



BEHAVIORAL ADAPTATIONS IN RELATION TO LONG-TERM RETENTION OF ENDOSYMBIOTIC CHLOROPLASTS IN THE SEA SLUG *Elysia timida* (OPISTHOBRANCHIA, SACOGLOSSA)

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Key words: Sacoglossa, endosymbiosis, chloroplasts, retention, phototaxis, photobehavior.

ABSTRACT

A comparative study was performed to analyze differences in evolutionary adaptations in two sea slug species, *Elysia timida* with long-term retention of endosymbiotic chloroplasts and *Thuridilla hopei* with short-term retention of endosymbiotic chloroplasts. Both sacoglossan species stem from the same habitat and show similar body sizes and structures with parapodial lobes whose position can be actively varied by the slugs. Ethological analyses were carried out concerning the positioning of parapodia and other photobehavioral parameters like phototaxis. In parallel, photosynthetic activity was measured with a Pulse Amplitude Modulated Fluorometer (PAM). In total, 252 *E. timida* individuals and 63 *T. hopei* individuals were included in the analysis. Slugs were collected diving in shallow depths up to 5 m in Banyuls sur mer, France, and kept in the laboratory

in basins with running seawater and natural light through a glass window. Behavioral observations and PAM-measurements were performed in 4 time intervals in the course of an observation day in daylight and dark-adapted conditions. Phototactic behavior was found to be present in both compared species, although the phototactic reaction was more pronounced in *E. timida*. Phototaxis was also observed in juvenile *E. timida* before sequestration of first *Acetabularia*-chloroplasts, which indicates no direct current influence of the endosymbiotic chloroplasts. Other parameters, however, like the positioning of the parapodia, were observed to be significantly different between the long-term and short-term storing species. While an adapted changing of the parapodia's position in reaction to light conditions was not observed in *T. hopei*, the typical specialized photobehavior of *E. timida* with active variation of parapodial positions including exposure and protection of integrated chloroplasts could be confirmed and analyzed in this study. Positioning of the parapodia in *E. timida* showed a significant relation to fluorescence values from PAM-measurements demonstrating the efficiency of exposure and protection of embedded chloroplasts.

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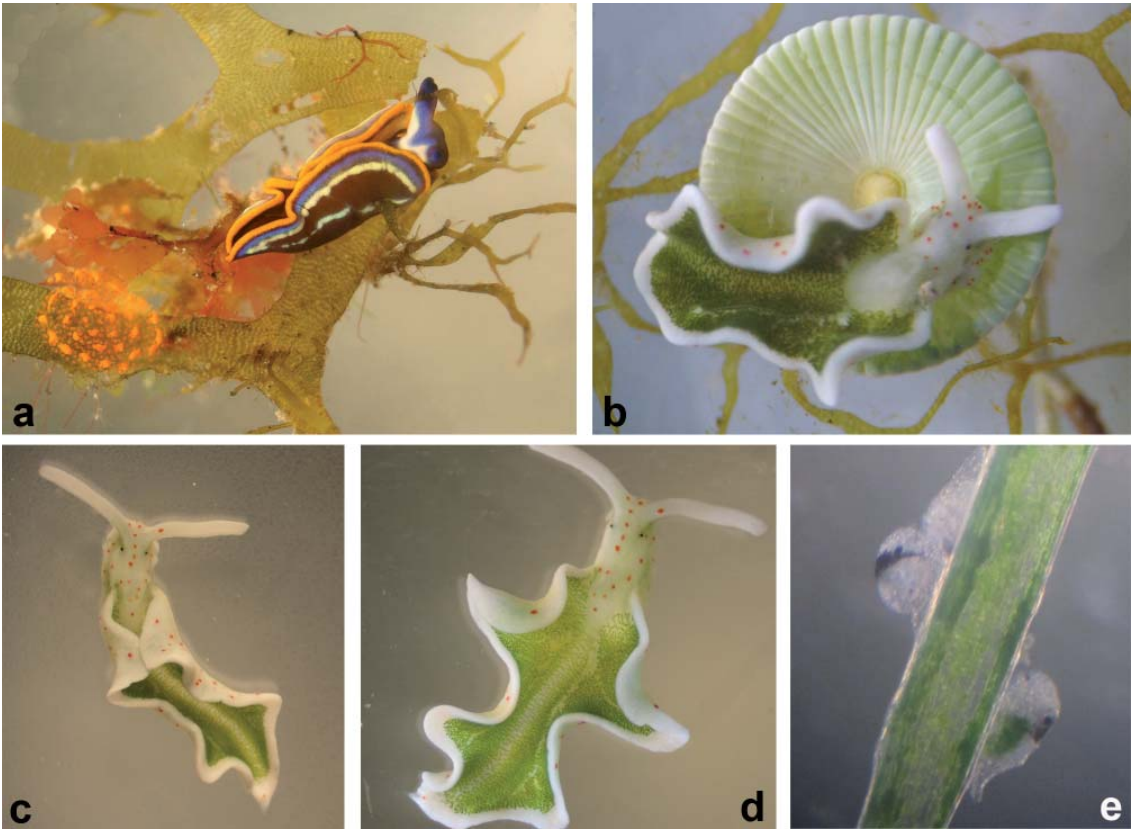


Figure 1:

a *Thuridilla hopei* on *Dictyota* (not a food organism of this species). *b* *Elysia timida* on its natural food alga *Acetabularia acetabulum*. *c* *Elysia timida*, parapodial opening level 2. *d* *Elysia timida*, parapodial opening level 3. *e* Three juveniles attached to a young *Acetabularia*: on the left two specimens before feeding, on the right one specimen after feeding

The specific photobehavior of *E. timida* with controlled exposure of parapodial lobes represents a highly specialized evolutionary adaptation in relation to long-term integration of chloroplasts and - state of the art - is only recorded for this species.

