Development of testes and differentiation of germ cells in water frogs of the *Rana esculenta* – complex (Amphibia, Anura)

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**Abstract.** The European water frog, *Rana esculenta*, is a hybrid whose genome is composed of haploid chromosome sets of its parental species *R. lessonae* and *R. ridibunda*. Prior to meiosis one of the parental sets is discarded and the other is duplicated (hybridogenesis). In the parental species sex differentiation begins at tadpole stages 28-30 (Gosner, 1960), at stages 30-36 the testes are composed of proliferating pale spermatogonia I°. At stages 36-39 a new class of spermatogonia I° (dark) appears. Before first hibernation, seminiferous lobules are filled with cysts containing germ cells at various stages of spermatogenesis up to elongating spermatids. In *R. esculenta* gonad development is affected from the earliest stages: the gonads are smaller and composed of reduced number of spermatogonia I°. The phase of pale spermatogonia I° proliferation is prolonged up to the second year of life. The structure of the gonads, as well as that of germ cells themselves, are often abnormal.

**Introduction**

*Rana esculenta* is a natural hybrid between *R. lessonae* and *R. ridibunda*. Its genome is composed of *lessonae-ridibunda* chromosome sets (for review see Berger, 1983, 1988; Graf and Polls-Pelaz 1989). In natural populations the hybrids originate as a result of a special way of reproduction called hybridogenesis (Schultz, 1969; Tunner, 1974) in which the parental chromosome sets are segregated during gametogenesis; one of them is discarded, while the remaining one is duplicated. Gonial cells with duplicated genome enter meiosis which results in production of clonal gametes. This hypothesis was supported by Tunner and Heppich (1981) and Tunner and Heppich-Tunner (1991) who analysed chromosome composition of oogonia and spermatogonia during metamorphosis in water frogs. The study of Ogielska and Wagner (1993) on the development and differentiation of water frog ovaries led to the conclusion that tadpole stages are crucial for female gamete
formation. The discarded chromosomes form unique structures called nuleus-like bodies (NLB) observed exclusively in gonial cells during tadpole stages (Ogielska, 1994).

Oogenesis and ovary differentiation in water frogs were studied rather extensively (Ogielska and Wagner, 1990, 1993; Wagner and Ogielska, 1990, 1993; Ogielska, 1994). Because the same process of chromosome elimination is expected to occur also in the male germ line, the aim of this paper was the description of testis development and differentiation in the hybrid, *Rana esculenta*, and in its parental species. This will help to focus further studies on precisely chosen stages of testis development.

**Material and methods**

Testes were studied in tadpoles, juvenile and adult *R. lessonae*, *R. ridibunda* and *R. esculenta*. All tadpoles were obtained by means of artificial fertilization (Berger et al., 1994). Parental individuals were collected in the vicinity of Wroclaw (Lower Silesia) and Poznań, Poland. Taxa were identified according to morphological features (shape of callus internus, tibia/callus internus and digitus primus/callus internus indices) (Berger, 1966, 1968). *Rana esculenta* tadpoles were the result of the following crosses (female × male): *lessonae × esculenta* (2 crosses), *esculenta × lessonae* (4 crosses), *ridibunda × lessonae* (2 crosses). The tadpoles were kept in a greenhouse in plastic containers and fed with frozen lettuce, dry nettle and commercial fish meal. The testes of tadpoles at stages 28-46 (Gosner, 1960) were fixed in Smith’s fixative, embedded in paraffin and stained with iron hematoxyline. Because of variation in developmental rate among tadpoles of the same age, only the most advanced individuals at a given stage were used in the study. In each group of tadpoles, juveniles and adults six to ten individuals were examined. Juveniles (before and after I and before II hibernation) as well as adults were collected in the field or reared outdoors. The size of testes and the diameter of germ cell nuclei were measured using a calibrated eyepiece.

**Results**

*Morphology of spermatogonia*

We started our investigation after tadpoles reached stage 28, when gonads are already sexually differentiated (Ogielska and Wagner, 1990, 1993). In the tadpoles under study no earliest stages of germ cells, i.e. primordial germ cells and gonial cells, were observed. Among primary spermatogonia we distinguished two cell types: pale and dark (figs. 7 and 8). Pale primary spermatogonia are characterized by a round shape with light cytoplasm and pale nuclei containing 2-5 nucleoli. In most sections nuclei are round, but sometimes bean-shaped nuclei are also seen. Nucleus diameter ranges from 10.3 μm to 13.5 μm (12.0 μm on average). Mitochondria are distributed around the nuclei and form
a cloud at one of their sides. In the bean-shaped nuclei the mitochondrial cloud is situated at the concave side of the nucleus. Dark primary spermatogonia are smaller than the pale ones, with denser cytoplasm and heterochromatin patches in their nuclei. The diameter of the nuclei ranges from 7.7 µm to 10.9 µm (8.5 µm on an average). Mitochondria form the same pattern around the nuclei, as in the pale spermatogonia.

