Gonadal Development and Germ Cell Kinetics in the Course of Sex Differentiation in the Salamander *Onychodactylus japonicus*

TAKASHI NOMURA AND HISAAKI IWASAWA

**Abstract:** Gonadal development was studied histologically in *Onychodactylus japonicus*, and changes in the number of germ cells in the course of sex differentiation were examined. The mode of sex differentiation is of the sexually differentiated type. Gonadal sex differentiation occurred directly from the sexually indifferent primordial gonad, and this differentiation was recognized in the larvae of 19–22 mm in snout-vent length (developmental stages 67–68). Slight local variations in external and histological structures were observed in the primordial gonad. No sexual difference in the number of germ cells was found when sexual differentiation was beginning. Just after sex differentiation, secondary oogonia appeared in the ovarian primordia, and proliferated temporarily. Germ cells in the ovarian primordia therefore became more numerous than those in the testicular primordia. When auxocytes appeared in the ovaries, oocytes in earlier stages decreased in number, and degenerative germ cells were seen. In the testes, a positive correlation was recognized between the number of germ cells and body length.

Key words: Gonadal development; Sex differentiation; Germ cell kinetics; Salamander; *Onychodactylus japonicus*

In the fetal or larval period of vertebrates, sexual difference in the number of germ cells in the primordial gonads has been reported (Hardisty, 1967). There is, however, little information on germ cell kinetics in the course of gonadal sex differentiation. In anurans, studies on this subject have been reported for *Rana nigromaculata* (Kobayashi, 1975), *Xenopus laevis* (Ijiri and Egami, 1975; Yamaguchi and Iwasawa, 1981) and *Bufo japonicus formosus* (Tanimura and Iwasawa, 1987), but no information is available on urodelans. Shinbo (1936) reported gonadal development in *Onychodactylus japonicus*, but his observations were rather incomplete. That is, he used a small number of specimens as materials, and the time of sexual differentiation and the time of occurrence of meiosis were unclear in his descriptions. The present studies were undertaken to clarify the gonadal differentiation of *O. japonicus* in more detail.

**Materials and Methods**

Larvae used in this study were collected at Iwamuro-mura (Altitude: 130 m; Population: 1), Niigata Prefecture in spring (April) and late autumn (October and November), and at Hinoemata-mura (Altitude: 1200 m; Population: H), Fukushima Prefecture, in early summer (June). Snout-vent length (SVL) was measured from the tip of the snout to the posterior angle of the vent. Measurement was made to the nearest 0.1 mm with slide calipers. For histological observations, the urogenital region of each animal was fixed in Bouin’s solution. Measurement was made to the nearest 0.1 mm with slide calipers. For histological observations, the urogenital region of each animal was fixed in Bouin’s solution. To determine gonadal size, the area of the gonads was measured from the ventral side with a Planimex 25 (Nikon Regulator Co., Ltd, Tokyo). The gonads were embedded in paraffin, sectioned serially at 15 μm, and stained with Masson trichrome. In each ovary, the diameter of 20 large auxocytes was measured with an eyepiece micrometer, and the mean was calculated. Germ cells were classified into four groups (primary oogonium, secondary oogonium, oocyte, auxocyte), and the number of cells belonging to each group was counted. For statistical treatment, correlation was examined by coefficient of correlation, and levels of statistical significance were determined by the analysis of covariance in each group. The developmental stages of larvae follow the normal table of development of this species (Iwasawa and Kera, 1980).

**Results**

Individual variation in snout-vent length was...
TABLE 1. Number of animals used in the present observations.

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Snout-vent length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50</td>
</tr>
<tr>
<td>Population I**</td>
<td>67 4 19 11</td>
</tr>
<tr>
<td>(Spring)</td>
<td>68 69 1</td>
</tr>
<tr>
<td>(Autumn)</td>
<td>68 69 68 1 5 3 3 1 1 4 5 5 1 1 1 1 2 1 1 3 2 2</td>
</tr>
<tr>
<td>Population H***</td>
<td>67 2 2 68 5 11 7 2 1</td>
</tr>
<tr>
<td>69 4 15 14 8 14 12 4 4 1 1 1 1 1 1 1 1</td>
<td></td>
</tr>
</tbody>
</table>

* Iwasawa and Kera (1980).
** Iwamuro-mura, Niigata Prefecture (elevation 130 m).
*** Hinoemata-mura, Fukushima Prefecture (elevation 1200 m).

particularly large in stage 69 (Table 1). In full-grown larvae, gonadal development corresponded more closely to SVL than to the stage number. Larval growth is therefore represented mainly by SVL in the following description.

