Oogenesis and the Ovarian Cycle in *Salamandra salamandra infraimmaculata* Mertens (Amphibia; Urodela; Salamandridae) in Fringe Areas of the Taxon’s Distribution

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ABSTRACT Reproductive cycle and oogenesis were studied in specimens of *Salamandra salamandra* infraimmaculata Mertens that inhabit fringe areas of the taxon’s distribution in the Mediterranean region. Both ovarian mass and length are correlated significantly with body mass and length. Ovarian length is also correlated with the number of oocytes. During the oogenetic cycle six stages in oocyte development were recognized. Three occur during previtellogenesis: stage 1, in which oogonia divide and form cell nests; stage 2, in which oogonia differentiate into oocytes; and stage 3, in which the oocyte cytoplasm increases in volume. In the vitellogenic phase two additional stages, 4 and 5, were recognized: stage 4, in which lipid accumulates in vacuoles in the periphery followed by the appearance of yolk platelets near the cytoplasmic margin; and stage 5, in which oocyte volume increases rapidly due to increased number of yolk platelets until it reaches its maximal size. During postvitellogenesis one stage was recognized: stage 6, in which the beginning of maturation is characterized by movement of the nucleus toward the animal pole. Oogenesis continues year-round. The first four stages were seen in all ovaries examined. The ovarian cycle is independent of season and reproductive stage apart from the number of mature, postvitellogenic oocytes that increases following gestation toward the beginning of spring (March–April). *J. Morphol.* 231:149–160, 1997.

The urodele Salamandra salamandra (Linnaeus) has a palearctic distribution, with the center in Central Europe. However, a few subspecies occur in fringe habitats that are generally drier and hotter than in the center. These habitats are in Spain, North Africa, West Africa, and northern Israel, which is the southern border. The subspecies *S.s.* infraimmaculata inhabits the latter region (Degani, '86). In these regions the populations are isolated from each other, and salamanders are extremely limited by the availability of water even during their winter breeding season (Warburg, '86, '92). On the other hand, in Europe, water is available during their entire spring and summer breeding season. Among the subspecies of *S. salamandra*, differences were found in reproductive strategies. Thus, in France, *Salamandra salamandra* terestriss has a 1-year reproduction cycle: vitellogenin accumulates in the oocytes from September, oocytes reach their maximum size and ovulate in June, gestation lasts 3–9 months, and parturition begins from April throughout the summer, while *Salamandra* salamandra fastuosa in the Pyrenees has a biennial cycle: vitellogenesis in the second year after parturition (Joly et al., '94). *S. salamandra* is generally ovoviviparous, but *S.s. bernardezi* in Spain sometimes produces postmetamorphic young (Wake, '93). *Salamandra s.* infraimmaculata in Israel delivers 17–200 larvae, mainly between November and March.

Various aspects of oogenesis in amphibians were described in *Chthonerpeton* (Beriois and De Sa, '88), *Ichthyophis* (Masood-Parveez and Nadkarni, '93a,b), *Necturus* (Kessel and Panje, '68), *Triturus* (Fischer, '32), *Pleurodeles* (Bonnafant-Jais and Men-
tre, '83), and several anuran species (e.g., Rugh, '51; Del Pino and Sanchez, '77; Jørgensen, '73, '74, '81, '84; Xavier et al., '70), where oogenesis was described in detail.

Only a few studies have dealt with the ovarian cycle in Salamandra, some of which were reviewed recently (Greven and Guex, '94; Joly et al., '94). Broek ('33) described the ovarian epithelium, and Joly and Picheral ('72) described the follicle before and after ovulation in S. s. terrestris. However, to date oogenesis has not been described in any detail in this species, and there has been no attempt to analyze quantitatively oocyte number in each stage in order to determine the ovarian cycle throughout the salamander’s reproductive cycle.

In addition to the description and quantitative analysis of oogenesis in this species, we wish to describe the extent to which xeric environments affect the reproductive cycle (mainly the ovarian cycle) of Salamandra salamandra.