Secondary spermatogonia are found in cysts whose walls are formed by somatic cells. All the secondary spermatogonia in each cyst constitute a clone originating from mitotic divisions of a primary spermatogonium, probably of dark type. The cell cycles within each cyst are synchronized. After the completion of mitotic divisions the secondary spermatogonia enter meiosis and form spermatocyte I (leptotene/zygotene, pachytene and diplotene) cysts (figs. 10 and 15). Then spermatocytes II, smaller than spermatocytes I, are formed. Spermatids are the smallest round cells arranged close to the inner wall of the cyst, with the lumen of the cyst remaining empty (fig. 12). The next step of spermatogenesis observed by us is spermatid elongation and formation of spermatozoa. During this phase of spermiogenesis heads of spermatozoa are still in contact with the inner wall, while their tails are directed towards the center of the cyst. Ripe spermatozoa are released from the cysts, and are found in the lumen of seminiferous lobules.

Development and differentiation of testes in R. lessonae and R. ridibunda

The rate of testis development is very similar in the two parental species. At stages 28-32 (fig. 1) first presumptive lobules are formed and 2-4 of them can be distinguished on cross sections of the testis. Mitotic activity of spermatogonia is rather high, and up to 8 primary spermatogonia are seen in the cross section of each lobule. Each spermatogonium is surrounded by 2-3 somatic cells. First pale primary spermatogonia with mitochondrial clouds were detected at stage 30. An external epithelium of testes is well differentiated and some empty spaces between the lobules can be noticed. At stages 33-37 (fig. 3) the number of presumptive lobules increases up to 10 on cross section. Occasionally pale spermatogonia with lobulated nuclei are seen. Mitoses are numerous and the number of spermatogonia increases significantly. At stage 36/37 in R. lessonae first dark primary spermatogonia appear. In the center of the gonad somatic cells form a cord, which will later give rise to rete testis. At stages 38-41 the gonad is approximately twice as large as at stage 33. The number of lobules still increases up to 30 on a cross section. The number of dark spermatogonia increases and is now greater than that of pale ones (fig. 7). Rete testis is more distinct. During metamorphosis (stages 42-46) both pale and dark primary spermatogonia are still present (fig. 8). They are single cells, each surrounded by somatic cells and sometimes form groups. First cysts with few secondary spermatogonia are seen at stages 44/45. After metamorphosis and before hibernation I pale primary spermatogonia are still numerous, and their mitotic activity is high. They are scattered among cysts containing leptotene/zygotene and pachytene spermatocytes (fig. 10). Only few cysts contain elongated spermatids and no spermatozoa are observed. Males of body
length ranging from 4.3 to 4.6 cm (only *R. lessonae* were examined) are sexually mature. During summer (June-August, figs. 12 and 14) testes display a high mitotic activity of pale primary spermatogonia. The most numerous cysts contain secondary spermatogonia and leptotene/zygotene spermatocytes. Degenerating germ cells in cysts were observed occasionally. In testes before hibernation (September) all the pale primary spermatogonia are in interphase and the lobules are filled with cysts containing all stages of meiosis, as well as spermatozoa in the lumen.

**Development and differentiation of testes in *R. esculenta***

Because we do not observe any differences in testes development among tadpoles resulting from various crosses, all individuals are described as *R. esculenta* tadpoles. Compared to the parental species in most cases testis differentiation is delayed and gonads have smaller size (fig. 20). At stages 28-32 the differentiation of gonads varies among individuals. In one tadpole at stage 29 gonads are still undifferentiated. In one case the differentiation of gonad at stage 30 is the same as in the parental species. The other tadpoles have abnormal testes. The gonads are small with empty spaces between presumptive lobules greater than in the parental species (fig. 2). The number of pale primary spermatogonia seen on cross sections of lobules is lower (up to 4). Numerous degenerating spermatogonia are observed. Their nuclei are swollen and often irregular in outline or fragmented. Among 9 tapoles at stages 33-37 only one (stage 37) has normal testes with a few dark primary spermatogonia and somatic sexual cord (fig. 4). The others have the same features as described for stages 28-32: lower number of spermatogonia, greater spaces between lobules and degenerating pale primary spermatogonia. A similar situation is also observed at stages 38-41. In one case testes were almost normal with a few dark primary spermatogonia (fig. 9), but in the rest of tadpoles the gonads were smaller, with few lobules on the cross sections and degenerating pale primary spermatogonia (fig. 6) or no well differentiated lobules and poorly developed rete testis (fig. 5). Rete testis is well developed. During metamorphosis (stages 42-46) testes of normal size are seen in only one tadpole, but with no dark primary spermatogonia.

