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Authors: Tazumi, Yoshimitsu, Nakamura, Osamu, and Watanabe, Tasuku

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Intraovarian Cavity Leucocytes of Viviparous Fish, *Neoditrema ransonneti* (Perciformes, Embiotocidae)

Yoshimitsu Tazumi, Osamu Nakamura* and Tasuku Watanabe

School of Fisheries Sciences, Kitasato University, Ofunato, Iwate 022-0101, Japan

ABSTRACT—To obtain basic information on the properties of the intraovarian cavity leucocytes (IOCLs) of the viviparous teleost, *Neoditrema ransonneti*, morphological characteristics and numerical changes of IOCLs during the reproductive cycle were investigated. In the ovaries of newborn females, leucocytes exuded into the lumen were observed first in November, prior to insemination of semen. These cells were primarily macrophages, neutrophils and lymphocytes. Among them, macrophages were invariably the largest population throughout the reproductive cycle. They began to phagocytize spermatozoa in December, when spermatozoa were first detected in the ovary. The number of IOCLs gradually increased from November in the newborn female. However, this increase is not ascribed to the effect of copulation or the presence of semen, because the number of leucocytes also increased in non-mating fish. While developing embryos were discharged into the ovarian lumen at the latest in January, a number of spermatozoa and spermatozoa-phagocytizing macrophages were seen until March. Even after the extinction of sperm cells, numerous IOCLs remained in the lumen and coexisted with fetuses until their parturition. These results suggest that IOCLs play roles in successful pregnancy, besides elimination of remaining spermatozoa.

Key words: viviparity, intraovarian cavity leucocytes, macrophage, teleostei, *Neoditrema ransonneti*

INTRODUCTION

In teleost, more than 500 species are known to be viviparous (Wourms, 1981). Females of almost all viviparous teleosts have a single hollow ovary. After an internal fertilization, embryos remain in the ovarian cavity where they retain a close relationship with the mother in terms of nutrition, respiration, excretion, and immunity.

Viviparous vertebrates are inevitably confronted with a conflict as their immune system has developed an accurate recognition system for distinguishing self and non-self. In mammals, elaborate mechanisms which protect spermatozoa and fetuses from immunological rejection by the maternal immune system have being elucidated (Wegmann *et al*., 1993, Sacks *et al*.; 1999, Piccini *et al*.; 2000, Chaouat *et al*., 2002). While a number of physiological studies on viviparity have been done in fish, little is known about the immunological aspects of this mode of reproduction.

Embiotocidae fish, so-called surfperch, stand out among viviparous species of teleost because of their long period of gestation (more than six months in general) and almost complete dependence on maternally supplied nutrients (Turner, 1938; Mizue, 1961; Igarashi, 1961; Gardiner, 1978). Maternal nutrients seem to be secreted into the ovarian cavity, where fetuses develop to juvenile. We found that a number of leucocytes were distributed in the ovarian cavity of *Neoditrema ransonneti*, a conventional Embiotocidae fish in Japan. In mammals, leucocytes in the genital tracts and the utero-placental tissues are known to play crucial roles in fertilization and gestation through sperm cell selection and secreting diverse kinds of immuno-modulating substances (Barrat *et al*., 1990; Hunt and Robertson, 1996; Bukulmetz and Arici, 2000). On the other hand, only a few studies have noted the presence of leucocytes in the ovarian cavity of teleost (Gardiner, 1978; Koya *et al*., 1997). In these studies, the leucocytes were described simply as scavengers for remaining spermatozoa after fertilization, and the possible other functions that they may play in reproduction were not considered. In addition, basic information about those leucocytes, such as the number of cells, cellular composition, and time-course of appearance and extinction are also lacking.

In this study, we investigated characteristics and numerical changes of ovarian cavity leucocytes during the reproductive cycle of *N. ransonneti*, expecting that those may provide clues to the functions of the cells in fertilization and pregnancy in this species. The effect of copulation on the infiltration of leucocytes into the cavity was also examined.

* Corresponding author. Tel. +81-192-44-1907;
FAX. +81-192-44-2125.
E-mail: naosamu@kitasato-u.ac.jp

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MATERIALS AND METHODS

Fish

Fish were collected by angling at Okirai Bay, Iwate, Japan in spring, 2001 (Group 0). The fish were held in FRP tanks with running seawater under natural conditions and fed a commercial diet for marine fish (Fuji-Seifun Co., Japan) ad libitum. Females gave birth in early August. Newborn fish were fed live brine shrimp nutritionally enriched with fish egg oil (Nisshin Marine Tech Co., Japan). After one month, a commercial diet for marine fish juveniles (Fuji-Seifun) was supplied. Newborn fish were then divided into two groups in September. One group was composed of females alone (Group1) and the other group consisted of males and females (Group2). Adult fish caught in Autumn 2001 (Group3) and Spring 2002 (Group4) were reared in the same manner and used for another experiment.

