REPRODUCTION, LARVAL GROWTH AND METAMORPHOSIS OF THE NUDIBRANCH MOLLUSCS "ONCHIDORIS BILAMELLATA" (L.) AND "GONIODORIS NODOSA" (MONTAGU)

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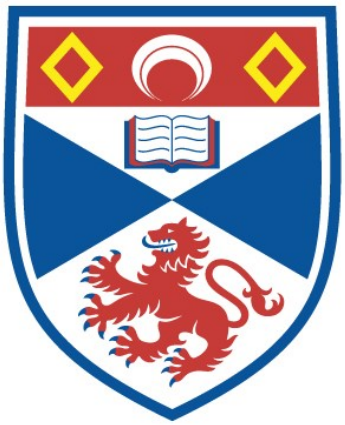

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Reproduction, Larval Growth and Metamorphosis of the Nudibranch Molluscs *Onchidoris bilamellata* (L.) and *Goniodoris nodosa* (Montagu).

by Isabel Lafuente

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at the University of St Andrews

School of Biological and Medical Sciences
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Signed Date 29th October 1997

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CONTENTS

ABSTRACT

This study investigated the reproduction, larval growth and metamorphosis of the nudibranchs *Onchidoris bilamellata* (L.) and *Goniodoris nodosa* (Montagu) under controlled laboratory conditions. In addition, the rare occurrence of spawning events of *O. bilamellata* taking place in the field outwith the winter and spring reproductive period was studied.

Onchidoris bilamellata and *Goniodoris nodosa* differ in the size and number of eggs produced per spawning event. In the present study *O. bilamellata* does not exhibit a clear spawning pattern, whereas *G. nodosa* lays increasingly smaller eggs and larvae as the season progresses. The diameter of the eggs of *O. bilamellata* is not correlated with the organic content per egg. The data available for *G. nodosa* were insufficient to analyse this relationship.

The rates of larval growth and development are reported for *Onchidoris bilamellata* and *Goniodoris nodosa*. These variables do not differ significantly for *O. bilamellata* between cultures maintained under various light regimes, ranging from continuous darkness to continuous illumination, and are comparable to those of *G. nodosa*. The shell growth pattern exhibited by larvae of *G. nodosa* is sigmoid, similar to that of other opisthobranchs.

Spawn masses laid during the winter and spring months were collected fresh from the field and compared to spawn masses collected from the field in July and September, outwith the typical spawning period of *Onchidoris bilamellata* in the British Isles. The shell size of the July and September hatchlings was significantly smaller than that of the winter and spring hatchlings. Furthermore, the larvae hatched from spawn masses laid in September cultured in the laboratory exhibited high mortality rates and the overwhelming majority did not survive through metamorphosis. The significance of the spawning activity of *O. bilamellata* past the typical spawning period of this species is discussed.

The induction of metamorphosis of *Onchidoris bilamellata* was investigated using seawater containing elevated concentrations of potassium ion, and the results indicate that the optimal concentration inductive of metamorphosis is 19 mM K^+ ASW. Metamorphosis experiments were also performed with the natural prey of *O. bilamellata*, the acorn barnacle *Semibalanus balanoides* (L.). The results suggest that pediveligers can be induced to metamorphose at a distance from the inductive substrata. Attempts were made to identify the natural inductive cue of *Goniodoris nodosa*, but this was not successful.

CHAPTER 1
GENERAL INTRODUCTION

Onchidoris bilamellata (L.) and *Goniodoris nodosa* (Montagu) are intertidal nudibranch molluscs common on the British coasts. *O. bilamellata* may reach 40 mm in length (Thompson & Brown, 1984), although 25 mm is more usual (Todd, 1979b), and feeds on species of the barnacles *Elminius* and *Chthamalus*, but especially *Semibalanus* (Thompson & Brown, 1984; Chia & Koss, 1988). *G. nodosa* (Montagu) has a smaller body size, reaching 27 mm in length (Fig. 1.1). In contrast with *O. bilamellata*, *G. nodosa* feeds upon encrusting bryozoans, such as *Alcyonidium polyoum* (Hassall), *Callopora dumerili* (Audouin) and *Flustrellidra hispida* (Fabricius) when young, whereas the adults feed preferentially on the ascidians *Diplosoma listerianum* (Milne Edwards), *Botryllus schlosseri* (Pallas) and *Dendrodoa grossularia* (van Beneden) (McMillan, 1942; Miller, 1961; Swennen, 1961).

These nudibranchs are primarily found in the intertidal zone in association with their prey items. *Onchidoris bilamellata* does not extend as high up the shore as its main prey species, *Semibalanus balanoides*, due to the desiccation risks inherent to this zone (Todd, 1979a), and is frequent in intertidal pools on the undersides of rocks, but it is also common at the lower tidal limit. *Goniodoris nodosa* is primarily located beneath rocks at the lower limits of the intertidal zone, but may also occur on *Fucus* sp. fronds on which the polyzoan and ascidian preys settle. Additionally, both of these nudibranchs can also be found subtidally at depths to 120 meters in their northernmost limits of distribution (Ohdner, 1939; Lemche, 1929).

The geographic distribution of *Onchidoris bilamellata* ranges from France to Norway, the White Sea and Spitzbergen, Iceland, Greenland, and the Atlantic and Pacific coasts of North America. *Goniodoris nodosa* is restricted to north-western Europe, from Norway and the Faeroes to the north west of Spain (Thompson & Brown, 1984). Both of these nudibranchs are common species in Britain.

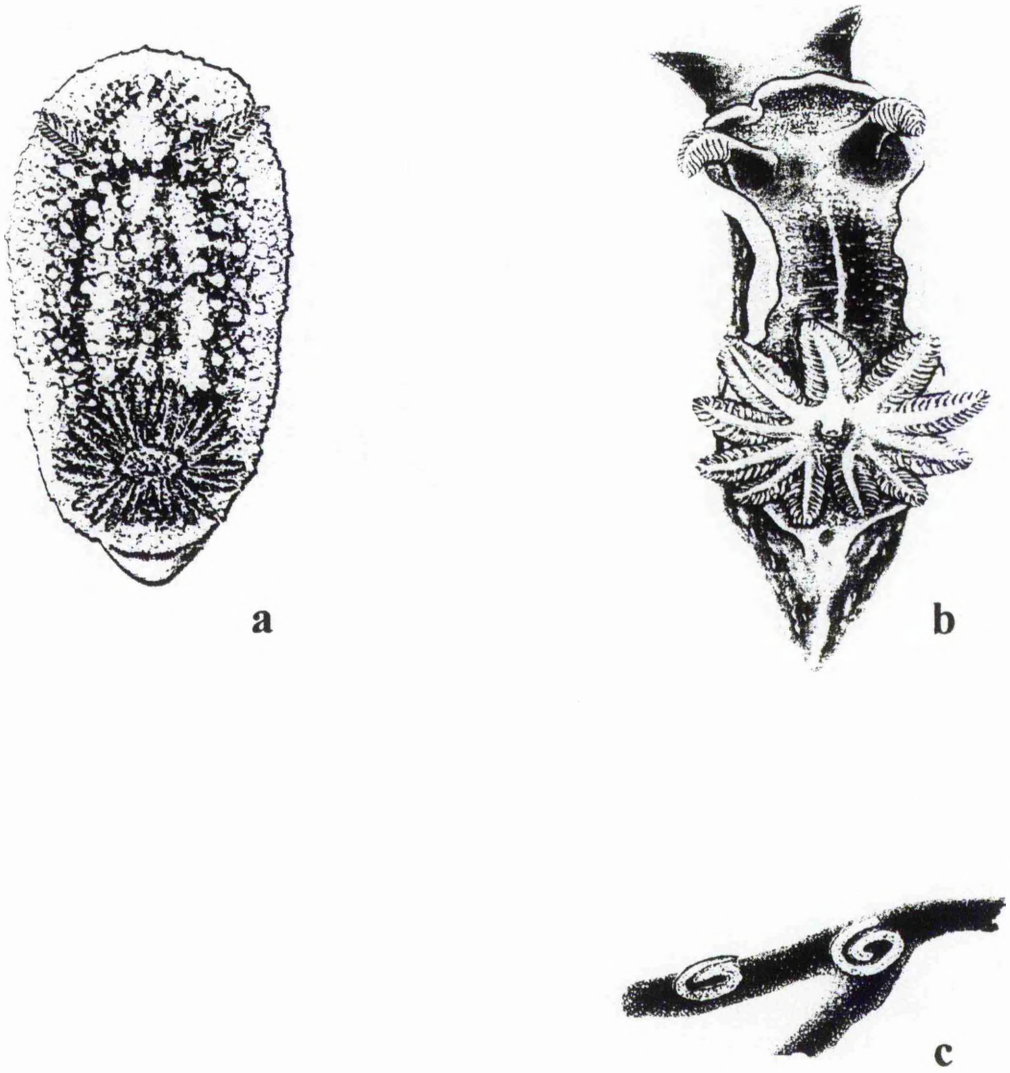


Fig. 1.1. a) Dorsal view of a 14 mm specimen of *Onchidoris bilamellata*.
b) Dorsal view of a 22 mm specimen of *Goniodoris nodosa*, c) spawn mass of the same attached to the polyzoan *Alcyonidium*. From Thompson & Brown (1984).

According to the classification system outlined by Thompson (1976), and later updated by Thompson & Brown (1984), the order Nudibranchia is included in the subclass Opisthobranchia (Class Gastropoda). In accordance with this classification, *Onchidoris bilamellata* and *Goniodoris nodosa* belong to the families Onchidoridídae and Goniodoridídae respectively, albeit at the present time the taxonomic position of a number of families within the subclass, including the Onchidoridídae (presently termed Onchidiidae) is controversial (see e.g. Haszprunar 1985, 1988; Haszprunar & Huber, 1990).

Relevant compendia on the Opisthobranchia include those by Ghiselin (1966) on the evolution of the reproductive system, Thompson (1976) on the natural history of the subclass, and a number of reference works contained in the series 'The Mollusca' (Wilbur, ed-in-chief, 1983-1985). Amongst the latter, of particular importance are the compilation of opisthobranch reproduction by Hadfield & Switzer-Dunlap (1984), on opisthobranch evolution by Schmekel (1985), and the articles by Bayne & Newell (1983) and Russell-Hunter & Buckley (1983) on the physiological energetics of molluscs. Calow (1983) reviewed molluscan life-history traits, whereas Bayne (1983) specifically considered the ecology of marine molluscan larvae. In addition, particular information on nudibranchs is provided in the reviews by Harris (1973), Clark (1975), Todd's (1977) PhD dissertation, and in his comprehensive reviews (Todd 1981, 1983, 1985a) of the ecology of nudibranchs. A further treatise on the Nudibranchia is the monograph by Thompson & Brown (1984).

More specific information on *Onchidoris bilamellata*, studied herein, comes essentially from the work of Potts (1970), and the thorough studies by Todd (refs. above and 1979a,b; 1991). Other relevant papers are those by Todd & Doyle (1981), Grant & Williamson (1985) and Todd (1985b) on a model of the optimal timing for reproduction and settlement in marine invertebrates, with particular reference to *O. bilamellata*. In addition, Chia & Koss (1988, 1989) and Arkett and co-workers (1989) have investigated the induction of settlement and metamorphosis in this species.

Works referring to *Goniodoris nodosa* include the life history studies of British populations by Gargstang (1890), Miller (1962), Thompson (1964), and Todd (1977), and Dutch populations by Swennen (1961). Other investigations include those by Alder & Hancock (1845-1855) on feeding, and Lloyd (1952) on the reproductive system. Further information on this species is provided in Thompson & Brown's (1984) monograph.

Onchidoris bilamellata and *Goniodoris nodosa* are simultaneous hermaphrodites, both with annual life cycles (Todd 1981, 1983). These species are semelparous (Todd, 1981), that is, they have a single reproductive period during which copulation and spawning take place repeatedly, followed by the death of the adults (Calow, 1983; Hadfield & Switzer-Dunlap, 1984). Fertilization is internal, with reciprocal transfer of sperm (Thompson, 1976; Hadfield & Switzer-Dunlap, 1984). These dorids generally produce one generation per year, although two generations have been reported occasionally (Miller, 1962; Thompson, 1964; Todd 1977, 1979a; Thompson & Brown, 1984; Lafuente, 1996). The eggs hatch as pelagic planktotrophic larvae (Thompson, 1967; Todd 1979a,b, 1991; Ros, 1981).

In addition to the field and laboratory estimates of reproductive effort for *Onchidoris bilamellata* carried out by Todd (1977, 1979a,b), information pertaining to the egg masses of this species and of *Goniodoris nodosa* are limited to measurements of egg diameters and counts of the number of eggs contained in each egg mass (Thompson, 1967; Thompson & Brown, 1984; Ros, 1981). Data of the organic content of the eggs in these and other nudibranchs is wanting (for a further discussion of the literature relevant to this research area see the introduction to chapter 2). In the present investigation the organic content of eggs of various sizes in the nudibranchs *O. bilamellata* and *G. nodosa* are analysed.

The laboratory culture of opisthobranch larvae through metamorphosis was initially limited to species with direct development, e.g., *Cadlina laevis* (Thompson, 1967), and species with lecithotrophic larvae, e.g., *Tritonia hombergi* (Thompson, 1962) and *Phestilla sibogae* (Bonar & Hadfield, 1974). Following the improvement of

phytoplankton culture techniques (described in Guillard, 1975), the methods for the culture of planktotrophic opisthobranchs were conceived. Amongst the first successful attempts were those of Kriegstein and co-workers (1974) and Switzer-Dunlap & Hadfield (1977) who cultured a number of aplysiid species, and Kempf & Willows (1977) who reared the nudibranch *Tritonia diomedea* Bergh through to metamorphosis.

The methodology ordinarily used in the laboratory culture of *Onchidoris bilamellata* was developed by Todd (1981). In this and other studies, Todd (1977, 1979a, 1991) provides detailed information of the embryonic and larval development of this dorid species. In contrast, no reports on the larval growth of *Goniodoris nodosa* in laboratory cultures had been published up to the present time. In the present work, the techniques described by Todd (1981) were employed for the culture through metamorphosis of *Goniodoris* larvae obtained from eggs laid by specimens maintained in captivity.

Competent larvae of *Onchidoris bilamellata* are induced to metamorphose in the presence of its natural prey organism, the acorn barnacle *Semibalanus balanoides* (Todd 1981, 1991). The first experiments on metamorphosis in this species were carried out by Todd (1981), where an adequate protocol for the larval culture of *O. bilamellata* was provided. In his study, Todd (1981) investigated the effect of light on metamorphosis. Later works have dealt with the nature of the inductive cue for metamorphosis (Chia & Koss, 1988), and with the identification of sensory cells (Arkett *et al.*, 1989) and propodial ganglia (Chia & Koss, 1989) in competent veligers.

The natural inducers of *Goniodoris nodosa* include members of the Bryozoa and Urochordata (Lambert & Todd, 1994). However, the metamorphosis induction in this species has not been treated in detail up to the present time. In order to gain a further insight into the metamorphic process of *Onchidoris bilamellata* and *G. nodosa*, laboratory metamorphosis experiments were conducted with these two species.

CHAPTER 2

SPAWNING PATTERNS OF *ONCHIDORIS BILAMELLATA* (L.) AND *GONIODORIS NODOSA* (MONTAGU)

INTRODUCTION

It is generally agreed that the egg size in marine invertebrates is highly related to the mode of larval development (Thorson, 1950; Vance, 1973a,b; Havenhand, 1994). Species with planktotrophic development generally lay high numbers of small, poorly nourished eggs which hatch at an early stage of development. Conversely, lecithotrophic species and 'direct' developers produce relatively fewer but larger, highly nourished eggs which hatch at a more advanced state (refs. above and Thorson 1946; Hadfield, 1963; Crisp, 1976; Thompson 1967, 1976; Strathmann, 1978; Todd 1981, 1983; Ros, 1981; Jablonski & Lutz, 1983; Hadfield & Switzer-Dunlap, 1984; Hadfield & Miller, 1987).

In early works, egg size was considered to be a good indicator of the energy content, but it was not until the late 1970's (Strathmann & Vedder, 1977; Turner & Lawrence, 1979) that this relationship was analysed. To date the relationship between the egg size and the egg organic content has mainly been addressed in species of commercial relevance, including fish (Vázquez *et al.*, 1994; Quattro & Weeks, 1991; Scott & Barbour, 1992), and crustaceans (Shakuntala & Reddy, 1982; Clarke *et al.*, 1991; Clarke & Gore, 1992; Clarke, 1993a,b), and other organisms such as echinoderms (Strathmann & Vedder, 1977; Turner & Lawrence, 1979; Lawrence *et al*, 1984; McClintock & Pearse, 1986; Mc Edward, 1986; Mc Edward & Carson, 1987; Mc Edward & Coulter, 1987; Mc Edward & Chia, 1991). Even though there appears to be a correlation between egg size and the total organic content among marine invertebrate groups (Hadfield & Switzer-Dunlap, 1984; McEdward & Chia, 1991), the size alone is not an accurate indicator of the egg contents at the intraspecific level (McEdward & Carson, 1987; Havenhand, 1994; but see Clarke, 1993a,b).

The study of reproductive output of marine gastropods has increasingly gained attention in the last two decades (Grahame, 1977; Goodwin, 1979; Havenhand, 1986; Havenhand & Todd, 1988b). Parallel to these studies, several comparative works on the differences in the egg size between species have been conducted (Hadfield & Miller, 1987; Lima & Lutz, 1990). Pandian (1969) observed the changes in the egg composition throughout the embryonic development of the gastropod *Crepidula fornicata*, but the relationship between the egg size and the organic content was not analysed. References to the egg energy allocation in relation to the egg size within species in marine gastropods were not found in the reviewed literature. Indeed, the studies on the egg energy allocation within species are also few for other marine invertebrate groups (Clarke, 1993a).

The ova in nudibranchs are enclosed, either individually or in groups, by membranous capsules that are then linked together to form strings within a gelatinous mass (Ghiselin, 1966; Thompson, 1976, Hadfield & Switzer-Dunlap, 1984), which is generally ribbon-shaped. The first ova extruded are deposited at one edge of the ribbon, followed by ova deposited in the central area, and ova deposited at the opposite extreme of the ribbon.

As already noted, *Onchidoris bilamellata* and *Goniodoris nodosa* are planktotrophic nudibranchs commonly found in intertidal areas of British coasts. The eggs of *O. bilamellata* are generally laid in individual capsules. The spawn mass is laid as a ribbon-shaped spiral (type A of Hurst, 1967) which is attached along one edge (Thompson, 1976; Hadfield & Switzer-Dunlap, 1984). Each spawn mass contains up to 125,000 ova, 80-100 μm in diameter, taking 17-19 days to hatch at 10°C (Thompson, 1967). The dimensions of the spawn masses vary greatly, but as an approximation it can be said that a spawn mass of 9 cm^2 surface area was in the upper size limits of the populations observed in this study.

The spawn masses of *Goniodoris nodosa* are smaller, contain less gel stroma than those of *Onchidoris bilamellata*, and have the shape of cylindrical cords attached along one side (type B of Hurst, 1967) (this study). The spawns are normally narrow (~3 mm in width), and of variable length (up to 60 mm approximately) (personal observations). Each spawn mass may contain 13,900-32,500 eggs; the white ova measured 73-103 μm in diameter (Thompson & Brown, 1984).

The spawning season of *Onchidoris bilamellata* in the British Isles extends from December to May (Todd, 1979a). Summer and early autumn spawnings have, however, been recorded in intertidal populations occasionally (Renouf, 1915; Miller, 1962; Todd 1977, 1979a; present study). The larval development of *O. bilamellata* has been previously studied on larvae hatching from eggs laid during the winter-spring spawning season. No information is available on the larval growth and development of summer and early autumn spawnings.

The first experiment of this work was conducted in order to determine whether the egg size in *Onchidoris bilamellata* decreases as the deposition progresses, or, on the contrary, if the egg size is not dependent upon the location in the spawn mass. The second experiment of this chapter examined the characteristics of spawnings laid throughout the reproductive period by *O. bilamellata* and *Goniodoris nodosa* adults maintained in captivity. In Experiment 3, two key points were addressed: the occurrence of individuals and spawn masses in the field throughout the year; and the identification of any distinctive traits of the July and September offspring.

MATERIALS AND METHODS

Collection, maintenance, and mating of the adults

Adults of *Onchidoris bilamellata* and *Goniodoris nodosa* were collected prior to the onset of the spawning season in the field. The animals were transported to the laboratory and maintained in containers with seawater prior to the start of the experiments. The slugs were provided with an excess of their prey items which were collected fresh from St Andrews Bay and changed regularly. The details concerning the collection sites and the specimens used in the experiments 1 and 2 are given in Tables 2.1 & 2.2 respectively.

The spawning season in the British Isles extends from February/March to the end of May in *Goniodoris nodosa* (Miller, 1962; Thompson, 1964; Todd, 1977), and, as noted earlier, from mid-December to May in *Onchidoris bilamellata* (Todd, 1979a). Once the mating season had begun, the animals were allowed to mate twice weekly. For this purpose, the slugs, distinguishable by size and pigmentation, were placed in pairs in small containers with seawater in the absence of food for a period of six hours after which they were returned to their original containers. Every effort was made to provide a different mate for each animal in consecutive encounters.

Sampling sites

Collections were carried out in the locations listed in Tables 2.1 & 2.2. Atlantic Bridge, Argyll (56° 17'N, 5° 37'W) is a sheltered narrow tidal strait on the west coast of Scotland; St Andrews East Sands (56° 20'N, 2° 47'W) and Kingsbarns, Fife (56° 18'N, 2° 39'W) are semi-exposed rocky shores on the east coast of Scotland; Robin Hood's Bay, North Yorkshire (54° 26'N, 01° 28'W), is characterised by boulder fields which leave large pools on low spring tides; and Loch Sligachan is a sea loch on the Isle of Skye.

Experiment 1

In this experiment the nudibranch *Onchidoris bilamellata* was bred in the laboratory in order to study the variation in egg size within the first spawn mass produced by each specimen. A total of 33 adults of *O. bilamellata* were collected in the field in November 1991 in four different locations (Table 2.1). Following collection, the animals were

maintained individually in tanks with circulating seawater at 10 °C until the commencement of the experiment, on 13 January 1992. The nudibranchs were fed the acorn barnacle *Semibalanus balanoides* growing on fragments of rock. Only ten specimens, which had not spawned prior to the beginning of the experiment, were used. From this date, the ovipository activity of the slugs was checked twice daily.

Table 2. 1. *Onchidoris bilamellata* adults collected in 1991 for Experiment 1.

