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Regulation of filter-feeding house components in response to varying food regimes in the appendicularian, *Oikopleura dioica*

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The frequent repetitive secretion of filter-feeding houses of the tunicate, Oikopleura dioica represents investment of a substantial proportion of total body carbon. Despite this investment, the filter-feeding strategy of pelagic tunicates has been proposed as an adaptation to oligotrophic environments. Here, we examined the capacity of O. dioica to modify its house renewal rate (HRR) and expression of component proteins, oikosins, as well as ingestion rates of different sized particles in response to varying food regimes. There were no significant changes in HRR (0.26 ± 0.07 SD house h^{-1}) with age or food concentration throughout the life cycle. Our data suggest that the complex pattern of endoreduplicating cycles in the oikoplastic epithelium probably limits the capacity to reduce the energetic output of house replacement as a response to a limiting food environment. On the other hand, at the molecular level, there was differential regulation of component house proteins when animals were cultured in standard versus limiting food regimes. Animals pre-conditioned in each of these regimes and subsequently challenged with an identical mixture of large and small particles exhibited different retention efficiencies of larger food particles. Taken together, these results raise the possibility that a limited ability to modulate house architecture may underlie the differential particle retention efficiencies observed.

INTRODUCTION

Appendicularians live inside a complex extracellular, gelatinous filter-feeding house composed of glycoproteins, mucopolysaccharides and cellulose (Kimura *et al.*, 2001; Spada *et al.*, 2001; Thompson *et al.*, 2001). The house is secreted from the oikoplastic epithelium, a monolayer of polyploid cells with distinctive nuclear morphologies characteristic of different cellular fields (Ganot and Thompson, 2002). Water is pumped through the filters of the house by sinusoidal movements of the tail (Fig. 1A). The fine filter mesh enables appendicularians to filter particle sizes down to 0.15 μm (Flood and Deibel, 1998), yielding a size ratio between prey and predator of 1:10 000 (Gorsky and Fenaux, 1998). Pelagic tunicates increase filtration rates exponentially with filter size (Harbison and Gilmer, 1976), and it has been proposed that the filter-feeding strategy of these organisms is adapted to survival in low food concentrations (Harbison, 1992; Acuña, 2001). However, among appendicularians, repetitive renewal of houses implies a substantial energy investment (Sato *et al.*, 2001) in generating the filter-feeding apparatus.

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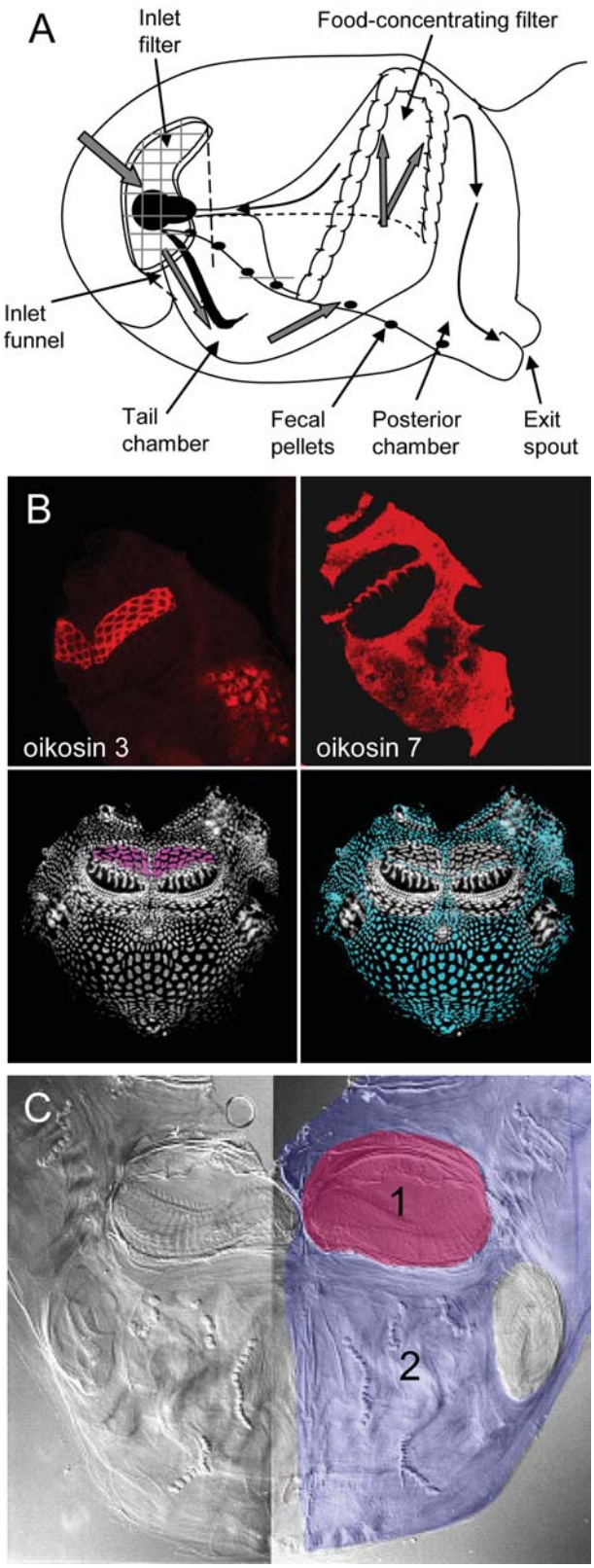

Questions regarding possible plastic responses in this structure are therefore of particular interest.

