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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

CHARACTERISTICS OF REPRODUCTION AND DEVELOPMENT OF A MARINE MOLLUSK, *Aplysia kurodai*

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Characteristics of reproduction and development of a marine mollusk, *Aplysia kurodai*

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국문요약

이 연구는 군소(*Aplysia kurodai*)의 번식과 발생 특성을 조사하기 위해, 번식체계의 형태조직적 특성, 생식주기 및 산란, 유생의 발달과 변태 유도를 수행하였다.

군소는 동시자웅동체로 복잡한 번식체계를 갖고 있고, 교미를 통해 체내수정으로 수정란을 생산하였다. 군소의 번식체계는 post-ampullar에서 생식공까지 1개의 관으로 연결된 monaulic type이지만 내부구조는 불완전하게 oviduct와 copulatory duct로 구분된 oodiaulic type이었다. 군소의 생식소는 ovotestis로 동일한 follicle에서 암수 배우자가 발달하였고, accessory genital mass는 3가지의 과립선(albumen, membrane, mucus gland)으로 구분되었다. albumen gland는 basophilic, neutral mucopolysaccharides를 형성하는 세포들로 구성되어 있었고, membrane과 mucous gland는 acidophilic, sulphated mucopolysaccharides를 형성하는 세포들로 구성되어 있었다.

군소의 생식주기를 조사하기 위해 제주도 함덕 연안에서 2002년 12월부터 2004년 1월까지 채집을 하였지만, 계절적 출현양상으로 2003년 10월부터 2004년 1월까지는 채집할 수가 없었다. 군소의 생식주기는 GSI와 배우자형성과정, 생식소 발달단계에 따라 Inactive stage (12-2월), Active stage (12-4월), Mature and Spawning stage (4-9월)로 구분할 수 있었다. 또한 암컷과 수컷의 배우자가 동일한 follicle에서 생산되지만, 암컷과 수컷 생식세포의 발달은 시기적인 차이가 있었다.

군소의 수정란은 젤라틴성 피막에 싸인 캡슐안에 있었고, 1개의 캡슐에 15-25개의 수정란이 있었다. 유생의 변태유도가 가능한 일령은 패각에 변태징후인 붉

은 반점이 출현하는 부화 후 80일(418±7.9 μm SL) 전후였다. 최장기간 생존한 유생은 부화 후 380일 이었다. 군소의 변태유도는 갈조류를 제외한 녹조류, 홍조류에 비특이적으로 일어났지만, 변태유도 후 먹이원은 홍조류인 가시비단풀과 모로우붉은실 2종으로 종특이적이었다. 변태 후 군소의 성장은 다른 종과 유사한 발달 경향을 보였고, 성 성숙은 변태 후 90일에서 120일 사이에 일어났다.

List of abbreviations

AG	atrial gland	AGM	accessory genital mass	AM	ampulla
AN	anus	BB	black band	BM	buccal mass
CC	ciliated cell	CI	cilia	CR	crop
CT	connective tissue	DG	digestive gland	DO	degenerative oocyte
EC	egg capsule	EM	egg masses	EO	early growing oocyte
ES	esophagus	EY	eye	FE	fertilized egg
FO	foot	GA	genital aperture	GG	genital groove
GI	gill	GP	grinding plates	HE	head
IN	intestine	IS	internal septum	IV	ink vesicles
JE	jelly matrix	JS	juvenile shell	LA	larva
LHD	large hermaphroditic duct	LS	larval shell	ME	membrane gland
MO	mature oocytes	MU	mucous gland	N	nucleus
OG	opaline gland	OO	oocytes	OV	oral veil
PA	parapodia	PR	prevelum	PS	penis
RH	rhinophore	RHD	red hemiduct	RP	rhinophore
RS	red spots	SC	secretory cell	SG	salivary gland
SH	shell	SHD	small hermaphroditic duct	SI	siphon
SO	spermatocyte	SP	spermatheca	SR	seminal receptacle
ST	spermatids	SZ	spermatozoa	TA	tail
UO	unyolked oocyte	USZ	undischarged spermatozoa	WHD	white hemiduct
WS	white spots	YG	yolk granule	VE	velum
VI	veliger				

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CHAPTER Ⅰ

Reproductive system of *Aplysia kurodai*

Abstract

This study was investigated structure and function of the reproductive system in *A. kurodai* by anatomical, histological, and histochemical observation. There reproductive system is consisted of ovotestis, small hermaphroditic duct, ampulla, accessory genital mass and large hermaphroditic duct. The ovotestis is embedded in the posterior dorsal surface of the digestive gland. The ovotestis is composed of a large number of follicles, and both oocytes and spermatocytes matured in the same follicle. The small hermaphroditic duct is a single tube leading from the ovotestis to the fertilization chamber. This duct contains a swelling, the ampulla, which functions as a storage organ for endogenous sperm and an oviduct. The accessory genital mass is connected both the small and large hermaphroditic duct, consisted of three glands: albumen, membrane (winding) and mucus gland. The albumen gland is consisted of granules cells producing basophilic and neutral mucopolysaccharides. The membrane and mucus gland are consisted of granules cells producing acidophilc and sulfated mucopolysaccharides. The large hermaphroditic duct is a single tubular gonoduct linking from the accessory genital mass to the common genital aperture but is consisted of two parallel compartments, i.e., the reddish-brown color and thin yellow (or white) color parts. Internally, these two compartments are incompletely divided by internal septum or fold, which are called as the red hemiduct and white hemiduct, respectively. The red hemiduct functions as an oviduct and the white hemiduct functions as a copulatory duct. The atrial gland is also located in the wall of the large hermaphroditic duct. The reproductive system of *A. kurodai* is externally comprised a single tube, i.e., monaulic

type. However, internal structure of duct is incompletely divided into oviduct and copulatory duct, i.e., the oodiaulic type.

The progress of egg covering in *A. kurodai* is as following. Mature oocytes are released from the ovotestis are moved through the ampullar duct into the fertilization chamber. Here, they receive all sperm released from seminal receptacle and fertilization occurs. Thereafter, the fertilized egg is encapsulated in accessory genital mass; beginning with the egg membrane secreted in the membrane gland, followed by mucus layers added in the mucus gland.

1. Introduction

Aplysia kurodai (Gunso in Korean) belongs to the family Aplysiidae, order Anaspidea, subclass Ophithobranchia, class Gastropoda, and is a major species in Korea. The genus *Aplysia* has 50 species that are distributed worldwide (Beeman 1968; Klussmann-Kolb 2004). Since *Aplysia* have a relatively simple nervous system with large neurons, they have been of a major interest to neurobiologists and physiologists (Kandel 1979; Kaang et al. 1993). *Aplysia* are generally the largest opisthobranchs, characterized by the presence of 2 pairs of head tentacles, lateral parapodia, defensive glands, and a greatly reduced shell (Klussmann-Kolb 2004). Aplysiidae have been classified into four subfamilies: Aplysiinae, Dolabellinae, Dolabriferinae and Notarchinae (Eales 1960). These subfamilies are distinguished by the shape of the parapodia, the shape of the radula teeth and the arrangement of the nervous system (Beenman 1968; Marcus 1972).

Almost all opisthobranchs such as Cephalaspidea, Anaspidea and Nudibranchia, etc., are simultaneous hermaphrodite animals and have a functionally and structurally complex reproductive system (Hadfield and Switzer-Dunlap 1984; Berry et al. 1992; Kress and Schmekel 1992; Painter et al. 1985; Klussmann-Kolb 2004). Most of opisthobranchs are capable of internal cross-fertilization, and perform both sex roles. The role of the male is the production and transference of autosperm (own sperm), and the role of the female is the storage of allosperm (sperm of another animal) and production of egg masses (Beeman 1970; Hadfield and Switzer-Dunlap 1984). Thus, opisthobranchs perform both function of male and female during copulation.

In order to understand how the sexual selection processes operate in a certain

animal, it is essential to know the function of its reproductive system. The reproductive system of opisthobranchs consists of ovotestis, small hermaphroditic duct, accessory genital masses, large hermaphroditic duct, seminal receptacle, spermatheca, genital aperture, and genital groove (Beeman 1970). The arrangement of the reproductive system, i.e., the pallial gonoduct from the post-ampullar to the common genital aperture varies greatly among species. According to the division of this duct, the reproductive system may be termed as monaulic, diaulic, or triaulic (Ghiselin 1966). Many literatures are described on the opisthobranch reproductive system (Gosliner 1981; Schrödl 2000), that is based mainly on the anatomical and taxonomic viewpoints but functional analyses are comparatively lacking. The understanding of reproductive system provides systematic and phylogenetic information of opisthobranchs (Wägele 2000; Dacosta et al. 2007; Ruthensteiner et al. 2007).

Like other opisthobranchs, *Aplysia* spp. have a complex reproductive system, and in particular, an interest because of an incompletely divided large hermaphroditic duct. Although the morphological feature and anatomy of *Aplysia* spp. have been studied previously, studies regarding the structure and function of reproductive system are comparatively lacking. This study was investigated structure and function of the reproductive system in *A. kurodai* by anatomical, histological, and histochemical observation.

2. Materials and Methods

2. 1. Anatomy

A. kurodai were sampled by scuba diving at a depth of 3–5 meters in the coastal waters of Hamdeok, northeast of Jeju Island, Korea. Samples were collected from March to June during spawning season. The anatomical investigations were performed on live specimens which are about 200 g body weights. For marcroscopic investigation, the specimens were anaesthetized using 10% $MgCl_2$ (Sigma Chemical Co., USA). The anatomy of *A. kurodai* was observed using a stereomicroscope and sketch morphological feature of reproductive system.

2. 2. Histology

For histological and histochemical analysis on the reproductive system, the specimens were anaesthetized in 10% $MgCl_2$, and each of the reproductive organs was dissected out of the animal. The pieces of the organs were fixed using 10% formaldehyde or Bouin's solution, embedded in paraffin, sectioned into 5–6 μm, and stained with Hansen's haematoxylin and 0.5% eosin. The ovotestis was stained with Azan. The sections of accessory genital mass were stained with Toluidine blue, and additional sections were also stained with standard histochemical stains such as periodic acid Schiff and Alcian Blue.

2. 3. Terminology

The terminology for the different part of the reproductive system of opisthobranchs is very inconsistent throughout the literature. Therefore, the terminology

used in this study mainly based on those of published by Hadfield and Switzer-Dunlap (1984), Painter et al. (1985), Gosliner (1994) and Klussmann-Kolb (2001a, b).

3. Results

3. 1. Morphology

3. 1. 1. External features

A. kurodai is bilateral symmetry, dark brownish or purplish-black in color and is covered with an irregular scattering of whitish spots. The sensory organs, such as the tentacles and eyes are located at head. The tentacles have 2 types: the cephalic tentacles in the form a fold of the body wall and conical rhiophores projecting dorsally from the surface of the neck. The eyes are located at the base of the each rhinophore. The long genital groove is located on the right dorsal side of the head. The mantle and mantle cavity are located between the two symmetrical parapodia that are separated posteriorly. The mantle forms the dorsal body wall of the visceral mass, and the shell is embedded in it. Under the shell is a single, large, folded gill. The siphon is folded to form a tubular structure posterior to the gill, with the anus in its center (Fig. 1, 2).

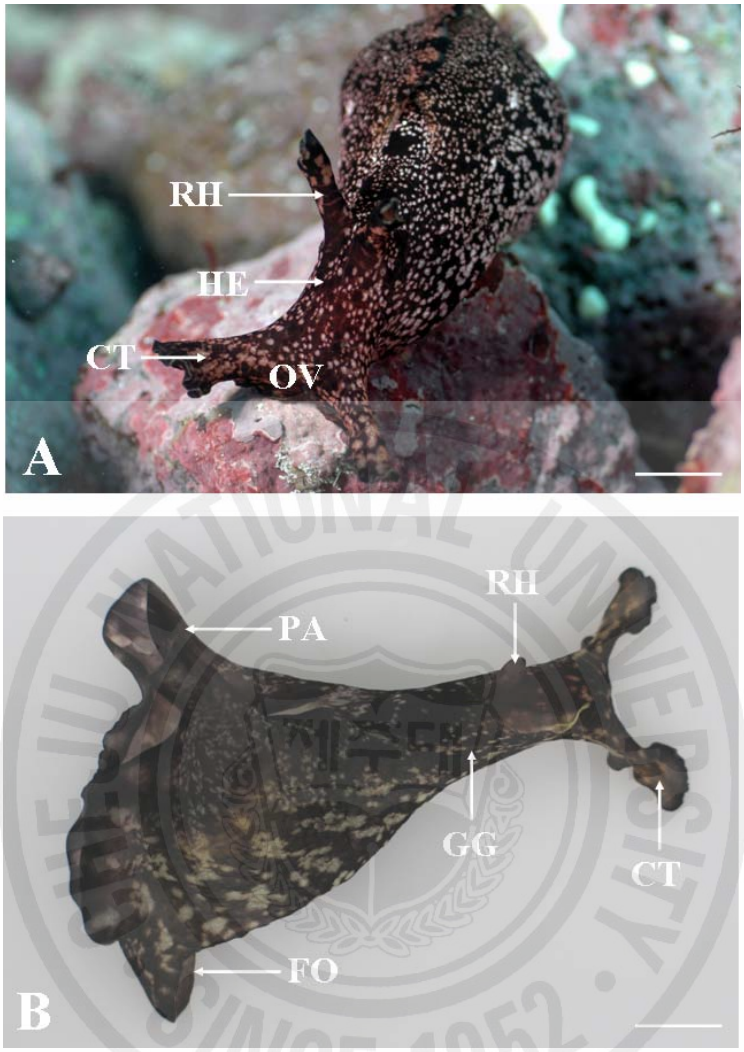

Fig. 1. External features of *Aplysia kurodai*. A, anterior view of head. B, dorsal view of body. CT, cephalic tentacle; FO, foot; HE, head; GG, genital groove; OV, oral veil; PA, parapodia; RH, rhinophore. Scale bars indicate 2.0 cm.

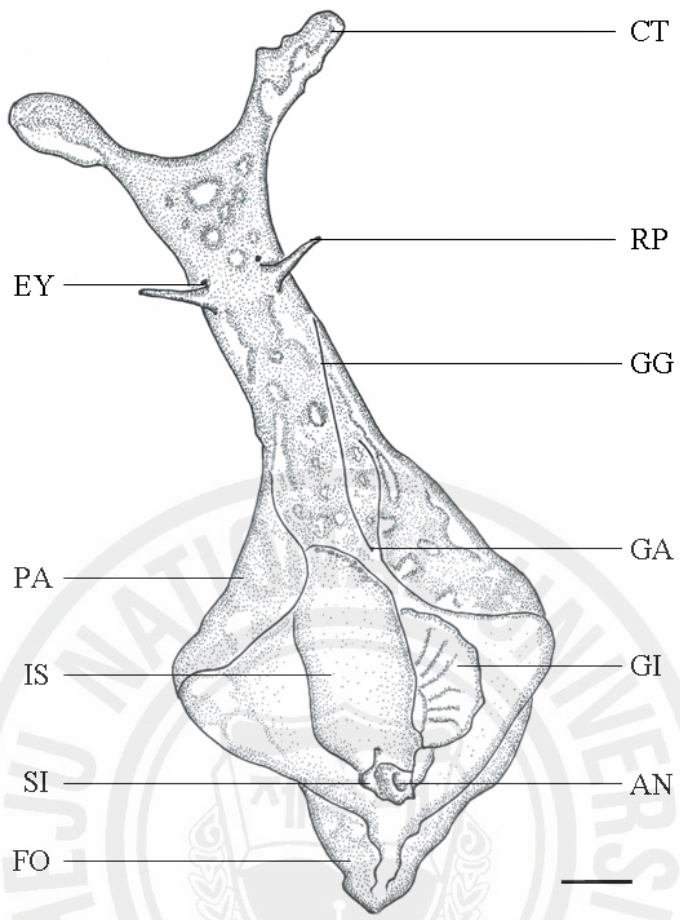

Fig. 2. Schematic outline of *Aplysia kurodai* showing main external features. AN, anus; CT, cephalic tentacles; EY, eye; FO, foot; GI, gill; GA, genital aperture; GG; genital groove; IS, internal shell; PA, parapodia; RP, rhinophore; SI, siphon. Scale bar indicates 1.0 cm.

3. 1. 2. Anatomical features

The major portion of body cavity of *A. kurodai* is occupied by the digestive tract that can be divided into a foregut, midgut and a hindgut. The foregut consisted of a buccal mass, two salivary glands, and the esophagus. The midgut consisted of a large crop, grinding plates, and a true stomach (digestive pouch). The hindgut is made up of an intestine and a rectum (Fig. 3A to E).

The buccal mass is large, dark red, and posterior to the buccal mass cerebral ganglia and other ganglia exist on top esophagus (Fig. 3C). The thin-walled crop that is contact with the esophagus is gray-brown in color and very large. It acts as a storage space and is filled with algae. The crop leads to the anterior gizzard (triturative stomach), which is composed of red muscle fibers, and its inner wall is lined with large pyramidal teeth. The posterior gizzard enters in the true stomach, i.e., a small digestive pouch. The stomach leads to a thin-walled, gray-brown intestine embedding into a large gray-brown digestive gland (Fig. 3D). The intestine makes several coils through the digestive gland and then contract into the anus (Fig. 3E).

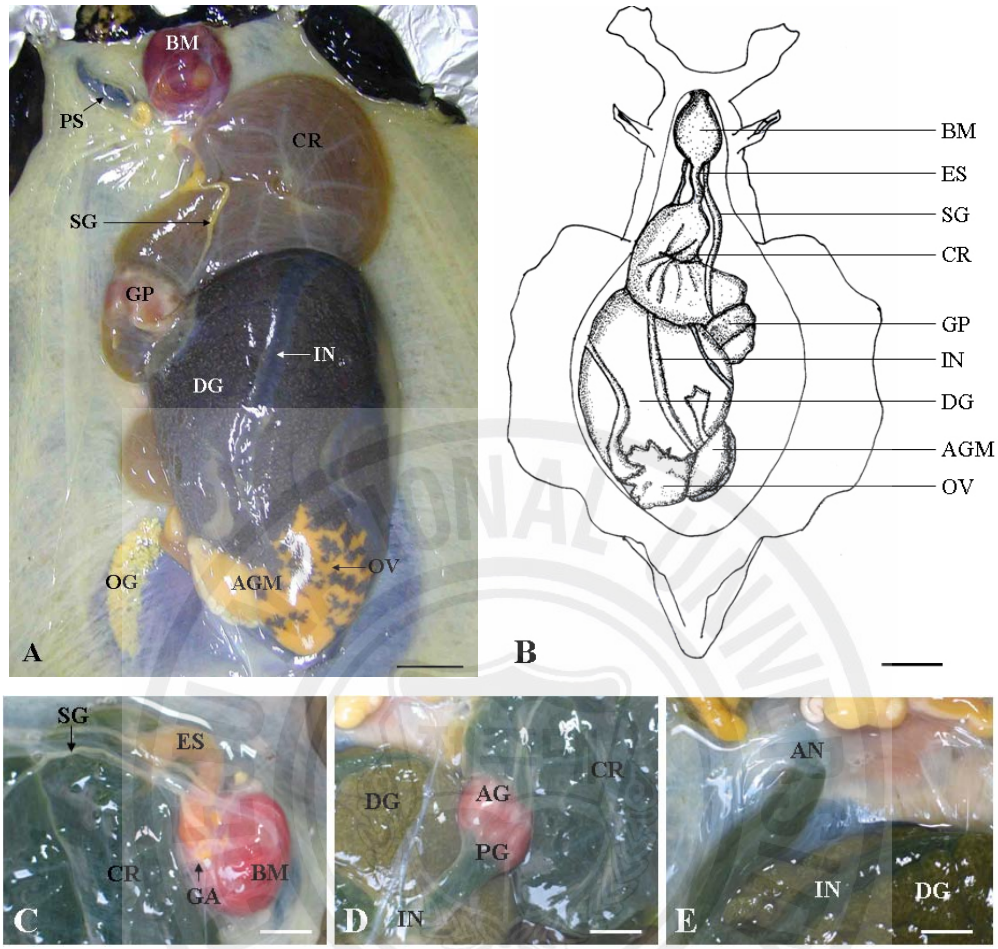

Fig. 3. Anatomical features of *Aplysia kurodai*. A, anatomical feature of digestive system. B, schematic outline of digestive system. C, foregut part of digestive systems. D, midgut part of digestive system. E, hindgut part of digestive system. AG, anterior gizzard; AGM, accessory genital mass; AN, anus; BM, buccal mass; CR, crop; Dg, digestive gland; ES, esophagus; GA, ganglia; GP, grinding plates; IN, intestine; OG, opaline gland; OV, ovotestis; PG, posterior gizzard; PS, penis; SG, salivary gland. Scale bars indicate 1.0 cm.

3. 2. Reproductive system

The reproductive system of *A. kurodai* is composed of ovotestis, small hermaphroditic duct, ampulla, accessory genital mass and large hermaphroditic duct (Fig. 4). Following ovulation, eggs are transported from a posteriorly located ovotestis through the small hermaphroditic duct to the accessory genital mass. The accessory genital mass is connected to the large hermaphroditic duct. The large hermaphroditic duct opens externally as the common genital aperture. The external genital groove runs forward along the right external body surface from the common genital aperture to the penis, ensheathed in the right side of the head. The egg masses are released from genital aperture and moves along the external genital groove to be deposited on the substrate (Fig. 5).