INTRODUCTION

Our knowledge on biology and evolution of functional kleptoplasty in various sacoglossan sea slugs has increased lately to a considerable extent (see e.g., Giménez Casalduero and Muniain, 2008; Händeler et al., 2009; Jesus et al., 2010 and literature herein). But when it comes to behavior,

our knowledge is still limited. Sacoglossans reveal a variety of evolutionary adaptations when it comes to retain endosymbiotic chloroplasts – especially with regard to behavior. First descriptions of specialized photobehavior in sea slugs were done by Fraenkel (1927) when he examined photomenotaxis in *Elysia viridis*. In a later study comparing five sacoglossan species, the focus was laid on the presence or absence of endosymbiotic chloroplasts in the sea slugs. Three symbiotic species with integrated chloroplasts (*Elysia tuca*, *Costasiella lilianae* (= *Costasiella ocellifera* after Clark (1984)), and *Elysia crispata*) and two aposymbiotic species (*Oxynoe antillarum* and *Berthelinia carribea*) were analyzed concerning their

photobehavior (Weaver and Clark, 1981). As one result, the symbiotic species oriented towards light while the aposymbiotic species avoided light which points to a possible relationship between symbiotic chloroplasts and phototaxis.

The chloroplast-hosting sacoglossan *Elysia timida* has a specially notable photobehavior, changing the position of its parapodial lobes from a contracted, closed posture to a spread, opened leaf-like posture (Rahat and Monselise, 1979). As *E. timida* varies the position of the parapodia as a reaction to light conditions, a possible nearby conclusion is that this photobehavior could have evolved in relation to the chloroplast-endosymbiosis. *E. timida* is a common Mediterranean species that lives in a close relationship to its food alga *Acetabularia acetabulum* from which it retains its endosymbiotic chloroplasts (Marin and Ros, 1992; Marin and Ros, 1993). With an extensive duration of approximately three months of retaining the endosymbiotic chloroplasts functional during starvation, *E. timida* belongs to the few species with the most extended capability of long-term retention of chloroplasts (Evertsen et al., 2007; Giménez Casalduero and Muniain, 2008; Händeler et al., 2009; Wägele et al., 2010). Recent literature defines long-term retention as lasting functionality of chloroplasts of more than a month opposed to short-term retention lasting about one week (Händeler et al., 2009).

As the special photobehavior of *E. timida* should be analyzed in more detail in this study with regard to its relation to the long-term integration of endosymbiotic chloroplasts, it was compared to a similar Mediterranean species with short-term retention of chloroplasts. The sacoglossan *Thuridilla hopei* is a species with short-term chloroplast endosymbiosis (Marin and Ros, 1989; Händeler et al., 2009) and was chosen as the most suitable comparative species, as both *E. timida* and *T. hopei* are common Mediterranean species that live sympatrically and have about the same body size and structure with parapodial lobes that can be actively closed and opened by the slugs - the basis for the comparison of

this behavior. During our studies we analyzed these varying positions in relation to irradiance and tested both species for the presence of phototaxis.

MATERIAL AND METHODS

In total, 252 *Elysia timida* and 63 *Thuridilla hopei* (Fig. 1a and b) were collected in the same habitat in Banyuls sur mer, France, by diving in shallow depths down to about 5 m, in July 2009 and September 2010. Individuals were kept in the laboratory (Observatoire Océanologique, Banyuls sur mer, France) in basins of about 160 cm x 60 cm with running seawater from the laboratory circulation system (21.2 ± 1.0 °C in July 2009 and 19.6 ± 0.9 °C in September 2010). It was attempted to provide the animals semi-natural conditions with exposure to natural (but not direct sun-) light through a window (orientated to the west) with a light intensity of up to 47 and 37 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (PAR: photosynthetic active radiation, highest single values measured in July 2009 and September 2010, respectively). Free access to an assortment of various algae from their natural environment, including the preferred food alga *Acetabularia acetabulum* (*E. timida*) and *Cladophora cf. vagabunda* (*T. hopei*) (Marin and Ros, 1989) collected from the same collection sites as the animals, was provided. For the various photobehavioral experiments, algae were removed from the basins and running sea water supply was stopped in order to exclude any additional influencing factors. Clutches laid by *E. timida* individuals in the laboratory were kept in petri dishes with artificial sea water and regular water exchange until hatching. Until experiments started, the juveniles were kept in artificial seawater with no food provided. In this state, juveniles are transparent (Fig. 1 e).

First phototaxis study: Elysia timida

The first observations on phototactic behavior included two groups of 50 individuals each in two separate basins. The two basins were both orientated parallel to the window side and for the trial were covered each half with black board. As a result, each

