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Source: Zoological Science, 18(7) : 909-918

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.18.909>

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# Organization of Carbohydrate Components in the Egg-Jelly Layers of the Newt, *Cynops pyrrhogaster*

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**ABSTRACT**—The egg-jelly of *C. pyrrhogaster* is composed of six layers: the J0, J1, J2, J3, J4 and sticky layers (from the innermost to outermost). In this study, we investigated the localizations of carbohydrate moieties in the egg-jelly using the FITC-conjugated N-acetylgalactosamine/galactose, glucose/mannose, N-acetylglucosamine and fucose group lectins. Eight of nine kinds of tested lectins belonging to every group were bound to the J1 and J3 layers, while *Dolichos biforus* agglutinine (DBA) were bound only to the J0 and the J2 layers. Though *Triticum vulgare* agglutinine was bound to all the four layers, the binding was inhibited only in the J0 and J2 layers by N-acetylglucosamine. Five kinds of lectins belonging to every group except for the fucose group uniformly bound to the J4 layer, while DBA and *Arachis hypogaea* agglutinine did not. *Ulex europaeus* agglutinine and *Glycine max* agglutinine bound only to the inner portion of the layer. These results suggest that the inner four layers are composed of two types of layers and that the J4 layer has a unique carbohydrate composition. In the oviduct, most lectins bound to the regions where the egg-jelly layers bound by the lectins were added. However, DBA bound to the anteriormost portion of the regions where the layers next to those bound by DBA was added. These results suggest that carbohydrate components of egg-jelly layers are fundamentally added in the corresponding regions of the oviduct while some molecules may be added after the matrices of the layer have already been accumulated.

**Key words:** egg-jelly, oviduct, fertilization, newt, lectin

## INTRODUCTION

Egg-jelly is significant for sperm-egg interaction in fertilization. It contains a large amount of carbohydrates (Bonnell *et al.*, 1993) and several factors for inducing chemotaxis (sea urchin: Ward *et al.*, 1985; *Xenopus laevis*: Al-Anzi and Chandler, 1998), initiation/activation of motility (*Discoglossus pictus*: Campanella and Gabbiani, 1979; sea urchin: Suzuki and Garbers, 1984; Suzuki, 1990; *Cynops pyrrhogaster*: Ukita *et al.*, 1999) and acrosome reaction (starfish: Hoshi *et al.*, 1988; sea urchin: Keller and Vacquier, 1994a; *Cynops pyrrhogaster*: Onitake *et al.*, 2000) in sperm. Egg-jelly also sustains cations that support the induction of the sperm-egg interaction (*Bufo japonicus*: Ishihara *et al.*, 1984). Recently, it was reported that a fertilization promotion factor exists in *Xenopus* (Olson and Chandler, 1999). These factors act at the correct time and place in or around egg-jelly, thereby leading to successful fertilization. Carbohydrate components are responsible for the activity of these molecules in some cases (SeGall and Lennarz, 1979; Keller and Vacquier, 1994a; Hoshi *et al.*, 1988; Hoshi *et al.*, 1990).

In amphibians, egg-jelly has several layers (*Triturus torosus*: Good and Daniel, 1943; *Bufo japonicus*: Katagiri, 1965; Omata, 1993; *Notophthalmus viridescens*: Humphries, 1966; MacLaughlin and Humphries, 1978; *Xenopus laevis*: Freeman, 1968; Gusseck and Hedrick, 1971; Yurewicz *et al.*, 1975; *Rana pipiens*: Shivers and James, 1970; *Ambystoma mexicanum*: Carroll *et al.*, 1992). In *Xenopus*, the layers are composed of a network of fibril structures including small globular structures, and the layers are morphologically and qualitatively different (Yurewicz *et al.*, 1975; Bonnell *et al.*, 1996; Bonnell and Chandler, 1996; Mazingo and Hedrick, 1999). Some sperm passing into the egg-jelly are blocked on the border of the layers (Reinhart *et al.*, 1998). The significance of each layer with regard to fertilization is under discussion.

Most newts, unlike anuran amphibians, undergo internal fertilization (Elinson, 1986; Wake and Dickie, 1998). The eggs are fertilized in the cloaca of the female before spawning into water. They are physiologically polyspermic. In *Cynops pyrrhogaster*, four sperm, on the average, are seen in the fertilized egg (Street, 1940). However, when too many sperm enter into an egg, the egg develops abnormally, which suggests that eggs are inseminated with the small population of sperm in nature.

The egg-jelly of *C. pyrrhogaster* contains sperm motility-inducing substances (Ukita *et al.*, 1999; Mizuno *et al.*, 1999)

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and sperm acrosome reaction-inducing substances (Nakai *et al.*, 1999). The egg-jelly structure is responsible for the sperm guidance to the egg surface of *C. pyrrhogaster*, because sperm cannot move forward unless the gel structures surround the egg (Itoh *et al.*, in press). Though the function of the sperm guidance can also be accomplished with a substitute, such as gelatin gel including egg-jelly substances, the intact egg-jelly is suggested to have the greatest ability to support fertilization. That would indicate that the egg-jelly of *C. pyrrhogaster* is composed of highly organized extracellular matrices that promote successful fertilization. Recently, we found that WGA inhibited the induction of sperm acrosome reaction by the egg-jelly extract (Onitake *et al.*, 2000). This suggests the participation of carbohydrate components in the sperm-egg interaction in egg-jelly of *C. pyrrhogaster*, much as has been demonstrated in other animals. In this study, we used lectins to investigate the localizations of carbohydrate moieties in the egg-jelly and the organization of egg-jelly in the oviduct.

## MATERIALS AND METHODS

### Eggs

Sexually mature newts, *C. pyrrhogaster*, were collected in Yamagata, Japan. Ovulation was induced by two injections of gonadotropin (Teikoku Zoki Inc., Tokyo) at a dose of 50 IU at room temperature every 24 hr and the oviduct was dissected from the newt. Eggs were obtained from the most posterior portion of the oviduct by surgical operation and stored in a moist chamber at room temperature without immersion in any solutions.

