The Journal of Experimental Biology 201, 2857–2865 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1593

FUNCTIONAL PARTITIONING OF ENERGY RESERVES BY LARVAE OF THE MARINE BRYOZOAN *BUGULA NERITINA* (L.)

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Accepted 27 July; published on WWW 22 September 1998

Summary

The effects of extended swimming on short-lived lecithotrophic larvae of the marine bryozoan *Bugula neritina* (L.) were examined. Larvae were forced to swim for 2 or 24 h by bath application of serotonin. Settlement and metamorphosis success were significantly reduced, larval dimensions were unaffected and ancestrulae were smaller after 24 h of swimming. Larvae settled predominantly on seawater-conditioned glass after 2 h, but became less discriminative after 24 h.

Lipid content in intact larvae and dissociated surface ciliated and interior cell fractions was analysed by thin-layer chromatography. Hydrophilic lipids were unaffected by swimming regime. The hydrophobic fraction contained triglyceride, confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) analysis and correlation spectroscopy (^{1}H-^{1}H COSY) patterns, which was significantly depleted after 24 h, and diacylglycerol, which was not. NMR spectra suggested no differences in fatty acid chain compositions between larvae swimming for 2 and 24 h. Triglyceride depletion was limited to the ciliated cell fraction.

We propose that the functional partitioning of lipid reserves has evolved in association with the costs and benefits linked with larval dispersal.

Key words: larval swimming, settlement, energetics, lipid, cell dissociation, thin-layer chromatography, Bryozoa, *Bugula neritina*.

Introduction

The free-living larvae of marine invertebrates demonstrate extreme variation in strategies employed to detect and secure suitable settlement substrata. Such variation is reflected in the range of larval morphologies observed and procedures of settlement-site procurement. The energy source, or sources, utilised by larvae to fuel their free-living existence will, to a certain extent depend on the developmental mode, the environmental conditions, the duration of larval lifetime and the behaviour patterns exhibited therein. Information linking the biochemical and physiological changes which occur during major life-history events is scarce. Many sessile marine invertebrates living in the shallow sublittoral zone produce non-feeding larvae, which swim for a brief period before they locate a suitable settlement site, where irreversible transformation to the adult stage occurs. The duration of the free-living larval stage depends on both physical and ecological factors (Crisp, 1974; Woollacott, 1984). However, since these larvae are dependent on limited energy reserves, the duration of larval life is essentially finite.

The cheilostomatid bryozoan *Bugula neritina* (L.) is an erect, dendritic form, which produces short-lived lecithotrophic larvae that are the presumed result of outcrossing (Silén, 1972). *B. neritina* is a cosmopolitan species, economically important through the impact of both biofouling (Gordon and Mawatari, 1992) and in the development of novel anti-carcinogenic pharmaceuticals (see Newman, 1996). Embryos are brooded within ovicells for approximately 3 weeks, where nutrition is provided by the parental autozooid by means of a rudimentary placenta (Woollacott and Zimmer, 1975). Under natural conditions, *B. neritina* larvae have been found to settle rapidly following release (Mawatari, 1951; Hunter and Fusetani, 1996), usually within 2–6 h, presumably in response to abundant field settlement cues. However, laboratory studies have demonstrated that these larvae can swim for 12 h or more while

still retaining competence for settlement and metamorphosis (Jaeckle, 1994; Wendt, 1996). Jaeckle (1994) suggested that the planktonic life of *B. neritina* larvae is regulated principally by the amount of endogenous energy stores, modulated by the ability of the larvae to absorb dissolved organic materials from sea water. However, both Jaeckle's (1994) work and a study of larvae of the ascophoran bryozoan *Celleporella hyalina* (Orellana *et al.* 1996) have demonstrated that bryozoan larvae contain total energy reserves surpassing their expected requirements even following an extended free-living existence. Since settlement is usually rapid under field conditions, the concept of bryozoan larval duration being constrained by total energy reserves becomes increasingly untenable (Orellana *et al.* 1996).

Hunter and Fusetani (1996) found that artificial prolongation of *B. neritina* larval swimming by mechanical agitation resulted in larvae with reduced settlement and metamorphosis capability when settlement followed a swimming period of over 6 h. In addition, larvae that had swum for 8–10 h produced primary zooids (ancestrulae) of reduced overall size, while feeding organ (lophophore) dimensions were unaffected. A subsequent study (E. Hunter, K. Shimizu and N. Fusetani, unpublished data) confirmed the results of these swimming experiments and also demonstrated that the effects of extended swimming were not propagated in adult colonies when metamorphosis was successfully completed. In a similar study, but using different methods, Wendt (1996) found that larval swimming duration did not show any significant effect on the numbers of larvae undergoing settlement and metamorphosis until 16 h had elapsed. Cystid variables were not measured in the latter study; however, lophophore size did decline as a function of enforced swimming time following swimming times in excess of 8 h.

