, larvae at day-3 post hatch were counted by means of a digital camera system (Canon 550 D) and the number of larvae was stocked up to 255 larvae/tank, where necessary. Fish larvae were exposed to five different feeding regimes, including Microdiet Minipro™ (Maripro AS), *Artemia salina* as well as *T. aceti*, either as exclusive feed or in combination with the other two feed sources over a 16-day period (Table 1). At least 2/3 of the culture water (>1L) was exchanged manually twice per day, once in the morning before feeding and once in the evening from a preheated tank.

Cysts of *A. salina* (Sanders Brine Shrimp Company, Ogden, UT) were decapsulated and hatched in cylindro-conical polyethylene containers according to Lavens and Sorgeloos (1996). Feeding of fish was performed manually on a daily basis at 9 : 00 in the morning. At 8 and 16 days post hatch a minimum of five larvae from each tank were sampled (Canon EOS 550D) for determination of total body length using the software package IrfanView (Version 4.30). Larvae were counted 8 dph and after the termination of the experiment at 17 dph using digital photography to calculate the mean survival per treatment.

Fatty acid analysis

Samples of all feed sources (*Artemia* nauplii, Minipro™, *T. aceti, T. aceti* + S.presso®) were freeze-dried and ground. Lipids were extracted twice from the different food items (1–10 mg freeze-dried material) using a mixture of dichloromethane/methanol (2 : 1, v/v). For the analysis of fatty acids, the cell-free extracts were dried under a stream of nitrogen and esterified with 3 mol L^{-1} methanolic HCl (60°C, 20 min). Next, fatty acid methyl esters (FAMEs) were partitioned into iso-hexane, dried under a stream of nitrogen, and resuspended in a volume of 50 μl iso-hexane. Lipids were then analyzed by gas chromatography on an HP 6890 GC (Agilent Technologies) equipped with a flame ionization detector and a DB-225 capillary column (J and W Scientific). Details of GC configurations are given elsewhere (Martin-Creuzburg et al., 2010). Fatty acids were quantified by comparison to internal standards (C17:0 and C23:0 methyl esters); the quantification limit was 20 ng of fatty acid. Fatty acids were identified by their retention times and their mass spectra, which were recorded with a gas chromatograph-mass spectrometer (Agilent Technologies, 5975C) equipped with a fused silica capillary column (DB-225MS, J and W Scientific). Mass spectra were recorded between 50 and 600

Table 1
Description of five different feeding regimes used in *C. maraena* 14-day feeding trial

Food treatment	Composition		
	Turbatrix aceti	*Artemia* nauplii	Minipro Microdiet
A	–	5 Ind. ml^{-1} day^{-1}	–
A+T	10 Ind. ml^{-1} day^{-1}	3 Ind. ml^{-1} day^{-1}	–
M	–	–	0.1 mg ml^{-1} day^{-1}
M+T	10 Ind. ml^{-1} day^{-1}	–	0.05 mg ml^{-1} day^{-1}
T	20 Ind. ml^{-1} day^{-1}	–	–

Dalton in the electron impact ionization mode and compared to mass spectra of reference substances purchased from Sigma-Aldrich.

Statistical analysis

Analysis of variance (ANOVA) was applied to compare average total length of *C. maraena* larvae fed on different feeding regimes after a rearing period of 5 and 13 days (8 and 16 dph) as well as for the fatty acid analysis. Data were log-transformed and checked for normality of residuals (Shapiro–Wilk's test) and equality of variance (Levene's test). Fatty acid data were arcsin transformed to ensure normal distribution and tested for equality of variance (Levene's test). Generalized linear modelling (GLM, family = binomial) was applied to test for significant differences in overall survival between the treatments at 8 and 17 dph. *Post-hoc* comparisons between treatments were performed for all measured variables according to the Tukey-Kramer method. All statistical tests were conducted using R Version 2.13.0 (R Development Core Team, 2013) at a significance niveau of $\alpha = 0.05$.

Results

Fatty acid composition of the nematodes

Nematodes contained significantly higher levels of total fatty acids than the MD Minipro™ and *Artemia* nauplii (ANOVA, $P < 0.005$; Table 2). Both groups of nematodes contained approximately twice the amount of fatty acids found in *Artemia* nauplii. Although *Artemia* nauplii contained significantly higher amounts of n-3 PUFA compared to nematodes (ANOVA, $P < 0.007$; Table 2), the relative contribution of EPA was significantly lower than in *T. aceti* (ANOVA, $P < 0.001$; Table 2) and DHA was completely absent in *Artemia*. Non-enriched specimens of *T. aceti* contained $0.8 \pm 0.6\%$ DHA and $18.3 \pm 2.1\%$ EPA prior to enrichment. After enrichment, DHA concentrations were 19-fold higher ($15.0 \pm 2.4\%$) in *T. aceti* and thus significantly higher than in any other food item (ANOVA, $P < 0.001$; Table 2).