1. External observation

A pair of sexually indifferent gonads appeared as thread-like cords along the inner edge of the kidneys, 2–3 mm in length and ca. 0.14 mm in width. Outlines of gonads are shown in Fig. 1. After sex differentiation, the size of the gonadal primordia increased with a high positive correlation to SVL (p < 0.001; Fig. 2). In both Population I (Pop. I) and Population H (Pop. H), the rate of increase in gonadal size was higher in the ovary than in the testis (p < 0.001). In specimens of the same size, the width of gonadal

![Fig. 1. Outline of gonads (ventral view). I: Sexually indifferent gonad. O₁-O₃: Ovary. T₁-T₃: Testis. The numerals affixed to O or T indicate the order of ovarian or testicular development.](image)

![Fig. 2. Correlation between SVL and gonadal size (ventral view).](image)
primordia of both sexes was larger and the rate of increase in testicular size was higher in Pop. H than in Pop. I (p < 0.001). The outline of a cross section of gonadal primordia of both sexes was not a depressed oval, but a circle. In Pop. I, numerous small constrictions appeared on the surface of the ovary with the enlargement of oocytes. In females 36 mm in SVL, the development of auxocytes was recognized from the surface under a binocular microscope. During the course of testicular differentiation, no remarkable change was seen. In males 47 mm in SVL, however, some ruggedness was seen on the surface of testicular primordia due to the
development of seminiferous tubules. After sex differentiation, gonadal primordia increased in size, especially at the anterior part of the ovary. This increase was more remarkable in Pop. H than in Pop. I. In the ovary of Pop. H, unlike Pop. I, constriction of the outline was not remarkable, and auxocytes were hardly seen externally even under a binocular microscope. The testes were slightly thicker in Pop. H than in Pop. I.

2. Histological observation

a. Sexually indifferent gonad.—Sexually indifferent gonadal primordia were located on both sides of the basal part of the mesentery, and jutted out into the abdominal cavity (Fig. 3). The number of germ cells in these rudimentary gonads was 1–3 in each cross section, and the diameter of these germ cells was 25–30 µm. At the time of sex differentiation, the gonadal primordia had a cortico-medullary structure. Testicular and ovarian differentiation occurred directly from these gonadal primordia. In Pop. I, this differentiation occurred in larvae 19–22 mm in SVL (stage 67). In Pop. H, on the other hand, sex differentiation occurred slightly earlier in females than in males, and the beginning of ovarian differentiation was recognized in larvae 22 mm in SVL (stage 68).

b. Ovary.—After the beginning of ovarian differentiation, germ cells remained in the cortical region, and the medullary tissue showed a tendency to degenerate. In larvae 20–24 mm in SVL (stage 67), secondary oogonia were seen, but the formation of ovarian cavities was still poor (Fig. 4). In larvae 24–26 mm in SVL, the ovarian cavities were clearly seen. Because of the proliferation of secondary oogonia and the appearance of a large number of oocytes in the
leptotene and zygotene stages, the thickness of the cortical region increased remarkably (Fig. 5). In Pop. I, auxocytes appeared in larvae 24 mm in SVL (Fig. 6). Owing to the appearance of auxocytes, oocytes in earlier stages were situated in the outer region of the ovarian primordia, and the ovarian cavities became narrow (Fig. 7). In the region where no auxocytes were seen, the cortical region was filled with oocytes in leptotene-pachytene stages (Fig. 8). A positive correlation was recognized between the diameter of the auxocytes and body size ($p < 0.001$). The diameter of auxocytes was 200-250 $\mu$m in larvae 36 mm in SVL (Fig. 15). In Pop. H, the cortical region was filled with secondary oogonia and oocytes, and thicker than that in Pop. I, though auxocytes were only seen in larvae larger than 30 mm in SVL (Fig. 9). In Pop. H, the growth of auxocytes was definitely retarded, and the diameter was only 70 $\mu$m on the average in larvae 36 mm in SVL.

