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THE SYMBIOSIS BETWEEN THE 'SOLAR-POWERED' NUDIBRANCH *MELIBE ENGELI* RISBEC, 1937 (DENDRONOTOIDEA) AND *SYMBIODINIUM* SP. (DINOPHYCEAE)

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ABSTRACT

For the first time, specimens of the nudibranch *Melibe engeli* hosting zooxanthellae (*Symbiodinium* spp.) were cultured for more than 9 months in aquaria in order to study this symbiotic system. *Melibe engeli*, in contrast to other previously studied 'solar-powered' nudibranchs, does not obtain its symbionts by feeding on prey that house *Symbiodinium*, but as by-catch from the water column. Specimens were exposed to different experimental conditions (nonfeeding *vs* feeding, light *vs* darkness) to estimate the efficiency of this putative mutualistic symbiosis. Photosynthetic efficiency of *Symbiodinium* measured by means of PAM fluorometry remained high, independent of experimental treatment. Specimens kept under nonfeeding conditions survived the whole experimental period, grew to modest size and laid fertile egg clutches continuously. Specimens fed additionally with crustaceans and turbellarians grew faster and larger and laid more egg clutches, implying higher fecundity. *Symbiodinium* density was higher in fed specimens, but is potentially regulated actively by *M. engeli* through various mechanisms. Fed specimens kept in continuous darkness died relatively soon, suggesting that light is crucial for survival. Histological analyses revealed specialized morphological structures of the digestive gland ('cisternae' and 'fine tubuli') that house *Symbiodinium*. These data suggest an advanced state of mutualistic symbiosis that enables *M. engeli* to survive times of food shortage.

INTRODUCTION

The mutualistic symbiosis between different taxa of the Nudibranchia and unicellular photosynthetic dinoflagellates of the genus *Symbiodinium* (often known as 'zooxanthellae') was described by Rousseau (1934, 1935). Most 'solar-powered' nudibranch species take up *Symbiodinium* from their prey of soft or hard corals and cultivate them inside the cells of their digestive gland (Rousseau, 1934, 1935; Rudman, 1981b, 1991; Kempf, 1984, 1991; Wägele & Johnsen, 2001; Burghardt *et al.*, 2005; Loh, Cowlishhaw & Wilson, 2006; Burghardt *et al.*, 2008a, 2008b; Wägele *et al.*, 2010). An exception to this rule are nudibranchs of the genus *Melibe*, which mainly feed on small crustaceans (amphipods, copepods, gammarids, caprellids, isopods and ostracods; Agersborg, 1919; Hurst, 1968; Ajeska & Nybakken, 1976; McDonald & Nybakken, 1978; Kempf, 1984; Willan & Coleman, 1984; Gosliner, 1987; Strathmann, 1987; Gosliner & Smith, 2003). Gosliner & Smith (2003) stated that all *Melibe* species from the tropical Indo-Pacific house *Symbiodinium*, without mentioning how they obtain these. They postulated that uptake of *Symbiodinium* is an adaptation for living in less productive tropical waters, since *Melibe* species might gain additional nutrients from these symbionts. No known crustaceans contain *Symbiodinium* as symbionts, therefore the source of *Symbiodinium* in *Melibe* species is still not known. *Symbiodinium* exists free-living within the ocean environment, providing aposymbiotic hosts with a pool of various genotypes for potential infection (Carlos *et al.*, 1999; Coffroth *et al.*, 2006; Stat & Gates, 2008). Thus, *Symbiodinium* found in *M. engeli* are most likely the result of by-catch from the water column during feeding or, alternatively, might be acquired by the veliger larvae prior to metamorphosis.

The general advantages to nudibranchs of a symbiosis with *Symbiodinium* have already been discussed by several authors: the incorporation of *Symbiodinium* offers cryptic appearance in colour (Rudman, 1987; Gosliner, 1987; Wägele & Johnsen, 2001; Wägele, 2004; Burghardt & Wägele, 2004; Burghardt *et al.*, 2005; Burghardt & Gosliner, 2006; Burghardt *et al.*, 2008a, 2008b) and translocated photosynthetic products serve as nutrients (Hoegh Guldberg & Hinde, 1986; Hoegh-Guldberg *et al.*, 1986; Rudman, 1991; Wägele & Johnsen, 2001; Burghardt & Wägele, 2004; Burghardt *et al.*, 2005; Burghardt *et al.*, 2008a). Additional metabolites produced by the symbionts allow 'solar-powered' nudibranchs to survive food shortage of weeks or up to several months (Burghardt & Wägele, 2004; Burghardt *et al.*, 2005, Burghardt & Gosliner, 2006; Burghardt *et al.*, 2008a, 2008b).

Wägele & Johnsen (2001) first introduced the now well established method of using a PAM (pulse amplitude modulated) fluorometer in sea slugs to distinguish digested from photosynthetically active *Symbiodinium* cells *in situ*. Burghardt & Wägele (2004), Burghardt *et al*. (2005), Burghardt & Gosliner (2006), Burghardt & Wägele (2006) and Burghardt *et al*. (2008a, 2008b) subsequently performed long-term experiments with different taxa of 'solar-powered' nudibranchs and showed intra- and interspecific differences in the efficiency of symbiosis by means of PAM data (see also review by Wägele *et al*., 2010). However, these studies have mainly focused on aeolid nudibranch species that obtain *Symbiodinium* by feeding on corals.

The current pilot study is the first to investigate a nudibranch species symbiotic with *Symbiodinium* that is apparently obtained from the free water column—the 'solar-powered' dendronotoid nudibranch *Melibe engeli* Risbec, 1937. We present results of long-term culture under various conditions (by means of PAM data), describe morphological structures adapted for the symbiosis and discuss the implications for overall symbiotic performance.

