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# *Ciona intestinalis* as an emerging model organism: its regeneration under controlled conditions and methodology for egg dechorionation*

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**Abstract:** The ascidian *Ciona intestinalis* is a model organism of developmental and evolutionary biology and may provide crucial clues concerning two fundamental matters, namely, how chordates originated from the putative deuterostome ancestor and how advanced chordates originated from the simplest chordates. In this paper, a whole-life-span culture of *C. intestinalis* was conducted. Fed with the diet combination of dry *Spirulina*, egg yolk, *Dicrateria* sp., edible yeast and weaning diet for shrimp, *C. intestinalis* grew up to average 59 mm and matured after 60 d cultivation. This culture process could be repeated using the artificially cultured mature ascidians as material. When the fertilized eggs were maintained under 10, 15, 20, 25 °C, they hatched within 30 h, 22 h, 16 h and 12 h 50 min respectively experiencing cleavage, blastulation, gastrulation, neurulation, tailbud stage and tadpole stage. The tadpole larvae were characterized as typical but simplified chordates because of their dorsal nerve cord, notochord and primordial brain. After 8~24 h freely swimming, the tadpole larvae settled on the substrates and metamorphosized within 1~2 d into filter feeding sessile juvenile ascidians. In addition, unfertilized eggs were successfully dechorionated in filtered seawater containing 1% Tripsin, 0.25% EDTA at pH of 10.5 within 40 min. After fertilization, the dechorionated eggs developed well and hatched at normal hatching rate. In conclusion, this paper presented feasible methodology for rearing the tadpole larvae of *C. intestinalis* into sexual maturity under controlled conditions and detailed observations on the embryogenesis of the laboratory cultured ascidians, which will facilitate developmental and genetic research using this model system.

**Key words:** *Ciona intestinalis*, Model organism, Laboratory culture, Embryogenesis
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## INTRODUCTION

*Ciona intestinalis* (subphylum Urochordata, class Ascidiacea, family Ciona) is hermaphrodite, sessile and filter-feeding marine solitary invertebrate. Its tadpole larvae possess a prototypical chordate body plan that includes a dorsal neural tube, an axial notochord flanked by muscle cells, and a ventral endodermal strand (Satoh, 1994). As a cosmopolitan species, they are ubiquitous throughout the world and also scatter along the Chinese coast (Zheng, 1995). *C. intestinalis* was used as a popular model for embryo biology and was also one of the animals which were first introduced for cell lineage research (Conklin, 1905; Satoh, 1994; Xiang, 2003); In the 1940s, Morgan (1940; 1945) studied self and cross fertilization of *C. intestinalis*; Tung *et al*.(1977) investigated the interaction between oocyte plasm and nucleus

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taking ascidians as material. In the last decades, researchers showed a surge of interest in ascidians because of their crucial evolutionary position and representative early developmental mode (Satoh and Jeffery, 1995; Simmen *et al*., 1995; Di Gregorio and Levine, 1998; Corbo *et al*., 2001; Dehal *et al*., 2002; Nishida, 2002). The whole genome of *C. intestinalis* has now been sequenced, analyzed and annotated (Sordino *et al*., 2001; Dehal *et al*., 2002; Satoh *et al*., 2003); Hundreds of thousands of ESTs (expressed sequence tags) were disclosed; a great number of development involved genes were cloned and hundreds of gene expression patterns were published (Nishikata *et al*., 2001; Satou *et al*., 2001a; 2002; Kusakabe *et al*., 2002; Inaba *et al*., 2002; Fujiwara *et al*., 2002; Shida *et al*., 2003; Satoh *et al*., 2003). As a potential model system of developmental and evolutionary biology, *C. intestinalis* gained so much attention that it was regarded as a "biology's rising star" (Pennisi, 2002).

