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Comparative morpho-physiological analysis between *Ciona robusta* and *Ciona savignyi*

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ABSTRACT

The seasquirt *Ciona robusta* and its co-generic *Ciona savignyi*, are two long-divergent species, sharing the same habitat and competing for the same spaces and resources. Their very similar morphology has been responsible for the misidentification of the two organisms, and, consequently, for the underestimation of their geographic distributions and new areas of co-occurrence. In spite of the large amount of knowledge built up by developmental biologists, few data are available regarding the morpho-physiology of the two sea squirts. The comparison of morphological and physiological features carried out in the present study (i.e. length-body weight correlation, tunic/organ ratio, tissue-specific retained water and metabolic rate), highlighted slight, but significant, differences strongly supporting the hypothesis of different ecological strategies for the two species. More precisely, *C. savignyi* invests more energy in growing faster and taller, likely to improve the quality and concentration of the filtered food, and sustains a faster metabolic/growth rate. Conversely, *C. robusta* invests more into tunic thickness, reducing the risk of predation, even if this likely means a slower metabolic/growth rate. In addition it was noted that in both species, the tunic absorbs more water than the other tissues, a peculiarity that may allow them to better counteract sudden fluctuations in environmental salinity.

1. Introduction

In the last decade, different research groups have shown that the species *Ciona intestinalis* is actually a complex of genetically differentiated types (Caputi et al., 2007; Iannelli et al., 2007; Nydam and Harrison, 2010; Suzuki et al., 2005; Zhan et al., 2010). Very recently (Brunetti et al., 2015), the "types" formerly denoted as A and B, were ascribed respectively to two distinct species: *Ciona robusta* Hoshino and Tokioka, 1967 and *C. intestinalis* (Linnaeus, 1767). On the basis of the different morphological features observed at the larval stage, Pennati et al. (2015) confirmed the new classification. Due to their very close morphological features, *C. robusta*, *C. intestinalis*, and *Ciona savignyi* Herdman, 1882 were wrongly considered for a long time to be the same species (Hoshino and Nishikawa, 1985; Lambert and Lambert, 1998; Smith et al., 2010), in spite of the fact that *C. robusta* and *C. intestinalis* were estimated to diverge approximately 20 Mya (Suzuki et al., 2005), while *C. robusta* and *C. savignyi* were estimated to diverge 180 Mya (Berná et al., 2009).

Attention was focused on the comparison of *C. robusta vs. C. savignyi*, since numerous reports stressed that the two species show overlapping distribution areas, competing for space and resources: southern California (Byrd and Lambert, 2000; Lambert and Lambert, 2003), New Zealand (Smith et al., 2010), the Korean peninsula (Taekjun and Sook, 2014), and Tokyo Bay (M. Yoshikuni, personal communication). Moreover, along the California coast, *C. savignyi* has been reported to replace *C. robusta* (Lambert, 2007; Lambert and Lambert, 1998; Lambert and Lambert, 2003).

Despite the interest of the scientific community towards sea squirts, few data are available about the physiology of these organisms. Early studies examined oxygen consumption in *C. intestinalis* embryos and adults (Holter and Zeuthen, 1944; Jørgensen, 1952). The measurements performed by Jørgensen were carried out according to the Winkler methodology, estimating a consumption rate of about 0.8 ml O_2/h. Markus and Lambert (1983), although corroborating the results of Jørgensen by using the same methodology (0.82 ml O_2/g dry weight/h), stated that former data "were reported as relative values and not as weight-specific rates". It should be noted that, while Markus and Lambert measured oxygen consumption on animals sampled in California, thus almost surely belonging to the new classified species *C. robusta* (*ex C. intestinalis* type A), the specimens used by Jørgensen were collected from Woods Hole (Massachusetts), thus most probably *C. intestinalis* (*ex* "type B"). As far as we know, no metabolic studies are available in the current literature for *C. savignyi*. Thus, in this study, morpho-

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physiological parameters, i.e. body size, tunic/organ dry weight ratio, tissue water retention, as well as the routine oxygen consumption rate as proxy for routine metabolic rate, were measured for adults of *C. savignyi* and compared to those of *C. robusta*.

2. Materials and methods

2.1. Specimens

The seasquirt *Ciona robusta* was provided by Kyoto University (Kyoto, Japan). The adult individuals were obtained from *in vitro* fertilization of wild gametes, which after settlement on Petri dishes, were reared in the field in Maizuru Bay by Maizuru Fisheries Research Station (Nagahama, Maizuru-shi, Kyoto, Japan). The co-generic *Ciona savignyi* was collected in two different areas: Tokyo Bay, by Tokyo University (Tokyo, Japan), and Sugashima Bay, by Nagoya University (Nagoya, Japan).

Individuals were classified on the basis of their morphological characters according to Smith et al. (2010) and Sato et al. (2012). The specimens reared in Maizuru Bay were identified as *C. robusta* since adults showed the siphon tubercles, as well as the peculiar pigmentation of the tip of the vas deferens. The specimens collected in Tokyo Bay were identified as *C. savignyi*, because of the lack of pigmentation on the vas deferens and the presence of marked orange pigmentation of the inhalant siphon.