House renewal rates (HRRs) of the appendicularian *Oikopleura dioica* have been reported to vary from 4 to 19 houses per day (temperature-dependent), corresponding to a daily energy investment of 63–290% of total body carbon (Fenaux, 1985; Sato *et al.*, 2001). The semelparous *O. dioica* has a very short generation time (1 week at 15°C) and upon maturity the reproductive organ attains ~50% of total body size, females producing about 300 eggs (Troedsson *et al.*, 2002). Large energy investment in both the feeding structures and total reproductive output implies a trade-off. To reduce energy investment in the house when food is scarce, it has been hypothesized that appendicularians could lower their HRR. However, some studies indicate that HRR is independent of food concentration (Acuña and Kiefer, 2000; Sato *et al.*, 2001), while others have reported an effect (Fenaux, 1985; Tiselius *et al.*, 2003).

A filter-feeding organism encountering varying particle concentrations in the water column can modify filtration rates to ingest a constant number of food particles, as exemplified by the negative correlation between increased food concentration and clearance rate in *Oikopleura* (Bochdansky and Deibel, 1999; Acuña and Kiefer, 2000; Selander and Tiselius, 2003). To investigate changes in filtration rate in *O. dioica*, behavioural studies have shown a decrease in tail beat frequency as well as an increase in tail arrest with increasing food concentrations (Selander and Tiselius, 2003). However, the behavioural response in *O. dioica* did not fully explain the increase in clearance rate with decreasing food concentration. The authors concluded that particles not accounted for in their model were trapped in the filters, an idea that was also proposed in the related appendicularian, *O. vanhoeffeni* (Bochdansky and Deibel, 1999). This suggestion was supported by Acuña and Kiefer (Acuña and Kiefer, 2000) monitoring radio-labelled algae as well as through molecular

Fig. 1. Construction of the filter-feeding house of *O. dioica*. (**A**) Water circulation (grey arrows) through, and general organization of, the oikopleurid house (Adapted from Thompson *et al.*, 2001). (**B**) Top panels: whole mount (trunk of the animal) *in situ* hybridization patterns for oikosins 3 and 7. Bottom panels: an epithelial spread of *O. dioica* showing nuclei stained with Hoechst 33342 with the spatial expression patterns of oikosin 3 and 7 superimposed on the spread with purple and blue pseudo-colours, respectively. (**C**) A prehouse rudiment spread of *O. dioica*. (Adapted from Thompson *et al.*, 2001.) (1) indicates the pre-inflation food-concentrating filters secreted directly on top of the region of Fol. and (2) indicates the outer wall of the house, coincident with the expression pattern of oikosin 7. The location of the food-concentrating filters and the general scaffold are indicated only on one half of the rudiment but are fully bilaterally symmetric.

detection of different sized food particles trapped in the house (Troedsson *et al.*, 2007a). However, considering the repeated energetic investment in appendicularian houses, an alternative hypothesis is that a plastic response in the filtering structure could increase the net energy gain from filtration in a low food environment. This has been shown in other filter-feeding organisms (Koza and Kořínek, 1985; Lampert, 1994; Lampert and Brendelberger, 1996). In *Oikopleura*, consideration of the template that produces houses, the oikoplastic epithelium, suggests that there may be fundamental cellular restrictions to the degree of modification of filter morphology in response to food availability. The complex, differential regulation of endoreduplicative cycles (partial or total replication of genomic DNA in a cell in the absence of cell division) making up the oikoplastic epithelium appears to be set early in development and may have a limited coordinated repertoire of response to environmental cues (Ganot and Thompson, 2002). However, changes in the expression level of different house components as a function of nutritional intake has never been tested previously and could reveal regulatory mechanisms in this complex extracellular structure.

Here we have tested the plasticity of the *O. dioica* filter-feeding apparatus in response to different food regimes experienced throughout the life cycle, in terms of the number of houses produced (HRR), the relative production of two molecular components contributing to different parts of the house structure and the ingestion rate. We previously identified several structural proteins, oikosins (Spada *et al.*, 2001; Thompson *et al.*, 2001), that compose the house and are secreted from specific cellular territories in the oikoplastic epithelium (Fig. 1B and C). Among these proteins, oikosin 3 is located in the food-concentrating filter, whereas oikosin 7 is expressed more broadly in the epithelium, corresponding to the outer wall of the house within which the filters and chambers are organized. Therefore, evaluation of the relative levels of expression of these genes and the corresponding gene products together with measurements of ingestion rates would provide an indication of whether changes in investment into different structures in the house occur in response to altered food conditions.

METHODS

Culture of algae

Fresh inoculates were made from static cultures of *Isochrysis sp.* (Prymnesiophyta, CCAP 927/14, 6 μm diameter) and *Chaetoceros calcitrans* (Bacillariophyta, CCAP 1010/11, 4 × 3 μm—length × width). The carbon content was 10.6 ± 0.3 SD pg C/cell for *Isochrysis sp.* and 4.6 ± 0.3 SD pg C/cell for *C. calcitrans*. Nutrients and silica (for *C. calcitrans*) were added to sterile-filtered sea water and they were grown in 2 L plastic bags under constant light (36W/20 cool white fluorescent light tube) with air bubbles for agitation (Bouquet *et al.*, 2009). Using this culturing system, we rarely see colonies or long spines in *C. calcitrans*. Further, we have previously demonstrated that both algae are ingested by *Oikopleura dioica* (Troedsson *et al.*, 2007a), and due to the complementary nutritional value between these two algae (Troedsson *et al.*, 2005), the animals have high growth and reproductive outputs (Troedsson *et al.*, 2002). The same strains were used throughout the experiments and only algae in the exponential growth phase were used to avoid fluctuations in nutritional value.