Survival and growth of *C. maraena* larvae

Larvae in all treatments were observed to actively feed on *T. aceti*, *Artemia* nauplii and MD. Survival rates of *C. maraena*

Table 2
Fatty acid composition of *Artemia* sp., Minipro™ microdiet and *T. aceti* prior to enrichment and after enrichment with commercial PUFA solution (*T. aceti*+). All values in percentage of total fatty acids unless otherwise stated

	Artemia sp.	Minipro™	*T. aceti*	*T. aceti*+
FA total (μg FA mg^{-1} DW)	54.9 ± 1.5^{a}	81.4 ± 3.7^{b}	111.1 ± 19.0^{c}	104.1 ± 7.5^{c}
14:0	1.3 ± 0.1^{a}	5.0 ± 0.2^{b}	2.1 ± 0.3^{c}	3.1 ± 0.4^{c}
15:0	nd	0.4 ± 0.0^{a}	nd	0.3 ± 0.0^{b}
16:0	17.0 ± 0.9^{a}	20.0 ± 0.7^{b}	4.8 ± 1.1^{c}	11.7 ± 1.1^{d}
18:0	8.5 ± 0.3^{a}	2.7 ± 0.1^{b}	9.0 ± 2.3^{a}	5.6 ± 0.2^{c}
20:0	0.2 ± 0.4^{a}	0.6 ± 0.0^{a}	0.6 ± 0.1^{a}	0.5 ± 0.0^{a}
21:0	1.0 ± 0.2^{a}	nd	nd	nd
22:0	0.5 ± 0.1^{a}	1.8 ± 0.2^{b}	nd	0.4 ± 0^{a}
SAFA total	28.5 ± 0.8^{a}	30.5 ± 1.3^{b}	16.5 ± 3.4^{c}	21.6 ± 1.7^{d}
16:1n-7	2.5 ± 0.1^{a}	1.9 ± 0.1^{ab}	2.5 ± 0.6^{a}	3.2 ± 0.3^{ac}
17:1n-7	1.0 ± 0.1^{a}	0.5 ± 0.0^{b}	nd	0.3 ± 0.0^{c}
18:1n-12/n-9	23.9 ± 0.9^{a}	10.6 ± 0.7^{b}	27.5 ± 5.0^{c}	11.9 ± 1.2^{d}
18:1n-7	6.3 ± 0.5^{a}	2.1 ± 0.1^{b}	0.7 ± 0.1^{c}	4.9 ± 0.3^{d}
20:1n-9	1.2 ± 0.1^{a}	2.6 ± 0.3^{b}	0.4 ± 0.1^{c}	0.4 ± 0.0^{c}
20:1n-7	0.6 ± 0.1^{a}	0.0 ± 0.0^{b}	3.3 ± 0.7^{c}	1.1 ± 0.1^{d}
22:1n-9	nd	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	nd
24:1n-9	0.0 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.1^{b}	0.2 ± 0.0^{b}
MUFA total	35.5 ± 1.4^{a}	18.2 ± 1.2^{b}	35.0 ± 6.1^{a}	22.0 ± 2.0^{c}
18:2n-6	4.1 ± 0.2^{a}	20.9 ± 1.1^{b}	12.2 ± 2.3^{c}	17.3 ± 1.4^{d}
18:3n-6	0.7 ± 0.0^{a}	nd	1.4 ± 0.2^{b}	0.8 ± 0.1^{a}
20:2n-6	0.1 ± 0.3^{a}	nd	1.0 ± 0.3^{b}	0.9 ± 0.1^{b}
20:3n-6	nd	3.7 ± 0.3^{a}	5.0 ± 0.9^{b}	2.2 ± 0.1^{c}
20:4n-6	0.5 ± 0.0^{a}	1.1 ± 0.1^{b}	2.4 ± 0.6^{c}	1.0 ± 0.1^{b}
n-6 PUFA	5.5 ± 0.5^{a}	25.7 ± 1.4^{b}	22.0 ± 4.1^{c}	22.3 ± 1.8^{c}
18:3n-3	24.1 ± 1.2^{a}	2.8 ± 0.1^{b}	0.7 ± 0.2^{c}	2.9 ± 0.2^{b}
18:4n-3	4.1 ± 0.2^{a}	1.8 ± 0.1^{b}	0.5 ± 0.2^{c}	0.4 ± 0.0^{c}
20:3n-3	nd	nd	0.4 ± 0.1^{a}	0.4 ± 0.0^{a}
20:4n-3	1.1 ± 0.1^{a}	0.6 ± 0.0^{b}	5.9 ± 1.5^{c}	2.6 ± 0.1^{d}
20:5n-3 EPA	1.3 ± 0.1^{a}	7.4 ± 0.5^{b}	18.3 ± 2.1^{c}	12.2 ± 0.7^{d}
22:5n-3	nd	0.5 ± 0.0^{a}	nd	0.7 ± 0.1^{b}
22:6n-3 DHA	nd	12.4 ± 0.1^{a}	0.8 ± 0.6^{b}	15.0 ± 2.4^{a}
n-3 PUFA	30.5 ± 1.6^{a}	25.5 ± 0.8^{b}	26.6 ± 3.6^{b}	34.1 ± 3.6^{c}
PUFA total	36.0 ± 2.1^{a}	51.2 ± 2.2^{b}	48.6 ± 7.7^{c}	56.4 ± 5.2^{d}