Sampling Site	Date of Collection	Specimens Collected	Specimens Used in the Experiment	Number Assigned to the Experimental Animals
Atlantic Bridge (Argyll) 56° 17'N, 5° 37'W	23 Nov. 1991	10	5	2,4,5,7,8
Robin Hood's Bay, North Yorkshire 54° 26'N, 01° 28'W	23 Nov. 1991	9	1	15
East Sands (St Andrews) 56° 20'N, 2° 47'W	8 Nov. 1991	7	1	24
Loch Sligachan (Isle of Skye) 57° 29'N, 6° 9'W	25 Nov. 1991	7	3	30, 32

The first spawn mass laid by each specimen was carefully removed from the walls of the tanks with forceps and a scalpel, and was observed under a binocular microscope in order to determine whether the embryonic divisions had commenced. Only those spawn masses that contained uncleaved zygotes were included in the experiment. The uncleaved spawn masses were transversally cut into three sections, and the diameters of 35 eggs of each one of the sections were measured with a graticule under an Olympus-CH light microscope to the nearest 3 μm. The lateral section with the smallest ova was considered the 'final' section, the opposite lateral section the 'initial' section, and the central area the 'central' section. The conversion factor (1 graticule unit ≈ 10.31 μm) was estimated by measuring under the same magnification a precalibrated scale.

Experiment 2

In this experiment *Onchidoris bilamellata* and *Goniodoris nodosa* were maintained and allowed to reproduce in captivity throughout the breeding season in order to permit monitoring of oviposition and the analysis of the egg number and size, in addition to the ash free dry weight of the spawn masses being laid. For this purpose, 35 *O. bilamellata* and 20 *G. nodosa* adults were collected from different sites in November and December 1992 (see Table 2.2), transported to the laboratory and maintained in two tanks at 10 °C with circulating seawater (one tank containing each species) at 10 °C. The nudibranchs were fed their natural prey organisms, *Semibalanus balanoides* L. growing on fragments of rock for *O. bilamellata*, and the bryozoan species *Flustellidra hispida* and *Alcyonidium polyoum* epiphytic on *Fucus* sp. fronds for *G. nodosa*.

Table 2. 2. *Onchidoris bilamellata* and *Goniodoris nodosa* adults collected in 1992 for Experiment 2.

Species	Sampling Site	Date of Collection	Specimens Collected	Specimens Used in the Experiment
O. bilamellata	Kingsbarns (Fife) 56° 18'N, 2° 39'W	15 Dec. 1992	35	10
G. nodosa	Atlantic Bridge (Argyll) 56° 17'N, 5° 37'W	25 Nov. 1992	9	4
	East Sands (Fife) 56° 20'N, 2° 47'W	10 Dec. 1992	3	3
	Kingsbarns (Fife) 56° 18'N, 2° 39'W	12 Dec. 1992	8	3

On 14th January 1993 ten *Onchidoris bilamellata* specimens were chosen at random, isolated in individual containers, and assigned a number from 1 to 10. This procedure was also followed for *Goniodoris nodosa* on 1st February 1993. During the course of the experiment the animals were allowed to mate at regular intervals. The experiment terminated on 8th June 1993 for *Onchidoris*, and on 10th August 1993 for *Goniodoris*, when all the animals had died.

The *Onchidoris bilamellata* adults had spawned prior to the commencement of the experiment, therefore the data on the first spawn masses of the reproductive season were not available. The first monitored spawn masses laid in captivity were considered instead. *Goniodoris nodosa*, on the contrary, had not spawned prior to the start of the experiment. Consequently, the first monitored spawn masses were the first of the reproductive season.

The spawn masses were collected immediately following deposition, before the first zygotic division occurred, and were maintained thereafter in a cold-room at 10 °C. It was originally intended to monitor three or four spawn masses laid at different points of the breeding season (e.g, the first, fifth, and tenth spawn mass). However, because on many occasions the individuals spawned during the night, the zygotic divisions had commenced prior to their discovery. Only spawn masses containing undivided ova were used for measurements, which explains why the order of deposition of the spawn masses analysed does not coincide in the majority of cases (see Tables 2.4 & 2.5). The spawn masses were carefully removed from the container walls with a scalpel. Following removal of excess water in a standardised manner with desiccating paper, the spawn masses were weighed on a digital balance to the nearest 0.1 mg.

In order to monitor ova of comparable size between spawn masses, a section was cut from the central zone of the spawn mass. This was further excised in two sections which were weighed on a digital balance to the nearest 0.1 mg, placed on a glass slide with a coverslip, and photographed under a Wild stereomicroscope. A precalibrated graticule was also photographed in order to obtain the conversion factor.

One of the sections was cultured, and the shell lengths of 40 newly hatched larvae were measured for each spawn mass. The remaining section was cut in three and rinsed in 0.9% (w/v) ammonium formate (HCO_2NH_4) in order to displace marine salts (Havenhand, 1986). Subsequently the material was frozen and then dried in a SF50 Vacuum Freeze-Drier (Chemlab Instruments), prior to being weighed on a Mettler ME22 analytical microbalance. The ash free dry weight of the dried samples was obtained by igniting the dried material for at least four hours at 550°C in a muffle furnace (Paine, 1964).

Experiment 3

The field observations were carried out on the south east coast of Scotland (56° 18' N, 2° 39' W) from July 1993 to August 1994. The surface seawater temperatures were recorded twice weekly at low tide from January 1993 to August 1994 in the intertidal zone of East Sands Beach, St Andrews (56° 20' N, 2° 47' W), 7 miles from the location under study (Fig. 2.1). A sample of the spawn masses observed in the field throughout this period was brought into the laboratory, weighed, and the stage of development of the embryos was determined. The spawn masses were cultured and the shell lengths of the larvae were measured at hatching.

In view of the information that could be gained from these rare findings, it was decided, though at a late stage, in September, to culture the egg masses up to metamorphosis in the laboratory. The results of four larval cultures set up from two egg masses (A and B) are described in Chapter 3. The cultures derived from spawn mass C were lost due to a technical failure. The larval cultures were fed on the flagellate *Rhodomonas* sp., and reared under ambient conditions (temperature falling from an initial 14°C to 6°C) in an attempt to estimate the likelihood of the successful completion of the larval period in the field. For logistic reasons, the egg masses collected during this period were transferred from the field temperatures to a culture temperature of 10°C, and the shell lengths of the hatchlings were assessed.

Statistical analysis

All data analysis was by Minitab (Release 7.1). Egg size data of Experiment 1 were tested for normality and followed by ANOVA of two factors, 'section' and 'parent', with 3 and 9 levels respectively. The interaction of these two factors was also examined. Tukey's multiple comparison procedure was employed to investigate significant differences among the levels of a factor (Sokal & Rohlf, 1981).

Model I regression analysis was used to examin the relationships between the spawning variables (weight, egg number and ash-free dry weight of spawn masses, egg diameter, duration of embryonic period, and shell length of hatchlings) considered in Experiment 2.

RESULTS

Experiment 1

In order to analyse the source of variation of egg size within and between spawn masses of *Onchidoris bilamellata*, data of egg diameters were grouped by 'section' and 'parent' (Table 2.3), and Analysis of variance (ANOVA) was conducted (Table 2.4). This analysis reveals that the egg diameters vary significantly between sections and between parents, but no significant interaction between section and parent is observed ($F = 1.46$, $p = 0.109$).

Table 2.3. Descriptive statistics of egg diameters of *Onchidoris bilamellata* for the main factors (section and parent) of Experiment 1.

Factor	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN
Parent No. 2	105	105.11	103.10	105.05	7.88	0.77
Parent No. 4	105	102.51	103.10	102.45	5.17	0.50
Parent No. 5	105	109.63	113.41	109.67	4.91	0.48
Parent No. 7	105	103.44	103.10	103.48	5.17	0.50
Parent No. 8	105	108.45	108.26	108.58	5.25	0.51
Parent No. 15	105	100.40	103.10	100.17	5.59	0.55
Parent No. 24	105	111.15	113.41	111.08	6.15	0.60
Parent No. 30	105	100.01	103.10	99.79	5.14	0.50
Parent No. 32	105	104.18	103.10	104.29	4.83	0.47
Initial section	315	105.73	103.10	105.78	6.79	0.38
Central section	315	105.54	103.10	105.56	6.59	0.37
Final section	315	103.69	103.10	103.59	6.71	0.38

Table 2.4. Crossed Analysis of Variance conducted to test for significant differences in egg diameter within spawn masses laid by *Onchidoris bilamellata* specimens.

Source	DF	SS	MS	F	P
section	2	802.51	401.25	13.05	0.000
parent	8	13291.12	1661.39	54.04	0.000
section*parent	16	716.51	44.78	1.46	0.109
Error	918	28221.61	30.74		
Total	944	43031.75			

The significant differences among the levels of the factors ('section' and 'parent') were investigated by Tukey's multiple comparison procedure with 95% confidence interval (Tables 2.5 & 2.6). In Tukey's test two means differ significantly when the confidence interval for their difference does not contain zero (Sokal & Rohlf, 1981). The confidence intervals, detailed in Table 2.5, reveal that the mean egg diameters of the central and initial sections of spawn masses of *Onchidoris bilamellata* cannot be considered to differ significantly, whereas the mean egg diameter of the final section differs significantly from the remaining two.

Table 2.5. ANOVA and Tukey test conducted to investigate differences among levels of the factor 'section' of Experiment 1 (see Table 2.4).

SOURCE	DF	SS	MS	F	p
Section	2	802.5	401.3	8.95	0.000
Error	942	42229.2	44.8		
Total	944	43031.8			

Level	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Final	315	103.69	6.71	(-------*------)
Central	315	105.54	6.59	(------*-------)
Initial	315	105.73	6.79	(------*-------)
POOLED STDEV =		6.70		103.0 104.0 105.0 106.0

TUKEY'S multiple comparison procedure. The contingency table below shows the 95% confidence intervals for the pairwise mean differences. * denotes significant differences between means.

	Final	Central
Central	-3.098* -0.601	
Initial	-3.294* -0.797	-1.445 1.052

The Tukey test conducted to investigate significant differences among parents indicates that, in general, the parental population do not represent a homogeneous group; a considerable number of individuals contain eggs of sizes significantly different from the rest (Table 2.6). Furthermore, there is no systematic pattern to the variation in this factor.

Table 2.6. ANOVA and Tukey test analysing the differences among levels of the factor 'parent' of Experiment 1 (see Table 2.4)

Source	DF	SS	MS	F	p
Parent	8	13291.1	1661.4	52.29	0.000
Error	936	29740.6	31.8		
Total	944	43031.8			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
2	105	105.11	7.88	(--*-)
4	105	102.51	5.17	(-*--)
5	105	109.63	4.91	(--*--)
7	105	103.44	5.17	(--*-)
8	105	108.45	5.25	(--*--)
15	105	100.40	5.59	(--*--)
24	105	111.15	6.15	(--*--)
30	105	100.01	5.14	(--*--)
32	105	104.18	4.83	(-*--)
POOLED STDEV =		5.64		100.0 104.0 108.0 112.0

TUKEY'S multiple comparison procedure. The contingency table below shows the 95% confidence intervals for the pairwise mean differences. * denotes significant differences between means.

	2	4	5	7	8	15	24	30
4	0.190* 5.014							
5	-6.928* -2.105	-9.530* -4.707						
7	-0.742 4.081	-3.344 1.479	3.774* 8.598					
8	-5.750* -0.927	-8.352* -3.529	-1.233 3.590	-7.419* -2.596				
15	2.301* 7.125	-0.301 4.523	6.818* 11.642	0.632* 5.456	5.640* 10.463			
24	-8.450* -3.627	-11.052* -6.229	-3.934 0.890	-10.120* -5.296	-5.112* -0.289	-13.164* -8.340		
30	2.694* 7.518	0.092* 4.916	7.211* 12.034	1.025* 5.848	6.033* 10.856	-2.019 2.804	8.733* 13.556	
32	-1.479 3.344	-4.081 0.742	3.038* 7.861	-3.148 1.675	1.860* 6.683	-6.192* -1.369	4.560* 9.383	-6.585* -1.761

Experiment 2

The spawning activity for *Onchidoris bilamellata* commenced on 10th January 1993 and continued until 6th June 1993. The last *Onchidoris* adult died two days later, on 8th June 1993. In *Goniodoris nodosa*, spawning commenced on 9th February, and terminated on 5th August 1993, with the last adult dying on 10th August 1993. The data obtained from these spawnings are summarised in Tables 2.7 & 2.8.

Onchidoris bilamellata adults laid some 12 spawn masses on average, although one individual (No. 2) laid as many as 18 spawn masses (Table 2.7). In contrast, the majority of the *Goniodoris nodosa* individuals spawned on 6 or 7 occasions, with a maximum of 17 spawn masses laid by individual No. 3 (Table 2.8). A correlation matrix of the characteristics mentioned in Tables 2.7 & 2.8 was derived, and is shown in Table 2.9.

Table 2.7. Summary of spawning data for *Onchidoris bilamellata* bred in captivity.

Specimen	Spawn Masses Monitored	Mean Egg Diameter (µm)	Estimated µg · Egg^{-1}	Number of Eggs in the Spawn Mass	Damp Weight of the Spawn Mass (mg)	Embryonic Period (days)	Mean Shell Length of Hatchlings (µm)
1	1st	101.6	0.347			17	
2	1st	99.3				21	
	6th	109.5	0.421	20,200	172.2	22	162.6
	9th	96.1	0.286	33,700	313.4	20	176.5
	13th	95.4	0.237	39,000	369	20	163.9
	18th	115.1			217.3	19	159.5
3	1st	105.1	0.102			18	
	4th	100.9	0.305	9,000	51.1	20	
4	4th		0.483	53,141	483.1	21	181.6
5	1st	104.7	0.245			17	
	4th	105.9	0.525	8,600	96.7	20	178.5
	9th	88.7	0.385	9,000	78.4	24	
	11th	124.3			101.5	21	
6	1st	93.5	0.153			19	
	4th	105.1	0.286	8,800	50.3		
	10th	88.8			11		
	11th	95.1			18.6	23	154.8
7	1st	103.4	0.704			25	
	4th	111.2	0.381	41,422	298	20	178.9
	9th	97.9			384.2	19	
	10th	98.2	0.505	46,569	554.4		
	11th	95.5			377		
	15th	96.1			270.9		

Table 2.7.(continued)

Specimen	Spawn masses monitored	Egg diameter (µm)	Estimated µg · Egg^{-1}	Number of Eggs in the Spawn mass	Damp Weight of the Spawn Mass (mg)	Embryonic Period (days)	Mean Shell Length of Hatchlings (µm)
8	1st	102.5				21	
	4th	106.5	0.444	26,660	191.8	21	186.6
	12th	102.7	0.6	2,131	29.5		
	14th	99.8			14.6	19	
9	1st	98.0				23	
	5th	102.8	0.429	41,855		22	180.1
	9th		0.438	25,600	220.4		
	15th	91.9	0.39	4,466			
10	1st	95.8	0.556			23	
	5th	94.0	0.267	24,717	149.8	20	160.5
	9th	90.5	0.375	2,000	20		

Table 2.8. Summary of spawning data for *Goniodoris nodosa* bred in captivity

Specimen	Spawn Masses Monitored	Mean Egg Diameter (µm)	Estimated µg · Egg^{-1}	Number of Eggs in the Spawn Mass	Damp Weight of the Spawn Mass (mg)	Embryonic Period (days)	Mean Shell Length of Hatchlings (µm)
1	1st	107.2	0.239	4,072	19.3	23	
1	4th	106.3			5.5		
1	6th	103.1			3.9		
2	1st	95.8			12.7	21	165.3
2	2nd					20	156.7
2	3rd	89.2			36.7	20	157.4
2	7th				50.6	23	142.3
3	1st	112.9			3		
3	3rd	102.1			8.6	21	170.8
3	7th	101.4			11.7	20	170.6
3	16th	89.1			9.1		
3	17th						158.3
4	1st	116.7	0.64	2,749	23.5	23	185.9
5	1st	117.5	0.537	12,325	72.5		
5	4th	115.6		2,855	11.8	23	180.6
6	1st	124.2	0.388	14,071	87.4	25	191.3
6	4th					23	183.9
6	5th	106.6	0.516	7,558	64.6	22	178.4
6	9th					18	152.1
7	1st					33	163.8
7	4th					23	182.5
7	7th	89.1			38.5	19	145.5
8	1st	120.2	0.39	2,619	7.1		

Table 2.8. (continued).

Specimen	Spawn Masses Monitored	Mean Egg Diameter (µm)	Estimated µg · Egg^{-1}	Number of Eggs in the Spawn Mass	Damp Weight of the Spawn Mass (mg)	Embryonic Period (days)	Mean Shell Length of Hatchlings (µm)
8	4th		0.271	1,700	4.6		
8	5th	107.0			7.5	23	
8	6th	116.5			8.1		
9	1st	130.2	0.643	1,700	16.5	25	194.7
9	5th	122.4	0.52	171	2.2		
10	1st	95.0	0.295	3,400	18.8	24	
10	4th	131.4	0.431	1,300	9.1		
10	8th	105.0	0.444	2,700	21.4		
10	10th				34.0	20	163.1
10	12th					15	145.3
10	13th	88.2	0.364	4,200	31.0		

Table 2.9. Correlation matrix of the spawning variables. Figures above the diagonal are correlation coefficients for *Onchidoris bilamellata* data, figures below the diagonal are for *Goniodoris nodosa* data. For the latter species, the data were insufficient to study some of the correlations, whose spaces in the table were left blank. Asterisks denote statistical significance for $p \leq 0.05$ (*); $p \leq 0.01$ (**); and $p \leq 0.001$ (***).

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) Damp weight of spawn mass	—	0.014	0.958***	0.005	0.063	0.302	0.435
(2) Order of deposition	0.072	—	0.203	0.076	0.111	0.044	0.656*
(3) Number of eggs · spawn mass^{-1}	0.966***	0.214	—	0.234	0.154	0.199	0.255
(4) Mean egg diameter	0.033	0.509*	0.009	—	0.157	0.182	0.244
(5) Ash-free dry weight · egg^{-1}	0.156	0.121	0.013	0.501	—	0.726***	0.627
(6) Duration of embryonic period	0.167	0.671**	0.223	0.798**		—	0.086
(7) Mean shell length of hatchlings	0.005	0.609**		0.961***		0.503*	—

In *Onchidoris bilamellata* specimens, the size of the spawn mass (expressed as damp weight) was positively correlated with the number of eggs (egg number = 3,808.58 + 95.32 · mg^{-1} damp weight; $r = 0.958$, $p < 0.001$). Additionally, the mean shell length of the newly hatched larvae was negatively correlated with the order of the spawning event ($r = 0.656$, $p < 0.05$). Even though the egg diameter was not correlated with the egg ash-free dry weight ($r = 0.157$, $p > 0.05$), the latter was positively correlated with the duration of the embryonic period ($r = 0.726$, $p < 0.001$).

Similarly to the pattern observed in *Onchidoris*, the damp weight of the spawn mass in *Goniodoris* was positively correlated with the number of eggs (egg number = 285.51 + 147.31 · mg^{-1} damp weight; $r = 0.966$, $p < 0.001$). Furthermore, the order of spawn deposition in *G. nodosa* was negatively correlated with the shell length of the newly hatched larvae ($r = 0.609$, $p < 0.01$). However, in contrast with *Onchidoris*, the order of spawn deposition was in addition negatively correlated with the egg diameter and the duration of the embryonic period. However, the latter two variables were also related to one another ($r = 0.798$, $p < 0.01$). To investigate whether both of these variables were independently correlated with the correlative order of spawn deposition, standardised partial regression coefficients ('beta-weights') between the variables were derived (Sokal & Rohlf, 1981).

Beta-weight analysis indicated that only the duration of the embryonic period was independently correlated with the order of spawn deposition.

The correlation between the egg ash-free dry weight and the duration of the embryonic period could not be studied in *Goniodoris nodosa* given that a large number of spawn masses were incubated through hatching, and therefore the destructive methodology used for the assessment of ash-free dry weight was not carried out. In contrast to *Onchidoris bilamellata*, the egg diameter in *G. nodosa* exhibited a high positive correlation with the shell length of the hatchlings ($r = 0.961$, $p < 0.001$). Nonetheless, the shell length of the newly hatched larvae was in turn related to the duration of the embryonic period ($r = 0.503$, $p < 0.05$). Moreover, as indicated above, the duration of the embryonic period was also correlated to the egg diameter.

Experiment 3

A total of six spawn masses found on *Fucus* sp. fronds in July and September 1993 were cultured in the laboratory at field temperatures (Table 2.10). No adults were found in the field simultaneously, all individuals present were juveniles (size range 0.7 mg

Table 2.10. Spawn masses found in the field in July and September 1993.

Collection Date	Spawning	Development	Damp wt (mg)	Eclosion Date	Mean shell length of hatchlings
23/07/93	1	Early trochophore	17	28/07/93	Not available
	2	Mid trochophore	225	29/07/93	Not available
	3	Early trochophore	155	30/07/93	Not available
02/09/93	A	Gastrula	181	16/09/93	135 µm n = 40 Stdev = 6.31
	B	Late trochophore	144	11/09/93	141 µm n = 40 Stdev = 7.17
	C	Postgastrula	108	13/09/93	138 µm n = 40 Stdev = 7.64

to 42.2 mg damp body weight, 2.4 mm to 7.2 mm mantle length whilst the usual adult size observed in the study area ranged from 250 to 500 mg in damp body weight, 12 mm to 20 mm in mantle length). In July, two of the spawn masses (nos. 2 and 3) were on adjacent fronds, and a third spawn (No. 1) was found at an approximate distance of 10-12 meters from the others.

Subsequent to September 1993, the spawning activity in the field was not resumed until January 1994, ceasing in April 1994. The larvae hatching in July and September 1993 were smaller than those hatching during the 'normal' (winter-spring) spawning season (Fig. 2.2).