### Chemicals

Nine kinds of fluorescein isothiocyanate (FITC)-conjugated lectins (Table 1) were used in the assay, from *Bauhinia purpurea* (BPA), *Canavalia ensiformis* (Con A), *Griffonia simplicifolia* (GS-I and GS-II), *Arachis hypogaea* (PNA), *Glycine max* (SBA), *Ulex europaeus* (UEA-I), *Triticum vulgare* (WGA) and *Dolichos biflorus* (DBA) (EY Lab. Inc., San Mateo). The lectins were divided into four groups according to carbohydrate specificity: for glucose/mannose group carbohydrates (ConA), for N-acetylgalactosamine/galactose group carbohydrates

(GS-I, BPA, DBA, and SBA), for fucose group carbohydrates (UEA-I) and for N-acetylglucosamine group carbohydrates (GS-II and WGA). For the competition of lectin-binding, carbohydrates that specifically bind to each lectin (Table 1) were used: N-acetylgalactosamine for BPA, DBA, MPA and SBA; D-mannose for Con A; galactose for GS-I and PNA; N-acetylglucosamine for GS-II and WGA; L-fucose for UEA-I (EY Lab. Inc., San Mateo).

### Histology

Eggs were independently obtained from ten females. Egg-jellies were mechanically removed with fine forceps, then immediately embedded with O. C. T. compound and frozen in liquid nitrogen. Transverse sections of 8  $\mu$ m were serially prepared. In some cases, egg-jelly was fixed in methanol fixative, then embedded in paraffin, sectioned at 20  $\mu$ m thickness, and stained with 0.5% toluidine blue.

The oviducts were independently dissected from ten females after two injections-separated by a 24-hr interval-of gonadotropin at a dose of 50 IU at room temperature. They were fixed in Bouin's fixative. The posterior regions of the oviduct, i.e., pars convoluta and uterus, were cut at intervals of about 1-cm lengths along the anterior-posterior axis and embedded in paraffin. Longitudinal sections of 6  $\mu$ m thickness were prepared from each fragment and stained with 0.5% toluidine blue.

### Fluorescein staining

Frozen sections were washed with 0.01% Tween 20 in 0.02 M phosphate buffered saline (pH 7.4: PBST). Paraffin sections were hydrated with alcohol series and washed with PBST. Both frozen and paraffin sections were then incubated with FITC-conjugated lectin for 2 hr at room temperature. The lectin solutions were prepared at a concentration of 50  $\mu$ g/ml except in the case of GS-II solution, which was prepared at 25  $\mu$ g/ml. In the control, carbohydrates that specifically bind to each lectin were added at a concentration of 50 mg/ml (fucose), 125 mg/ml (mannose) or 25 mg/ml (the other carbohydrates). After incubation, the sections were washed with PBST and fluorescence was observed by luminescence microscopy (BH2-rfk; Olympus Co.).

Nine serial sections from ten individuals were stained with the nine kinds of lectins.

## RESULTS

The egg-jelly of *Cynops pyrrhogaster* has been shown to be composed of six layers that can be observed microscopically when the egg-jelly is hydrated (Onitake *et al.*, 2000). These layers were here labeled the J0, J1, J2, J3, J4 and sticky (st) layers (from the innermost to outermost). Hematoxylin has been shown to strongly stain the J0 and J2 layers, and the J1 and J3 layers are known to have a layered composition. The sticky layer is known to have high viscosity in solution and be strongly stained with eosin.

### Lectin-binding in egg-jelly

Staining with FITC-conjugated lectins showed various patterns of localizations of carbohydrate moieties in egg-jelly layers (Fig. 1, 2, 3, Table 2). The J1, J3 and J4 layers were stained by BPA, Con A (Fig. 1a), GS-I and GS-II (Fig. 1b, Table 2). Though SBA or UEA-I also stained these layers, they did not stain the outer portion of the J4 layer (Fig. 1c, Table 2). This result indicates that qualitative differences exist between the inner and the outer portions of the J4 layer despite the fact that this layer was morphologically uniform.

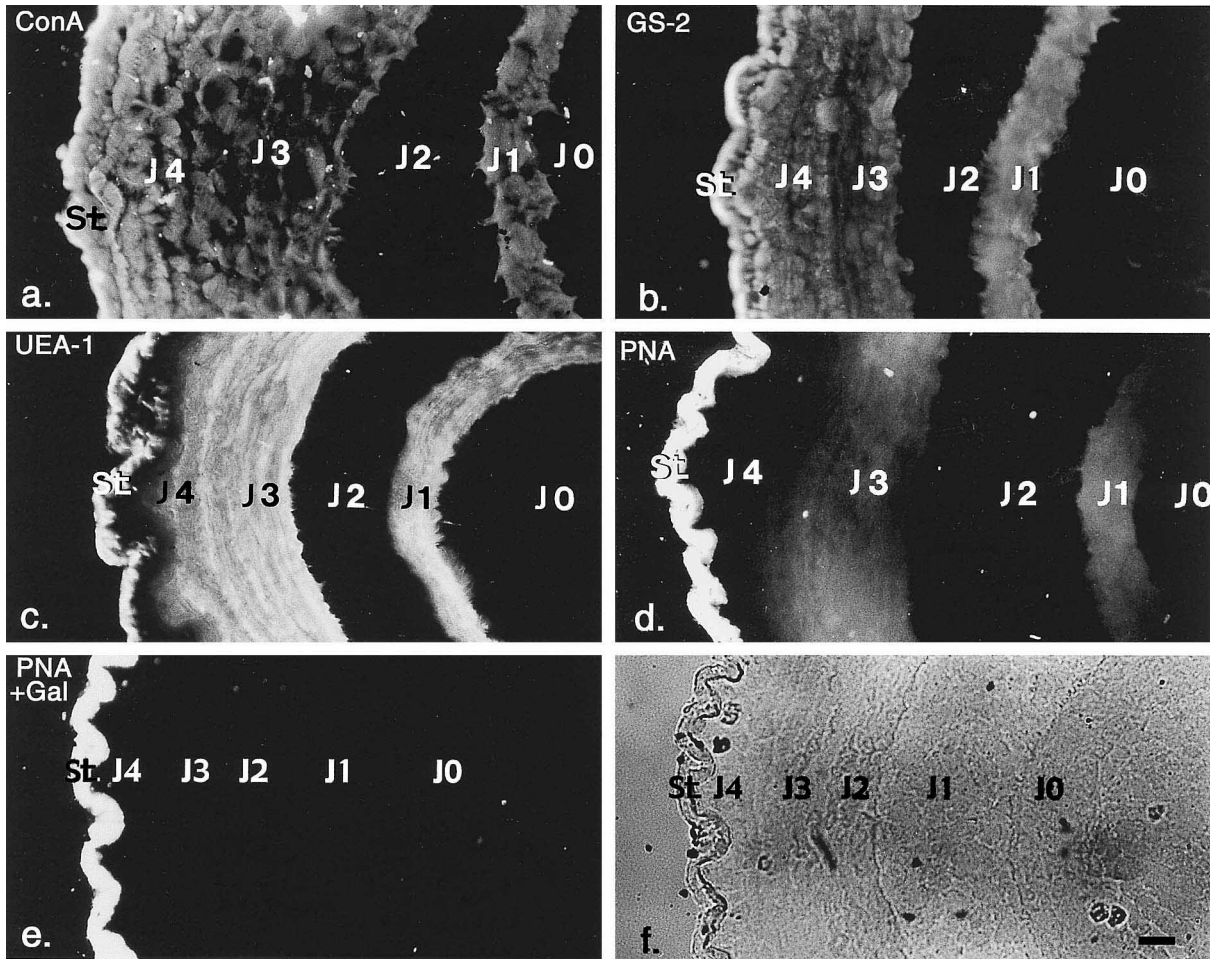
**Table 1.** Sugar specificity<sup>a</sup> in the tested lectins