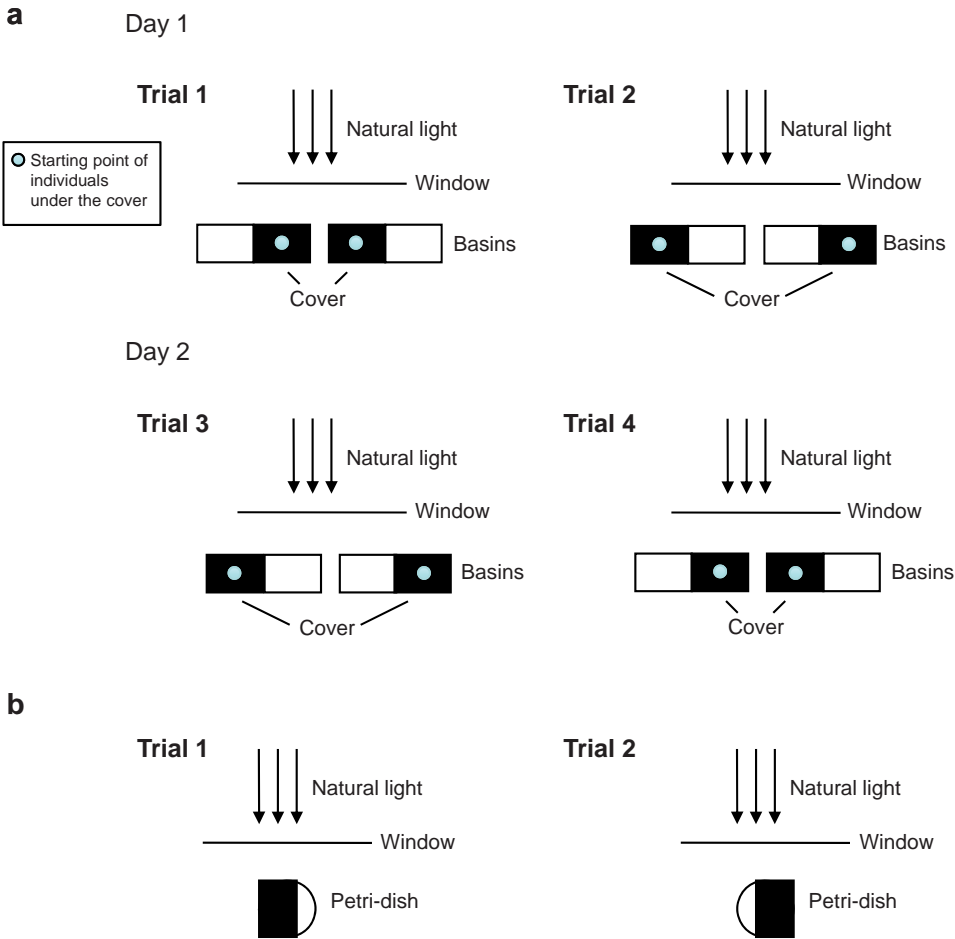


Figure 2:

Schemata of phototaxis experiments. a First and second phototaxis study.

The first phototaxis study with 100 *Elysia timida* was started by covering the inner sides of the basins and putting 50 individuals each in the middle of the dark covered side of the respective basin (indicated with a grey dot). Cover was changed after 3.5 h to the other side of the basin for the second trial. The next day the experiment was repeated with reversed sides. For the second phototaxis experiment with *E. timida* and *Thuridilla hopei*, the procedure of the first day of the first experiment was performed again in the same way.

b Phototaxis experiment with juvenile *E. timida*.

20 juvenile *E. timida* were put into one half of a petri-dish which was covered with black paper leaving only a gap of about 1cm for light incidence of natural light through a glass window. The cover was first put on the one side for the first trial, and then changed to the other side for the second trial.

half of the basins was shaded while the other half was illuminated by natural light through the window in the same angle. The first trial was started with covering the right half of the left basin and the left half of the right basin (Fig. 2 a). After 3.5 hours the cover was changed to the respective other side of the basin and observations were continued for another 3.5 hours. On the second day, the same procedure was

performed in the reversed way starting with covering the outer sides of the basins first, then changing after 3.5 hours. Thus, in total four trials were performed in two days. This experimental design was chosen in order to equalize any influence from different angles of light incidence or potential other influences from position conditions. The basins were covered at 11 a.m. at each observation day. Before starting the

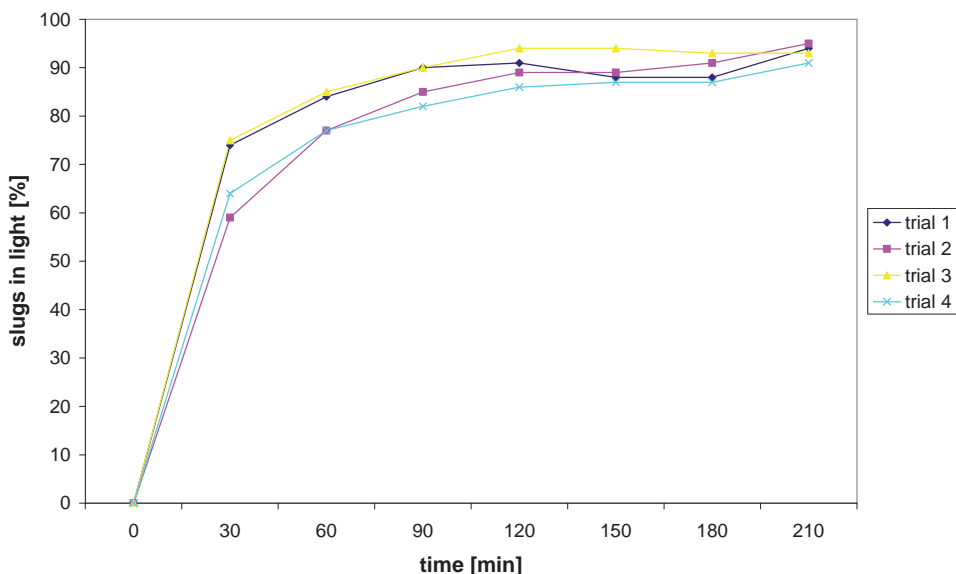


Figure 3:

Phototaxis in *Elysia timida*.

Two 160 x 60 cm basins were each covered half with black board and 50 *Elysia timida* individuals were placed under the cover on the dark side of each basin. Every 30 min. until 210 min. locations of specimens were recorded (trial 1). Then the cover was put to the other half of the basin and location recorded until after 210 min. (trial 2). Trial 3 and 4 were performed in the same way.

experiment, the 50 individuals were placed each in the middle of the shaded half of the basin. Starting with 30 minutes after the basins were covered, individuals that had crawled into the illuminated side were counted. The census was repeated every 30 minutes for 3.5 hours for each trial – in total 7 counts.

Second phototaxis study: Elysia timida – Thuridilla hopei

The same trial was performed another day to compare the phototactic behavior in *E. timida* and *T. hopei*. For this, 77 *E. timida* and 48 *T. hopei* were allocated into a group of 40 *E. timida* and 15 *T. hopei* in one basin and a group of 37 *E. timida* and 33 *T. hopei* in the other basin. The basins were covered with black board in the same way as in the first phototaxis trial and the trial was also started at 11 a.m. Again individuals were placed in the middle of the shaded half of the basin. Observation intervals were shortened to 15 minutes and the cover was changed to the other side already after 90 minutes in adaptation to the results of the first phototaxis trial, which had shown that the examination of the phototactic reaction is possible in a short observation period.

Third phototaxis study: juvenile Elysia timida

Six days after hatching of veliger larvae had started in the clutch, 20 juveniles which had turned into the crawling juvenile state were put into a small petri-dish and observed through a stereomicroscope. The petri-dish was covered on the sides and from upside with black paper so that only a small gap of approximately 1 cm was left open to natural light through a glass window (Fig. 2 b). In correspondence with the former phototaxis studies, the juveniles were put under the cover on the dark side and after 30 minutes it was counted how many individuals had moved to the light-exposed area. The cover was then changed to the other side without moving the petri-dish to repeat the trial in the reverse way. Again, the number of individuals which had moved into the light after 30 minutes was evaluated.