Similar results have been reported for related *B. stolonifera* larvae (Woollacott *et al.* 1989), whose immediate post-metamorphic growth rate was negatively affected by mechanically enforced swimming periods in excess of 6 h. Negative impacts on growth potential have also been noted following non-enforced extended swimming by bryozoan larvae (Nielsen, 1981; Orellana and Cancino, 1991; Orellana *et al.* 1996). These and the above studies suggest that a framework may operate whereby the fuelling of larval swimming is partitioned such that the negative impacts of extended free-living existence are restricted to specific life-history events. Hunter and Fusetani (1996) have proposed that for *B. neritina* this is a means by which farther-dispersing larvae minimise the costs associated with isolation. Keough (1984) has shown that *B. neritina* larvae not only settle rapidly, but settle preferentially in clumps of related individuals. Keough (1989) was further able to demonstrate that the presence of adult colonies had no negative effect on the growth of proximally settling colonies under field conditions and proposed that clumped settlement of siblings provided a means to minimise the impact of predation by fishes. Furthermore, there appears to be a trade-off between larval swimming time and metamorphosis capability, such that extended larval swimming time is inversely related to metamorphosis success (Hunter and Fusetani, 1996; Wendt, 1996). E. Hunter, K. Shimizu and N. Fusetani (unpublished data) found no evidence that the negative effects of prolonged larval swimming were perpetuated in adult colonies. Since the majority of *B. neritina* larvae choose to settle within 3 h, this suggests a selective advantage to rapid settlement; however, by minimising the loss of energy reserves available to the developing ancestrula, it is possible that a small proportion of larvae may persist, increasing the fitness of resultant colonies by taking advantage of the benefits associated with extended dispersal (Crisp, 1974).

Bryozoan larvae comprise an outer layer of transitory larval tissues and an inner reserve of cells destined for incorporation into the post-larval body (Woollacott and Zimmer, 1971). Zimmer and Woollacott (1977) suggested that a protracted larval existence would decrease the energy content of larval transitory cells, in turn reducing the energy available to the developing juvenile. However, the deleterious effects reported following a prolonged larval existence, as documented above, are pertinent principally to major ontogenetic events, such as settlement and metamorphosis. We propose that energy depletion from larvae is not generalised, but that localised depletion of larval energy reserves occurs within larval tissues. Such a localised effect would be almost impossible to measure, especially where larvae have been treated as homogeneous energetic units (Jaeckle, 1994; Orellana *et al.* 1996). Even small differences in biochemical composition in such studies would be masked by variability in total larval volume. Support for this idea arises from the lack of impact on post-metamorphosis colony growth of extended larval swimming (E. Hunter, K. Shimizu and N. Fusetani, unpublished data) suggesting that, while protracted larval lifetime may reduce settlement success, energy stores reserved for post-settlement growth remain largely untapped, at least below a certain threshold (Hunter and Fusetani, 1996; Wendt, 1996).

Bath application of the monoamine neurotransmitter serotonin (5-HT) has been found to inhibit settlement of *B. neritina* larvae (K. Shimizu, E. Hunter and N. Fusetani, unpublished data). This provided a means during the present experiments whereby semi-natural swimming patterns could be retained in larvae, while greatly extending their free-living phase. This innovation, coupled with recently developed cell dissociation techniques (Okano *et al.* 1996) allowed us to examine the energetics of larval swimming both in intact larvae and within larval tissues. Dissociation allows the partitioning of the surface ciliated cells responsible for larval swimming (Okano *et al.* 1996) from the interior cell fraction, which is mainly involved in structural reorganisation following settlement. Ciliated cells contain many refractile granules, which may be lipid in content (Okano *et al.* 1996). Large energy reserves are also presumed to be located within the larval interior, notably in the parenchymal tissue (Woollacott and Zimmer, 1971; Okano *et al.* 1996). Protein metabolism by *B. neritina* during larval swimming has been discounted (E. Hunter, K. Shimizu and N. Fusetani, unpublished data), and carbohydrates have been reported to occur at low levels in larval tissues (Woollacott and Zimmer, 1971; Loeb and Walker, 1977). We decided, therefore, to make a detailed analysis of the

role of lipids in larval swimming. Given the partitioning of larval and post-larval tissues, we hypothesised that this dichotomy would also be mirrored in the energy pool, such that energy reserves allocated to swimming did not encroach on those supplies reserved for post-settlement growth.

Materials and methods

Biological material

Mature *Bugula neritina* (L.) colonies bearing ovicells were collected from floating rafts at Aburatsubo and Nagai on the Miura Peninsula, Eastern Japan (35°20′N 139°60′W) during spring 1996. These were transported to the laboratory in cooled insulated boxes, where they were transferred to 200 l tanks containing vigorously aerated, coarsely filtered running sea water at 20±1 °C. The cryptophyte alga *Rhodomonas* sp. was added three times weekly to enrich the semi-natural diet (Hunter and Hughes, 1993). This regime allowed the production of large quantities of larvae for up to 2 weeks, following which colonies were discarded and replaced.

Colonies to be used for larval release were transferred to 10 l aquaria containing filtered (2 μm) sea water and placed in total darkness at least 12 h before experimentation. Larval release was stimulated by exposure to bright overhead light (Ryland, 1959). All experiments were performed at a water temperature of 20±1 °C. Larvae were collected by pipette 1 h after exposure and were considered to be 1 h old at this stage, irrespective of the exact time of larval release.

Enforced larval swimming

Samples containing approximately 2000 larvae were transferred immediately to 500 ml beakers containing 10^{-4} mol l^{-1} serotonin (5-HT) (Sigma Chemical Company, St Louis, MO, USA) solution prepared in filtered sea water (FSW), where they were forced to swim for 1 h or 23 h (2 h and 24 h total swimming time, respectively). These swimming times were chosen on the basis of settlement rates of non-manipulated Japanese *B. neritina* larvae. Hunter and Fusetani (1996) have shown that 52 % of larvae settle within 2 h of release, with 98 % settlement occurring by 8 h. Although 24 h is well in excess of expected settling times within this population, we wanted to compare a semi-natural settlement time with a greatly extended one. The 24 h swimming period also fell within swimming times reported from other studies, beyond which settlement and metamorphosis capability was retained (Keough, 1989; Jaeckle, 1994; Wendt, 1996).