MATERIAL AND METHODS

Six specimens of *Melibe engeli* (as identified by morphology) were collected in May 2003 in Dahab Lagoon (Gulf of Aqaba, Red Sea, Egypt) in shallow water from 0.51.5 m depth. Specimens were found on a heavily damaged coral reef between large pieces of coral rubble, which were overgrown by green and brown algae. Light intensity at the locality was not measured, but appeared high due to the clarity and shallowness of the water. The water temperature was about 26°C. Specimen P (Table 1) was directly preserved after capture and was used only for morphological and histological investigation. All other five specimens were first kept under natural light conditions outside in the shade (up to 150 μmol quanta $m^{-2} s^{-1}$) during the first 10–14 d in Egypt. They were exposed to a natural diurnal light cycle (about 12 h light/12 h darkness) in aquaria with natural and unfiltered sea water that was changed completely every day. Due to the lack of cooling systems in Egypt, water temperature in the aquaria was higher than in the natural environment (up to about 30°C). The slugs were not fed, although tiny planktonic organisms might have been available as food in the unfiltered sea water. Specimens were transported from Egypt to Germany in a 1-l plastic bottle with natural seawater. After transfer the specimens were kept in the laboratory at Ruhr-University Bochum under controlled artificial light conditions, produced by an aquarium lamp (Osram Lumilux '11' light tubes) emitting an average of 70 μmol quanta $m^{-2} s^{-1}$ in 20 cm water depth. Slugs were acclimated to the new conditions in Bochum for 1 week. After acclimation, specimens were subjected to different controlled experimental setups (Table 1). Regular PAM measurements were first performed after acclimation in Bochum, thus day '0' in the experiment reflects conditions in Bochum.

Artemia sp. larvae and unidentified *Symbiodinium*-free turbellarians (maximum size 3 mm) were provided during the feeding experiments in Bochum twice a week (Table 1). Since *M. engeli* was kept in sterilized artificial sea water in Bochum, the uptake of additional organisms and nutrients was excluded or minimized. For preparing artificial sea water 'Tropic Marine' sea salt was used. Two specimens were initially kept under a diurnal light cycle (12 h light/12 h darkness) and then subsequently kept in constant darkness for 24 h a day as described in Table 1, with only a short interruption of few minutes for cleaning in dimmed light. All other specimens were exposed to a diurnal light cycle (12 h light/12 h darkness) throughout the experiment. Aquaria were cleaned regularly every third day to remove faeces and possible algae. Faeces and removed algae were carefully sucked up after settling on the ground by using a plastic hose, in order to avoid complete water exchange. The aquaria were afterwards topped up with sterilized artificial water. Larger water changes were performed once a week, half of the aquarium water was then replaced by sterilized artificial seawater.

The state of health and size of specimens were documented by taking digital photos of their external appearance. Measurements of body length were made when slugs were maximally stretched while crawling on the glass wall of the aquarium.

Cerata were either cut off for later histological investigations or freshly autotomized cerata were collected immediately after natural detachment. After preservation in 9% formalin and transfer to 70% ethanol, samples were embedded and histological analysis was performed. After long-term experiments all specimens were preserved in the same way as cerata. Specimens 3 and 4 were preserved before natural death due to their weak appearance and to avoid loss for later histological analysis. All other specimens were preserved after their natural death. Samples were embedded in hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer, Germany) for histological serial sections (2.5 μm, stained with toluidine blue) and photographically documented by using a digital camera (Olympus DP 50) on an Olympus microscope (BX).

Fertility of egg clutches was determined by placement in petri dishes filled with sterilized sea water. Veliger larvae hatched from all egg clutches, but did not metamorphose and subsequently died.

A PAM fluorometer (Diving-PAM, Walz, Germany) was used to detect *in vivo* photosynthetic efficiency of *Symbiodinium*

Table 1. Synopsis of investigated specimens of Melibe engeli exposed to various experimental conditions.

Specimen	Collection date	Experimental conditions	Egg clutches	Death/ preservation
P	12.05.2003	Directly preserved in the field; only used for histological investigations	--	Preserved 12.05.03
1	12.05.2003	Nonfed under ALC: Day 0–22 Fed under ALC: Day 23–263	On days 58, 95, 122, 140 and 246	Natural death after 263 d of culture
2	12.05.2003	Nonfed under ALC: Day 0–22 Fed under ALC: Day 23–101 Fed under darkness: Day 102–133	On day 58	Natural death after 133 d of culture
3	12.05.2003	Nonfed under ALC: Day 0–270	On days 56, 58, 61, 122, 224 and 246	Debilitated animal preserved after 270 d of culture
4	12.05.2003	Nonfed under ALC: Day 0–101 Fed under ALC: Day 102–254	On days 58, 122 and 140	Debilitated animal preserved after 254 d of culture
5	12.05.2003	Nonfed under ALC: Day 0–48 Fed under darkness: Day 49–74	On days 61 and 66	Natural death after 74 d of culture

ALC, artificial light conditions in laboratory in Bochum (see text).

inside *M. engeli* by measuring fluorescence emitted by photosystem II (PSII) of chlorophyll *a*. This method allows actively photosynthetic *Symbiodinium* cells to be distinguished from digested ones inside the nudibranch (Wägele & Johnsen, 2001; Burghardt & Wägele, 2004; Burghardt *et al*., 2005; Burghardt & Gosliner, 2006; Burghardt *et al*., 2008a, 2008b).

The PAM fluorometer consists of a main instrument, a cosinus-corrected light collector and an optic fibre. The fibre detects the fluorescence of the *Symbiodinium* cells inside the nudibranch by measuring the ground fluorescence (F_0) using a very low light source. F_0 is defined as fluorescence measured in dark-acclimated tissues. A flash of *c*. 10 000 µmol quanta m^{-2} s^{-1} for 0.8 s is applied via the optic fibre to obtain maximum fluorescence (F_m). The maximum quantum yield of fluorescence for PSII (F_v/F_m) is defined as $F_v/F_m = (F_m - F_0)/F_m$. Measurements of F_v/F_m were taken in darkness. Between the usual three measurements per individual session, breaks of at least 10 min in complete darkness allowed the reaction centres of PSII to recover after light saturation. F_v/F_m of symbionts in individual specimens of *M. engeli* was plotted *vs* time in diagrams in order to investigate photosynthetic efficiency and the influence of additional food uptake on photosynthetic performance of *Symbiodinium* (Burghardt & Wägele, 2004; Burghardt *et al*., 2005; Burghardt & Gosliner, 2006; Burghardt *et al*., 2008a, 2008b). Due to absence of the Diving-PAM fluorometer from day 58 to day 93, measurements of F_v/F_m were taken by a PAM 102 and a PAM 103 (Walz, Germany) in the Department of Plant Biochemistry at the University of Bochum. These data were recorded by an oscilloscope (Gould Classic 6100 Digital Storage Oscilloscope 200 MHz). Following day 93, measurements were continued with the aforementioned Diving-PAM.