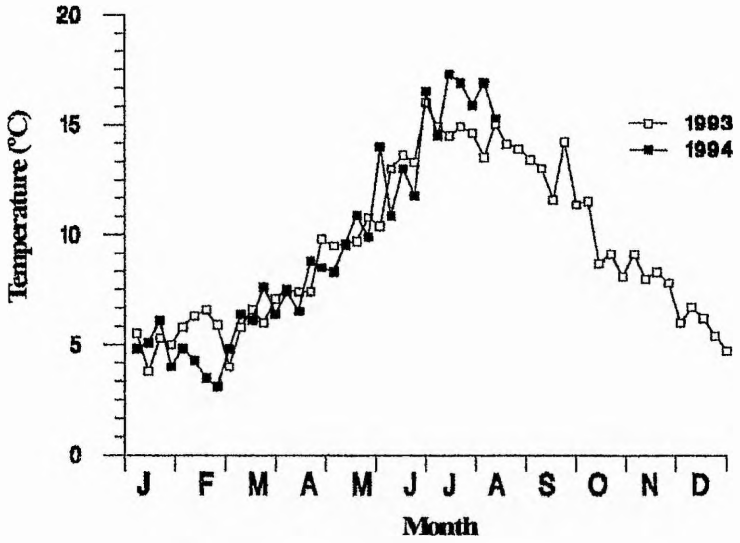


Figure 2.1. Surface sea-water temperatures recorded from January 1993 to August 1994 at East Sands, St Andrews (56°20'N 2°47'W) at low tide. Each data point represents the average value of twice-weekly measurements.

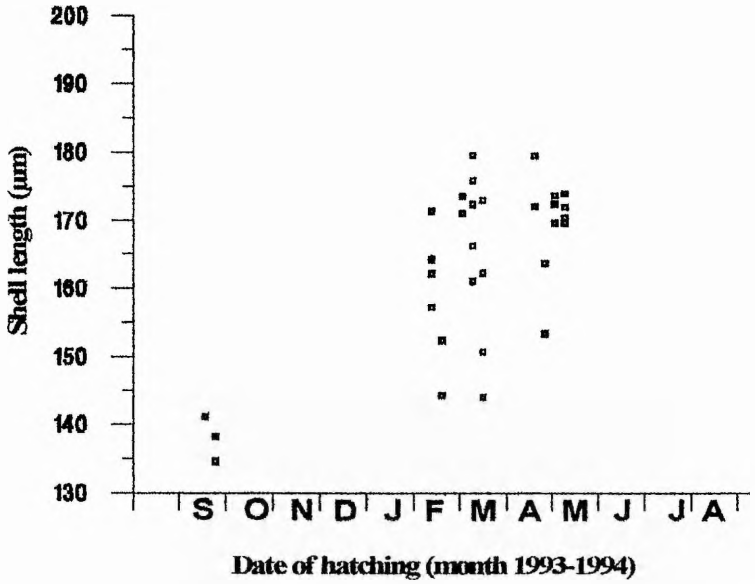


Figure 2.2. Shell length of *Onchidoris bilamellata* larvae hatching between September 1993 and May 1994 from spawn masses collected in the field. In 1993 the spawn masses were cultured at the field temperatures. In 1994 all the spawn masses were cultured at 10°C. Each data point represents the mean value of larvae (N = 40) hatching from a single spawn mass.

DISCUSSION

Studies of nudibranch reproduction in captivity are numerous, some examples of which are those by Todd (1979c), Havenhand (1986), and Todd & Havenhand (1988) for *Adalaria proxima* and *Onchidoris muricata*; Todd (1979a) for *Onchidoris bilamellata*; and Hall & Todd (1986) for *Aeolidia papillosa*. Whilst all of the above investigated the parental investment in reproduction, in the present study the egg features throughout the spawning season were analysed in the dorids *O. bilamellata* and *Goniodoris nodosa*. A recent study by Jones *et al.* (1996) also investigates this question within and between populations of *A. proxima*.

The spawning activity for *Onchidoris bilamellata* and *Goniodoris nodosa* exceeded the spawning period normally encountered in the field by about two months. This phenomenon has been observed previously for *Adalaria proxima* and *Onchidoris muricata*, (refs. above), and for *O. bilamellata* by Todd (1979a), who attributes this fact to the exceptionally protected conditions encountered in the laboratory.

In the present study *Onchidoris bilamellata* individuals had spawned prior to their isolation in individual containers, and therefore the first spawning event of the season could not be monitored. In contrast, all the *Goniodoris* adults commenced spawning subsequently to the start of the experiment, at approximately the same body size (about 15 mm in length). The first spawn mass laid was not the largest of the season for half of the individuals (Table 2.5). In addition, the size of the spawn masses did not decline as the season progressed, and did not follow any obvious pattern (Table 2.5).

These results do not concur with those obtained by Havenhand (1986) and Havenhand & Todd (1988b) for the annual nudibranchs *Onchidoris muricata* and *Adalaria proxima*, Jones *et al.* (1996) for *A. proxima*, and Todd (1979a) for *O. bilamellata*, who reported a larger than usual first spawn mass in those nudibranchs. Conversely, Clark (1975), in a study of North-West Atlantic nudibranchs, observed seasonal peaks of egg production albeit not at the commencement of the spawning period for the nudibranchs *Catriona aurantia* Alder & Hancock, *Tergipes tergipes* Johnston, and *Stiliger fuscatus* Gould. Nevertheless, the latter are subannual species, with overlapping of generations throughout the

year, and cannot be compared to annual species with only a predetermined time-period for egg production. Indeed, annual nudibranchs lay, in general, smaller spawn masses as the season progresses (Todd, 1979a; Havenhand, 1986), although this spawning behaviour is notably less pronounced in some species (e.g., in *O. muricata*, Havenhand & Todd, 1988b). The lack of a definite spawning pattern in the present study for *Goniodoris*, although possibly attributable to experimental conditions, may represent a behaviour comparable to *O. muricata*, whereby the adults do not compromise their entire reproductive output in the first spawn mass. Nevertheless repetition of this experiment with comprehensive records of adult body weight changes in order to establish the species reproductive effort would prove necessary to clarify this point.

A decrease of body size in association with spawning has been reported by a number of authors (Todd 1979a, 1981, 1983; Clark, 1975; Hadfield & Switzer-Dunlap, 1984), although occasionally alternating with periods of growth (Todd, 1979a; Havenhand, 1986). In *G. nodosa* the size of the spawning individuals was not monitored. However, feeding was observed to proceed normally throughout the reproductive period, and the likelihood is that the 'regrowth' of some specimens explains the production of large spawn masses towards the end of the reproductive period.

In addition to damp weight measurements, in the present investigation the size of the spawn masses for *Onchidoris bilamellata* and *Goniodoris nodosa* was estimated through numbers of eggs, determined from photographic records. The egg numbers thereby obtained were highly correlated with the damp weights of the spawn masses ($r = 0.958$, $p < 0.001$ for *O. bilamellata*; $r = 0.966$, $p < 0.001$ for *G. nodosa*, Table 2.6). These results, expressed in eggs · mg^{-1} damp weight correspond to 180.18 eggs · mg^{-1} damp weight for *G. nodosa*, and 120.72 eggs · mg^{-1} damp weight for *O. bilamellata*. The figure obtained for the latter species compares favourably with the results of Todd (1979a), who estimated 138.7 eggs · mg^{-1} damp weight for *O. bilamellata*. The relevance of the relationship between egg number and damp weight is of a practical nature, given that it allows the observer to obtain good estimates of egg numbers without resorting to tedious and time-consuming traditional counting methods.

An acknowledged problem with regard to egg diameters in marine invertebrates is the frequent disagreement in the figures provided for a single species by different authors, but also on occasions between publications of the same author (Todd, 1983; Hadfield & Switzer-Dunlap, 1984; Hadfield & Miller, 1987; Clarke, 1993a). Notwithstanding measurement errors, this variation may not only be to a certain extent attributable to true geographical variations (Clark & Goetzfried, 1978) but also to the nutritional state of the adult (DeFreese & Clark, 1983).

The estimated measurement error introduced in the assessment of egg diameters in the present work was ± 3 µm. The results from Experiment 1 indicate that the egg size of *Onchidoris bilamellata* is subject to two sources of variation: parent (i.e., 'source' of spawn mass) and section of the spawn mass in which the egg is contained (Table 2.4). The ova with the largest diameters were found in the 'initial' and 'central' sections of the spawn mass (Table 2.5), and were significantly different from those of the 'final' section (Table 2.5). Nonetheless, the ova developed and hatched successfully irrespectively of their size and their location in the egg mass.

Significant differences in egg volume from single spawning events within individual females have been reported for crustaceans (Clarke, 1993a), echinoids (George, 1990) and starfish (Mc Edward & Carson, 1987). Whilst the present results may partly reflect within-individual variability, the different location in the spawn mass of eggs of different sizes suggests that other factors are involved. Todd (1979a) reports that *Onchidoris bilamellata* spawn masses swell following deposition until the 8-cell stage of development is reached. The length of time it takes to lay a complete egg mass in nudibranchs is a function of egg mass size, which is often correlated with the size of the adult (Hadfield & Switzer-Dunlap, 1984), and a time period of about 7 hours was estimated for the largest specimens of *O. bilamellata* (~ 40 mm) in the present work. Consequently, the actual time elapsed between the commencement and termination of oviposition is considerable, and the likelihood is for eggs located in the (herein termed) 'initial' and 'central' zones to have absorbed more water than those of the 'final' zone by the time measurements were taken. In conclusion, a high variability in egg size can be at least in part attributed to water intake, further increased by the inevitable variation of the time elapsed between end of deposition and assessment of the

egg diameters. An alternative hypothesis would be to consider that larger eggs are laid at the commencement of oviposition, and that these gradually decrease in size as oviposition progresses. To clarify which portion of the egg volume is attributable to the level of hydration, damp and dry weights of eggs ascribed to the initial, central, and final zones of the spawn mass should be compared with mean egg diameters averaged for each zone.

The variability in egg size among first spawn masses laid by *Onchidoris bilamellata* individuals in the present study was considerable but did not exhibit a consistent pattern (Table 2.6). Intraspecific variability in egg size both within and between populations is a phenomenon which has also been reported for other marine invertebrates such as echinoderms (e.g., McClintock & Pearse, 1986; Mc Edward & Carson, 1987; Mc Edward & Chia, 1991) and crustaceans (e.g., Clarke, 1993a,b). In a recent work, Jones *et al.* (1996, in press) report considerable variation in egg size both within and between first spawn masses of six British populations of the nudibranch *Adalaria proxima*, and ascribe a significant proportion of the total observed variance to the effect of population. The *O. bilamellata* individuals used in Experiment 1 of the present work were collected at different sampling sites (Table 2.1), which may, at least in part, explain the variability observed among parents. Nonetheless, between population comparisons could not be conducted in this study due to the low numbers of individuals visualized (and collected) at the sites.

The egg diameters obtained for *Onchidoris bilamellata* in Experiments 1 and 2 averaged 105 µm and 100.50 µm respectively, ranging between 89 µm and 115 µm, which is slightly above the figures reported by Thompson (1967), ranging between 80 µm and 100 µm. The overall egg diameter obtained for *Goniodoris nodosa* in the present investigation was 108 µm, with considerable size variations, ranging between 88 µm and 131 µm. Again, these figures are above the values obtained from literary sources, from 73 µm to 107 µm (Thompson, 1967; Kress, 1971). It is relevant to note at this point, however, that the eggs measured in the present work were obtained from adults maintained in captivity, a detail which is often not specified in publications. The observed differences between these data and data obtained from previous works, are well above the estimated values attributable to error, and consequently may reflect either between population differences, or differences in the

nutritional state of the adults as a consequence of the *ad libitum* food supplied to the captive individuals, in addition to the differences in hydration level already discussed.

Life-history models, put forward to predict the optimal offspring size under different environmental conditions (e.g. Thorson, 1950; Mileikovsky, 1971; Vance, 1973a,b, 1974; Stearns 1976, 1977, 1980; Grahame & Branch, 1985) were developed on the assumption that size is indicative of the organic content of eggs. This presupposition, nonetheless, has been shown to fail for a number of marine invertebrates. McEdward & Carson (1987) and McEdward & Coulter (1987), for example, showed that egg volume and energetic content are not correlated among sibling offspring for the starfishes *Solaster stimpsoni* and *Pteraster tesselatus*. Similarly, McEdward & Chia (1991) found no significant correlations between these traits in two holothuroid and five asteroid echinoderms when the data were considered intraspecifically. Turner & Lawrence (1979) found no evident relationship between egg volume and percentage organic composition among 11 echinoderm species. The assessment of organic content as an alternative to egg dimensions is, in fact, defended by an increasing number of authors (e.g., Hadfield & Miller, 1987; Baur, 1994 and refs. above).

The correlation between egg diameter and organic content in aquatic invertebrates is frequently investigated in species with large ova which are either laid freely in the water column or easily isolated (e.g., in echinoderms, George 1990, 1994a,b; George *et al.* 1990, 1991; McEdward & Carson, 1977; isopods Clarke & Gore, 1992; and decapods, Clarke *et al.* 1990, 1991; Clarke, 1993a,b). In contrast, nudibranch eggs are small, with the majority of planktotrophic species falling in the range of 70µm to 100 µm (Hadfield & Switzer-Dunlap, 1984), and the eggs are embedded in a gelatinous matrix (Ghiselin, 1966). For this reason, no studies analysing isolated egg contents in nudibranchs have been conducted so far.

Estimates of organic content calculated from entire egg masses inevitably result in caloric values being obtained for the investing layers as well as that of the eggs (Gibson & Chia, 1991). To circumvent this problem in the present study, a methodology to isolate *Onchidoris bilamellata* eggs was developed by boiling the spawn mass in distilled water, which caused the gel matrix to disintegrate but left the egg capsules intact. Nonetheless, high numbers of eggs were lost in this technique through translocation from the boiling container

to the pre-weighed foil pans used for ignition, and consequently the technique was dismissed. In addition, the assessment of organic content for isolated eggs was not feasible for either *O. bilamellata* or *Goniodoris nodosa* given their small egg sizes (about 100 µm in diameter).

For the reason already noted, the variation in organic content in relation to egg size within spawn masses was not studied, and the values under comparison correspond to averaged diameters and ash-free dry weight derived from whole spawn masses. The ash-free dry weight for eggs of *Onchidoris bilamellata* averaged 0.39 $\mu g \cdot egg^{-1}$, ranging between 0.10 $\mu g \cdot egg^{-1}$ and 0.70 $\mu g \cdot egg^{-1}$ (Table 2.4). Mean egg diameters did not correlate with egg ash-free dry weight in *O. bilamellata* ($r = 0.157$, $p >> 0.05$, Table 2.6). In *Goniodoris nodosa* the overall mean ash-free dry weight was 0.44 $\mu g \cdot egg^{-1}$, ranging between 0.24 $\mu g \cdot egg^{-1}$ and 0.64 $\mu g \cdot egg^{-1}$ (Table 2.5). Even though there was a tendency for ash-free dry weight to increase with increasing egg diameter ($r = 0.501$, $p > 0.05$), the data obtained for this species were insufficient to establish a linear correlation between the variables (Table 2.5).

The remainder of the results obtained refer to the positive correlation observed between egg ash-free dry weight and the duration of the embryonic period in *Onchidoris bilamellata* ($r = 0.726$, $p < 0.001$). In *Goniodoris nodosa* the data were insufficient to analyse this relationship, nevertheless there was a significant relationship between egg diameter and embryonic developmental period ($r = 0.798$, $p < 0.01$) in this species. Published studies conclude that developmental times are correlated with egg size between several opisthobranch species (Spight, 1975; Ros, 1981; Thompson & Jarman, 1986), however within species differences were not considered in those publications. In the study of *Adalaria proxima* by Jones *et al.* (1996), in which intraspecific differences were investigated, no correlation was observed between egg size and embryonic developmental period. Therefore, whilst egg diameter may be indicative of the duration of embryonic period in some species, at the present time the available data are clearly insufficient for any conclusions to be drawn.

Despite the observation that in *Onchidoris bilamellata* neither the spawn masses nor the eggs exhibited a tendency to increase or decrease in size or ash-free dry weight throughout the spawning season (Table 2.6), the larvae were increasingly smaller as the

season progressed ($r = 0.656$, $p < 0.05$). The results obtained for *Goniodoris nodosa* follow to some extent the expected model: smaller eggs (albeit not smaller spawn masses) with an increasingly brief embryonic period gave rise to smaller larvae ($r = 0.609$, $p < 0.01$) as the season progressed.

To summarise, the egg diameter is not a reliable predictor of ash-free dry weight for *Onchidoris bilamellata*. This species did not exhibit a clear spawing pattern in captivity, however, this result must be treated with caution given the lack of information regarding the first spawn mass produced by this species. In contrast, the results obtained for *Goniodoris nodosa* show a distinct spawning pattern, with an unclear tendency for spawn mass size but a clear trend for reduction in egg and larval size throughout the season.

Furthermore, the size of the eggs in either species proved to be insufficient for individual calorific estimates. Possibly lecithotrophic nudibranch species with larger, richer eggs, such as *Cadlina laevis* whose eggs reach ~ 380 μm in diameter (Thompson, 1967), would prove more appropriate for this type of study.

In addition to investigating the spawning pattern of *Onchidoris bilamellata* in the laboratory, Todd (1979a) reports the spawning activity of *O. bilamellata* in the field, and concludes that this nudibranch lays smaller spawn masses as the breeding season progresses. The spawning activity of *Onchidoris* was not monitored from the commencement of the spawning period in the present investigation (Experiment 2). If the pattern observed by Todd (1979a) is constant in the field situation, the July spawns No. 2 (225 mg) and No. 3 (155 mg) of Experiment 3 were possibly laid by the same individual on consecutive days, and the spawn No. 1 (17 mg) was laid by a different adult. This possibility is also consistent with the location of the spawn masses and the reduced adult population to be expected at that time of the year. In September, the three spawn masses collected were on different *Fucus* sp. fronds within a surface area of 1 squared meter (Table 2.7). The dates on which hatching occurred and the sizes of the spawn masses suggest that spawns B (144 mg) and C (108 mg) were successive depositions of one individual; whilst the largest spawn (A; 181 mg) was laid by a different adult.

In the present study the larvae hatching in July and September 1993 were smaller than those hatching during the 'normal' (winter-spring) spawning season (Fig. 2.2). The substantial changes in temperature to which the spawn masses collected between February and March were exposed, may account at least partially for the variation observed. However, the field temperature in April (Fig. 2.1) coincided with the culture temperature in the laboratory (10°C), and the larvae haching from these egg masses were also substantially larger than the July and September larvae. This suggests that factors other than temperature affect the size of the larvae. The duration of the spawning activity of *Onchidoris bilamellata* results in individuals reaching maturity over a long period of time. This explains the wide range of adult sizes occurring simultaneously in the field in the present study, which in turn may result in the variation observed in the sizes of larvae.

No satisfactory explanation has yet been found to account for the existence of spawning outwith the 'normal' spawning season. Although originally attributed to long lived adults (Renouf, 1915; Miller, 1962), Todd (1979a) presented evidence strongly suggesting that summer-autumn spawnings were due to the precocious development of individuals from the succeeding generation. This author postulated that the warm weather of that year affected the growth and early maturation of the offspring. In the present study the high temperatures reached in 1994 during the summer months did not coincide with the presence of egg masses, indicating that factors other than temperature are involved. In Todd's study (1979a) ten small *Onchidoris bilamellata* individuals were found in the field in July, together with spawn masses; the five larger specimens (9-13 mm) had mature oocytes and spermatozoa, while the rest (2.1-5.2 mm) were immature individuals. This, together with the interruption of spawning in late spring (egg masses have not been recorded in the literature in either May or June) supports Todd's hypothesis.

The fact that adults 'degrow' as the spawning season progresses (Todd, 1979a) could account for the comparatively small size of the adults found by Todd in the summer (9-13 mm in July *vs.* 14-29 mm in January). To rule out the possibility of long

lived adults being responsible for the 'out of season' spawnings, a means of establishing the age of individuals accurately could provide the answer. It has been pointed out in studies by Todd (1977), Havenhand (1986) and Havenhand & Todd (1988b) that approximately 45-50% of *Onchidoris* dry weight is inorganic ash. It is likely that the proportion of inorganic ash (constituting the hard body structures of the animal, such as the spicules) would be higher in degrown individuals due to the higher catabolism of the body tissues. Thus, the inorganic ash content could be used to discriminate a degrown animal from a younger individual.

The 'out of season' spawning reveals the species plasticity in time patterns; however, the persistence of the 'normal' annual life cycle in known populations suggests that the larvae hatching outwith the 'normal' spawning period do not reach maturity in high enough numbers to cause a shift in the reproductive seasonality of *Onchidoris bilamellata*, or if a shift is taking place, it is happening in timescales that cannot (as yet) be observed.

CHAPTER 3

LARVAL GROWTH AND DEVELOPMENT IN *ONCHIDORIS BILAMELLATA* AND *GONIODORIS NODOSA*.

INTRODUCTION

The processes involved in the embryonic, larval and metamorphic development can proceed according to different patterns, which were first outlined by Thorson (1946, 1950) and later revised by Thompson (1967). Both of these schemes have been the basis for ulterior classifications (see e.g., Hadfield, 1963; Crisp 1976, 1984; Hadfield & Miller, 1987, Strathmann, 1978; Jablonski & Lutz, 1983; Jablonski, 1986). According to Thompson (1967) the development patterns may be classified into Type 1, species with planktotrophic veliger larvae, which undergo an obligatory pelagic phase during which feeding is essential; Type 2, species with lecithotrophic larvae, with a short pelagic life during which the larvae do not require to feed; and Type 3, species with direct development, without a pelagic larval phase, and whose benthic juvenile hatches on completion of a lengthy embryonic period.

Numerous studies have investigated the origin and evolution of these larval types (e.g., Chia, 1971; Jablonski & Lutz, 1983; Hadfield & Switzer-Dunlap, 1984; Jablonski, 1986; Strathmann 1978, 1985, 1986; Kempf & Todd, 1989; Havenhand 1993, 1994; Haszprunar et al., 1995), their ecological significance (refs. above and Thorson 1946; 1950; Mileikovsky, 1971; Clark, 1975; Todd, 1981; Hadfield & Switzer-Dunlap, 1984), as well as their implications on dispersal (see reviews by Crisp 1976, 1984; Palmer & Strathmann, 1981; Levin, 1990; Young, 1990), and energetics and fecundity (Vance 1973a,b; 1974, Underwood, 1974; Hermans, 1979; Stearns, 1976, 1977, 1980; Caswell, 1981; Hart & Begon, 1982; Grahame & Branch, 1985).

The development of laboratory culture of planktotrophic larvae relied to a great extent upon the improvement of the techniques employed in the culture of phytoplankton (Crisp, 1984; Todd, 1981). In fact, although some field studies have been conducted, the majority of the information regarding the larval phase of marine invertebrates

originates primarily from laboratory culture attempts (see reviews by Thompson, 1959; Thorson 1946, 1950; Hadfield, 1963; Todd, 1981; Bayne, 1983; Crisp, 1984; Day & McEdward, 1984; Hadfield & Switzer-Dunlap, 1984; Jablonski, 1986; Pechenik, 1987; Bhaud, 1988; Young, 1990; Havenhand 1991, 1993, 1994; Grosberg & Levitan, 1992). Even though results both in culture and from the field exhibit a great degree of variation in rates of growth and development (Todd & Doyle, 1981), laboratory culture has provided data otherwise inaccessible on the duration of the larval period under controlled conditions (Crisp, 1984).