From each batch of larvae, three replicate samples of 60–215 larvae (129±64; mean ± S.D.) were removed after 2 h and 24 h for settlement trials, and further samples of approximately 500 larvae were removed for lipid analysis. Two further subsamples of approximately 50 larvae from each condition were randomly selected and transferred to a 4 % formalin solution in FSW, from which larval diameter measurements were taken (N=50) using an inverted microscope (Nikon TMD300) equipped with a CCD TV camera (Hitachi).

Larvae for settlement trials were washed twice in FSW then transferred to Petri dishes containing glass slides conditioned in coarsely filtered running sea water for 2 weeks, where they were allowed to settle. Settled and metamorphosed larvae were counted after 3 days. Metamorphosis was considered to be complete only on formation of an active, feeding lophophore. A note was made of whether larvae had settled on the conditioned glass slide (S), the untreated glass surface (G) or at the air–water interface (W). Where possible, ancestrular dimensions were recorded using an inverted dissection microscope.

Larval and post-larval dimensions after 2 and 24 h of enforced swimming were compared using Student's t-tests, while settlement and metamorphosis success and settlement location were compared using log–linear analysis (analysis of deviance). Values are presented as means ± S.D.

Lipid extraction

Lipid extraction was performed using the methods of Bligh and Dyer (1959) with some modification. Following 2 or 24 h swimming times, batches of 500–1000 larvae were cooled slowly on ice to prevent further movement. Larvae were counted and transferred to glass centrifugation tubes containing chilled Van't Hoff artificial sea water (ASW) (460 mmol l^{-1} NaCl, 10.1 mmol l^{-1} KCl, 9.2 mmol l^{-1} $CaCl_2$, 35.9 mmol l^{-1} $MgCl_2$, 17.5 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} Hepes; pH adjusted with 1 mol l^{-1} NaOH to 7.7), where they were left undisturbed for 30 min at 4 °C. All ASW was then removed, and 2 ml of cold methanol, 1 ml of chloroform and 0.8 ml of distilled water were added sequentially. The solution was mixed by vortexing followed by ultrasonication to destroy larval tissue structure and to ensure complete lipid extraction. Samples were then stored in the chloroform–methanol–water solution at −30 °C for between 1 week and 6 months in light-protected sealed tubes.

The solution was next separated from tissue debris by centrifugation (Hitachi 05 PR-22, 3500 revs min^{-1} or 2300 ***g*** for 10 min). To the collected supernatant, 1 ml of chloroform and 1 ml of distilled water were added, and the mixture was vortexed. The lower layer containing lipids was then collected and evaporated, following which lipids were dissolved in chloroform. The solution contained 1–2 larvae μl^{-1} chloroform. The extracted lipids were then ready for analysis by thin-layer chromatography (TLC) (see below).

Isolation of cell fractions

Isolation of the ciliated and interior cell fractions was performed using methods reported previously (Okano *et al.* 1996). Following enforced swimming, larvae were slowly cooled over ice to stop further movement. The larvae were then transferred to Mg^{2+}-substituted nominally Ca^{2+}-free sea water (MSCF; containing 462 mmol l^{-1} NaCl, 8 mmol l^{-1} KCl, 32 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes, pH adjusted with 1 mol l^{-1} Tris base to 7.7) (for details, see Okano *et al.* 1996) for 5–10 min. Larvae were then transferred to a solution containing 5 mg ml^{-1} preactivated papain (Okano *et al.* 1996). Dissociating larvae were monitored using a dissection microscope. After 3 h, most ciliated cells had dissociated and were swimming vigorously in solution, leaving denuded phase 2 larvae,

composed mainly of interior cells (Okano *et al.* 1996). These were collected by pipette and washed with MSCF, and lipid extraction was performed as described above. The residual solutions containing ciliated cells were combined and diluted with cold MSCF, then maintained for 30 min at 4 °C to reduce ciliary action. Following 5 min of centrifugation (2300 ***g***) at 4 °C, the ciliated cells were pelleted and were then ready for analysis.

Thin-layer chromatography

Commercially available thin-layer chromatography (TLC) plates (Merck silica gel plate 60 F254) were used in all experiments. The following lipids were used as standards: monoglyceride mixture (MG, lipid standard), stearic acid (FA standard, C18:0), L-α-phosphatidyl L-serine (PS, from bovine brain), L-α-phosphatidylethanolamine (PE, dipalmitoyl), L-α-phosphatidylcholine distearoyl (PC, C18:0), 1,2-dioleoyl-3-stearoyl-rac-glycerol (triglyceride, TG, C18:1, cis-9/C-18:1, cis-9/C18:0), 1,3-diolein (DG, C18:1, cis-9). Chemical standards were obtained from Sigma.

The solvent system used to analyse levels of phospholipids, MG and FA was composed of chloroform:methanol:acetic acid:water (50:10:2:1). Two other solvent systems employed for the more hydrophobic lipids, notably diglycerides and triglycerides, were composed of (1) chloroform, and (2) hexane:ether:acetic acid (80:30:1). Each plate consisted of 6–8 lanes, to which lipid extracts (in chloroform solution) were applied (equivalent to 1–2 larvae for whole-larva samples or 3–6 larvae for cell-fraction samples and standard lipids). Visualisation and quantification of lipid classes were achieved using molybdophosphoric acid. The resulting spot densities were analysed using a TLC scanner (CS-9000, Shimadzu Corp., Kyoto, Japan).