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**Figure 1.** Cross section of testes of a *R. lessonae* tadpole at stage 31. Presumptive seminiferous lobules (L) with primary pale spermatogonia are shown (bar = 25 μm).

**Figure 2.** Cross section of a testis of a *R. esculenta* tadpole at stage 33. Note large empty spaces (S) between presumptive seminiferous lobules (L) (bar = 25 μm).

**Figure 3.** Cross section of a testis of a *R. lessonae* tadpole at stage 36. (L) — presumptive seminiferous lobules. Somatic cells with dark nuclei (C) form a cord (future rete testis) (bar = 25 μm).

**Figure 4.** Cross section of a testis of a *R. esculenta* tadpole at stage 40. Note a single primary dark spermatogonium (arrow) and rete testis (RT) in the center of the gonad (bar = 25 μm).

**Figure 5.** Cross section of a testis of a *R. esculenta* tadpole at stage 40. Lobules and rete testis are not well developed (bar = 25 μm).

**Figure 6.** Cross section of a lobule with a group of degenerating primary pale spermatogonia (D) in a testis of *R. esculenta* tadpole at stage 37 (bar = 30 μm).
In the rest of individuals the number of pale primary spermatogonia is lower than in the parental species, many of them degenerating. No dark primary spermatogonia are detected. After metamorphosis and before hibernation I testes remain small with a low number of pale primary spermatogonia. No dark spermatogonia were seen. Many of pale spermatogonia undergo degeneration, sometimes forming clumps of abnormal cells. The lower number of germ cells in lobules, the higher number of somatic cells filled the lumen of the lobules. The degree of germ cells differentiation at the end of metamorphosis can be compared to that of stages 30-36 in the parental species. In all cases rete testis is well differentiated. After hibernation I the majority of germ cells are still pale primary spermatogonia and first cysts containing secondary spermatogonia appear (fig. 11). After hibernation II lobules are filled with cysts containing all stages of meiosis up to spermatocytes II; in one case spermatids and spermatozoa are present, and the gonads have a normal appearance. In sexually mature fertile hybrids the structure of testes is similar to that described for the parental species (fig. 13). In most cases the size of gonads is comparable to that of parental species (fig. 20), but individuals collected in one of the localities (Wroclaw-Nowy Dom) are sterile or have very few motile spermatozoa. It should be mentioned that in this population the sex ratio is extremely biased in favour of males (Ogielska et al., 1994). Testes of these males were irregular in shape and composed of lobules of various diameters containing mostly degenerating germ cells (figs. 16 and 17).

Testis-ova

In one adult male *R. lessonae* collected in summer and in one juvenile *R. esculenta* we found 2 and 3 testis-ova, respectively (figs. 18 and 19). The composition of the testes in *R. lessonae* is normal, whereas they are abnormal in *R. esculenta*. The gonad of the latter is divided into lobes resembling ovarian sacs with irregular lumen, but the cortex is composed of seminiferous lobules containing cysts with leptotene/zygotene, pachytene and diplotene spermatocytes.

Figures 7 and 8. *R. lessonae* tadpole at stage 39 and 42, respectively. Section of seminiferous lobules containing two classes of primary spermatogonia: pale (P) and dark (D) (bar = 30 μm).

Figure 9. *R. esculenta* tadpole at stage 41. Section of seminiferous lobules with few dark primary spermatogonia (D) (bar = 30 μm).

Figure 10. *R. lessonae* before I hibernation. Seminiferous lobules containing cysts with leptotene/zygotene (Z) and pachytene (Pa) spermatocytes. Single primary pale spermatogonia (P) are scattered among cysts (bar = 25 μm).

Figure 11. *R. esculenta* after hibernation I. Most of germ cells within a lobule are primary pale spermatogonia (P). A few cysts with secondary spermatogonia (S) are also visible (bar = 25 μm).

Figure 12. Summer testis of adult *R. lessonae*: cysts with all stages of spermatogenesis and spermatozoa (bar = 25 μm).

Figure 13. Summer testis of adult fertile *R. esculenta*: all stages of spermatogenesis are present (bar = 25 μm).
Discussion

The development and differentiation of testes in *R. lessonae* and *R. ridibunda* is similar to that process in the Japanese pond frog, *R. nigromaculata* (Iwasawa et al., 1987; Tanimura and Iwasawa, 1988, 1991). However, the rate of development of *R. nigromaculata* is slightly delayed compared to the species described in this paper. One of the differences is the appearance of secondary spermatogonia: stages 44-46 in *R. lessonae* and *R. ridibunda* and 2 weeks after metamorphosis in *R. nigromaculata*. These differences may reflect various environmental conditions, such as feeding or temperature, and are not crucial.