The technique for the successful larval culture of *Onchidoris bilamellata* was developed and further improved by Todd (1981, 1991), based on that of Kempf & Willows (1977) for *Tritonia diomedea*. In the present study this methodology was used in the culture of *O. bilamellata* under different light regimes in order to determine whether growth and development proceed normally. The algal concentration was varied in accordance to the light regime in order to ensure that the larval food supply was plentiful.

The aims of this study were to investigate the following in *Onchidoris bilamellata*: i) whether larval growth and development is affected by the light regime, ii) the difference in shell size between newly hatched larvae from spawn masses laid by captive adults and larvae hatching from spawn masses collected in the field, and iii) the growth and development of larvae hatching from spawn masses laid in the field in September, outwith the winter-spring (December to May) reproductive period typical of the British Isles (Todd, 1979a). These cultures were maintained under ambient illumination and temperature regimes in an attempt to assess the viability of the larvae in conditions resembling those encountered in nature.

Because the methodology developed by Kempf & Willows for *Tritonia diomedea* was found appropriate for the culture of other nudibranchs (e.g., *Adalaria proxima* and *Onchidoris muricata* [Havenhand, 1986]), in the present work this technique was employed for the larval culture of *Goniodoris nodosa*. In this respect, this study is the first report of larval growth of *G. nodosa* in the laboratory.

MATERIALS AND METHODS

Collection and maintenance of the adults

A number of *Onchidoris bilamellata* and *Goniodoris nodosa* individuals were collected in 1991 and 1992 in St Andrews Bay (56° 20'N, 2° 47'W) and maintained in the laboratory in 5 litre containers with through-flow seawater for the production of spawn masses. Larvae of *Onchidoris bilamellata* were reared in the laboratory in 1991 and 1992. The spawn masses used for the cultures reared in 1991 were obtained from 10 adults collected on 18 January 1991. Those larval cultures reared in 1992 were from spawn masses laid by 13 adults collected on 22 November 1991. Larval cultures of *G. nodosa* were set up in 1992 from spawn masses laid by eight adults collected on 22 January 1992. In addition to this, in September 1993 two *O. bilamellata* spawn masses collected from Kingsbarns (56° 18'N, 2° 39'W) were cultured through metamorphosis.

The *Onchidoris* adults maintained in captivity were fed the barnacle *Semibalanus balanoides* (L.) attached to rock fragments. *Goniodoris* was fed the bryozoans *Flustellidra hispida* and *Alcyonidium polyoum* epiphytic on *Fucus serratus* fronds. Both water and food were replaced twice weekly for both of these nudibranchs, and post-spawning dead nudibranchs were removed as they occurred.

Spawn mass culture

The spawn-masses laid by the adults collected in winter and maintained in captivity thereafter were excised from the walls of the containers as they were laid using a scalpel and fine forceps and were transferred to 50 ml porcelain crucibles containing 0.2 µm filtered seawater (FSW). The water was changed daily, but aeration was not provided. The spawn masses were incubated at 10° C. The duration of embryonic period for all spawn masses was recorded, and the newly hatched larvae were pipetted from the incubation crucibles into larval culture containers. The spawn-masses collected in September 1993 were also maintained in 0.2 µm FSW in porcelain crucibles at 10° C.

Algal culture

The cryptophyte *Rhodomonas sp.* was chosen as food supply for the larval cultures of *Onchidoris bilamellata* and *Goniodoris nodosa* in view of the satisfactory results obtained by Todd (1988) in the previous laboratory culture of *O. bilamellata*. Inocula of this flagellate were obtained from the Culture Collection of Algae & Protozoa, Cambridge. New inocula were purchased in 1992, following contamination of the extant algal cultures. *Rhodomonas* sp. was cultured in the laboratory in Provasoli's Enriched Seawater (P.E.S.) medium (Provasoli, 1968), which was passed through a sterile 0.2 µm Millipore filter to preclude bacterial contamination and stored at 4°C in sterile screw-cap bottles. The medium was protected from light by covering the storage bottles with aluminium foil. The algal culture medium consisted of 30 ml of P.E.S. and 1000 ml of 0.2 µm FSW. The final mixture was filtered through a 0.2 µm Millipore filter.

Two types of algal cultures were prepared; stock cultures, to ensure a continuous supply of algae throughout the experiment, and batch cultures, set up sequentially in order to yield a continuous production for the larval cultures.

The purchased stock algal culture of *Rhodomonas* sp. was transferred to P.E.S. medium upon arrival and divided between three sterile 125 ml screw-cap bottles. The stoppers were left loose to allow for gas exchange. The cultures were maintained at room temperature under constant fluorescent light, but aeration was not provided in order to hinder the growth of these stocks.

The stock cultures were used to inoculate the new algal cultures. Inoculations of the routine batch cultures were done from other batch cultures in order to prevent contamination of the stock cultures. In the event of contamination occurring in the batch cultures, fresh cultures could be prepared from stock cultures. Once every two weeks new stock cultures were prepared by inoculating a small volume of algae (approximately 2 ml) from the batch cultures into sterile screw-cap flasks containing 75 ml of sterile algal medium.

Batch cultures were prepared by inoculation of ~ 2 ml of stock culture suspension in 300 ml of the algal culture medium. The batch cultures were maintained in 500 ml conical

flasks which were stoppered and aerated. The air input was filtered through cotton wool and the air vent tubing also was filled with cotton wool to prevent contamination (Fig. 3.1). The algae were incubated at room temperature under constant fluorescent light. After seven to 10 days, the cultures acquired a bright red coloration, indicative of algae being in the log-phase growth and providing a usable concentration for feeding purposes. New batch cultures were prepared on a weekly basis using previous batch cultures for inoculation. The old batch cultures not used for feeding purposes were disposed of, except for one, which was kept as a back up in the event of a culture failure.

The glassware employed in the algal culture had been thoroughly cleaned in hot water only (no detergents were used) and, with the exception of rubber stoppers, all the materials had been autoclaved prior to use. The bungs were sterilised in a warm oven (200°C approximately) for 20 minutes.

Previous larval culture (Todd, 1988) had indicated that the P.E.S. algal medium was noxious or toxic to mollusc larvae and thus could not be added to the larval cultures. Accordingly, all the algal suspensions that were used for feeding purposes were centrifuged, resuspended in FSW and re-centriguged prior to addition to the larval cultures.

Algal aliquots were centrifuged at 2,500 g for 10 minutes at 10°C. The supernatant was discarded, the pellet resuspended in 0.2 μm FSW, and the suspension centrifuged a second time. The concentration of cells in the suspension was determined using a haemocytometer. Preliminary observations indicated that *Rhodomonas* concentration of 5 x 10^4 cells · ml^{-1} and 10^5 cells · ml^{-1} were the optimal concentrations required for feeding larval cultures maintained under constant light and constant dark regimes respectively.

Larval culture

All the glassware utilized in the larval culture was either new or had been only used previously for live material. No detergents, soap or cleaning agents were used. The glassware was cleaned with hot tap water, left to dry, placed in an oven at 200°C for at least one hour and autoclaved (121°C; 15 min) prior to use. The larval cultures were maintained in 1L Pyrex glass beakers, and the transfer of the larvae were carried out using Pasteur pipettes.

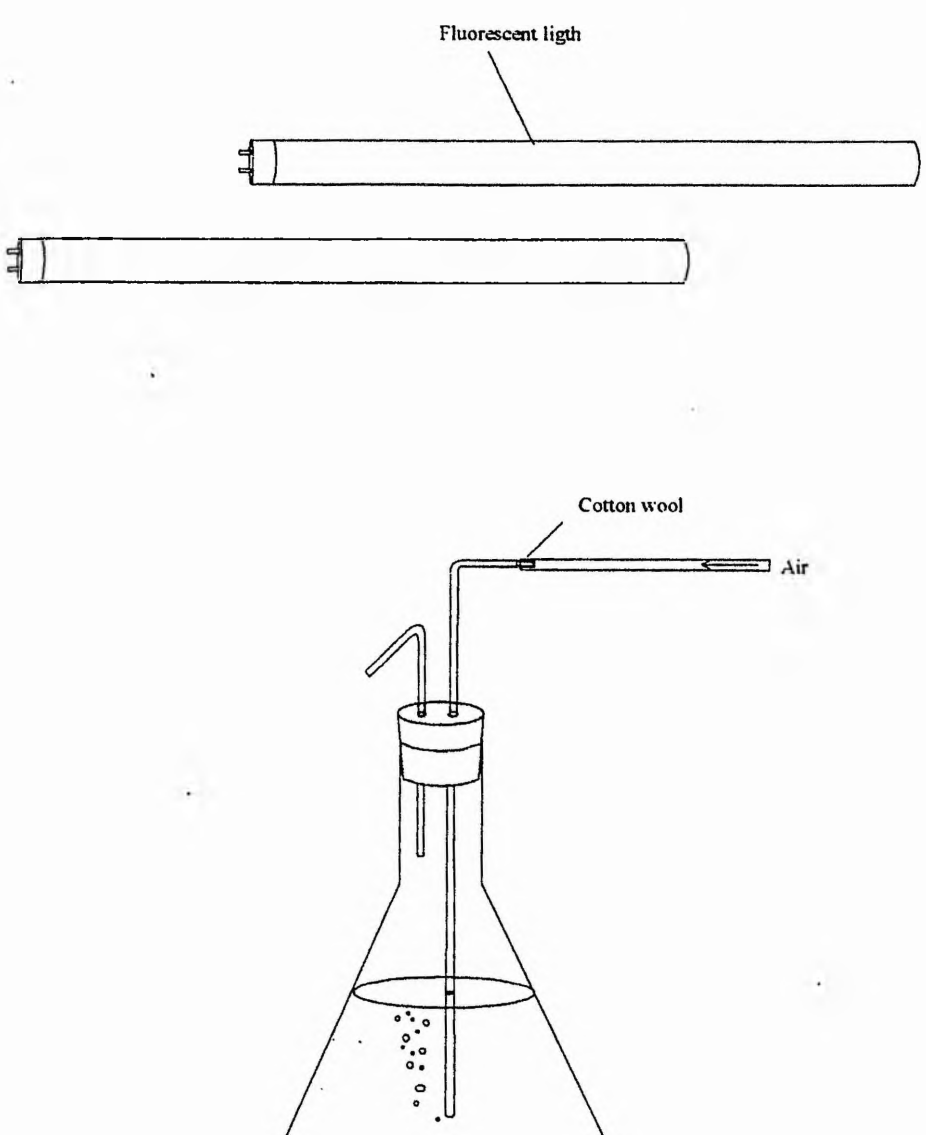


Figure 3.1 Set up used in the algal batch culture

In order to control bacterial and ciliate infestations, an antibiotic solution containing streptomycin sulphate (755 units · mg^{-1}) and penicillin G (1,602 units · mg^{-1}) (Sigma) was utilized for all the larval cultures, at final concentrations of 50 µg · ml^{-1} and 60 µg · ml^{-1} respectively.

The larval cultures were reared under five different illumination regimes, outlined in Table 3.1. The maximal concentrations of *Rhodomonas* in the culture medium were determined by observations of the larvae, whose left digestive diverticulum acquires a dark-red coloration when feeding is satisfactory (Todd, 1991). The *Rhodomonas* concentrations were, i) 5 x 10^4 cells · ml^{-1}; ii) 10^5 cells · ml^{-1}; iii) 5 x 10^4 cells · ml^{-1}; iv) and v) 2.5 x 10^4 cells · ml^{-1}. The concentrations used in regimes i) and iii) coincide because these cultures were carried out in different years and the algae were renewed in 1992 (see materials and methods).

Table 3.1. Summary of conditions of the *Onchidoris bilamellata* larval cultures throughout the present study.

Year	Origin Of the Spawn Masses	Locality of the Adults	Light Regime	Temperature	*Rhodomonas* Concentration
1991	laid by winter adults maintained in captivity	St Andrews	i) 24 L/0 D	15°C	5 x 10^4 cells · ml^{-1}
			ii) 0 L/24 D	15°C	10^5 cells · ml^{-1}
1992	laid by winter adults maintained in captivity	St Andrews	iii) 8 L/16 D	15° C	5 x 10^4 cells · ml^{-1}
			iv) 16 L/8 D	15° C	2. 5 x 10^4 cells · ml^{-1}
1993	Collected in the field	Kingsbarns	v) ambient illumination	ambient temperature (from 14° C to 6° C)	2. 5 x 10^4 cells · ml^{-1}

For the preparation of the larval cultures 700 ml of 0.2 µm FSW was transferred into the culture beakers, and the required volumes of algal suspension and antibiotic solution were added to obtain the final *Rhodomonas* concentration. The density of the newly hatched larvae in the spawn mass cultures was determined, and the required volume was added to the culture beakers to provide a final concentration of 2,500 larvae per culture.

The culture beakers were filled to 800 ml with 0.2 µm FSW. Any larvae entrapped on the water surface were resuspended by sprinkling 0.2 µm FSW on the floating larvae. Prior to placement in the incubators, all the culture beakers were covered with parafilm.

Onchidoris bilamellata and *Goniodoris nodosa* larvae may be successfully reared at any temperature ranging from 5 to 17°C. Several incubators (Gallenkamp instruments) were set at the required temperature and illumination regimes prior to the comencement of the experiment. The cultures were not aerated, to prevent the entrapment of larvae at the water surface, and fresh larval culture media were prepared and changed every four or five days. At each change of the larval culture medium the larvae were isolated by means of mesh filters and pipetted into fresh culture beakers.

The mesh filters were constructed by replacing the base of tripour plastic beakers with plankton mesh of the desired pore size. Epoxy resin was used to attach the mesh screens. A filter assembly consisted of a mesh filter held in a glass beaker (Fig. 3.2), allowing water to be poured through the filter and to run to waste.

For the maintenance of the larval cultures an 80 µm mesh filter was immersed in a 250 ml glass beaker containing 0.2 µm FSW. Three quarters of the volume of the larval culture was poured through the filter assembly, and the remainder of the culture was gently swirled to resuspend any larvae entrapped on the beaker walls or lying on the bottom, and poured through the filter. In order to prevent 'rafting' of the larvae, the filter was maintained below the water surface at all times.

The filter-beaker assembly was placed on a light box (Fig. 3.2), and the filter was rotated gently by hand. Once the water column was circulating, the filter was steadily raised at short intervals. In this way the larvae aggregated at the centre of the filter. The larvae were transferred by pipetting into a clean beaker containing 800 ml of the culture media. At every culture change the maximal shell lengths of ten larvae were measured to the nearest 3 µm using an ocular micrometer fitted to an Olympus-CH light microscope prior to their transfer to the clean vessel. Those larvae which had become entrapped on the water surface were resuspended by sprinkling 0.2 µm FSW.

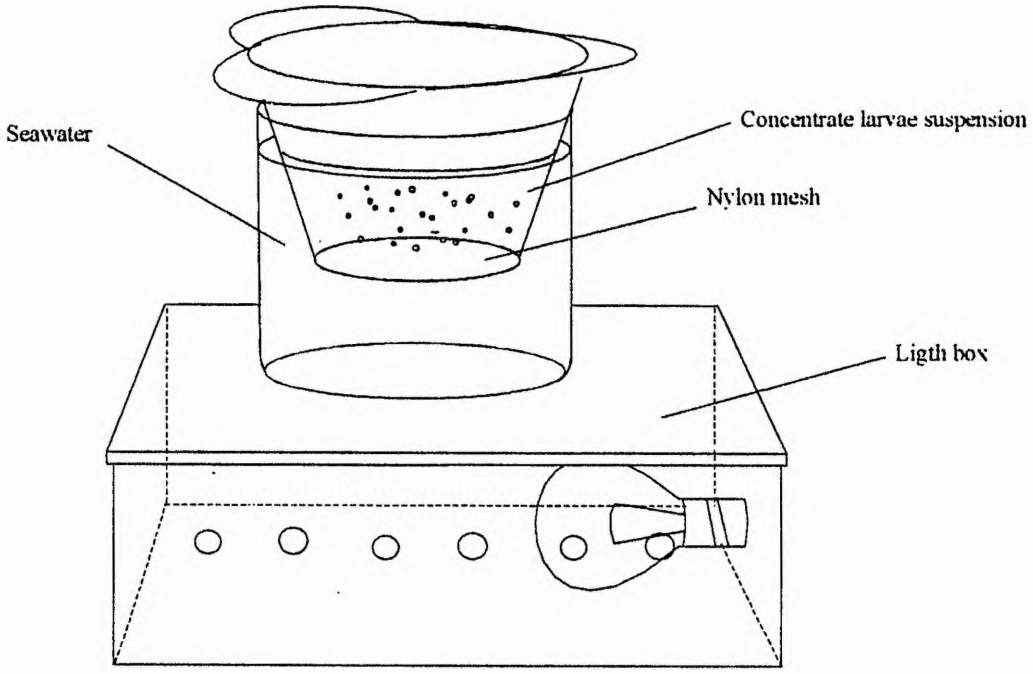


Figure 3.2 Ligth box used for the visualization of larvae.

Statistical analysis

All data analysis was by Minitab (Release 7.1). Shell lengths of larvae hatching from spawn masses laid at different times of the spawning season were tested for normality and followed by ANOVA.

RESULTS

Onchidoris bilamellata

A total of 17 separate spawn masses of *Onchidoris bilamellata* were incubated and their larvae cultured, the results of which are summarised in Tables 3.2, 3.3, & 3.4.

Table 3.2. Summary of data for *Onchidoris bilamellata* larval cultures set up from spawn masses laid by adults collected in January 1991 and maintained in captivity thereafter.

Light Regime	Month of Hatching	Mean Shell Length At Hatching (µm)	Mean Maximum Shell Length Attained (µm)	Achievement Of Competence (Days)
24 D/0 L	February	181.46	339.2	27
24 L/0 D	April	177.33	339.2	31
24 L/0 D		177.33	338.17	31
24 L/0 D		177.33	323.73	31
24 L/0 D		177.33	333.01	31
24 L/0 D		168.05	328.89	31
0 L/24 D		168.05	340.23	31
0 L/24 D	May	165.99	337.14	37
0 L/24 D		160.84	324.77	31
0 L/24 D		160.84	327.86	36
0 L/24 D	June	150.01	296.93	22
0 L/24 D		159.81	——	Not achieved
0 L/24 D		——	291.63	32
0 L/24 D	July	160.84	252.6	Not achieved
0 L/24 D		157.74	350.54	Not achieved
0 L/24 D		168.05	324.77	Not achieved

Rhodomonas concentration: 24 L/0 D, 5 x 10^4 cells · ml^{-1}; 0 L/24 D, 10^5 cells · ml^{-1}.

Table 3.3. Summary of data for *Onchidoris bilamellata* larval cultures set up in 1992 from spawn masses laid by adults collected in November 1991 and maintained in captivity thereafter.

Light Regime	Month of Hatching	Mean Shell Length at Hatching (µm)	Mean Maximum Shell Length Attained (µm)	Embryonic Period	Achievement of Competence (Days)
8 L/16 D	April	159.3	303.63	29	49
8 L/16 D		159.3	303.63	29	49
16 L/8 D		158.8	304.66	30	48
16 L/8 D	May	162.4	311.89	28	38
16 L/8 D		162.4	320.13	28	39
16 L/8 D		162.4	310.31	28	39
16 L/8 D	June	162.4	304.15	24	28
16 L/8 D		162.4	305.18	24	28
16 L/8 D		162.4	315.49	24	28

Rhodomonas concentration: 16 L/8 D, 2.5 x 10^4 cells · ml^{-1}; 8 L/16 D, 5 x 10^4 cells · ml^{-1}.

Table 3.4. Summary of data for *Onchidoris bilamellata* larval cultures set up in September 1993 from two spawn masses collected in the field.

Month	Light Regime	Mean Shell Length at Hatching (µm)	Mean Maximum Shell Length Attained (µm)	Achievement of Competence (Days)
September	ambient	141.12	244.35	Not achieved
		141.12	238.68	Not achieved
		134.67	245.38	Not achieved
		134.67	256.2	43

Mean shell size of hatching larvae was found to vary significantly between spawn masses laid at different times of the spawning season (one-way ANOVA, $p < 0.001$, Table 3.5), ranging between 150 µm and 180 µm (grand mean = 163.09, stdev = 11.98). This overall mean shell size is significantly larger than that for the spawn masses collected outwith the winter spawning season, in September 1993 (grandmean = 138.03 µm, stdev = 7.52), (ANOVA, $F = 330.74$, $p < 0.001$; Table 3.6).

Table3.5. One way ANOVA comparing the shell lengths of newly hatched larvae of *Onchidoris bilamellata* hatching in February, May, June, and July 1991. The spawn masses were obtained from adults collected in the field in January 1991 and maintained in captivity thereafter.

Source	DF	SS	MS	F	p
Factor	7	5909.7	844.2	11.21	0.000
Error	72	5423.8	75.3		
Total	79	11333.5			

Level	N	MEAN	STDEV
February	10	181.46	13.04
May	10	165.99	9.03
May	10	160.84	8.69
June	10	150.01	5.13
June	10	159.81	8.76
July	10	160.84	8.69
July	10	157.74	8.49
July	10	168.05	4.98

POOLED STDEV = 8.68

Table 3.6. ANOVA conducted to test for significant differences in size between newly hatched larvae of spawn masses laid from February-July 1991 in the laboratory, and spawn masses collected from the field in September 1993.