In the course of preliminary experiments, the relationship between the quantity of TG and DG and their corresponding spot densities was investigated. The 'background subtraction' function of the TLC scanner was used to remove baseline drifts. No significant differences were observed between default and manually set values. Spot densities were less than 0.5 units (arbitrary machine units), which was considered to be adequately linear, indicating that baseline changes would not influence the quantification of DG and TG levels. Quantification of experimental samples was therefore performed by two consecutive TLC trials (1) to set the sample concentration in the appropriate quantitative range and (2) to compare actual density values with those of standards. Quantities presented are equivalent to the same quantity of standard producing the same staining intensity. The areas corresponding to TG and DG were extracted and eluted with chloroform. After thorough drying, purified lipids were dissolved in $CDCl_3$ and analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR; Bruker ARX-500). NMR spectra were obtained under the following operating conditions: field strength 500.13 MHz; bandwidth 7142.86 Hz; data points 16 384; resolution 0.4 Hz; eight repetitions. For correlation spectroscopy (^{1}H-^{1}H COSY; Bruker ARX-500) analysis, the following conditions were used: pulse programme COSY45; accumulation data points 2k×512; sweep width 4505 Hz; 16 repetitions; repetition time 1000 ms; flip angle 45 °. Values are presented as means ± S.D.

Results

Effect of swimming time on larval settlement and metamorphosis

After 2 h, 95.9±2.3 % of larvae were able to settle compared with only 59.2±11.4 % after 24 h (P<0.001, Table 1; Fig. 1). Of those larvae that settled after 2 h, 87.2±12.1 % went on to complete metamorphosis compared with only 54.73±17.5 % after 24 h (P<0.05, Table 1; Fig. 1). No significant differences in mean larval length (2 h, 323.2±17.7 μm, N=47; 24 h, 322.6±17.7 μm, N=47; Student's t-test, P>0.05) or larval diameter (2 h, 292.3±15.7 μm; 24 h, 293.2±16.9 μm, Student's t-test, P>0.05) were observed. However, following a 24 h swimming period, the mean ancestrular length (850±4.9 μm, N=58) was significantly smaller than that reached by the 2 h swim group (882±11.4 μm, N=60) (Student's t-test, P<0.05). It was further noted that settlement locations differed significantly between the two groups (Fig. 2; Table 2). Following a 2 h swimming period, larvae settled predominantly on the conditioned glass surface (81.4±15.2 %), and a significant portion of larvae settled at the air–water interface (13.7±18.6 %). Following the extended swimming time, larvae appeared less discriminative, with only 65.2±13.9 % of larvae settling on conditioned glass, while no larvae settled at the air–water interface.

Larval lipid composition

A comparison of the hydrophilic and hydrophobic lipid fractions extracted from *B. neritina* larvae after 2 and 24 h of enforced swimming is shown in Fig. 3. The hydrophilic lipid composition was relatively complex (Fig. 3A) and included spots corresponding to the fatty acids monoacylglycerol (MG), phosphatidylethanolamine (PE) and phosphatidylserine (PS). However, comparison of the lipid traces between larvae

Table 1. *Statistical comparison (analysis of variance) of the proportions of settled and metamorphosed larvae following 2 or 24 h of enforced swimming in 10^{-4} mol l^{-1} serotonin solution in filtered seawater*

	Deviance	d.f.	P
Replicate	153.3	5	0.043
Settlement	107.6	1	0.008
Swim time×Settlement	289.8	1	0.001
Residual	18.35	4	
Replicate	183.9	5	0.087
Metamorphosis	161.3	1	0.012
Swim time×Metamorphosis	67.5	1	0.046
Residual	33.08	4	

The metamorphosis group included only those larvae that settled successfully.

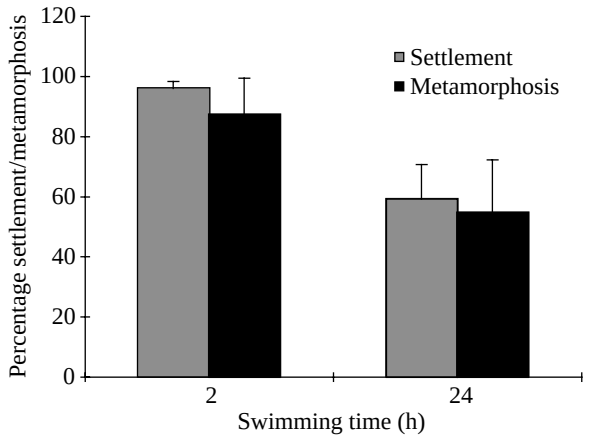

Fig. 1. The effect of swimming time on larval settlement and metamorphosis of *Bugula neritina*. Each bar represents the mean percentage settlement or metamorphosis (± S.D.) from three replicate samples for 60–215 larvae for each swimming time (129±63.8; mean ± S.D.). The metamorphosis group includes only those larvae that settled successfully.

swimming for 2 and 24 h demonstrated no significant changes in overall composition (Fig. 3A).

In contrast, only two broad conspicuous spots were observed in the hydrophobic fraction (Fig. 3B), the most hydrophobic of which was noticeably depleted following 24 h of enforced swimming. The flow rate (R_f) values of this spot (R_f=0.58–0.73 with chloroform as a solvent, R_f=0.5–0.55 with hexane:ether:acetic acid, 80:30:1, as a solvent) corresponded to those of the 1,2-dioleoyl-3-stearoyl-rac-glycerol (C18:1, cis-9/C-18:1,cis-9/C18:0) which was used as a standard for triglyceride (TG) (R_f=0.63–0.77 for chloroform as a solvent, R_f=0.52 for hexane:ether:acetic acid, 80:30:1). The less hydrophobic band corresponded to 1,3-diolein (DG), which was unaffected by the duration of enforced swimming.