lectin	specificity
<i>N-acetylgalactosamine/galactose group lectins</i>	
BPA	$\alpha$ and $\beta$ GalNAc > $\alpha$ and $\beta$ Gal
GS-I	$\alpha$ GalNAc > $\alpha$ Gal
SBA	$\alpha$ and $\beta$ GalNAc > $\alpha$ and $\beta$ Gal
DBA	GalNAc ( $\alpha$ 1, 3GalNAc) >> $\alpha$ GalNAc
PNA	Gal ( $\beta$ 1, 3GalNAc) > $\alpha$ and $\beta$ Gal
<i>glucose/mannose group lectin</i>	
Con A	Man ( $\alpha$ 1, 2 Man) <sub>1-2</sub> > Man ( $\alpha$ 1, 2Man) > $\alpha$ Man > $\alpha$ Glc > $\alpha$ GlcNAc
<i>N-acetylglucosamine group lectins</i>	
GS-II	$\alpha$ and $\beta$ GlcNAc
WGA	GlcNAc ( $\beta$ 1, 4GlcNAc) <sub>1-2</sub> > GlcNAc ( $\beta$ 1, 4GlcNAc) >> GlcNAc > NeuAc
<i>fucose group lectin</i>	
UEA-I	$\alpha$ L-Fuc

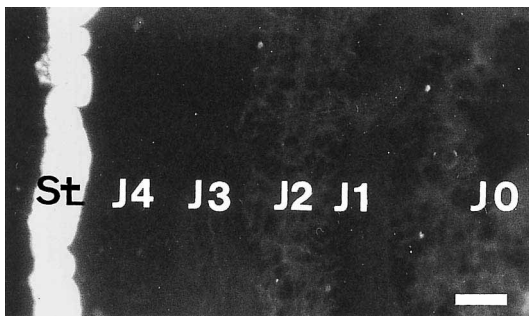
GalNAc: N-acetylgalactosamine, Man: Mannose, Fuc: Fucose

GlcNAc: N-acetylglucosamine, NeuAc: neuraminic acid.

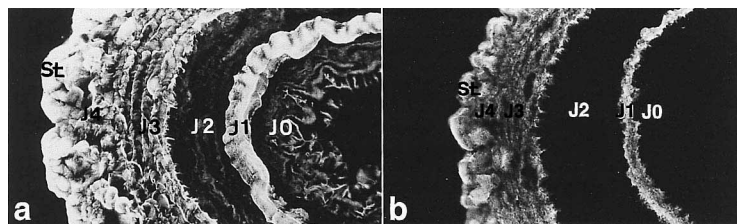
<sup>a</sup> (Goldstein and Poretz (1986)).



**Fig. 1.** Lectin bindings to egg-jelly of *Cynops pyrrhogaster*. Egg-jelly was mechanically dissected without immersion in solution. It was immediately embedded in O.C.T. compound, and frozen sections of 8  $\mu$ m thickness were prepared. The sections were stained with 50  $\mu$ g/ml FITC-conjugated Con A (a), GS-II (b), UEA-I (c), and PNA (d). (e) Control. 25 mg/ml galactose (Gal) was added to 50  $\mu$ g/ml FITC-conjugated PNA and applied to a section. (f) Bright field of (e). J0, J1, J2, J3, J4 or St indicates the corresponding layer of egg-jelly. Bar: 100  $\mu$ m.



**Fig. 2.** DBA binding to egg-jelly of *Cynops pyrrhogaster*. Egg-jelly was mechanically dissected without immersion in solution. Frozen sections of 8  $\mu$ m thickness were prepared and stained with 50  $\mu$ g/ml FITC-conjugated DBA. J0, J1, J2, J3, J4 or St indicates the corresponding layer of egg-jelly. Bar: 100  $\mu$ m.



**Fig. 3.** WGA binding to egg-jelly of *Cynops pyrrhogaster*. Egg-jelly was mechanically dissected without immersion in solution. Frozen sections of 8  $\mu$ m thickness were prepared and stained with 50  $\mu$ g/ml FITC-conjugated WGA (a). In the control, 25 mg/ml N-acetylglucosamine was added to the 50  $\mu$ g/ml FITC-conjugated WGA (b). J0, J1, J2, J3, J4 or St indicates the corresponding layer of egg-jelly. Bar: 100  $\mu$ m.

**Table 2.** Lectin binding to egg-jelly layers of *Cynops pyrrhogaster*

	J0	J1	J2	J3	J4		St
					inner	outer	
SBA	–	+	–	+	+	–	ND
UEA-I	–	+	–	+	+	–	ND
PNA	–	+	–	+	–	–	ND
BPA	–	+	–	+	+	+	ND
Con A	–	+	–	+	+	+	ND
WGA	+	+	+	+	+	+	ND
GS-I	–	+	–	+	+	+	ND
GS-II	–	+	–	+	+	+	ND
DBA	±	–	±	–	–	–	ND

Egg-jelly was removed without immersing any solution. It was immediately frozen in O.C.T. compound. Frozen sections were prepared and treated with nine kinds of FITC-conjugated lectins. + and ± indicates the strong and faint staining by the FITC-conjugated lectin. ND means 'not determined'. See text in details.

The J1 and J3 layers were stained by PNA, but no staining was seen in the J4 layer (Fig. 1d). The J0 and J2 layers were faintly stained by DBA (Fig. 2). The staining was inhibited by the addition of a specific carbohydrate for each lectin (Fig. 1e), indicating that there was specific binding of each lectin to carbohydrate moieties in the egg-jelly layers. The outermost layer, the st layer, was strongly stained with all tested lectins, and the stainings were not inhibited by the addition of specific carbohydrates (Fig. 1d, e). This result suggests that carbohydrates with a stronger affinity with the lectins exist in the st layer. However, the st layer tends to bind most proteins nonspecifically because of its high viscosity in solution, and thus were unable to determine the localizations of carbohydrate moieties in the st layer.