However, it is not always very convenient to obtain *C. intestinalis* as experimental materials because their sources vary among seasons and regions. Almost all the biological materials of the published articles were collected from the seaside (Corbo *et al*., 1997; Dehal *et al*., 2002; Inaba *et al*., 2002; Tsuda *et al*., 2003; Awazu *et al*., 2004; Kawai *et al*., 2005). On the other hand, there are always requirements on the population in regard to some special experimental designs, for example genetic mutation, inbred strain construction, micromanipulation and so on. With the rapid increasing interest in ascidian research, it is necessary to establish a laboratory culture mode as in the case of other model organisms, such as fruit fly, *Caenorhabditis elegans*, zebra fish and mouse. Therefore, it is essential to carry out the whole life span laboratory culture of *C. intestinalis* under controlled conditions (Kano *et al*., 2001; Sordino *et al*., 2001). Up to date, there are no informative references on this. This paper presents methodology for artificial propagation, removal of the egg chorion and laboratory cultivation of *C. intestinalis*. In addition, some new observations on the development of the laboratory cultured eggs and dechorionated eggs together with some details of the biological characteristics of the juvenile ascidians were also provided in order to better exploit this emerging model organism.

## MATERIALS AND METHODS

### Source of experimental animals

Healthy adult ascidians, more than 6 cm in height, with tunica semitransparent and integral in appearance and both incurrent siphon and outcurrent siphon naturally spread, were collected from Jiaozhou Bay and maintained in seawater aquariums with constant illumination at 18 °C. They were fed with microalgae and egg yolk till sexual maturity. The eggs were fertilized and hatched in filtered seawater at 20 °C. Several batches of hundreds of hatched tadpole larvae were settled on plastic dishes for cultivation purpose.

### Laboratory culture method

1. Food

Dried powder of several microalgae species, namely *Spirulina* sp., fresh *Nitzschia* sp., *Chlorella* sp., *Dicrateria* sp., and egg yolk, nauplius of brine shrimp, a commercial micro pellet diet (Seafood™, Qingdao, China), a commercial weaning food for shrimp (HighSciTech™, Qingdao, China), soybean milk, egg yolk, edible yeast were selected as food for the juvenile ascidians. Among them, *Nitzschia* sp., *Chlorella* sp., *Dicrateria* sp., and soybean milk were fed directly. The dried powder of *Spirulina* sp. and edible yeast were rehydrated for 30 min before feeding. The solid diets were triturated to make the particles less than 50 μm.

2. Culture management

Different groups of juvenile ascidians were fed using the above diets with adjustments according to the fullness in the intestines. Food efficiency was evaluated and the diet combination was optimized based on the surviving rate and growth rate resulting from different diet. The ascidians were cultured at 15~18 °C with the optimized diet combinations as final food. When the juvenile ascidians grew up to about 1 cm, they were maintained in 200 L aquariums at a density of not more than 500 individuals/m$^3$. The diet remnants were removed by plastic siphons and half of the seawater was changed every day. After a 60 d culture, the ascidians reached sexual maturity.

3. Collection and culture of fertilized eggs from the artificially cultivated ascidians

Mature artificially cultured ascidians with eggs and sperm visible in the gonoducts were used. The

animals were dissected to expose gonoducts. Excess seawater and body fluid were removed with tissue paper. After their oviduct was cut open by sharp scissors, their eggs were collected by a Pasteur pipette and transferred immediately into a petri dish containing filtered seawater. The sperm duct was cut and sperms (from all individuals) were collected using a Pasteur pipette in a 1.5 ml micro centrifuge tube and stored at 4 °C. Only sperm, but not seawater or body fluid were collected as neatly as possible. With all things prepared, the sperms suspension was diluted with into about 1 ml (depending on the original volume of sperm suspension) filtered and sterile seawater. One to two drops of diluted sperm suspension were added into a petri dish containing eggs. The dish was allowed to stand for 10 min to complete fertilization and then the eggs were washed three times to prevent polyspermy. The fertilized eggs were cultured at 10, 15, 20, 25 °C respectively with 3 replicates. The time for different development stages and their different hatching rates at different temperature were recorded.

4. Removal of the follicle cells and test cells

Mature eggs are covered with follicle cells in the outer layer, and test cells in the inner layer, between them are the chorions. These two layers of cells should be removed for better observations. Unfertilized eggs were repeatedly pipetted to remove follicle cells. After two times of washing, the eggs were transferred to a 15 ml centrifuge tube containing 10 ml of dechorionation solution of filtered seawater containing 10 ml of 1% Tripsin (Sigma) and 0.25% EDTA (Gibco) at pH of 10.5, and incubated on a shaker at 25 °C. Normally, both the chorion and test cells would be removed within 40 min. Once the eggs were dechorionated, they were collected swiftly into petri dishes, with floor coated with 1% agarose, and washed several times with filtered seawater. After that, the dechorionated eggs were fertilized and cultured at 20 °C.