Histological observation of ovaries

For light microscopy, 3 female individuals in Group 0 were sacrificed every month from April 2001 to March 2002. The fish were anaesthetized with 2-phenoxyethanol. After blood had been collected from the caudal vein with a heparin-coated 27G needle attached to a syringe, ovaries were removed, fixed in Bouin's fluid, and embedded in paraffin. Paraffin sections 3–5 μm thick were prepared and stained with Mayer's hematoxylin and eosin.

For transmission electron microscopy, the tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C for 2 h. The specimens then were rinsed in three changes of 0.1 M PB and postfixed in 2% osmic acid in 0.1 M PB at 4°C for 1 h. All tissues were rinsed again, dehydrated with ethanol, and embedded in Quetol 651 epoxy resin (Nisshin EM, Japan). Sections for electron microscopy were cut approximately at 90nm on an ultramicrotome. The sections were stained with 1% uranyl acetate and 0.1% lead citrate, and observed using a JEM-100S TEM (JEOL Ltd, Japan) operating at 80 kV.

Sampling of IOCLs

Periodic sampling of intraovarian cavity leucocytes (IOCLs) of Groups 1 and 2 was conducted from October 2001 to January 2002. To investigate the further seasonal changes of IOCLs during gestation, sampling was continued from December 2001 to March 2002 (Group 3). Because all of these fish were killed by accident in April, another group (Group 4) was used for the observation from May to September 2002. All of the fish of Groups 3 and 4 which were sacrificed from January to July were pregnant.

At each sampling, 3 fish of each group were sacrificed in the manner mentioned above. The gonads were removed and weighed. Then, intraovarian cavity cells were obtained as described in the following section.

Cell preparation and microscopy

The removed ovaries were washed with 0.01 M phosphate buffered saline (PBS, pH 7.2) and cut longitudinally in 1 ml of PBS. Cells were suspended in the liquid through gentle shaking of the ovary. After total cell numbers had been counted using Bürker-Turk Counting chambers, cells were cytocentrifuged at 80 *g*×5 min (SC-2, TOMY, Japan) onto glass slides. The cells were dried and fixed in 3.5% glutaraldehyde in 80% ethanol. To detect peroxidase activity, the slides were incubated in a substrate solution containing 0.02% 3,3´-diaminobenzidine (Wako Pure Chemicals, Japan) and 0.03% H_2O_2 in 0.05 M Tris-HCl (pH 7.6) and stained with Giemsa's solution (Kamei, 1999). The cells were classified into several types according to morphology and stainability. Percentages of each cell type were determined by counting 500 cells on each slide. Blood smears were also stained in the same manner for comparison.

For transmission electron microscopy, intraovarian cavity cells were fixed by glutaraldehyde and osmic acid as aforementioned. The fixed cells were embedded in 3% agarose. Then, small pieces of the gelatinized cakes were dehydrated, embedded, sectioned and observed in the same manner.

Statistics

The differences in the number of IOCLs between Groups 1 and 2 in the same month was analyzed by t-test. Differences of percentages of each cell type among months and groups (1 and 2) were analyzed using the one-way ANOVA and Bonferroni's Multiple Comparison Test.

RESULTS

Seasonal changes in the ovary

The ovary of *N. ransonneti* is composed of three parts; the ovarian wall, ovigerous folds and ovarian cavity. The ovigerous folds consist of 6 lamellae projecting from the inner dorsal surface of the ovarian cavity. The lamellae have a connective tissue, developed blood vessels, oocytes and follicle cells, and are covered on both surfaces by epithelial cells.

The epithelial cells of the ovigerous lamellae can be seen to go through three stages during one annual reproductive cycle, in content with the observations in *Cymatogaster aggregata* (Gardiner, 1978). During October to February, most of the epithelial cells became tall and were separated laterally by voluminous intercellular dilations (Fig. 1). In these spaces, penetrating leukocytes were occasionally observed. During March to early August, when fetuses develop in the ovarian lumen with maternally supplied nutrition, most of the epithelial cells became columnar. The intercellular dilations progressively decreased in volume. After birth, the ovigerous folds underwent rapid shrinking and flattened out.