Although R_f values provided strong evidence that TG was the primary candidate for the depleted lipid, the broadness of the spots meant that three other possibilities had to be investigated: (1) that the lipid was not TG despite similar R_f values; (2) that the spot contained a mixture of lipids other than TG; and (3) that, even if the spot was composed of TG, a specific fatty acid component of TG might have been the primary energy source during enforced swimming.

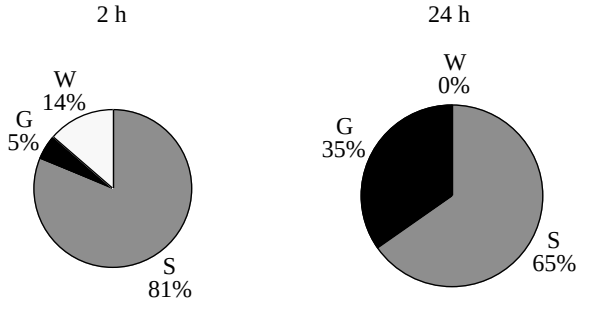

Fig. 2. Percentage of total settled larvae on seawater-conditioned glass slides (S), untreated glass (G) and at the air–water interface (W) following 2 and 24 h of enforced swimming time in 10^{-4} mol l^{-1} serotonin solution in filtered sea water. N=350 larvae (2 h) and 233 larvae (24 h).

Table 2. *Statistical comparison of the proportions of larvae settling on different substrata following 2 or 24 h of enforced swimming in 10^{-4} mol l^{-1} serotonin solution in filtered sea water*

	Deviance	d.f.	*F*	*P*
Replicate	153.3	5	4.1	0.021
Site	439.8	3	19.5	<0.001
Swim time×Site	324.4	3	14.4	<0.001
Residual	90.22	12	4	

Therefore, to confirm and characterize the identity of the depleted spot and to estimate its purity, large-scale preparations of those spots from the 2 h and 24 h treated lipid samples were obtained by preparative TLC. The purified samples were then analyzed using ^{1}H-NMR (Fig. 4). The NMR spectrum in both swimming treatments indicated the presence of glycerol proton chemical shifts, in both cases at 4.12 p.p.m. (double doublet, 2H), 4.27 p.p.m. (double doublet, 2H) and 5.24 p.p.m. (multiplet, ^{1}H), identical to that of 1,2-dioleoyl-3-stearoyl-rac-glycerol (C18:1, cis-9/C-18:1,cis-9/C18:0). The correlation spectroscopy (^{1}H-^{1}H COSY) patterns of these protons (see inset in Fig. 4) were identical to that of the standard irrespective of swimming time. The major spectral differences between the larval TG and the 1,2-dioleoyl-3-stearoyl-rac-glycerol were (1) the number of olefinic protons at 5.3–5.4 p.p.m. and (2) the presence of methylene groups at 2.8 p.p.m. Depleted lipids had approximately 10 olefinic protons (10.4 for 2 h, 9.0 for 24 h), indicating that the mean number of double bonds per triglyceride molecule was five in each case.

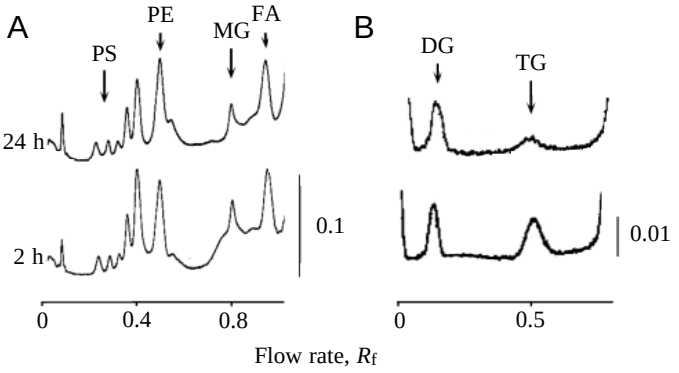

Fig. 3. Comparison of (A) hydrophilic and (B) hydrophobic lipid fractions extracted from *Bugula neritina* larvae after 2 and 24 h of enforced swimming in 10^{-4} mol l^{-1} serotonin solution in filtered sea water using thin-layer chromatography. (A) MG, monoglyceride mixture; FA, stearic acid (standard for fatty acid); PS, L-α-phosphatidyl L-serine; PE, L-α-phosphatidylethanolamine (standard for dipalmitoyl). (B) Solvent chloroform. TG, 1,2-dioleoyl-3-stearoyl-rac-glycerol; DG, 1,3-diolein. The ordinate gives spot densities in arbitrary units. The abscissa gives flow rate (R_f) values.