5. Embryogenesis observation and biological traits of juvenile ascidians

The eggs from the artificially cultured ascidians and the dechorionated eggs were fertilized with the methods mentioned above. The embryo development process and juvenile ascidians were observed and recorded using ZEISS Axioplan microscope and Kodak MDS digital camera.

6. Data analysis

Data were documented as mean±standard error. *T*-test was applied to verify the resulting differences.

## RESULTS AND DISCUSSION

### Effect of diets on growth performance

Our priliminary test, revealed that soybean milk, nauplius and micropellet diet were not fit food for *C. intestinalis* because of shortcomings such as bad growth performance, trouble in preparation and difficulty for storage. If we fed *C. intestinalis* with egg yolk, weaning diet of only crustacea and yeast, the ascidians grew slowly at low surviving rate (data not shown). Feeding the *C. intestinalis* larvae with *Spirulina* powder, fresh *Nitzschia* sp., *Chlorella* sp., *Dicrateria* sp., could meet their need for growth in the early stage (body height less than 1 cm), but resulted great growth diversity and increasing mortality rate in the late culture period. At last, we optimized a diet combination of *Dicrateria* sp., dry *Spirulina* powder, egg yolk, weaning diet for shrimp, edible yeast, which yielded best results (Fig.1). After 45 d culture with the optimized diet, white sperms appeared in the spermaduct; after 50 d, orange eggs were found in the oviduct. In about 2 months, the ascidians reached average body height of 59 mm, survival rate rose to 85%, and most of them matured (Fig.1).

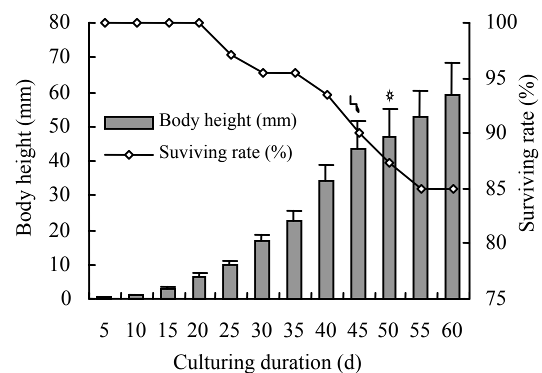


**Fig.1 Growth performance and surviving rate of laboratory cultured *Ciona intestinalis***
↳: Sperm emerged; ⁎: Oocyte emerged

### Developmental timetable and hatching rate at different temperatures

Water temperature has obvious effect on the developmental process (Fig.2). At 10, 15, 20, 25 °C, it

required 3 h 10 min, 1 h 20 min, 50 min, 40 min for fertilized eggs to become two cells, and 30 h, 22 h, 16 h, 12 h 50 min to become tadpole larvae, respectively. At 10 °C, fertilized eggs cleaved one time at approximately an hour, while at 20 °C, it required 20~30 min. At 10 °C, eggs could be normally fertilized but the hatching rate was obvious lower than that at 15, 20 and 25 °C ($P<0.01$) (Fig.3). In the aquariums, matured ascidians could spawn at 12 °C, but the hatching rate was high (average 93%) only at water temperature of 15~25 °C (Fig.3).