Collection and culture of animals

Appendicularians were collected and cultured as previously described (Bouquet *et al.*, 2009). To establish a generation of animals, 40–45 mature females and 30–35 mature males were placed in 4 L of seawater. The animals were monitored at full maturity and forced to release their gametes into the water by gentle aspiration in a Pasteur pipette. This was done to synchronize the population. After fertilization, embryos were transferred, using 1 L beakers, into two fresh 6 L beakers with the designated food regimes. After 24 h, the content of each beaker was distributed into three new 6 L beakers, and after a further 24 h the content of each of these beakers was distributed into two 6 L beakers, resulting in a total of 12 beakers. After each dilution, the assigned food regime was added. These serial dilutions allowed addition of fresh sea water every 24 h, and promoted consistently better survival than a single, more extensive, initial dilution. Each successive 24 h after this, the animals were transferred with a 5 or 10 mL Sterilin pipette with a cut tip, the diameter of which exceeded the maximum width of the house, to a fresh 6 L beaker under the same experimental conditions.

The animals were cultured under standard or limited food regimes. In the standard food regime, animals were fed *Isochrysis sp.* and *Chaetoceros calcitrans* each at a concentration of 2000 cells/mL (2.54 μM C, or 30.4 μg C/L) prior to 96 h and 4000 cells/mL (5.08 μM C, 60.8 μg C/L) after. Although spring bloom conditions can far exceed these values in Norwegian coastal waters, these food regimes have empirically been tested to yield stable culture conditions with normal reproductive output (Paffenhöfer, 1973;

Bouquet *et al.*, 2009). Exceeding these food concentrations can at times yield significant mortalities, a complicating factor that was undesirable in our experiments. In the limited food regime, the animals were fed 1/6 of the standard food regime, which has previously been recorded to significantly reduce oocyte number while having no effect on somatic growth or mortality rates (Troedsson *et al.*, 2002, 2005). The differential reproductive output between these two food regimes in *O. dioica* provides a platform for testing potential trade-offs in energy allocations, e.g. HRR and reproductive output. All experiments were done at 15°C and 30 psu. Due to the relatively long spawning period of the animals (Troedsson *et al.*, 2002), experiments were not conducted for more than 5.5 days post-fertilization as this would bias the population towards the end of the life cycle.

House renewal rate

The experiment was conducted with two seed beakers per food regime cultured throughout the life cycle. Starting at 48 h, and thereafter each 24 h, 20 animals were transferred from each seed beaker to a fresh 4 L beaker (Cambro camwear®) with the corresponding food regime. After 4 h, the animals were transferred with a 10 mL pipette, into new 4 L beakers with assigned food regimes and the discarded houses in the old beakers were counted. This was repeated every 4 h for a total duration of 12 h. To facilitate house detection following transfer, 10–20 μL of Quink (Solv-x®) was added to the water for contrast. The number of houses produced per individual and hour was then plotted throughout the development and the significance of the slope was tested. A non-significant slope indicate that there were no changes in HRR throughout the development and all time points could be pooled and the effect of food regime was analysed using a one-factor ANOVA analysis (Statistica 8.0; StatSoft, Inc.) with HRR as the dependent variable and food as the categorical predictor after verifying homogeneity of variance by Levene's test.

RT–PCR of oikosin genes in response to different food regimes

To investigate whether there is a response to different food regimes in the transcription of oikosin genes, semi-quantitative PCR (qPCR) specific for oikosin 3 and oikosin 7 were performed. Animals were cultured under standard and limited food regimes and sampled every 12 h, beginning at 24 h of development. The number of animals per sample was dependent on the developmental stage (Table I). Sampled animals were prodded to escape their house and left in the sedative 3-aminobenzoic acid ethyl ester (MS222, Sigma A5040; 0.125 mg/mL) on ice until the requisite number of animals had been collected (<15 min). Animals were transferred to an Eppendorf tube, seawater removed and the animals frozen in liquid nitrogen until further analysis.

Table I: Number of animals sampled per mRNA, protein and ingestion rate time points

Time (h)	No. of animals/ mRNA	No. of animals/ protein	No. of animals/ ingestion
24	1200	–	–
36	1000	–	–
48	800	250	–
60	500	–	–
72	200	150	80
84	90	–	–
96	60	75	50
108	40	–	–
120	30	40	30

Total RNA was extracted with guanidinium thiocyanate/acid phenol and first-strand cDNA synthesis was done by incubating 1 μg of DNaseI-treated (PCR-grade, GibcoBRL, Life Technologies) total RNA for 1 h at 37°C with 100 pmol random hexamers, 10 mM DTT, 1 U/μL RNasin (Promega), 0.5 mM dNTP, 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$ pH 8.3, 400 U M-MLV RT (GibcoBRL, Life Technologies). As a negative control, the reaction was run without M-MLV RT. Semi-quantitative PCR was performed using cDNA corresponding to 25 ng of total RNA, 2 μL of LightCycler-FastStart DNA Master SYBR Green I (Roche), 0.2 μM primers, 4 mM Mg^{2+}, in 20 μL total volume. Amplification included an initial 10 min at 95°C followed by 40 cycles [94°C, 10 s; annealing, 5 s (see Table II); 72°C, 10 s] and finally a 10 min elongation step at 72°C. Each reaction was followed by a melting curve profile from 65 to 95°C to confirm primer specificity. Quantification was done using Roche LightCycler software.

To determine whether the standard and limited regimes exerted any differential effects on the relative mRNA levels of oikosin 3 to oikosin 7, five independent replicate experiments were performed. The transcription level of the oikosin genes throughout *O. dioica* development was measured, and normalized to transcripts for the ribosomal protein L-23 (RpL23), an indicator of general metabolism [equation (1)]:

$$\text{Normalized mRNA level} = \frac{\text{oikosin X}}{\text{RpL23}}, \qquad (1)$$

Table II: Primers sequences, amplicon lengths and specific annealing temperatures

Target	F-primer	R-primer	Amplicon (bp)	Annealing (°C)
Od oikosin 3	CCACCTACGATGAGTTCCAC	CACCAGAGCAAGAAAGATCG	229	55.0
Od oikosin 7	ACAGCACAGCCAACCTCAT	GAATCCGAAGTAGGCGACAT	215	60.0
Od RpL 23	ACAGCACAGCCAACCTCAT	AGGTCGATTGAACCGACTC	380	55.0
Isochrysis sp. 18S	TCCGGTTGCGTGCTGAGTCA	TCGCCAGCGTAAAGCCGTGC	57	60.5
E. coli 16S	GGAAACTGCCTGATGGAG	CCTACTAGCTAATCCCATCTG	129	60.5

The values were plotted as a function of age and the significance of slope was tested. For both oikosin 3 and oikosin 7 data sets, the slope was not significant and a one-factor ANOVA analysis (Statistica 8.0; StatSoft, Inc.) with mRNA levels (log-transformed in the case of oikosin 3) as the dependent variable and food as the categorical predictor were performed after verifying homogeneity of variance by Levene's test.