Studies on specialized photobehavior

Two trials were performed to analyze the correlation of the parapodial opening and the ground fluorescence: the first contained 25 adult specimens of *E. timida* together with 15 adult specimens of *T. hopei*, the second trial was performed with 50 individuals

of *E. timida* by measuring with a higher sensitivity of the PAM (see below). For the trials, individuals were kept in the basins separated individually in conform containers made out of transparent plastic bottles. Wholes were pierced equally into three rims of each bottle in distances of about 1 cm, permitting exchange of water from the running seawater (mean temperature during the hours of observation 21.5 ± 0.4 °C in July 2009 and 19.8 ± 0.1 °C in September 2010). Each container was stabilized with a stone, which also provided an opportunity for the slugs to hide underneath. Behavioral observations were performed along with PAM-measurements 4 times during an observation day during the time spans 9 a.m. – 12, 12 – 3 p.m., 3 p.m. – 6 p.m. and 6 p.m. – 9 p.m.. Opening level of the parapodial lobes was defined in the following 6 levels and documented in correlation of light intensity (measured in $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$):

- 0 – parapodia completely closed, the inside of the parapodia is totally covered, slug may be contracted
- 1 – parapodia are mainly closed with rims of both parapodia coming together over the body for the most part, but opened only a small part so that a little area of the dorsal body can be seen (Fig. 1a)
- 2 – parapodia are mainly opened, but still the rims of the opposing parapodia touch at least at one, often at two areas, the usual position while crawling (Fig. 1c)
- 3 – parapodia are opened, the rims of the opposing parapodia do not touch, but still the angle of the parapodia is more upward than sideward ($<45^\circ$), hence the insides of the parapodia are only partly exposed (Fig. 1d)
- 4 – parapodia are fully opened, the angle of the parapodia is more sideward than upward ($>45^\circ$), the rims of the parapodia are still either a little upward or undulated (in contrast to 5)
- 5 – parapodia are fully opened and absolutely outstretched and flat, angle is totally sideward (90°), the rims of the parapodia are smooth and fully expanded, sometimes even pointing downwards ($>90^\circ$)

In parallel, fluorescence was measured with the help of a PAM to examine the relation between opening level of parapodia and efficiency of exposure of the chloroplasts.

PAM-measurements

The maximum quantum yield of fluorescence for Photosystem II and ground fluorescence was measured with a Pulse Amplitude Modulated Fluorometer (Diving PAM, WALZ, Germany) during the experiments for the observation of specialized photobehavior. Measurements were performed 4 times per observation day (during the 4 time spans 9 a.m. – 12, 12 – 3 p.m., 3 p.m. – 6 p.m. and 6 p.m. – 9 p.m.). Animals were not dark acclimated before measurements in order to obtain the actual fluorescence with regard to actual light intensity and parapodia positions. The maximum quantum yield of fluorescence for PSII in ambient light can be defined as $(F_m' - F_0')/F_m'$ (Wägele and Johnsen, 2001; Jesus et al., 2010) and shows the photosynthetic activity in the actual light regime as a relative value. During measurement, the maximum fluorescence (F_m) is induced by a saturation light pulse triggered by the PAM. The ground fluorescence (F_0) measured directly before the saturation pulse reflects the actual fluorescence under the given light regime. Both values depend on quality and quantity of chloroplasts. But it has to be kept in mind that accurate estimations of fluorescence values may be difficult to obtain and are influenced by other factors (see Wägele and Johnson, 2001). Only two measurements after 6 p.m. in the second study were performed dark-acclimated for comparison.

The fibre optic was held above the animal with a distance of 1 cm in the region of the body part with the parapodia. Since the size of the measured animals was around 10 mm and the head has not to be included in the measurements, the sensor with a cross section of 5 mm covered the body area with the parapodia well.

The second study on the relation of parapodial opening was performed with increased sensitivity

of the PAM by putting the parameters 'outgain' and 'measure-int' from level 2 (default) to level 8 during the whole study.

Ambient light conditions were measured with the light sensor of the PAM.

Statistical analysis

Statistical analysis was performed using Excel and SPSS.

RESULTS

Phototaxis

The first four observational trials to investigate phototaxis in 100 *E. timida* individuals revealed a very distinct and fast phototactic reaction for *E. timida* (Fig. 3). In the first census, 30 minutes after the slugs had been put under the cover in the basin, the majority of individuals (ranging from 59-75% in the four trials) had already moved from the dark covered side of the basin into the light.

The slugs then stayed in the light-exposed areas while the remaining individuals from the dark followed subsequently. When the cover was changed to the other side of the basin, the same fast movement into the light was observed again. Repeating the trial with reversed sides in trial 3 and 4, the reaction was identical. After 3.5 hours of observation in each of the four trials, nearly all of the individuals (ranging from 91-95% in the four trials) were positioned in the light-exposed area of the basin. Only a small percentage did not enter the light side or moved back under the cover. Those individuals were found to be in the border area directly under the rim of the cover where a small amount of light was falling in.

As in this first phototaxis study it became obvious that the phototactic reaction is performed fast and can be examined in a short observation period, the time spans of the second phototaxis study were adapted and shortened to observation intervals of 15 minutes and an overall duration of 90 minutes per trial. In this second phototaxis study with the aim

to compare phototactic reactions in *E. timida* and *T. hopei*, phototactic behavior was also seen in *T. hopei* although it was obviously more pronounced in *E. timida* (Fig. 4 a and b).

While after 30 minutes the phototactic reaction of *E. timida* was similar as in the first phototaxis study (mean value of 63% in the two trials compared to 68% in the four trials of the first study), it was slightly lower in *T. hopei* with 50% of individuals counted on the light-exposed side. After 90 minutes, *E. timida* revealed again a comparable result to that in the first study with 81% of the individuals located on average in the light area compared to 86% in the first four trials. In *T. hopei*, however, the phototactic reaction was clearly less pronounced with only 59% of individuals positioned on the light side. Similar as in the first study with exclusively *E. timida*, also in this experiment remaining individuals of *E. timida* and *T. hopei* were found to be in the partly illuminated border area directly under the rim of the cover. Thus *T. hopei* showed a stronger tendency to prefer this border area with only a small amount of light falling in while *E. timida* showed a stronger tendency to prefer the area which was fully illuminated with moderate natural light.