MATERIALS AND METHODS

This investigation was based on a rather limited number of Salamandra s. infraimmaculata females (n = 18) collected from two geographically close habitats, Tel-Dan and Mt. Meron. (The reason for this is that this species is protected. Consequently, we received a special permit from the Board of Nature Conservation to collect and dissect a limited number of females during the course of this study). The study lasted for 18 months, during which salamanders were collected in the vicinity of water or on the way to their breeding sites during the rainy season (Degani and Warburg, '78). Average monthly temperature and monthly precipitation are presented in Table 1. The number of females collected monthly and their reproductive stage are presented in Figure 1.

Salamanders were weighed, snout-vent length and body mass were measured, and the ovaries were excised. Both the length and weight of the ovaries were taken. Following a longitudinal cut of the ovarian envelope (which enables us to expose the oocytes that are connected to the inner side of the envelope), the oocyte number was determined and their diameters measured. Three groups of oocytes were distinguished by color: transparent or previtellogenic, yellow or vitellogenic, and orange or atretic.

The ovaries were divided into a number of sections that were fixed in Bouin, Smith, Carnoy, Altmann, and Karnowsky fixatives (Karnowsky, '65; Humason, '72). Paraffin blocks were prepared, sectioned at 5–8 µm, and stained with the Hematoxylin and Eosin (H+E), Azan, Mason trichrome and Barret's as well as Periodic Acid Schiff + Alcian Blue (PAS + AB), Bromo-Phenol Blue (BPB), and Para-aldehyde Fuchsin (PF) procedures. Haematoxylin, Azan, Mason trichrome and Barret, stain cellular organelles (nucleus, cytoplasm, yolk, collagen etc). PAS-AB stains polysaccharides, BPB stains proteins and PF stains neurosecretory cells (Humason '72, Ewen '62). After staining, the oocyte oogeneric stage was determined. In order to calculate the effect of the treatment, the diameter was measured again, and, based on both parameters (ovarian mass and oocyte diameter), an oogenetic scale per female was determined, thereby obtaining quantitative data. These data were later analyzed by the Mann-Whitney test and the Student’s t-test to describe ovarian cycle and its relationship to season and reproductive cycle.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>MONTH</th>
<th>PRECIPITATION (mm)</th>
<th>TEMPERATURE (°C)</th>
<th>Maximum</th>
<th>Minimum</th>
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<td>17.2</td>
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</tr>
<tr>
<td></td>
<td>X</td>
<td>22</td>
<td>15.8</td>
<td>11.1</td>
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<td></td>
<td>XII</td>
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<td>14.5</td>
<td>8.5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>231.3</td>
<td>11.1</td>
<td>6.5</td>
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<td>118.3</td>
<td>10.2</td>
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<td>69.8</td>
<td>14.4</td>
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<td>0</td>
<td>28.3</td>
<td>16.5</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1.** Temperature and precipitation in study habitats.
Fig. 1. Number of females collected monthly and their reproductive stage.
RESULTS

Ovary

Ovaries are wrapped in a thin, transparent envelope, and oocytes are attached to the inner side of the ovaries (Fig. 2). The envelope consists of connective tissue interfolded with collagen fibers. Both large, rounded, granular cells and nongranular flat cells which comprise the germinal epithelium are seen between the collagenous fibers.

Allometric elements of the ovaries are presented in Table 2. Ovarian length ranges

Fig. 2. Ovary. A: Ovary enclosed in a thin, transparent envelope (oocyte can be seen through envelope). Scale bar = 100 μ. B: Ovary after the transparent enveloping membrane was slit open. Oocytes can be seen attached to the membrane. Scale bar = 100 μ. P, previtellogenic oocyte; Po, postvitellogenic oocyte; V, vitellogenic oocyte.
With body mass (r = 0.741; P < 0.01) are also significant. However, no significant correlation is found between ovary mass and oocyte number, although significant correlation is found between ovarian mass and ovarian length (r = 0.583; P < 0.05) (see Table 2 for data).

Oogenesis

We distinguished six stages in oogenesis which we separated into three main phases in the oogenetic cycle: 1) previtellogenic, 2) vitellogenic, and 3) postvitellogenic oocytes (maturation).