nd, not detected; FA, Fatty acids; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n3-PUFA total, total amount of Omega n-3 polyunsaturated fatty acids; n-6-PUFA total, total amount of Omega n-6 polyunsaturated fatty acids; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid.
Values within each row with different superscript letter denote significance ($P < 0.05$; $n = 3$).

larvae reared on food treatments containing *Artemia* (A and A+T) were significantly higher (>60%) than in any other treatment (GLM, $P < 0.0002$) at the end of the experiment (Fig. 1a), while at 8 dph only feeding regimes M and T were significantly different from each other (GLM, $P < 0.04$). Lowest survival was observed in regime T (74 ± 1.5%), while highest survival was found in treatment M (86 ± 2.7%) (Fig. 1a).

Fish larvae growth was generally higher in treatments containing *Artemia* nauplii compared to all other treatments (Fig. 1b). At 8 dph fish fed the combination of *Artemia* sp. and *T. aceti* (Treatment A+T) were significantly larger than fish from any other treatment (ANOVA, $P < 0.002$). At 16 dph highest growth in length was found in treatments A and A+T (ANOVA, $P < 0.001$).

Discussion

Survival and growth of *C. maraena* larvae

The nematode *T. aceti* was found to contain higher levels of EPA and DHA compared to *Artemia* nauplii, which transferred to a higher growth rate of *C. maraena* larvae in combination with *Artemia* nauplii at day 8 post hatch, but not to higher survival or better growth at the end of the experiment (Fig. 1). This finding suggests that *C. maraena* larvae benefit from increased levels of n-3 PUFA during first feeding, as also found for juvenile *C. maraena* of approx. 3 months of age (Watanabe et al., 1989). Furthermore, results reveal that *T. aceti* seem to be beneficial especially within the earliest rearing period of fish larvae due to their relative small size compared to *Artemia* nauplii, which might facilitate the onset of exogenous feeding in *C. maraena*. The maximum size of prey is determined mainly by the ability of a given fish larvae to ingest particles of a certain size (Lavens and Sorgeloos, 1996). In contrast, the minimum size is limited by the fact that at some point the energy expenditure for searching, capturing and ingesting is surpassing the energetic gain from this small food item (Lubzens, 1987), suggesting that the larger *Artemia* nauplii become increasingly available for the growing larvae between 8 and 16 dph, while the energetic gain from consumption of nematodes is decreasing. Although *C. maraena* is principally capable of digesting *T. aceti*, the low survival rate and slow growth in feeding treatments, where *T. aceti* was fed exclusively (T) or in combination with microdiet (M+T) (Fig. 1), indicates that this species is unsuitable in replacing *Artemia* nauplii as the main food source, but could serve as a potential vector of essential fatty acids in co-feeding regimes. For example, the nematodes may be fed with a gradual transition towards increasingly larger proportions of *Artemia*, similar to the use of the rotifer *Brachionus plicatilis* in the aquaculture of marine fish larvae (Conceição et al., 2010).