Determination of oikosin 7 and 3 protein levels under different nutrient conditions

To determine the levels of oikosin 7 and 3 proteins in response to different food regimes, animals were sampled as in the above section. Four replicates were sampled per time point. Total protein extracts were obtained by heating the samples for 10 min at 98°C in a loading buffer containing 100 mM Tris–HCl pH 6.8, 4% SDS, 10% Glycerol, 40% β-mercaptoethanol. Samples were centrifuged at 20 000*g* at room temperature for 15 min, and the extracts were run on gradient (5–20%) SDS–PAGE gels (Laemmli, 1970). Separated proteins were transferred to PVDF membranes (Immobilon P, Millipore) in a transfer buffer containing 25 mM Tris–HCl, 192 mM glycine, 10% methanol and 0.1% SDS. To evaluate transfer, membranes were stained with 0.5% Ponceau red (MERCK) in 0.01% acetic acid and de-stained with 100% methanol for 20 min followed by PBS for 1 h. Subsequent steps were performed at room temperature. Membranes were blocked for 3 h in 3% BSA (Sigma, fraction V) in PBS containing 0.1% Tween (PBS-T). Primary antibodies were diluted 1:1000 in 0.5% BSA/PBS-T and incubated on membranes for 1 h. Membranes were washed 3 × 10 min and incubated for 1 h with secondary antibodies diluted in PBS-T; those conjugated with horse radish peroxidase (HRP) were diluted 1:10 000, whereas antibodies conjugated with alkaline phosphatase (AP) were diluted 1:5000. Membranes were washed 3 × 10 min in PBS-Triton (0.1%). For AP detection, membranes were incubated 5 min with 2 M Tris–HCl pH 9.5 and antibody detection was done with CDP-star diluted 1:100 in 2 M Tris–HCl pH 9.5. For HRP detection, membranes were incubated with ECL™ western blotting Analysis System (Amersham Biosciences). Detection was with a Fuji Film FPM-100A developer using Kodak Scientific Imaging Film. Primary antibodies for oikosin 7 were hybridized on PVDF membranes and the HRP detection system was used. After detection, membranes were washed for 4 h in PBS-Triton (0.1%) and re-blocked (as above) overnight at room temperature. Primary antibodies for oikosin 3 were then hybridized and detected with the AP system. Films were scanned and densitometry was performed using Gene Tool Analysis Software (v 3.03.03).

In order to investigate possible differential regulation between the two house components, oikosin 7 normalized to oikosin 3 was plotted for the two food regimes over age. This was done to eliminate any bias of the total amount of protein loaded in each lane of the SDS–PAGE. After testing the significance of the slopes, a two-way ANOVA with the normalized oikosin 7 to oikosin 3 protein level as the dependent variable and food and age as the categorical predictor was conducted after verifying homogeneity of variance by Levene's test (Statistica 8.0; StatSoft, Inc.). To further investigate any significance in the ANOVA, a multiple pairwise comparison (*post hoc*, unequal-N HSD) was performed.

Ingestion rates

To test whether any change in house morphology will affect ingestion, two analyses were made. First, we investigated if there was a differential ingestion of 5 μm particles between animals conditioned to standard versus limiting food regimes. Second, we investigated whether there was a differential ingestion of 1 μm particles between animals conditioned to the two food regimes. Ingestion rates were measured using a molecular gut content method (Troedsson *et al.*, 2007a). An underlying assumption using this method is that there is no digestion of prey genomic DNA between animals of the two food regimes. Although, digestion of prey genomic DNA is a significant factor when applied to other mesozooplankton, e.g. copepods (Troedsson *et al.*, 2009), previous studies on appendicularians indicate

that digestion is very limited and can therefore be neglected here (Troedsson *et al.*, 2007a). For mixed feed dosages, *Isochrysis sp.* cell counts were made as previously described (Bouquet *et al.*, 2007a). For bacteria (kanamycin resistant strain), serial dilutions were made, optical densities of the dilutions were measured at 600 nm and the dilutions plated on agar-kanamycin plates to make counts. Standard curves of optical density to counts were then generated and systematically used to calibrate feed dosages. Appendicularians were collected and cultured in standard and limited food regimes at 15°C, as previously described (Troedsson *et al.*, 2002, 2005). In replicates of five, animals from each food regime were sampled at 24 h intervals from 3 to 5 days post-fertilization (Table I) according to Troedsson *et al.* (Troedsson *et al.*, 2007a). Each sample was transferred separately to 4 L beakers (Cambro camwear®), containing 0.2 μm filtered sea water, for 20 min to ensure complete gut evacuation (López-Urrutia and Acuña, 1999). Houses were not removed from the animals in order that we randomly sampled different aged houses (~4 h). This was done because it is known that house filtration efficiency varies with time post inflation (Acuña and Kiefer, 2000). Thus, the ingestion rates measured represent averaged snapshots over the lifetime of a house. The animals were then transferred to fresh 4 L beakers with 1000 cells mL^{-1} of *Isochrysis sp.* and 1000 000 cells mL^{-1} of *Escherichia coli* stain HT115 (Timmons *et al.*, 2001) as prey for 5 min. Immediately following food incubation, animals were quickly transferred to new 4 L beakers with 0.2 μm filtered sea water for rinsing, transferred again to a 3 mL deep cavity staining block (Hecht Assistant, 2020/1), removed from their houses, anaesthetized in 0.125 mg mL^{-1} MS222 and finally rinsed with freshly filtered sea water. The animals were pooled in 1.5 mL Eppendorf tubes, and quickly centrifuged to remove excess seawater. The animals were frozen in liquid nitrogen and stored at −80°C until further analysis. Capture and transfer of animals was accomplished using a wide bore pipette. The time from capture to anaesthesia was <2 min for each replicate. Gut evacuation was not detected during this period. Previous studies indicated that particle passage time from the inlet filter to the mouth was 10.4 ± 2.8 SD seconds and will therefore not significantly affect estimation of particle ingestion (Troedsson *et al.*, 2007a).