Juvenile *E. timida*, which had reached a crawling state, but had no possibility yet to feed on *Acetabularia acetabulum*, also revealed a distinct phototactic behavior. In both trials with changing the cover from one side to the other like in the studies before, 90% and 95% (respectively) of the 20 juveniles had moved into light after 30 minutes which reflects a very fast and distinct phototactic reaction.

Specialized photobehavior

The individuals of *E. timida* varied their parapodial positions from a nearly closed condition to fully spread leaf-like positions ranging from parapodial opening level 1-5 (Fig. 5 a and b). A complete closure (level 0) was not observed during the trials, but during night and extreme light exposure

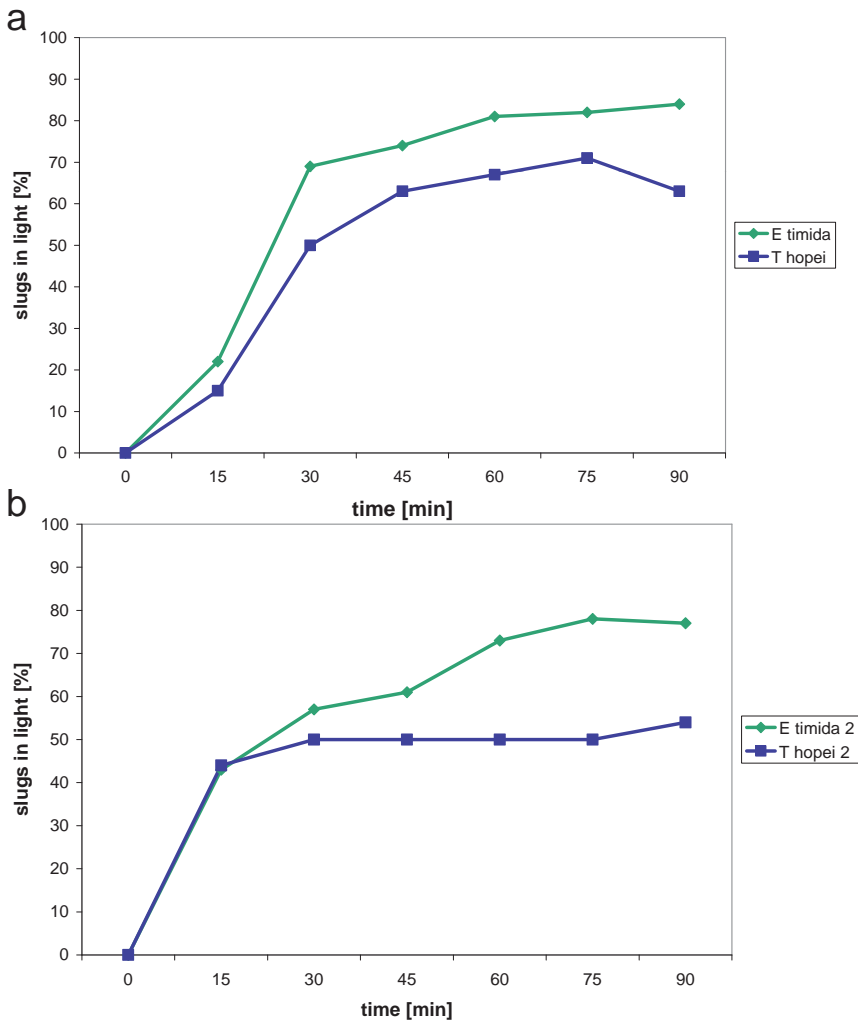


Figure 4:

Phototactic reaction in *Elysia timida* and *Thuridilla hopei*.

The experiments were performed with 77 *Elysia timida* and 48 *Thuridilla hopei* in two basins. a First trial with cover on the inner side. b Change of cover to the outer side (after 90 min). Observation intervals were shortened to 15 minutes and duration of one series was limited to 90 minutes.

(not figured here). *T. hopei*, however, did not show a higher parapodial opening level than 1 (Fig. 1a) during the observations irrespective of irradiance (Fig. 5 a). In the majority of cases (112 out of 120 observational cases), the parapodia were closed (level 0). To examine the ability to open the parapodia, *T. hopei* was also observed in dark conditions, where the slugs sometimes showed an opening level of 3 to 4. Additionally, opening was observed as a reaction to a tactile stimulus by carefully touching the slug's body.

E. timida revealed a tendency of broader exposure of the chloroplasts (parapodia opening levels 3-5) with higher light irradiances, but in the frame of the moderate lux values of the natural light spectrum (and in accordance the reduced photosynthetic active radiation PAR) through a window in the laboratory and the short momentous recordings of behavior, a clear significant correlation between current light intensity measurements and parapodial position in *E. timida* could not be inferred.

The momentary fluorescence values in the PAM-measurements (F_0'), however, increased in strong correspondence with increasing parapodial opening level of *E. timida* individuals which constituted a significant correlation ($p < 0.01$ in both of the studies, Spearman rank-order correlation test) (Fig. 6a and b). While with a low parapodial opening level of 1, the momentary fluorescence measured in *E. timida* was similar to that in *T. hopei*, the fluorescence values rose with every higher level of parapodial opening in *E. timida*, reflecting the higher exposure of the imbedded chloroplasts. In contrast, corresponding yield values, which represent relative values, stayed constant irrespective of parapodia position (Fig. 7a and b). This can probably be explained by the increasing measurable maximum fluorescence (F_m) when parapodia show a higher level of opening. No remarkable variances in the ground fluorescence were observed in the measured *T. hopei* individuals (Fig. 6a) and yield values were lower than in *E. timida* (Fig. 7a).