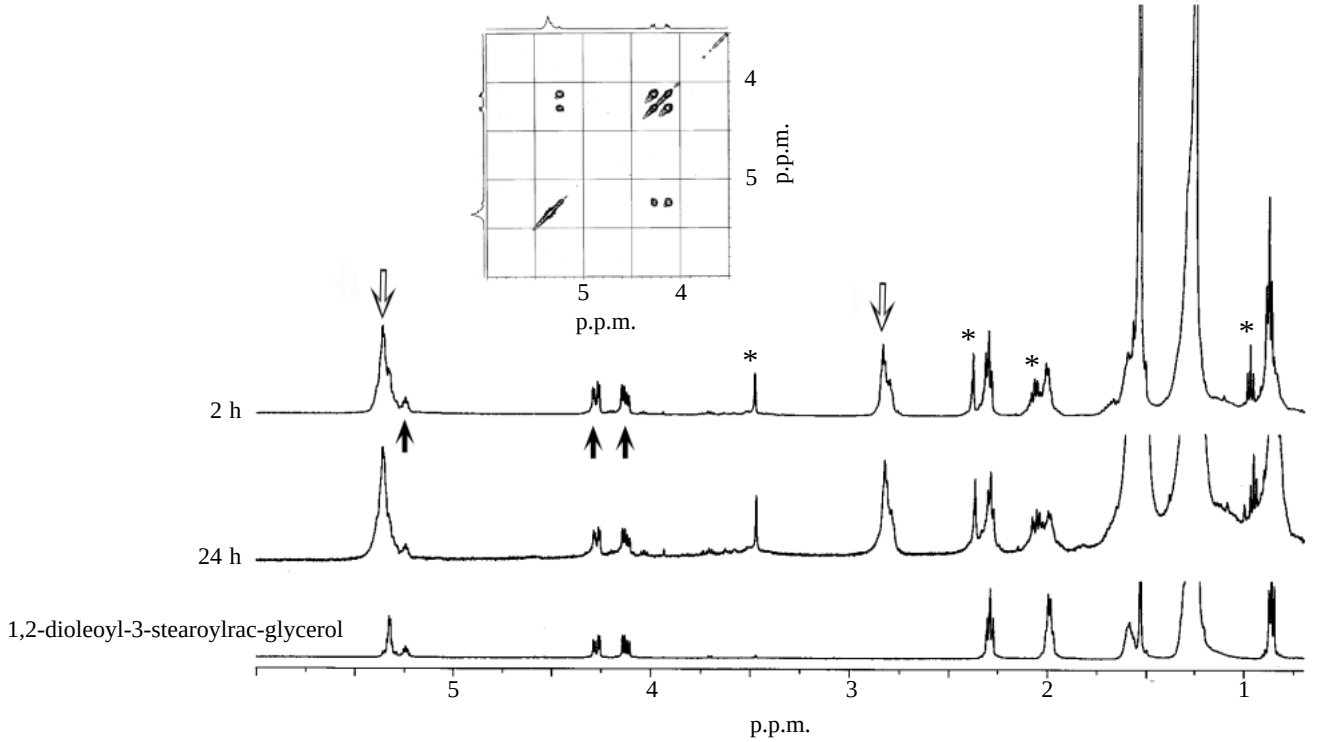

Fig. 4. Proton nuclear magnetic resonance (^{1}H-NMR) spectra from *Bugula neritina* larvae after 2 and 24 h of enforced swimming in 10^{-4} mol l^{-1} serotonin solution in filtered sea water. 1,2-dioleoyl-3-stearoyl-rac-glycerol was used as a standard for triglyceride. Filled arrows indicate signals from glycerol protons (4.12, 4.27 and 5.24 p.p.m.). Open arrows indicate signals from olefinic protons (5.3–5.4 p.p.m.) and methylene protons flanked by double bonds (2.8 p.p.m.). An asterisk indicates impurities from the preparative thin-layer chromatography plates. Residual chloroform was used as a chemical shift standard (7.24 p.p.m.). Inset: correlation spectroscopy (COSY) analysis of glycerol protons from the 2 h sample.

The peaks observed at 0.9 and 2.1 (ethyl groups), 2.4 and 3.5 p.p.m. were believed to represent impurities from the preparative TLC, since extraction from preparative TLC plates without the addition of any test sample caused the same peaks. These results provide clear evidence that the principal component of the depleted spot was TG. Furthermore, the NMR spectra did not suggest differences in fatty acid chain compositions between larvae swimming for 2 h and 24 h.

Table 3. *Comparison of diglyceride and triglyceride content in intact larvae, surface ciliated cells and interior cells of* Bugula neritina *larvae following 2 h and 24 h of enforced swimming in 10^{-4} mol l^{-1} serotonin solution in filtered sea water*

	2 h swim			24 h swim		
	Intact	Ciliated	Interior	Intact	Ciliated	Interior
Diglyceride (ng larva^{-1})						
1	192	31	53	174	36	57
2	182	36	46	193	42	54
3		43	41		32	42
4		43			33	
Mean±S.D.	187±7.1	38.3±5.9	46.7±6.0	183.5±13.4	35.8±4.5	51±7.9
Triglyceride (ng larva^{-1})						
1	493	73	180	305	21	148
2	456	61	146	169	15	135
3		149	143		66	136
4		113			43	
Mean±S.D.	474.5±26.1	99±40.1	156±20.6	237±96.2*	36.25±23.2*	139.7±7.2

Asterisks indicate significant differences (P<0.05) between the 24 h and 2 h mean values.

Larval lipid content

Following quantification of spots obtained by molybdophospholic acid staining, DG and TG contents of *Bugula neritina* larvae were measured using a Shimadzu flying spot scanner (Table 3). Total TG content per intact larva was reduced by approximately 50 % after an extended larval swimming period, from 474.5±26.1 ng larva^{-1} after 2 h to 237±96.2 ng larva^{-1} after 24 h (P<0.05, Table 3), while DG levels remained relatively constant (P=0.31, Table 3).