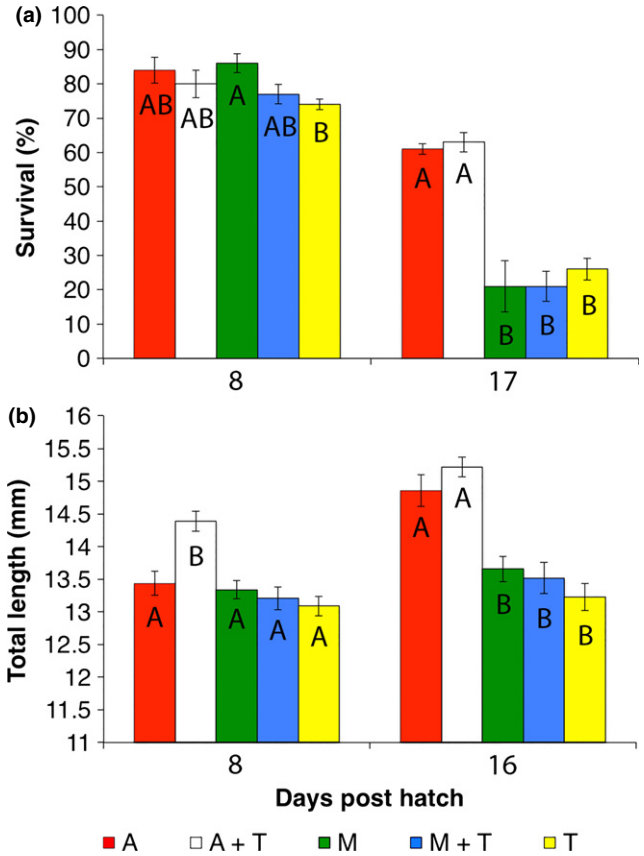

Fig. 1. (a) Relative survival (%) and (b) length-increment (mm) of *C. maraena* during a 14-day feeding trial. Five different feeding regimes in five replicates were investigated (A: Artemia sp., A+T: Artemia sp. + *T. aceti*, M: microdiet, M+T: microdiet + *T. aceti*, T: *T. aceti*; see Table 1 for detailed description of each feeding regime). Significant differences according to the Tukey-Kramer method denoted by capital letters. All values displayed as mean ± standard error. For determination of length a minimum of five larvae per replicate was analysed

Fatty acid composition

The DHA levels (Table 2) of PUFA-enriched nematodes were more than twice the levels reported for PUFA-enriched *Artemia* nauplii, whereas the proportional contribution of EPA (Table 2) was comparable to those found in enriched *Artemia* nauplii (Shields et al., 1999). The measured DHA contribution furthermore surpasses values reported for the free-swimming nematode *Panagromlaimus* sp., that has been enriched comparably with S.presso® at different concentrations (Honnens et al., 2013). The mean EPA fraction of non-enriched specimens of *T. aceti* found in our study nearly doubles those values reported by Krusberg (1972) and also surpasses the maximum reported amount of EPA for non-enriched

P. redivivus specimens (Schlechtriem et al., 2004). Therefore, the suitability of *T. aceti* should be investigated with particular regard to marine fish species known to have higher DHA requirements (Tocher, 2010). In larvae of freshwater or anadromous fish a sufficient supply with n-3 PUFA in combination with suitable vitamins can also improve survival in high stress situations, as shown for the two fish species *Stizostedion vitreum* and *Acipenser persicus* during exposure to salinity and temperature changes or when facing elevated levels of nitrite and ammonia, situations typically encountered during stocking (Kolkovski et al., 2000; Noori et al., 2011a). Therefore, co-feeding of *T. aceti* can be considered particularly useful in situations where fish larvae are stocked directly after the first feeding period, as is, for example, performed in restocking programmes for the Allis shad (*Alosa alosa*) in the Rhine River (Klinger, 2011).

Conclusions

Most likely due to its solid cuticle, *T. aceti* as a solitary feed or in combination with MD cannot match the performance levels provided by *Artemia* nauplii in the early rearing period of *C. maraena*. Nevertheless, incorporation of *T. aceti* into the feeding regime of *C. maraena* in co-feeding with *Artemia* nauplii enhanced growth significantly within the first 5 days of the rearing period. Therefore, *T. aceti* might be suitable for improving rearing protocols at least of *C. maraena* as a cost-effective vector of essential fatty acids. Due to the easy production, advantageous fatty acid composition and active swimming ability, the application of *T. aceti* as larval live food should be evaluated for other fish, particularly crustacean species, which have shown the ability to digest nematodes more efficiently than fish at an early developmental stage (Brüggemann, 2012).

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