RESULTS

PSII quantum yield vs *time curves* (F_v/F_m – *T-curves*)

Maximum quantum yield *vs* time curves (Fig. 2) of all specimens are relatively uniform, with no significant intraspecific differences irrespective of the conditions under which they were kept (in light or darkness and in combination with feeding versus nonfeeding (see Table 1). The narrow range of the F_v/F_m values remained at a high level for all specimens between 0.55 and 0.75 over the whole experimental period (average approximate level 0.65).

Body growth

At time of capture in May 2003 all specimens were between 13 and 20 mm in length. Figure 3 shows growth for individuals 1–4 (day 0 to 270). The nonfed specimen 3 showed an increase in body length from ~20 mm on day 0 to 65 mm on day 78. Afterwards size fluctuated slightly before decreasing from day 260 onwards. The size of the permanently fed specimen 1 increased strongly from ~15 mm on day 0 to 145 mm on day 239. After a slight increase in body size (similar to the growth of specimen 3) at the beginning of the nonfeeding period, individual 4 was fed from day 102 onwards with *Artemia* and turbellarians and consequently grew quickly to 110 mm (day 228) after the start of feeding (see Fig. 1b for comparison with individuals 3 and 4). Nevertheless, that individual stayed smaller than specimen 1. Specimen 2 (fed with *Artemia* and turbellarians) grew from a size of 13 mm on day 0 to 60 mm on day 118 and showed a similar growth rate to the other fed specimen 1.

Biological notes and behaviour

When first collected, all specimens were small, transparent and only the ducts of the digestive gland were slightly coloured cream-olive (Fig. 1A). Due to body transparency, colour and shape, specimens resembled rotten algae and were well camouflaged on the original substrate. At the time of capture globular ovotestis bodies (Fig. 1A) were already developed, but sexual maturity was unlikely since none of the specimens laid eggs at that stage. After transfer to the laboratory in Bochum all specimens grew relatively fast and overall the body became more olive-coloured. Due to transparency of the body the fine diffuse reticulate pattern of digestive glandular ducts pervading the whole body remained visible (Fig. 1B, C). This dense reticulum of tubule tissue contained numerous *Symbiodinium* cells and was most dense in the centre of cerata (Fig. 1C), producing the overall olive colour of the slugs.

In the aquarium the feeding behaviour characteristic of many *Melibe* species could be observed: the oral hood (Fig. 1C) was held out horizontally, just above the bottom, and moved gradually along, searching for small prey with the tentacles around the edge of the hood. Fed specimens caught *Artemia* larvae and turbellarians by waving the inflated oral hood over the bottom and wall of the aquaria. Interestingly even nonfed animals behaved in a similar way, despite the absence of potential food. Nevertheless, when offered food for the first time after several weeks of nonfeeding, these animals only slowly reacted when sensilla of the hood were touched by swimming *Artemia*. All specimens frequently detached from the substratum when disturbed to swim through the water column by lateral flexion of the body, a behaviour also known in other *Melibe* species (Mills, 1994).

After a few weeks in captivity all specimens (fed and nonfed) produced fertile, spirally coiled, whitish to yellowish transparent egg clutches. Even specimens kept isolated from other individuals for months were still able to produce fertile egg clutches until the end of their lifespan (Table 1). Free-swimming veliger larvae hatched after some days but did not metamorphose. Number and size of egg clutches and egg-laying frequency appeared considerably higher in fed individuals. Additionally, egg clutches of fed specimens had a more intense yellow colour than those of nonfed specimens, possibly indicating that they contained more yolk. Since both eggs and veliger larvae were more or less transparent, we checked them for the potential presence of *Symbiodinium* under the light microscope, but neither appeared to contain any of these brownish symbionts.

The shape of cerata of *M. engeli* changes as they grow to maturity; cerata of juveniles are lanceolate, whereas those of older specimens are more cylindrical, covered with large blunt papillae or pustules and ending distally in palm-like extensions with long, narrow and pointed papillae (Fig. 1). The cerata even of full-grown specimens could change slightly in size and shape, depending on activity state. We often observed gas bubbles inside cerata, apparently lying within digestive glandular ducts (Fig. 1B, C). Specimens with gas bubbles in their cerata shook their whole body more often than others—by contracting, retracting and waving the body from one side to the other.

In general the colour intensity (the brownish colour apparently reflecting the concentration of *Symbiodinium*) of all specimens varied over time, dependent on culture conditions, resulting growth rates and possibly changes in fluid volume inside the body. In some cases cerata became dark brown in colour, shrank and were afterwards detached (Fig. 1B, arrow). Interestingly, cerata that contained large gas bubbles were often darker in colour (possibly indicating higher *Symbiodinium* density) and were more often detached than cerata with no or only smaller gas bubbles. *Melibe engeli* is able to regrow detached cerata, although regenerated cerata were generally smaller and of slightly different shapes than the original ones. Regeneration of detached cerata was fast; for instance one regenerated ceras of specimen 1 grew from 6 to 19 mm in length within 20 d. In most individuals the elongate triangular papillae at the distal end of the cerata also occasionally became dark brown and, similar to whole cerata, were often detached and afterwards regenerated (Fig. 1B, C).