Distribution of spermatozoa and leucocytes in the ovarian cavity

Mating behavior was observed in October to December in Group 0. It was not possible to confirm whether females copulate only once or repeatedly. During this period, spermatozoa were found in the ovarian cavity in masses. Around them, many leucocytes were found (Fig. 2a). There appears no sperm storage structure such as the "sperm pocket" described in the study of *C. aggregata* (Turner, 1938) in the ovarian epithelia. Instead, a part of spermatozoa were also observed between epithelial cells and beneath epithelia in October at the latest (Fig. 2b), suggesting that a small percentage of spermatozoa had successfully penetrated the subepithelia soon after copulation. They appeared to fertilize ova in the follicle as in other Embiotocidae fish (Turner, 1938), though it could not be ascertained when fertilization had been completed.

In December, embryos discharged into the ovarian cavity were seen. Fig. 2c shows the developing embryo in March, which were still covered by a fertilization membrane attached with leucocytes. After hatching, a number of leuco-

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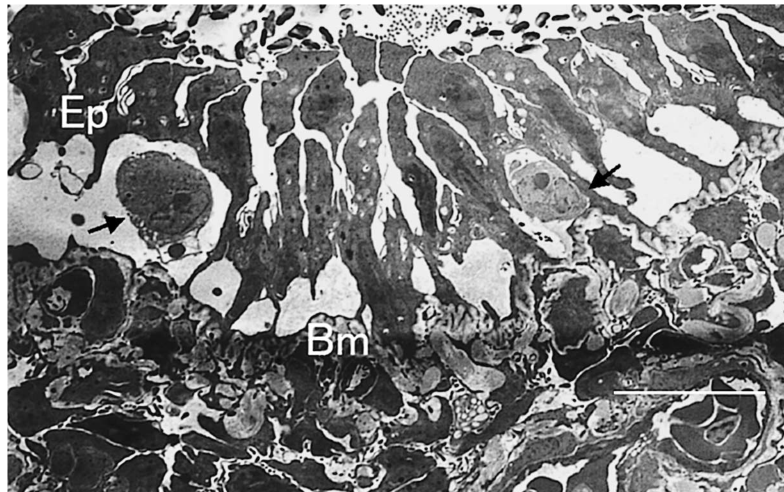

Fig. 1. TEM of the epithelium of ovigerous lamellae in November. Note the leucocytes (arrow) have infiltrated between epithelial cells. Ep: epithelial cells, Bm: basal membrane. Bar=10 μm.