Source	DF	SS	MS	F	P
Factor	1	30159.1	30159.1	330.74	0.000
Error	198	18054.7	91.2		
Total	199	48213.8			

Level	N	MEAN	STDEV
February-July 1991	80	163.09	11.98
September 93	120	138.03	7.52

POOLED STDEV = 9.55

The larval batches obtained from adults collected in the winter months achieved competence to metamorphose under all the light regimes (Figs. 3.3 & 3.4). In all the cultures

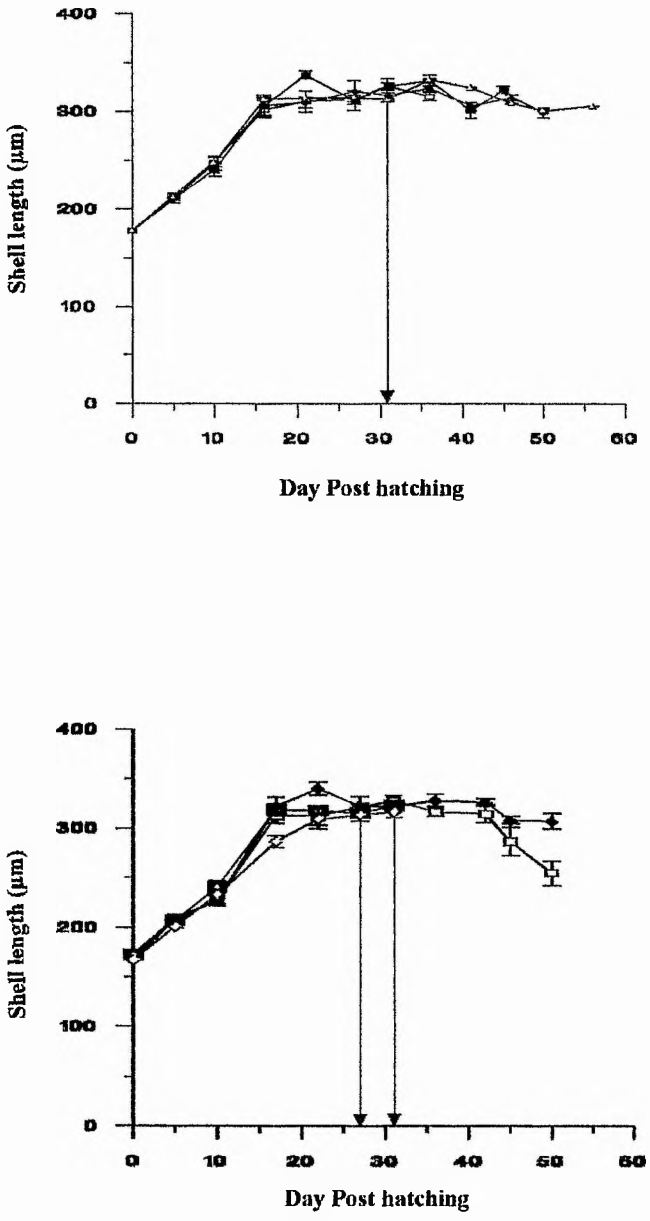


Fig 3.3 Larval *Onchidoris bilamellata* cultures reared under continuos illumination (Top graph) and in the absence of ligth (bottom graph).

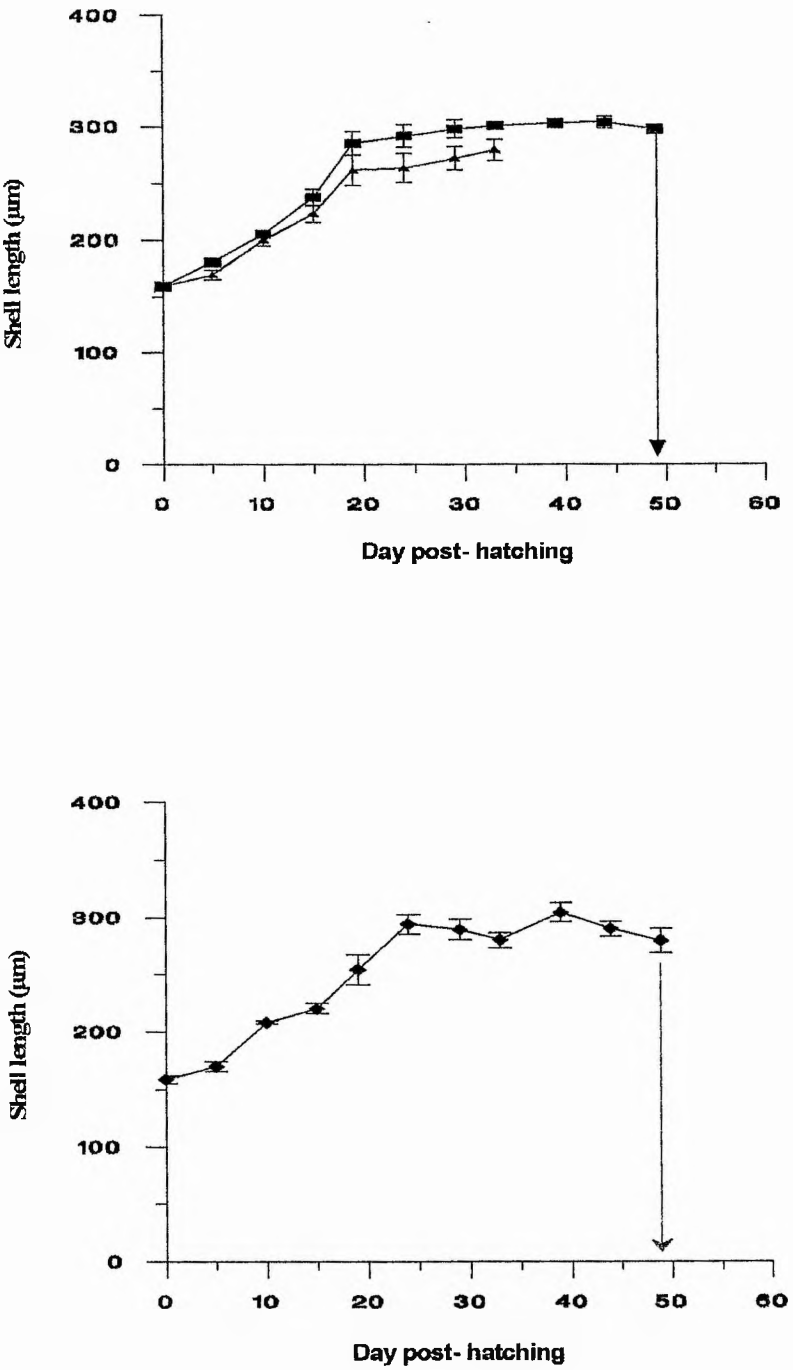


Figure 3.4. Larval growth of *Onchidoris bilamellata* cultured under different light regimes, 8 hours light/16 hours darkness (top graph) and 16 hours light/8 hours darkness (bottom graph).

the shape of the growth curves is curvilinear. The time to attain metamorphic competence was used as an estimate of developmental rate, and varied considerably between and within ligth regimes, ranging between 22 days and 49 days (Fig. 3.5).

The growth curves of the cultures reared in September 1993 are illustrated in Figure 3.6. With the exception of one larva, which was observed to crawl on day 43 and which metamorphosed successfuly when exposed to 19 mM K^+ ASW, larvae in the cultures commenced in September did not achieve competence.

Goniodoris nodosa

Two spawn masses of *Goniodoris nodosa* (separated into six individual larval cultures) were cultured through metamorphosis, the results of which are summarised in Table 3.7. Of the six larval cultures, four survived to achieve metamorphic competence.

Table 3.7. Summary of larval culture data for *Goniodoris nodosa*.

Month	Light regime	Mean Shell length at hatching (μm)	Mean maximum shell length attained (μm)	Achievement of competence (Days)
April	16L/ 8D	162.9	293.84	32
	16L/ 8D	162.9	300.02	32
	16L/ 8D	162.9	277.34	32
May	16L/ 8D	175.27	309.3	Not achieved
	16L/ 8D	175.27	307.24	40
		175.27	307.75	Not achieved

As for those of *Onchidoris* (Figs. 3.3. & 3.4), the shape of the growth curves in *Goniodoris nodosa* (Fig. 3.7) is sigmoid, characterized by a period of shell growth which arrests prior to the achievement of metamorphic competence.

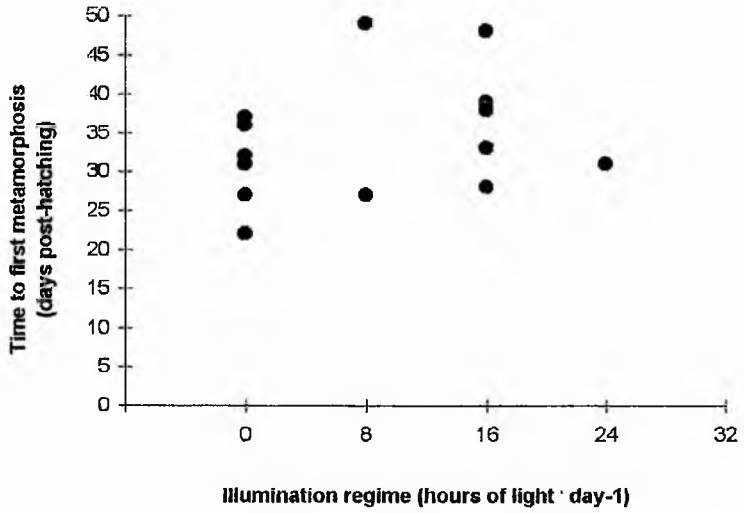


Fig. 3.5. Time to first achievement of competence to metamorphose of *Onchidoris bilamellata* larvae in culture under different illumination regimes, ranging between continous illumination and continuous darkness.

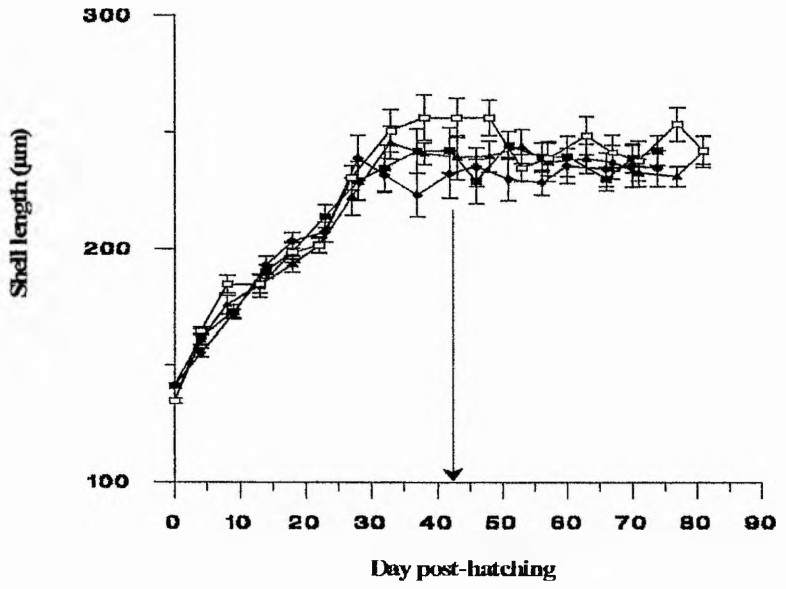


Fig. 3.6. Sibling larval cultures of *Onchidoris bilamellata* reared in September 1993 from two spawn masses collected in the field. An arrow denotes first competence observed in culture.

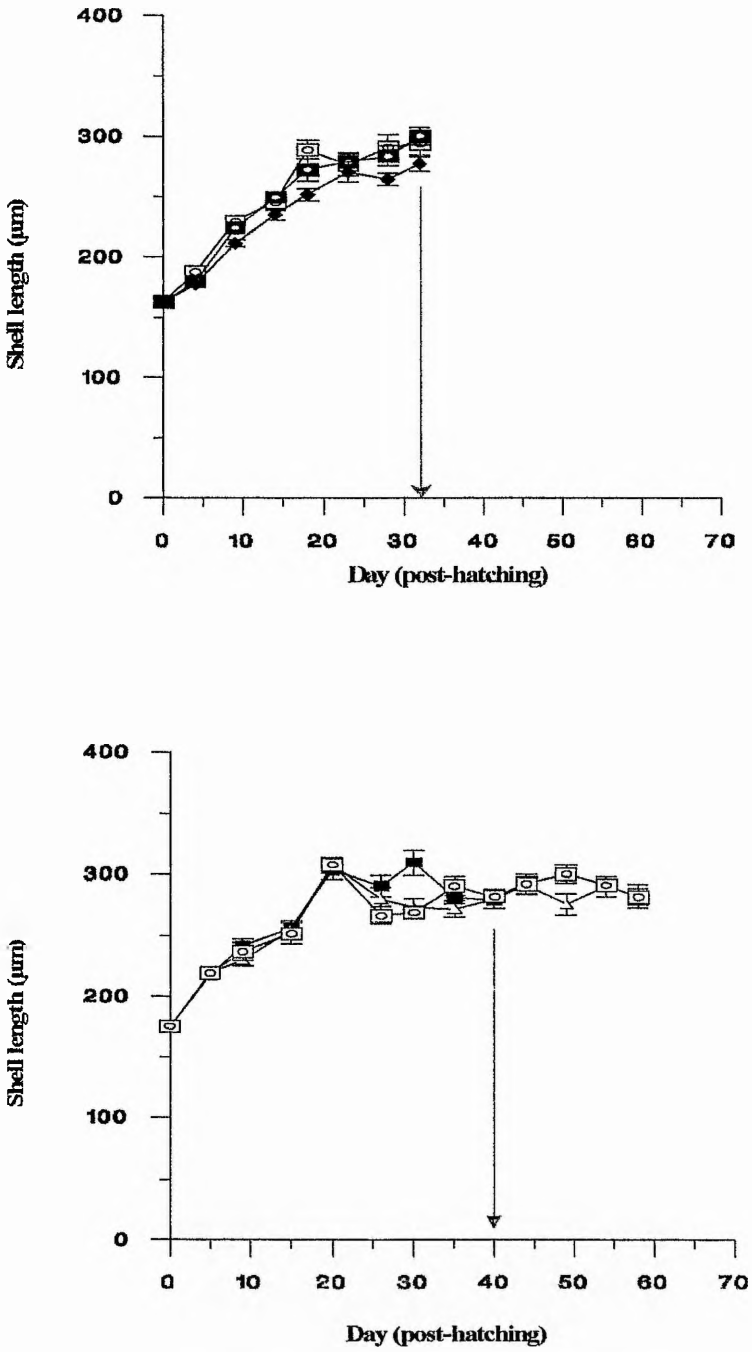


Fig. 3.7. Sibling larval cultures of *Goniodoris nodosa* reared in April 1992 (top graph) and May 1992 (bottom graph). The cultures set up in April were used on day 32 for metamorphosis experiments. An arrow denotes time to first competence observed in culture.

DISCUSSION

The embryonic development of *Onchidoris bilamellata* has been studied by Thompson (1967), Hadfield (1963), and Todd (1979a). These authors report a duration of the embryonic period for *O. bilamellata* ranging between 8 to 9 days at 10 to 12 °C (Hadfield, 1963), 17 to 19 days at 10 °C (Thompson, 1967; Todd, 1979a), and 22 days at 7°C (Todd, 1979a). The duration of the embryonic period at 10 °C for *O. bilamellata* in the present study was greater than the above figures, ranging between 24 and 30 days (Table 3.3), which indicates that some degree of variation may be present.

Similarly, there exists a great degree of variability in the growth and development rates between and within cultures during the larval phase in marine invertebrates (e.g., Pechenik 1980, 1984; Lima & Pechenik, 1985; Pechenik *et al.*, 1990; Zimmerman & Pechenik, 1991; Gonçalves *et al.*, 1995). In nudibranchs this phenomenon has also been reported (e.g, Havenhand, 1986) and has been primarily attributed to the entrapment of the larvae on the air-water interface due to the hydrophobic nature of the veliger shell (Todd 1981, 1991). In the present investigation the larval culture of *Onchidoris bilamellata* and *Goniodoris nodosa* exhibited this characteristic variation in the time to achieve competence to metamorphose (Tables 3.2 to 3.5).

The first successful attempt to culture *Onchidoris bilamellata* through metamorphosis was reported by Todd (1981), who in a later study (Todd, 1991) provided detailed information on the larval growth and development of this nudibranch, which he cultured on flagellate mixtures of *Isochrysis*, *Pavlova*, *Rhodomonas* and *Tetraselmis* at 5, 10 and 15 °C. The results obtained in the present study agree in general with those of Todd (1991). The shell sizes of the larvae at competence, for example, average 350 µm in his investigation, which is comparable to ~300 µm of the present study. Likewise, sigmoid growth curves were obtained in both studies.

The effect of illumination in nudibranch larvae has been mainly addressed from the view point of changes in photic response with larval age (Thompson, 1976; Todd, 1983; Hadfield & Switzer-Dunlap,1984; Miller & Hadfield, 1986), it being a generally accepted

notion that young larvae are strongly photopositive, whereas phototaxis decreases with incresasing age. However, the effects of illumination on the larval growth and development have not been assessed in nudibranchs. The tendency of young nudibranch larvae to become entrapped on the air-water interface (Todd, 1981), can be lessened when cultures are maintained in continuous darkness (personal observations). Whether this is related to larval photic responses is unknown, but non-illuminated cultures can provide a useful tool to circumvent the rafting of larvae.

In the present work larvae were cultured under different light regimes in order to determine the influence of light on the time to achieve competence (as an estimate of development rate). Light conditions ranged between constant illumination and constant darkness (Table 3.1). No substantial differences in development or achievement of competence were observed in larvae reared with different illumination regimes (this study).

The variability in the larval quality throughout the spawning season has received little attention; nonetheless data from other studies indicate that 'better quality' eggs are produced early in the spawning season (see for example, Kraeuter et al, 1982; Havenhand & Todd, 1988b). This presumably would result in a reduced viability of the later offspring. In the present study the *G. nodosa* and *O. bilamellata* spawn masses laid in captivity rendered viable larval cultures until May and June respectively, but the cultures set up in May, June, and July contained many larvae which evacuated the shell spontaneously, but without subsequent completion of metamorphosis. These larvae continued feeding and swimming in the normal manner, but they were discarded from the cultures. Although this phenomenon had been observed previously in our laboratory (Todd, personal communication), the reasons for the casting of the shell have as yet not been ascertained, and whether these 'spontaneous evacuees' eventually attain competence or die is also an interesting subject matter which requires further investigation.

In a comprehensive study on the biology of *Onchidoris bilamellata* Todd, (1979a) demonstrated that many more spawn masses are laid by adults maintained in captivity than by adults of field populations. However, he did not conduct comparative studies on the larval development between laboratory and field-produced offspring. In order to ascertain whether

the larval viability is influenced by the season, or is a laboratory artefact, cultures set up from spawn masses laid in the field at different stages of the reproductive season would prove necessary. Large samples of spawn masses collected on several occasions throughout the reproductive season would be necessary in order to account for the between adult variation which would ultimately reflect on larval viability.

The larval cultures set up from spawn masses collected in September 1993 did not achieve competence to metamorphose, with the singular exception of one larva in one of the cultures (on day 48 post-hatching, shell length 294 μm). The mean maximum shell length attained in the September cultures was below 300 μm (grand mean pooled from the four cultures = 242 μm). These results are in marked contrast with the laboratory cultures set up during the winter and spring months in the present study (Tables 3.2 & 3.3), as well as with previous studies on this species, where competence to metamorphose was consistently achieved by larvae of shell lengths above 320 μm (Todd 1981, 1991; Todd & Doyle, 1981), and the numbers of metamorphs ranged from 30 to 70 per culture (personal observations).

Juvenile stages are subject to prey size constraints (Todd & Doyle, 1981), and therefore the ability to attain a size when *Semibalanus balanoides* can be tackled is determined by the grazing capacity of the juveniles. According to Todd & Doyle (1981) the duration of the larval period for *Onchidoris bilamellata* is timed so that it coincides with the settlement of the prey spat, the barnacle *Semibalanus balanoides*, and thereby the time during which the juveniles must subsist on an intermediate diet is minimized. The results obtained from the present study suggest that the September offspring exhibits low viability; however, the data derive from only two larval batches, and therefore can only be regarded as preliminary results. In the event of some of these larvae surviving to metamorphosis, the grazing capacity of the newly-metamorphosed juveniles would have to suffice to sustain the energy requirements until a suitable size is attained.

The present study is the first report of larval growth of *Goniodoris nodosa*. This nudibranch, cultured through metamorphosis at 15 °C, exhibited shell growth which ceased some days prior to achievement of competence. The completion of shell growth has been

attributed to the retraction of the mantle fold from the shell margin (Bonar, 1976). The result is a sigmoid growth curve, which has been observed in nudibranchs (e.g., *Berghia verrucicornis*, Carroll & Kempf, 1990; *Tritonia diomedea* Bergh, Kempf & Willows, 1977; *Onchidoris muricata* Müller, Havenhand, 1986) as well as in other opisthobranchs (e.g., aplysiids, Kriegstein *et al*, 1974; Switzer-Dunlap, 1977; Switzer-Dunlap & Hadfield, 1977; Kempf, 1981; Paige, 1986; Nadeau *et al*, 1989) and is possibly a feature of opisthobranchs (Todd, 1991).

CHAPTER 4

INDUCTION OF METAMORPHOSIS IN *ONCHIDORIS BILAMELLATA* (L.) AND *GONIODORIS NODOSA* (MONTAGU)

INTRODUCTION

On completion of the larval development, most opisthobranch molluscs pass through a metamorphosis which transforms the larva, whose body plan is specialized for a pelagic existence, into a benthic juvenile with an entirely different mode of life (Thompson 1962, 1967, 1976; Bonar 1976, 1978a; Switzer-Dunlap, 1978; Ros, 1981; Todd, 1981; Miller & Hadfield, 1986; Hadfield & Miller, 1987).

The general morphology of the opisthobranch veliger larvae prior to metamorphosis is illustrated in Fig. 4.1 (from Bonar, 1978a). Competence to metamorphose is often marked by a set of recognizable morphological features (Chia, 1978), which in opisthobranch molluscs includes the development of the propodium, the appearance of eyes and the retraction of the mantle fold (Todd, 1983). In addition, the competent veliger is easily recognized in laboratory cultures by the alternation of swimming activity with periods in which the larva sinks and crawls on the bottom of the culture container (Thompson, 1976; Todd 1981, 1983, 1991). The changes involved at metamorphosis in the Nudibranchia (illustrated in Fig. 4.2) include the regression of the velar lobes, the loss of the shell, operculum and mantle cavity, and the fusion of the visceral mass with the foot, resulting in a flattened and elongated juvenile (Bonar & Hadfield, 1974; Thompson, 1976; Bonar, 1978a; Todd, 1983; Hadfield & Switzer-Dunlap, 1984).

Many physical and biotic factors elicit or inhibit the settlement and metamorphosis of marine invertebrate larvae (see reviews by Crisp 1974, 1984; Scheltema, 1974; Chia & Rice, 1978; Burke, 1983a; Bakus et al., 1986; Morse, 1990; Pawlik & Hadfield, 1990; Pawlik, 1992). The induction of metamorphosis of *Onchidoris bilamellata* has been investigated by Todd (1979a, 1981), Chia & Koss (1988, 1989) and Arkett *et al.* (1989).