c. Testis.—After sex differentiation, germ cells migrated to the medullary region and proliferated, and the cortical region became degenerative (Fig. 10). The seminiferous tubules developed in larvae 25 mm in SVL (Fig. 11). In this period, 3-5 rudimentary seminiferous tubules, in which 3-4 primary spermatogonia were recognized, were seen in each cross section. With the advance of testicular development, the arrangement of seminiferous tubules became fan-like in the cross section (Fig. 12). This arrangement was more clearly seen in Pop. I, i.e., the number of seminiferous tubules was 6-7 in a cross section, and the formation of these tubules was recognized as some ruggedness on the surface of the testes (Fig. 13). The number of germ cells was 4-5 per tubule in each cross section. In Pop. H, although testicular development was well advanced, the arrangement of seminiferous tubules was not clear, and the surface of the testicular primordia was smooth and flat in most specimens (Fig. 14). The number of germ cells was 6-7 per tubule in the cross section. In both Pops. I and H, no secondary spermatogonia were recognized during metamorphosis.

3. Numerical observations

a. Changes in the number of germ cells in the course of gonadal differentiation.—Changes in the number of germ cells in the course of gonadal differentiation are shown in Fig. 16. In the rudimentary gonads, the number of germ cells was 400-600 in Pop. I and ca. 100 in Pop. H. In both populations, sexual difference in this
number was not clear at the early stages of sex differentiation. In the rudimentary ovaries, just after the differentiation from the sexually indifferent primordium, the number of germ cells increased rapidly, and reached 1000–2000 in Pop. I. When oocytes in the meiotic prophase appeared, individual variation in the number of germ cells tended to be greater.

In Pop. I, germ cells did not exceed 4000 in number even in the largest ovaries in the spring specimens. In the autumn specimens, on the other hand, the number was 4000–6000 even in larvae 24–30 mm in SVL. There was a positive correlation between the number of germ cells and body length in the spring specimens \( (p < 0.001) \), but no correlation was found in the autumn specimens. In the male gonads, the number of germ cells reached ca. 1000 after sex differentiation, and tended to be greater as the larvae grew up. In metamorphosing larvae, the number of germ cells was ca. 6000 in the testes, and was greater than that in the ovaries. In the spring and autumn specimens, there was a positive correlation between the number of germ cells and SVL \( (p < 0.001) \). The increase in the ratio of this number to SVL was larger in the autumn specimens than in the spring ones \( (p < 0.005) \). Just after sex differentiation, the number of germ cells was greater in ovarian primordia than in testicular ones, and the increase in the ratio of the number to SVL tended to be great in male gonads \( (p < 0.01) \). Changes in the number of degenerative germ cells in the course of sex differentiation are shown in Fig. 17. In spring, degenerative germ cells were few in both sexes. In autumn, there was a positive correlation between the number of degenerative germ cells and SVL in male gonads \( (p < 0.001) \). The ratio of degenerative germ cells to total germ cells was about 1: 10 in males, and 1: 20 to 3: 10 in females.

In Pop. H, degenerative germ cells were scarce. In females, a great number of oocytes were seen, and over 20000 germ cells were counted in a larva 33 mm in SVL. Among the larvae of the same size, the number of germ cells and the increase in the ratio of the number to SVL were both greater in Pop. H than in Pop. I \( (p < 0.025) \). In males, the number of germ cells

![Fig. 17. Correlation between SVL and number of degenerating germ cells in population I.](image)

![Fig. 18. Correlation between SVL and ratio of germ cells of each stage to total germ cells.](image)
was correlated with SVL (p < 0.001). The number of germ cells was similar to that in Pop. I, but the increase in the ratio of the number to SVL was greater in Pop. H than in Pop. I (p < 0.005).

b. Oogenesis and number of germ cells.—The ratios of different kinds of germ cells to the total number of germ cells are plotted in Fig. 18. In the spring specimens of Pop. I, the ratio of primary oogonia was 80-90% in larvae over 30 mm in SVL. In these larvae, the ratio of secondary oogonia was less than 10%, and the ratio of oocytes in the meiotic prophase was about 10%. Few oocytes were seen in smaller specimens. In the autumn specimens, on the other hand, the ratio of oocytes was very high, and oocytes was constituted for a great portion of germ cells. Concerning the ratio of secondary oogonia, a similar tendency was found in Pop. H. In both populations, the number of auxocytes was 40-100 in metamorphosing larvae.