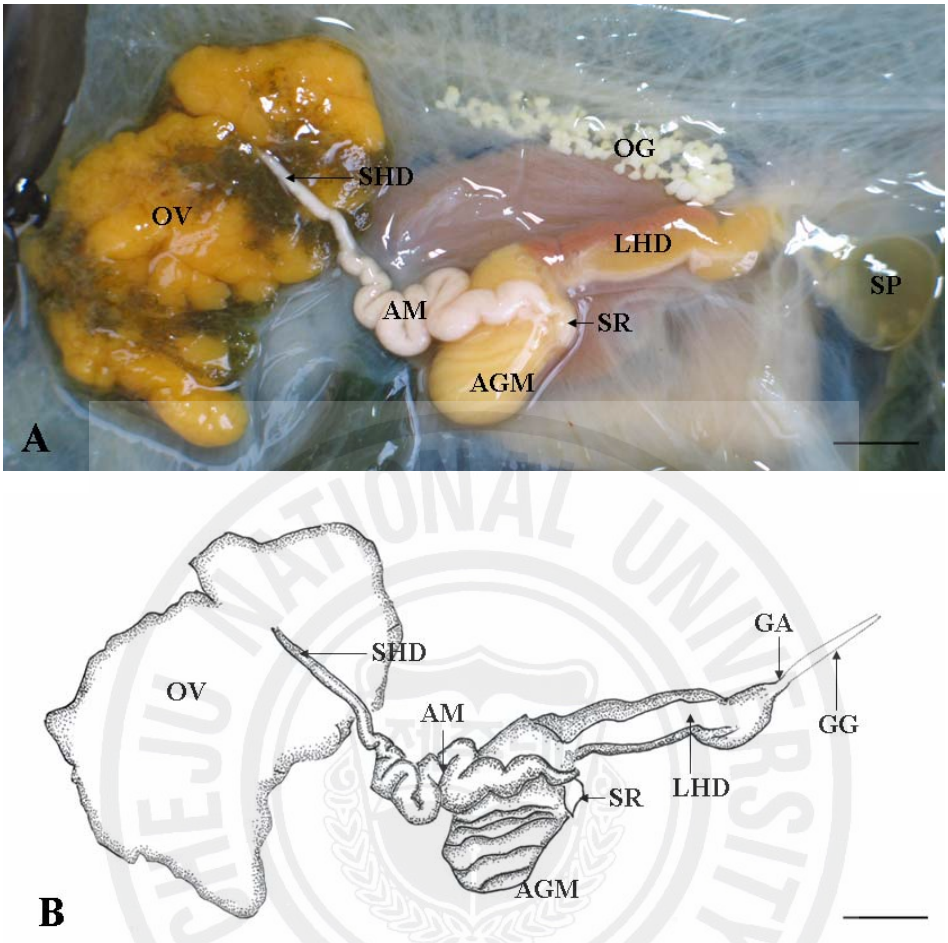

Fig. 4. Reproductive system of *Aplysia kurodai*. A, anatomical feature of reproductive system. B, schematic outline of reproductive system. AGM, accessory genital mass; AM, ampulla; GA, genital aperture; GG, genital groove; LHD, large hermaphroditic duct; OG, opaline gland; OV, ovotestis; SHD, small hermaphroditic duct; SR, seminal receptacle; SP, spermatheca. Scale bars indicate 1.0 cm.

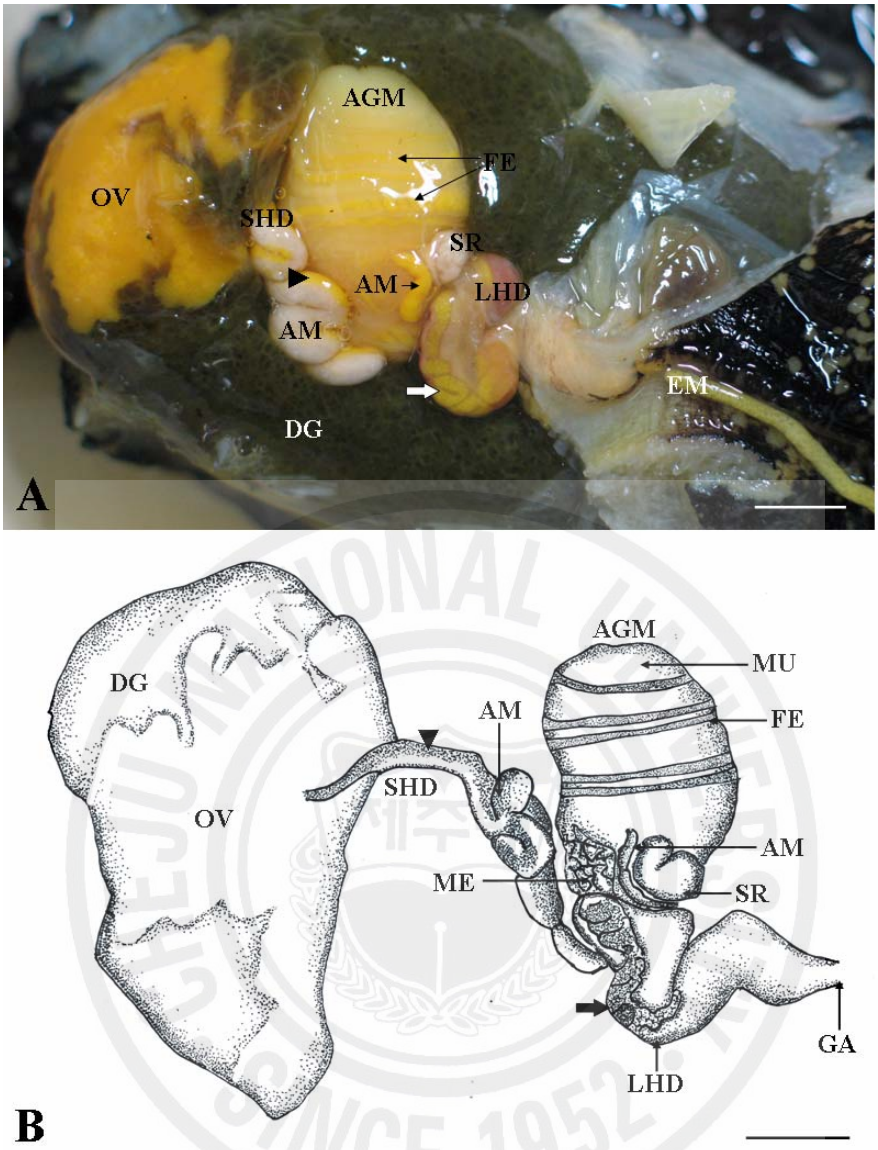

Fig. 5. Process of deposition of the egg coats in *Aplysia kurodai*. AGM, accessory genital mass; AM, ampulla; DG, digestive gland; EM, egg masses; FE, fertilized egg; GA, genital aperture; LHD, large hermaphroditic duct; ME, membrane gland; MU, mucous gland; OV, ovotestis; SHD, small hermaphroditic duct; SR, speminal receptacle. Arrowhead indicates mature oocytes within ampulla. Arrow indicates egg masses within LHD. Scale bars indicate 2.0 cm.

3. 2. 1. Ovotestis

A. kurodai have a single unpaired gonad, the ovotestis. In mature specimens the ovotestis is the largest of the reproductive organs and is orange-yellow in color. It is generally embedded in the posterior dorsal surface of the brownish digestive gland (Fig. 4). The ovotestis is composed of a large number of follicles and the follicles are surrounded by a basement membrane. Each of follicles opens into a division of the small hermaphroditic duct (Fig. 6A). Both oocytes and spermatozoa are developed in the same follicles, and oocytes are mainly observed membrane of the follicle and numerous spermatozoa are observed the lumen of follicle (Fig. 6B). When oocytes and spermatozoa are matured, they gather into a division of the small hermaphroditic duct (Fig. 6C).

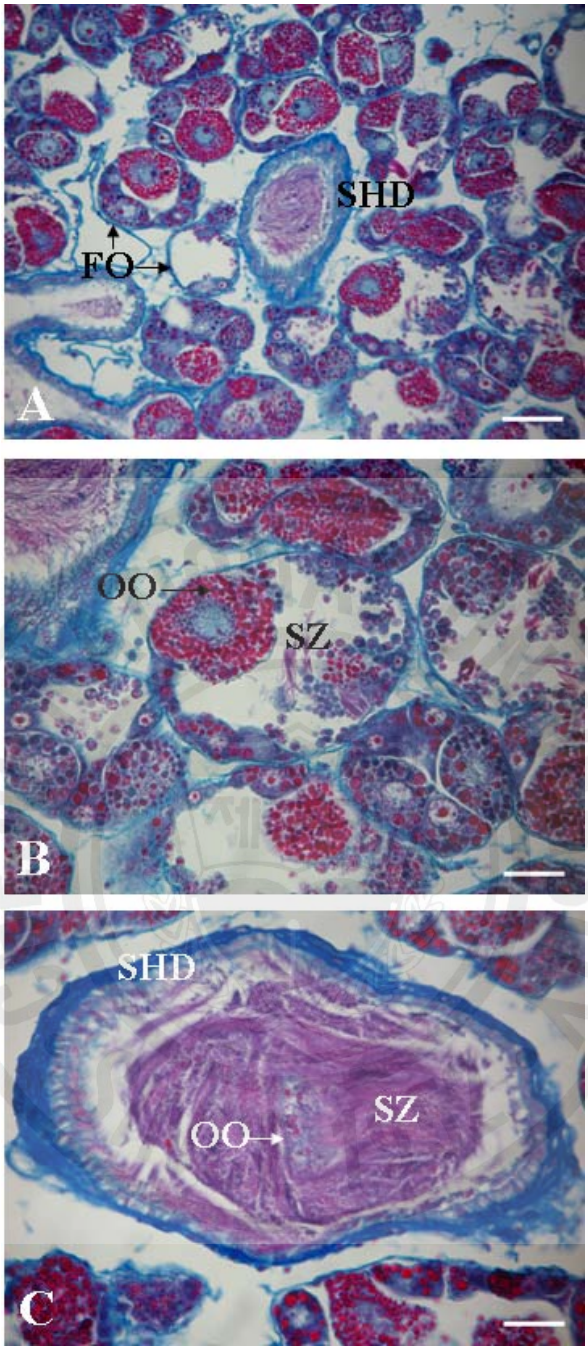

Fig. 6. Histological sections of ovotestis stained with Azan. A, ovotestis of composing a large number of follicles. B, oocytes and spermatozoa of developing in the same follicles. C, oocytes and spermatozoa of collecting in small hermaphroditic duct. FO, follicle; OO, oocytes; SHD, small hermaphroditic duct; SZ, spermatozoa. Scale bars indicate 50 μm.

3. 2. 2. Small hermaphroditic duct

The small hermaphroditic duct is relatively straight and narrow at its origin from the ovotestis, but becomes progressively wider and more tortuous as it approaches in the accessory genital mass (Fig. 4, 5). The wide part of small hermaphroditic duct is called as the ampulla, and function as a storage organ for endogenous sperm (autosperm; the animal's own sperm) and an oviduct (Fig. 7A, B). The wall of ampulla consists of a narrow epithelium with long ciliary tracts and is surrounded by muscular and connective tissue layers (Fig. 7C).

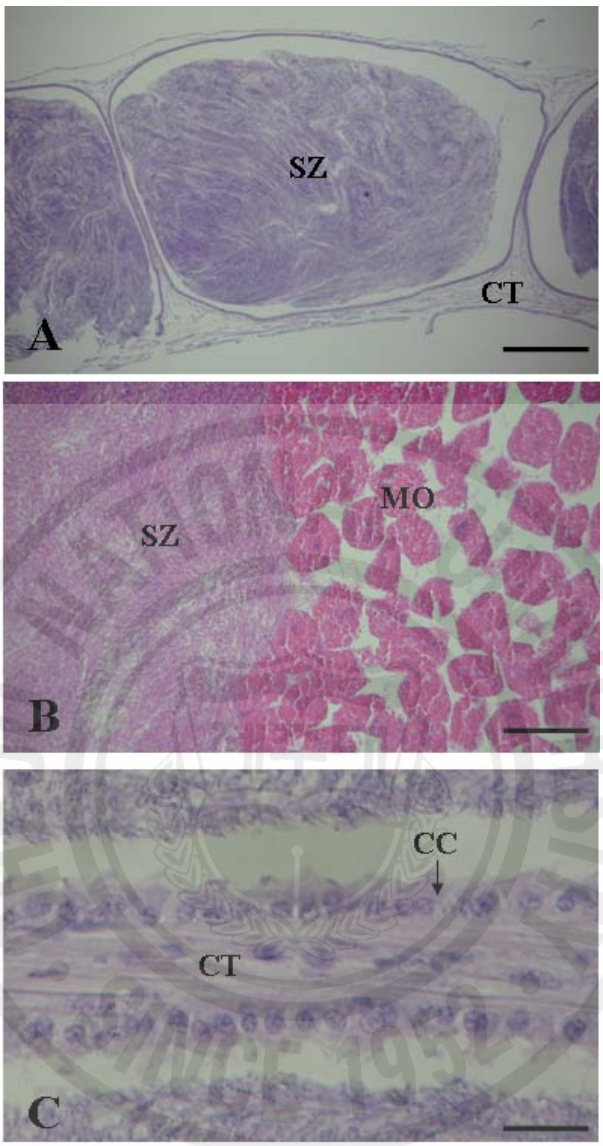

Fig. 7. Histological sections of small hermaphroditic duct. A and B, ampulla full of spermatozoa and mature oocytes. C, ciliated cells of the luminal surface in the ampulla. CC, ciliated cell; CT, connective tissue; MO; mature oocytes; SZ, spermatozoa. Scale bars indicates 100 μm (A and B) and 40 μm (C).

3. 2. 3. Accessory genital mass

The accessory genital mass is a large hemispherical organ located on the floor of the hemocoelom and is orange-yellow in color (Fig. 4, 5). The accessory genital mass consists of three glands: albumen, membrane (winding) and mucus gland. This mass is connected to both the small and large hermaphroditic duct (Fig. 8).

Albumen gland

The albumen gland is located in the central part of the accessory genital mass and is yellowish-white color. The albumen gland is tubular (or sac-like) in shape and can be recognized by its granular structure. It is surrounded by a distinct basal lamina (Fig. 9A). The secretory cells have large oval nuclei situated basally, and is filled with spherules 2.4–2.6 μm diameter staining blue-purple in Toluidine Blue, red in PAS, but is not stained Alcian Blue (Fig. 9B to D). Histochemical staining indicate production of neutral mucopolysaccharides in the albumen gland (Table 1).

Membrane gland (Winding gland)

The membrane gland (winding gland) is located to the right of the base of the accessory genital mass and is the shape of a tubule, mostly narrowly coils (Fig. 8). The secretory cells are distinctly round in shape and contain irregular-shaped granules and basal small nuclei. The wall consists of ciliated cells with extremely long cilia (Fig. 10A, B). The granules are stained violet with Toluidine Blue (Fig. 10C) and blue in Alcian Blue (Fig. 10D). Histochemical staining indicate production of acidic, sulfated mucopolysaccharides (Table 1).

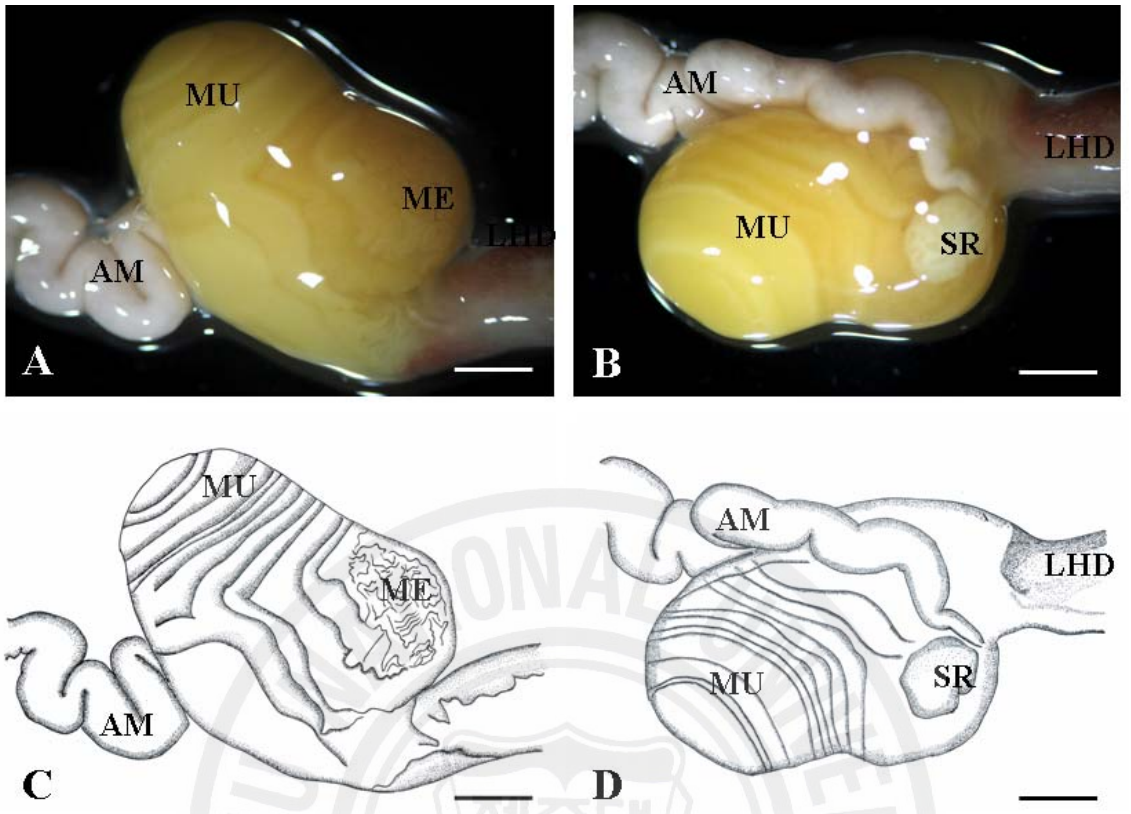

Fig. 8. Accessory genital mass in *Aplysia kurodai*. A and B, anatomical feature of accessory genital mass. C and D, schematic outline of accessory genital mass. AM, ampulla; LHD, large hermaphroditic duct; MU, mucous gland; SR, seminal receptacle; ME, membrane gland. Scale bars indicate 1.0 cm.

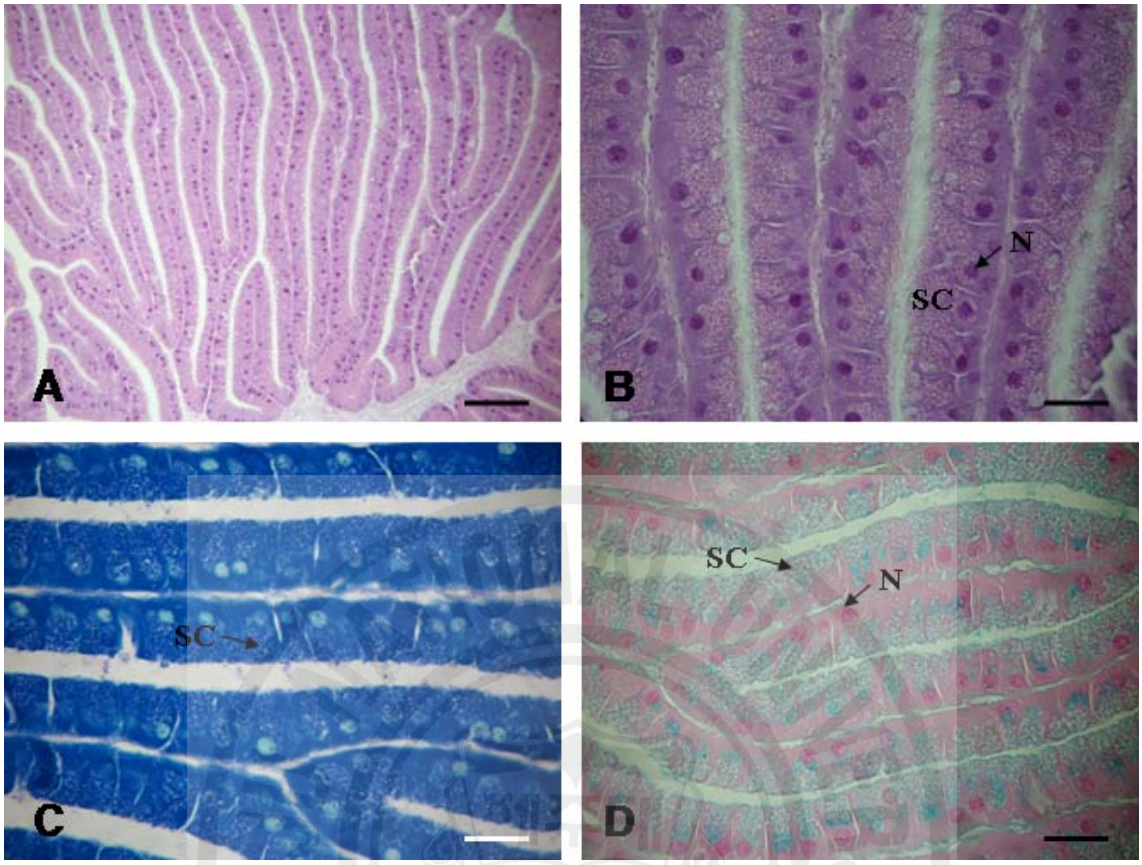

Fig. 9. Histological sections of albumen gland. A and B, albumen gland stained with haematoxline and eosin. C, secretory cell stained with Toluidine blue. D, secretory cell stained with Alcian blue. N, nucleus; SC, secretory cell. Scale bars indicate 200 μm (A) and 50 μm (B to D).

Table 1. Histochemical staining reactions of accessory genital mass

Accessory genital mass	Alcian blue pH 2.5	Alcian bule pH 1.0	PAS	Toluidine blue
Albumen gland	-	-	++	Blue
Membrane gland	+	++	+	Violet
Mucous gland	+	++	++	Violet-red

– Not stained, + weakly stained, ++ moderately stained, +++ brightly stained (PAS periodic acid-Shiff)

Mucous gland

The mucous gland is white in color and comprised the largest part in the accessory genital mass (Fig. 8). The mucous gland is also folded like the albumen gland. Grossly, the mucous gland is observed spiral tubes that encircle the albumen gland (Fig. 11A, B). The granules is stained red-violet in Toluidine blue (Fig. 11C) and blue in Alcian blue (Fig. 11D). Histochemical staining indicate production of neutral and acidic, sulfated mucopolysaccarides (Table 1).

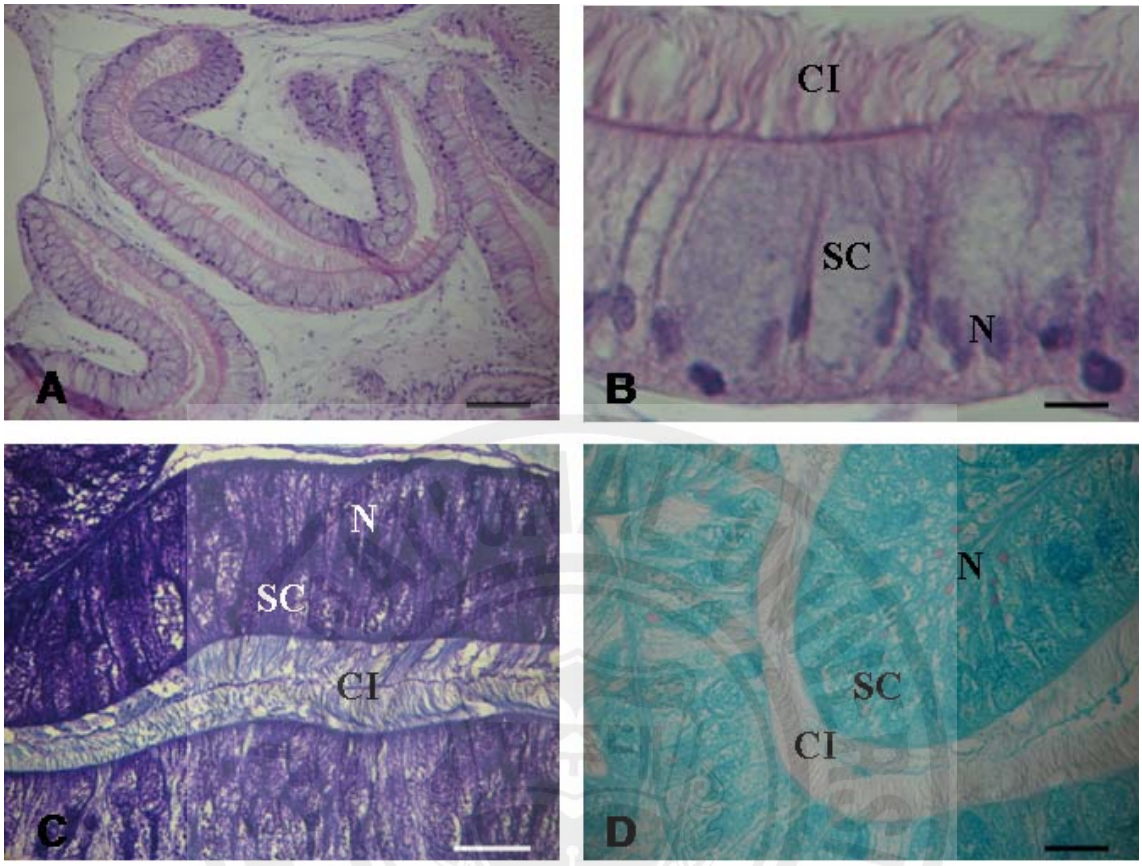

Fig. 10. Histological sections of membrane gland. A and B, membrane gland stained with haematoxline and eosin. C, secretory cells stained with Toluidine blue. D, secretory cells stained with Alcian blue. Scale bars indicate 100 μm (A), 20 μm (B) and 40 μm (C and D). CI, cilia; N, nucleus; SC, secretory cell.