Significant depletion of DG was not observed from either the ciliated cell fraction or the interior cell fraction (Table 3). TG content from the ciliated cell fraction was depleted from 99±40.1 ng larva^{-1} after 2 h to 36.25±23.2 ng larva^{-1} after 24 h of swimming (P<0.05, Table 3); however, a similar decrease was not observed from the interior cell fraction, which showed a non-significant decrease from 156.3±20.6 ng larva^{-1} after 2 h to 139.7±7.2 ng larva^{-1} (P=0.13, Table 3).

Discussion

When settlement was prevented by bath application of serotonin, the resulting greatly extended larval lifetime of 24 h had the following physical effects. Settlement success was significantly reduced from 96 % after 2 h to 59 %. Metamorphosis success was reduced from 87 % to 55 %. Larval dimensions appeared to be unaffected by enforced swimming; however, the resultant ancestrulae were significantly smaller after 24 h. In general, these results confirmed previous studies using Japanese *Bugula neritina* larvae in which larval settlement was prevented using mechanical agitation (Hunter and Fusetani, 1996; E. Hunter, K. Shimizu and N. Fusetani, unpublished data). These studies reported impaired metamorphosis success (E. Hunter, K. Shimizu and N. Fusetani, unpublished data) and reduced zooid size (Hunter and Fusetani, 1996) after enforced swimming times in excess of 6 h.

The prevention of settlement by the application of serotonin provides a more biologically realistic situation in that larvae swim in a manner directly comparable with that observed in freshly released larvae, at least in terms of surface contact time (E. Hunter, K. Okano and N. Fusetani, unpublished data). The number of temporary attachments to substrata has been suggested to represent a potential and unrecorded energy drain during larval swimming (E. Hunter, K. Shimizu and N. Fusetani, unpublished data). Assuming that larvae also swim continuously in response to prolonged fluorescent illumination, the present results should be directly comparable with those of Wendt (1996), who used *B. neritina* larvae from Cambridge, MA, USA, which were prevented from settlement by continuous exposure to fluorescent light. Wendt (1996) sampled larvae at 1 h intervals, following which settlement was induced by the application of KCl. Wendt (1996) reported significant decreases in settlement initiation (from 88 % to 60 %) and in the percentage of larvae going on to complete metamorphosis (from 85 % to 55 %) after 16 h. It would appear, therefore, that larvae forced to swim by the bath application of serotonin demonstrate biologically realistic swimming activity and are, therefore, metabolically comparable with naturally occurring larvae.

Previous experiments examining the ciliary behaviour of isolated *B. neritina* cells (K. Okano, E. Hunter and N. Fusetani, unpublished data) have demonstrated effective concentrations of 5-HT in the range 10^{-5} to 10^{-7} mol l^{-1}, while comparable effects in intact individuals were observed only at 10^{-4} mol l^{-1}. These results suggested the presence of a diffusion barrier to the penetration of larvae by 5-HT. Effects of 5-HT on *B. neritina* behaviour have been observed at similar concentrations in other studies (Pires and Woollacott, 1997; K. Shimizu, E. Hunter and N. Fusetani, unpublished data). To separate the effects of 5-HT from the effects of swimming, preliminary experiments were carried out using cooled (immobilised) larvae. However, prolonged cooling caused deciliation and degradation of larvae, making a concise evaluation of the effects of 5-HT impossible. However, although we cannot exclude the possibility that 5-HT had a stimulatory effect on lipid metabolism, the similarity of our data to those of Wendt (1996) and the localised time-dependent lipid depletion observed suggest that this was not the case.

Behavioural differences were observed following a swimming time of 24 h. Freshly released larvae demonstrate a preference for glass surfaces which have been conditioned previously in sea water, allowing the formation of a bacterial film (Kitamura and Hirayama, 1987; Hunter and Fusetani, 1996). While 2-h-old larvae chose to settle predominantly on the conditioned glass surface, 24 h swimmers appeared to become less discriminating, with 30 % more larvae settling on unconditioned glass. In settlement experiments, young non-feeding barnacle *Balanus amphitrite* cyprid larvae are reported as being discriminating with regards to settlement site (Rittschof *et al.* 1984; Crisp, 1988) but become less able to discriminate with age, presumably because depletion of energy reserves may reduce their chances of successful metamorphosis (Lucas *et al.* 1979) or may compromise their post-metamorphic growth (Pechenik *et al.* 1993; Satuito *et al.* 1996). Reduced chances of metamorphosis success would clearly apply to *B. neritina*; however, post-metamorphic growth rates have been demonstrated to be unaffected by extended larval swimming time (E. Hunter, K. Shimizu and N. Fusetani, unpublished data). In addition, a significant proportion of larvae (14 %) settled at the air–water interface following 2 h of swimming, whereas no settlement was observed at the water surface following 24 h of swimming, suggesting that these larvae may have experienced difficulty in maintaining their position in the water column.

Holland (1978) suggested that quantity and ease of mobilisation make lipids the primary candidates for the major energy reserve in marine invertebrate larvae. Protein has been ruled out as an energy source for *B. neritina* larvae (E. Hunter, K. Shimizu and N. Fusetani, unpublished data). We found that lipid depletion was restricted to the hydrophobic lipids; the R_f values corresponded to those expected for triglyceride (TG) compounds. This was confirmed by comparison of the NMR spectra obtained from the 24 h swimming sample with those obtained from the 2 h sample and the TG standard. The similarity of the spectra (Fig. 4) indicated that the decrease in

staining intensity following extended swimming reflected a decrease in the quantity of TG present. Although we cannot exclude the possibility that some of the minor hydrophilic lipids are used during enforced swimming, any such depletion was insignificant in comparison with that of TG. The neutral lipids that form the principal energy reserve in cyprids of the barnacle *Balanus balanoides* (Holland and Walker, 1975) are made up predominantly of triglycerides (Waldock and Holland, 1978). Triglyceride levels decreased from 8 % of total dry mass in cyprids to only 2 % following metamorphosis (Waldock and Holland, 1978).