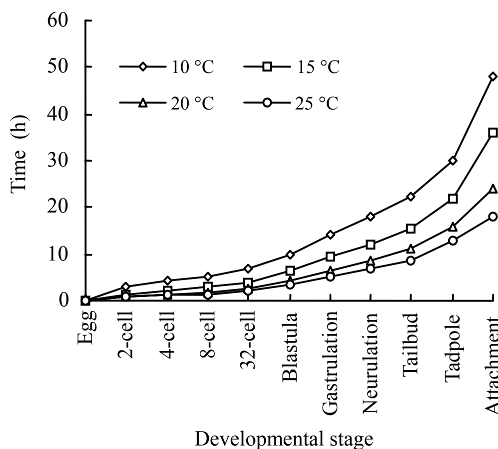


**Fig.2 Timetable of embryogenesis of *Ciona intestinalis* at different culture water temperature**

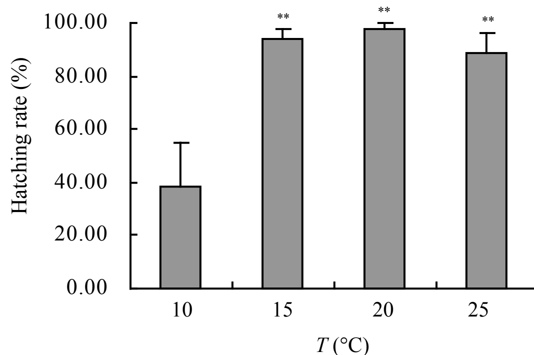


**Fig.3 Effect of water temperature on the hatching rate of *Ciona intestinalis* embryos**
$^{**}P<0.01$

## Embryogenesis and characteristics of tadpole larvae

The eggs of *C. intestinalis* were floating. The first two blastomeres, destined to form the two sides of the tadpole larvae, were equal in size. The eggs ooplasm rearranged within 10 min post fertilization. The fertilized eggs developed underwent egg cleavage, blastula, gastrula, neurula stage, tailbud stage and then hatched as tadpole larvae (Figs.4a~4i).

The 0.7 mm long tadpole larvae were composed of head and tail (Fig.4j). The head had a cerebral ganglion with black ocellus and black otolith; the tail contained notochord, muscles and a hollow dorsal neural tube connecting with the anterior cerebral ganglion. Tunica was formed in the tadpole larva stage and enwrapped the larva. The tadpole larvae were phototactic and could swim for a short distance by intermittent swinging of the tail. During this period, the larva did not feed. The tadpole larvae then attached to substrates by the attachment villi. In the following days, notochord and muscles of the tail disappeared, neural tube and sensory organs transformed into cerebral ganglion; With the appearance of the incurrent and outcurrent siphons, the freely swimming tadpole turned into filter feeding and sessile juveniles.

After dechorionation, the fertilized eggs could develop into tadpole larvae (Fig.5). The dechorionated eggs were sinkable and developed at the same speed at that of the normal fertilized eggs (Mita-Miyazawa *et al*., 1985), while the hatching rate decreased and a few embryos developed abnormally, especially after neurulation.

## Observations on the juvenile ascidians

Microscopic observations showed that after metamorphosis, some adult organs such as incurrent siphon, outcurrent siphon, branchial sac, intestine, heart appeared, while other advanced characteristics of the tadpole such as notochord and dorsal neural tube disappeared, with only neural complex left positioned between the incurrent siphon and outcurrent siphon. Chiba *et al*.(2004) presented detailed observations of development of the tissues and organs in *Ciona intestinali*s larva and juveniles. The blood cells of the juvenile ascidians are distinguishable flowing in the body and are countable in number. The circulation mode is unique because the circulation direction is reversible with a specific frequency. Under our laboratory culture conditions, the ascidians showed evident growth diversity (Fig.1); A few of the cultivated asicidians grew slowly, with the tunica broken, viscera became whitish and the incurrent siphon and outcurrent siphon not extending freely, and finally died.