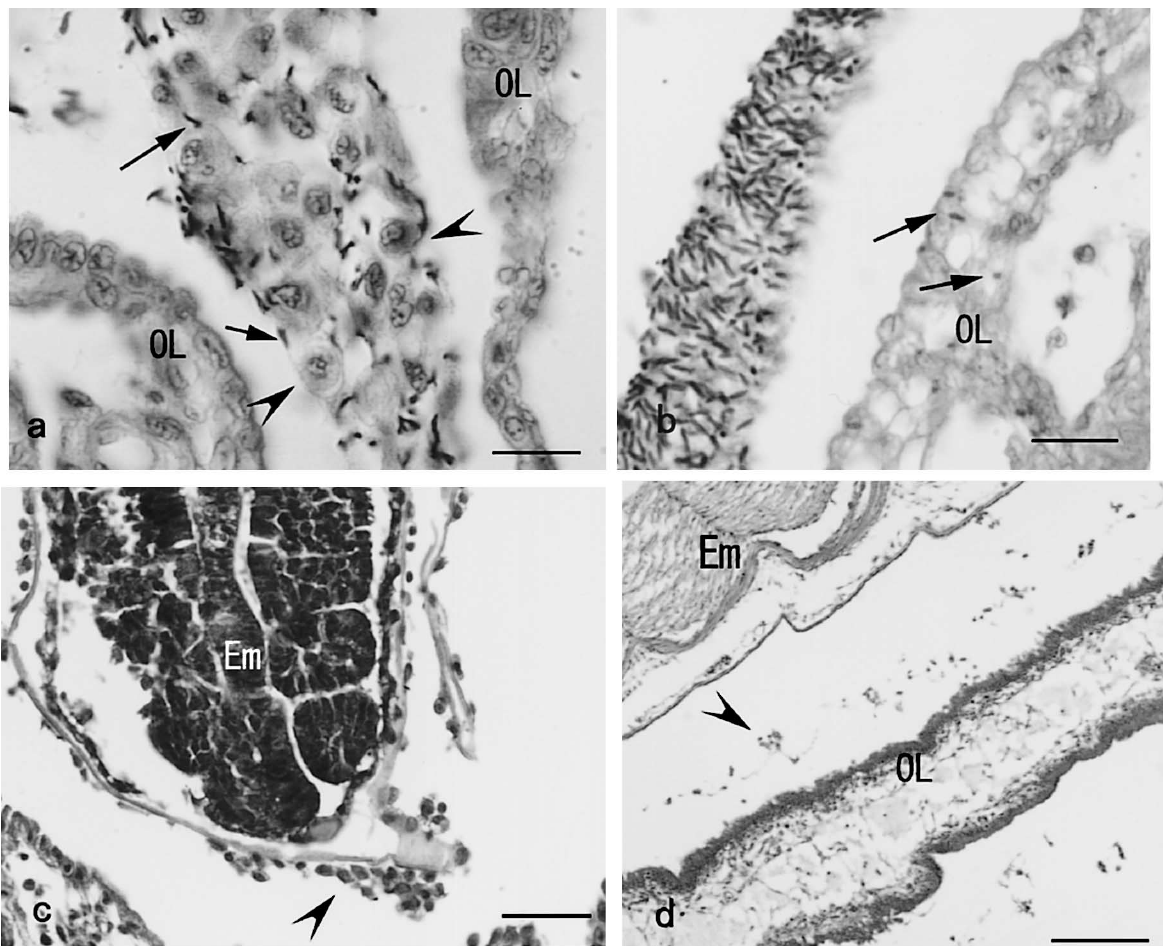

Fig. 2. Paraffin sections of an ovary. H.E. staining. (a) After mating. A number of spermatozoa (arrow) and surrounding leucocytes (arrowhead) were seen in the ovarian cavity. Bar=10 μm. (b) In October. A part of spermatozoa (arrow) penetrated into epithelia of ovigerous lamellae. Bar=10 μm. (c) In March. Note the leucocytes (arrowhead) have adhered to the fertilization membrane. Bar=25 μm. (d) In May. After hatching. IOCLs suspending around a developing fetus. Bar=100 μm. OL: Ovigerous lamellae, Em: embryo.

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cytes were seen to be suspended adjacent to the conceptus in the ovarian lumen (Fig. 2d).

Identification of IOCLs

Since there was no available criteria or cell markers for identification of leucocytes in surfperch, we tried to make a rudimentary classification of IOCLs according to light and electron microscopy. As a result, it appeared that they could be classified into several types, including monocytes/macrophages, neutrophils and lymphocytes. Macrophages were large cells having an oval nucleus and pale blue cytoplasm (Fig. 3a). Their cytoplasm was highly vacuolated in general, peroxidase-negative, and often contained ingested spermatozoa (Fig. 3b, d). The cells which were smaller than typical macrophages and possessed a kidney-shaped or ovoid nucleus and higher nucleus/cytoplasm ratio appeared to be monocytes (Fig. 3a). They were also peroxidase-negative. Since they can be regarded as of the same lineage, they were represented as macrophages in the differential count. The cells which were replete with peroxidase-positive granules were referred to as neutrophils in this study (Fig. 3a, e). They possessed an ovoid, and in some cases a segmented nucleus. Cells with the same characteristics were also seen in the blood. They also engulfed sperm cells but very rarely did so. Lymphocytes were small, round cells with short cell processes. They had round nuclei and scant cytoplasm (Fig. 3c, f). No eosinophilic or basophilic granulocytes were seen. In all specimens, a variable proportion of small mononuclear leukocytes could not be characterized by light microscopy with confidence and were documented as unidentified cells. Besides them, one curious kind of cells was seen. They contained a bulk of eosinophilic vacuoles and compressed nuclei in their marginal cytoplasm (Fig. 3g). The lineage of these cells, which were never seen in the blood, is unknown. Because these cells may not be leucocytes and occupy only a small portion of intraovarian cells (less than 0.5%), they were neglected in the differential cell counting. Leucocytes in mitosis were found in some specimens (Fig. 3h), showing