Although it was our intention only to determine the class or classes of lipids involved in larval metabolism in *B. neritina*, comparison of the NMR spectra (Fig. 4) revealed some interesting features of the depleted lipids. The major spectral differences between the larval TG and the 1,2-dioleoyl-3-stearoyl-rac-glycerol were (1) the number of olefinic protons at 5.3–5.4 p.p.m. and (2) the presence of methylene groups at 2.8 p.p.m.. Depleted lipids had approximately 10 olefinic protons (10.4 for 2 h, 9.0 for 24 h), indicating that the mean number of double bonds per triglyceride molecule was five in each case. The presence of 6–8 methylene groups at 2.8 p.p.m. suggested that most of these double bonds were flanked by a methylene group.

Having identified the principal larval energy source as TG, we were then able to investigate whether depletion of TG was localised within larval tissues as predicted. Coronal cells are vigorously active cigar-shaped ciliated cells which cover 90 % of the outer larval body (Woollacott and Zimmer, 1971; Okano *et al.* 1996). They are known to contain many refractile granules which may be lipid in content (Okano *et al.* 1996). Parenchymal tissue from the inner cell fraction is also known to contain large quantities of yolk-like material (Woollacott and Zimmer, 1971; Okano *et al.* 1996). Separate analyses on the surface ciliated cell fraction and interior cell fraction showed that both tissues contained TG; however, only TG from the ciliated cell fraction was depleted significantly following extended swimming.

In Wendt's (1996) study, settlement rates levelled off after 16 h of swimming, while successful completion of metamorphosis continued to decrease. It is suggested that a threshold occurs at approximately 16 h of free swimming (or 6–8 h during mechanical agitation; E. Hunter, K. Shimizu and N. Fusetani, unpublished data), at which coronal triglyceride reserves become exhausted and internal reserves come into play. We are unable from the present results to demonstrate which compounds are responsible for the reduction in autozooid size following extended swimming, although it seems reasonable to speculate that lipid depletion must have a role. Further work will be required to demonstrate whether the two distinct TG sources (from surface ciliated cells or interior cells) correspond directly to larval and post-larval reserves. Wendt (1996) suggested that it is metamorphosis, not settlement, that is dependent on energy reserves, and therefore on swimming time. Our results are largely in agreement with this statement; however, we have provided further evidence that post-metamorphosis growth rates are largely independent of swimming time.

While recognising that bryozoan larvae are composites of larval and post-larval tissues (Jaeckle, 1994; Orellana *et al.* 1996), previous biochemical analyses have treated larvae as homogeneous energetic units. Orellana *et al.* (1996) found that the total energy reserves (both lipid and protein) in larvae of *Celleporella hyalina* clearly surpassed total requirements, even after protracted larval swimming, and concluded that larval duration must be limited by factors other than energy supply. Assuming a similar situation for *B. neritina*, whose larvae usually settle within hours of release from the maternal brooding chamber, this suggests that under most natural conditions it is unlikely that *B. neritina* larvae would either exhaust endogenous reserves or have to utilize alternative extrinsic energy sources as suggested by Jaeckle (1994). While a range of larvae, including those of *B. neritina*, demonstrate the ability to take up and metabolize dissolved organic materials (Jaeckle, 1994), there is as yet no clear evidence that they do this as a matter of routine (Manahan, 1990). Indeed, Jaeckle's (1994) inferred potential duration of larval lifetime [based on 34 % of total energy reserves as proposed by Lucas *et al.* (1979) for cyprid larvae of the barnacle *Balanus balanoides*] of 12 h even before active uptake of metabolites is still well in excess of the probable larval duration for *B. neritina* (Hunter and Fusetani, 1996). However, the partitioning of energy reserves as demonstrated in the present study does illustrate a potential situation in naturally occurring biological confines within which energy reserves might become limiting.

The present study has demonstrated that, following a greatly extended larval swimming time effected by the application of 5-HT, larval settlement rates are reduced, the successful metamorphosis of settled individuals is impaired and larvae become less discriminative regarding settlement site. When metamorphosis was successful, the ancestrulae produced were of reduced overall size. We have demonstrated that these effects are at least partially due to the depletion of TG and that this depletion was limited to the outer layer of ciliated cells, which function only during the larval phase of the life cycle. We propose that the partitioning of lipid reserves between larval and post-larval tissue components ensures that, even following a long swimming duration, larvae that successfully settle and complete metamorphosis will have sufficient energy reserves to take advantage of the benefits of enhanced dispersion (Crisp, 1974), increased outcrossing potential (Keough, 1984) and colonisation of unexploited habitat (Keough, 1987). As far as we are aware, this is the first demonstration of functional partitioning of energy reserves within the tissues of a non-feeding marine invertebrate larva.

We wish to extend our thanks to M. Yamazaki and K. Natoyama who maintained algal cultures throughout the duration of this work. We are grateful to the staff of Tokyo University Marine Station, Misakiguchi, Japan, who helped in the collection of specimens, and M. Nicholson and D. Maxwell (CEFAS Laboratory, Lowestoft, UK), who provided guidance in the statistical analysis of the results. Drs K. Shimizu, H. Hirota, C. G. Satuito and S. Kawaii and Professor R. N. Hughes provided useful discussions and advice.

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