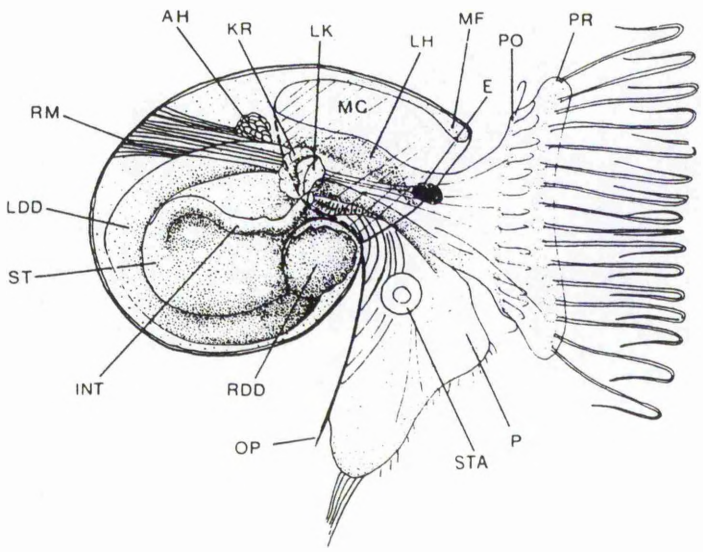


Fig. 4.1. Diagrammatic representation of a premetamorphic opisthobranch veliger. AH, adult heart rudiment; E, eye; INT, intestine; KR, adult kidney rudiment; LDD, left digestive diverticulum; LH, larval heart; LK, larval kidney; MC, mantle cavity; MF, mantle fold; OP, operculum; P, propodium; PO, postoral velar ridge; PR, preoral velar ridge; RDD, right digestive diverticulum; RM, retractor muscles; ST, stomach; STA, statocyst. From Bonar (1978a).

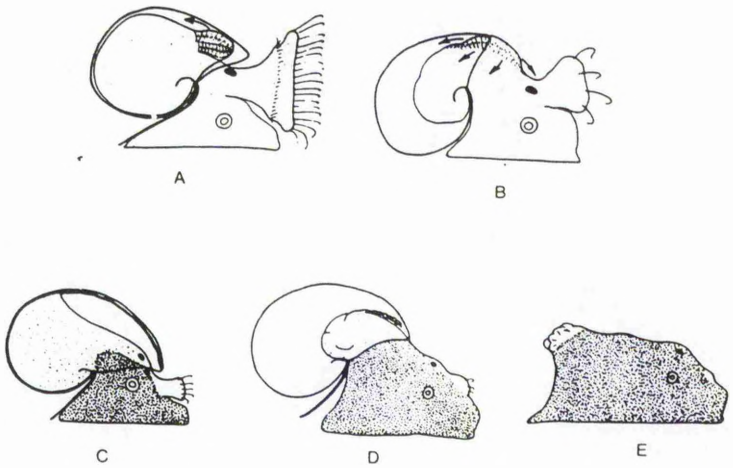


Fig. 4.2. Diagrammatic representation of the main events taking place at metamorphosis. A.B, reflection of the mantle fold. C-E, Resorption of the velum and loss of the shell, operculum and mantle fold. From Bonar (1978a).

Competent larvae of *O. bilamellata* are induced to metamorphose in the presence of the adult prey species *Semibalanus balanoides* (Todd, 1979a) and *Chthamalus dalli* (Chia & Koss, 1988). Todd (1981) examined the influence of light on metamorphosis of *O. bilamellata*, and concluded that light does not influence metamorphosis in this nudibranch. Chia & Koss (1988) studied the inductive properties of live and dead prey, and their results indicated that larvae must establish physical contact with either living or dead barnacles in order to be induced to metamorphose. Subsequent work focused on the identification of sensory cells (Arkett *et al.*, 1989) and propodial ganglia (Chia & Koss, 1989) in competent veligers. Given that metamorphosis is induced by water-borne substances in a number of nudibranchs (Hadfield & Pennington, 1990; Pires & Hadfield, 1991a; Bahamondes-Rojas & Dherbomez, 1990; Lambert & Todd, 1994), and in order to verify the results obtained by Chia & Koss (1988), in the present study the metamorphic activity of *O. bilamellata* was tested allowing larvae to establish physical contact with the natural inductive substratum, and at a distance from it.

Along with natural inducers, many marine invertebrate species can be stimulated to metamorphose by elevated concentrations of the potassium ion above that normally found in seawater (Yool *et al.*, 1986; Baloun & Morse, 1984; Pechenik and Heyman, 1987; Eyster & Pechenik, 1988; Todd *et al.*, 1991). Nonetheless, elevated K^+ does not induce metamorphosis of all the marine invertebrates for which it has been assayed (e.g., *Mytilus edulis*, Eyster & Pechenik, 1987; the barnacle *Balanus amphitrite*, Rittschof *et al.*, 1986). In order to ascertain whether *Onchidoris bilamellata* metamorphoses in response to elevated concentrations of potassium, and to establish the optimal concentration of excess potassium at which metamorphosis may occur, a potassium dose-response test was carried out, with concentrations ranging from 0 mM excess K^+ to 30 mM excess K^+.

In the absence of the appropriate environmental stimuli, many species are capable of delaying metamorphosis (Crisp, 1974, Scheltema, 1974; Pechenik, 1990). The duration of the delay phase has been investigated in many marine invertebrates, including echinoids (Rumrill, 1989), annelids (Knight-Jones, 1953; Qian *et al.*, 1990), and gastropods (bivalves, Coon *et al.*, 1990; prosobranchs, Pechenik 1980, 1984; Pechenik & Lima, 1984; Lima & Pechenik, 1985; and nudibranchs, Kempf & Hadfield, 1985; Kempf & Todd, 1989; Miller &

Hadfield, 1990; Miller, 1993). The ability of *Onchidoris bilamellata* to delay metamorphosis has not been investigated up to date, and the present work aimed at assesing the delay potential in this nudibranch.

With respect to the induction of metamorphosis of *Goniodoris nodosa*, Lambert & Todd (1994) provide succinct information regarding this species, and indicate that *G. nodosa* larvae may metamorphose in response to species of Bryozoa and Urochordata. However, no additional studies have further analysed this problem. The dorid nudibranch *Goniodoris nodosa* preys upon encrusting bryozoans (e.g., *Alcyonidium polyoum* and *Flustrellidra hispida*) when young, whereas the adults feed preferentially on the ascidians *Diplosoma listerianum*, *Botryllus schlosseri* and *Dendrodoa grossularia* (McMillan, 1942; Miller, 1961; Swennen, 1961). In the present study metamorphosis of *G. nodosa* in response to the juvenile prey *Alcyonidium polyoum* and the adult prey *Botryllus schlosseri* were investigated.

MATERIALS AND METHODS

The competent larvae of *Onchidoris bilamellata* and *Goniodoris nodosa* utilized in the present work were obtained from eggs laid by adults collected in St Andrews (56° 20'N, 2° 47'W) and maintained in groups in aquaria at 7 °C (±1 °C) with food in excess (for further details see Chapter 3). Adult *O. bilamellata* were collected in November 1991, except for the specimens whose larvae were used in Experiment 6, which were collected in January 1991. Adult *G. nodosa* were collected in January 1992. The spawn masses were incubated at 10 °C and transferred to 15 °C at hatching to accelerate growth and developmental rates. The larvae were cultured in the laboratory following the techniques outlined in Chapter 3. Those veligers which exhibited a crawling behaviour, regarded as competent (Todd, 1981), were selected for the experiments.

Wherever possible, only sibling larvae were used for any one experiment, otherwise larvae obtained from more than one spawn mass were grouped and then mixed to ensure randomization. The number of larvae available for experimental purposes was low due partly to bacterial and ciliate contaminations, and partly to losses inherent to the technique employed to change the culture medium (see Chapter 3). Moreover, the tendency of opisthobranch veligers to become entrapped on the air-water interface impedes their feeding activity and causes a reduction in growth and ultimately the demise of many of the larvae (Todd, 1991). This in turn, accentuates between and within-cultures variations of the rates of growth and development - a common phenomenon in the culturing of marine invertebrates (e.g. Pechenik 1980, 1984; Zimmerman & Pechenik, 1991). Consequently, the numbers of larvae that attained competence at any one time in the present study were restricted (for example, the time required to attain competence to metamorphose at 15 °C ranged from 25 to 40 days in *O. bilamellata*, and from 33 to 40 days in *G. nodosa*). In view of this scarcity, it was decided to include the totality of the competent larvae in the treatments, which resulted in unequal numbers of pediveligers in some of the replicates (ranging from 5 to 7 larvae per replicate).

The experiments were carried out in covered glass petri dishes maintained in an incubator (Gallenkamp) at 10 °C in those experiments which included prey treatments (Experiments 4 to 6) in order to conform with the temperature tolerances of the preys. Other

experiments were run at 15 °C (Experiment 1), when prey treatments were not included. The test dishes had been previously rinsed in hot water, heated for one hour at 200 °C and autoclaved for 15 minutes at 121 °C. The dishes employed for all the experiments were 50 mm diameter and 18 mm height, except for Experiment 2. On this occasion the fragments of mussel shells included in the treatments exceeded the height of the smaller petri dishes, and larger glass dishes (77 mm diameter and 45 mm height) covered with parafilm were used instead. For the smaller dishes a volume of 20 ml of artificial seawater (ASW) was used, which resulted in a maximal depth of 10 mm. The larger dishes contained 80 ml of ASW, yielding a water depth of 30 mm. In Experiment 6 ASW was substituted by culture medium containing the flagellate *Rhodomonas* sp. and an antibiotic mixture in 0.2 µm filtered seawater (FSW) (see Chapter 3).

The treatments of every experiment were triplicated, and each replicate was scored every 24 hours using a Wild M8 stereomicroscope. The larvae were recorded as either metamorphs (those individuals which exhibited resorption of the velar lobes and loss of the shell) or pediveligers (those with shell and/or non-reduced velar lobes), and any dead individuals were removed. The experiments were terminated after 3 or 4 days, and the cumulative percentages of metamorphs after this period were considered.

Preparation of artificial seawater

The procedure followed to prepare the artificial seawater (ASW) was that described by Baloun & Morse (1984), based on the 'MBL' recipe (Woods Hole Marine Biological Laboratory). A stock solution was prepared by adding $CaCl_2$, $MgCl_2$, $MgSO_4 \cdot 7H_2O$ and $NaHCO_3$ to deionized water (Table 4.1). The stock solution was thoroughly mixed, and the required amounts of NaCl and KCl were added in order to obtain 9 mM K^+ ASW, which is the potassium concentration of natural seawater.

For Experiment 1, six additional solutions of ASW with potassium ion in excess were prepared by varying the amount of KCl added to the stock solution, and reducing NaCl accordingly in order to maintain the ionic content and osmolarity (Table 4.2).

Table 4.1. Stock solution recipe of Baloun & Morse (1984).

	Molecular Weight	N mM in M.B.L.*	$g \cdot l^{-1}$
$Ca\ Cl_2$	110.99	9.27	1.0289
$Mg\ SO_4 \cdot 7\ H_2O$	246.5	25.50	6.2858
$Na\ H\ CO_3$	84.01	2.15	0.1806
Mg Cl solution 4.9 M	95.21	22.94	4.6816 ml

* millimolar concentration in 'Woods Hole Marine Biological Laboratory' seawater
** made up with deionised water

Table 4.2. Artificial seawater solutions with different concentrations of potassium ion, obtained by adding the amounts of Na Cl and K Cl stated below to one litre of the stock solution.

Potassium concentration in ASW	NaCl (g)	KCl (g)
9 mM	24.72	0.67
14 mM	24.42	1.04
19 mM	24.14	1.42
24 mM	23.86	1.8
29 mM	23.56	2.16
34 mM	23.26	2.54
39 mM	22.98	2.92

Collection and preparation of live and dead prey

Both live and exoskeleton remains of *Semibalanus balanoides* settled on either unoccupied molluscan shells (*Mytilus* sp. and *Patella* sp.) or fragments of rocks were freshly collected from St Andrews Bay. Bare shells and small rocks were also collected for the controls.

For Experiment 2 unoccupied shells of the mussel *Mytilus* sp. bearing live *Semibalanus balanoides*, and shells bearing dead (exoskeleton remains) of *S. balanoides* were fragmented into smaller pieces with a hammer and a chisel. The presence of bacterial films, which induces many marine invertebrates to settle and metamorphose (Cameron & Schroeter, 1980; Crisp, 1984; Todd & Keough, 1994; Keough & Raimondi, 1995) was minimized by brushing the shells with a toothbrush, following which they were rinsed in natural seawater. In Experiments 3 and 4 the mussel shells were substituted by unoccupied limpet (*Patella sp.*) shells as the settling substrata for *S. balanoides*, for the reason that limpet

shells were more readily available at St Andrews Bay and easier to cleave. The limpet shells were fragmented and treated similarly to those of Experiment 2. In Experiment 5 live colonies of *Alcyonidium polyoum* and *Botryllus schlosseri* growing epiphytically on *Fucus* sp. fronds, together with bare fronds of *Fucus* (for the control) were collected from Argyll (56° 17'N, 5° 37'W) on the day prior to the start of the experiment and maintained in an aquarium of running seawater at 7 °C. The fronds, which were not brushed, were excised in small sections and transferred to the test dishes. For Experiment 6 the barnacle *S. balanoides* was collected settled on rocks which were fragmented into smaller pieces using a hammer and a chisel, brushed with a toothbrush and rinsed in natural seawater.

Preparation of bags and separation chambers

Flat envelope-bags of plankton net (150 μm) were used to preclude physical contact between the inductive substratum and the larvae (Experiment 3). For their construction a small section (~40 x 25 mm) of nylon mesh was folded in half and sealed with heat on three of the sides. The inductive substratum was introduced in the bag, after which the sealing of the bag was completed. In the control the bag was substituted by a piece of plankton net to account for any effects of the nylon material.

The separation-chambers used in Experiment 4 were made with a finer plankton net (80 μm) than that used for the bags to prevent the escape of debris originating from the prey. To construct a chamber, the soles of two plastic cups were cut off and a plankton net was entrapped between the cups at 5 mm from the bottom. The chambers were placed in test dishes (50 mm diameter and 18 mm height) containing 20 ml of water each, which yielded a water depth of 5 mm above and below the plankton net (Fig. 4.3.).

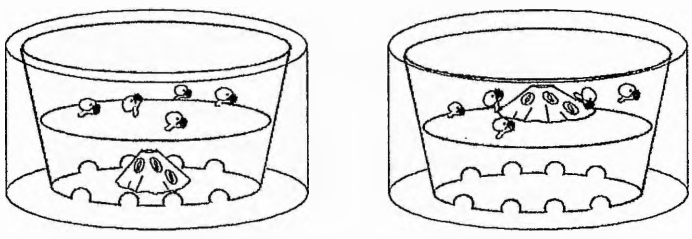


Fig. 4.3. Filter chambers used to preclude contact between larvae and the inductive substrata.

Experiment 1. Potassium ion dose-response of *Onchidoris bilamellata*

The aim of this experiment was to determine the concentration of potassium ion in excess to that found in natural seawater (9 mM) which induces the maximal metamorphic response of *Onchidoris bilamellata*. The risk of contamination by bacteria or ciliates was estimated to be low given that ASW was used, hence antibiotics were not utilized in this experiment. The larvae were exposed to seven treatments (with 7 larvae per replicate dish), (i) 9 mM K^+ ASW (control), (ii) 14 mM K^+ ASW, (iii) 19 mM K^+ ASW, (iv) 24 mM K^+ ASW, (v) 29 mM K^+ ASW, (vi) 34 mM K^+ ASW, and (vii) 39 mM K^+ ASW. The experiment was started on 16 June 1992 and terminated after 4 days.

Experiments 2 to 4. Induction of Metamorphosis in *Onchidoris bilamellata* on contact with and at a distance from the natural prey.

This set of experiments was carried out to establish a) whether physical contact between competent *O. bilamellata* larvae and the natural inducer, the adult prey *Semibalanus balanoides*, is necessary to induce metamorphosis; and b) whether the inductive cue is present in live barnacle tissues or associated with the barnacle exoskeleton. In the tests conducted to this effect, the larvae were not allowed to establish physical contact with the prey but were exposed to any soluble components emanating from the barnacles. The flagellate *Rhodomonas* sp. (25×10^3 cells ml^{-1}) was added to the ASW in all the treatments to prevent starvation of the prey. In Experiments 3 and 4 the antibiotics streptomycin sulphate 755 units $\cdot$ mg^{-1}) and penicillin-G (1,602 units $\cdot$ mg^{-1}) (Sigma) were added to the ASW at final concentrations of 50 $\mu g \cdot ml^{-1}$ and 60 $\mu g \cdot ml^{-1}$ respectively, in view of the larval mortality observed in Experiment 2. Experiment 2 started on 2 July 1992, Experiment 3 on 29 July 1992, and Experiment 4 on 24 September 1992. Experiments 2, 3, and 4 were terminated after 4 days.

In Experiment 2 the tendency of nudibranch veligers to become entrapped at the water surface (Todd, 1991) was used to preclude physical contact between the larvae and the prey. The larvae were entrapped at the air-water interface by manipulation with a fine needle. All the replicates (a, b, & c) contained 6 larvae, except for replicates (i) c and (ii) c which had 5. The treatments were (i) larvae rafted above fragments of mussel shells bearing exoskeleton

remains of *S. balanoides* (control), and (ii) larvae rafted above fragments of mussel shells bearing live *S. balanoides*. The height of the shell fragments was ~ 20 mm (maximal water depth = 30 mm) which resulted in the larvae being ~ 10 mm from the inductive substratum. Only those rafted metamorphs whose shells were also on the air-water interface were considered to have metamorphosed at a distance from the substratum.

The methodology followed in Experiment 2 proved unsatisfactory because a high percentage of the larvae (ranging from 33 to 83%) did not remain rafted to the end of the experiment. To overcome this difficulty, an alternative approach was followed in Experiment 3. The treatments of Experiment 3, in which physical contact between larvae and prey were impeded, were enclosed in plankton net-bags (150μm mesh) and immersed in the test dishes. The larvae were sunk and allowed to crawl freely on the bags but were not able to establish physical contact with the prey. In the treatments where contact was allowed, the bags were substituted by plankton net situated beside the prey at the bottom of the dish. All of the replicates (a, b, & c) contained 7 larvae, except for the replicate (ii) c which had 8 larvae. The treatments in Experiment 3 were (i) larvae sunk with bags containing dead *Semibalanus balanoides* on *Patella* shell, (ii) larvae sunk with bags containing live *S. balanoides* on *Patella* shell, and controls comprising (iii) larvae sunk with live *S. balanoides* on *Patella* shell adjacent to plankton net, and (iv) larvae sunk with dead *S. balanoides* on *Patella* shell adjacent to plankton net.

The larvae were difficult to visualize in Experiment 3 due partly to the debris expelled by the prey, and partly to the fact that the larvae crawled beneath the bags. To overcome this problem, in Experiment 4 the bags were substituted by separation-chambers of 80 μm mesh. In the treatments in which contact between larvae and prey was precluded, the inductive substratum was placed under the plankton net and the larvae above it. In those in which contact was permitted both the prey and the larvae were placed above the net (Fig. 4.3). The prey used in this experiment was *Semibalanus balanoides* growing on *Patella* shells. The treatments in Experiment 4 were (i) larvae contacting live prey, (ii) larvae contacting dead prey, (iii) larvae contacting bare *Patella* shell (control), (iv) larvae at a distance from live prey, and (v) larvae at a distance from dead prey.

Experiment 5. Metamorphosis induction of *Goniodoris nodosa*

This experiment was conducted to establish whether the juvenile bryozoan prey *Alcyonidium polyoum* or the adult ascidian prey *Botryllus schlosseri* induce metamorphosis of *Goniodoris nodosa*.

Neither food nor antibiotics were added to the ASW. Competent *G. nodosa* larvae (5 larvae per replicate dish) were allowed to contact the inductive substrata in all the treatments. The treatments were (i) a small section of *Fucus* frond (control), (ii) live colony of *Alcyonidium polyoum* epiphytic on *Fucus*, and (iii) live colony of *Botryllus schlosseri* epiphytic on *Fucus*. The experiment started on 23 May 1992 and terminated after 3 days.

Experiment 6. Delay of metamorphosis in *Onchidoris bilamellata*

The aim of this study was to investigate whether *Onchidoris bilamellata* can delay metamorphosis, and the time period during which larvae can postpone this event without losing the ability to metamorphose.

The totality of the competent larvae used in this experiment (140 larvae) were obtained from two cultures which had undergone a larval phase of 30 days (post-hatching). Because the larvae were not individually-reared due to the extended pre-competent period of *O. bilamellata* (25 to 40 days at 15°C, Todd, 1991; the present study), it was not ascertained whether these larvae were at day 0 of their competent period. However, in view of the high numbers of larvae used in this work, and their concomitant logistic difficulties, a time leeway of 5 days was considered justifiable.

The protocol followed in this experiment is illustrated in Fig. 4.4. The larvae were mixed and divided into groups of 7 in 25 ml beakers which were sequentially numbered from 1 to 20. The beakers were placed in an incubator (Gallenkamp) at 15 °C containing 20 ml of culture medium consisting of 0.2 µm (millipore) filtered natural seawater (NSW), *Rhodomonas* sp. (25×10^3 cells $\cdot$ ml^{-1}), and a mixture of streptomycin sulfate and Penicillin-G (Sigma) at final concentrations of 50 µg ml^{-1}, and 60 µg $\cdot$ ml^{-1} respectively). The culture medium was changed on days 5 and 8.

The date on which the experiment was initiated, 27 June 1991, was considered as day 1. After 24 hours of the beginning of the experiment (on day 2) those beakers which had been assigned an even number were scored, and those with odd numbers were scored on the following day (48 hours subsequent to the commencement of the experiment). Consequently, each beaker was scored every 48 hours. The larvae were examined under a Wild M8 stereomicroscope. The numbers of spontaneous metamorphs were recorded and any dead individuals removed. The first two beakers of the series (i.e. numbers 2 and 4 on day 2) were selected for the larval assays, which were conducted at 10 °C to ensure the viability of the live prey. Five larvae from each beaker were chosen at random and transferred to a petri dish. Two assays were conducted, (i) culture medium (control for spontaneous metamorphosis), and (ii) *Semibalanus balanoides* settled on fragments of rocks, immersed in culture medium (positive control). The dishes were scored after 24 or 48 hours, and the numbers of metamorphs and deaths were recorded. The two remaining larvae from each beaker that were not used in the larval assays were maintained in the original beakers to be used in duplicate or alternative treatments on subsequent days. A total of 15 larvae was available for duplicates, and were used as follows: on day 4, in addition to treatments (i) and (ii) described above, a third and fourth treatment (containing 5 larvae per treatment) were carried out, (iii) 20 ml of 19 mM K^+ ASW (positive control), and (iv) 0.2 µm (Millipore) filtered natural seawater (control for spontaneous metamorphosis). On day 7, treatment (iii) was a duplicate of treatment (ii), *S. balanoides* settled on fragmented rocks. The experiment was terminated on day 16, when the results of the last larval assays conducted had been scored.