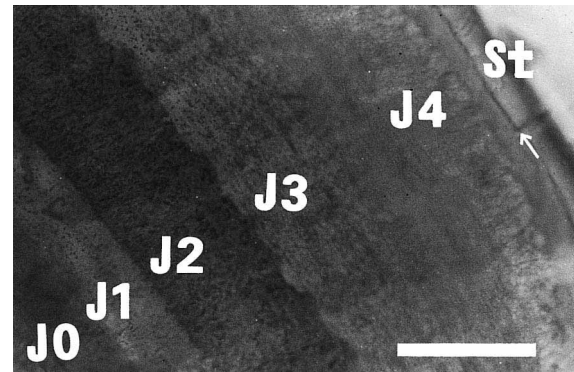
The J0, J1, J2, J3 and J4 layers were stained by WGA (Fig. 3). The staining in the J0 and J2 layers was inhibited by the addition of N-acetylglucosamine, while that in the J1, J3 and J4 layers was not (Fig. 3b). The affinity of WGA to N-acetylglucosamine is higher in oligomers than in monomers (Bains *et al.*, 1992; Table 1), which means that the oligo-N-acetylglucosamine moiety may be localized in the J1, J3 and J4 layers, and the other carbohydrate moieties that have specific affinity with WGA may be localized in the J0 and J2 layers.

Though the J1, J3 and J4 layers were stained with many lectins, each lectin showed the unique staining pattern. Small clusters of staining were observed in the J1 and J3 layers by Con A, GS-II or WGA (Fig. 1, 3), while those layers were smoothly stained by BPA, PNA or SBA (Fig. 1). Layered staining was observed by GS-I or UEA-I (Fig. 1). The strength of both SBA and PNA staining differed between the J3 and J4.

### Comparison of egg-jelly layers and the oviductal regions

In this study, the J0 and J2 layers were also strongly stained with toluidine blue, while the other three layers were only faintly stained. The inner portion of the sticky (st) layer was strongly stained with toluidine blue (Fig. 4), suggesting that this layer is composed of two sublayers, a st 1 and st 2 layers.

The oviduct of *C. pyrrhogaster* is about 20 cm in length



**Fig. 4.** Toluidine blue staining of egg-jelly of *Cynops pyrrhogaster*. Mature egg was fixed in methanol fixative. Paraffin sections of 20 µm thickness were prepared and stained with 0.5% toluidine blue solution. J0, J1, J2, J3, J4 or St indicates the corresponding layer of egg-jelly. The arrow indicates the stained region in the sticky layer. Bar: 100 µm.

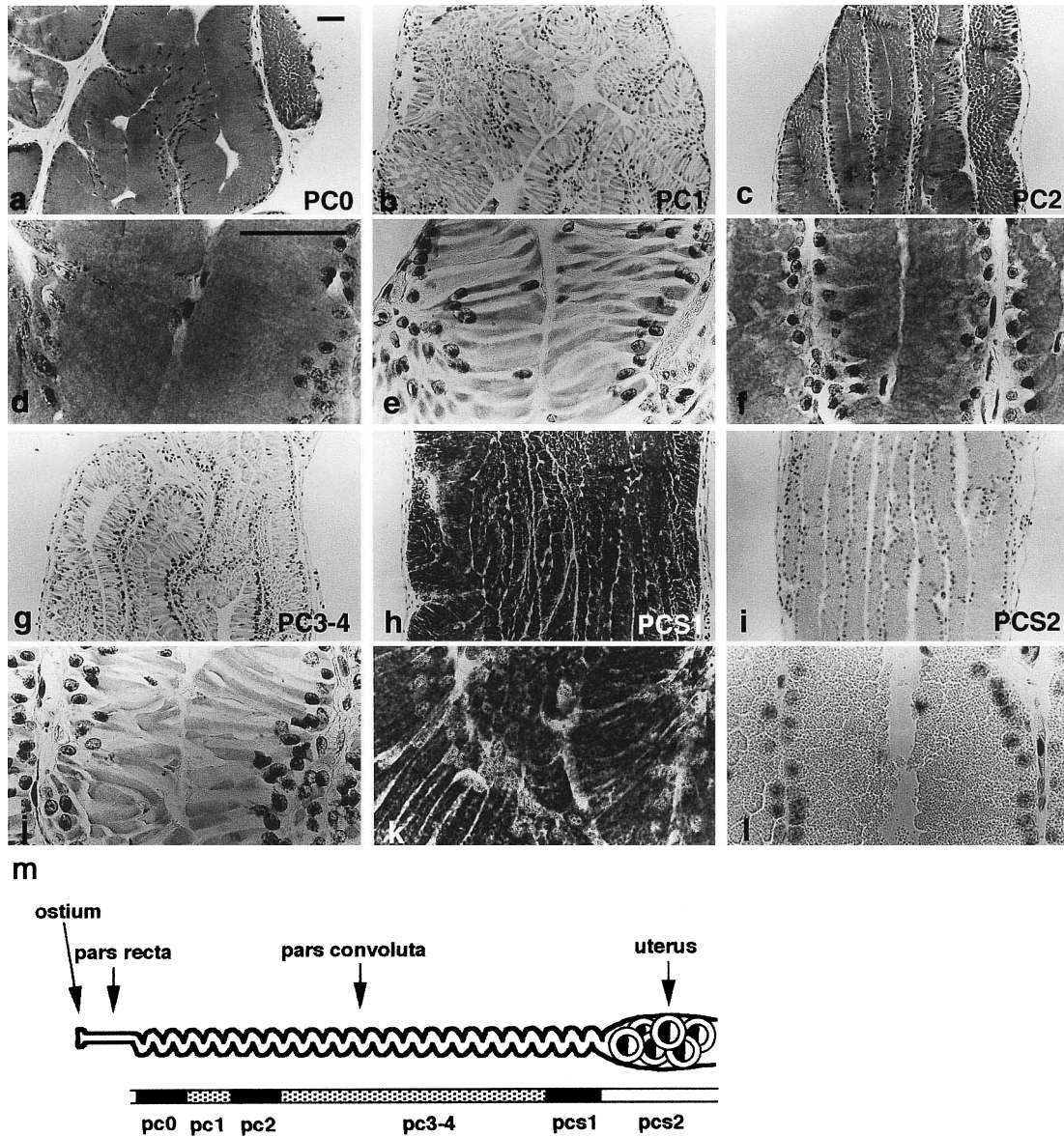
and morphologically divided into four regions: the ostium, pars recta, pars convoluta and uterus (Fig. 5m). Jelly layers are known to be added to the egg surface at the posterior regions. In the present study, large secretory cells were faced to the inner space of the pars convoluta and uterus, and their nuclei were located in the basal area (Fig. 5). Small ciliated cells were sometimes observed on the surface of the secretory cells. Fertilizable eggs were deposited in the uterus (Matsuda and Onitake, 1984b).