DNA was extracted and purified from the samples using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, including treatment with 4 μL of 100 μg/mL of RNase A for 10 min at 37°C. Primers for qPCR targeting *E. coli* were designed using the GeneTool Lite 1.0 (BioTools Inc.), while the primers targeting *Isochrysis sp.* were the same as previously described in Troedsson *et al.* (Troedsson *et al.*, 2007a) (Table II). Primers were evaluated using qPCR for specificity and quantification by comparing cross reaction with *O. dioica* and the two prey particles. All qPCR reactions for ingestion rate estimates were performed using 96-well plates with 12 μL of 2 × QuantiTech SYBR Green Master Mix (Qiagen), 0.8 μM of primers and 5 μL extracted total genomic DNA from *O. dioica*. Amplification included an initial 15 min at 95°C followed by 40 amplification cycles [94°C, 15 s; annealing, 30 s (Table II); 72°C, 60 s] and finally a 10 min elongation step at 72°C. Primer specificities were confirmed by melting curve analysis. All real time qPCR reactions investigating ingestion rates were run using the MJResearch Opticon® 2 real-time thermal cycler. Cycle threshold (C_t) values obtained from total DNA extracted from appendicularian tissues were compared to standard curves prepared from known numbers of algal and bacterial cells such that prey abundance associated with appendicularian tissues could be expressed as cells mL^{-1}.

The rational behind these experiments was to compare animals conditioned to standard versus limited food regimes for large (5 μm) prey sizes and separately for small prey sizes (1 μm). To test this, the ingestions were analysed using a two-factor ANOVA (Statistica 8.0; StatSoft, Inc) with ingestion as a dependent variable, food and age as the categorical predictors (after transforming data to fulfil the criteria for homogeneity of variance) for the two particle sizes separately. To further evaluate any difference after the ANOVA, a multiple pairwise comparison (*post hoc*, unequal N-HSD) was performed on data from *Isochrysis sp.* feeding, while on data for *E. coli* feeding a pairwise comparison with age as a factor was performed (because food regimes were not significant).

RESULTS

House renewal rate

Regression lines were fitted to the HRR over age and none of the slopes were significant (standard food, $P = 0.76$; limited food, $P = 0.62$). This indicates that there was no change in HRR throughout development. Further, as no significant slopes were found, a one-factor ANOVA was performed with food as the categorical predictor yielding no significant difference ($F_{(1,43)} = 1.05$; $P = 0.31$) after verification of homogeneity of variance (Levene's test; $F_{(1,43)} = 0.93$; $P = 0.34$), indicating that the food regimes tested had no effect on

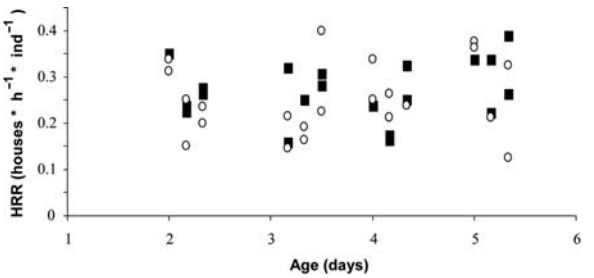

Fig. 2. House production at 15°C throughout development of *O. dioica*. Measurements were done on animals raised in a standard (filled squares) versus a limited (open circles) food regime. The rate is given as the number of houses per hour. There where no significant difference between the two food regimes. There were also no significant changes in HRR throughout development.

HRR (Fig. 2). The pooled mean HRR was found to be 0.26 ± 0.07SD houses h^{-1} at 15°C and 30 psu, corresponding to approximately 44 ± 12 SD houses in a 7 day generation time (Troedsson *et al.*, 2002). These estimates are consistent with previous independent results of Sato *et al.* (Sato *et al.*, 2001), estimating 47 ± 9.9 SD houses in a generation time. That there was no change in HRR throughout development was also confirmed by the steady state mRNA levels of oikosins 3 and 7 relative to RpL23, a general marker for cellular metabolism, indicating that investment in growth and house production had a linked relationship at the gene transcriptional level (data not shown).

Differential regulation of oikosin genes in response to limited food

As no difference was observed in HRR as a function of food availability, we next examined whether this environmental factor exerted any effects on house composition, particularly with respect to the food-concentrating filter. The food-concentrating filter is a complex three-dimensional structure that is constantly expanding and collapsing as a result of the pumping action of the tail. Consequently, it is not trivial to obtain repetitive morphometric measurements that would accurately reflect alterations of this structure as a function of food regime. Instead, we developed molecular tools to assess potential differential investment in house structures as a function of nutritional regime. We evaluated both mRNA and protein levels of oikosin 3, a component of the fine meshes of the food-concentrating filter (Sagane Y and Thompson EM, unpublished), compared to oikosin 7, that has an expression pattern characteristic of a protein implicated in building the outer wall of the house.