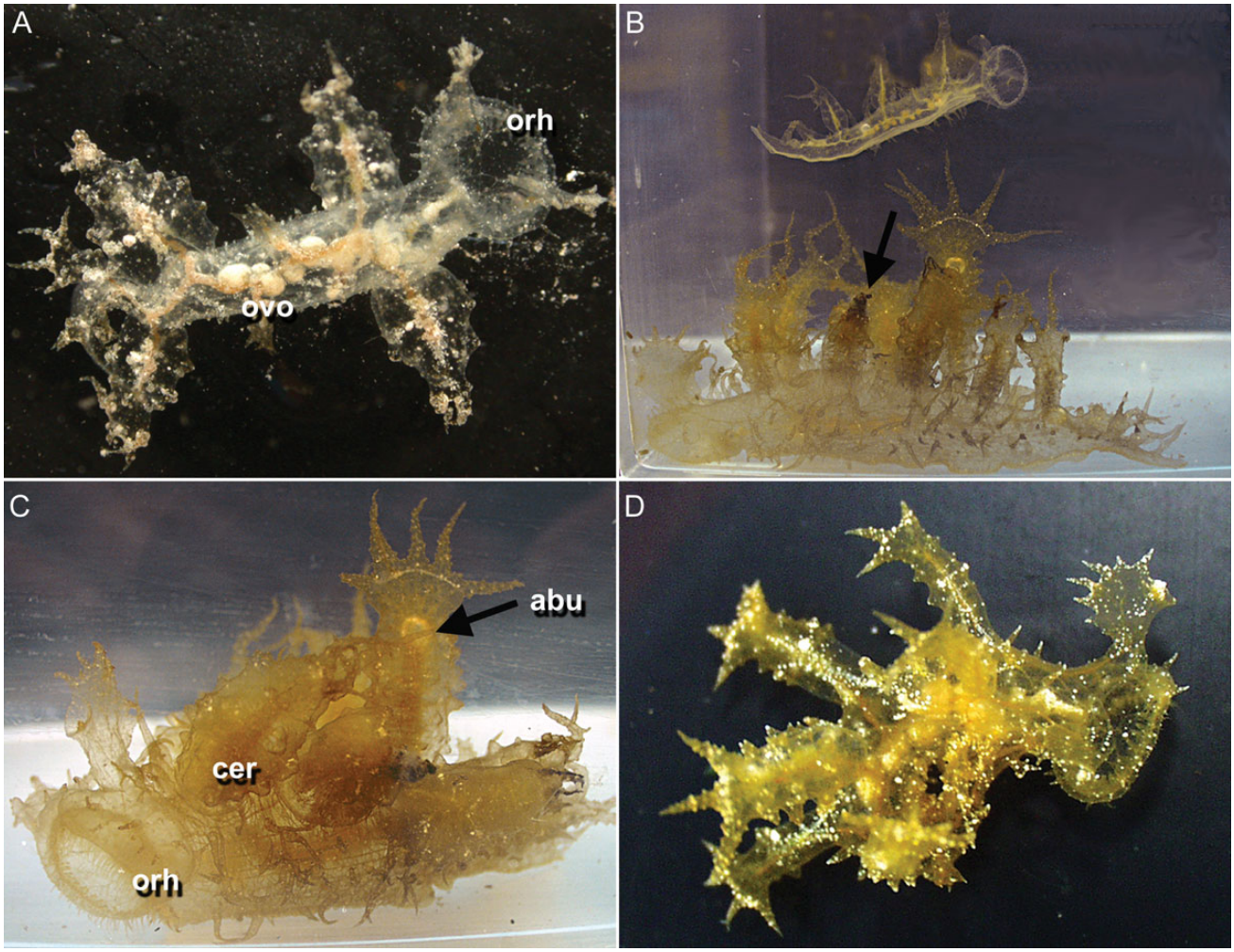

Figure 1. Different specimens of *Melibe engeli*. **A.** Specimen P, directly after capture in May 2003. Length approximately 20 mm. Note the transparent body and already developed ovotestis bodies. **B.** Comparison between the upper nonfed specimen 3 (body length 50 mm) and lower fed specimen 4 (body length 95 mm) on day 156. Note dark-coloured ceratal tips of specimen 4 (arrow). **C.** Specimen 4 showing feeding behaviour on day 156. Note dense reticulate pattern of digestive glandular structures all over body and dark coloured cerata with large air bubble (arrow). **D.** Specimen 2 on day 53. Abbreviations: abu , air bubble; cer, ceras; orh, oral hood; ovo, ovotestis bodies.

All specimens produced brownish faeces, which were composed of undigested remains of *Artemia* larvae (only in fed individuals) and intact-appearing *Symbiodinium* cells (confirmed for all specimens by microscopic examination; Fig. 5E). Measurements of these faeces with the Diving-PAM showed a high maximum quantum yield (F_v/F_m) of approximately 0.6 for at least 3–4 d following defaecation.

The individuals kept in the light (fed or nonfed, numbers 1, 3 and 4) died or were preserved about 8.5–9 months after capture. The specimens kept in darkness after a certain light period (2 and 5) died much sooner, about 1 month after light deprivation, despite being fed with *Artemia* (Table 1).

Histology of the digestive gland

The structure of the digestive gland in *M. engeli* is complex and profoundly branched. One central main duct of the digestive gland originates in the posterior part of the stomach and runs caudally throughout the body (Fig. 4A). From this main duct one central duct branches off into each ceras (Fig. 1B; upper specimen). The main ducts with typical digestive glandular tissue (Fig. 4A) continue and give way to either large balloon-shaped 'cisternae' (surrounded by a very thin membrane; Fig. 4D–F) and/or they continue in an extremely fine and diffuse reticulate pattern of digestive glandular ducts ('fine tubuli') that pervades the whole body (Figs 4B, 5A–D, F). Gradations from one digestive gland form to the other exist and contain intracellular *Symbiodinium* cells (Figs 4, 5). *Symbiodinium* cells are present intracellularly in all digestive glandular cells, as well as in the lumen of the digestive gland, irrespective of the cultivation period (Fig. 4A–C). The density of *Symbiodinium* in the lumen of typical digestive glandular ducts is nevertheless low compared to the density inside cisternae (Fig. 4D–F) and inside the cells of fine tubuli (Fig. 5A, B). These cisternae appear to be unique to *M. engeli* and have not been described in any other 'solar-powered' nudibranch species. The fine tubuli form continuous ribbons and mainly consist of 'carrier' cells (Kempf, 1984) containing *Symbiodinium* (Fig. 5A, B). The tubuli are often thin, with *Symbiodinium* cells arranged like a string of pearls (Fig. 5A, B). They penetrate the whole body, including the foot, notum (Fig. 4A), cerata (Figs 4B, 5A, B), rhinophores (Fig. 5C), muscles (Fig. 5D) and even the ovotestis (Fig. 5F) and penis. The oocytes and oogonia in the gonad do not contain any *Symbiodinium* cells (Fig. 5F).