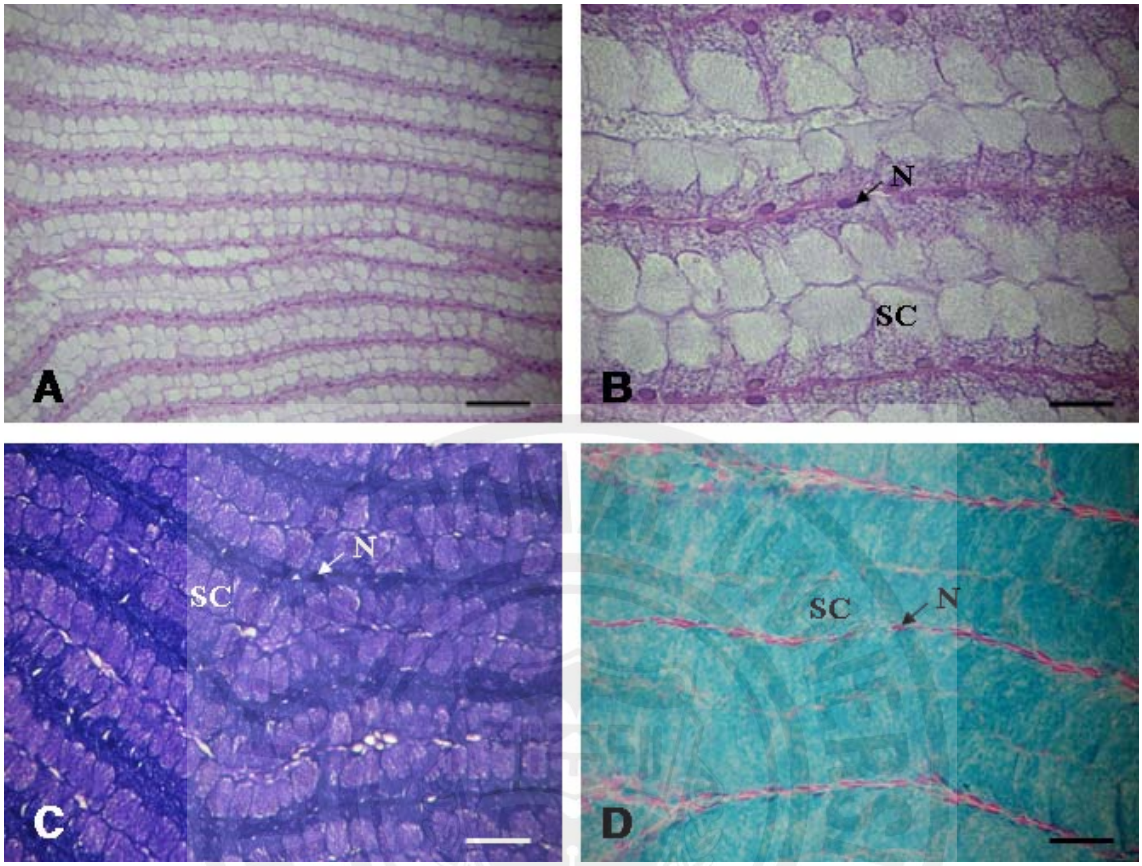

Fig. 11. Histological sections of mucous gland. A and B, mucous gland stained with haematoxline and eosin. C, secretory cell stained with Toluidine blue. D, secretory cell stained with Alcian blue. N, nucleus; SC, secretory cell. Scale bars indicate 100 μm (A) and 40 μm (B to D).

3. 2. 4. Large hermaphroditic duct

The large hermaphroditic duct is a single tubular gonoduct linking from the accessory genital mass to the common genital aperture but consists of two parallel compartments, i.e., the reddish-brown color and thin yellow (or white) color parts, which can be clearly distinguished from each other (Fig.12). Internally, these two compartments longitudinally divided by internal septum or fold, which are called as the red hemiduct and white hemiduct, respectively (Fig. 13A). The red hemiduct functioned as an oviduct and the white hemiduct functioned as a copulatory duct (Fig. 13B, C).

The RHD is lined by epithelium composed of two kinds of epithelial cells. The first type includes non-ciliated columnar epithelial cells (approximately 70 μm in height and 10 μm in width) which a basal nucleus staining dark with haematoxline. These cells also contain large eosinophilic secretory granules (1-2 μm in diameter). The second type includes irregular-shaped capping cells that cover most of the luminal surface of the RHD. These cells lie between the apices of the columnar epithelial cells and extend long, narrow processes between them; these cells are in contact with the basal surface of the epithelium. These cells have apical cilia (approximately 15 μm long) and nuclei that stain weakly with haematoxyline. The capping cells do not contain large secretory granules, which are detectable by light microscopy (Fig. 14A, B).

The atrial gland is lined by pseudostratified epithelium composed of non-ciliated columnar epithelial cells and irregular-shaped cpapping cells (Fig. 15A). The capping cells with the apical cilia cover most of the lumenal surface of the artial gland (Fig. 15B).

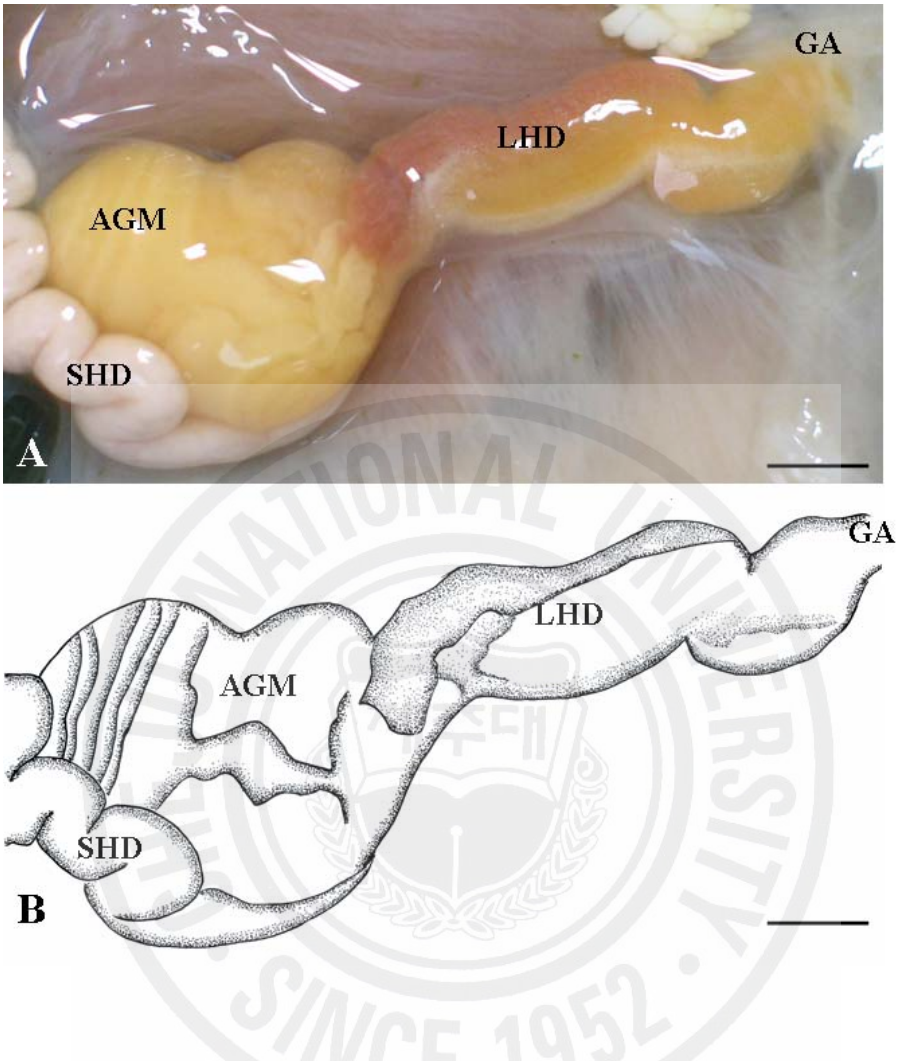

Fig. 12. Large hermaphroditic duct in *Aplysia kurodai*. A, anatomical feature of large hermaphroditic duct. B, schematic outline of large hermaphroditic duct. AGM, accessory genital mass; GA, genital aperture; LHD, large hermaphroditic duct; SHD, small hermaphroditic duct. Scale bar indicate 1.0 cm.

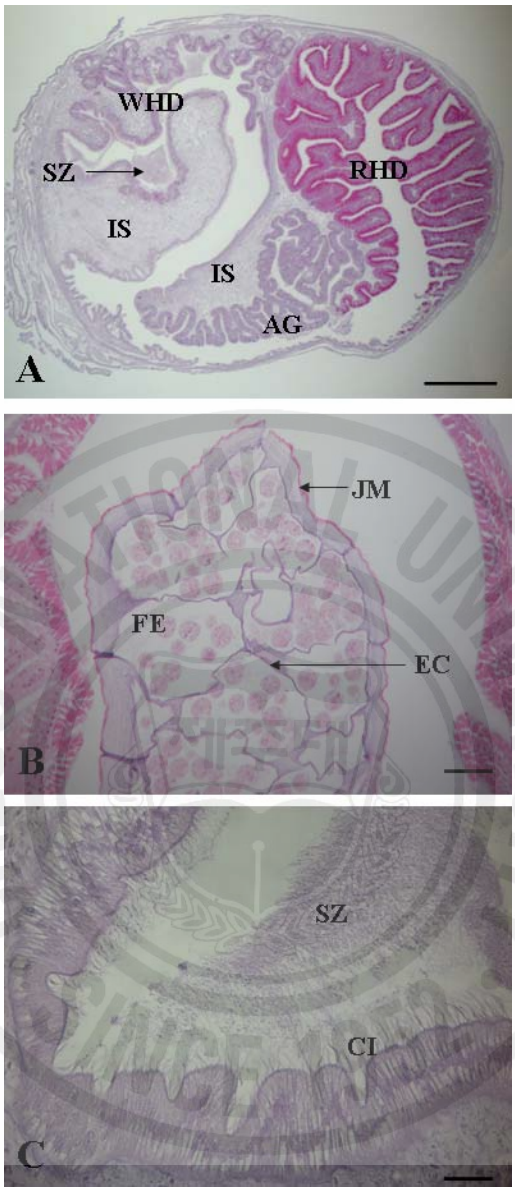

Fig. 13. Histological sections of large hermaphroditic duct. A, large hermaphroditic duct divided as red hemiduct and white hemiduct by internal septum. B, egg masses within red hemiduct. C, spermatozoa within white hemiduct. AG, atrial gland; CI, cilia; EC, egg capsule; FE, fertilized egg; IS, internal septum; JM, jelly matrix; RHD, red hemiduct; WHD, white hemiduct; SZ, spermatozoa. Scale bars indicate 100 μm.

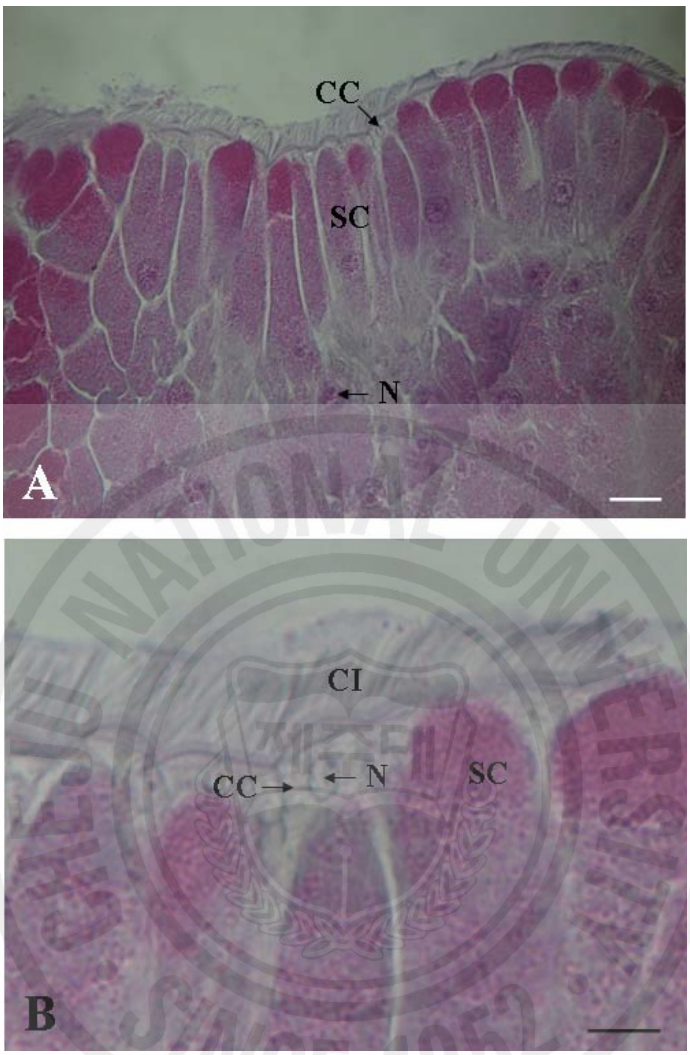

Fig. 14. Histological sections of red hemiduct. A, non-ciliated secretory cell epithelium of the red hemiduct. B, ciliated capping cells of the luminal surface in the red hemiduct. CC, capping cell; CI, cilia; N, nucleus; SC, secretory cell. Scale bars indicate 10 μm.

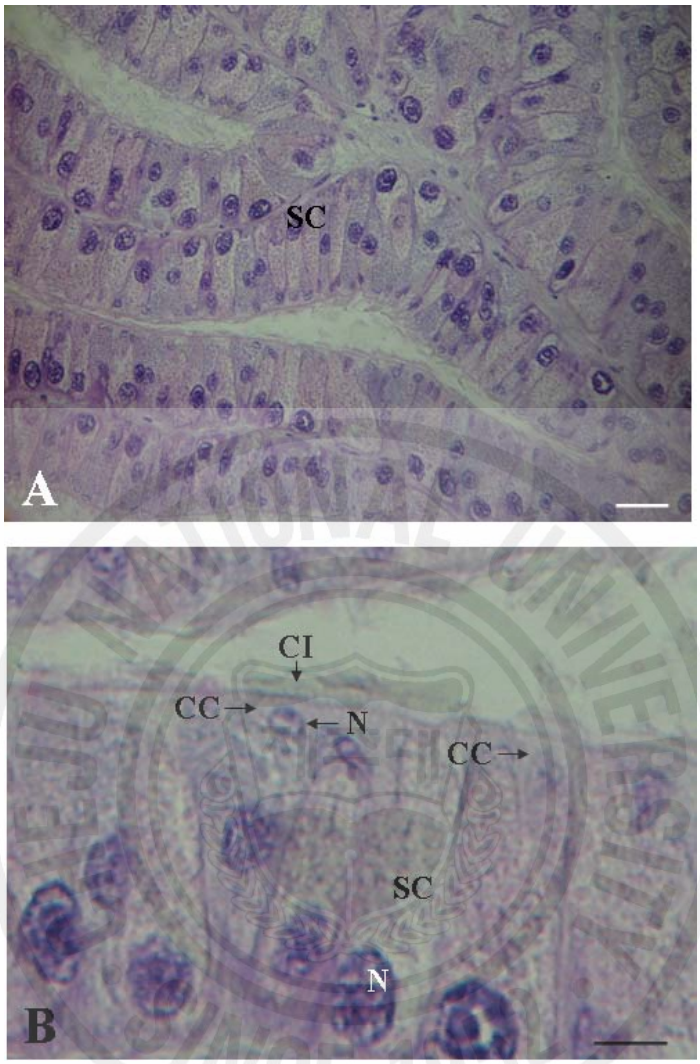

Fig. 15. Histological sections of atrial gland. A, non-ciliated columnar epithelial cells lined the atrial gland. B, capping cells of the lumenal surface on the artial gland. CC, capping cell; CI, cilia; N, nucleus; SC, secretory cell. Scale bars indicate 10 μm.

4. Discussion

Almost all opisthobranchs are simultaneous hermaphroditic animals that possess structurally and functionally complex reproductive systems consisting mainly of an ovotestis, a complex gonoduct and accessory genital gland. According to the division of gonoduct, the reproductive system is termed as monaulic, diaulic, or triaulic (Ghiselin 1966). The monaulic type represents a single undivided large hermaphroditic duct and diaulic or triaulic types represent that the large hermaphroditic duct is divided into two or three ducts, respectively. The diaulic type can also be further subdivided into androdiaulic and oodiaulic types (Ghiselin 1966; Hadfield and Switzer-Dunlap 1984; Wägele 2000). This arrangement of reproductive system differs among opisthobranchs and variations can be observed within same family. Nudibranchs and Notaspids generally have diaulic or triaulic systems (Willan 1987; Cervera et al. 2000; Schrödl 2000), and Pulmonata and Cephalasidea have monaulic or diaulic systems (Visser 1988; Kress and Schmekel 1992). In particularly, aplysiid species have complex and incompletely divided reproductive tracts. Externally, although the tract may appear as a single tube, it is internally divided by at least two incomplete internal folds. Thus, most aplysiids species have either a monaulic or diaulic reproductive system (Ghiselin 1966; Beeman 1970). In this study, the reproductive system of *A. kurodai* was externally a single tube, i.e., monaulic type. However, internal structure of duct is incompletely divided into oviduct and copulatory duct, i.e., the oodiaulic type.

In mature opisthobranchs, the ovotestis is generally the largest of the reproductive organs. It is generally located posteriorly in the body and is closely interdigitated with the digestive gland. The ovotestis is composed of individual follicles

or acini that may be widely spaced (*Acteonia cocksi*; Gascoigne 1956) or closely packed (*Phyllaplysia taylori*; Beeman 1970). In aplysiid, each follicle is covered by a simple epithelial layer and is surrounded by small muscle filaments (Dudek et al. 1980). In many opisthobranchs, oocytes and sperm are produced in the same follicles, however in only a few species, complete separate male and female follicle are found (Reid 1964), and separate male and female gonads are also found in some Acochlidiacea (Morse 1976). In this study, *A. kurodai* also have a single unpaired gonad, the ovotestis, which is composed with numerous follicles. The follicles either partially or completely embedded in the digestive gland tissue, from which they are separated by the basal lamina. Each follicle contains a mixture of both male and female cells in different stage after the onset of sexual maturation. Previtellogenic oocytes lie in the periphery of the follicle, and male cells fill the lumen of follicle; thereafter, the full-grown oocytes are preferentially located in the center of the follicle. This phenomenon is similar to those reported in case of other *Aplysia* spp. (Beeman 1970; Dudek et al. 1980). This arrangement may be due to the displacement of preexisting oocytes by the young oocytes.

The small hermaphroditic duct, referred to by some authors as the coelomic gonoduct or ampulla, is a single tube leading from the ovotestis to the fertilization chamber. This duct can be divided into three regions: a preampullar region, an ampulla and a postampullar portion. The autosperm (the animal's own sperm) formed in the ovotestis is collected and transported to the ampulla, the main part of the small hermaphroditic duct, and mature autosperm are stored prior to copulation (Beeman 1970). In this study, this duct is narrow in immature individuals, but wider in mature animals and is convoluted to form a seminal vesicle for storage of autosperm. In mature

specimens, this tube is creamy-white in appearance due to the presence of sperm. The seminal vesicle is the proximal hermaphroditic duct itself, and not a diverticulum. The autosperm are inactive until ejaculated into a partner. The wall of small hermaphroditic duct is usually composed of ciliated epithelium surrounded by a thin layer of muscle and connective fibers. These results are similar to the results of a previous study regarding characteristics of autosperm and morphology of this duct (Thompson and Bebbington 1969; Beeman 1970). The ciliated cells may be responsible for the propulsion of the autosperm and oocytes rapidly through the ampullar during copulation and oviposition.

The accessory genital mass consists of a fertilization chamber and a series of glandular tubes or folds that provide the eggs with the protective layers such as egg capsule and gelatinous matrix. The accessory genital mass has been variously referred to as the female gland mass, anterior genital mass or nidamental glands (Beeman 1970; Coggeshall 1972; Klussmann-Kolb 2001a, b). A three-part accessory genital mass with a proximally located albumen gland, a membrane gland, and a distally located mucous gland is found in most opisthobranchs (Klussmann-Kolb 2001a, b). However, pleurobranchoidea and nudibranchia possess only two glandular parts (Hadfield and Switzer-Dunlap 1984) and aeolid nudibranchia possess three distinct glandular parts (Gosliner 1994). *Aplysia* spp. generally presents three glandular parts within the accessory genital mass (Marcus and Marcus 1957; Thompson and Bebbington 1969; Beeman 1970; Coggeshall 1972). In this study, the accessory genital mass of *A. kurodai* was composed with albumen gland, the membrane gland and the mucous gland. The albumen gland was large, yellowish-white in color, and its anterior end formed most of the forward part of the accessory genital mass. The membrane gland formed the right-anterior part of the

accessory genital mass. The mucus gland was a semi-translucent white organ forming the posterior part the accessory genital mss.