The animals were shipped to Kyushu University Fishery Research Laboratory (Fukutsu, Fukuoka, Japan) with minimal temperature rise during transportation and transferred to 50-l aquaria with running filtered seawater and continuous aeration. In the case of the reared *C. robusta*, the animals were manually removed from the Petri dishes. Individuals attached to each other were separated, and epibionts on the tunic surface were removed by tweezers. Water temperature (ranging from 16.5 °C to 18.0 °C) and salinity (on average 33.5 ppt) were checked twice a week. The animals were supplied once a day in the early morning with 10 ml of commercial algae mix (Shellfish Diet 1800, Reed Mariculture Inc., USA) and 5 ml of a 50×10^6 cells/ml commercial solution of *Chaetoceros calcitrans* (Higashimaru-marinetech PLC, Japan). During feeding, the water flow was stopped for 3 to 4 h to allow the animals to filter enough algae. The tanks were siphoned every two days and checked for dead individuals. After 6 days of acclimation to the laboratory conditions specimens were moved into an experimental tank. Temperature was maintained at 17 °C by a cooling/heating system, and animals were left to fast for 48 h prior to the experiments. The water in the experimental tanks was completely replaced weekly. All experimental procedures were approved by a Kyushu University committee and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Japan.

2.2. Respirometry & morphometric measurements

The semi-closed method of Yagi and Oikawa (2014) was used to determine the effective oxygen rate consumption, as a proxy for basal metabolic rate (MR), in both species. Rate of oxygen consumption in resting condition was measured using oxygen electrode probes. To control for background respiration, a bottle that received water flowing out of the respiration chamber was used as a blank chamber. The bottle was sealed at the beginning of the oxygen consumption experiment, placed in the water-bath of the respiration chamber during the experiment, and the oxygen concentration determined at the end of the experiment (C_0). Specimens were introduced into the respiration chambers (250 ml each) and left undisturbed to acclimate to the new conditions under a constant air-saturated water flow from 1 h up to 1.5 h after their siphons opened and extended. The chambers were then positioned in closed flow tanks with fine-controlled temperature (T = 17 °C). Closing time ranged from 45 min up to 3 h, depending on the size of the animals. Upon opening, two volumes of 50 ml were sampled from the respiration chamber and the oxygen concentration was determined via a DO electrode (C_1 and C_2). The oxygen consumption was calculated as:

$$O_2cons = \left[C_0 - \left(\frac{C_1 + C_2}{2}\right)\right] \times \text{chamber volume}$$

Values of $(C_1 - C_2) \geq 0.1$ ppm were discarded.

The final data set consisted of 58 measurements for *C. robusta* and 44 for *C. savignyi*. After the respirometry experiments, the total length at maximum extension (BL) of each individual was measured. The tunic and the internal organs were dissected, rinsed in distilled water, drained with absorbent paper, and weighed separately to determine the wet weight (wW). Then all tissues were dried in a drying oven for at least 48 h at 60 °C to determine the dry weight (dW). The water retention of the separated tissues, as well as whole body water retention of the two species, were calculated as the percentage of wW to which the total dW has been subtracted (i.e. the evaporated water during the drying period).

The mass specific MR was calculated as follows:

$$\text{Mass specific MR} = \left[\frac{O_2cons}{\text{whole body wW (or dW)}}\right] \div \text{Closing time}$$

2.3. Statistical analysis

2.3.1. Morphometrics

To compare the interspecific morphological relationships between *C. robusta* and *C. savignyi*, known allometric equation models were applied. In particular, regarding the relationship of whole body wW (or dW) *vs.* BL, we referred to the power equation $Y = aX^b$ proposed by Carver et al. (2003) for *C. intestinalis*, where $Y =$ whole body wW (or dW), $X =$ BL. The equations were log-log linearized and fitted with the least squares method. Each distribution was checked for the normality of residuals (Shapiro-Wilk test, $\alpha < 0.01$). The F-test was used to evaluate the best fit model: *i*) the model in which one curve fits all data sets (p-value > 0.05), or *ii*) the model in which each species is fitted by a different curve (p-value < 0.05).

Regarding the tunic wW (or dW) *vs.* organ wW (or dW) relationship, it has been shown to be linear (Carver et al., 2006), following the equation $Y = a + bX$, where $Y =$ weight of the tunic and $X =$ weight of the organs. The r^2 and p-value were also calculated ($\alpha < 0.05$). Each distribution was checked for the normality of residuals and the F-test was used to evaluate the best-fit model.

Regarding the tunic/organ ratio (expressed as grams of tunic divided by grams of organs), the statistical significance of the differences were assessed by the Mann-Whitney test. When multiple comparisons were performed, the Bonferroni correction for multiple tests was applied.

2.3.2. Water retention

The amount of retained water in the tissues was calculated as a fraction of the total wW (p). According to Bartlett (1947), for n number of observations near the higher limit, i.e. p near to one, the values were transformed by applying $Y = 2\sin^{-1}\sqrt{p - \frac{1}{2n}}$. The differences were assessed by the Mann-Whitney test.

2.3.3. Metabolic rate

The relationship between body mass and MR has been massively studied (see Agutter and Wheatley, 2004 for a review), and is known to follow the equation $Y = aX^b$, where $Y = MR$, and $X =$ body mass (whole body wW, or dW regarding the data here analyzed). As already seen above, the obtained equations were log-log linearized, and fitted with the least squares method. Each distribution was checked for the