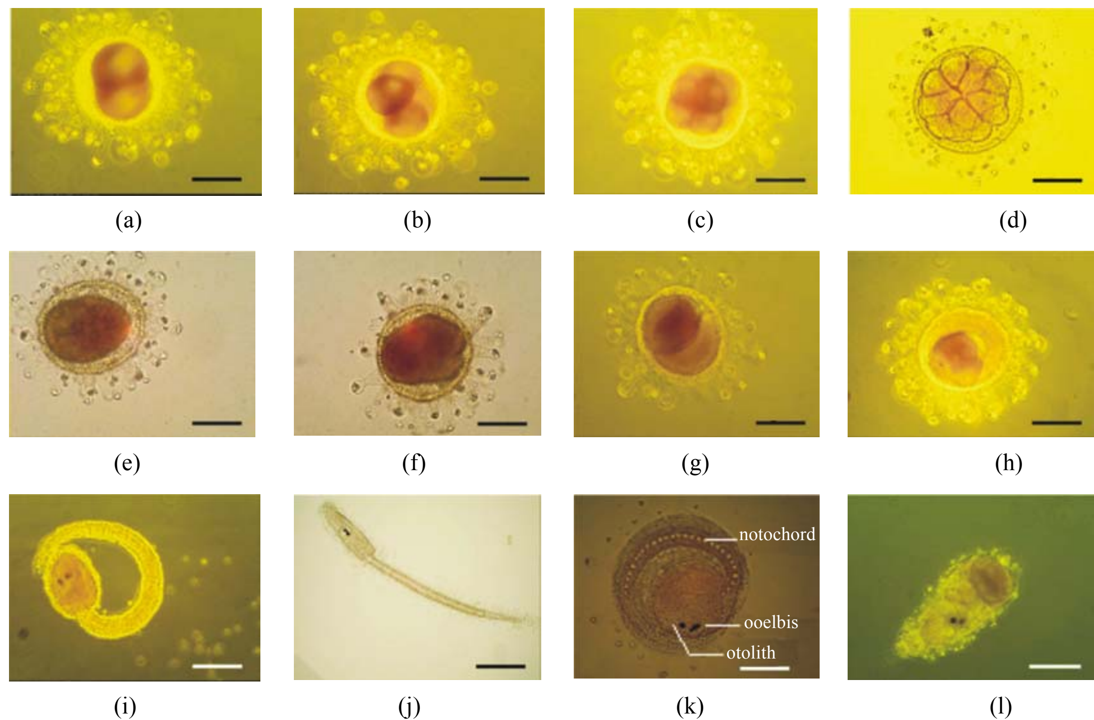


**Fig.4 Embryogenesis of *Ciona intestinalis* using artificially cultured ascidians as materials. (a) 2-cell; (b) 4-cell; (c) 8-cell; (d) 16-cell stage; (e) Gastrula; (f) Neurula; (g) Early tailbud stage; (h) Late tailbud stage; (i) Newly hatched tadpole larva; (j) Tadpole of *Ciona intestinalis*; (k) Late tailbud stage, showing notochord and sensory organs; (l) Metamorphosis**
Scale bar=100 μm

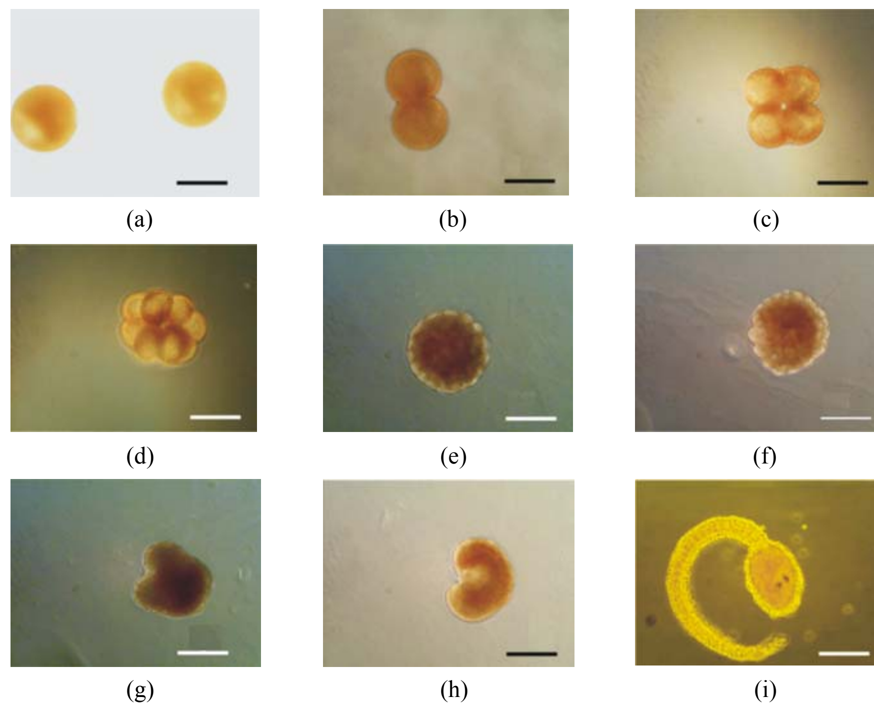


**Fig.5 Development of the dechorionated eggs. (a) Unfertilized eggs with follicle cells and test cells removed; (b) 2-cell; (c) 4-cell; (d) 8-cell; (e) 64-cell; (f) Gastrula; (g) Neurula; (h) Early tailbud stage; (i) Tadpole**
Scale bar=100 μm

