

The relative energetic costs of the larval period, larval swimming and metamorphosis for the ascidian *Diplosoma listerianum*

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Abstract

Variation in larval quality has been shown to strongly affect the post-metamorphic performance of a wide range of marine invertebrate species. Extending the larval period of non-feeding larvae strongly affects post-metamorphic survival and growth in a range of species. These 'carry-over' effects are assumed to be due to changes in larval energetic reserves but direct tests are surprisingly rare. Here, we examine the energetic costs (relative to the costs of metamorphosis) of extending the larval period of the colonial ascidian *Diplosoma listerianum*. We also manipulated larval activity levels and compared the energy consumption rates of swimming larvae and inactive larvae. Larval swimming was, energetically, very costly relative to either metamorphosis or merely extending the larval period. At least 25% of the larval energetic reserves are available for larval swimming but metamorphosis was relatively inexpensive in this species and larval reserves can be used for post-metamorphic growth. The carry-over effects previously observed in this species appear to be nutritionally mediated and even short (<3 h) periods of larval swimming can significantly deplete larval energy reserves.

Keywords: Larva, ascidian, metamorphosis, energy, metabolism, delayed metamorphosis

Introduction

For a wide variety of marine invertebrates and some fish, events during the larval period can have profound effects on adult performance (Marshall et al. 2003a,b; Pechenik et al. 1998; Shima & Findlay 2002; Wendt 1998). Effects of larval nutrition, exposure to pollutants, length of the larval period, and larval size have all been shown to 'carry-over' to affect adult survival, growth, and even reproduction (Marshall et al. 2003a,b; Ng & Keough 2003; Phillips 2002). For species with non-feeding larvae, the length of the larval period is thought

to affect adult performance because the larval stage depletes nutritional resources that are required after metamorphosis (Maldonado & Young 1999; Marshall et al. 2003b; Wendt 1996, 2000). There are several indirect lines of evidence that support this suggestion. Larger larvae (presumably with greater nutritional reserves) swim for longer than smaller larvae in the absence of appropriate settlement cues suggesting that larval nutritional reserves are depleted by swimming (Marshall & Keough 2003). Furthermore, delaying the metamorphosis of feeding larvae (that can continue to replenish their energetic stores) does not affect the performance of the adults as severely as delays of non-feeding larvae (Highsmith & Emler 1986). Consequently, most effects of delayed metamorphosis are interpreted as driven by energetic effects (Gebauer et al. 1999; Maldonado & Young 1999; Marshall et al. 2003b; Pechenik & Cerulli 1991; Pechenik & Eyster 1988; Pechenik et al. 1998; Vaitilingon et al. 2001).

Despite being essentially an energetic argument, there have been few direct measures of the energetic costs of extending the larval period to support the suggestion that swimming is significantly costly, especially in species with non-feeding larvae (Moran & Manahan 2003). Some studies have shown that larval swimming consumes little of the energetic reserves relative to those consumed during metamorphosis suggesting that in some species, larval swimming is not extremely costly (Wendt 2000). However, in those same species, strong effects of larval swimming on adult performance have been found (Wendt 1998). More tests of the relative costs of larval swimming and metamorphosis are needed to determine if the carry-over effects of larval swimming are due to energetic depletion or some other mechanism.

One species in which strong carry-over effects of delayed metamorphosis have been observed is the colonial ascidian *D. listerianum* (Marshall et al. 2003b). Relatively small increases in the larval period strongly affect adult growth in this species and larval activity levels during the larval period determine the strength of the carry-over effects (Marshall et al. 2003b). Like all ascidian larvae, *D. listerianum* have tadpole larvae with large tails for locomotion (Young & Svane 1989). Swimming with a tail may be particularly costly relative to other types of locomotory structures such as cilia. Alternatively, the surprisingly strong effects of extending the larval period in this species may be due to *D. listerianum* larvae having high metabolic rates, regardless of swimming activity. *Diplosoma listerianum* is an ideal species for examining the effects of larval activity levels because larval swimming activity can be manipulated by placing larvae in constant light or alternating between light and shadow (Young & Chia 1985; Young & Svane 1989). When a shadow passes over the larvae, they swim vigorously for up to 20 s after the shadow and this response can be induced repeatedly for up to 3 h (Marshall et al. 2003b). We manipulated the larval period and larval activity levels of *D. listerianum* larvae and compared their energetic contents (measured here as oxidizable carbon, indicative of available energy) to those of newly released larvae and newly metamorphosed larvae. Given larval swimming can be extremely costly in terms of adult performance in this species, we also examined whether larvae adjust the amount of swimming activity as they age.