During previtellogenesis we found three stages (Fig. 3): stage 1 in which oogonia divide and form cell nests (Fig. 3A,B); stage 2 in which oogonia differentiate into oocytes which possess a large central nucleus containing nucleoli but relatively little ooplasm, and follicle envelopes are formed which consist of two layers of theca cells and one layer of granulosa cells differentiated from the germinal epithelium (Fig. 3C); and stage 3 in which oocytes increase in volume due to addition of cytoplasm. Two types of cytoplasm are distinguishable: 1) dense cytoplasm at the periphery with a pigmented ring separating it from 2) the less dense cytoplasm surrounding the nucleus (Fig. 3D).

In the vitellogenic phase (2) we recognized two stages (Fig. 4). In stage 1, lipid accumulates in vacuoles forming lipid droplets in the periphery. Oocyte diameter ranges between 0.2 and 0.6 mm (substage I) (see Fig. 4A). Later, yolk platelets appear at the cytoplasm edges, and the theca fold to form a space that separates it from the granulosa (Fig. 4D). Cytoplasm of two densities still exists. Mean oocyte diameter is 1.12 ± 0.07 and ranges between 0.4 and 1.6 mm (substage II). In stage 2, oocyte volume increases rapidly due to the increased number of yolk platelets; they are smaller around the edge and increase in size toward the center. The cytoplasm becomes homogenous, uniformly dense, throughout the cell. Nucleolus still surrounds the nucleus. Mean oocyte diameter is 2.0 ± 0.2 mm and ranges between 1.0 and 2.9 mm (substage III) (Fig. 4B). At the end of this stage the oocyte reaches its maximum size. A ring of cytoplasm surrounds the nucleus. Yolk platelets fill the oocyte. Mean oocyte diameter is 2.66 ± 0.32 mm and ranges between 2.1 and 3.6 mm. The follicle envelopes attach to the oocyte, and the granulosa cells flatten (substage IV) (Fig. 4D).

The last stage observed is the beginning of the third phase, the maturation (postvitellogenic) stage, characterized by movement of the nucleus toward the animal pole. Mean oocyte diameter is 3.19 ± 0.29 mm, and ranges between 2.0 and 3.9 mm (Fig. 4C).

Ovarian mass and length are independent of the season and the reproductive cycle (Fig. 5). In ovaries of all females examined we observed the first four stages of oogenesis. The percentage of oocytes reaching the vitellogenic stage is constant, independent of season and reproductive stage (Fig. 6). Over half of the vitellogenic oocytes are at substage II. Most of the oocytes that reach substage III continue to substage IV, but only half of them mature (Table 3). Mature oocytes increase in number toward March in gravid and nongravid females (Fig. 7).

**TABLE 2. Measurements of body and ovaries**

<table>
<thead>
<tr>
<th>N</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snout-vent length (cm)</td>
<td>18 animals</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>18 animals</td>
</tr>
<tr>
<td>Ovarian length (cm)</td>
<td>36 ovaries</td>
</tr>
<tr>
<td>Ovarian mass (g)</td>
<td>36 ovaries</td>
</tr>
<tr>
<td>Oocyte number (per female)</td>
<td>36 ovaries</td>
</tr>
</tbody>
</table>

**Ovarian mass is significantly correlated with body mass (r = 0.697; P < 0.05). Correlations between ovary and snout-vent length (r = 0.817; P < 0.05) and between ovary length and number of oocytes (r = 0.741; P < 0.01) are also significant. However, no significant correlation is found between ovary mass and oocyte number, although significant correlation is found between ovarian mass and ovarian length (r = 0.583; P < 0.05) (see Table 2 for data).**

**DISCUSSION**

**Ovary**

In other amphibians the relative weight of the ovaries is positively correlated with the
Fig. 3. Previtellogenic oocyte development. 

A: Division of oogonia (arrow). H & E. Scale bar = 10 µ. 

B: Creation of “cell nest” Arrow, cell nest; g, germinal epithelium. Azan. Scale bar = 10 µ. 