**Prospect of *Ciona intestinalis* as a future model organism**

Ascidians are some of the prevailing marine sessile organisms, among which are many ascidians with bioactivities (He and Cheng, 2002). As far as evolutionary theory and developmental biology and genetic domain are concerned, *C. intestinalis* is considered as an attractive simple experimental system. For evolutionists, phylogenetics inferred by molecular evidences and cladistic analysis suggested (Wada and Satoh, 1994) that among the three subphyla of chordata, subphylum urochordata (including ascidians) are the basal group; the other two subphyla, namely subphylum cephalochordate (the amphioxus are the most famous representative species) and vertebrata are sister groups. Furthermore, it is now widely believed that all the chordates originate from the same deuterostome ancestor, thus, ascidians facilitate understanding of the origins and evolutionary diversification of the chordates (Satoh and Jeffery, 1995; Satoh *et al*., 2003). For developmental biologists and geneticists, the fertilized egg develops rapidly into a tadpole larva, with a small number of organs including epidermis, central nervous system with two sensory organs, endoderm and mesenchyme in the trunk, and notochord and muscle in the tail. This configuration of the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan. Disclosure of the molecular mechanisms underlying ascidian embryogenesis is essential for understanding the ancestral developmental program of human beings and other chordates (Corbo *et al*., 2001; Satoh *et al*., 2003).

There are several potential advantages in taking ascidians as material. (1) Voluminous data including hundreds of thousands of ESTs and the whole genome sequences were published, which are free for use in academic research (Dehal *et al*., 2002; Satoh *et al*., 2003); (2) The body of *C. intestinalis* is transparent regardless of its size and the embryos develop quickly; the whole life span is only 2~3 months; therefore it is convenient to conduct mutation and genetic screening (Nakatani *et al*., 1999; Moody *et al*., 1999; Sordino *et al*., 2001); (3) Every blastomere of the early developmental stages is distinguishable in appearance and is named based on the nomenclature of Conklin (1905), and the fate of the blastomeres before gastrulation was documented (Conklin, 1905; Nishida, 1987); also each lineage leading to the formation of epidermis, central nervous system, mesenchyme, tail muscle and notochord was well characterized (Nishida, 1987); (4) Exogenous DNA can be introduced into fertilized eggs using simple electroporation methods (Corbo *et al*., 1997; Di Gregorio and Levine, 1998; Bertrand *et al*., 2003) enabling transforming enough synchronously developing embryos. By these means, the upstream regulatory sequences can be quickly identified. Besides, whole-mount in-situ hybridization techniques were successfully applied to embryos, even to juvenile ascidians, thus temporal and spatial expression patterns of specific genes could be obtained (Satoh *et al*., 2003; Tsuda *et al*., 2003; Tokuoka *et al*., 2004); (5) The only 2500~2600 cells of the tadpole larvae made up of epidermis, nervous system (including cerebral neural complex and spinal cord with two sense organs), muscle, mesenchyme, notochord and endodermal strand, which represent the main traits of the chordates (Satoh and Jeffery, 1995; Corbo *et al*., 2001; Satoh *et al*., 2003); (6) A newly emerging technique-gene knockdown, which includes morphlinos (Satou *et al*., 2001b) to identify gene functions could be applied in ascidian embryos because they are large enough and easy to be handled (Nishida, 2002; Tsuda *et al*., 2003; Inada *et al*., 2003; Tokuoka *et al*., 2004); (7) *C. intestinalis* has a small genome (1/17 of that of human) of about $1.55\times10^8$ bp/haploid containing approximately 15800 genes, which share 62% similar genes with human and fruit fly. In addition, 17% of the genes are exclusively similar to human (Dehal *et al*., 2002). The compact genome is easy for gene regulatory network assays (Awazu *et al*., 2004; Yagi *et al*., 2004).

In conclusion, this paper presents methodology for artificial propagation, removal of egg chorion, and laboratory cultivation of *C. intestinalis*. Moreover, some new observations on the development of normal and dechorionated eggs together with some details of biological characteristics of the juvenile ascidians were also provided in order to better understand and exploit this emerging model organism. In addition, the application of various molecular techniques to the ascidian system highlights its advantages as a future experimental system for exploring the molecular mechanisms underlying the expression and function of developmental genes as well as genetic circuitry responsible for the establishment of the basic chordate body plan.

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