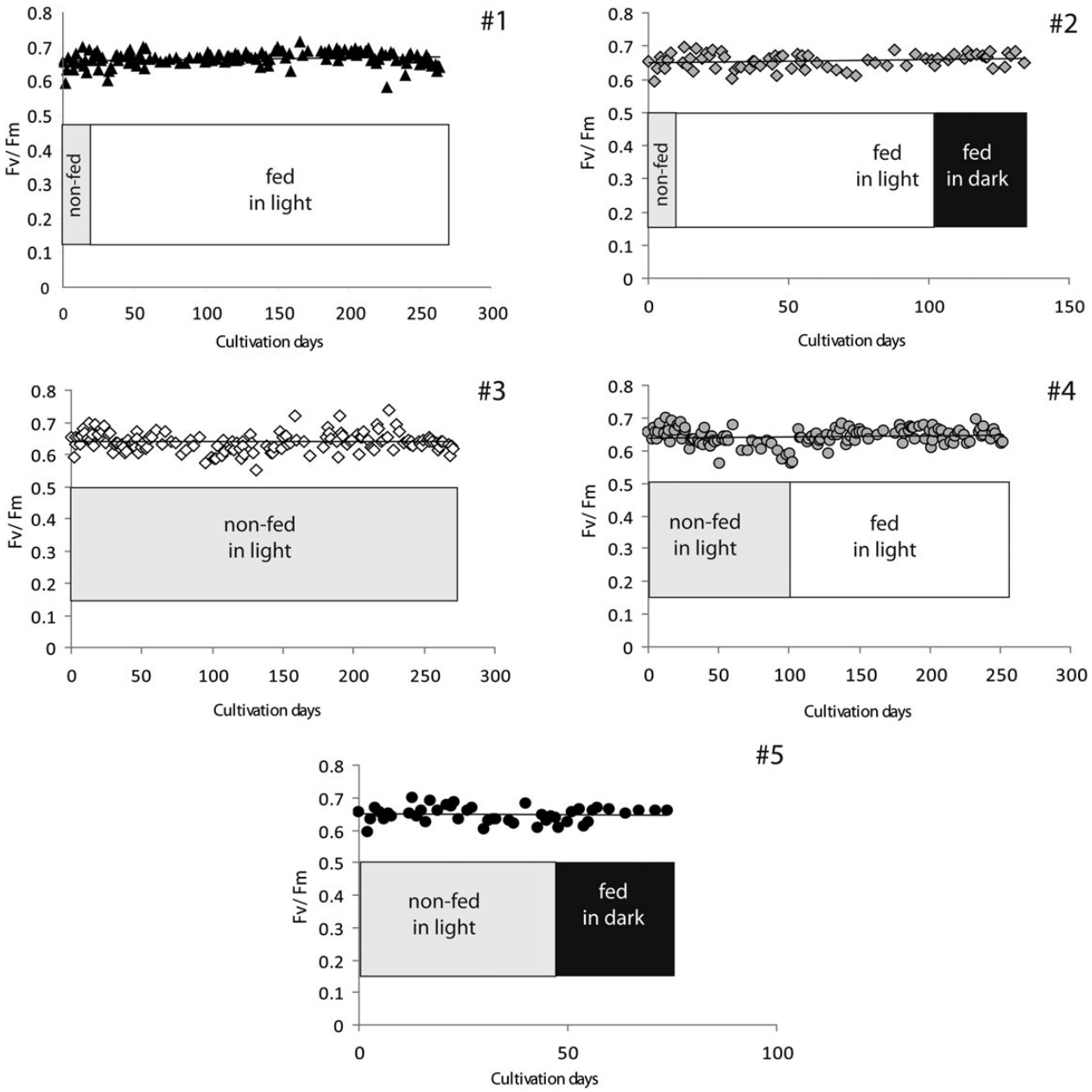

Figure 2. Maximum quantum yield of photosynthesis (F_v/F_m) of specimens 1–5 of *Melibe engeli* versus time, from day 0 to day 270. Daily values are the average for each specimen. Note the different scales of the x-axis for specimens 2 and 5. Bar length below the plotted data reflects length of culture of individual specimens with white bars indicating feeding periods, grey bars nonfeeding periods, and black bars the time spent in darkness while fed with *Artemia* and turbellarians.

Apart from the slightly higher density of digestive glandular structures in fed specimens the impact of feeding/nonfeeding on the digestive gland seems to be minor, since no major intraspecific differences could be detected, even after long periods of nonfeeding. Dividing *Symbiodinium* cells are found in nonfed as well as fed specimens (Figs 4F, 5B). Intact *Symbiodinium* cells could be found even within the lumen of the hindgut/anus of the nonfed specimen 3 (Fig. 5E).

DISCUSSION

Melibe engeli was identified by means of morphology. Until now, *M. viridis* Kelaart, 1858, *M. megaceras* Gosliner, 1987 and *M. bucephala* Bergh, 1902 were the only *Melibe* species known from the Red Sea (Bergh, 1902; O'Donoghue, 1929; Gosliner & Smith, 2003). This is the first record for *Melibe engeli* in the Red Sea, extending its distributional range significantly. So far, *M. engeli* has been known only from New Caledonia (Risbec, 1937, 1953; Catala, 1986), Japan, Hawaii and the Philippines (Gosliner & Smith, 2003).

High values of the maximum quantum yield of chl *a* fluorescence (F_v/F_m) between 0.55 and 0.75 (Fig. 2) for up to 9 months with minor fluctuations indicate well-functioning photosynthesis performed by *Symbiodinium* and long-term retention with stable symbiosis between *M. engeli* and its symbiont. Culture conditions (fed or nonfed) seem to have almost no influence on F_v/F_m values and therefore on the photosynthetic performance of incorporated *Symbiodinium* over the whole experimental time.