Methods

Collection and manipulation of larvae

We obtained *D. listerianum* larvae from reproductively mature colonies that were growing on perspex plates at Breakwater pier, Williamstown (Melbourne). The perspex plates were brought in to the laboratory and maintained in constant darkness for at least 24 h.

The colonies ($n=20$) were exposed to bright light and we collected the larvae that were released. Colonies generally began to release larvae within an hour of exposure to bright light. Collected larvae were then randomly allocated into one of four treatment groups: No Delay, Metamorphosed, Delayed, and Swimming.

For larvae allocated to the 'No Delay' treatment, aliquots of newly spawned larvae were transferred to chilled (approximately 4°C) 0.2 µm filtered seawater to slow swimming activity. This was an effective method of slowing swimming activity, and larvae swam normally when returned to room temperature seawater. They were then immediately rinsed twice in distilled water and placed into pre-ashed glass vials (50 larvae/vial × 4 vials). All water was removed from the vials, and the vials were frozen at -20°C until required for analyses.

For larvae allocated to the 'Metamorphosed' treatment, larvae were placed onto glass petri dishes containing filtered seawater and were allowed to metamorphose immediately. The metamorphosing larvae were maintained at 22°C. After 18 h, the metamorphosing larvae had almost completed metamorphosis but had not yet begun to feed. We then carefully removed the metamorphs from the glass surface (they were not strongly adhered) rinsed the larvae twice in distilled water and frozen in pre-ashed glass vials (5 larvae/vial × 3 vials) as described above.

For larvae allocated to the 'Delayed' treatment, individual larvae were pipetted into their own sterile cell culture well containing 1 mL of 0.2 µm filtered seawater. The larvae were then exposed to continuous bright light, which inhibits the metamorphosis of *D. listerianum* larvae but the larvae remain quiescent under bright light (Marshall et al. 2003b). We maintained the larvae under the light for 3 h, after which we removed larvae, rinsed them twice in distilled water and then froze them as described above (5 larvae/vial × 5 vials).

For larvae allocated to the 'Swimming' treatment, larvae were treated in the same manner as those in the delayed treatment except the cell culture wells were placed under an alternating bright light/shade regime. Larvae were exposed to light:dark cycle of 75 s under light, 5 s in the dark. This regime causes larvae to swim vigorously throughout the 3 h period (Marshall et al. 2003b; Young & Chia 1985). After 3 h of this light/dark cycle the larvae were rinsed twice in distilled water and then frozen as described above (5 larvae/vial × 4 vials).

Comparison of swimming activity

We were interested in whether larvae decreased their swimming activity in the presence of a constant stimulus (i.e. repetitive shadows) over time. Therefore, for larvae in the Swimming treatment we examined the length of time over which larvae swam once the light went out 30 min after larvae were spawned ($n=9$) and 120 min after the larvae were spawned ($n=6$). Larvae were chosen at random each time, and the length of the subsequent swimming period was observed.

Dichromate oxidation

Samples were analysed using the dichromate micro-oxidation technique as described by Parsons et al. (1984) and according to the recommendations described by Gosselin & Qian (1998) with several modifications. All glassware to be used in the storage of larvae and during the assay were pre-ashed at 550°C for 4 h to remove the residual organic material.

Reagents

Acid dichromate oxidant was prepared by dissolving 0.08 g potassium dichromate in 4 mL distilled water. This was added dropwise to 196 mL AR grade sulfuric acid. The oxidant reagent was prepared immediately prior to use.