C: Differentiation into oocyte. Arrow, granulosa; g, germinal epithelium. Azan. Scale bar = 10 µ. 

D: Volume of oocyte increases. Arrows, pigmentation; Cy, cytoplasm; N, nucleus. Azan. Scale bar = 50 µ.
Fig. 4. Vitellogenesis (A, B), Postvitellogenesis (maturation) (C). Changes in oocyte envelope (D). A: Substage I. Lipid accumulates at the periphery of the cytoplasm. Arrows, pigmentation; arrowhead, lipid drops; Cy, cytoplasm; N, nucleus. Azan. Scale bar = 50 µ. B: Substage III. Oocyte is half-filled with yolk. Cy, cytoplasm; N, nucleus. Azan. Scale bar = 50 µ. C: Nucleus has shifted to animal pole. Y, yolk. Azan. Scale bar = 10 µ. D: Postvitellogenic oocyte (P). Envelope adheres tightly to the oocyte. It is difficult to differentiate between the layers. Vitellogenic oocyte (V). The theca (arrowheads) is folded, and the granulosa cells (arrows) are large and rounded. Azan. Scale bar = 10 µ.
female's body weight (Bufo: Reading, '86; Rana: Jørgensen, '81; Kyriakopoulou-Sklavounou and Loumbourdis, '90). There is seasonal variation in ovarian mass of Rana spp. (Jørgensen, '81; Elmberg, '91). The increase in the relative weight of Ichthyophis ovary is related to the increase in oocyte diameter (Masood-Parveez and Nadkarni, '93a). The relative ovarian weight in Triturus torosus (= Taricha torosa) ranges between 0.5 and 4.9% (Miller and Robbins, '54), and in Triturus (= Notophthalmus) viridescens between 2.49 and 11.73% (Adams, '40). Prior to the present study there were no comparable data available for S. salamandra ovaries.

In the present study the ovarian length rather than ovarian mass is better correlated with snout-vent length and oocyte number. There is no seasonal variation in ovarian mass and length.

Oogenesis

Oogenesis is the process whereby oogonia that multiply by mitosis are transformed into mature oocytes (Rugh, '51; Lofts, '74; Saidapur, '82). Lofts ('74) recognized in Amphibia primary and secondary phases characterized by the accumulation of yolk and by growth. Most studies on amphibian oogenesis recognize three main stages: previtellogenesis, vitellogenesis, and postvitellogen-
Vitellogenic follicles grow and bulge out of the ovarian wall (Lofts, '74). The vitelline membrane envelopes the egg after ovulation until fertilization takes place (Wischnitzer, '66).

Detailed descriptions of oocyte development were given by Masood-Parveez and Nadkarni ('93b) for the caecilian Ichthyophis beddomeii, by Saidapur ('82) and Berois and De Sa ('88) for Chthonerpeton indistinctum, and by Xavier et al. ('70) for Rana nectophrynaides. In Urodela the most detailed description of oogenesis was given by Bonnafant-Jais and Mentre ('83) for Pleurodeles waltlii. They distinguished three stages in oogenesis: previtellogenic with oocytes smaller than 500 µ in diameter, vitellogenic oocytes containing yolk up to 1,000 µ in diameter, and postvitellogenic stages with oocytes larger than 1,000 µ and more intensely pigmented due to their denser cytoplasm. Each stage was divided into substages.

### TABLE 3. Vitellogenic and mature oocyte number and percentage

<table>
<thead>
<tr>
<th>Development stage</th>
<th>Number ± SD</th>
<th>Percentage from total number of vitellogenic + mature oocytes</th>
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<tr>
<td>Vitellogenesis</td>
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</tr>
<tr>
<td>Stage 1 Substage II</td>
<td>82.1 ± 27.9</td>
<td>55.0</td>
</tr>
<tr>
<td>Stage 2 Substage III</td>
<td>27.9 ± 17.7</td>
<td>18.7</td>
</tr>
<tr>
<td>Substage IV</td>
<td>25.1 ± 21.6</td>
<td>18.7</td>
</tr>
<tr>
<td>Maturation</td>
<td>14.1 ± 17.6</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Fig. 6. Percentage of vitellogenic oocytes from total number of oocytes by months and during the reproductive cycle.
Descriptions of oocytes before and after ovulation in Salamandra salamandra were given by Joly and Picheral ('72). The dimensions of yolk platelets increase with oocyte development (Grodzinski, '75), and consequently oocyte diameter is 5 mm (Joly and Picheral, '72). In our study the maximum oocyte diameter is 3.9 mm.