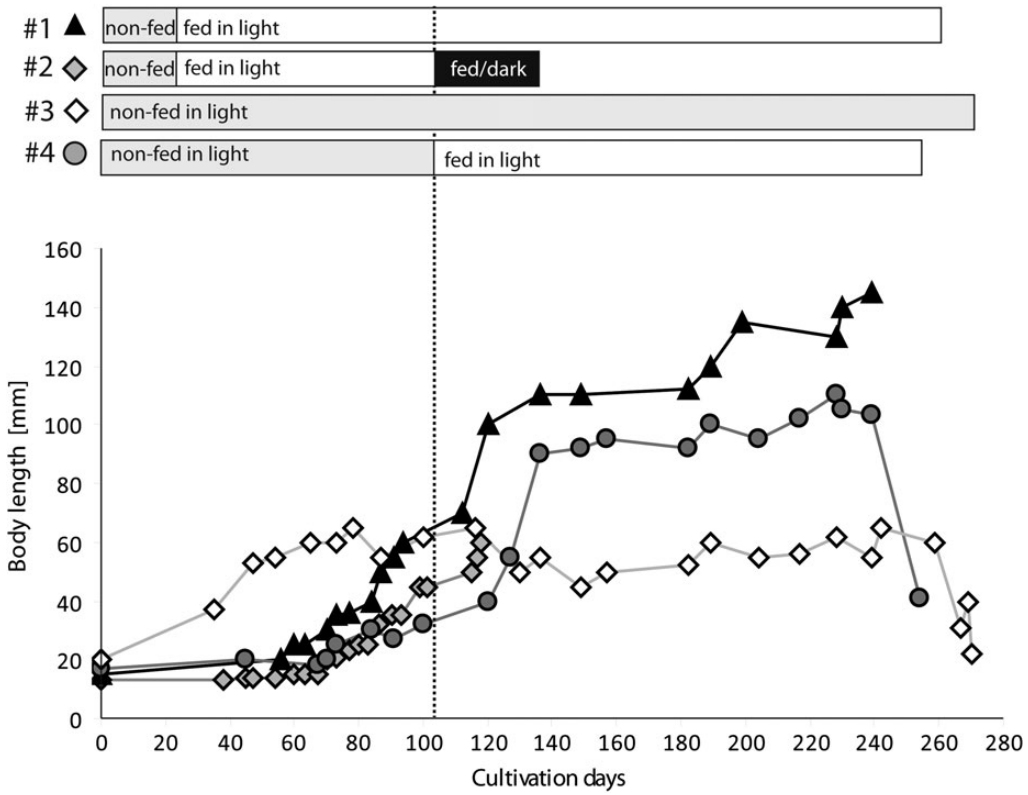

Figure 3. Course of the body length of different specimens of *Melibe engeli* versus time from days 0–270. Black triangles, specimen 1; pale grey diamonds, specimen 2; white diamonds, specimen 3; pale grey circles, specimen 4. Bar length above diagram reflects length of culture of individual specimens with white bars indicating feeding periods, grey bars nonfeeding periods and black bars time spent in darkness while fed with *Artemia* and turbellarians. The dotted line marks simultaneous start of feeding (specimen 4) and beginning of culture in darkness (specimen 2).

Although no exchange of metabolites from symbionts to host was measured, an exchange seems evident as indicated by the capability of specimens to survive and even grow under nonfeeding conditions (e.g. specimen 3).

Histological sections of fed and nonfed animals show no significant differences in the appearance of *Symbiodinium*. Dividing *Symbiodinium* cells could be observed in all specimens (Figs 4F, 5B), indicating suitable conditions for proliferation inside *M. engeli*. Special digestive glandular structures like extremely thin 'fine tubules' with 'carrier' cells that penetrate almost all body parts (Figs 4A, B, 5A–D, F) and 'cisternae' densely filled with *Symbiodinium* (Fig. 4C–F) confirm the assumption of a highly evolved symbiosis in *M. engeli* and suggest a possible adaptation for 'farming' *Symbiodinium*. Fine tubuli in *M. engeli* look similar to analogous structures that have been described in the aeolid nudibranch *Pteraeolidia ianthina* (Kempf, 1984; Wägele & Johnsen, 2001; Burghardt *et al*., 2005), which is often referred to as an example of a highly efficient symbiosis. Nevertheless, fine tubuli in *M. engeli* penetrate more body regions than those in *P. ianthina*. As for the cultivation of *Symbiodinium* our histological investigations did not reveal whether *Symbiodinium* inside cisternae of *M. engeli* are farmed in a matrix of digestive glandular cells or whether cisternae just represent the lumen of large balloon-shaped structures where high concentrations of *Symbiodinium* are stored. Dividing *Symbiodinium* (Fig. 4F, arrow) suggest the former hypothesis, although the latter one is supported by the existence of similar structures in the giant clam *Tridacna*, where *Symbiodinium* cells are cultivated extracellularly in the lumen of digestive-glandular ducts (Norton *et al*., 1992).

The presence of highly branched and specialized digestive-glandular structures in combination with high photosynthetic efficiency of the symbionts is in agreement with the hypothesis of Rudman (1991), that there is a correlation between the extent of branching of the digestive gland and the efficiency of symbiotic relationship in 'solar-powered' nudibranchs. According to Rudman an extensive branching of the digestive gland significantly enlarges the surface area for storage of *Symbiodinium* and allows higher photosynthetic light absorption and utilization. His hypothesis has been corroborated for the nudibranch *Phyllodesmium* (Burghardt *et al*., 2005; Burghardt *et al*., 2008a) and can also be confirmed here for *M. engeli*.

Additionally, the transparent body of some *Melibe* species allows good light transmission (Gosliner & Smith, 2003) and the alternate arrangement of the cerata minimizes shading and optimizes control of light intensity, thus enhancing optimal conditions for the endosymbionts (Gosliner & Smith, 2003). The combination of transparency and the brownish colour originating from *Symbiodinium* also offers additional cryptic appearance to the animal, probably another driving force in the evolution of this symbiotic system (Wägele *et al*., 2010).

Corals and bivalves are able to control population size of *Symbiodinium* through several mechanisms, e.g. ammonium concentration (reviewed by Gordon & Leggat, 2010). For nudibranchs no similar control mechanisms have been documented in the literature. Nevertheless, we observed a variation of *Symbiodinium* density between specimens exposed to different experimental conditions, as reflected in body colour (Fig. 1B). The additional available nutrients through food uptake might be one reason for a higher growth rate and therefore higher density of *Symbiodinium*. We observed detachment of dark brown cerata with an exceptionally high density of *Symbiodinium*. This might be one mechanism for regulation of the number of *Symbiodinium*