DISCUSSION

In our analyses of phototaxis we observed phototactic behavior in *E. timida* with long-term integration of functional chloroplasts as well as in *T. hopei* with short-term chloroplast integration. In the first phototaxis study with 100 individuals of *E. timida*, approximately all individuals had moved from the dark into the light-exposed area at the end of each of the four trials. The remaining individuals were located in the border area under the rim of the cover where some light was falling in. Thus it can be concluded that *E. timida* in general has an automatic strong and direct phototactic behavior. The second phototaxis study revealed phototactic behavior also in *T. hopei*, but the reaction was less pronounced than in *E. timida*. In comparison, individuals of *T. hopei* showed a stronger tendency to stay in the border area under the rim of the cover with only a slight light incidence or crawl back into this area while individuals of *E. timida* showed a stronger preference of the light-exposed area. With still the majority of slugs choosing the light-exposed area and

most remaining individuals staying in the border area with some light incidence, we consider *T. hopei* as a phototactic species, but with a gradual difference of stronger tendency to more shaded areas in contrast to *E. timida*. This corresponds to observations of localities in the sea when collecting the animals. While *E. timida* was found mainly on horizontal, light-exposed rocks, *T. hopei* was found mainly on vertical, half-shaded rocks, often even in little holes in the rock surface. Future experiments with regard to phototaxis may help to elucidate the distinct behavior concerning sensitivity in various light regimes.

Fraenkel (1927) wrote that he chose *Elysia viridis* for his observations on photomenotaxis out of many tested opisthobranch species as *E. viridis* showed the fastest and clearest reaction. Unfortunately he did not describe which other species exactly he compared and in which way. Weaver and Clark (1981) compared the three sacoglossan species *Elysia tuca*, *Elysia crispata* and *Costasiella lilianae* (= *Costasiella ocellifera* after Clark (1984)) with endosymbiotic chloroplasts and the two sacoglossan species *Oxynoe antillarum* and *Berthelinia carribea* without endosymbiotic chloroplasts concerning their photobehavior. They found that the symbiotic species oriented towards light while the aposymbiotic species avoided light. This indicates a possible correlation of chloroplasts' sequestration and phototaxis. The results of our phototaxis analyses correspond in so far that both investigated species are symbiotic and both show phototactic behavior. As furthermore the phototactic behavior was stronger in *E. timida* with long-term chloroplast retention as in *T. hopei* with short-term retention, the question arises, if species with long-term functional chloroplast retention reveal stronger evolutionary adaptations in relation to endosymbiotic chloroplasts. The phototactic behavior is more probably to be regarded as such an evolutionary adaptation, not as an immediate, direct influence of the chloroplasts on their host. The finding of our study that juvenile *E. timida* already revealed strong phototaxis before the first integration of chloroplasts from *A. acetabulum* supports this assumption.

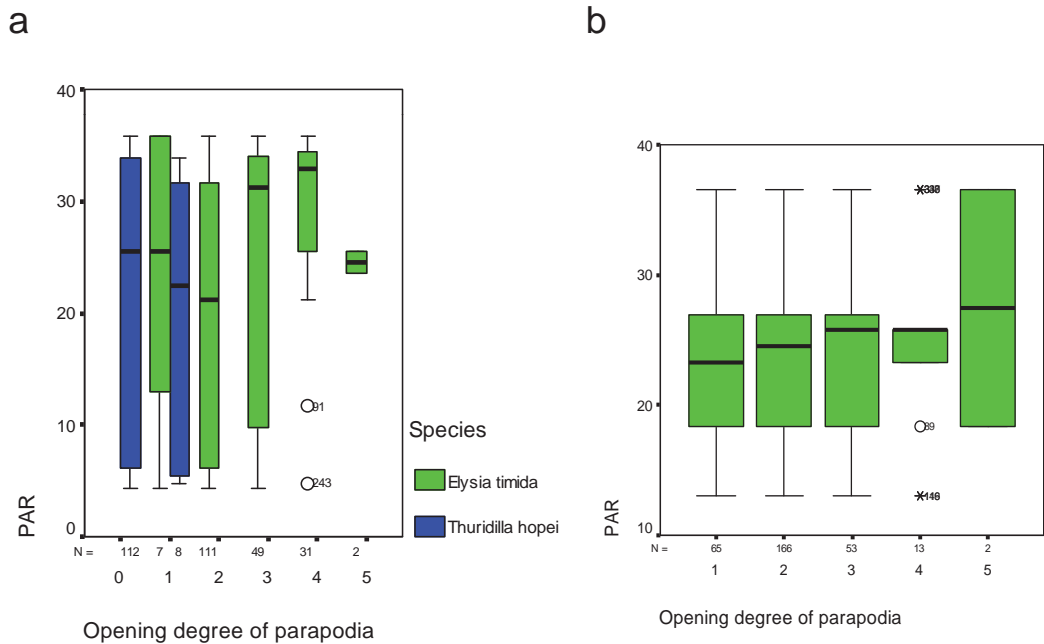


Figure 5:

Current irradiance [PAR: $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$] in relation to opening level of parapodial lobes.

a First trial with 25 *Elysia timida* and 15 *Thuridilla hopei* measured 4 times on 2 days respectively in July 2009. b Second trial with 50 *E. timida*, measured 4 times on 2 days, respectively, in September 2010. Due to seasonal effects, light incidence in the laboratory reached higher values in the measurements in July than in September. *T. hopei* was not observed to open the parapodia more than level 1 (only if touched) and therefore not included in the second analysis. N displays the number of incidences this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.

Importance of photosynthesis of the endosymbiotic chloroplasts as source of nutrients for *E. timida* was shown in experiments, in which *E. timida* was kept in the dark and thus deprived of the photosynthetic products of their chloroplast. These individuals had lower survival rates and stronger size decreases opposed to those kept in light (Giménez Casalduero and Muniain, 2008). The need of exposure to light for the function of the photosynthetic endosymbionts stands in conflict with potential dangers connected to exposure, e.g. bigger vulnerability through greater exposure to predators, waves and currents and especially damage of photosynthetic endosymbionts through exposure to irradiances higher than a well tolerated maximum (Monselise and Rahat, 1980). The predator problem can be reduced by mechanisms like producing toxic or irritating secretions and cryptic

colorations in sacoglossan sea slugs (Cimino and Ghiselin, 1998; Marin and Ros, 2004), even if not fully eliminated. The potential damage of photosynthetic functions through extreme light intensities still poses a difficult problem (Jesus et al., 2010). It seems evident that *E. timida* has evolved an efficient protection mechanism against this photodamage problem with the specialized photobehavior. By closing the parapodia, *E. timida* can react directly to threatening light intensities and form a natural protection shield for the embedded chloroplast in the inside of the parapodia. This mechanism enables *E. timida* to be located permanently in shallow light-exposed areas and adapt to current light irradiances. Opening of the parapodia exposes the chloroplasts to higher irradiation, whereas the closure reduces light penetration. This specialized photobehavior of