In *A. kurodai*, the glandular part of the accessory genital mass could be divided into two parts based on their histochemical staining properties and mode of secretion; the basophilic albumen gland (1), so called because the glandular tissue stained blue with toluidine blue and not stained with alcian blue, and the gland contained neutral polysaccharides and the membrane and mucous glands (2), which stained blue with alcian blue and red to violet (acidophilic) with toluidene blue, and the gland contained neutral polysaccharides and acidic, sulphated mucopolysaccharides. These results correspond with those of the histochemical investigation of egg mass in some opisthobranchs (Klussmann-Kolb and Wägele 2001); it was shown that egg mass of opisthobranchs is composed of various layers, i.e., albumen, albuminous, membrane and mucous layers. This information may provide some understanding of the formation of egg messes in the accessory genital mass.

In simultaneous hermaphrodites, the large hermaphrodite duct is a very complex organ and has variously been referred to as the large, wide, distal or anterior hermaphroditic duct (Hadfield and Switzer-Dunlap 1984). This duct plays reproductive functions, including transportation of the egg cordon during oviposition, transportation endogenous sperm during copulation as a male, and to receive the penis and exogenous sperm during copulation as a female (Thompson and Bebbington 1969). Externally, it appears as a single duct in *Phyllaplysia taylori*, but is actually composed of two distinct and parallel ducts. Internally, these two ducts longitudinally divided by at least two incomplete internal septa or folds, which are called as the spermoviduct and the copulatory or vaginal ducts (Beeman 1970). Incomplete internal separation of these

ducts has been reported in *A. fasciata*, *A. punctata*, and *A. depilans* (Thompson and Bebbington 1969), and *A. californica*, *A. brasiliana* and *A. dactylomela* (Painter et al. 1985). In the case of *A. kurodai* species, large hermaphroditic duct was a single tubular which is composed with two parallel compartments, i.e., the reddish-brown and the thin yellow color parts. Internally, this duct was divided with the red and white hemiduct by internal septa. The red hemiduct functioned as a spermoviduct transporting the jelly-coated string of encapsulated eggs. The white hemiduct functioned as a copulatory or vaginal duct carrying endogenous and exogenous sperm.

The atrial gland is a secretory organ located primarily in the wall of the large hermaphroditic duct. Its secretion, when injected into a sexually mature animal, stimulates the process of egg laying (Beeman 1970; Arch et al. 1980). The atrial gland differs in its shape and location among aplysiids; for example, large hermaphroditic duct of *A. brasiliana* lacks an atrial gland but has a pea-shaped gland located anterior to the gametolytic gland stalk. *A. dactylomela* has both a pea-shaped gland with a location and morphology similar to that of *A. brasiliana* and an atrial gland similar to that of *A. californica*. In this study, *A. kurodai* was also observed to possess the atrial gland in the wall of the large hermaphroditic duct. On histological observation, it can be observed that the atrial gland, like the RHD and most secretory areas in the reproductive tract, consists of pseudostratified epithelium which is composed of nonciliated columnar epithelial cells and irregular-shaped capping cells. As in the RHD, the capping cells cover most of the lumenal surface of the atrial gland. The columnar epithelial cells have large eosinophilic secretory granules. This result is similar to the results of the study of other *Aplysia* spp. (Arch et al. 1980; Beard et al. 1982; Painter et al. 1985). In a previously study on function of atrial gland, Heller et al. (1980) and Strumwasser et al.

(1980) reported that the atrial gland might function to temporally link the act of egg laying with copulation and thus, secrete peptides into the hemocoel in response to mechanical or chemical stimulation by the penis. However, morphological and physiological evidence indicates that the atrial gland is an exocrine organ that secretes into the lumen of the large hermaphroditic duct rather than into the hemoceol (Arch et al. 1980; Beard et al. 1982). In the present study, the atrial gland was confirmed to be an exocrine organ located in the wall of the large hermaphroditic duct and was presumed to play a direct role in oviductal function of large hermaphroditic duct.

The progress of egg covering in *A. kurodai* is as following. Mature oocytes released from the ovotestis are moved through the ampullar duct into the fertilization chamber. Here, they receive all sperm released from seminal receptacle and fertilization occurs. Thereafter, the fertilized egg is encapsulated in accessory genital mass; beginning with the egg membrane secreted in the membrane gland, followed by mucus layers added in the mucus gland.

CHAPTER Ⅱ

Reproductive cycle of *Aplysia kurodai*

Abstract

This study investigated the reproductive cycle based on monthly changes of gonadosomatic index, gametogenesis, and stage of gonadal development in order to characterize the reproductive biology of *Aplysia kurodai* inhabiting the coastal waters of Jeju Island, Korea. *A. kurodai* was simultaneous hermaphrodite and the ovotestis was composed of a large number of follicles, and both oocytes and sperm were produced in the same follicles. In the sampling periods, the adult *A. kurodai* population have characteristic of seasonal pattern present during only 10 months. The gonadal development of *A.kurodai* coincided with rising temperature in spring, and kept on spawning from April to September during warm season. These results suggest that the gonadal development and spawning of this species are closely changed with temperature.

1. Introduction

Sea hares of the species belonging to the genus *Aplysia* are benthic herbivores residing in the intertidal and subtidal zones throughout the world. They are widely used as model organism for neurobiological and behavioral studies (Kandel 1979). Their biological and ecological characteristics are short lives, rapid growth, and abundance (Gev et al. 1984; Carefoot 1987). Many studies have concentrated upon seasonal variation in weight or size (Carefoot 1967; Usuki 1970; Audesirk 1979), reproductive activity (Yusa 1996), seasonal change in population (Plaut et al. 1998), and recruitment into the population by recently metamorphosed juveniles (Sarver 1979).

The aplysiids have a distinct seasonal occurrence and are a seasonal breeder (Carefoot 1967; Lederhendler et al. 1975; Audesirk 1979; Sarver 1979; Gev et al. 1984; Achituv and Suswein 1985; Pennings 1991; Strenth and Blankenship 1991; Plaut 1993). Spawning period differs according to the geographical position, and spawning event showed species specificity (Usuki 1970). The reproductive activity of aplysiids is controlled by nuroendocrine regulation (Kupfermann 1967; Strumwasser et al. 1969; Pinsker and Dudek 1977) and environmental factor, water temperature (Pinsker and Parsons 1985; Wayne and Block 1992; Wayne et al. 1996). Thus, initial investigations of the reproductive cycle of *Aplysia* spp. were conducted from the standpoint of its neurohormonal control.

As described above, the reproductive strategies of *Aplysia* spp. includes species specificity, and due to the variations in their ecological distribution, their morphological and biological characteristics are different. Therefore, studies on the reproduction of *Aplysia* are essential in order to gain an understanding of their ecological roles. The

present study investigated the reproductive cycle based on monthly changes of gonadosomatic index, gametogenesis, and stage of gonadal development in order to characterize the reproductive biology of *A. kurodai* inhabiting the coastal waters of Jeju Island, Korea.

2. Materials and Methods

A. kurodai were sampled by scuba diving at a depth of 3–9 meters in the coastal waters of Hamdeok, northeast of Jeju Island, Korea. Samples were harvested monthly from December 2002 to January 2004, but were not harvested during periods from October 2003 to January 2004. For histological analysis on the ovotestis development the animal anaesthetized in 10% $MgCl_2$ (Sigma Chemical Co., USA), thereafter, pieces of the ovotestis were fixed in Bouin's solution and then embedded in paraffin. Paraffin-embedded gonadal tissues were sectioned at 5–6 μm and stained with Hansen's haematoxylin and 0.5% eosin, and the specimens were examined under a light microscope.

The gonadosomatic index (GSI) was calculated for each individual, by using the following equation ($GSI=W_{ovotestis}/W_{body} \times 100$).

Where $W_{ovotestis}$ is weight of ovotestis and W_{body} is total weight of body. The developmental stage of ovotestis was grouped following as; inactive, active, and mature and spawning stage. Statistical analysis was conducted using the computer package SYSAT (SPSS, IL USA) and differences between means were determined using Tukey's test.

3. Results

3. 1. Monthly changes of gonadosomatic index (GSI)

The GSI was lower between December and February and ranged 0.14 ±0.06 to 0.23±0.03%. Subsequently, the GSI began to increase and reached 1.37±0.30% in April. The GSI then slightly decreased and it increased again, reached maximum values (2.12±0.11%) in July. Then, GSI decreased from August to September. The specimen was not harvested from October 2003 to January 2004.

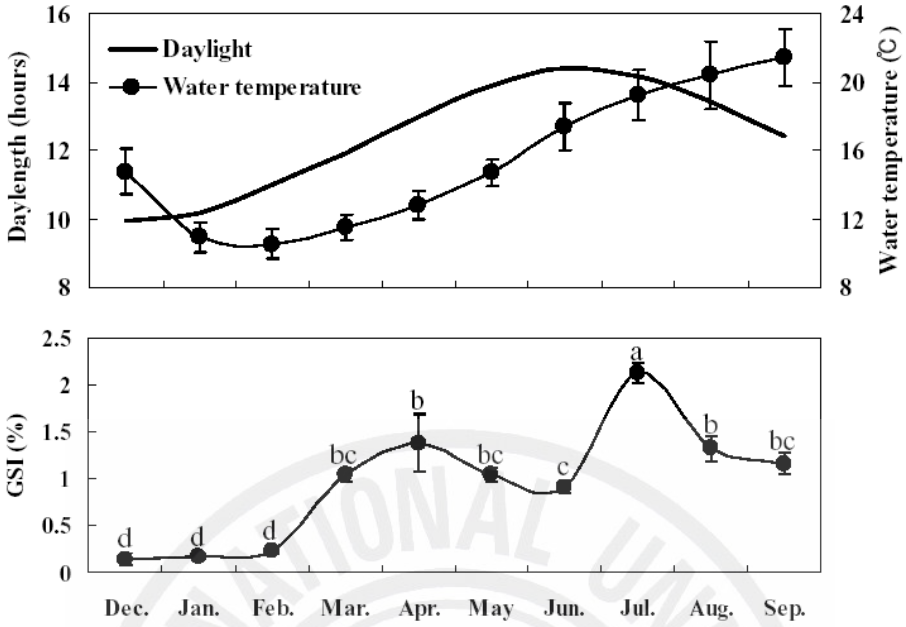

Fig. 1. Monthly changes in daylength, water temperature, and gonadosomatic index (GSI). Different letters indicate difference between monthly significant ($P < 0.05$).

3. 2. Gametogenesis

The ovotestis was composed of a large number of follicles, and the follicles were surrounded by basement membrane. Both oocytes and sperm were produced in the same follicles. The unyolked oocytes were approximately 5.0–7.5 μm in diameter, and had a small cytoplasmic volume, since the 4.0–6.0 μm nucleus occupies the majority of the intracellular space. Unyolked oocytes were mainly observed in the basement membrane of follicle (Fig. 2A). Unyolked oocytes accumulated uniform granular material in the cytoplasm and attained a diameter of approximately 15.0 μm, containing a voluminous nucleus with a nucleolus (Fig. 2B). The early growing oocytes were approximately 30.0–40.0 μm in diameter, and their cytoplasm began to accumulate yolk granules (Fig. 2C). The mature oocytes were approximately 62.5–75.0 μm in diameter, with numerous yolk granules homogeneously distributed in the cytoplasm (Fig. 2D). Thereafter mature oocytes were released from the ovotestis via small hermaphroditic duct, and the undischarged oocytes degenerate and follicles were constricted (Fig. 2E).

The spermatocytes and spermatids clustered around unyolked oocytes or inside the basement membrane of follicle (Fig. 3A). As the ovotestis develops, numerous spermatozoa occupied the lumen of follicle (Fig. 3B). The mature sperm were collected into a division of the small hermaphroditic duct and were stored prior to copulation (Fig. 3C). Thereafter, the undischarged spermatozoa degenerated, and the follicles were constricted (Fig. 3D).

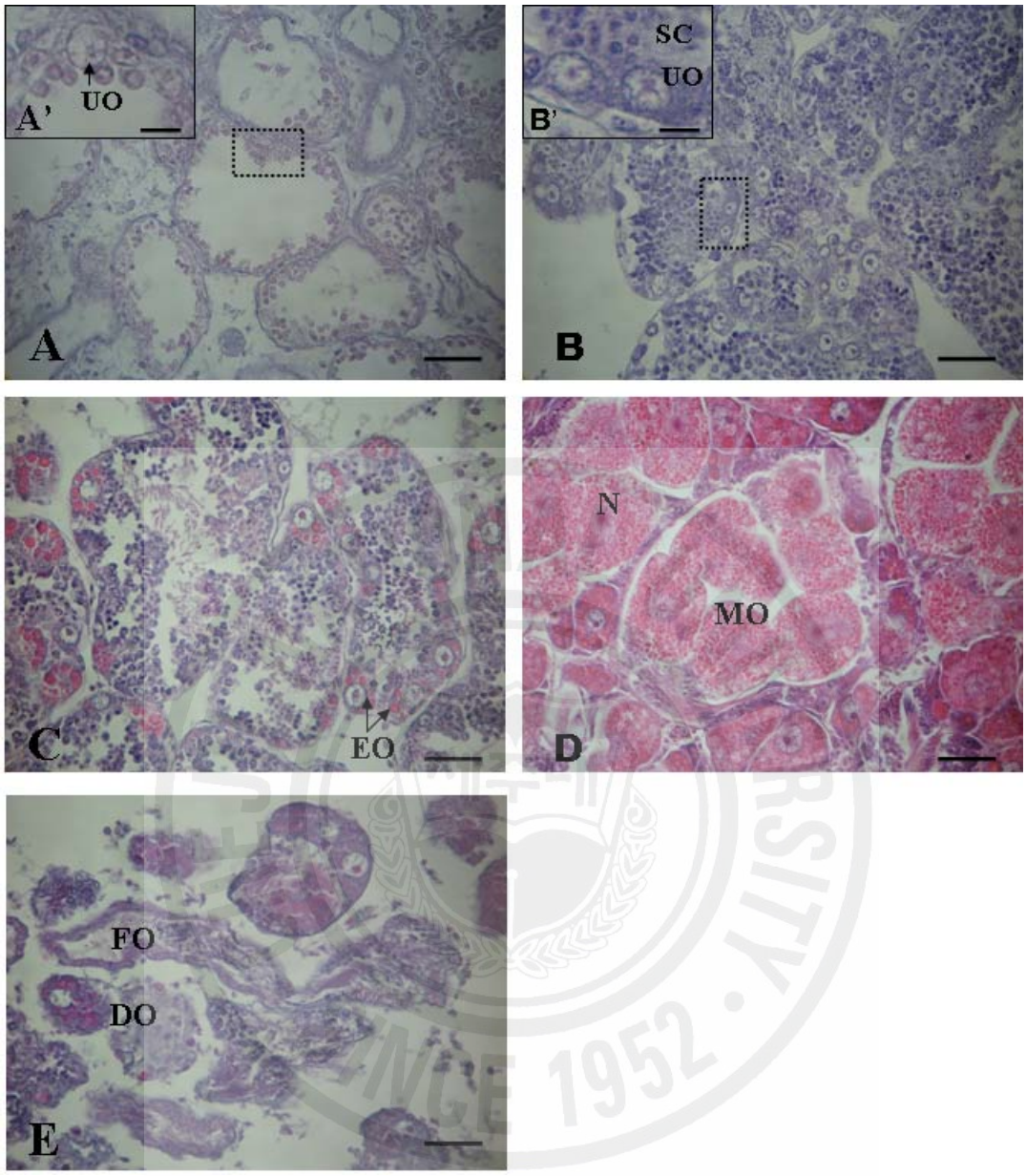

Fig. 2. Oogenesis of *Aplysia kurodai*. A and B, unyolked oocytes stage. C, early growing oocytes stage. D, mature oocytes stage. E, degeneration of undischarged oocytes. DO, degenerative oocyte; N, nucleus; MO, mature oocyte; YG, yolk granule; FO, follicle; UO, unyolked oocyte. Scale bars indicate 50 μm. Inset of A and B showing unyolked oocytes in basement of follicle. Scale bars indicates 5 μm.

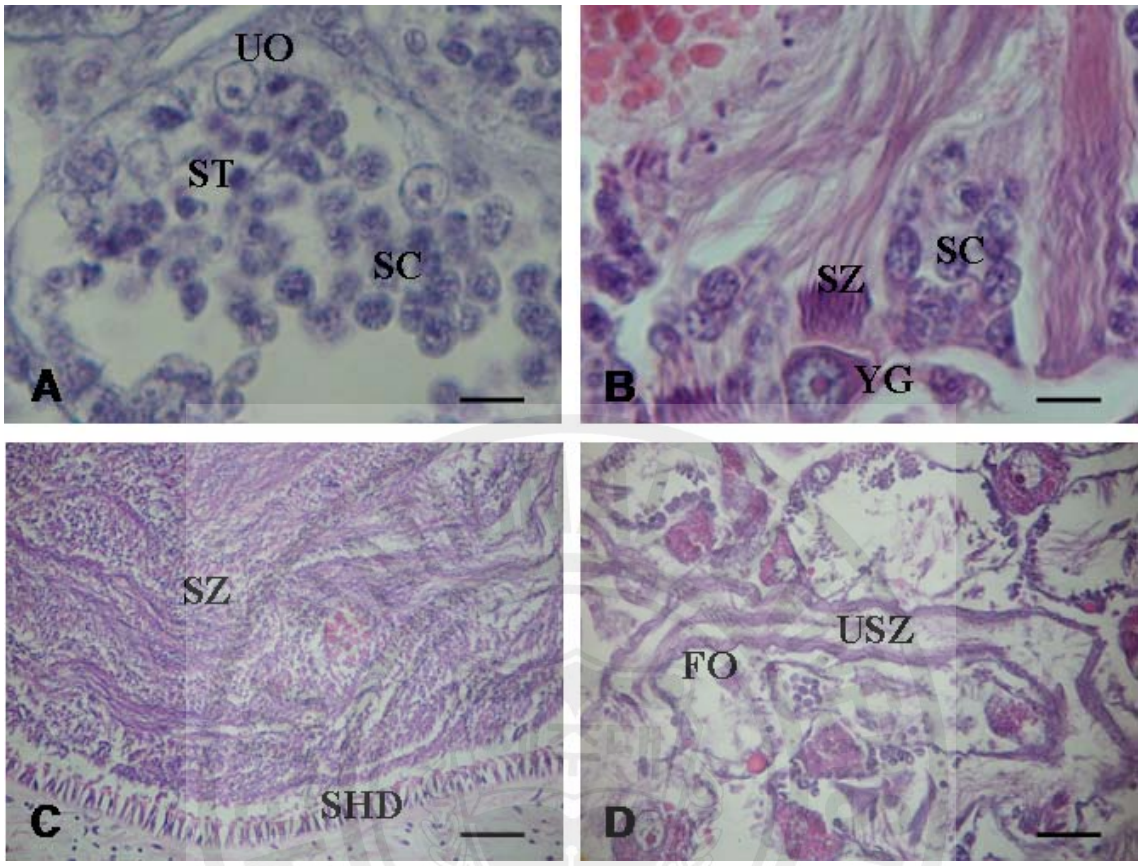

Fig. 3. Spermatogenesis of *Aplysia kurodai*. A, spermatocytes and spermatids stage. B, spermatozoa stage. C, numerous spermatozoa within a division of the small hermaphroditic duct. D, degeneration of undischarged spermatozoa. FO, follicle; SC, spermatocytes; SHD, small hermaphroditic duct; ST, spermatids; SZ, spermatozoa; YG, young oocytes; USZ, undischarged spermatozoa. Scale bars indicates 10 μm (A and B) and 50 μm (C and D).

3. 3. Reproductive cycle

Based on gametogenesis and the stages of gonadal development, the reproductive cycle of *A. kurodai* can be divided into three stages: inactive, active, and mature and spawning (Fig. 4, 5).

3. 3. 1. Inactive stage

The ovotestis of inactive stage had a few unyolked oocytes with about 5.0–7.5 μm in diameter and sperm bundles in follicle, but most of the follicles were emptied (Fig. 4A). This stage was observed from December to February (Fig. 5).

3. 3. 2. Active stage

The ovotestis of active stage had unyolked ocytes and a few early growing oocytes. The early growing oocytes were about 20.0–30.0 μm in diameter and began to accumulate yolk granules in their cytoplasm. Also, the follicle was observed spermatocytes, spermatids and sperm bundles (Fig. 4B). This stage was observed from December to April (Fig. 5).

3. 3. 3. Mature and spawning stage

The ovotestis of mature and spawning stage had a few early growing oocytes but mature oocytes occupied the majority of the lumen. The mature oocytes were approximately 62.5–75.0 μm in diameter with numerous yolk granules that were homogeneously distributed in the cytoplasm. The follicles were also contained spermatocytes, spermatids and sperm bundles (Fig. 4C). Particularly, a numerous sperm could be observed within a division of the small hermaphroditic duct (Fig. 4D). This stage was observed from April to September (Fig. 5).

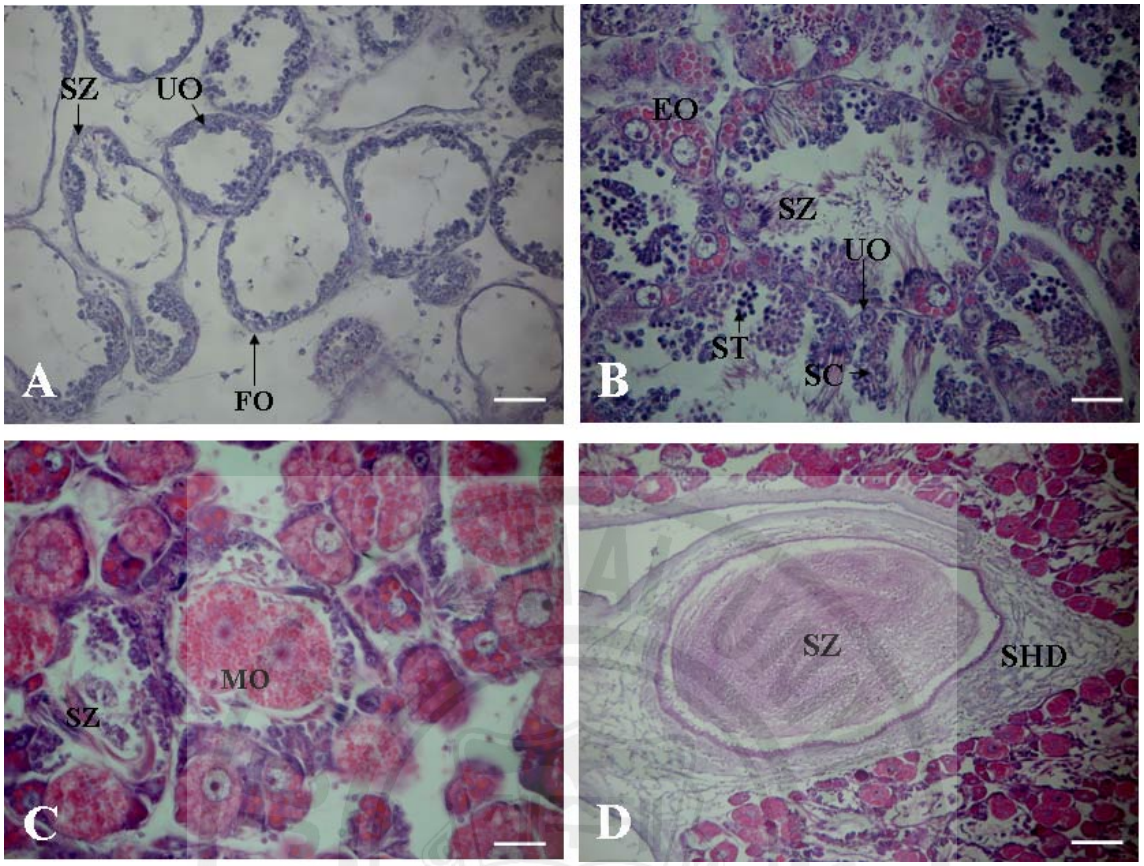

Fig. 4. Histological sections according to developmental ovotestis. A, inactive stage. B, active stage. C, mature and spawning stage. D, numerous sperm within a division of the small hermaphroditic duct. EO, early growing oocyte; FO, follicle; MO, mature oocyte; SHD, small SO, spermatocyte; ST, spermatid; SZ, spermatozoa. UO, unyolked oocyte. Scale bars indicate 40 μm (A to C) and 100 μm (D).