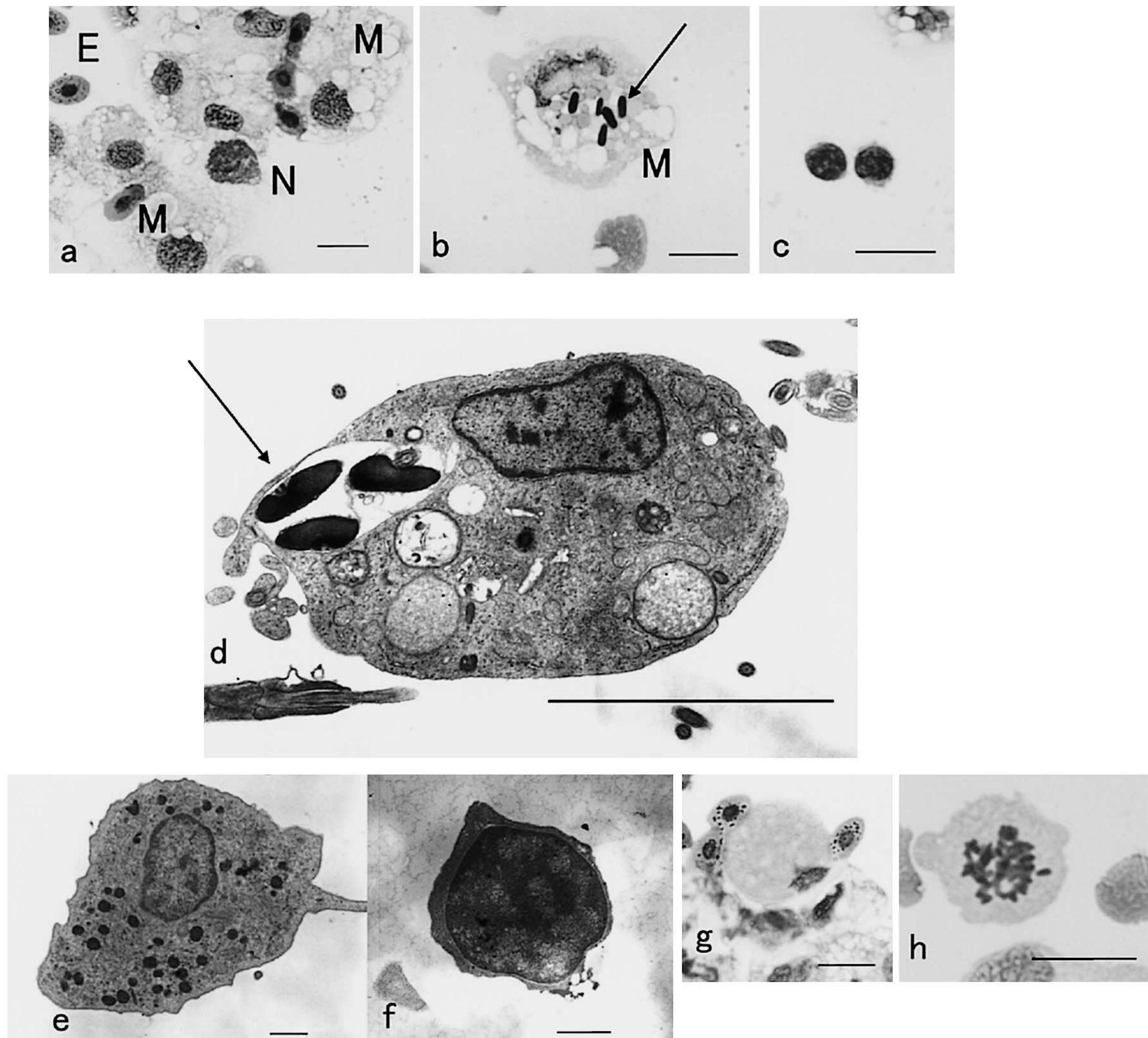

Fig. 3. Giemsa staining (a, b, c, g, h) and TEM (d, e, f) of IOCLs. a: Macrophage (M), monocyte (Mc), neutorphils (N). b, d: macrophage ingesting spermatozoa (arrow). c, f: lymphocytes. e, neutrophil. g: large eosinophilic cell. h: mitosing cell. Bars=10 µm in a, b, c, d, g, h and 1 µm in e and f.

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that a part of IOCLs proliferate in the ovarian cavity. A small number of erythrocytes were seen among IOCLs in most specimens. Though this seems to be a result of contamination of the blood in cell preparation, a few red blood cells were also observed in the ovarian cavity in the paraffin section of the ovary (not shown).

Numerical changes of IOCLs in the reproductive cycle

In newborn females, no spermatozoa or leucocytes were found in the ovarian cavity until October, about 2 months after birth. In November, when no inseminated sperm cells had been seen yet in the ovarian lumen of Group 2 individuals, a small number of IOCLs was observed in Groups 1 and 2 (Fig. 4c). Spermatozoa were first observed in the ovarian lumen in Group 2 in December, when some of them were ingested by macrophages that had infiltrated the cavity. A few other spermatozoa were observed in the subepithelial region, suggesting that they had headed successfully toward fertilization. In some specimens of Group 2 in this period, bacteria, some of which were ingested by macrophages, were found. Embryo discharged into the ovarian cavity was not found even in January in Group 2. In both groups, the IOCLs significantly increased in number by January 2002. At each sampling, however, there was no significant difference in the number of IOCLs per ovary between the two groups.