Cadmium iodide (CdI_2) starch reagent was prepared by dissolving 1.4 g CdI_2 in approximately 70 mL distilled water and boiling for 20 min. Soluble starch (0.33 g) was dissolved in about 70 mL distilled water and boiled for 5 min. The two solutions were combined and subsequently boiled for 5 min. The CdI_2 -starch solution was passed through a 0.22 micron glass fiber filter, and the solution was brought to 500 mL with distilled water. The solution is stable for several months if stored in the dark at room temperature.

Standard curve

AR grade glucose (1 g glucose = 0.4 g carbon) was dissolved in distilled water and serially diluted to the desired concentration for the standard curve range (e.g. 0, 4, 8, 12, 16, 20 $\mu\text{g C mL}^{-1}$). The standard curve was repeated in triplicate and values averaged for the calculation of the standard curve equation. There was a strong relationship between glucose concentration and absorbance with little variation about the curve ($R^2 = 0.991$, S.E. of estimate: 0.023).

Oxidation

The addition of phosphoric acid prior to the oxidation step was omitted from the procedure, as this was found to be ineffective in the removal of chlorine from the analysis (Gosselin & Qian 1999). One mL acid dichromate was dispensed from a syringe dispenser to each standard tube and each sample tube. Tubes were left to stand at room temperature for 5 min to allow the acid-water reaction to subside and cool. Tubes were then incubated at 115°C for 15 min, and then left to cool for 5 min. Tubes were then mixed, and incubated for a further 15 min at 115°C. The volume of each tube was brought to 7 mL with distilled water (5 mL in standard tubes, 6 mL in sample tubes). Aliquots of 0.5 mL of the oxidized solution were then transferred to a new set of clean tubes.

Colorimetry

Four mL of CdI_2 -starch reagent was sequentially added to the new aliquots of oxidized solution at fixed time intervals (e.g. every 20 s) and mixed thoroughly. Tubes were incubated for 20 min at room temperature, and 6 mL of distilled water was then added to the first tube and mixed, this was repeated for the remaining tubes at a similar 20 s interval.

Spectrophotometer readings were taken immediately afterward at 575 nm wavelength in a 1-cm cuvette. Readings were taken in the same sequence as processed in the colorimetry step, and at approximately 40 s time intervals.

Calculation of energy requirement

Amount of carbon ($\mu\text{g C}$ /individual larva) was calculated according to the equation of the glucose standard curve. The amount of organic carbon was converted to the equivalent joules using the conversion $1 \mu\text{g C} = 3.95 \times 10^{-2}$ Joules (Gosselin & Qian 1999).

Data analysis

Following initial ANOVA on the effects of the treatment on the energetic content of the larvae, we tested the effects of delaying/swimming of larvae and the metamorphosis of larvae with planned comparisons (Quinn & Keough 2002). First, we compared the energetic contents of larvae that had not been delayed to metamorphosed settlers. There was no significant difference between these groups ($F_{1,13} = 1.58$, $P = 0.231$). Second, we compared the energetic content of delayed larvae to swimming larvae, which were significantly different (see Results). We then separately compared swimming larvae and delayed larvae to the pooled no delay and metamorphosed larvae. Each planned comparison used the error term from the original ANOVA and in most cases 1-tailed tests were used because we expected swimming larvae to have less energy than non-delayed or delayed larvae.

Results

Increasing the activity levels of *D. listerianum* larvae greatly reduced their energetic content (Table I, Figure 1). Swimming was by far the most energetically expensive process with a reduction in the energetic content of individual larvae of 31 mJ (~24% of total energetic reserves). There was a significant difference between the energy content of larvae that had been swimming compared to the pooled non-delayed and metamorphosed groups and compared to delay group (Table I). There was no significant difference between the energetic contents of pooled non-delayed and metamorphosed larvae and that of delayed larvae (Table I).

Larvae that had been in the Swimming treatment for 120 min decreased the length of time for which they swam compared to larvae that had been swimming for only 30 min ($F_{1,13} = 5.03$, $P = 0.043$, Figure 2).