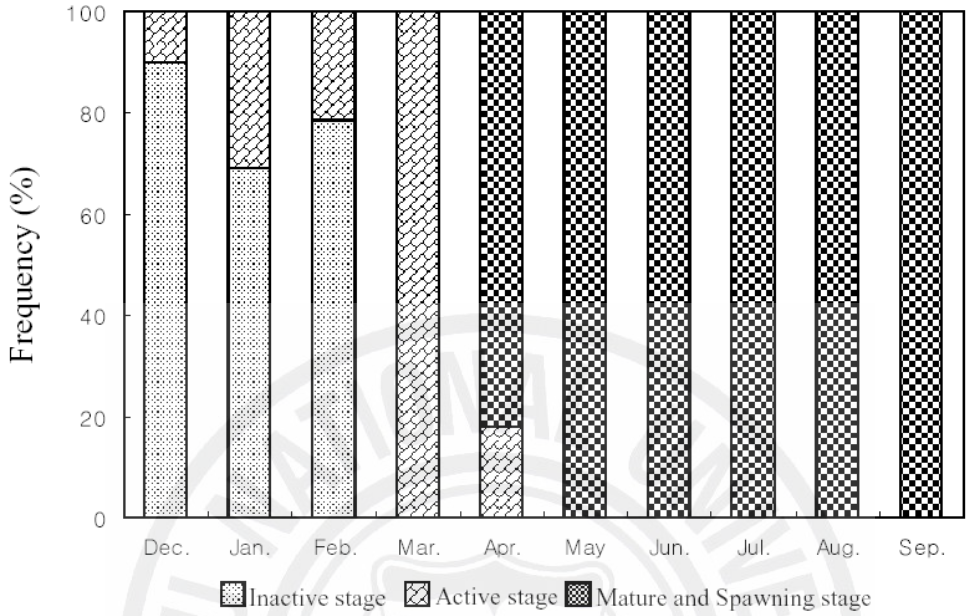

Fig. 5. Frequency of ovotestis developmental phase of *Aplysia kurodai* from December 2002 to September 2003.

4. Discussion

Aplysia spp. are simultaneous hermaphrodite, i.e., adult animals having a functional female as well as male reproductive system, and lay egg masses by internal cross-fertilization after copulation (Kandel 1979; Beeman 1970; Blankenship et al. 1983; Switzer-Dunlap et al. 1984). Most of the previous studies on the reproduction of *Aplysia* spp. have reported the periodicity of occurrence and high variability in abundance and population structure at different sites (Usuki 1970; Susswein et al. 1987; Pennings 1991; Strenth and Blankenship 1991). Particularly, the adult *Aplysia* population has characterized that seasonal pattern present during only 5–6 months every year and this pattern is related with abundance of food such as algae (Usuki 1970; Sarver 1979; Audesirk 1979; Gev et al. 1984; Susswein et al. 1987; Strenth and Blankenship 1991). For instance, adult *A. oculifera* inhabit the intertidal and subtidal zones for 5 months every year, during winter and spring, when the green algae *Enteromorpha intestinalis* and *Ulva* spp. are abundant (Susswein et al. 1987; Plaut 1993; Plaut et al. 1998). This supports the results of Plaut et al. (1995) that these algae induce the settlement and metamorphosis of *A. oculifera* larvae. Strenth and Blankenship (1991) suggested that *A. brasiliana* larvae metamorphosis in the presence of the red alga *Callithamnion*, and the seasonal peak of this alga could be a major contributing factor in the timing of the spring recruitment of juvenile *A. brasiliana*. In this study, the occurrence of *A. kurodai* followed seasonal pattern, i.e., the adults were present from December 2002 to September 2003, but we failed to collect any specimens from October 2003 to January 2004. Carefoot (1987) suggested that the disappearance of the populations during a part of the year could be related to many factors. First,

recruits of the small body size, which becomes distinguished only after a long growth period. Second, larvae delay their settlement and metamorphosis until they find suitable conditions that will allow successful development of the adult populations. Third, adult animal migrate from the intertidal zone to more distant locations in deeper waters. In the case of *A. kurodai*, the seasonal pattern of occurrence may be due to relatively long larval period and ability to delay metamorphosis (as described Chapter 3).

The aplysiids are a seasonal breeder and the reproductive activity is controlled by nuroendocrine regulation (Kupfermann 1967; Strumwasser et al. 1969; Pinsker and Dudek 1977) and environmental factor, water temperature (Pinsker and Parsons 1985; Wayne and Block 1992; Wayne et al. 1996). The reproductive season is also related to the geographic distribution of the species and its specific reproductive strategy (Usuki 1970). In *Aplysia* spp. the period of spawning is relatively long; for instance, spawning of *A. californica* in southern California occurs between late spring and early fall with a peak in summer, and the increasing water temperature in spring provides a synchronizing cue for the initiation of gonadal development (Audesirk 1979). Usuki (1970) suggested that *A. kurodai*, *A. parvula* and *A. juliana* found in the Sado of the Japan have almost similar spawning patterns, two distinct spawning periods in a year. The spawning periods of these species are quite similar, and include the periods from April to July and that from November to December or January. However, the spawning period in each species was determined on base of discovery of the egg masses in the field or aquaria.

The gonadal development of *A. kurodai* coincided with rising temperature in spring, and kept on spawning from April to September during warm seasion. The structure and developmental tendencies of ovotestis in *A. kurodai* was similar to those

previously reported for other *Aplysia* spp. (Beeman 1970; Dudek et al. 1980). The ovotestis of *A. kurodai* is composed of numerous follicles, and oocytes and sperm are produced in the same follicles. However, sperm maturation just preceded oocytes maturation. The mature sperm is then stored in the ampulla prior to copulation, after which they entered into the seminal receptacle of the other animal and stayed until the oocytes mature (as described Chapter 1). These results suggested that the spawning of *A. kurodai* does not always coincide with copulation, and inconsistency of oocytes and sperm development in *A. kurodai* is specific reproductive strategy.

CHAPTER Ⅲ

Larval development and metamorphosis of *Aplysia kurodai*

Abstract

This study investigated (1) whether the larval settlement and metamorphosis of *Aplysia kurodai* is a specific response or a non-specific response and (2) the prey source after the larvae have been induced to metamorphose, and also described the development of *A. kurodai* from its larvae stage to the attainment of reproductive maturity.

The fertilized eggs of the *A. kurodai* were packaged in capsules that were embedded in layers of mucopolysaccharide jelly to form a cylindrical string called an egg masses. The larval age for attainment of metamorphic competent was at 80 days after hatching, and shell size was approximately 418±7.9 μm, and the red spots had appeared at this time. Thereafter, the larvae did not grow further before settlement and metamorphosis, and their shell size remained constant. The long-surviving larvae maintained the ability to metamorphosis, and spontaneous metamorphosis was not observed. The maximum longevity of the larvae was 380 days after hatching. The larvae of *A. kurodai* also induced to metamorphose all red and green algae, but not brown algae, among the tested algae. This result suggested that larvae of *A. kurodai* settlement and metamorphosis appear to be less specificity or non-specific substrate. The post-metamorphic larvae feed only specific algae (*Polysiphonia morrowii* and *Centroceras clavulatum*) and develop into adult and attained sexual maturity within 3 to 4 months after metamorphosis and began to laying egg mass.

1. Introduction

Although most gastropods exhibit gonochorism, some species such as opithobranchia and pulmonatea generally are functional simultaneous hermaphrodites. In simultaneous hermaphrodites, the reproductive systems are composed of form a single gonad, i.e., the ovotestis and a common genital gonoduct. These animals simultaneously produce eggs and sperm but do not normally self-fertilize; they cross-fertilization by copulation with another individual's sperm (Hadfield and Switzer-Dunlap 1984). Many gastropod species in the intertidal zone enclose their fertilized eggs within capsular or gelatinous egg masses to provide protection against extreme changes in the surrounding environment, such as desiccation, temperature, salinity, ultraviolet radiation and water flow (Pawlings 1999; Przeslawski 2004; Przeslawski and Benkendorff 2005). The encapsulation of fertilized eggs is a common phenomenon among many marine invertebrate groups, and the structure and composition of egg masses differ among species.

Most marine invertebrates develop into adults via settlement and metamorphic stage from planktonic larva. The planktonic stage of the larvae may range minutes to day and sometimes, they may remain in the larval phases for months before settling on suitable substrata for juvenile growth and reproduction (Kriegstein et al. 1974; Lewis 1978). Larval settlement and metamorphosis are important stage during the development of the larvae into reproductive adult and are influenced by chemical, physical, and biological cues in the environment (Crisp 1974; Hadfield 1977; Burke 1983; Hadfield and Scheuer 1985; Bonar et al. 1990; Hadfield and Paul 2001).

The settlement cues originate from a variety of sources, including conspecific individuals, suitable substrate surfaces, specific prey, and films of microorganisms. The chemical settlement cues can be divided into the cues related gregarious settlement and those related to associative settlement (Pawlik 1992). Gregarious settlement responds to specific chemical cues is associated with conspecific juvenile or adults (Pawlik 1992; Toonen and Pawlik 1994). Associative settlement responds to cues is associated with non-conspecific, prey, symbiont, and requisite substratum (Hadfield 1984; Hadfield and Koehl 2004). It has bee demonstrated that during associative settlement chemical cues originating form algae induce the settlement and metamorphosis among species such as tubeworm, chitons, asteroids, echinoderms, corals and gastropods (Morse 1992; Boettcher and Targett 1996; Huggett et al. 2005).

Exception of *Phyllaplysia taylori* which develops directly, most of aplysiids develop through planktotrophic larvae, which must feed on microalgae to develop into competent metamorphosed forms (Bridges 1975; Kriegstein et al. 1974). Aplysiid larvae can prolong their larval period until they encounter suitable substrata for settlement and metamorphosis (Kempf 1981). Regarding larval settlement and metamorphosis of aplysiids, it has been shown that when the larvae of *Aplysia californica* are exposed to a variety of red algae (*Plocamiun* sp., *Laurencia* sp., *Polysiphonia* sp., *Daysia* sp., *Chondrus* sp.) and green alga (*Ulva* sp.), they preferentially crawled on *Laurencia* and metamorphosed only on it (Kriegstein et al. 1974). Also Switzer-Dunlap and Hadfield (1977) suggested that four species of aplysiidae including *A. juliana*, *A. dactylomela*, *Dolabella auricularia* and *Stylocheilus longicauda* metamorphose preferentially on a particular species of benthic algae. However, Pawlik (1989) reported that *A. californica* metamorphosed on all the examined algae (9 red algae, 7 brown algae and 2 green

algae). The larvae of *A. oculifera* induced metamorphosis on red and green algae, but not on brown algae (Plaut et al. 1995). Larval settlement and metamorphosis of aplysiids have revealed differences among species and have reported specific or less specific responses to algae; however, little is known regarding the larval biology and metamorphosis of aplysiids. Larval settlement and metamorphosis of *A. californica* and *A. oculifera* was less specificity than had been previously reported; however, after metamorphosis, the prey was highly specificity (Pawlik 1989; Plaut et al. 1995).

The several aplysiids have provided information on settlement and metamorphosis. In particular, the life cycle of *A. californica* is well documented in the National Institute of Health (NIH), National Center for Research Resources (NCRR), National Resource for *Aplysia* at the University of Miami; however, the settlement, metamorphosis and growth of *A. kurodai* remain uninvestigated.

The present study was investigated (1) whether the larval settlement and metamorphosis of *A. kurodai* is a specific response or a non-specific response and (2) the prey source after the larvae have been induced to metamorphose, and also described the development of *A. kurodai* from its larvae stage to the attainment of reproductive maturity.

2. Materials and Methods

2. 1. Rearing conditions

Adult *A. kurodai* were collected in the intertidal zone along the coastal waters of the Hamduk, northeast of Jeju Island, Korea, between March and August during each year from 2003 to 2006. Adult animals were kept in 5 ton aquaria with an open seawater circulation system, and fed daily fresh algae (*Ulva* sp.). Newly spawned egg masses were harvested from the aquaria, rinsed, and inserted into 1-L flasks containing filtered seawater with filter holes diameter 0.45 μm under continuous aeration. The filtered seawater was daily changed and was maintained at room temperature (20±0.5℃).

2. 2. Larval culture

The hatched larvae were placed in a 2-L bottle containing the culture medium. The culture medium was prepared from fresh filtered seawater containing 60 $\mu g \cdot ml^{-1}$ penicillin G, 50 $\mu g \cdot ml^{-1}$ streptomycin sulfate (Sigma Chemical Co., USA), and 10^4 $cells \cdot ml^{-1}$ of the unicellular algae (*Isochrysis galbana*). The bottle was filled to the brim and covered with parafilm to exclude air. The culture bottles were placed on a continuously rotating roller culture apparatus (Wheaton Industries Inc., USA) at a constant temperature of 20±0.5℃. Once a week, the culture medium was filtered through various mesh sizes (60–250 μm) in order to retain the larvae of different sizes. The larvae were placed in 5 ml PVP-Ⅰ (2–5 $mg \cdot ml^{-1}$) for 10 min. During this time, the shell length (SL) of 20–30 larvae from each bottle was measured using an ocular micrometer, and the larvae were returned to fresh culture medium.

2. 3. Metamorphosis induction

2. 3. 1. Metamorphic competence

The larval age of *A. kurodai* in relation to the metamorphic competence was researched following the metamorphosis induce of aplysiids reported by Kriegstein et al. (1974), Pawlik (1989), and Plaut et al. (1995). From 40 days after hatching, settlement and metamorphic competence of the larvae were examined at interval of 10-days until metamorphosis occurred. The macroalgae used in this experiment were green (*Ulva* sp. and *Enteromorpha linza*), brown (*Sargassum thunbergii*), and red (*Gelidium amansii* and *Chondria crassicaulis*) algae. Twenty larvae were placed in 50-mL conical tube with filtered seawater and 0.15–0.20 g of each alga. The larvae were considered metamorphosed when they lost velar cilia and crawling behavior. Each experiment was replicated three times. There experiments were repeated five to ten times for each algae species.

2. 3. 2. Metamorphosis of larvae by marine macroalgae

Fifteen algae species from the natural habitat of adult sea hares were examined as metamorphosis inducers for *A. kurodai* larvae. The macroalgae used in this experiment were green (*Ulva* sp., *Enteromorpha linza* and *Codium* sp.), brown (*Ecklonia cava*, *Undaria pinnatifida* and *Sargassum thunbergii*) and red (*Gelidium divaricatum*, *Gelidium amansii*, *Callophyllis japonica*, *Hypnea* sp., *Ahnfeltisopsis flabelliformis*, *Lomentaria hakodatensis*, *Centroceras clavulatum*, *Laurencia* sp. and *Polysiphonia morrowii*). To induce metamorphosis, twenty larvae (SL > 400 μm, age > 90 d) were placed in 50-mL conical centrifuge tube containing filtered seawater and 0.15–0.20 g of

the test algae. The control tube contained only filtered seawater. Each experiment was replicated three times. There experiments were repeated three to five times for each algae species.

2. 3. 3. Ability to delay metamorphosis

Ability of delay metamorphosis of *A. kurodai* larvae were researched following the metamorphosis induces Experiment 2.3.1 and 2.3.2. This experiment used age of *A. kurodai* larvae were 100, 150, 200, 250, 300 and 350 days after hatching. The macroalgae used in this experiment were red algae (*Polysiphonia morrowii*). Twenty larvae were placed in 50-mL conical centrifuge tube which contained filtered seawater and 0.15–0.20 g of the test algae. Larvae were counted metamorphosis when they lost velar cilia and crawling behavior. Each experiment was replicated three times.

2. 4. Development and morphological changes after metamorphosis

Development and morphological change after metamorphosis of *A. kurodai* was described on the basis of behavioral and morphological characteristics visible in living specimens. According to categorization described by Kriegstein (1977), the developmental stage of *A. kurodai* classified into six stages: fusion of the velar lobes, development of pink color, appearance of the parapodia, development of the rhinophores, development of the genital groove, and sexually maturity stage.

The sexually maturity of juvenile was confirmed histological observation of ovotestis, reproductive behavior and deposited egg masses.

2. 5. Statistical analysis

Statistical analysis was conducted using the computer package SYSAT (SPSS, IL USA) and differences between means were determined using Tukey's test. Results are given as mean ± standard error.

3. Results

3. 1. Reproductive behavior and spawning characteristics

A. kurodai produced fertilized egg by internal fertilization via copulation. Unilateral copulation occurred in *A. kurodai*, i.e., both animals of a pair facing in the same direction. When a pair of *A. kurodai* copulated, the first animal (A) attaching to the substrate acted as a female, and the second animal (B) that is in close contact with the dorsal surfaces of A acted as a male. Then, B protruded penis and inserted it into the common genital aperture of A and sperm was released (Fig. 1A). Frequently, if the other animals join the initial copulating pair, a coupling chain was formed and only the first animal acted as a female, deposited egg string on the walls of the rearing tank (Fig. 1B).

3. 2. Morphological feature of egg mass

The fertilized eggs were packaged in capsules that were embedded in jelly to form a cylindrical string called an egg masses (Fig. 2A). The number of capsule per cm of the egg masses was 55 to 60 capsules and each capsule within the egg masses held 15 to 25 eggs (Fig. 2B, C).

After spawning, the egg masses were bright yellow or orange in color (Fig. 3A). This egg masses color not changed until embryos developed into trochophore stage (Fig. 3B). Thereafter, as embryo developed from trochophore stage to veliger stage the egg masses color became brownish (Fig. 3C, D).

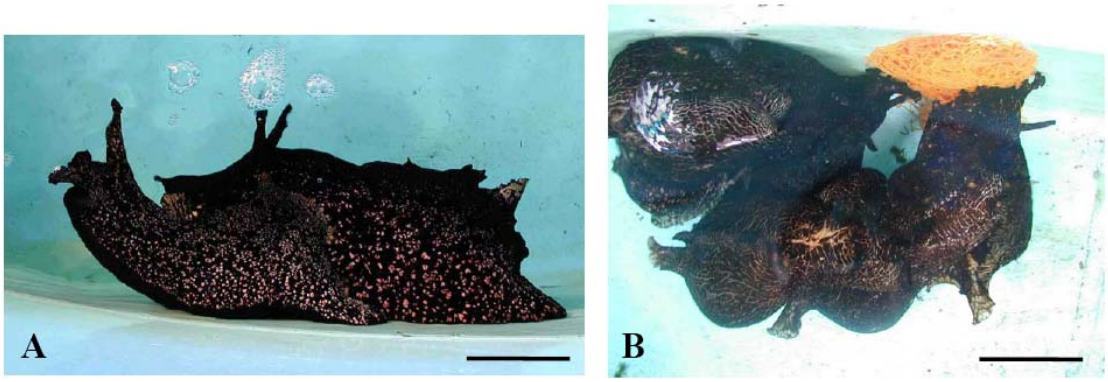

Fig. 1. Copulation and egg masses of *Apysia kurodai* in rearing tank. A, copulation a pair of *A. kurodai*. B, formation of coupling chain. Scale bars indicate 10.0 cm.

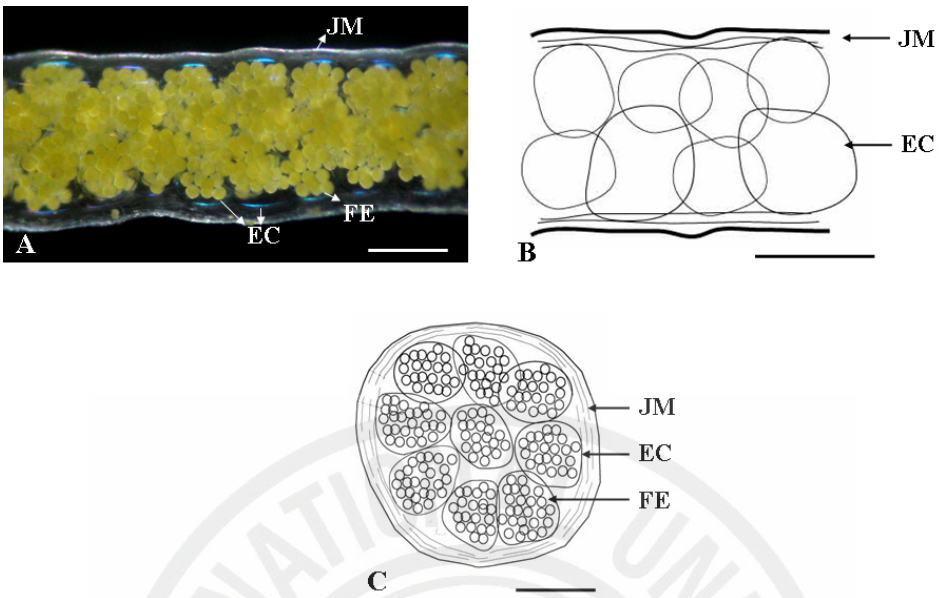

Fig. 2. Egg masses of *Aplysia kurodai*. A, morphological feature of egg masses. B and C, schematic outlines of egg masses. FE, fertilized egg; Ec, egg capsule; JM, Jelly metrix. Scale bars indicate 500 μm.