Differential cell counts showed that the macrophage was the dominant cell type in IOCLs, comprising more than 90% of IOCLs in every observation (Table 1). The percentages of the other three types remained low. There was no significant difference among groups and months. From September to January, body weight and length were augmented, but GSI remained low as shown in Fig. 4b.

In Group 3, the number of IOCLs increased significantly between December and January, and between February and March (Fig. 5). Macrophages composed the largest population among the IOCLs unchangingly. There was no significant change in the proportions of the leucocytes excepting that the ratio of neutrophils in July to September was significantly higher ($P<0.05$) than in other months (Table 2). Discharged embryo was found in cyto-spin specimen in December in Group 3. A small number of sperma-

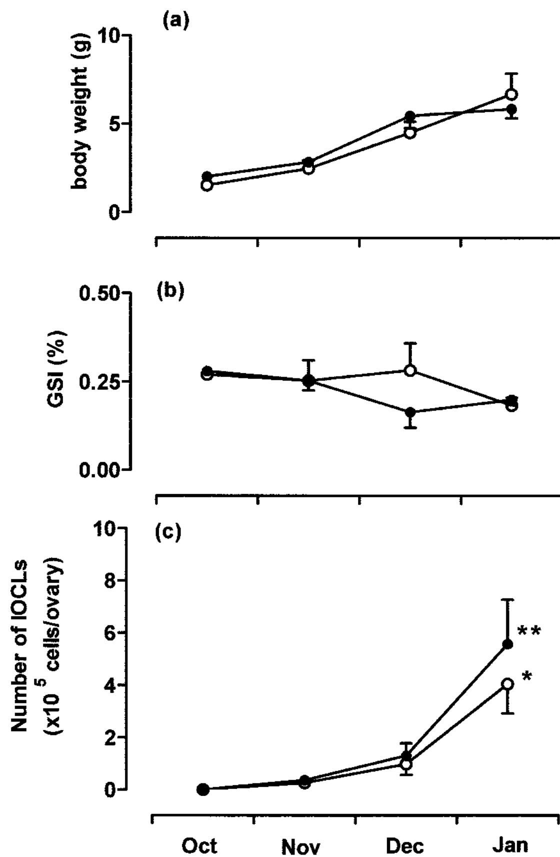

Fig. 4. Changes in body weight (a), GSI (b), and number of IOCLs (c) of the female in the copulating period (mean ± SD, *n*=3). Closed circle: Group1, Open circle: Group2. Significant increase of IOCLs number compared to the last month was represented as *(p<0.05) and **(p<0.001).