In this study, all secretory cells were strongly stained with toluidine blue in the most anterior region of the pars convoluta (Fig. 5a, d). In the next region, secretory cells were randomly stained (Fig. 5b, e), and all of them were stained again in the anterior third region (Fig. 5c, f). They were stained in the large portion of the middle region (Fig. 5g, j), and all of them were randomly stained in the most posterior region (Fig. 5h, k). No staining was observed in the uterus (Fig. 5i, l). The pattern of toluidine blue staining of the pars convoluta and uterus along the anterior-posterior axis (Fig. 5) coincided with that of egg-jelly from the inner to the outer layer (Fig. 4), suggesting that each layer was added in the corresponding region of the oviduct. We named these regions pars convoluta (pc) 0, 1, 2, 3-4, s1 and s2 beginning with the most anterior region, in correspondence with the egg-jelly layers J0, J1, J2, J3 and J4, st 1 and st 2, respectively.

### Lectin-binding in the oviduct

Longitudinal sections of the pars convoluta and uterus were stained with the nine kinds of FITC-conjugated lectins that were tested in egg-jelly. The pc1 and the pc3-4 regions were stained by PNA (Fig. 6), UEA-I, SBA, BPA, Con A, GS-I and GS-II (Table 3). Though strong autofluorescence was observed in the nuclei of secretory cells, no staining was observed by the addition of carbohydrate specifically binding to each lectin.

Secretory cells were randomly stained in the pc1 and the pc3-4 regions by PNA (Fig. 6), UEA-I and BPA, while all secretory cells were stained in the same regions by Con A. The



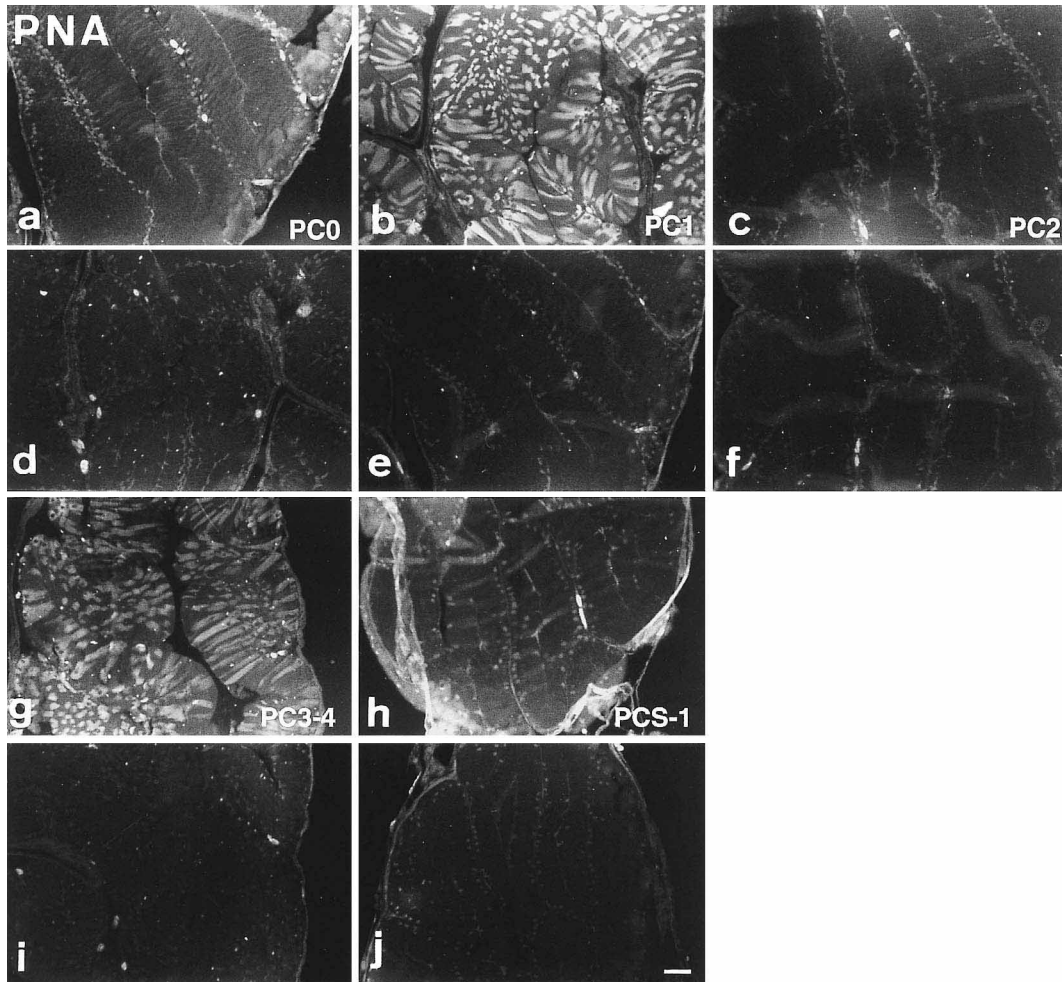
**Fig. 5.** Toluidine blue staining of pars convoluta and uterus of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative. Longitudinal sections of 6  $\mu\text{m}$  thickness were prepared and stained with 0.5% toluidine blue solution. (a), (b), (c), (g), (h) and (i) show sections of the pars convoluta from the anterior region to the posterior region and the uterus, that is, regions pc0, pc1, pc2, pc3-4, pcs1 and pcs2. (d), (e), (f), (j), (k) and (l) are the magnified views of (a), (b), (c), (g), (h) and (i), respectively. (m) is a diagram of the oviduct divided into four regions, the ostium, pars recta, pars convoluta and uterus, from anterior to posterior. The column below the oviduct indicates each region of the pars convoluta estimated by the reconstruction of the sections. Bar: 100  $\mu\text{m}$ .

cells were randomly stained in the pc1 region and the anterior portion of the pc3-4 region by SBA, GS-I and GS-II (Fig. 7), and all of them were stained in the posterior portion (Table 3). No staining was observed in the pc0 and the pc2 regions by any of these seven kinds of lectins. Although each lectin showed the unique staining pattern, each pattern fits with that for the corresponding layer of egg-jelly.