Statistical analysis

The cumulative percentages of metamorphs were transformed into the arc-sine of $\sqrt{p}$, with p = ratio of metamorphs (Todd *et al.*, 1991) and analysed by a one-way ANOVA on Minitab (Release 7.1). The treatments concerning potassium ion manipulations were further analyzed by a least significant difference test (LSD) in order to establish which treatments were significantly different from each other (Sokal & Rohlf, 1981).

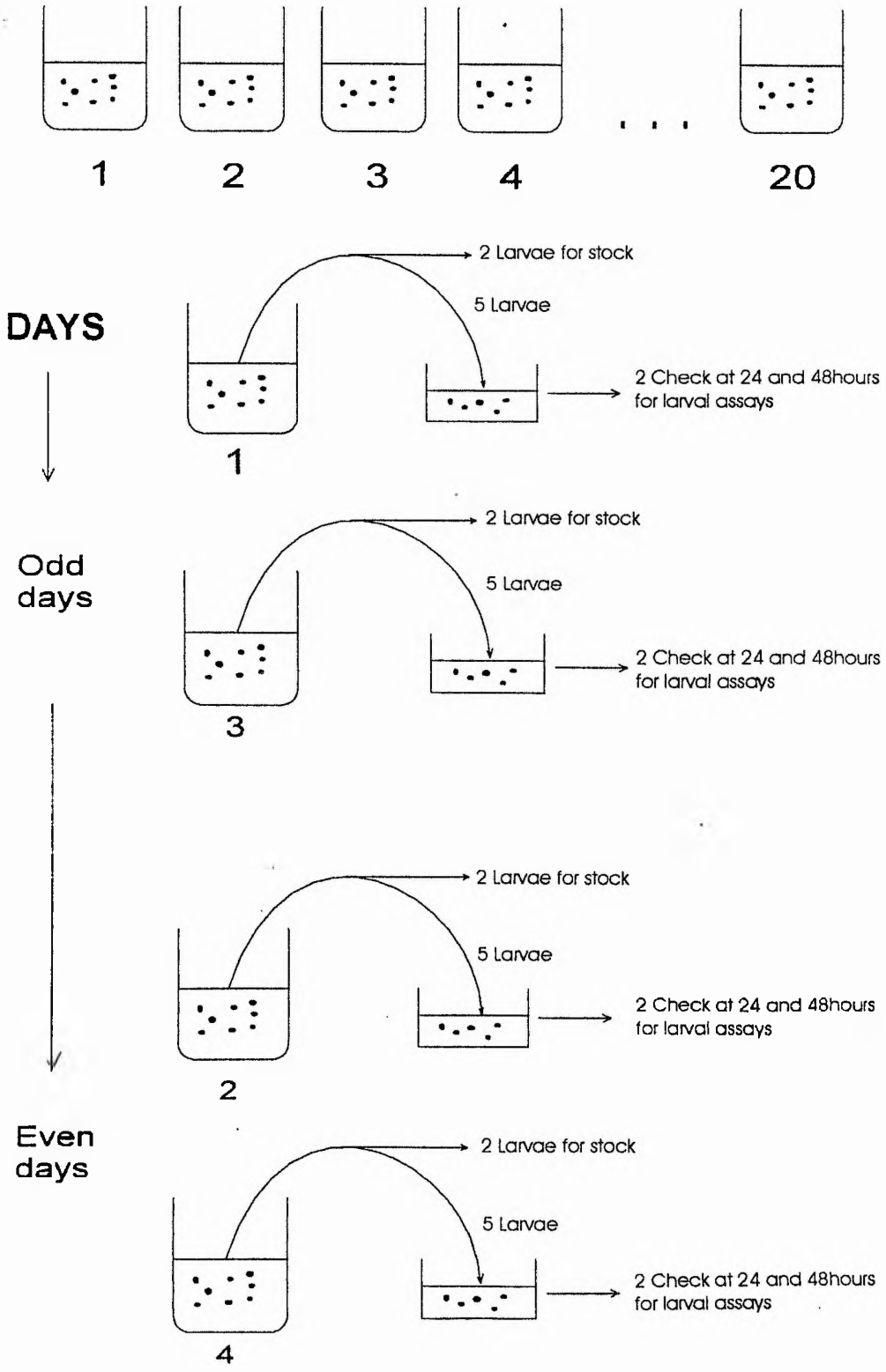


Fig. 4.4. Protocol followed in Experiment 6 (see text for details).

RESULTS

Experiment 1. Potassium ion dose-response of *Onchidoris bilamellata*

No metamorphosis occurred in the 9 mM K^+ ASW control, the 34 mM and 39 mM K^+ treatments. Essentially no metamorphosis (4.8%) was observed at 14 mM K^+. Maximal metamorphosis success (67%) was obtained in the 19 mM treatment after 4 days, followed by 43% metamorphosis in the 24 mM treatment after 4 days, and 19% metamorphosis at 29 mM K^+ after 3 days. Overall mortality for the totality of the treatments was 20.4%. The highest survivorship (90.5%) was obtained in the 9 mM K^+ ASW control, and the lowest (71.4%) in the 14 mM and 29 mM treatments (Table 4.3).

Table 4.3. Final percentages of *Onchidoris bilamellata* larvae metamorphosed in 9 mM K^+ ASW (control) and in elevated potassium ion concentrations.

Treatment	Cumulative Percentage Of Metamorphosis After +1 Day	+2 Days	+3 Days	+4 Days	Cumulative Percentage Of Mortality After +4 Days
9 mM	0	0	0	0	9.52
14 mM	0	0	0	4.76	28.57
19 mM	9.52	28.57	52.38	66.67	14.29
24 mM	0	9.52	38.1	42.86	19.05
29 mM	0	0	19.05	19.05	28.57
34 mM	0	0	0	0	23.81
39 mM	0	0	0	0	19.05

A one-way ANOVA analysis indicated that the treatments were significantly different ($F = 30.30$, $P < 0.001$; Table 4.4). The data were further analysed by a LSD test, which indicated 19 mM, 24 mM and 29 mM K^+ treatment responses to be significantly different from one another, whilst the remainder of the treatments were not significantly different (Table 4.5).

The results from this experiment indicate that 19 mM K^+ is at or near the optimum concentration for inducing metamorphosis of *Onchidoris bilamellata*. Lower concentrations of potassium have essentially no effect on the larvae (only one larva metamorphosed in the 14 mM treatment), whilst there is a decline in induction with concentration (Fig. 4.5).

Furthermore, metamorphosis occurs more rapidly at 19 mM K^+, where the first metamorphosis was observed after 24 hours. The larvae in the remainder of the treatments underwent first metamorphosis on day 2 (19 mM and 24 mM treatments), day 3 (29 mM treatment) or day 4 (14 mM K^+ treatment).

Table 4.4. One-way ANOVA comparing the effects of the excess K^+ concentrations on the metamorphosis induction of *Onchidoris bilamellata*. The data were recorded as the metamorphosis ratios (p) observed in each treatment after 4 days, and transformed into the arsins of $\sqrt{p}$ for analysis.

Source	DF	SS	MS	F	p
Factor	6	8810.7	1468.5	30.30	0.000
Error	14	678.6	48.5		
Total	20	9489.3			

Level	N	MEAN	STDEV
9 mM K^+	21	0.000	0.000
14 mM K^+	21	7.403	12.823
19 mM K^+	21	55.337	10.785
24 mM K^+	21	38.030	4.954
29 mM K^+	21	25.577	5.831
34 mM K^+	21	0.000	0.000
39 mM K^+	21	0.000	0.000

POOLED STDEV = 6.962

Table 4.5. Summary of results obtained in the LSD test comparing the effects of the excess K^+ concentrations in the induction of metamorphosis of *Onchidoris bilamellata*.

Treatment (K^+ mM)	Transformed Mean (arc-sine $\sqrt{p}$)	Homogeneous Groups			LSD	α
9	0	X			7.984	0.05
14	7.40	X				
19	55.34			X*		
24	38.03		X*			
29	25.58		X*			
34	0	X				
39	0	X				

*denotes significant difference

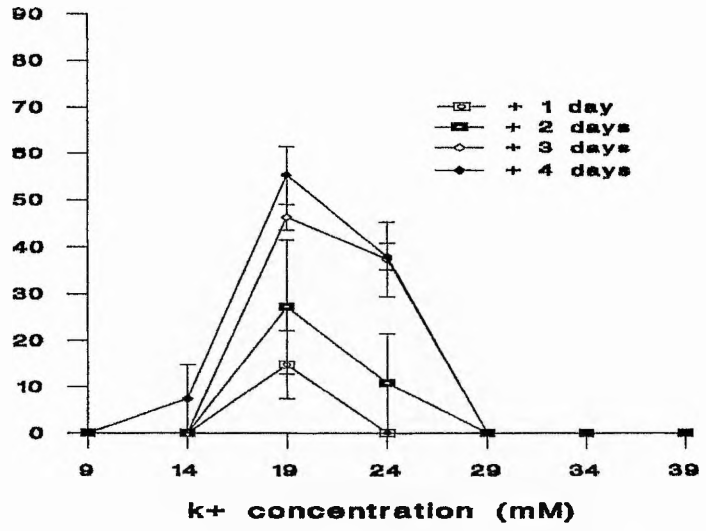


Figure 4.5. Potassium-ion dose response in *Onchidoris bilamellata*, showing metamorphosis after +1, +2, +3 and +4 days. Data are for back-transformed means and standard errors for each triplicated treatment.

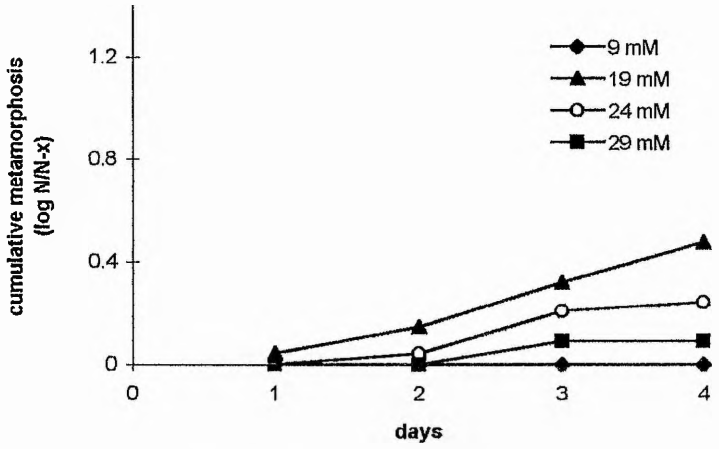


Fig. 4.6. Time-courses of metamorphosis in 9 mM K^+ ('normal' ASW) and ASW with potassium ion in excess. Data are for the summed triplicates, with cumulative metamorphosis plotted as the log (N/N-x) transform, where N = number of larvae at start of experiment and x = cumulative number of larvae that had metamorphosed in each treatment.

The temporal patterns of metamorphosis of *O. bilamellata* larvae in 19 mM, 24 mM and 29 mM treatments along with the 9 mM K^+ ASW control are shown in Fig. 4.6. (the 14 mM K^+ treatment is not included in the graph given that only one larva metamorphosed in this treatment). From this figure it is apparent that metamorphosis proceeds at a constant *per capita* rate (indicated by a straight line, Todd *et al.*, 1991) only at 19 mM K^+. In the 24 mM and 29 mM treatments, however, the majority of the larvae that metamorphosed did so on day 3, but no substantial increase of metamorphosis induction was observed thereafter (see Table 4.3).

At 34 mM and 39 mM K^+, where there was no induction of metamorphosis, swelling of the larval foot occurred in some of the larvae. These larvae were generally inactive and retracted within the shell, but despite this all of them survived to completion of the experiment. This indicates that even though 34 mM and 39 mM potassium concentrations are not toxic to the larvae, they exert an inhibitory effect of metamorphosis in *O. bilamellata*.

Experiments 2 to 4. Induction of Metamorphosis in *Onchidoris bilamellata* on contact with and at a distance from the natural prey.

In experiment 2 veligers of *Onchidoris bilamellata* metamorphosed in both treatments. The mortality rate was 5.9 % in treatment (i), and 35.3 % in treatment (ii). Larvae rafted above live *Semibalanus balanoides* (treatment ii) metamorphosed at a higher rate (23.5 %) than those larvae rafted above dead *S. balanoides* (11.8%) (treatment i) (Table 4.6). Despite this difference, an F-test followed by Student's t-test showed no significant difference (F = 5.62; t = 1.44; Table 4.7) between the two treatments.

Table 4.6. Final percentages of *Onchidoris bilamellata* larvae metamorphosed in Experiment 2. The percentages of larvae sunk prior to the end of the experiment and the rates of mortality are also indicated.

Treatment	Metamorphs	Larvae Sunk	Mortality
i) Larvae rafted above dead *Semibalanus balanoides*	11.8	52.9	5.9
ii) Larvae rafted above live *S. balanoides*	23.5	47.1	35.3

Table 4.7. F- and t-test conducted to analyse the results obtained in Experiment 2 for *Onchidoris bilamellata*. All the data, recorded as the ratio of metamorphosis occurred at the end of the experiment in each treatment, were square rooted and transformed into arc-sine for analyses. N refers to the total number of larvae per treatment.

Treatment	N	MEAN	STDEV		p
i) Larvae rafted above dead *Semibalanus balanoides*	17	16.06	13.91	**F = 5.62**	> 0.1
				t = 1.44	> 0.1
ii) Larvae rafted above live *S. balanoides*	17	28.64	5.87		

The low numbers of competent larvae available at the commencement of the experiment made it impossible to conduct parallel controls of larvae rafted above mussel shell in ASW and larvae rafted in ASW. The main difficulties encountered in this experiment were the high mortality rates and the elevated percentage of larvae that sunk to the bottom of the dish throughout the experiment (52.9 % in treatment (i) and 47.1 % in treatment (ii) (Table 4.6).

In Experiment 3 veligers underwent metamorphosis in all the treatments. Maximal metamorphosis (100%) was observed in one of the triplicates in treatment (ii), containing live prey enclosed in a mesh bag. The final percentages of metamorphosis obtained in this experiment are shown in Table 4.8. A one-way ANOVA test indicated that the treatments were not significantly different ($F = 1.46$, $P > 0.1$; Table 4.9). The overall mortality rate was 5.9 %. Nonetheless the actual rate may have been higher considering that an elevated percentage of the larvae (32.9 %) were not found when the treatments were scored. The mesh bags used in the treatments hindered the visualization of the larvae and the metamorphs. This difficulty was overcome in Experiment 4 by substituting the bags by separation chambers.

Table 4.8. Final percentages of *Onchidoris bilamellata* larvae metamorphosed in response to live and dead prey in Experiment 3. The rates of mortality and the percentage of larvae that were lost to view when the treatments were scored are also shown.

Treatment	N	Percentage of Metamorphs	Percentage of Larvae Lost to View	Percentage of Mortality
(i) larvae sunk with bags containing dead *Semibalanus balanoides* on *Patella* shell	21	23.8	38.1	9.5
(ii) larvae sunk with bags containing live *Semibalanus balanoides* on *Patella* shell	22	54.5	31.8	22
(iii) larvae sunk with live *Semibalanus balanoides* on *Patella* shell adjacent to plankton net	21	23.8	42.9	4.8
(iv) larvae sunk with dead *Semibalanus balanoides* on *Patella* shell adjacent to plankton net	21	23.8	19	9.5

Table 4.9. One way ANOVA comparing metamorphosis ratios of *Onchidoris bilamellata* in response to cues emanating from live and dead barnacles settled on limpet shell fragments (Experiment 3). Data are metamorphosis ratios square rooted and transformed into arc-sine for analyses All the treatments, triplicated, contained seven larvae except for one replicate in treatment 2 with eight larvae.

Source	DF	SS	MS	F	p
Factor	3	1410	470	1.46	0.297
Error	8	2577	322		
Total	11	3986			

Level	N	MEAN	STDEV
Treatment i)	21	28.94	5.83
Treatment ii)	22	53.63	31.96
Treatment iii)	21	28.44	10.78
Treatment iv)	21	28.44	10.78

POOLED STDEV = 17.95

In Experiment 4 metamorphosis was induced in all the treatments, i.e., on contact with and at a distance from both live and dead prey. Maximal metamorphosis (72.2 %) occurred in treatment (ii) containing larvae in contact with dead prey (Table 4.10). Although the rate of metamorphosis in treatment (iii) (control) was the lowest, a one-way ANOVA test indicated that the treatments were not significantly different from one another ($F = 2$, $p > 0.1$; Table 4.11).

Table 4.10. Final percentages of *Onchidoris bilamellata* larvae metamorphosed in Experiment 4 in response to contacting and distant live and dead prey. The rates of mortality and the percentage of larvae that were lost to view when the treatments were scored are also shown.

Treatment	N	Percentage of Metamorphs	Percentage of Larvae Lost to View	Percentage of Mortality
(i) larvae contacting live prey	17	58.82	0	5.82
(ii) larvae contacting dead prey	18	72.2	5.5	0
(iii) larvae contacting bare *Patella* shell (control)	18	27.7	0	5.82
(iv) larvae at a distance from live prey	18	66.7	11.1	0
(v) larvae at a distance from dead prey	18	38.8	5.82	0

Table 4.11. One way ANOVA comparing the metamorphosis of *Onchidoris bilamellata* larvae in response to contacting and distant metamorphic cues (Experiment 4). The data, recorded as the cumulative ratio of metamorphosis occurring in each treatment after + 4 days, were square rooted and arc-sine transformed prior to analysis.

Source	DF	SS	MS	F	p
Factor	4	2542	636	2.00	0.171
Error	10	3185	318		
Total	14	5727			

Level	N	MEAN	STDEV
Treatment i)	17	50.17	4.90
Treatment ii)	18	63.72	27.44
Treatment iii)	18	26.75	23.68
Treatment iv)	18	55.22	10.46
Treatment v)	18	8.03	12.07

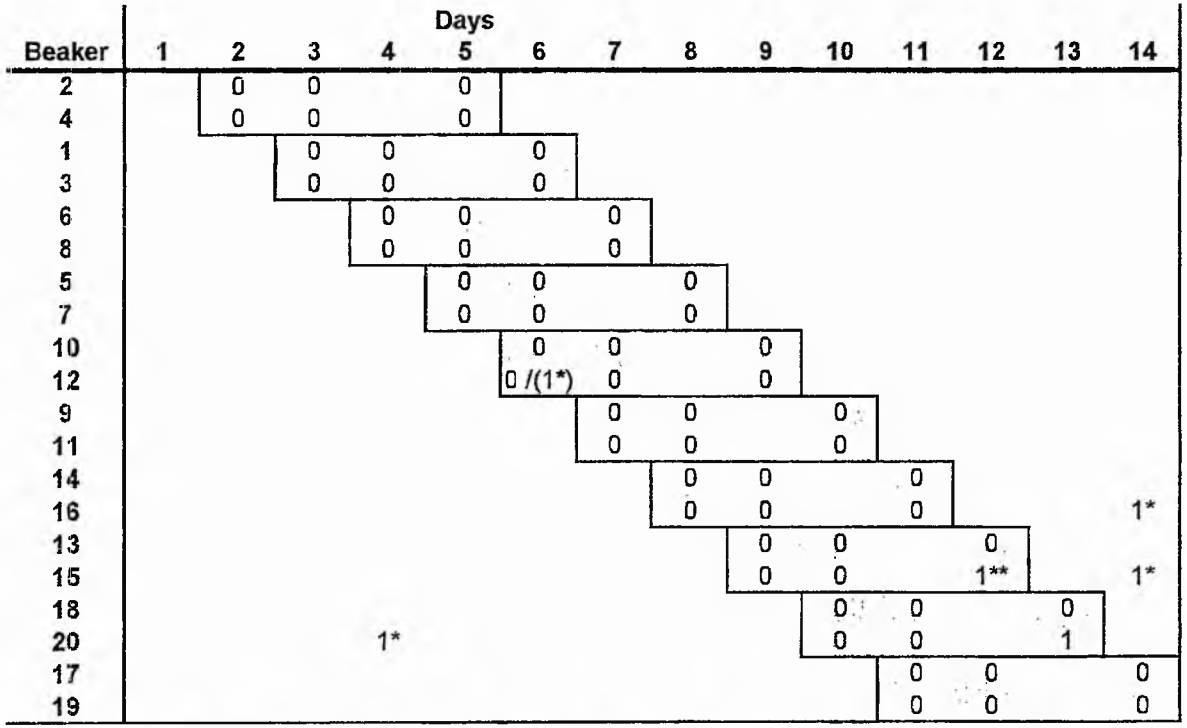
POOLED STDEV = 17.85

Experiment 5. Metamorphosis induction of *Goniodoris nodosa*

In Experiment 5 the larvae did not metamorphose in any one treatment. The only exception was one larva in the control (i) which 'evacuated' the shell. In this instance even though the shell was cast, the velum was not resorbed and the operculum was not lost and therefore metamorphosis did not proceed to completion. The overall mortality was 51 %. No food was added to the seawater in this experiment, which may have caused the starvation of the prey items and this in turn may have produced the mortality of the larvae.

Experiment 6. Delay of metamorphosis in *Onchidoris bilamellata*

The majority of the larvae survived to the termination of the experiment, with only 3.6 % overall mortality. However, the larvae did not metamorphose in the metamorphosis assays after exposure to either the natural inducer or to 10 mM excess K^+. This lack of response occurred throughout the duration of the experiment, with only one metamorph on day 15 (beaker 20) in the presence of live prey, and one metamorph (from beaker 15) in the control (FSW) on day 14. Four larvae metamorphosed 'spontaneously' on days 4, 6, and 14 (Table 4.12).



Spontaneous metamorphs; (1) spontaneous metamorph outwith the metamorphosis assay.
** This larva metamorphosed in the control treatment.

Table 4.12. Summary of results obtained in Experiment 6. The number of metamorphs in the metamorphosis assays are indicated in the shaded boxes, except for one larva in beaker 12.