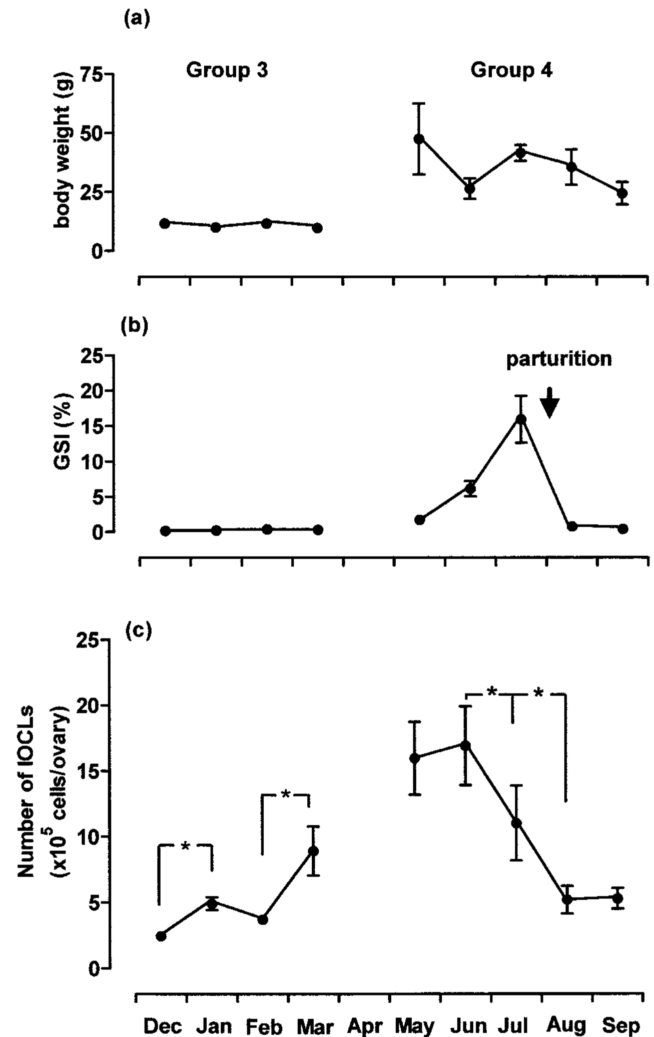

Fig. 5. Changes in body weight (a), GSI (b), and number of IOCLs(c) of the female in the gestating period (mean ±SD, *n*=3). * represents significant difference ($p<0.05$).

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Table 1. Composition (mean percent±S.D. for each cell type, n=3) of leucocyte populations recovered from ovarian cavity of Groups 1 and 2.

Month	Group	Macrophages	Neutrophils	Lymphocytes	Unidentified
Oct	1	ND	ND	ND	ND
	2	ND	ND	ND	ND
Nov	1	91.6±3.7	2.9±1.9	1.1±0.8	4.4 ± 1.0
	2	93.2±1.3	5.1±1.6	0.7±0.1	1.0±0.4
Dec	1	97.2±0.6	1.5±0.2	0.2±0.3	1.1±0.3
	2	95.5±3.2	1.5±1.3	0.3±0.3	2.6±1.7
Jan	1	94.8±2.3	0.9±0.2	0.8±0.7	3.5±2.0
	2	94.0±2.3	2.3±2.6	0.6±0.5	3.1±1.0

ND: not detected

Table 2. Composition (mean±S.D. for each cell type, n=3) of leucocyte populations recovered from ovarian cavity of Groups 3 and 4. Values designated by the different letter are significantly different from each other (*P*<0.05).

Month	Group	Macrophages	Neutrophils	Lymphocytes	Unidentified
Dec	Group3	94.3±1.6	1.3±0.5 ab	0.9±0.8	3.5±1.5
Jan		91.5±2.6	1.1±0.6 ab	1.5±1.8	5.9±1.4
Feb		91.8±3.3	0.5±0.2 a	1.7±1.4	6.0±1.7
Mar		92.5±2.1	1.9±1.1 ab	1.1±0.5	4.6±1.0
Apr			no data		
May	Group4	94.9±2.4	0.5±0.7 ab	1.0±0.0	3.6±3.0
Jun		97.3±1.5	0.9±0.3 ab	0.2±0.3	1.7±1.0
Jul		91.3±3.8	5.8±2.9 c	2.7±2.1	2.4±0.9
Aug		90.4±3.9	4.8±1.5 bc	1.6±0.7	5.0±1.2
Sep		87.9±3.9	7.2±0.4 c	1.5±1.4	3.4±2.2

tozoa still remained in the cavity even in March, though macrophages ingesting spermatozoa were observed every month from December to March. In spite of the disappearance of the spermatozoa in the lumen by May, the number of IOCLs remained high until June. The percentages of neutrophils and lymphocytes were low throughout the observation period.

IOCLs decreased in number significantly from June to July, prior to parturition. In August, post-parturition, the number further diminished.

DISCUSSION

The present study showed that a number of leucocytes were present in the ovarian cavity of *N. ransonneti* throughout its reproductive cycle. They phagocytized spermatozoa that had been inseminated the cavity, attached to the fertilization membrane of the discharged embryo, and coexisted with the developing fetuses until birth. Among IOCLs, macrophages were invariably predominant. Spermiophagy by them began in December, when the sperm were first observed in the ovarian lumen in Group 2, and continued until March in Group 3. This suggests that one of the roles of the exuded macrophages is to eliminate spermatozoa that do not participate in fertilization. In fish, only a few studies have ever reported spermiophagy by intraovarian leucocytes (Gardiner, 1978; Koya *et al*., 1998). In those studies, the role of these leucocytes was defined as the elimination of residual spermatozoa that had not participated in fertilization. In many animal species, however, phagocytes exuded into the genital tract begin to engulf spermatozoa when fertilization has not been completed as reviewed by Cohen (1984). They seem to be involved in sperm selection, though how they select spermatozoa to be phagocytized is not well understood (Cohen, 1984; Cohen and Adeghe, 1987; Eisenbach, 2003). Igarashi (1961) reported that fertilization occurred just prior to extrusion of the embryo into the lumen in *N. ransonneti*. Since no discharged embryo was found even in January in Group 2, it appears that fertilization was not finished in Dec in this group. Thus, it seems not likely that macrophages of female ingest only “remaining” spermatozoa following fertilization. Such delay in reproductive cycle in newborn female was also observed in *C. aggregata* (Turner, 1938). As some spermatozoa successfully invaded the epithelia of ovigerous lamellae soon after copulation, it may not be a major obstacle to fertilization if macrophages begin to ingest gametes prior to fertilization. On the other hand, significant numbers of spermatozoa remained in the