All secretory cells were stained in the pc1 and the pc3-4 regions by WGA, while they were randomly stained in the pc0 and the pc2 regions (Fig. 8). Though strong autofluorescence was observed in the nuclei of secretory cells, no staining was observed by the addition of N-acetylglucosamine.

Secretory cells were randomly stained by DBA in the anteriormost portions of the pc1 and pc3-4 regions (Fig. 9). No staining was observed in the other portions of the regions and the pc0 and the pc2 regions. Though strong autofluorescence was observed in the nuclei of secretory cells, no staining was observed by the addition of N-acetylgalactosamine.

In the pcs1 and pcs2 regions, secretory cells were stained by ConA (Fig. 10), while the inner space of oviductal tubules, which ovulated eggs would pass through, was stained by SBA, BPA, WGA and GS-II (Fig. 10, Table 3). Though strong autofluorescence was observed in the nuclei and the gran-



**Fig. 6.** PNA binding to pars convoluta of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative, embedded in paraffin, and cut into 6- $\mu$ m thick longitudinal sections. The sections were stained with 50  $\mu$ g/ml FITC-conjugated PNA. (a), (b), (c), (g) and (h) show the pc0, pc1, pc2, pc3-4 and pcs1 sections, respectively. (d), (e), (f), (i) and (j) show the sections of the pc0, pc1, pc2, pc3-4 and pcs1 regions co-treated with 25 mg/ml galactose, respectively. Autofluorescence was observed in the nuclei of secretory cells. Bar: 100  $\mu$ m.

**Table 3.** Lectin binding to the oviduct of *Cynops pyrrhogaster*

	pc0	pc1	pc2	pc3-4	pcs1	pcs2
SBA	-	+	-	+ <sup>c</sup>	-	- <sup>a</sup>
UEA-I	-	+	-	+	-	-
PNA	-	+	-	+	-	-
BPA	-	+	-	+	-	- <sup>a</sup>
Con A	-	+	-	+	+	+
WGA	+	+	+	+	- <sup>a</sup>	- <sup>a</sup>
GS-I	-	+	-	+ <sup>c</sup>	-	-
GS-II	-	+	-	+ <sup>c</sup>	-	- <sup>a</sup>
DBA	-	+ <sup>b</sup>	-	+ <sup>b</sup>	-	-

Longitudinal sections of oviduct were treated with nine kinds of FITC-conjugated lectins. + indicates FITC-staining in secretory cells. a: Fluorescence was observed in the inner surface of oviduct. b: Fluorescence was observed only in the anterior portion of the region. c: Staining pattern differed between the anterior portion and the posterior. See text in details.

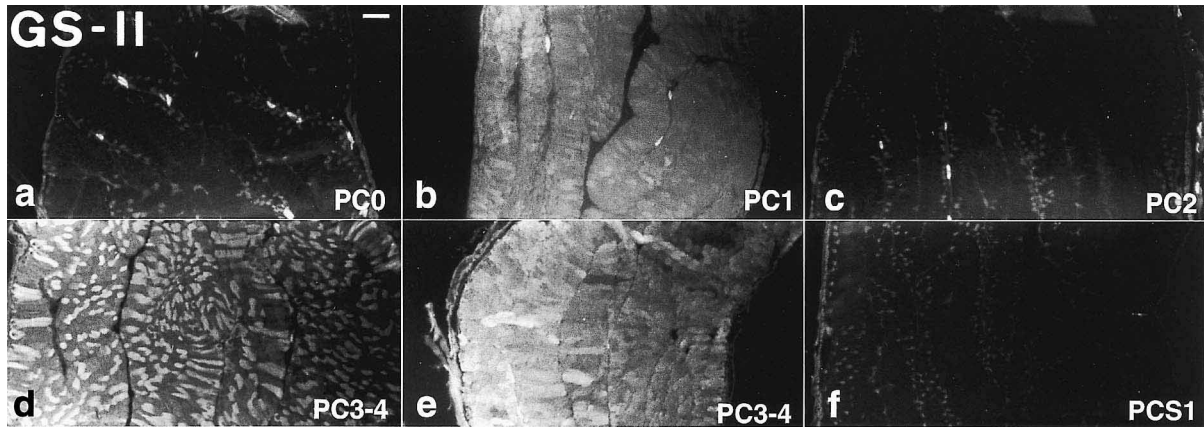
ules of the secretory cells, no staining was observed by the addition of carbohydrate specifically binding to each lectin.

## DISCUSSION

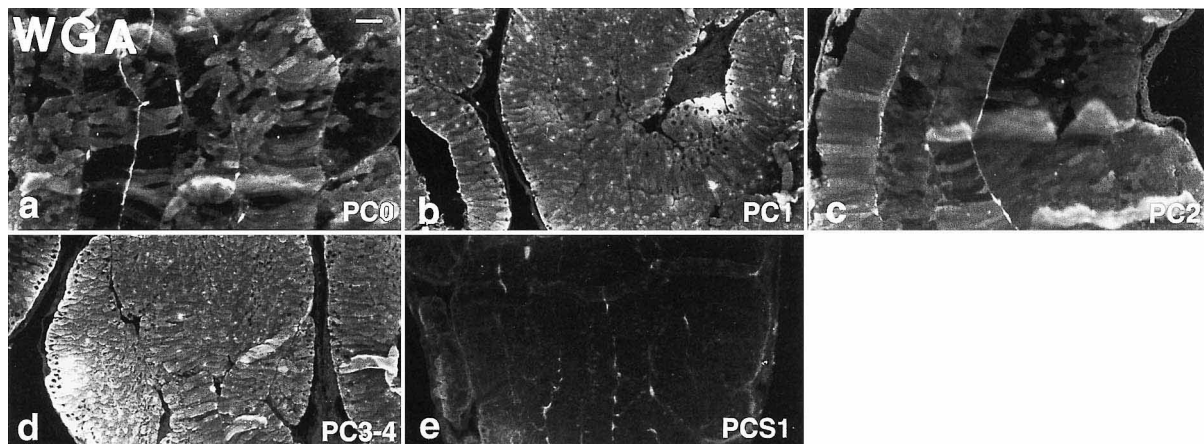
### Lectin-binding in egg-jelly

Egg-jelly of amphibians contains some diffusible components, which is an important feature for fertilization (Ishihara *et al.*, 1984; Al-Anzi and Chandler, 1998; Ukita *et al.*, 1999; Olson and Chandler, 1999; Onitake *et al.*, 2000). Newts undergo internal fertilization (Elinson, 1986), and sperm are directly inseminated onto the surface of egg-jelly at the exit of the oviduct. Fertilization is completed in the cloaca without immersion in any solution (Street, 1940; Elinson, 1986). When the egg of *C. pyrrhogaster* is placed in water, the egg-jelly swells by hydration, which appears to destroy the organized structure of egg-jelly, and the egg loses its ability to be fertilized (Matsuda and Onitake, 1984a). For that reason, we prepared frozen sections of egg-jelly without immersing them in any solution.