DISCUSSION

Neuroactive agents such as GABA, DOPA, catecholamines and choline, along with monovalent cations, have variously been found to be effective in the induction of metamorphosis of many marine invertebrates (Baloun & Morse, 1984; Coon et al., 1985; Coon & Bonar, 1987; Pires & Hadfield, 1991a,b; Hirata & Hadfield, 1986; Todd et al, 1991; Pawlik, 1992). The artificial induction of metamorphosis with elevated concentrations of potassium ion has been successful in the molluscs *Adalaria proxima* (Todd *et al.*, 1991), *Phestilla sibogae*, *Haliotis rufescens*, *Astraea undosa* (Yool *et al.*, 1986), *Crepidula fornicata* (Pechenik and Heyman, 1987; Eyster & Pechenik, 1988), and *Onchidoris bilamellata* (this study). It is uncertain whether potassium acts via depolarization of external chemosensory cells, or whether it acts directly on target tissues (Todd *et al.*, 1991). Nonetheless, potassium ion appears to act through membrane depolarization at least in some cases. In the red abalone, *Haliotis rufescens*, for example, potassium acts circumventing the receptors that specifically mediate recognition of the natural inducer (reviewed by Morse, 1990).

In the present work the response of *Onchidoris bilamellata* to potassium coincides with *Adalaria proxima*, *Haliotis rufescens*, and *Astraea undosa* in the optimal concentration for the induction of metamorphosis, 10 mM excess K^+, although the ionic levels beyond which excess K^+ are inhibitory vary between these molluscs. Interestingly, the optimal concentration inductive of metamorphosis in *P. sibogae* and *C. fornicata*, 20 mM excess K^+, is inhibitory for *Onchidoris*. With respect to the time-courses of metamorphosis, *Onchidoris* necessitated 3 days to reach 50 % metamorphosis (67 % after 4 days, Fig. 4.5), which was comparable to that of *Haliotis* (with 70 % metamorphosis after 3 days) and *Adalaria* (with 90 % metamorphosis after 3 days) at the same potassium concentration. This is in marked contrast with *Crepidula*, which only requires ~ 5 hours exposure to attain nearly 100 % metamorphosis (Pechenik & Heyman, 1987). In sum, the responsiveness to potassium ion in marine invertebrates varies between species; nonetheless, its use is justifiable inasmuch as providing a tool for assessing competence to metamorphose of a range of species with widely differing natural metamorphic stimuli (Yool *et al.*, 1986; Pechenik & Heyman, 1987).

The natural metamorphic inductive cues in marine invertebrates are of very different nature. Gregarious and sedentary species are generally induced to metamorphose by conspecifics (Chia, 1978), (e.g., the gregarious polychaete *Phragmatopoma californica*, Jensen, 1987; the oyster *Crassostrea virginica*, Crisp, 1967; the sand dollar *Echinarachnius parma*, Pearce & Scheibling, 1990), whereas specialist species are likely to be induced to metamorphose in response to the prey species. Some examples of the latter are the nudibranchs *Rostanga pulchra*, whose inducing factor is the encrusting sponge, *Ophlitaspongia pennata* (Chia, 1978), *Phestilla sibogae*, which is induced to metamorphose by the coral *Porites compressa* (Hadfield & Miller, 1987), *Eubranchus doriae* by the hydrozoan *Kirchenpaueria pinnata* (Bahamondes-Rojas & Dherbomez, 1990), and *Adalaria proxima* induced by *Electra pilosa*, the bryozoan species on which the adults feed (Thompson, 1958). Moreover, the larvae of *P. sibogae*, *E. doriae* and *A. proxima* metamorphose in response to water-soluble factors (Hadfield & Pennington, 1990; Pires & Hadfield, 1991a; Bahamondes-Rojas & Dherbomez, 1990; Lambert & Todd, 1994). Other molluscs (e.g. bivalves, Coon *et al*,. 1985; Fitt *et al.*, 1990; and prosobranchs, Eyster & Pechenik, 1988; McGee & Targett, 1989) as well as members of different marine invertebrate phyla (e.g. ascidians, Durante, 1991; and echinoids, Cameron & Schroeter, 1980; Highsmith, 1982; Pearce & Scheibling, 1990) are also responsive to cues that are free in the water column, which appears to indicate that physical contact with the inductive substratum is not a general requirement for the initiation of metamorphosis of marine invertebrates (Lambert & Todd, 1994).

In the present study *O. bilamellata* larvae metamorphosed upon contact with and at a distance from the inductive substrata. Larvae were capable of metamorphosing at a distance from the inductive substratum separated by a plankton mesh (Exps. 3 & 4), but also when rafted on the water surface, in Experiment 2. The plankton mesh does not appear to be the causative factor eliciting metamorphosis, because in Experiment 2 larvae were also capable of metamorphosing in the absence of mesh, and at a distance from the cue. In the study by Lambert & Todd (1994), larvae of *Adalaria proxima* did not

metamorphose in the control (rafted larvae in ASW), and therefore it is unlikely that rafting alone may explain the ubiquitous metamorphosis observed in the present study.

Furthermore, metamorphosis occurred when larvae were exposed to live and dead prey (Exps. 2, 3, and 4). The results from the present study agree only in part with those obtained by Chia & Koss (1988). These authors envision metamorphosis as a two-step process: a first phase of settlement in which the larva sinks and explores the substratum, and a second and irreversible phase of metamorphosis, culminating in the benthic juvenile. This distinction is not applicable in the present work given that all the larvae included were observed to crawl intermittently in clean seawater, prior to exposure to inductive substrata, and therefore settlement assays were not conducted.

With regard to the responsiveness to live and dead prey, Chia & Koss (1988) obtained metamorphosis by exposing the larvae to dead *Chthamalus dalli*, but only if combined with seawater which had previously contained living barnacles ('conditioned' seawater). In contrast, in the experiments conduced in our laboratory, *Onchidoris bilamellata* larvae metamorphosed in the presence of dead *Semibalanus balanoides* in seawater that was not pre-conditioned. The outcome of the experiments of the present study were unexpected. The larvae used in Experiments 2, 3, and 4 were obtained from late spawnings (hatching in May, June, and August, respectively), which is near the end of the reproductive season of *O. bilamellata* in British waters (Todd, 1979a).

Larvae of a number of marine invertebrates, such as annelids (Knight Jones, 1953), echinoids (Rumrill, 1989; Burke, 1983), and gastropods (Coon *et al.*, 1990, Fitt *et al.*, 1990) increasingly lose their specificity for metamorphic cues once they have entered the delay phase (reviewed by Hadfield, 1978; Morse, 1990; Pechenik, 1990), which may, at least in part, account for the responses observed in the present investigation. Furthermore, larval developmental rates (Pechenik 1980, 1984; Lima & Pechenik, 1985) show marked variation among cultures held at different temperatures but also within single cultures (Pechenik & Lima, 1984). For example, the length of the larval phase in *Crepidula plana* ranged from 58 to 86 days among cultures at 12 °C, and from 13 to 23 days among cultures maintained at 29 °C (Pechenik & Lima, 1984). The patterns of metamorphic activity

of larvae hatched at different points in the reproductive period have not been investigated, but in view of the differences in developmental rates that occur between sibling larvae (Pechenik & Lima, 1984; Miller, 1993), it is likely that a divergence in responsiveness between larvae hatched from early spawnings and larvae hatched from later spawnings will occur (Lambert & Todd, 1994).

With regard to *Goniodoris nodosa*, the larvae exposed to *Alcyonidium* and *Botryllus* did not metamorphose in the present work. It is evident from the high overall mortality observed (51 %), that the larvae were not under appropriate conditions and thus the results obtained from this experiment are inconclusive. The deprivation of food may have accounted for the demise of the prey, and this in turn, may have resulted in larval mortality. Further trials using algal food should be conducted to ascertain which are the natural inductive cues of *Goniodoris*.

The capacity to delay metamorphosis in the absence of the required inducer is notably variable across different phyla but also between members of the same group. For example, the echinoid *Strongylocentrotus droebachiensis* may prolong the period of competence for 52 days in laboratory cultures (Rumrill, 1989); similarly, the molluscs *Crassostrea gigas* retained competence to metamorphose for at least 30 days (Coon *et al.*, 1990), *Crepidula fornicata*, *Ilyanassa obsoleta*, and *Bittium alternatum* remained competent to metamorphose for 30, 60, and 73 days respectively (Lima & Pechenik, 1985). This delay period extends for as long as 311 days in the sea hare *Aplysia juliana* (Kempf, 1981). Planktotrophic larvae generally have a higher potential to delay metamorphosis than those species which depend entirely on their yolk reserves (Pechenik, 1990). The planktotrophic polychaete *Polydora ligni*, for example, may postpone metamorphosis for 6 days in fed cultures (Qian *et al.*, 1990). The obligatory lecithotrophic *Spirorbis borealis*, in contrast, may only delay metamorphosis for one day, followed by death of the larvae (Knight-Jones, 1953). Furthermore, lecithotrophs capable of feeding in the pelagic phase may postpone metamorphosis for a longer time in the presence of food than in the absence of food. The nudibranch *Phestilla sibogae*, for instance, attains competence to metamorphose 3 or 4 days after hatching, and dies if deprived a metamorphic stimulus in the absence of food, but is able to complete metamorphosis after 42 days in fed cultures (Hadfield & Scheuer, 1985). As mentioned previously, the duration of the delay

phase is related in many cases to larval developmental rates which vary between and within cultures (see also Todd, 1991). In addition to the absence of appropriate environmental stimuli, metamorphosis may be postponed in different situations. Reversible inhibition of metamorphosis (habituation) may occur in some marine invertebrate larvae when exposed to an inducer prematurely (Hadfield, 1984). Larvae of *Phestilla sibogae* that have been maintained in the presence of the coral prey *Porites compressa* prior to achieving competence to metamorphose, do not metamorphose unless they are transferred to clean seawater for a period of 1 to 5 hours before being re-exposed to the cue (Hadfield & Scheuer, 1985). Similarly, habituation occurs in larvae of the gastropod *Haliotis rufescens* by exposing pre-competent larvae to inducing substances (GABA or GABA analogs) 4 days prior to the achievement of competence (Trapido-Rosenthal & Morse, 1986). Habituation has been suggested to be a mechanism for increasing larval dispersal through the prolongation of the pelagic phase (Hadfield 1977, 1984; Trapido-Rosenthal & Morse, 1986).

In contrast with the above, 'spontaneous' metamorphosis, that is, metamorphosis in the absence of any apparent stimulus, has been reported for a number of marine gastropods (Pechenik 1980, 1986, 1990) and other marine invertebrate phyla (e.g., an echinoid, Rumrill, 1989), and is attributable to the loss of specificity for the inductive cue (already discussed), which in turn Pechenik (1980) ascribes to a genetically predetermined end for a larval life that determines the stimulation of metamorphosis once the larvae reach this point. In the present study one larva metamorphosed spontaneously after 14 days in the competent period, which may be indicative of the delay period of *O. bilamellata*. However, the larvae did not respond either to the natural cue (live *S. balanoides*) or to 19 mM K^+ ASW in Experiment 6. The reasons of this lack of response are unclear. The differences of the larvae hatching at a late stage in the spawning season already discussed may explain the outcome of this experiment, given that the experiment was conducted in June (see materials and methods). Nevertheless, it is uncertain why larvae metamorphosed in Exps. 3 & 4 in the control (dead *S. balanoides* and bare *Patella* shell, respectively), but the larvae used in Experiment 6 failed to metamorphose when exposed to the positive control.

CHAPTER 5

GENERAL DISCUSSION

In the present study several aspects pertaining to the reproduction of these nudibranchs were assessed, and several conclusions derive from it. The number of eggs produced per spawning event in opisthobranchs is largely dependent on animal size (Thompson, 1976; Ros, 1981; Hadfield & Switzer-Dunlap, 1984). This generalization is true for *Onchidoris bilamellata* and *Goniodoris nodosa*, with the larger of the two species, *O. bilamellata*, laying higher numbers of eggs (Tables 2.4 & 2.5). The size of eggs of both species, nonetheless, is not related to the adult sizes in these planktotrophic nudibranchs. *G. nodosa* lays larger eggs (grand mean diameter = 108 μm) than *O. bilamellata* (grand mean diameter = 103 μm). The ash-free dry weight per egg derived from intact spawn masses is also higher for *G. nodosa* (grand mean = 0.44 $\mu g \cdot egg^{-1}$) than for *O. bilamellata* (grand mean = 0.39 $\mu g \cdot egg^{-1}$). Nonetheless the data of the present study include gel stroma, and the ratio gel stroma/egg was not ascertained, consequently the true allocation of resources per egg for both of these species remains to be determined.

The above figures are considerably lower than those reported for species with larger eggs. The marine gastropod *Crepidula fornicata*, for example, whose eggs average 160 μm in diameter, contain 4.32 $\mu g \cdot egg^{-1}$ (Pandian, 1969). In marked contrast are the results obtained for eggs of other aquatic invertebrate groups. The mean organic content of the eggs from the caridean shrimps *Chorismus antarcticus* and *Eualus gaimardii*, for example, is 960 $\mu g \cdot egg^{-1}$ and 141 $\mu g \cdot egg^{-1}$, respectively (Clarke, 1993a), which is illustrative of the difficulties inherent to this type of work with species with small sized eggs.

Gibson & Chia (1991) estimated the organic content per egg in the planktotrophic cephalaspidean *Haminaea vesicula*, subsequent to removal of the covering jelly layers of the egg mass. The eggs of *G. vesicula*, averaging 90 μm in

diameter, were estimated to contain 0.026 μg C · egg^{-1} (Gibson & Chia, 1991), which differs substantially from the estimated ash-free dry weight per egg derived from intact spawn masses in the present study (0.44 μg · egg^{-1} for *Goniodoris nodosa*, and 0.39 μg · egg^{-1} for *Onchidoris bilamellata*). Notwithstanding that the data provided by Gibson & Chia (1991) account for carbon alone and not total organic content of eggs, their data and the data of the present work differ by an order of magnitude, and outstand the importance of removing the gel stroma prior to analysis. The relative energy and nutrient contents of eggs, capsule walls, and gel matrix have not been separately determined for opisthobranch molluscs, and probably vary substantially in adaptation to different habitats (De Freese & Clark, 1983). Studies in the line of Gibson & Chia's (1991) are necessary to determine accurately the organic content of individual eggs.

Three different groupings of *O. bilamellata* larvae are distinguishable in the present study; larvae hatched from spawn masses laid by adults maintained in captivity throughout the spawning season (Chapters 2 & 3), larvae hatched from spawn masses collected in the field in winter and spring (Chapter 2), and larvae hatched from spawn masses collected in the field in autumn (Chapter 2). The shell length of these larval groups was measured at hatching, and is illustrated in Figure 5.1. The differences in size observed between larvae hatching in autumn and larvae hatching in winter and spring months have already been discussed (Chapter 2).

The larvae hatched from spawn masses laid in captivity show a clear trend to decrease in size as the season progresses. This tendency, nonetheless is not exhibited by the larvae obtained from field collected spawn masses (Fig. 5.1). The primary reason that accounts for the observed divergence, with most likelihood, is the duration of the spawning activity in the field and in the laboratory. Whereas post-spawning death has occurred for the majority of adults by the end of May (Miller, 1962; Todd, 1979a), in the laboratory the spawning activity of some individuals continues until July (Todd, 1979a; this study). Todd (1979a) estimated a mean total spawn production of ~ 6 masses in the field for *Onchidoris bilamellata*, (in contrast to ~ 16 spawn masses in captivity), which implies that the larvae hatched from spawn masses laid in captivity in the present work

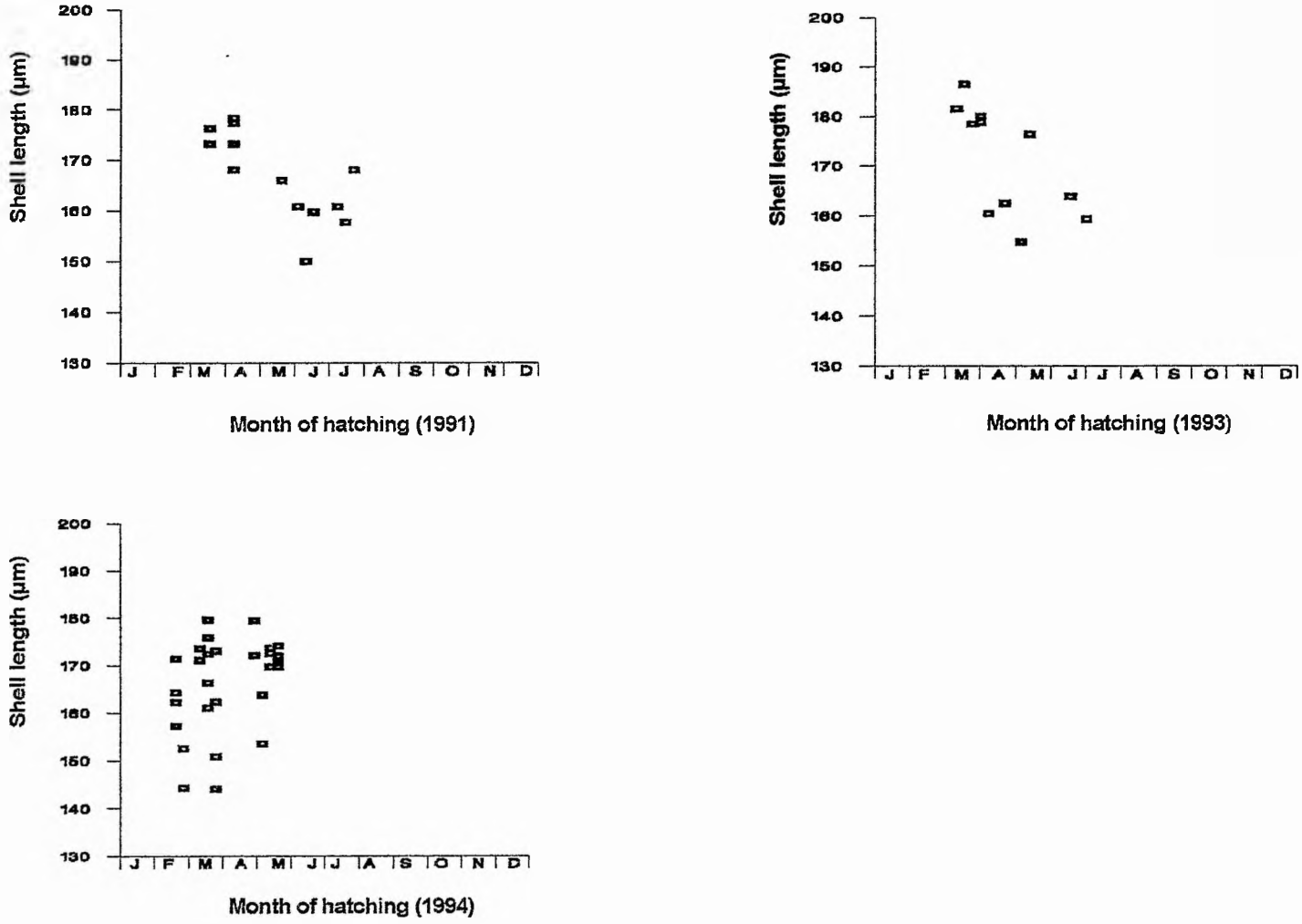


Figure 5.1. Newly hatched larvae of *Onchidoris bilamellata* obtained from spawn masses laid by adults maintained in captivity in 1991 and 1993 (top graphs) and from spawn masses collected from the field in 1994 (bottom graph). Each data point represents the mean shell length of thirty larvae hatching from a single spawn mass.

encompass early as well as late spawning events, whereas those collected from the field comprise only early spawn masses. The immediate consequence of these observations is that results derived from individuals maintained in captivity for long time-periods may not reflect the natural situation, and therefore field observations parallel to experimental conditions are necessary to interpret results in a realistic manner.

The larval growth and development of *Onchidoris bilamellata* and *Goniodoris nodosa* follow the characteristic pattern observed in other opisthobranch molluscs, irrespective of the illumination regime, featured by the arrest of shell growth, but not of tissues, prior to the achievement of metamorphic competence (Todd, 1981; Hadfield & Switzer-Dunlap, 1984). This is in contrast with prosobranch gastropods whose shell and tissues continue growing in competent larvae, although a shift in shell morphology occurs upon achievement of competence in at least some species (e.g., *Crepidula plana* and *C. fornicata*, Lima & Pechenik, 1985). The cessation of shell growth constitutes a constraint for opisthobranch veligers, and although it could suggest a limitation to the delay period, no data in support of this hypothesis are as yet available. Conversely, the longest delay period in laboratory culture reported up to the present time for a marine gastropod is that of the opisthobranch *Aplysia juliana*, which is able to postpone metamorphosis for at least 300 days in laboratory culture (Kempf, 1981). This time period largely exceeds the delay periods reported of some prosobranch gastropods, (e.g., Pechenik, 1980; Lima & Pechenik, 1985; Miller & Hadfield, 1990; Zimmerman & Pechenik, 1991), which generally are below 100 days.

The results obtained in the present investigation are indicative of a delay potential of at least 14 days for *O. bilamellata*. Nonetheless, the low percentage of metamorphosis achieved by competent larvae in the presence of a natural and an artificial inducer, requires the repetition of this experiment. Similarly, low rates of metamorphosis were obtained throughout the present study, with a maximum of 67 % metamorphosis after 4 days (Experiment 1, Chapter 4), in contrast with rates above 90 % for published reports on this (Chia & Koss, 1988) and other nudibranch species (Todd *et al.*, 1991). Due to logistic difficulties, the larvae used in the present work were obtained from spawn masses laid after the peak winter months (Todd, 1979a). Comparative studies of

metamorphosis in response to known inducers between larvae hatching from spawn masses laid early in the season and larvae hatching from spawn masses laid towards the end of the reproductive period, are necessary to clarify the low rate of metamorphosis herein observed.

CONCLUSIONS

In sum, several conclusions derive from the present study:

1) A pattern of spawning for *Goniodoris nodosa* reproducing in captivity was identified.
2) Larval growth and development of *Onchidoris bilamellata* and *Goniodoris nodosa* proceeds to completion under different light regimes, ranging from continuous illumination to continuous darkness, providing food is abundant. No significant differences in differentiation rates are observed in these illumination regimes.
3) *Onchidoris bilamellata* is induced to metamorphose by 19 mM K^+ ASW.
4) *Onchidoris bilamellata* larvae do not require to establish physical contact with *Semibalanus balanoides*, the natural inducer, to be induced to metamorphose.

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