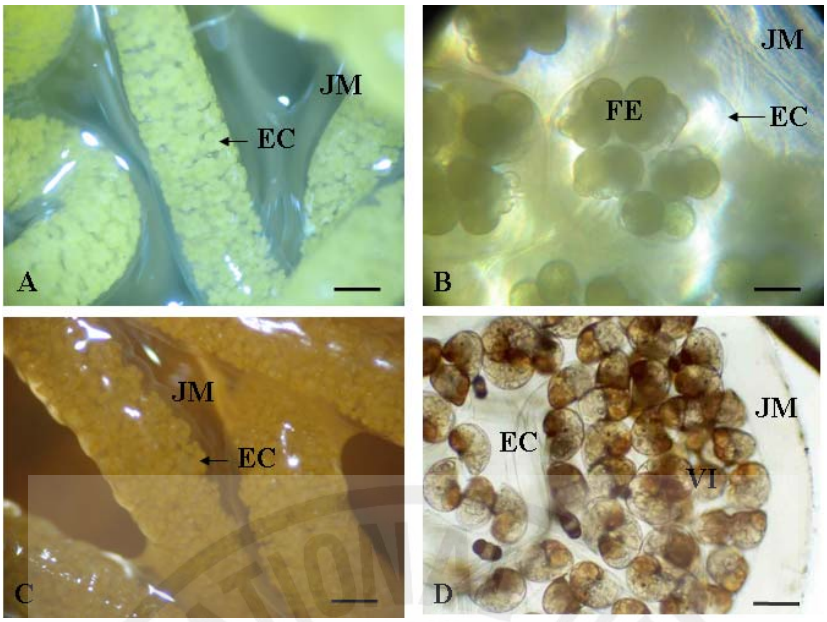

Fig. 3. Change of egg masses in color according to developmental stage. A and B, egg masses just after spawning. C and D, egg masses of 10 days after spawning. FE, fertilized egg; EC, egg capsule; JM, jelly matrix; VI, veliger. Scale bars indicate 200 μm (A and C) and 100 μm (B and D).

3. 3. Embryogenesis

The fertilized eggs were spherical, with a diameter of approximately 80±1 μm at spawning (Fig. 4A). The cell division underwent unequal spiral cleavage. The first cleavage passed through opposite poles of the embryo and split it into two unequal blastomeres and the formation of a compact 2-cell embryo took about 12hr after spawning (Fig. 4B). In the second cleavage, the cleavage of the two blastomeres was not synchronous; that of the smaller blastomere occurred at 5hr after the formation of 2 cell embryo and that of the larger one occurred 1hr later (Fig. 4C). Thereafter, the formation of a compact 4-cell embryo took about 17hr after spawning (Fig. 4D). The third cleavage occurred spirally, and the four small blastomeres were divided from the animal pole of each large blastomeres. These small blastomeres called the first quartet. The formation a compact 8-cell embryo occurred at 6hr after the formation of 4-cell embryo and took about 23hr after spawning (Fig. 4E). The fourth cleavage occurred in a counterclockwise direction, and the four large blastomeres divided into four small blastomers, which called the second quartet. The first quartet also divided into four small blastomers. The formation a compact 16-cell embryo occurred at 7hr after the formation of 8-cell embryo and took about 30hr after spawning (Fig. 4F).

At 5 to 6 days after spawning, the embryo developed into trochophore stage and began to rotate within the egg capsule (Fig. 5A). In the trochophore stage, the precursor of the velum, called the prototroch or prevelum, developed. At 10 days after spawning, the prevelum is transformed into the velum, and the trochophore developed into veliger stage (Fig. 5B). Between 10 to 15 days after spawning, the veligers broke out of the egg capsule, and hatched as free-swimming larvae (Fig. 5C, D).

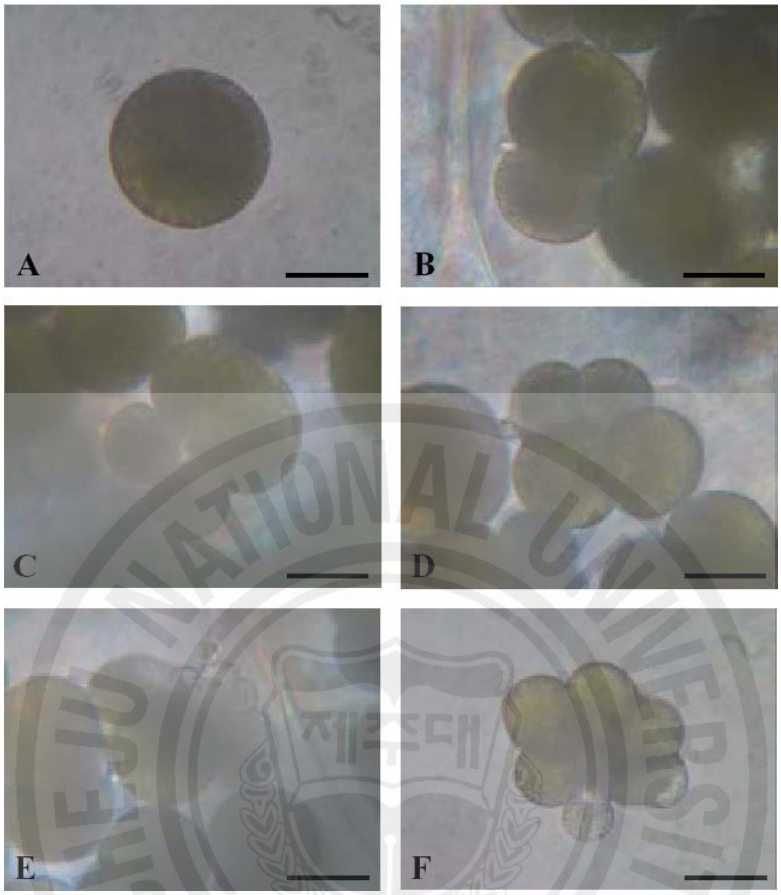

Fig. 4. Early developmental stage of *Aplysia kurodai*. A, fertilized egg. B, 2-cell stage. C, beginning of 4-cell stage. D, 4-cell stage. E, 8-cell stage. F, 16-cell stage. Scale bars indicate 50 μm.

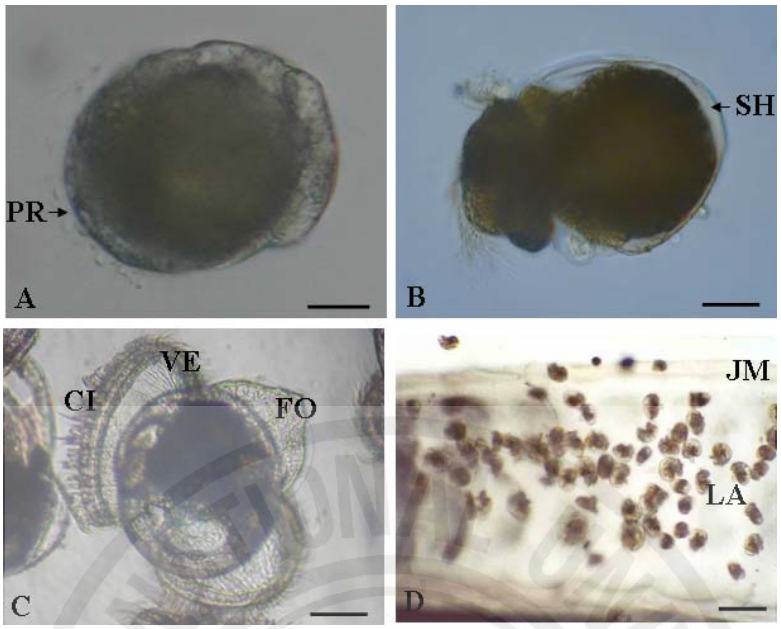

Fig. 5. Early developmental stage of *Aplysia kurodai*. A, trochophore stage. B, veliger stage. C and D, hatching larvae. CI, cilia; FO, foot; JM, jelly matrix; LA; larva; PR, prevelum; SH, shell; VE, velum. Scale bars indicate 20 μm (A to C) and 500 μm (D).

3. 4. Larval development and larval age of metamorphic competence

The size of newly hatched larvae was 107±11.65 μm shell length (SL). Thereafter, the growth of larvae stopped at a maximum 418±8.01 μm SL from 70 days after hatching (DAH). After this time point, the size of the larvae did not increased until metamorphosis or death. If larvae were not exposed to the substrate necessary for metamorphosis, the maximum longevity of larvae was > 380 DAH (Fig. 6).

In the experiment for determining the possible larval age of metamorphosis, it was observed that settlement and metamorphosis of larvae occurred form 80 DAH (Table 1). At this time, 11–15 irregularly shaped red spots appeared on the right side of the outer perivisceral membrane as indicators of competent metamorphosis (Fig. 7).

3. 5. Metamorphosis of larvae by marine macroalgae

The settlement and metamorphosis of larvae was occurred in 11/15 tested algae (Fig. 8). All red algae and 2/3 green algae induced settlement and metamorphosis; however, the brown algae and control did not induced metamorphosis. The metamorphosis rate of *Polysiphonia morrowii* and *Centroceras clavulatum* were 82.7±1.7% and 80.3±4.2%, respectively, and were relatively higher than that of any other algae, although there was no significant difference as compared to *Callophyllis japonica*, *Hypnea* sp., *Ulva* sp. and *Laurencia* sp. (P>0.05). The metamorphosis rate of *Lomentaria hakodatensis* was 3.3±4.7 %, which was relatively lower value than that of any other algae (P<0.05).

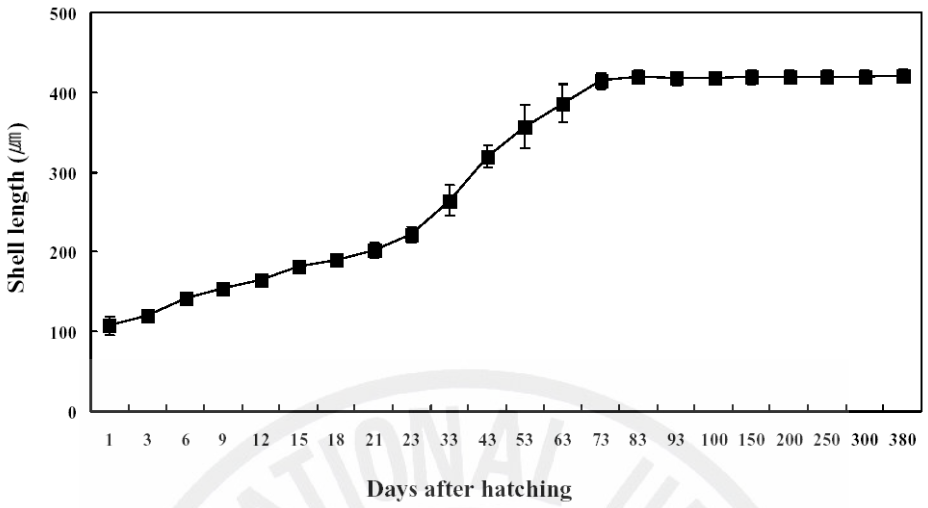

Fig. 6. Larval growth of *Aplysia kurodai* reared in the laboratory, fed on unicellular algae *Isochrysis galbana* at ambient temperature of 20±0.5℃ (mean ± standard error).

Table 1. Larval age of *Aplysia kurodai* in relation to the metamorphic competence

DAH[a]	Shell length (mean ± SE)	Indicator[b]	% of metamorphosis (mean ± SE)		
			Green algae[c]	Brown algae[d]	Red algae[e]
40 d	319.6±13.9 μm	-	-	-	-
50 d	357.1±26.9 μm	-	-	-	-
60 d	386.5±24.1 μm	-	-	-	-
70 d	414.6±10.0 μm	-	-	-	-
80 d	419.2±7.6 μm	+	54.8±5.4	-	50.9±5.3
90 d	417.7±8.3 μm	+	56.7±6.5	-	53.6±6.8

[a]DAH represent days after hatching

[b]Indicator represent red spots as indicators of metamorphic competence

[c]Green algae are *Ulva* sp. and *Enteromorpha intestinalis*

[d]Brown algae are *Sargassum thunbergii* and *Hizikia fusiforme*

[e]Red algae are *Gelidium amansii* and *Chondria crassicaulis.*

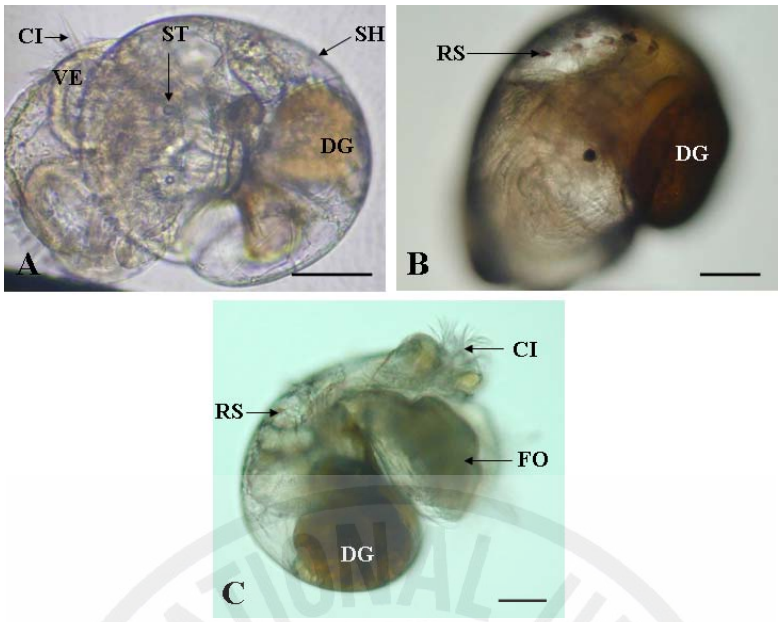

Fig. 7. Appearance of red spot as indicators of metamorphic competent. A, larvae of 70 DAH. B and C, larvae over 80 DAH and irregularly shaped red spots appeared on the right side of the outer perivisceral membrane. CI, cilia; DG, digestive gland; FO, foot; RS, red spots; SH, shell; ST, statocyst; VE, velum. Scale bars indicate 100 μm.

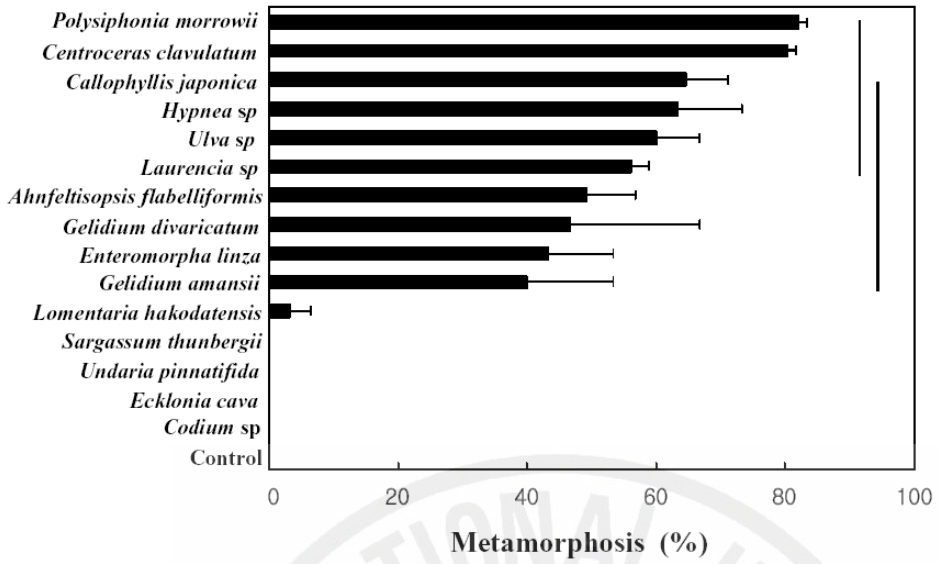

Fig. 8. Metamorphosis induction in larvae by 15 macroalgal species at 90 DAH (mean ± standard error).

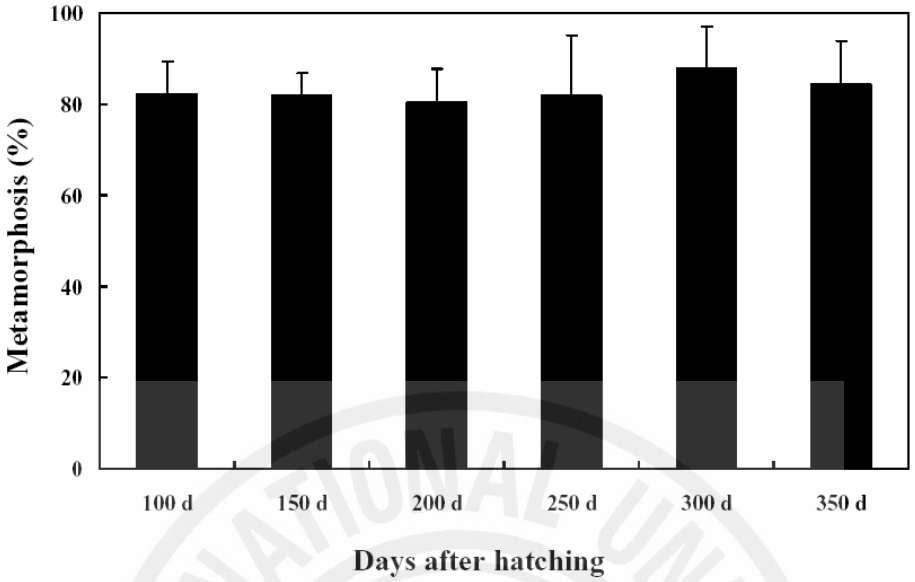

Fig. 9. Ability to delay metamorphosis of *Aplysia kurodai* larvae (mean ± standard error).

At 100, 150, 200, 250, 300 and 350 DAH, the metamorphosis rates were 82.1±7.1, 81.8±5.0, 80.3±7.5, 81.9±13.3, 87.5±9.3 and 84.2±9.4%, respectively (Fig. 9). There was no significant difference in metamorphosis rate according to age (P>0.05).

3. 6. Juvenile development

The larvae that metamorphosed on *P. morrowii* and *C. clavulatum* survived for longer than one month and developed into juvenile. But, the larvae that metamorphosed on other algae species died approximately 7 to 20 days after metamorphosis (DAM).

3. 6. 1. Fusion of velar lobes stage (3 days after metamorphosis)

The size of larvae was approximately 0.40–0.45 mm total length (TL). This stage began to metamorphosis and lost the velum. The larvae shed their velar cilia and stopped swimming and showed crawling behavior. The fused velar lobes gradually extended forward from beneath the shell to form the anterior tentacles. The larvae partially retracted the buccal mass but did not grazed algae, and at the end of this stage animals began to graze on seaweed (Fig. 10A, B).

3. 6. 2. Development of pink color stage (7–10 days after metamorphosis)

The size of larvae increased to approximately 0.9–1.0 mm TL. From this time point, the growth of the shell and body length of the larvae could be observed clearly, and began to turn pink in color (Fig. 11A). At 10 DAM, the size of juveniles increased to appro-ximately 1.2–1.5 mm body length. The body color of the juvenile changed to pink, and the color spread throughout the skin. The metapodium continued to grow, the

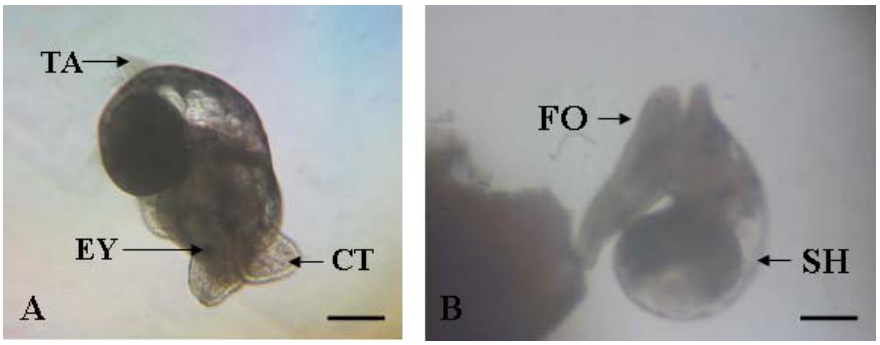

Fig. 10. Fusion of the velar lobes stage. CT, cephalic tentacle; EY, eye; FO, foot; SH, shell; TA, tail. Scale bars indicate 100 μm.

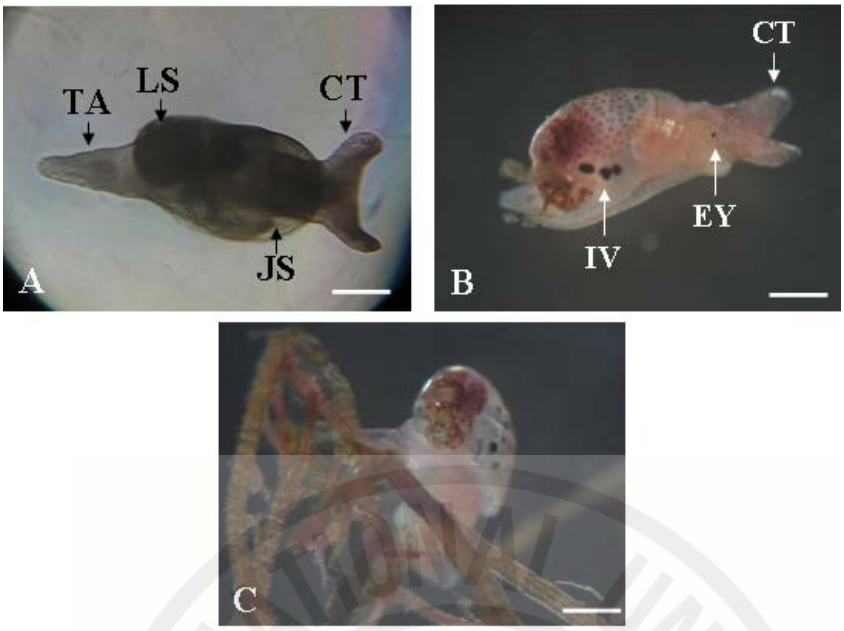

Fig. 11. Development of pink color stage. CT, cephalic tentacle; EY, eye; FO, foot; IV, ink vesicles; JS, juvenile shell; LS, larval shell; TA, tail. Scale bars indicate 150 µm.

color spread throughout the skin. The metapodium continued to grow, and the rudimentary parapodia began to develop as outgrowths of the lateral margins of the metapodium. At the right anterior edge of shell, four to six purple ink vesicles appeared in a row. The red spots located on the right in earlier stages became evenly distributed over the shell. From this stage onward, the animals began to graze on seaweed (Fig. 11B, C).

3. 6. 3. Appearance of parapodia stage (15 days after metamorphosis)

The size of the juveniles increased to approximately 2.0–2.5 mm TL. The body color of juveniles changed completely pink, and the red spots were observed to distribute more evenly over the entire shell. The purple ink vesicles also increased in number. The parapodia grew further and covered half of the shell. White spots appeared in the middle and at the margin of the parapodia and in small clusters at the tip of the anterior tentacles above the eyes (Fig. 12A). At 21 DAM, the size of juveniles increased to approximately 2.1–3.0 mm body length. At this stage the parapodia grew further and completely covered the entire shell. A black band appeared at the margin of parapodia, and the rhinophores began to develop on either side above the eyes (Fig. 12B).