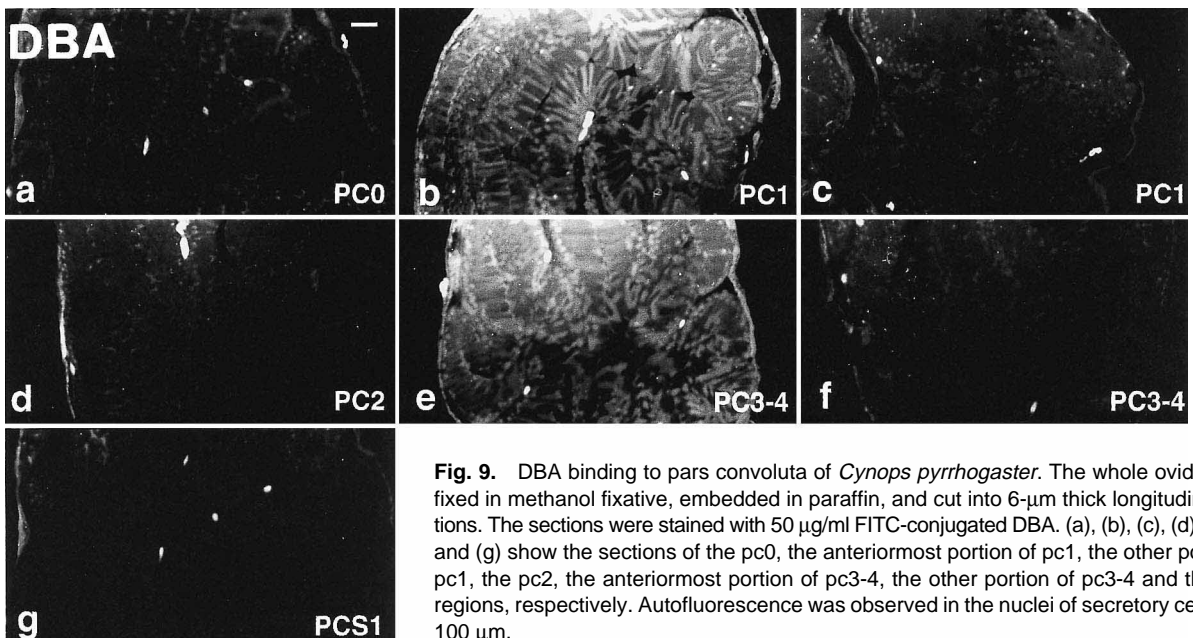
Egg-jelly of *C. pyrrhogaster* is composed of six layers. In the inner four layers, both the J0 and J2 layers are strongly stained by toluidine blue (Fig. 4) or hematoxylin (Onitake *et*



**Fig. 7.** GS-II binding to pars convoluta of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative, embedded in paraffin, and cut into 6- $\mu$ m thick longitudinal sections. The sections were stained with 25  $\mu$ g/ml FITC-conjugated GS-II. (a), (b), (c), (d), (e) and (f) show the sections of the pc0, pc1, pc2, anterior portion of pc3-4, posterior portion of pc3-4 and pcs1 regions, respectively. Autofluorescence was observed in the nuclei of secretory cells. Bar: 100  $\mu$ m.

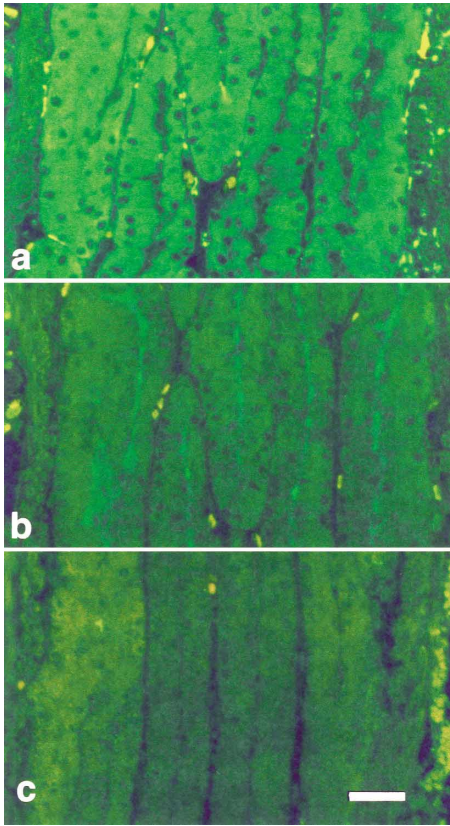


**Fig. 8.** WGA binding to pars convoluta of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative, embedded in paraffin, and cut into 6- $\mu$ m thick longitudinal sections. The sections were stained with 50  $\mu$ g/ml FITC-conjugated WGA. (a), (b), (c), (d) and (e) show the sections of the pc0, pc1, pc2, pc3-4 and pcs1 regions, respectively. Autofluorescence was observed in the nuclei of secretory cells. Bar: 100  $\mu$ m.



**Fig. 9.** DBA binding to pars convoluta of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative, embedded in paraffin, and cut into 6- $\mu$ m thick longitudinal sections. The sections were stained with 50  $\mu$ g/ml FITC-conjugated DBA. (a), (b), (c), (d), (e), (f), and (g) show the sections of the pc0, the anteriormost portion of pc1, the other portion of pc1, the pc2, the anteriormost portion of pc3-4, the other portion of pc3-4 and the pcs1 regions, respectively. Autofluorescence was observed in the nuclei of secretory cells. Bar: 100  $\mu$ m.





**Fig. 10.** Lectin bindings to the uterus of *Cynops pyrrhogaster*. The whole oviduct was fixed in methanol fixative, embedded in paraffin, and cut into 6- $\mu$ m thick longitudinal sections. The sections were stained with 50  $\mu$ g/ml FITC-conjugated lectins. (a) A section treated with FITC-conjugated Con A. Fluorescence was observed in all secretory cells. (b) A section treated with FITC-conjugated SBA. Fluorescence was observed in the inner space of the oviductal tubule. (c) A section treated with FITC-conjugated DBA. No fluorescence staining was observed. Bar: 100  $\mu$ m.