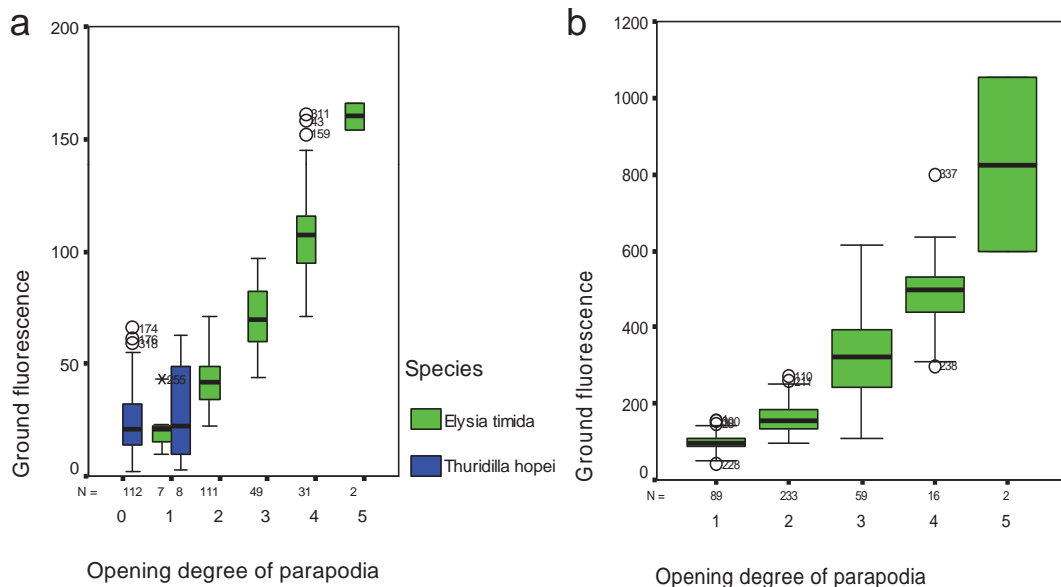


Figure 6:

Ground fluorescence (F_0') in relation to opening level of parapodial lobes.

a First trial with 25 *Elysia timida* and 15 *Thuridilla hopei* measured 4 times on 2 days respectively. b Second trial with 50 *E. timida*, measured 4 times on 2 days, respectively; PAM-settings were increased to high sensitivity (consequently values of momentary fluorescence are higher). N displays the number of times this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.

E. timida first described by Rahat and Monselise (1979) could be confirmed as a general mechanism by our observations and analyzed in more detail. In our experiments, we used the emission of the fluorescence through the parapodia as a factor to indirectly measure the exposure of the chloroplasts. The closure of the parapodia unambiguously shows that less light penetrates the parapodia and therefore protects the underlying chloroplasts of higher irradiances. With increasing parapodial opening level the momentary ground fluorescence values (F_0') in individuals of *E. timida* increase in strong correspondence, which constituted a significant correlation in our measurements. This reflects the efficiency of the behavior to expose the inlaying chloroplasts to light by opening the parapodia and thus enhancing photosynthetic activity in the integrated chloroplasts.

We assume that the maximum fluorescence (F_m') rises also with higher parapodial opening levels, which equalizes the higher values of ground fluorescence. As the overall effective yield value of photosynthetic activity is calculated from $(F_m' - F_0')/F_m'$, the effective yield therefore stayed relatively constant with the varying parapodial opening levels.

Concerning the specialized photobehavior of *E. timida* with light-adapted changing of the position of the parapodial lobes, the examined behavioral reactions were very different in the two compared species. The light-adapted gradual opening of the parapodia as in *E. timida* is apparently not present in *T. hopei*. Although *T. hopei* individuals were observed to actively open their parapodia in reaction to touch or sometimes in darkness, they did not open them wider

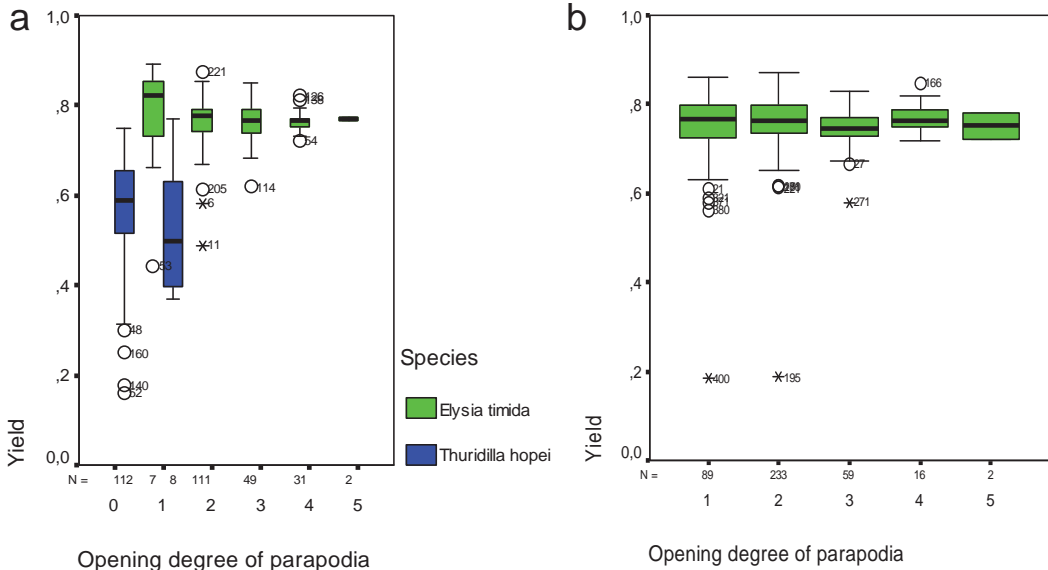


Figure 7:

Yield ($F_m' - F_0/F_m'$) in relation to opening level of parapodial lobes.

a) First trial with 25 *Elysia timida* and 15 *Thuridilla hopei* measured 4 times on 2 days respectively. b) Second trial with 50 *E. timida*, measured 4 times on 2 days, respectively; PAM-settings were increased to high sensitivity. N displays the number of times this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.

than level 1 in the moderate natural light conditions in the laboratory. The special photobehavior of *E. timida* is also related to the characteristic structure of integrating the chloroplasts into the body. In *E. timida*, the embedded chloroplasts can well be seen as a green area covering the inside of the parapodia while the outsides of the parapodia and the rest of the body are full of white pigment with only another small green stripe on the lower sides of the slug. In contrast *T. hopei*, which exhibits a similar arrangement of branched digestive gland and incorporated chloroplasts, seems to prevent photosynthesis of chloroplasts by shading them permanently with the help of the parapodia. Additionally, the rather dark body coloration may enhance this shielding of sunlight.

E. timida revealed a tendency of increasing exposure of the chloroplasts with higher light irradiances, but in the frame of the moderate lux

values of natural light through a window in the laboratory and the short momentous recordings of behavior, a clear significant correlation between current light intensity measurements and parapodial position in *E. timida* could not be inferred. The parapodial position is always connected to the current active state of the individual. Individuals usually start to open their parapodia to higher parapodial opening levels only while sitting in one position for a while. The opening level 2, which was observed in the majority of cases in both experiments, is the characteristic position while crawling. Thus more observations are necessary for detailed results on the relation between light conditions and behavior.

It is not explained so far how exactly the specialized photobehavior of *E. timida* functions. In general, the slug's behavior is in discrepancy anyway: When it exposes itself to higher irradiances, then chloroplasts

suffer from photodamage and can not be repaired, due to lack of genomic equipment (Wägele et al., 2010). When it hides from sunlight, photosynthesis is reduced and contribution to live maintenance is probably minor. Jesus et al. (2010) described that *E. timida* is capable of combining the behavioral photo-regulation mechanism (opening/closing the parapodia) with a functional physiological photo-regulation mechanism (xanthophyll cycle) increasing their photo-regulation capacity as a mechanism to keep their maximum photosynthetic capacity for longer periods. The exact mechanisms of the specialized photobehavior in *E. timida*, however, remain unclear. According to our observations until now, this specialized photobehavior is rather specific for *E. timida*. It represents a highly specialized evolutionary adaptation in relation to long-term retention of chloroplasts with efficient exposure of endosymbiotic chloroplast for high photosynthetic benefit as well as efficient protection of endosymbiotic chloroplasts from photo-damage, enabling functionality of chloroplast endosymbiosis in *E. timida* for one of the most extended durations known so far.

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REFERENCES

- Evertsen J, Burghardt I, Johnsen G, Wägele H (2007). Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. *Marine Biology*, 151: 2159-2166.
- Cimino G, Ghiselin MT (1998). Chemical defence and evolution in Sacoglossa (Mollusca: Gastropoda: Opisthobranchia). *Chemoecology*, 8: 51-60.
- Clark KB (1984). New records and synonymies of Bermuda Opisthobranchs (Gastropoda). *The Nautilus*, 98: 85-97.
- Fraenkel G (1927). Über Photomenotaxis bei *Elysia viridis* Mont.. *Zeitschrift für vergleichende Physiologie*, 6: 385-401.
- Giménez Casalduero F, Muniain C (2008). The role of kleptoplasts in the survival rates of *Elysia timida* (Risso, 1818): (Sacoglossa: Opisthobranchia) during periods of food shortage. *Journal of Experimental Marine Biology and Ecology*, 357: 181-187.
- Händeler K, Grzybowski Y, Krug PJ, Wägele H (2009). Functional chloroplasts in metazoan cells – a unique evolutionary strategy in animal life. *Frontiers in Zoology*, 6: 28.
- Jesus B, Ventura P, Calado G (2010). Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanism in the solar-powered sea slug *Elysia timida* (Risso 1818). *Journal of Experimental Marine Biology and Ecology*, 395: 98-105.
- Marin A, Ros J (1989). The chloroplast-animal association in four Iberian sacoglossan opisthobranchs: *Elysia timida*, *Elysia translucens*, *Thuridilla hopei* and *Bosellia mimetica*. *Scientia Marina*, 53: 429-440.
- Marin A, Ros J (1992). Dynamics of a plant-herbivore relationship: the photosynthetic ascoglossan *Elysia timida* and the chlorophycean *Acetabularia acetabulum*. *Marine Biology*, 112: 667-682.
- Marin A, Ros J (1993). Ultrastructural and ecological aspects of the development of chloroplast retention in the sacoglossan gastropod *Elysia timida*. *Journal of Molluscan Studies*, 59: 95-104.
- Marin A, Ros J (2004). Chemical defenses in Sacoglossan Opisthobranchs: Taxonomic trends and evolutive implications. *Scientia Marina*, 68: 227-241.
- Monselise EBI, Rahat M (1980). Photobiology of *Elysia timida* (Mollusca: Opisthobranchia): observations in the sea. *Israel Journal of Zoology*, 29: 125-128.
- Rahat M, Monselise EBI (1979). Photobiology of the chloroplast-hosting mollusc *Elysia timida* (Opisthobranchia). *Journal of Experimental Biology*, 79: 225-233.
- Wägele H, Deutsch O, Händeler K, Martin R, Schmitt V, Christa G, Pinzger B, Gould SB, Dagan T, Klussmann-Kolb A, Martin W (2010). Transcriptomic evidence that

longevity of acquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobranthus ocellatus* does not entail lateral transfer of algal nuclear genes. *Molecular Biology and Evolution*, 28: 699-706.

Wägele H, Johnsen G (2001). Observations on the histology and photosynthetic performance of “solar-powered” opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. *Organisms Diversity and Evolution*, 1: 193-210.

Weaver S, Clark KB (1981). Light intensity and color preferences in five ascoglossan (=sacoglossan) molluscs (Gastropoda, Opisthobranchia): A comparison of chloroplast symbiotic and aposymbiotic species. *Marine and Freshwater Behavior and Physiology*, 7: 297-306.