DISCUSSION

In the two populations examined, gonads differentiated directly from the sexually indifferent primordial gonads; therefore, *O. japonicus* belongs to the differentiated type in the mode of sex differentiation. Concerning this point, the present results agree with the results presented by Shinbo (1936) who used the larvae of *O. japonicus* collected in Mikawa-mura and Yamakoshi-mura, Niigata Prefecture and Tateiwa-mura, Fukushima Prefecture. Geographic variation in the mode of sex differentiation is well known in *Rana temporaria* (Witschi, 1930) and *Ambystoma maculatum* (Witschi, 1933). Furthermore, a slight variation in the mode of sex differentiation is often found in the same species inhabiting the same place. The causes of this variation seem to be the time of spawning and the water temperature during the larval period as well as genetic factors (Iwasawa, 1969; Iwasawa and Oyanagi, 1971; Michibata, 1973; Iwasawa and Takasawa, 1974). In the present study, a slight local variation was seen between Pop. I and Pop. H in the mode of development of gonads. It is conceivable that this is merely local variation in clutch size as already known in many amphibian species (Takahashi and Iwasawa, 1988). The mean clutch size in *O. japonicus* was 11.0 halfway up Mt. Komagatakage, Hinoemata-mura, Fukushima Prefecture (Pop. H; Iwasawa and Kera, 1978), and 22.0 halfway up Mt. Houdatsu, Ishikawa Prefecture (300 m in altitude; Akita, 1982).

In the present study, sexual difference in the number of germ cells was not clear at the time of gonadal sex differentiation. In the rudimentary ovaries, the number of secondary oogonia increased rapidly. Then the germ cells in the ovary became more numerous than in the testis. Sexual difference in the number of germ cells in the developing amphibian gonads has been reported in *Rana nigromaculata* (Kobayashi, 1975), *Xenopus laevis* (Ijiri and Egami, 1975; Yamaguchi and Iwasawa, 1981) and *Bufo japonicus formosus* (Tanimura and Iwasawa, 1987). In *B. j. formosus*, the main cause of the sexual differentiation in the number of germ cells was a remarkable proliferation of secondary oogonia. Numerical changes in each stage of the germ cells were not seen in *R. nigromaculata* or *X. laevis*. Judging from the histological description in these papers, however, this sex difference seemed to result from the proliferation of secondary oogonia in these two species also. In the metamorphosing larvae of *R. nigromaculata*, ovaries were filled with auxocytes and the proliferation of germ cells stagnated at this time (Kobayashi, 1975). In *B. j. formosus*, on the other hand, the number of auxocytes increased at 24-30 days after metamorphosis, but the total number of germ cells also decreased in this period (Tanimura and Iwasawa, 1987). In Pop. I, the degeneration of germ cells occurred with the appearance of auxocytes. As a result, all the stages of germ cells except auxocytes decreased in number. The decrease in the number of early oocytes was remarkable, so most degenerating germ cells might be early oocytes.

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LITERATURE CITED


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要旨 ハコネサンショウウオの生殖腺の発生と性分化過程における生殖細胞の動態

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ハコネサンショウウオの生殖腫の性分化過程とそれに伴う生殖細胞数の変化を調べた。性分化様式は、未分化生殖腺原基から卵巢と精巢が分化する、いわゆる分化型であった。性分化は頭胴長 19-22 mm（発生段階67-68）で起こった。

生息地域によって性分化中的生殖腺原基に外観と組織構造に若干の違いが認められた。性分化が起こった時点では生殖細胞数に性差は認められなかった。卵巣では性分化直後に2次卵原細胞の一時的な増殖によって、生殖細胞数が精巣におけるそれよりも増加した。肥大卵母細胞が現れると、より若い卵母細胞の数は減少し、退化像がみられた。精巣では体長と生殖細胞数との間に正の相関が認められた。

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