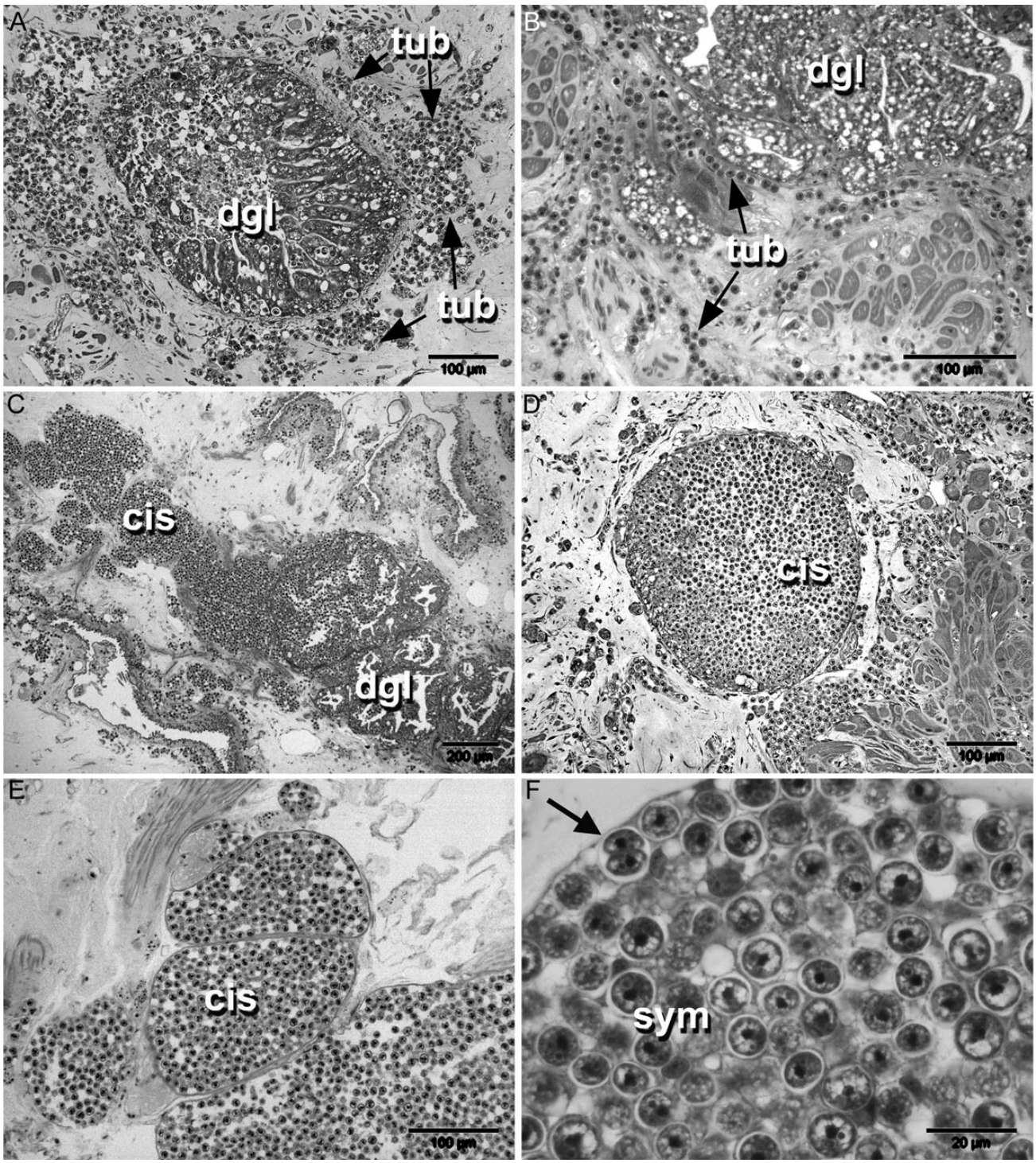

Figure 4. Histological sections of digestive-glandular structures in *Melibe engeli*. **A.** Cross-section through typical duct of digestive gland inside body of specimen 1 (day 263). Note *Symbiodinium* cells inside lumen of ducts and inside digestive glandular cells. Duct surrounded by fine tubuli (arrows) with intracellular *Symbiodinium*. **B.** Cross-section through ceras with transition zone between typical digestive glandular tissue and fine tubuli (arrows) in specimen 1 (day 157). **C.** Longitudinal section through ceras with transition zone between typical digestive glandular tissue and cisternae inside ceras of specimen 4 (day 254). **D.** Cross-section through cisternae inside body of specimen 1 (day 263). **E.** Cross-section through cisternae inside ceras of specimen 4 (day 254). **F.** High-magnification image of cross-section of cisternae that are densely packed with *Symbiodinium* inside body of specimen 3 (day 270). Note dividing *Symbiodinium* (arrow). Abbreviations: cis, cisternae; dgl, typical digestive gland; sym, *Symbiodinium*; tub, fine tubuli.

cells. Detachment of cerata also might help slugs to get rid of large gas bubbles inside the digestive gland (Fig. 1B, C). Gas bubbles increase buoyancy and result in more energy being required to keep attached to the substratum. In addition, specimens with bubbles are in danger of floating towards the surface when swimming. Due to the high density of photosynthesizing *Symbiodinium* cells, it seems likely that these gas bubbles consist to a large degree of oxygen. If that is the case these