3. 6. 4. Development of rhinophore stage (30 days after metamorphosis)

The size of juveniles increased to approximately 3.0–4.5 mm body length (BL). The white spots on the parapodia and body epidermis appeared and the rhinophores grew further (Fig. 13A). At 40 DAM, the size of juveniles increased to approximately 6.0–7.0 mm body length. The number of white spots on the parapodia and body epidermis increased, and the purple ink vesicles on the shell also increased in number

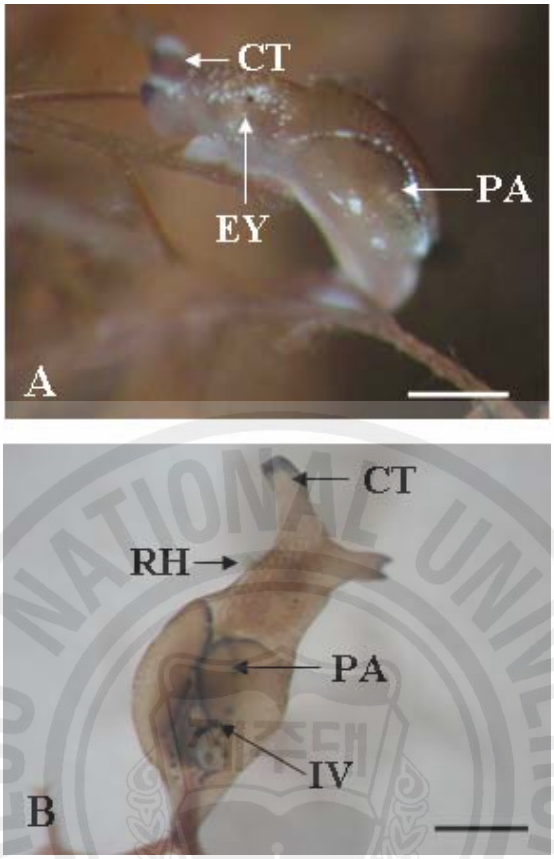

Fig. 12. Development of parapodia stage. BB, black band; CT, cephalic tentacle; EY, eye; IV, ink vesicles; PA, parapodia; RH, rhinophore; WS, white spots. Scale bars indicate 1.0 cm.

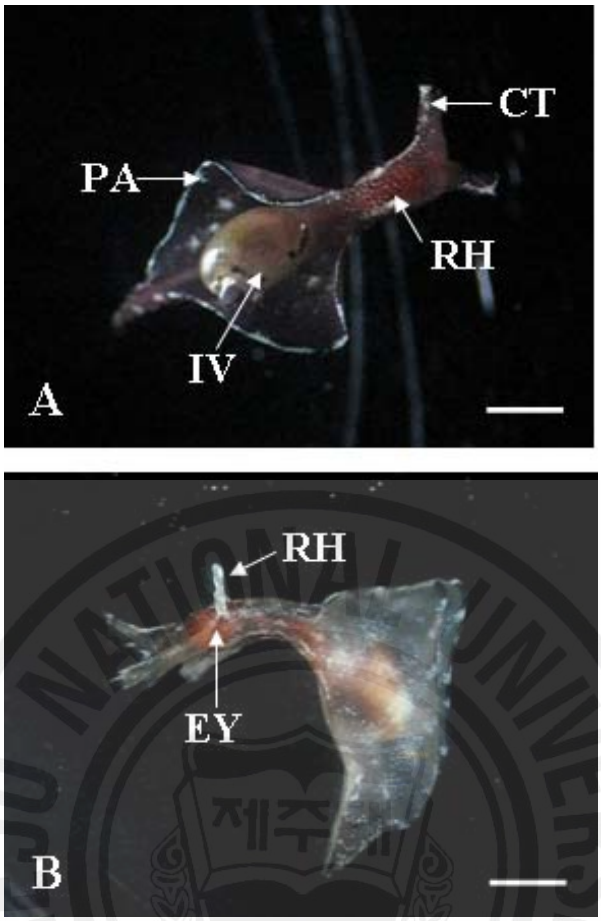

Fig. 13. Development of rhinophore stage. CT, cephalic tentacle; EY, eye; IV, ink vesicles; PA, parapodia; RH, rhinophore. Scale bars indicate 1.0 cm.

(Fig. 13B).

3. 6. 5. Development of genital groove stage (60 days after metamorphosis)

The size of the juveniles increased to approximately 10.0–12.0 mm body length. The genital groove was located on the right of the head. The white spots were observed to form well-defined clusters on the body epidermis, parapodia, and head. The body color of animal began to change to brown (Fig. 14A, C).

3. 6. 6. Sexual maturity stage (90 to 120 days after metamorphosis)

The size of juveniles increased to approximately 60.0–70.0 mm body length. The body color of the animal changed to brown, and it appeared like an adult animal (Fig. 15A). At this stage the ovotestis composed of mature oocytes and sperm. The mature oocytes are about 62.5-75.0 μm in diameter, with numerous yolk granules homogeneously distributed in the cytoplasm. The mature sperm were collected through a branching net of ciliated small hermaphroditic duct (Fig. 15B). The animals began to copulate and spawn eggs. The number of capsules per cm of egg-string was 60 to 100 capsules and each capsule within the egg-string hold 1 to 5 eggs (Fig. 15C, D). At spawning, the diameter of the embryo was approximately 80±1 μm.

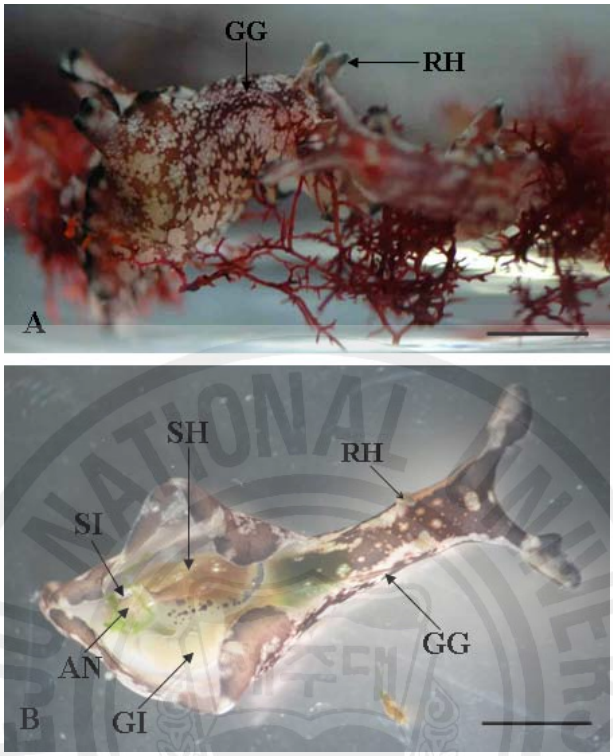

Fig. 14. Development of genital groove stage. AN, anus; GG, genital groove; GI, gill; RH, rhinophore; SI, siphon; SH, shell. Scale bars indicate 2.0 cm.

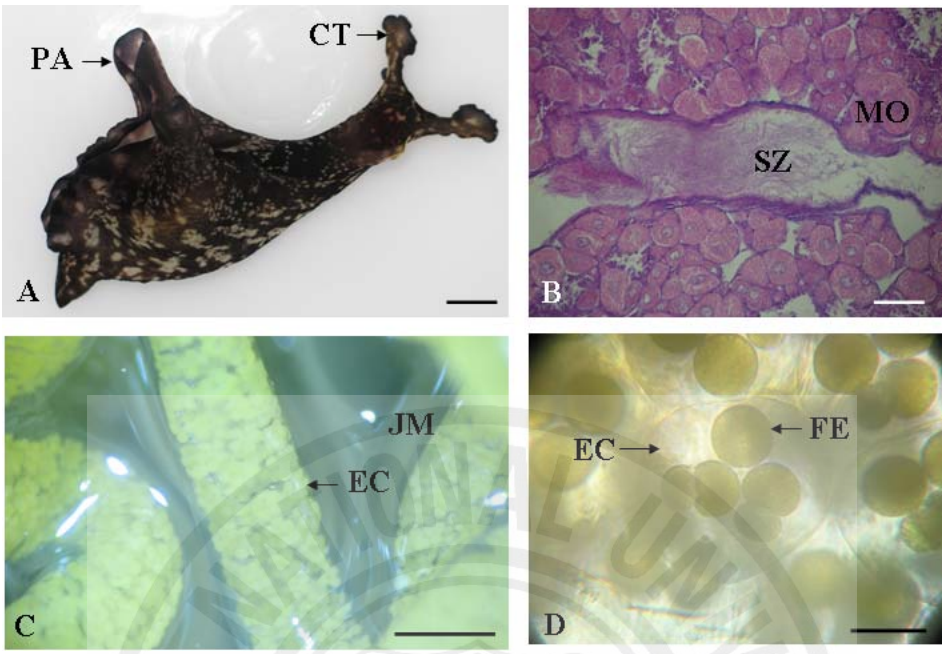

Fig. 15. Sexual maturity stage. A, morphological feature. B, histological section of ovotestis. C and D, deposited egg masses. CT, cephalic tentacles; EC, egg capsule; FE, fertilized egg; JM, jelly matrix; MO, mature oocyte; PA, parapodia; SZ, spermatozoa. Scale bars indicate 1.0 cm (A and C), and 100 μm (B and D).

Table 2. Characteristics of egg masses in Anaspidea

Subfamily species	Diameter of egg (μm)	Number of capsule per cm	Number of eggs per capsule	Embryonic period (days)	Water temperature (℃)	Reference
Aplysiinae						
Aplysia kurodai	80	55-60	15-25	10-15	20	This study
	73-85	-	15-135	8-9	20-23	Usuki 1970
Aplysia californica	85	100	4-100	8-11	22	Kriegstein et al. 1974
Aplysia depilans	-	160	25	14-16	25	Thompson and Bebbington 1969
Aplysia fasciata	-	118	43	14-16	25	Thompson and Bebbington 1969
Aplysia punctata	-	532	4	20-22	15	Thompson and Bebbington 1969
Aplysia parvula	70-80	-	1-4	7-10	19-24	Usuki 1970
Aplysia oculifera	75-80	-	1	8-10	22-24	Usuki 1970
Aplysia dactylomela	90	177	4-7	8-9	24-26	Switzer-Dunlap and Hadfield 1977
Aplysia juliana	75-80	-	10-55	10-11	18-20	Usuki 1970
	77	52	25-50	7-8	24-26	Switzer-Dunlap and Hadfield 1977
Dolabellinae						
Dolabella auricularia	92	924	1	9-10	24-26	Switzer-Dunlap and Hadfield 1977
Petalifera punctulata	78-88	-	1	6-7	20-24	Usuki 1970
Notarchinae						
Stylocheilus longicauda	63-68	-	1-8	6-7	25-28	Usuki 1970
	66	419	3-5	6-7	24-26	Switzer-Dunlap and Hadfield 1977

Table 3. Characteristics of larvae in Anaspidea

Subfamily species	Shell size at hatching (μm)	Larval period (days)	Shell size at settling (μm)	Water temperature (℃)	Reference
Aplysiinae					
Aplysia kurodai	107±11.6	80±1.0	419±7.6	20±0.5	This study
Aplysia californica	125	35	400	22	Kriegstein et al. 1974
Aplysia pulmonica	128	24	330-340	24-26	Switzer-Dunlap 1978
Aplysia parvula	105	-	500	24-26	Switzer-Dunlap 1978
Aplysia oculifera	102±2	45-60	385±11	24±1	Plaut et al. 1995
Aplysia dactylomela	114	30	310-315	24-26	Switzer-Dunlap and Hadfield 1977
Aplysia juliana	77	28	315-330	24-26	Switzer-Dunlap and Hadfield 1977
Aplysia brasiliana	111±7	34	382±14	24±1	Paige 1986
Dolabellinae					
Dolabella auricularia	148	31	290-300	24-26	Switzer-Dunlap and Hadfield 1977
Notarchinae					
Stylocheilus longicauda	103	30	325-340	24-26	Switzer-Dunlap and Hadfield 1977
Bursatella leachii plei	160±4	19	286±9	24±1	Paige 1986

4. Discussion

Most opisthobranchs, including aplysiid, are simultaneous hermaphrodites, i.e., an adult animal has both a functional female as well as a male reproductive system, and lay egg masses by internal cross-fertilization through copulation. The types of copulation in opisthobranchs differ into reciprocal or unilateral according to facing direction. The reciprocal copulation occurs mainly in gymnosomata (*Paedoclione doliiformis*; Lalli and Conover 1973) and nudibranchs (*Tenellia pallida*; Eyster 1979). The unilateral copulation occurs mainly in cephalaspidea (*Navanax inermis*; Leonard and Lukowiak 1991) and anaspidea (*Aplysia spp.*; Kandel 1979, Yusa 1996). However, reciprocal copulation also occurs in *Phyllaplysia taylori* (Beeman 1970) and *A. brasiliana* (Blankenship et al. 1983). The mating behavior of *A. kurodai* occurred in the form of unilateral copulating with chain formation. In chain copulation, only the first animal acted as a female; the second and succeeding animals acted as males (sperm donors) to the animals in front and as females to the animals in behind.

The fertilized eggs of the aplysiid are packaged in capsules that are embedded in layers of mucopolysaccharide jelly to form a cylindrical string called an egg masses. The egg masses of the aplysiid species is quite similar in shape and structure, but the number of capsules per unit length of egg masses and the number of eggs per capsule vary among aplysiid species. There exists an inverse relationship between the size of the eggs and the number of eggs per capsule (Bridges 1975), but the relationship is not clear for many species that have smaller eggs. Also, among *A. californica* (Kriegstein 1974; Capo et al. 2002), *A. brasiliana* and *Bursatella leachii plei* (Paige 1986), the number of eggs per capsule was increase with increasing body size of animal. In this study, the

number of eggs per capsule in case of *A. kurodai* collected from nature (400–700 g body weight) was approximately 15–25 eggs, while the number of eggs of *A. kurodai* in laboratory culture (from hatching to attainment of reproductive maturity, 4–6 g body weight) was 1–5 eggs. These results suggested that the number of eggs per capsule depended on the size of animal and was species specific.

Most mollusks undergo spiral holoblastic cleavage, and embryonic development varies by temperature and the egg size. In opisthobranchs, the egg diameter correlates positively with the size of the hatched veliger larvae, and hatching size also increases with increasing embryonic duration (Hadfield and Switzer-Dunlap 1984). In a study of the development of four aplysiid species, it was observed that at the same temperature, the embryonic periods are shorter among species with smaller eggs and longer among species with larger eggs (Switzer-Dunlap and Hadfield 1977). Although the exact developmental period from egg laying to hatching varies among aplysiid species, its range does not vary among species, i.e., it is generally <16 days (Table 2). In the case of *A. kurodai* species, the fertilized eggs, like those of other mollusk, underwent spiral cleavage, but with unequal cell division. The eggs hatched at 10 days after spawning, within the range of other aplysiid species.

Most benthic marine animals include a planktonic larval stage in their life cycles (Crisp 1984; Wilson et al. 1994; Levin and Bridges 1995). The larval development of most marine invertebrate can be divided into two types, namely, lecithotrophic (non-feeding) and planktotrophic (feeding) larval development. The most of aplysiids, including *A. californica, A. juliana* and *A. oculifera*, pass through a planktonic larval stage prior to settlement and metamorphosis (Pawlik 1989; Plaut et al. 1995). However, *Phyllaplysia taylori* has a lecithotrophic larval development and non-

pelagic veliger simultaneously loses its velum and develops an active radula, metapodium and propodium before hatching (Bridges 1975). In aplysiid species with planktotropic larval development, the minimal larval periods form hatching to metamorphosis is approximately 19 to 34 days; however, among aplysiid species, *A. oculifera* has a relatively long larval period (Table 3). In this study, *A. kurodai* was planktotrophic larval developmental type and minimal larval period from hatching to metamorphosis was 80 days after hatching under this culture conditions.

The settlement and metamorphosis of marine gastropod larva can be influenced of biological, physical, and chemical factors in the environment (Burke 1983; Hirata and Hadfield 1986; Morse 1990; Hadfield and Paul 2001). The metamorphic response of larva depends on the larval age, nutrition, and size (Hadfield 1977; Switzer-Dunlap and Hadfield 1977). The larvae of most *Aplysia* spp. occurs settlement and metamorphosis at 30 to 60 days after hatching, and shell size at settlement and metamorphosis is approximately 300 to 400 μm SL (Kriegstein et al. 1974; Paige 1986; Pawlik 1989; Plaut et al. 1995). Further, irregularly-shaped red spots appear on the perivisceral membrane of the larvae as indicators of competency for metamorphosis (Kriegstein et al. 1974; Nadeau et al. 1989). However, the timing and significance of the appearance of these red spots differs among *Aplysia* spp. In *A. oculifera* larvae, these pigment spots did not appeared at 60 days after hatching when larvae were competent to metamorphosis (Plaut et al. 1995). Pawlik (1989) and Paige (1988) reported that larvae lacking these spots frequently underwent normal settlement and metamorphosis. Therefore, these morphological criteria may not be adequate predictors of metamorphic competence. Generally, a shell growth plateau is associated to the proximity of metamorphosis in opisthobranch larvae (Kriegstein et al. 1974; Kempf and Willows

1977; Switzer-Dunlap and Hadfield 1977; Chia and Koss 1978; Kempf 1981; Todd 1981; Hubbard 1988; Hansen and Ockelmann 1991); however, the larvae keep growing after becoming competent to metamorphose (Pechenik 1980, 1984). In case of *A. kurodai*, the larval age for attainment of competent for metamorphosis was at 80 days after hatching, and shell size was approximately 400 μm, and the red spots had appeared at this time. Thereafter, the larvae did not grow further before settlement and metamorphosis, and their shell size remained constant. The long-surviving larvae maintained the ability to metamorphosis, and spontaneous metamorphosis was not observed. The maximum longevity of the larvae in which metamorphosis was not induced was 380 days after hatching. This result is similar to that of *A. juliana* (Kempf 1981) and *A. oculifera* (Plaut et al. 1995) in laboratory culture, which survived up to 316 days and 330 days, respectively. The ability to delay metamorphosis and the duration of this delay varies widely among various gastropod species and also among individuals within the same species (Pechenik 1980, 1984; Miller and Hadfield 1990).

When the larvae of gastropods become competence for metamorphosis, they enter into a ceasing-growth stage, and this stage can be sustained for long periods (Kempf 1981; Avila 1998). This ceasing-growth stage during the delay in metamorphosis may be caused by a decrease in either the rate of ingestion or efficiency of assimilation of ingested food, increased rates of energy demand relative to energy accumulation, or by some combination of these factors (Pechenik 1980). The ability of larvae to delay metamorphosis is not restricted to only gastropods, but has also been reported in many other groups of marine invertebrates (Lewis 1978). The ability of larvae to prolong planktonic stage without losing metamorphic competence enables the dispersion of larvae to new suitable habitats. This also has the advantage of a high

survival rate of juveniles and possibility for establishment of the reproductive adult population.

Larval metamorphosis of most benthic marine invertebrates occurs to non-randomly on substrates of suitable habitats. In case of most nudibranchs, the prey of the adult is necessary to induce metamorphosis (Thompson 1962). Prey-dependent metamorphosis has been observed in nudibranchs feeding on sponges, corals, hydrozoans and branacles (Hadfield 1977; Chia and Koss 1978; Harris and Alkon 1978; Todd 1981). For many opisthobranchs, including anaspideans and sacoglossans, a specific algal species is necessary to stimulate settlement and metamorphosis (Switzer-Dunlap and Hadfield 1977). Competent aplysiid larvae metamorphose preferentially on one or a few species of algae. In particularly, the competent larvae metamorphose preferentially on red algae; *A. californica* (*Laurencia pacifica*), *A. parvula* (*Cbondrococcus bornemanni*) and *A. dactylomela* (*Laurenica* sp.), but *A. juliana* metamorphose preferentially on green algae (*Ulva fasciata* and *U. reticulate*) (Kriegstein et al. 1974; Switzer-Dunlap and Hadfield 1977; Switzer-Dunlap 1978). Unlike species of the family Aplysiidae, which preferentially settle on macroalgae, *Bursatella leachii plei* (Paige 1988), *Dolabella auricularia* and *Stylocheilus longicauda* (Switzer-Dunlap and Hadfield 1977) metamorphose on blue green algae; however, *D. auricularia* and *S. longicauda* metamorphose on red and brown algae. In this study, the larvae of *A. kurodai* also induced to metamorphose on all red and green algae, but not on brown algae, among the tested algae. This result is similar to the findings of Pawlik (1989) and Plaut (1993), suggesting that metamorphosis may be induced by a common chemical, and metamorphic specificity is low. In the case of *A. kurodai*, settlement and metamorphosis appear to be less specificity or non-specific substrate.

Unlike sessile species, the juveniles of invertebrate that have the ability to move immediately after metamorphosis are also enabled to settle and metamorphose near suitable habitats and to move toward it later. In the present study, larvae of *A. kurodai* settled and metamorphosed on 11/15 tested algae; however, the larvae that metamorphosed on *P. morrowii* and *C. clavulatum* survived for longer than 1 month and developed into juveniles. In contrast, the larvae that metamorphosed on the other algae species died at approximately 7 to 20 days after the induction of metamorphosis. Similar to our results, Pawlik (1989) and Plaut et al. (1995) reported that the juveniles of *A. californica* (feeding on *Laurencia pacifica* and *Plocamium cartilagineum*) and *A. oculifera* (feeding on *Enteromorpha intestinalis*) that metamorphosed on algae that were not suitable as food survived a few days without feeding or feeding small amounts of the algae, but spent most of their time crawling on the bottom or side of the experimental dish and finally died. Those results suggested that the induction of metamorphosis in aplysiid larvae is non-specificity, and post-metamorphic juvenile are capable of searching until suitable food is found. However, it is thought that the discrepancy among aplysiid species with regard to the prey of the larvae after metamorphosis indicates species specificity. Although competent larvae of aplysiid species settle and metamorphose preferentially on a few species of algae, post-metamorphic larvae feed only specific algae and develop into adult, and the cause for this behavior remain unknown.

After metamorphosis, the growth and morphological development of *A. kurodai* was similar to those reported for other *Apysia* spp. (Kriegstein et al. 1974; Kriegstein 1977; Switzer-Dunlap and Hadield 1977; Switzer-Dunlap 1978; Paige 1988). The striking characteristic of post-metamorphosis stage is the development of pigmentation.