Discussion

Larval swimming consumed more carbon than metamorphosis or increasing the larval duration in *D. listerianum* larvae. Swimming for 3 h consumed almost a quarter of the energetic reserves of the larvae. Most of the difference in energetic contents was due to larval swimming rather than the basal metabolism of larvae. These results support previous studies on this species that show that larval swimming impinges on post-metamorphic performance more strongly than delayed metamorphosis alone (Marshall et al. 2003b). Furthermore, given the surprisingly large reduction in energetic content due to larval

Table I. ANOVA and planned comparisons for the effect of larval swimming duration and settlement on energetic content of individual *D. listerianum* larvae.

Source	df	MS	F	P
Treatment	3	654	4.07	0.031
Error	13	160		
Planned comparisons*				
Undelay vs settlers				0.231
Undelay & Settlers vs Swimming				0.004
Undelay & Settlers vs Delay				0.138
Swimming vs Delay				0.011

* All planned comparisons were done using 1, 13 degrees of freedom and 1-tailed tests.

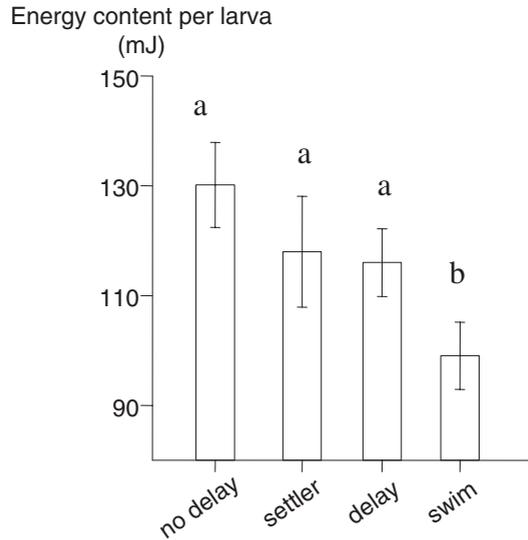


Figure 1. Mean energetic contents (mJ Larvae⁻¹ ± S.E.) of *D. listerianum* larvae that were newly released, metamorphosed, 3 h old or had been swimming for 3 h. Different letters indicate significance differences using *post-hoc*, planned comparisons.

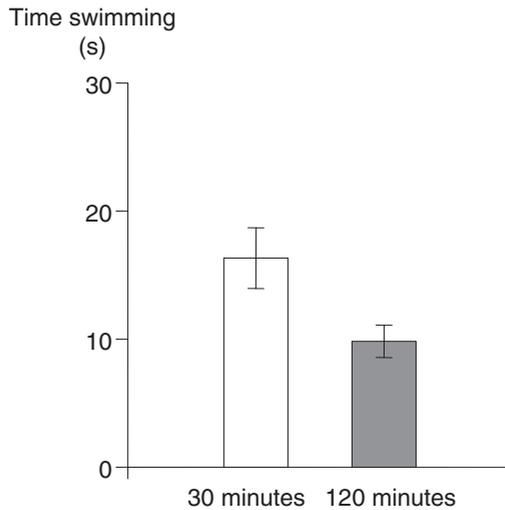


Figure 2. Mean time spent swimming (± S.E.) of *D. listerianum* larvae responding to a shadow. Open bar represents larvae that were 30 min old, shaded bar represent larvae that were 2 h old.

swimming, it is unsurprising that increased larval swimming causes reductions in the size of juvenile feeding structures in *D. listerianum* (Marshall et al. 2003b). Comparing our results to those of other studies, the percentage of energetic reserves utilised per hour in *D. listerianum* is much higher (4.5–10% per hour) than those of non-feeding bryozoan larvae (Table II). Given that *D. listerianum* larvae can swim for at least 6 h (Marshall et al. 2003b), a large proportion of energy may go into larval swimming relative to other species (e.g. Wendt 2000: 7–26% of energy reserves are consumed during the ‘maximal’ swimming period of

Table II. Comparison of energy consumption rates of swimming larvae and metamorphosis for marine invertebrate larvae.