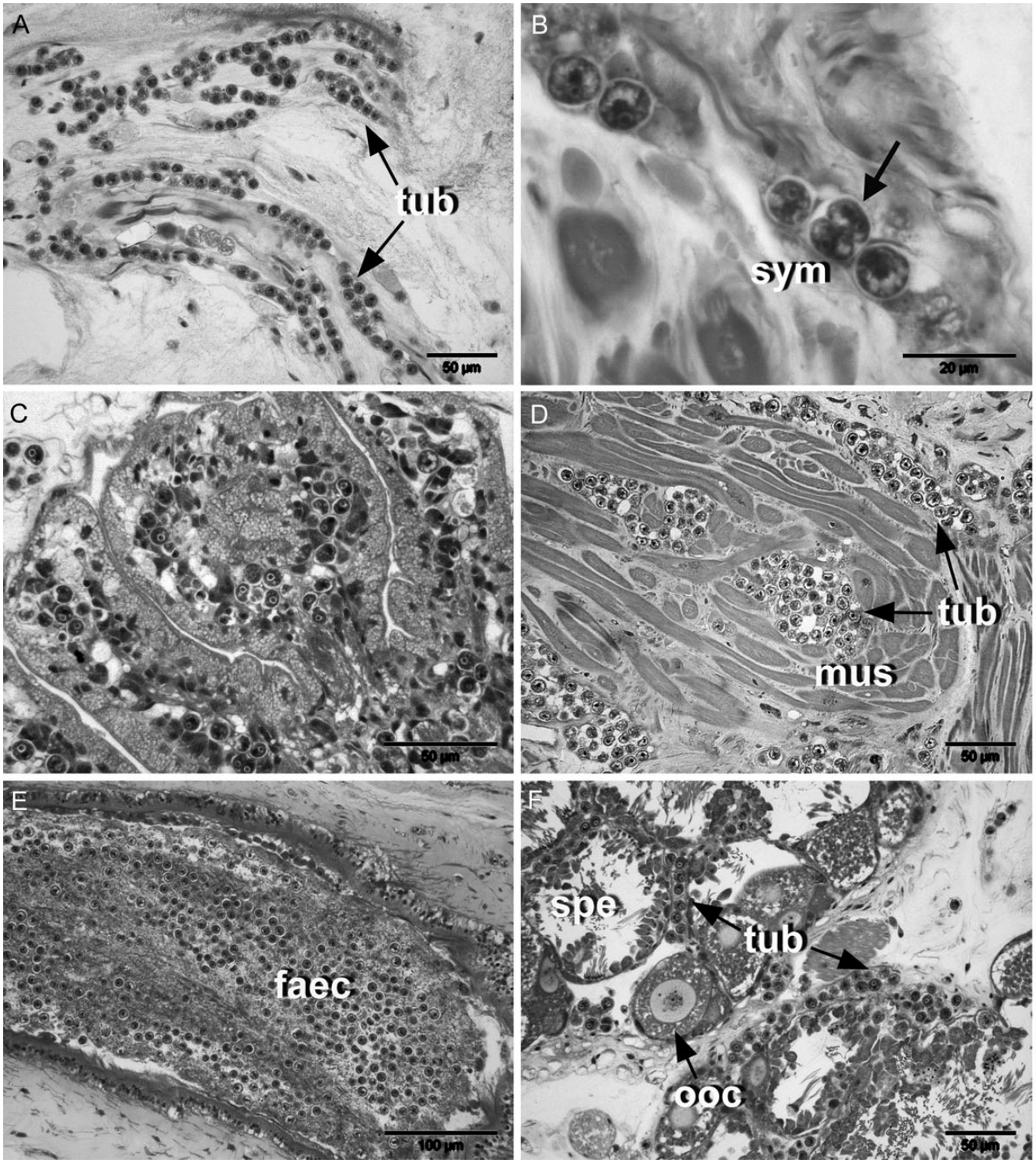

Figure 5. Histological sections of digestive glandular structures in *Melibe engeli*. **A.** Longitudinal section through fine tubuli (arrows) of digestive gland inside ceras of specimen 1 (day 157). **B.** Detail of fine tubuli inside specimen 3 (day 263). Note dividing *Symbiodinium* (arrow). **C.** Longitudinal section through rhinophore of specimen P with fine tubuli containing *Symbiodinium* inside rhinophore. **D.** Muscular structures (specimen 1) penetrated by fine tubuli (arrows; day 263). **E.** Hindgut/anus of specimen 3 with intact *Symbiodinium* in lumen (day 270). **F.** Ovotestis of specimen P with fine tubuli (arrows) containing *Symbiodinium*. Note absence of *Symbiodinium* within oocytes. Abbreviations: faec, faeces; musc, musculature; ooc, oocyte; spe, spermatozoa; sym, *Symbiodinium*; tub, fine tubuli.

bubbles could be a threat to the holobiont; oxygen in high concentrations can form reactive oxygen species (ROS), such as singlet oxygen ($^1O2$) and superoxide (O_2^-; Lesser, 2006). ROS causes major cellular damage including oxidizing membranes, denaturing proteins and damaging nucleic acids (Lesser, 2006). Along with possible biochemical adaptations of hosts and symbionts for managing ROS to prevent cellular damage (known from other symbioses with *Symbiodinium*; Richier *et al.*, 2005;

Lesser, 2006; Merle *et al*., 2007), detachment of cerata with gas bubbles might be an additional adaptation for this purpose. *Symbiodinium* density might also be actively regulated by the nudibranch through defaecation; faeces of all specimens were mainly composed of intact *Symbiodinium* cells that were expelled while still photosynthetically active. Intact-looking *Symbiodinium* cells were also found in the rectum of one nonfed specimen in histological examination (Fig. 5E); thus, even nonfed specimens might regulate *Symbiodinium* density.

Positive effects of hosting *Symbiodinium* obviously outweigh possible drawbacks since even nonfed specimens are able to grow and reproduce. Photosynthetic products of symbionts probably enable nonfed individuals of *M. engeli* to survive for a long period of time (at least 9 months) and facilitate production of fertile egg clutches in times of food shortage (Table 1). Our data on *M. engeli* seem to confirm a previous report that *M. pilosa* and an undescribed species of *Melibe* incorporate a substantial portion of photosynthetically-fixed carbon into their egg masses (Crossland & Kempf, 1985), although actual exchange of metabolites between *M. engeli* and *Symbiodinium* still needs to be confirmed by proper analysis. Results for specimens of *M. engeli* kept in darkness support previous studies on *Phyllodesmium* (Burghardt *et al*., 2005) that indicated *Symbiodinium* might switch to a heterotrophic lifestyle when photosynthesis is prohibited.

Despite the presence of high concentrations of *Symbiodinium* close to the ovotestis of *M. engeli*, oogonia and oocytes of this species do not contain *Symbiodinium* cells. Thus, acquisition of symbionts is 'horizontal'. This might seem surprising considering the high efficiency of this symbiosis, but it probably offers more flexibility since each new generation can take up *Symbiodinium* genotypes that are suitable under current environmental conditions. Horizontal (*de novo*) symbiont acquisition is common in symbioses of invertebrates with *Symbiodinium*, including all 'solar-powered' nudibranchs (Burghardt *et al*., 2008b), most Hexacorallia and Octocorallia (Fadlallah, 1983; Wallace, 1985), the jellyfish *Cassiopea* (Hofmann & Kremer, 1981; Pochon *et al*., 2010) and *Tridacna* (Norton *et al*., 1992). It seems to offer more benefits than drawbacks, especially in a changing and ecologically unstable environment. *Symbiodinium* genotypes differ in ecophysiology and bleaching susceptibility. Symbiont acquisition mode and genetic identity of *Symbiodinium* have therefore been discussed previously especially in relation to coral bleaching and the 'adaptive bleaching hypothesis' (Buddemeier & Fautin, 1993; Baker, 2003; Berkelmans & van Oppen, 2006). *Symbiodinium* diversity and resulting differences in bleaching susceptibility probably influence the symbiosis of *M. engeli* as well, but this aspect was beyond the scope of the present study.

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