Ovarian cycle

The present study gives for the first time quantitative data about oocyte numbers and their size and distribution in Salamandra salamandra. To date most researchers based their description of the ovarian cycle on ovarian mass (growth curve) or oocyte diameter. In the present study we have shown great variation in the size of oocytes belonging to the same stage of development in different females. We conclude that in order to give an accurate description of the ovarian cycle, all oocytes should be measured and each animal scaled separately for oocyte size at each stage histologically (see Materials and Methods), and then oocyte number in each stage of the oogenic development should be counted.

The ovarian cycle in amphibians differs among orders, families, and species. The factors affecting the ovarian cycle vary. In Gymnophiona, Masood-Parveez and Nadkarni ('93b) showed a gestation-dependent ovarian cycle in Ichthyophis beddomei. In Anura, Jörgensen ('73, '74, '81) and Jorgensen et al. ('79) stated that oocyte development is synchronized in Bufo bufo and Rana temporaria, and that the ovarian cycle is seasonal, depending on temperature or pre-
cipitation as well as being gestation-dependent. Redshaw ('72) stated that about a third of the oocytes in the ovary develop into vitellogenic oocytes and that the percentage is steady irrespective of ovarian mass. Bragg ('61) stated that oocytes in Scaphiopus toads are ready all year-round, waiting for heavy rains and favorable temperature. They are not season-dependent but depend on favorable conditions. In Urodela, Bonnafant-Jais and Mentre ('83) showed in Pleurodeles waltlII that the ovary contains oocytes at all stages all year-round. In Triturus torosa, yolk deposition takes place during aestivation and migration to the breeding sites, 5–6 months before ovulation (Miller and Robbins, '54). Adams ('40) stated that in T. viridescens oocyte development is synchronized; thus, ovarian mass is low during summer and high from fall to spring. The ovarian cycle in other subspecies of Salamandra salamandra depends on season (as in S.s. terres-tris) or on gestation (as in S.s. fastuosa), and oocyte development is synchronized (Joly et al., '94). On the other hand, in S.s. infraimmaculata oocytes of previtellogenic and vitellogenic stages are found throughout the year and throughout the reproductive cycle, and the percentage of vitellogenic oocytes remains constant. Therefore, ovarian cycle is not dependent on season or gestation, apart from the oocyte maturational cycle. Oocyte maturation depends on the season, starting at the onset of rainfall and increasing toward the end of winter (March–April). It is an adaptation for extreme and unpredictable climatic conditions when the female salamander has to be ready to ovulate practically at any time starting toward the end of the hot and dry summer, when temperatures drop and rain starts falling. It is a different way to enable survival in a xeric environment for a nonviviparous urodele.

Ovulation in Salamandra salamandra has been described only once (J oly, '86) but apparently has not been recorded (= observed?) since. This strengthens our impression that the process of ovulation is extremely rapid and thus difficult to observe and record. Fertilization takes place when oocytes pass through the glandular region of the oviduct (Siebold, 1858; Schwalbe, 1896; W hahlert, '54; Greven, '80).

In Salamandra s. infraimmaculata, ovulation probably takes place during spring when temperatures are not too low or too high. Since water is available only during winter and parturition occurs mainly between November and March (Sharon et al., '96), gestation is presumably shorter (less than 1 year) compared with the European S. salamandra, in which parturition takes place during the following summer (J oly et al., '94).

ACKNOWLEDGMENTS

The authors wish to acknowledge the valuable assistance of Mira Rosenberg during all phases of this study. Partial financial support came from the J. & A. Taub Biological Research Fund.

LITERATURE CITED