No significant regression slopes were fitted to the data for oikosin 3 (standard food, $P = 0.73$; limited food, $P = 0.74$) or oikosin 7 as a function of age

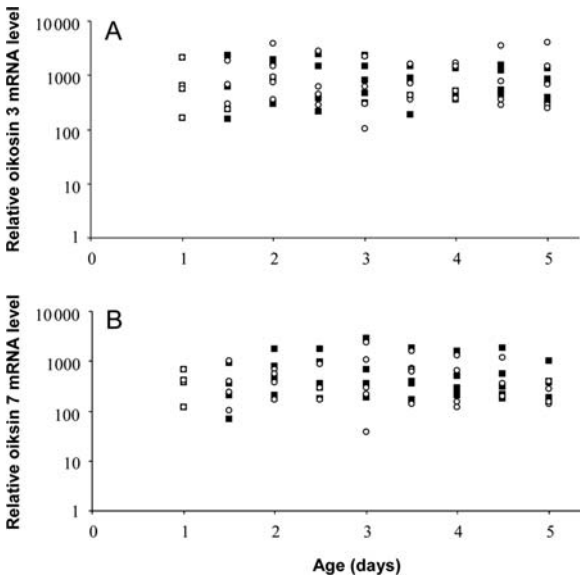

Fig. 3. Relative mRNA expression level of oikosin 3 (**A**) and oikosin 7 (**B**) compared with RpL23 which is a general marker for cellular metabolism. There were no significant differences between the food regimes, nor was there an effect of time post-fertilization. Measurements were done on animals raised in a standard (filled squares) versus a limited (open circles) food regime.

(standard food, $P = 0.90$; limited food, $P = 0.98$) indicating that there was no change in the house transcripts relative to RpL23 throughout development. Because no significant slopes were found, a one-factor ANOVA was performed with food as the categorical predictor yielding no significant difference, indicating that the food regimes tested here had no effect on the relative oikosin 3 ($F_{(1,81)} = 0.26$; $P = 0.61$) or oikosin 7 ($F_{(1,79)} = 0.53$; $P = 0.47$) mRNA levels (Fig. 3). Tests were performed after verification of homogeneity of variance by Levene's test for log-transformed oikosin 3 mRNA level ($F_{(1,81)} = 0.1.05$; $P = 0.31$) and oikosin 7 mRNA level ($F_{(1,79)} = 1.18$; $P = 0.28$). Thus, there were no detectable differences in oikosin transcriptional levels in response to different food regimes.

To test whether there was any differential regulation at the translational level, we performed quantitative analyses of the oikosin protein content between two food regimes throughout development. In the representative western blots shown in the top panel (Fig. 4), the multiple closely spaced bands revealed by the anti-oikosin 7 antibody reflect the polymorphism previously described for this protein (Thompson *et al.*, 2001). There was a significant change over time in the relative oikosin 7 to oikosin 3 expression level between the two food regimes (Table III). Additional multiple pairwise comparison (*post hoc*, unequal N-HSD) revealed that there was a significant difference between the food

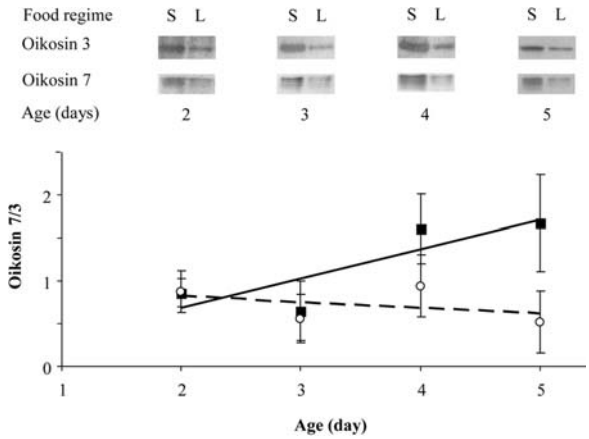

Fig. 4. Variation in to the normalized oikosin 7 to oikosin 3 protein expression level under standard (filled squares, solid line) and limited (open circles, broken line) food regimes with age. The top panel shows representative western blots used to determine the protein levels of oikosins 3 and 7. Standard deviations are indicated by bars.

Table III: Two-factor ANOVA with normalized Oikosin 7 to Oikosin 3 protein expression level as the dependent variable and food regime and age as the categorical predictor

	SS	df	MS	*F*	*P*
Age	1.849	3	0.616	3.883	0.019
Food	1.865	1	1.865	11.748	0.002
Food*Age	2.336	3	0.779	4.906	0.007
Error	4.604	29	0.159		

Tests were preformed after verification of homogeneity of variance by a Levene's test: oikosin 7 to oiksin 3 level; MS effect=0.059, MS error=0.039, $F = 1.502$, $P = 0.206$.

regimes at day 5 and further between the standard food regime at day 4 and limited food regime at day 5 (Table IV). This change at the protein level showed that the relative production of these two oikosins varied as a function of food concentration.

Ingestion rate

To understand whether changes in the relative house components as a function of feeding history had an effect on ingestion rate, we investigated the ingestion rate of two different sized prey particles. There was a significant increase in the ingestion rate of *Isochrysis sp.* by animals raised in a standard food regime compared to a limited food regime (Fig. 5A; Table V). However, the difference in food regime was not consistent over age and multiple pairwise comparisons revealed that at day 5, ingestion of *Isochrysis sp.* was significantly higher in animals raised in standard food compared to all

Table IV: Pairwise multiple comparison (post hoc, unequal-N HSD) on the effect of food regimes and age on the normalized oikosin 7 to oikosin 3 protein expression level

	2d, S	2d, L	3d, S	3d, L	4d, S	4d, L	5d, S	5d, L
2d, S		1.00	0.99	0.98	0.18	1.00	0.12	0.92
2d, L			0.99	0.97	0.20	1.00	0.13	0.90
3d, S				1.00	0.04	0.99	0.02	1.00
3d, L					0.05	0.93	0.03	1.00
4d, S						0.46	1.00	0.01
4d, L							0.35	0.90
5d, S								<0.001
5d, L								

Table is given with age (e.g. day 2=2d) and food regimes (S=Standard and L=Limited).