Species	Swimming (% total h ⁻¹)	Metamorphosis (% total)	Study
<i>Bugula neritina</i>	0.36	28	(Wendt 2000)
<i>Bugula simplex</i>	0.84	40	(Wendt 2000)
<i>Bugula stolonifera</i>	1.6	52	(Wendt 2000)
<i>Balanus amphitrite</i>	–	38–58	(Thiyagarajan et al. 2003)
<i>Haliotis rufescens</i>	–	39	(Shilling et al. 1996)
<i>Ostrea chilensis</i>	–	64.5	(Videla et al. 1998)
<i>Diplosoma listerianum</i>	4.5–10	~10	(this study)

Bugula spp. larvae). These differences in energy consumption rates are most likely due to differences in locomotion. The use of a muscular tail in ascidian larvae is probably energetically expensive compared to other forms of locomotion, such as cilia used by bryozoan larvae, and therefore consumes more energy. However, metamorphosis was far less energetically costly in *D. listerianum* than for other species with non-feeding larvae (~10% change in energetic reserves compared to 28–65% for other species; Videla et al. 1998; Wendt 2000). These differences may be due to the fact that in this colonial ascidian species, the juvenile colony structures are relatively well developed in the tadpole larvae (Young & Svane 1989). Consequently, the metamorphosis of *D. listerianum* may be relatively less energetically expensive than for those organisms with more dramatic metamorphic events (e.g. bryozoans, bivalve larvae). It will be interesting to compare the costs of metamorphosis for other colonial ascidian species (with complex, well developed larvae) compared to that of solitary ascidians which typically have more simple, less developed larvae (Young & Svane 1989).

It appears that *D. listerianum* larvae are provisioned with far more energy reserves than necessary for metamorphosis. Larvae that were kept swimming for 3 h would still be capable of successful metamorphosis and growth in the field (Marshall et al. 2003b). These excess energy reserves may be used for increased larval swimming or post metamorphic growth, whereby increasing the former reduces the latter. It appears that as larvae age (and deplete their nutritional reserves) they decrease their energetic expenditure by reducing the duration of their 'shadow response.' Davis & Butler (1987) directly observed ascidian larvae in the field and found that those larvae that had just been released swam vigorously and continuously but older larvae drifted passively. It appears that larval behaviour is dynamic, and varies according to nutritional condition. If so, in the field, older larvae should behave more like passive particles or 'dropped eggs' (Keough & Downes 1982) whereas more actively swimming younger larvae should better influence their position in the water column.

The dichromate oxidation method has been used widely to quantify the amount of organic carbon in marine invertebrate larvae (McEdward 1997; McEdward & Carson 1987; McEdward & Coulter 1987). Limitations of the technique for marine samples, however, have been attributed to the apparent interference of chloride ions in the assay, including the amount within samples themselves (Gosselin & Qian 1998). In this study, we deliberately minimised the amount of organic material in each sample in order to exclude any excess chloride with the larval tissue, and our sample regime was assumed to pool across any potential effect chloride may have on the assay. It appears that if care is taken to minimise sample volumes and remove excess seawater, this technique can reliably estimate

organic carbon contents in small marine samples. The fact that our methods could distinguish between larvae that had been delayed and larvae that had been swimming for the same period suggests that this method is relatively sensitive across the range of the assay.

Overall, larval swimming strongly affects larval energetic reserves and increasing the larval period of *D. listerianum* has the potential to significantly decrease the energetic reserves of the larvae. Carry-over effects appear to be nutritionally mediated in this species. At least 25% of the total energetic reserves in this species are available for larval swimming but from previous results it appears that they can also be used for post-metamorphic growth (Marshall et al. 2003b). Other studies examining the effects of delayed metamorphosis in lecithotrophs have delayed metamorphosis for periods far greater than those used here (e.g. Wendt 1996, 1998). For *D. listerianum*, it appears that only a relatively short larval period is required for significant depletion of larval energetic reserves (Wendt 2000). Given that there is limited evidence for naturally occurring delayed metamorphosis in the field (Marshall & Keough 2003), shorter delays seem more likely than longer delays and mortality rates in the plankton are high (Morgan 1995), it seems likely that most periods of metamorphic delay in non-feeding larvae will be short. Therefore we suggest that delayed metamorphosis effects will be more prevalent in the field for ascidians than for other organisms where larval locomotion is less expensive; this remains to be tested.

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