In case *A. californica* (Kriegstein 1977), *A. brasiliana* and *A. parvula* (Switzer-Dunlap 1978) species whose juveniles start with feeding on red algae, the overall body color is initially pink and progressively changed darker with continued growth and feeding. In other species, specific pigmentation also develops. In *Stylocheilus longicauda* (Switzer-Dunlap 1978) and *Bursatella leachii plei* (Paige 1988) juveniles had longitudinal pigmented stripes with dark (white with dark bands) on the head and the lateral surfaces of the foot. In this study, juveniles of *A. kurodai* were also observed normally pink color within 10 days after induction of metamorphosis. It appears that the differences in the pigmentation among species depend on the food source after metamorphosis. The maturation of aplysiids, including *A. californica* (Kriegstein 1974), *A. brasiliana* and *B. leachii plei* (Paige 1986, 1988) is reached 2 to 3 months after metamorphosis. In the laboratory, *A. kurodai* attained sexual maturity within 3 to 4 months after metamorphosis and began to laying egg mass.

The use of a defined process for the culture of *A. kurodai* now provides researchers with an opportunity to investigate the chemical requirements of larval development and the effects of changing environments on adult neurophysiology and behavior.

References

Achituv Y, Susswein AJ (1985) Habitat selection by two Mediterranean species of *Aplysia*: *A. fasciata* Poiret and *A. depilans* Gmelin (Mollusca: Opisthobranchia). J Exp Mar Biol Ecol 85: 113-122

Arch S, Lupatkin J, Smock T, Beard M (1980) Evidence for an exocrine function of the *Aplysia* atrial gland. J Comp Physiol 141: 131-137

Avila C (1998) Competence and metamorphosis in the long-term planktotrophic larvae of the nudibranch mollusc *Hermissenda crassicornis* (Eschasholtz, 1831). J Exp Mar Biol Ecol 231: 81-117

Audesirk TE (1979) A field study of growth and reproduction in *Aplysia californica*. Biol Bull 157: 407-421

Beard M, Millecchia L, Masuoka C, Arch S (1982) Ultrastructure of secretion in the atrial gland of a mollusk (*Aplysia*) Tissue Cell 14: 297-308

Beeman RD (1968) The order Anaspidea. The veliger 3(Suppl.): 87-102

Beeman RD (1970) The anatomy and functional morphology of the reproductive system in the opisthobanch mollusk *Phyllaplysia taylori* Dall, 1900. The veliger 13: 1-31

Berry AJ, Purvis J, Radhakrishnan KV (1992) Reproductive system and spermatogenesis in the opithobranch gastropod *Retusa Obtusa* (Montagu). J Moll Stud 58: 357-367

Blankenship JE, Rock MK, Robbins LC, Livingston CA, Lehman HK (1983) Aspects of copulatory behavior and peptide control of egg laying in *Aplysia*. Fed Proc Fed Am Soc Exp Biol 42: 96-100

Boettcher AA and Targett NM (1996) Induction of metamorphosis in queen conch,

Strombus gigas Linnaeus, larvae by cues associated with red algae from their nursery grounds. J Exp Mar Biol Ecol 196: 29-52

Bonar DB, Coon SL, Walch M, Weiner RM and Fitt W (1990) Control of oyster settlement and metamorphosis by endogenous and exogenous chemical cues. Bull Mar Sci 46:484-498

Bridges CB (1975) Larval development of *Phyllaplysia taylori* Dall, with a discussion of development in the Anaspidea (Opistobranchiata: Anaspidea). Ophelia 14: 161-184

Burke RD (1983) The induction of metamorphosis of marine invertebrate larvae: stimulus and response. Can J Zool 61: 1701-1719

Capo TS, Fiber LA, Stommes DL, Walsh PJ (2002) The effect of stocking density on growth rate and maturation time in laboratory-reared California sea hares. American Association for Laboratory Animal Science 41: 18-23

Carefoot TH (1967) Studies on a sublittoral population of *Aplysia punctata*. J Mar Biol Assoc UK 47: 335-350

Carefoot TH (1987) *Aplysia*: Its biology and ecology. Annual review of Oceanography and Marine Biology 25: 167-284

Cervera JL, Gosliner TM, Gomez JCGG, Ortea JA (2000) A new species of *Berthella blainville*, 1824 (Opisthobranchia: Notaspidea) from the Canary Island (Eastern Atlantic Ocean), with a re-examination of the phylogenetic relationships of the Notaspidea. J Moll Stud 66: 301-311

Chia FS, Koss R (1978) Development and metamorphosis of the planktotrophic larvae of *Rostanga pulchra* (Mollusca: Nudibranchia). Mar Biol 46: 109-119

Coggeshall RE (1972) The structure of the accessory genital mass in *Aplysia californica*.

Tissue Cell 4: 105-127

Crisp DJ (1974) Factors influencing the settlement and marine invertebrate larvae. In: Grant PT, Mackie AM (eds) Chemoreception in marine organisms. Academic press, New York, pp 177-265

Crisp DJ (1984) Overview of research on marine invertebrate larvae. In: Costlow JD, Tipper RC (eds) Marine biodeterioration: an interdisciplinary study, E & FN, Spon, London, 103-126

Dacosta S, Cunha CM, Simone LRL, Schrödl M (2007) Computer-based 3-dimensional reconstruction of major organ system of a new aeolid nudibranch subspecies, *Flabellina engeli lucianae*, from Brazil (Gastropoda:Opisthobranchia). J Mollus-can Stud 73: 339-353

Dudek FE, Soutar B, Tobe SS, (1980) Reproduction in *Aplysia californica*: correlations between egg laying in vivo and egg release *in vitro*. Can J Zool 58: 2163-2166

Eales NB (1960) Revision of the world species *Aplysia* (Gastropoda, Opithobranchia). Bull Br Mus (Nat. Hist). Zool 5: 276-404

Eyster LS (1979) Reproduction and developmental variability in the opisthobranchs *Tenellia pallida*. Mar Biol 51: 133-140

Gascoigne T (1956) Feeding and reproduction in the Limaphntiidae. Trans R Soc Edinburgh 63: 129-151

Gev S, Achituv Y, Susswein AJ (1984) Seasonal determinants of the life cycle in two species of Aplysia found in shallow waters along the Mediterranean Coast of Israel. J Exp Mar Biol Ecol 74: 67-83

Ghiselin MT (1966) Reproductive function and the phylogeny of opisthobranch gastro-pods. Malacologia 3: 327-378

Gosliner TM (1981) Origins and relationships of primitive members of the Opisthobranchia (Mollusca: Gastropoda). Biol J Linn Soc 16: 197-225

Gosliner TM (1994) Gastropoda: Opisthobranchia. In: Harrison FW, Kohn AJ (eds) Microscopic anatomy of invertebrates, V. 5: Mollusca Ⅰ. Wiley-Liss, New York, pp 254-355

Hadfield MG (1977) Chemical interactions in larval settling of a marine gastropod. In: Faulkner DJ, Fenical WH (eds) Marine natural products chemistry. Plenum press, New York, pp 403-413

Hadfield MG (1984) Settlement requirements of molluscan larvae: new data on chemical and genetic roles. Aquaculture 39: 283-298

Hadfield MG, Koehl MAR (2004) Rapid behavioral response of an invertebrate larva to dissolved settlement cue. Biol Bull 207: 28-43

Hadfield MG, Paul VJ (2001) Natural chemical cues for settlement and metamorphosis of marine invertebrate larvae. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, FL, pp 431-461

Hadfield MG, Scheuer D (1985) Evidence for a soluble metamorphic inducer in *Phestilla*: ecological, chemical and biological data. Bull Mar Sci 37: 556-566

Hadfield MG, Switzer-Dunlap M (1984) Opithobranchs. In: Tompa AS, Verdonk NH, Van Den Biggelarr JAM (eds) The Mollusca, vol 7. Academic Press, New York, pp 209-350

Hansen B, Ockelamann KW (1991) Feeding behavior in larvae of the opisthobranch Philine aperta. 1. Growth and functional response at different developmental stages. Mar Biol 111: 255-261

Harris JF, Alkon DL (1978) Larval rearing, metamorphosis, growth and reproduction of

the eolid nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) (Gastropoda: Opisthobranchia). Biol Bull 154: 539-550

Heller E, Kaczmarek L, Hunkapiller M, Hood L, Strumwasser F (1980) Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in *Aplysia*. Proc Natl Acad Sci USA 77: 2328-2332

Hirata KY, Hadfield MG (1986) The role of choline in metamorphic induction of *Phestilla* (Gastropoda, Nudibranchia). Comp biochem Physiol C 84: 15-21

Hubbard EJA (1988) Larval growth and the induction of metamorphosis of a tropical sponge-eating nudibranch. J Moll Studies 54: 259-269

Huggett MJ, Nys R, Williamson JE, Heasman M, Steinberg PD (2005) Settlement of larval blacklip abalone, *Haliotis rubra*, in response to green and red macroalgae. Mar Biol 147: 1155-1163

Kaang BK, Kandel ER, Grant SGN (1993) Activation of cAMP-Reponsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons. Neuron 10: 427-435

Kandel ER (1979) Behavioral biology of *Aplysia*. W.H. Freeman and Co., San Fransisco

Kempf SC (1981) Long-lived larvae of the gastropod *Aplysia juliana*: Do they disperse and metamorphose or just slowly fade away? Mar Ecol Prog Ser 6: 61-65

Kempf SC, Willows AOD (1977) Laboratory culture of the nudibranch *Tritonia diomedea* Bergh (Tritoniidae: Opisthobranchia) and some aspects of its behavioral development. J Exp Mar Biol Ecol 30: 261-276

Klussmann-Kolb A (2001a) Comparative investigation of the genital systems in the Opithobranchia (Mollusca, Gastropoda) with special emphasis on the nidamental glandular system. Zoomorphology 120: 215-235

Klussmann-Kolb A (2001b) The reproductive systems of the Nudibranchia (Gastropoda, Opisthobranchia): Comparative histology and ultrastructure of the nidamental glands with aspects of functional morphology. Zool Anz 240: 119-136

Klussmann-Kolb A (2004) Phylogeny of the Aphysiidae (Gastopoda, Opisthobranchia) with new aspects of the evolution of seahares. Zoologica Scripta 33:439-462

Klussmann-Kolb A, Wägele H (2001) On the fine structure of opisthobranch egg masses (Mollusca, Gastropoda). Zool Anz 240: 101-118

Kress A, Schmekel L (1992) Structure of the female genital glands of the oviduct in the opithobranch mollusk, *Runcina*. Tissue and Cell 24: 95-110

Kriegstein AR, Castellucci V and Kandel ER (1974) Metamorphosis of *Aplysia californica* in laboratory culture. Proc Nat Acad Sci USA 71: 3654-3658

Kriegstein AR (1977) Stage in the post-hatching development of *Aplysia californica*. J Exp Zool 199: 275-288

Kupfermann I (1967) stimulation of egg laying: possible neuroendocrine function of bag cells of abdominal ganglion of *Aplysia californica*. Nature (London) 216: 814-815

Lalli CM, Conover RJ (1973) Reproduction and development of *Paedoclione doliiformis*, and a comparison with *Clione limacine* (Opisthobranchia : Gymnosomata). Mar Biol 19: 13-22

Lederhendler II, Bell L, Tobach E (1975) Preliminary observations of the behavior of *Aplysia dactylomela* (Rang, 1828) in Bimini waters. The Veliger 17: 347-353

Leonard JL, Lukowiak K (1991) Sex and the simultaneous hermaphrodite: Testing models of male-female conflict in a sea slug, *Navanax inermis* (Opisthobranch). Animal Behaviour 41: 255-266

Levin LA, Brides TS (1995) Pattern and diversity in reproduction and development. In: McEdward (ed) Ecology of marine invertebrate larvae, CRC Press, Boca Raton, FL, pp 1-48

Lewis CA (1978) A review of substratum selection in free-living and symbiotic cirripeds. In: Chia FS, Rice ME (eds) Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York, pp 207-218

Marcus E (1972) On the Anaspidea (Gastropoda: Opithobranchia) of the warm waters of the western Atlantic. Bull Mar Sci 22: 841-874

Marcus ED, Marcus E (1957) Notes on *Aplysia*. Bol Inst Oceanogr Univ Sao Paulo 8: 3-22

Morse ANG (1992) Role of algae in the recruitment of marine invertebrate larvae. In: John DM, Hawkins SJ, Price JH (eds) Plant-animal interactions in the marine benthos. Systematics Association Special, 46, Clarendon Press, Oxford, pp 385-403

Morse DE (1990) Recent progress in larval settlement and metamorphosis: closing the gap between molecular biology and ecology. Bull Mar Sci 46: 645-483

Morse MP (1976) *Hedylopsis riseri* sp. n., a new interstitial mollusc from the New England coast (Opisthobranchia, Acochlidiacea). Zool Scr 5: 221-229

Miller SE, Hadfield MG (1990) Developmental arrest during larval life and life-span extension in a marine mollusc. Science 248: 356-358

Nadeau L, Paige JA, Starczak V, Capo T, Lafler J, Bidwell JP (1989) Metamorphic competence in *Aplysia californica* Cooper. J Exp Mar Biol Ecol 131: 171-193

Paige J (1986) The laboratory culture of two aplysiids, *Aplysia brasiliana* Rang, 1828, and *Bursatella leachii plei* (Rang, 1828) (Gastropoda: Opisthobranchia) in artificial

seawater. The Veliger 29: 64-69

Paige JA (1988) Biology, metamorphosis and postlarval development of *Bursatella leachii plei* Rang (Gastropoda: Opisthobranchia). Bull mar Sci 42: 65-75

Painter SD, Kalman VK, Nagle GT, Zuckerman RA, Blakenship JE (1985) The anatomy and functional morphology of the large hermaphroditic duct of three species of *Aplysia*, with special reference of the atrial gland. J Morphol 186:167-194

Pawlik JR (1989) Larvae of the sea hare *Aplysia californica* settle and metamorphose on an assortment of macroalgal species. Mar Ecol Prog Ser 51: 195-199

Pawlik JR (1992) Induction of marine invertebrate larval settlement: evidence for chemical cues. In: Paul VJ (ed) Ecological roles of marine natural products. Comstock Pub. Associates, Ithaca, New York, pp 189-236

Pawlings T (1999) Adaptations to physical stresses in the intertidal zone: the egg capsules of neogastropod molluscs. Amer Zool 39: 230-243

Pechenik JA (1980) Growth and energy balance during the larval lives of three prosobranch gastropods. J Exp Mar Biol Ecol 44: 1-28

Pechenik JA (1984) The relationship between temperature, growth rate and duration of planoktonic life for larvae of the gatropoda *Crepidula fornicate* (L.). J Exp Mar Biol Eco 74: 241-257

Pennings PC (1991) Spatial and temporal variation in recruitment of *Aplysia californica* Cooper: patterns, mechanisms and consequences. J Exp Mar Biol Ecol 146: 253-274

Pinsker HM, Dudek FE (1977) Bag cell control of egg laying in freely behaving *Aplysia*. Science 197: 490-493

Pinsker HM, Parsons DW (1985) Temperature dependence of egg laying in *Aplysia brasiliana* and *A. californica*. J Comp Physiol B 156: 21-27

Plaut I (1993) Ecology and ecophysiology of the sea hare, *Aplysia oculifera* (Adams and Reeve, 1850) in the Gulf of Eilat (Aqaba). Ph. D. thesis, Hebrew University, Jerusalem (in Hebrew with English abstract)

Plaut I, Borut A, Spira ME (1995) Growth and metamorphosis of *Aplysia oculifera* larvae in laboratory culture. Mar Biol 122: 425-430

Plaut I, Borut A, Spira ME (1998) Seasonal cycle and population dynamics of the sea hare *Aplysia oculfera* in the northern Gulf of Elat (Aquaba), Red Sea. J Moll Stud 64: 239-247

Przeslawski R (2004) A review of the effects of environmental stress on embryonic development within intertidal gastropod egg masses. Molluscan Research 24: 43-63

Przeslawski R, Benkendorff K (2005) The role of surface fouling the development of encapsulated gastropod embryos. J Moll stud 71: 75-83

Reid JD (1964) The reproduction of the sacoglossan opisthobranch *Elysia maoria*. Proc Zool Soc London 143: 365-393

Ruthensteiner R, Lodde E, schopf S (2007) Genital system development of *Williamia radiate* (Gastropoda, Siphonariidae). Zoomorphology 126: 17-29

Sarver DJ (1979) Recruitment and juvenile survival in the sea hare *Aplysia juliana* (Gastropoda: Opisthobranchia). Mar Biol 54:353-361

Schrödl M (2000) Revision of the nudibranch genus *Cadlina* (Gastropoda: Opisthobranchia) from the Southern Ocean. J Mar Biol Ass UK 80: 299-309

Strenth NE, Blankenship JE (1991) Reproductive patterns and seasonal occurrence of

the sea hare *Aplysia brasiliana* Rang (Gastropoda, Opithobranchia) at South Padre Island, Texas. Am Malac Bull 9: 85-88

Strumwasser F, Jacklet JW, Alvarez RB (1969) A seasonal rhythm in the neural extract induction of behavioral egg-laying in *Aplysia*. Comp Biochem Physiol 29: 197-206

Strumwasser F, Kaczmarek LK, Chiu AY, Heller E, Jennings KR, Viele DP (1980) Peptides controlling behavior in *Aplysia*. In: Bloom FE (ed) Peptides: Intergrators of cell and tissue function. New York Raven Press, pp 197-218

Susswein AJ, Achituv Y, Markovich S (1987) *Aplysia* from shallow waters along the coasts of Israel. Cah Biol Mar 28: 97-110

Switzer-Dunlap M (1978) Larval biology and metamorphosis of aplysiid gastropods. In: Chia FS, Rice ME (eds) Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York, pp 197-206

Switzer-Dunlap M, Hadfield MG (1977) Observations on development, larval growth and metamorphosis of four species of aplysiidae (Gastropoda: Opisthobranchia) in laboratory culture. J Exp Mar Biol Ecol 29: 245-261

Switzer-Dunlap M, Meyers-Schulte K, Gardner EA (1984) The effect of size, age, and recent egg laying on copulatory choice of the hermaphroditic mollusk *Aplysia juliana*. Int J Invert Reprod Dev 7: 217-225

Thompson TE (1962) Studies on the ontogeny of *Tritonia homberigi* Cuvier (Gastropoda, Opisthobranchia). Philos Tran R Soc London Ser B 245: 171-218

Thompson TE, Bebbington A (1969) Structure and function of the reproductive organs of three species of *Aplysia* (Gastropoda: Opisthobranchia). Malacologia 7: 347-380

Todd CD (1981) The ecology of nudibranch molluscs. Oceanogr Mar Biol Annu Rev 19: 141-234

Toonen RJ, Pawlik JR (1994) Foundations of gregariousness. Nature 370: 511-512

Usuki I (1970) Studies on the life histology of Aplysiae and their allies in the Sado district of the Japan Sea. Sci Rep Niigata Univ Ser D (Biology) 7: 91-105

Visser MC (1988) The significance of terminal duct structures and the role of neoteny in the evolution of the reproductive system of Pulmonata. Zoologica scripta 17: 239-252

Wägele H (2000) Phylogeny of the nudibranchia. Zoological Journal of the Linnean Society 130:83–181

Wayne NL, Block GD (1992) Effects of photoperiod and temperature on egg-laying behavior in a marine mollusk, *Aplysia californica*. Biol Bull 182: 8-14

Wayne NL, Nick T, Block GD (1996) Effects of temperature on reproductive neuroendocrine function in *Aplysia californica*. General and Comparative Endocrinology 102: 351-359

Wilson Jr WH, Stricker SA, Shinn GL (1994) Reproduction and development of marine invertebrates, Johns Hopkins University Press, Baltimore, MD.

Willan RC, Bertsch H (1987) Description of a new pleurobranch (Opisthobranchia: Notaspidea) from Antarctic waters with a review of notaspideans from southern polar seas. Veliger 29: 292-302

Yusa Y (1996) The effects of body size on mating features in a field population of the hearmaphroditic sea hare *Aplysia kurodai* Baba, 1937 (Gastropoda: Opisthobranch). J Moll Stud 62: 381-386

감사의 글

석사학위를 마치고 박사과정에 들어온 지 언 8년이란 긴 시간이 흘렸습니다. 지난하게 끌어왔던 논문이 막상 이렇게 끝을 맺고 보니 허전함 마음도 없지 않지만, 무엇보다 스스로 대견하고 감개무량하며, 끝을 낸 후의 뿌듯함은 무엇과도 비교할 수 없는 소중한 경험이라 생각이 듭니다. 이 모든 결과는 항상 관심과 애정으로 저를 이끌어 주신 많은 분들의 덕분이며 항상 감사한 마음을 잊지 않겠습니다.

제가 여기까지 올 수 있도록 지도해 주신 이영돈 교수님께 먼저 머리 숙여 깊은 감사를 드립니다. 부족함이 많은 저를 항상 믿고 격려해 주신 점 감사하고 한편으로 죄송스러울 뿐입니다. 교수님의 가르침 마음 속 깊이 새기고 앞으로 더 열심히 하겠습니다. 또한 바쁘신 와중에도 부족한 저의 논문을 심사해주시고 날카로운 지적과 소중한 조언을 아끼지 않으신 이기완 교수님, 강봉균 교수님, 김형배 교수님, 최광식 교수님께 진심으로 깊은 감사를 드립니다.

부족한 저가 박사과정을 무사히 마칠 수 있었던 것은 연구실의 선배님과 후배님들이 있었기에 가능한 일이었습니다. 긴 시간 생사고락(??)을 같이하며 모든 일을 맡아 해주고 지원을 아끼지 않은 영보형, 봉수형, 지금은 일본에서 박사학위를 하고 있는 창범이, 묵묵히 연구실의 궂은 일을 도맡아 한 상우, 성표, 용운, 한때 내 수제자가 될 뻔한 형철, 없으면 연구실이 삭막할 것 같은 막둥이 수용, 병훈, 그리고 학문의 끝없는 열정을 보이신 김성훈 선배님, 모두 감사하고 고맙단 말을 전하고 싶습니다. 항상 따뜻한 배려와 관심을 기울어 주신 병호형, 오수형, 성립이형, 봉원이형께 깊은 감사를 드리며, 그리고 해성회의 모든 분들께 감사의 말씀을 드리고 싶습니다.

부족한 둘째 아들을 위해 끝까지 변함없는 믿음을 주시고, 학업에 방해가 될까 바 연락하시는 것도 참으셨다는 아버지, 어머니께 깊이 감사 드립니다. 아버지, 어머니의 깊은 사랑과 믿음이 있었기에 지금의 제가 있을 수 있었습니다. 공부라는 핑계로 못난 동생을 대신해 집안의 대소사를 말없이 이끌어 나간 형과 형수님, 멀리서 내게 큰 힘이 되어 준 하나뿐인 내 동생, 그리고 사랑스런 우리 조카 왕우, 가족 모두에게 정말 이루 말할 수 없이 감사 드립니다.

일일이 언급하지 못했지만 늘 마음속으로 응원을 보내준 친구들과 학교 선배님과 후배님들께도 고마운 마음을 전합니다. 논문 작성을 위해 많은 시간을 할애해주신 서울대학교 신경생물학 연구실원들과 제주대학교 해양과환경연구소 선생님들께도 깊은 감사를 드립니다.

2008년 7월 어느 이른 새벽에...