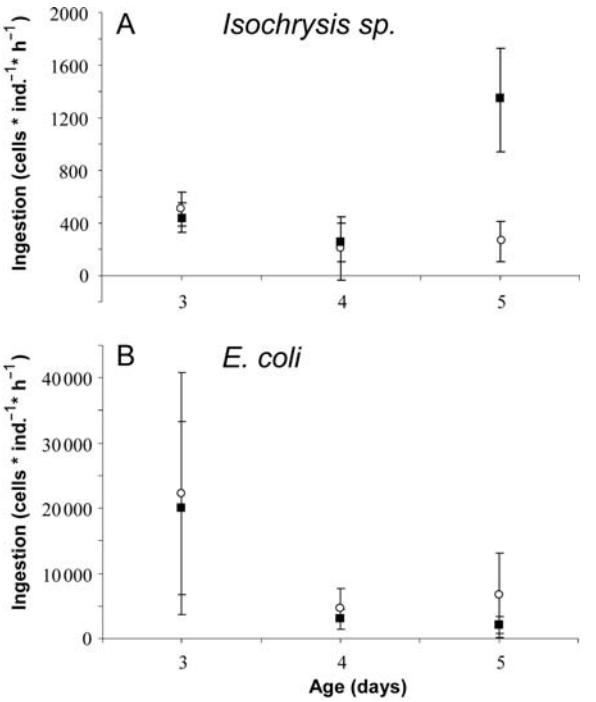

Fig. 5. Ingestion rate estimates determined in *O. dioica* cultured under standard (filled squares) and limited (open circles) food regimes on a mixed diet of *Isochrysis sp.* and *E. coli*. (**A**) Ingestion rate estimates of *Isochrysis sp.* at 3, 4 and 5 days post-fertilization. There was a significant difference in ingestion rates between animals cultured under the two food conditions at Day 5 (Table IV). (**B**) Ingestion rate estimates of *E. coli* at 3, 4 and 5 days post-fertilization. There was no effect of feeding history on ingestion, while there was a significant decrease of ingestion in both food regimes from Day 3 to Day 4 (Table IV).

other groups (Table VI). Furthermore, there were no significant differences in ingestion rates of *E. coli* as a function of feeding history (Fig. 5B; Table V). Nonetheless, a *post hoc* (unequal N-HSD) comparison in the ANOVA for age revealed a significant decrease in

Table V: Two-factor ANOVA with log-transformed ingestion rates as the dependent variable, food and age as the categorical predictors

	SS	df	MS	*F*	*P*
Isochrysis sp.					
Age	1.452	2	0.726	13.662	<0.001
Food	0.765	1	0.765	14.397	<0.001
Food*Age	1.016	2	0.508	9.558	<0.001
Error	1.488	28	0.053		
E. coli					
Age	3.680	2	1.840	17.053	<0.001
Food	0.280	1	0.280	2.596	0.119
Food*Age	0.300	2	0.150	1.388	0.267
Error	2.805	26	0.108		

Tests were preformed after verification of homogeneity of variance by a Levene's test: log-transformed *Isochrysis sp.* ingestion rate; MS effect = 0.034, MS error=0.014, $F = 2.370$, $P = 0.065$; log-transformed *E. coli* ingestion rate; MS effect = 0.012, MS error = 0.029, $F = 0.427$, $P = 0.826$.

Table VI: Pairwise multiple comparison (post hoc, unequal-N HSD) on the effect of food regimes and age on the ingestion rate of Isochrysis sp

	3d, S	3d, L	4d, S	4d, L	5d, S	5d, L
3d, S		1.00	0.40	0.02	0.01	0.37
3d, L			0.21	0.01	0.04	0.20
4d, S				0.77	<0.001	1.00
4d, L					<0.001	0.80
5d, S						<0.001
5d, L						

Table is given with age (e.g. day 2 = 2d) and food regimes (S = Standard and L = Limited).

E. coli ingestion after 3 days post-fertilization reflecting the increase in mesh size as the overall filtering house grows with age resulting in reduced filtration efficiency for these small particles (Table VII).

Table VII: Pairwise comparison (post hoc, unequal-N HSD) on the effect of age on ingestion of E. coli

	Day 3	Day 4	Day 5
Day 3		<0.001	<0.001
Day 4			0.96
Day 5			

DISCUSSION

The frequent renewal of filter-feeding houses, combined with the observation that each house represents a significant proportion of total body carbon, means that *Oikopleura dioica* invests considerable resources in its filter-feeding strategy. In response to changing nutrient conditions, it is conceivable that *O. dioica* could adapt its HRR, or modify filtering structures, or both. We found, however, that the mean HRR was 0.26 ± 0.07SD houses h^{-1} throughout the life cycle, and that this rate was maintained independent of food concentration (Fig. 2). It is possible that the complex cell cycle regulation involved in patterning and growth of the oikoplastic epithelium imposes constraints on the capacity to manipulate rates of house renewal (Ganot and Thompson, 2002). A fixed HRR at varying food regimes is in agreement with Acuña and Kiefer (Acuña and Kiefer, 2000) and Sato *et al.* (Sato *et al.*, 2001), using a similar culturing protocol, but contrasts with reports of a plastic response in the HRR by Fenaux (Fenaux, 1985) and Tiselius *et al.* (Tiselius *et al.*, 2003). The latter group used a different culture technique where short-term incubations were done on a plankton wheel. It is possible that the discrepancy in results is due to the short-term incubations of appendicularians. Indeed, appendicularians have several house rudiments covering the trunk ready to be inflated, and clogging of inlet filters due to high food can lead to increased short-term shredding and inflation of houses (personal observations). However, long-term incubations or careful monitoring of house rudiments covering the trunk would reveal any changes in house secretions for the oikoplastic epithelium, which would further indicate any plastic response in the energetic investment into the house structure. Our data do not support such a plastic response. Indeed, our data suggest a coupling to the general metabolism of the animals throughout the life cycle, as the expression level of the oikosin genes compared with the metabolic component RpL23 was fixed throughout development (Fig. 3). In previous studies, data suggest that there is a fixed somatic growth independent of food over a minimal threshold required for maintaining survival (Troedsson *et al.*, 2002). Our study has demonstrated that HRR is not responsive to different food regimes and further indicate that the transcriptional expression level of Oikosins over time when normalized to a general metabolic marker (RpL23) yields a close relationship. This suggests that there is a link between these two cellular components.