*al.*, 2000). While, both the J1 and J3 layers have layered structures which does not observed in the other two layers. All tested lectins except for DBA were bound to the J1 and J3 layers (Fig. 1, 3, Table 2). Although some of the tested lectins are known to have affinities with the same kind of carbohydrate, in the present study, the binding patterns in the layers were unique to each lectin. This may have been due to the differential interactions of each lectin with the carbohydrate moieties, suggesting that these layers had a complex composition of carbohydrate components. DBA and WGA bound to the J0 and J2 layers (Fig. 2). Although DBA is one of the N-acetylgalactosamine/galactose group lectins, it showed a unique affinity among the tested lectins (Table 1). The DBA-binding pattern suggests the localization of unique carbohydrate moieties in these layers. The WGA-binding pattern also suggests the localization of carbohydrate moieties specific for these layers, because WGA binding was inhibited only in these layers by N-acetylglucosamine (Fig. 3). These results suggest that the inner four layers comprise two types of distinct layers with respect to both morphology and carbohydrate contents.

The J4 layer showed a unique feature with respect to lectin binding (Fig. 1, Table 2). Among the four kinds of N-acetylgalactosamine/galactose group lectins, three types of binding pattern were observed: binding in all regions (BPA and GS-I), binding in the inner portion (SBA) and no binding (PNA and DBA) (Fig. 1). Although the mannose/glucose group lectin, Con A, and the N-acetylglucosamine group lectins, GS-II and WGA, bound in all regions, the fucose group lectin, UEA-I, bound in the inner portion only. These results indicate that the inner and outer portions of the J4 layer have different carbohydrate compositions, especially in the N-acetylgalactosamine/galactose and the fucose moieties, despite the fact that this layer was morphologically uniform (Fig. 1, 4, Table 2). Recently we found that the activity for the induction of sperm acrosome reaction was localized in the outer layer, that is, in the J4 layer and/or the sticky layer, of the egg-jelly in *C. pyrrhogaster* (Sasaki *et al.*, in press). The unique carbohydrate composition in the J4 layer may be involved in the sperm-egg interaction during the fertilization process.

#### Lectin-binding in the oviduct

In this study, toluidine blue staining showed that the egg-jelly of *C. pyrrhogaster* is divided into 7 layers (Fig. 4). The staining patterns from the inner layer to the outer layer fit with that from the anterior region of the pars convoluta to the uterus (Fig. 4, 5). All tested lectins except for DBA bound to secretory cells only in the corresponding regions of the pars convoluta and the uterus that were detected by toluidine blue staining (Fig. 6, 7, Table 3). Though the regions where the J3 and J4 layers were added (pc3-4) were stained in the same manner by the toluidine blue staining, the binding patterns of SBA, GS-I and GS-II to secretory cells in the anterior portion differed from those in the posterior portion (Fig. 7). These results suggest that carbohydrate components localized to each jelly layer were fundamentally added in the corresponding region of the pars convoluta and uterus.

DBA bound to secretory cells only in the anteriormost portions of the pc1 and the pc3-4 regions, while it bound to the J0 and J2 layers in egg-jelly (Fig. 2, 9). It is unknown why carbohydrate moieties bound to DBA in the pc1 and pc3-4 regions were not detected in the J1, J3 or J4 layers. One possibility is that some molecules that have an affinity with DBA are added after the jelly layers have already organized and that these molecules then diffuse into the jelly matrices. It has been reported that some components secreted from one region of the amphibian oviduct are precipitated by lectins secreted from another region (Jego and Joly 1983; Jego *et al.*, 1983). This result may reflect in part the organization processes of the egg-jelly in *C. pyrrhogaster*.

The toluidine blue staining divided the outermost layer of egg-jelly, the sticky layer, into two layers (Fig. 4). In the corresponding regions of the pars convoluta (pcs1) and uterus (pcs2), Con A bound to secretory cells (Fig. 10, Table 3), and SBA, BPA, GS-I and WGA bound to the matrices in the inner space of the oviduct, which ovulated eggs would pass through (Fig. 10, Table 3). This result suggests that carbohydrate

moieties of the mannose, N-acetylgalactosamine/galactose and N-acetylglucosamine groups may be localized in the sticky layer, though localization of carbohydrate moieties could not be determined by the lectin-bindings to egg-jelly (Fig. 1). The latter pattern of the lectin-bindings was observed only in these regions. We hypothesize that a large amount of the carbohydrate components is deposited in the inner space of the oviductal tubule, which may cause the unique quality of the sticky layer.

In *C. pyrrhogaster*, the egg-jelly structure makes it possible for motile sperm to reach the egg surface, since they cannot move forward in solution (Itoh *et al.*, in press). The egg-jelly guides motile sperm to the egg surface while it eliminates more than half of the sperm in the egg-jelly, suggesting that the organization of egg-jelly components is involved in the successful fertilization in *C. pyrrhogaster*. In this study, two types of layers were repeatedly observed in the inner four layers (Fig. 1, 4, Table 2). This construction may have a role in the sperm guidance and/or the sperm elimination in egg-jelly.

The outer layer of egg-jelly is important for the sperm-egg interaction in the internal fertilization of *C. pyrrhogaster* (Ukita *et al.*, 1999; Sasaki *et al.*, in press). Recently we found that sperm acrosome reaction-inducing activity is localized to the outer layer — that is, the J4 layer and/or the sticky layer — of egg-jelly (Sasaki *et al.*, in press). It appears that the substances to induce sperm acrosome reaction in egg-jelly extract can be precipitated by WGA, MPA, GS-I, and PNA (Onitake *et al.*, 2000; Sasaki *et al.*, in press). In this study, carbohydrates that had specific affinity with WGA and GS-I were detected in the outer layers (Fig. 3, 8, Table 2, 3). These N-acetylglucosamine group and/or N-acetylgalactosamine/galactose group carbohydrate moieties may be involved in the induction of the sperm acrosome reaction of *C. pyrrhogaster*.

In amphibians, several types of fertilization modes are seen among species (Wake and Dickie, 1998). Most anurans and an ancient group of urodeles undergo external fertilization, while most other urodeles undergo internal fertilization. There are some species that take the viviparous mode (Greven, 1998). Their oviductal secretions are thought to be suitable for the fertilization mode. The result of this study demonstrated the complex organization of carbohydrate components in the egg-jelly layers of *C. pyrrhogaster*. However, further study will be needed to clarify the internal fertilization system in the newt egg-jelly.

#### ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid (No. 12680710) from the Ministry of Education, Science and Culture of Japan.

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(Received May 2, 2001 / Accepted June 18, 2001)