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cavity until March, notwithstanding elimination by phagocytes. The physiological implications of sperm-phagocytosis by macrophages and the differences between the sperm which are phagocytized and those which are not need to be investigated.

The influx of leucocytes into the ovarian lumen in November to January was not an immune or inflammatory response to spermatozoa, because it occurred regardless of copulation as shown in Fig. 4. This is quite different from the case in mammals. There are usually few leucocytes in the genital tract fluids but, after intercourse, a number of leucocytes migrate into the lumen of the uterus and cervix in mice (Austin, 1957), calves (Howe and Black, 1963), rabbits (Howe, 1967), ewes (Mattner, 1969), and humans (Thompson *et al*., 1992). The numerical increase of IOCLs observed from November in *N. ransonneti* seems to be a spontaneous and physiological rather than inflammatory phenomenon. In this period, the intercellular spaces in the epithelium of the ovigerous lamellae grew markedly larger. This structural change in the epithelium, which may be regulated by hormonal control, may facilitate the infiltration of leucocytes into the lumen.

Neutrophils accounted for only minor population among IOCLs, though the percentage of neutrophils in July to September was elevated owing to unknown reason (Table 2). In mammals, phagocytes for sperm in female genital tract are primarily polymorphonuclear leucocytes, but in hen, they are macrophages (Koyanagi and Nishiyama, 1981). Also in *N. ransonneti*, spermatozoa were ingested exclusively by macrophages, and neutrophils seemed not to contribute to elimination of sperm virtually.

It is intriguing that a number of maternal leucocytes filled the ovarian cavity even after the spermatozoa had extinguished completely, and coexisted with fetuses until just prior to birth. Although the functions of the macrophages in this situation are unclear, it seems reasonable to assume that they play roles other than in the elimination of sperm cells in a successful pregnancy. In mammal, distribution patterns of leucocytes in matero-fetal interface seem to be largely different among species. In mouse, macrophages are virtually absent in decidua during pregnancy (De and Wood, 1991; Brandon, 1995). On the other hand, a large number of macrophages are distributed in endometrium and decidua in pregnant women (Vince and Johnson, 1999; Laird *et al*., 2003). Vince and Johnson (1999) suggested that utero-placental macrophages have vital roles in host defence against infection in pregnancy, because immunocompetent cells in specific defence is largely absent in those tissues. With marked paucity of lymphocytes, intraovarian cavity macrophages, and maybe neutrophils as well, may impart protection against pathogenic invasion in the ovarian lumen of *N. ransonneti*. Mammalian macrophages may also contribute to the sustainment of pregnancy by regulating immune responses (Hunt and Robertson, 1996; Hunt *et al*., 1998). Macrophages in pregnant human uterus are major sites of production of prostaglandin E_2 (Norwits *et al*., 1991), which suppresses lymphocyte activation and proliferation in placenta (Parhar *et al*., 1988; Lala *et al*., 1988; Parhar *et al*., 1989; Kvirkvelia *et al*., 2002). Though cytotoxic T cells and natural killer cells have not been identified distinctively in fish, it has been demonstrated that cytotoxic cells recognizing allogenicity also exist (Yoshida *et al*., 1995; Stuge *et al*., 1995; Fisher *et al*., 1998; 1999; Hasegawa *et al*., 1998; 1999). In the viviparous poeciliid fish, *Xiphophorus hellerii*, the fertilization membrane which surrounds fetuses during gestation may protect them from the maternal rejection (Hogarth, 1973). In *N. ransonneti,* fetuses appeared to be in contact with maternal IOCLs directly. Nevertheless, the ratio of lymphocytes among IOCLs was low and never increased throughout the reproductive cycle. Therefore, it seems plausible that suppressive mechanisms, in which macrophages may be involved, hamper immunological rejection of embryos by maternal cytotoxic cells in the ovarian cavity of *N. ransonneti.*

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