Although replacement rate of the filter-feeding house was non-responsive to different food concentrations, and a trade-off between investment in the house structure and reproductive output was not apparent, *O. dioica* could respond to varying nutritional regimes by changes in house morphology leading to alterations in filtering

efficiency. It has been proposed that the large surface of tunicate filters is an adaptation to low food concentrations (Harbison, 1992; Acuña, 2001). Interestingly, fresh water Daphniid crustaceans develop larger filters when grown at low food concentrations (Koza and Kořínek, 1985; Lampert, 1994; Lampert and Brendelberger, 1996). This is a plastic response, where the same genotype responds with different phenotypes depending on environmental conditions. The same theoretical principles that apply to the plastic response of Daphniid crustaceans may also be implicated in the gelatinous body of pelagic tunicates (Acuña, 2001). Considering the repeated energetic investment of appendicularian houses, a plastic response of house morphology might provide an efficient way of increasing the net energy gain from filtration in a low food environment.

We observed a differential regulation between the two house components oikosin 7 and oikosin 3 in animals raised in limiting compared with standard food regimes. This altered regulation was not apparent in mRNA levels (Fig. 3), but quantitative measures of respective protein levels (Fig. 4; Table III) as well as changes in ingestion rates (Fig. 5, Table V) support translational regulation of oikosins. This indicates that continuous incubation in different food regimes drives a plastic response in house morphology. The protein data suggest that the differential regulation as a function of food regime is predominantly significant at day 5, which is also supported by the ingestion rate data (Tables IV–VI). The observation that we could only observe differences in older animals indicates a lag response time and suggests that the animals may not respond immediately in patchy food environments.

To understand whether the plastic response of house protein components had an effect on feeding efficiency, we conducted a series of ingestion studies. The ingestion rate estimates indicated no significant difference in ingestion of small particles (*E. coli*) in animals that were raised in standard versus limited food regime (Fig. 5; Table V). Our data suggest a general reduction of ingestion rates of small particles from day 3 to day 5 due to the age-related shifts in pore size of the pharyngeal filter (Deibel and Lee, 1992) as it has been suggested that mesh size is relatively constant in relation to house size (Flood and Deibel, 1998). This is also consistent with independent studies indicating a reduction of retention efficiencies of 1 μm particles with size of *O. dioica* (Fernández *et al.*, 2004). Further, our data indicate that there is a general increase in ingestion of larger particles in the standard food regimes from day 3 to day 5. This is also consistent with independent studies suggesting an increased retention efficiency of 6 μm particles with increasing size (Fernández *et al.*, 2004). Curiously, Fernández *et al.* (Fernández *et al.*, 2004) reported a small tendency for reduced retention efficiency in 348 μm (trunk length) animals compared with 169 and 699 μm animals. Comparing age with previous growth data (Troedsson *et al.*, 2002), a 348 μm trunk size represents a day 4 animal and we also observed the same trend in our study. The biological mechanism behind this is not clear at this point, but it is possible that the increase in clearance rate with size (or age) does not compensate for the increase in pharyngeal mesh size at this age.

In conclusion, from the literature, it has been reported that food-concentrating-filter mesh size is relatively constant (Flood and Deibel, 1998) and that there is a negative correlation between retention efficiency and animal size (Fernández *et al.*, 2004). Further, previous data indicate that somatic and endostyle growth is not food-dependent (Troedsson *et al.*, 2002, 2007b) suggesting that pharyngeal filter size is also food-independent, which the data on *E. coli* indicate in our study. Our data further indicate a drive towards higher ingestion of *Isochrysis sp.* and a differential regulation of oikosin proteins in animals raised in the standard compared with the limiting food regime. An interpretation of the data is therefore that animals raised in the standard food regime produce a larger house than in the limiting food regime. A possible biological mechanism for this behaviour is that animals raised in the limited food save energy by producing a smaller house. A smaller house will yield the same ingestion rates of small particles as a larger house. There is, however, an energetic advantage to using larger prey particles during bloom situations, and increased house size will yield higher ingestion rates of large particles. The additional energy investment in producing a larger house is overcompensated by the increased ingestion rates. However, further experiments will have to be performed to investigate this hypothesis.

In contrast to the terrestrial environment, filter-feeding is common in the aquatic milieu. Our data indicate that one prominent filter-feeding species *O. dioica* can make small changes in its filter structure as a response to varying nutritional levels. The appendicularian house is among the most complex extra cellular structures produced by an organism and we now have an array of molecular tools to aid in the understanding of its assembly. The development of isogenic strains of *O. dioica* coupled with the controlled culture conditions already established (Bouquet *et al.*, 2009) should permit increased understanding of the interactions of environmental forcing factors and this elegant filtering structure at both the genetic and phenotypic levels. This should allow improved insight into the evolution and

optimization of this important way of making a living in the often dilute nutrient conditions of the oceans.

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