Spermatogenesis in water frogs is potentially continuous, i.e. spermatozoa are present in seminiferous tubules (lobules, as recommended by Pudney, 1995) of adult males throughout the year (Galgano, 1934, according to Bustos-Obregon et al., 1973). However, the slight seasonal variation in germ cell composition within seminiferous lobules is observed and is similar to that described for *R. nigromaculata* and *R. porosa porosa* (Iwasawa and Asai, 1959). First spermatozoa observed in the parental species after hibernation I are probably precocious, as was described for *R. nigromaculata* (Iwasawa et al., 1987), because sexual maturity is achieved by these species mostly after hibernation I.

Dark and pale primary spermatogonia: dark and pale spermatogonia were first described in adult male *R. esculenta* and *Pachymedusa dacnicolor* (Rastogi et al., 1985, 1988). The pale ones (called by the authors “undifferentiated”) were interpreted as stem cells, while the dark ones (“differentiated”) were believed to be destined for spermatogenesis. Our observation supports this hypothesis. In *R. lessonae* dark primary spermatogonia were first observed at stage 36 and were abundant at stage 39, while in the hybrids the dark spermatogonia were almost absent. As a result spermatogenesis in the parental species before hibernation I was advanced up to spermatids and in the hybrids still pale primary spermatogonia were the only germ cells observed.

Kawamura and Nishioka (1986) performed detailed studies on testis development in the male progeny of European water frogs *R. lessonae*, *R. ridibunda* and *R. esculenta* from various localities. The abnormal spermatogenesis was described for *lessonae × ridibunda* and *lessonae × esculenta*. They described degeneration of both spermatogonia and spermatozoa. In some cases testes were irregular in outline, had lobules of various size or lower number of germ cells. These abnormalities were similar to those described by us. However, their observations differ from ours in that the size of second-year animals was larger than the largest observed by us. The reason was probably the rearing without hi-

**Figures 14 and 15.** Adult *R. lessonae* testis before hibernation: seminiferous lobules are regular and all stages of spermatogenesis are present (bar = 100 μm in fig. 14 and 30 μm in fig. 15).

**Figures 16 and 17.** Adult non fertile *R. esculenta* testis before hibernation: note the irregular shape of seminiferous lobules filled with degenerating germ cells, mostly primary pale spermatogonia (bar = 100 μm in fig. 16 and 25 μm in fig. 17).
Figures 18 and 19. Testis-ova (0) in *R. esculenta* and *R. lessonae*, respectively: note the abnormal composition of seminiferous tubules in *R. esculenta* (bar = 25 μm).

Figure 20. Size differences of testes between *R. lessonae* and *R. esculenta* (tadpoles and adults); *adults: R. lessonae* of body length 43-55 mm; *R. esculenta* of body length 41-76 mm. Scale bar = 1 mm.

Degenerating spermatocytes and spermatozoa were also described in natural hybrids between Japanese species *R. nigromaculata* and *R. porosa porosa*, closely related to water frogs used in our study (Sumida and Ishihara, bereviation and with continuous feeding. It should be pointed out that the results presented by us more precisely reflect natural conditions. Degenerating spermatocytes and spermatozoa were also described in natural hybrids between Japanese species *R. nigromaculata* and *R. porosa porosa*, closely related to water frogs used in our study (Sumida and Ishihara,
These hybrids were sterile or had a low fertility, like *R. esculenta* from Wroclaw-
Nowy Dom. Degeneration of primary spermatogonia was described as frequent in adult
*Pachymedusa dacnicolor* (Rastogi et al., 1988), but rare in *R. esculenta* (Rastogi et al.,
1985) and *Bufo spinolosus* (Bustos-Obregon et al., 1973).

Testis-ova were described in more than a half of male *R. nigromaculata* studied by
Iwasawa and Kobayashi (1976) and Kobayashi and Iwasawa (1988). They were also
observed in progeny obtained by a crossing experiment between female *R. les-
sonae* and male *R. esculenta* who produced two types of spermatozoa: one containing the
*ridibunda*, and the other containing the *lessonae* chromosomes. In male progeny resulting from
*ridibunda*-containing sperm testis-ova were common (Kawamura and Nishioka, 1986). In
our material we found testis-ova only in one *R. lessonae* and one *R. esculenta*.

Our results show that development of testes and differentiation of germ cells in the
natural hybrid *R. esculenta* are delayed compared with the parental species, *R. ridibunda*
and *R. lessonae*. Beginning with the earliest stages examined, in most cases the testes in
*R. esculenta* were smaller, presumptive lobules had lower diameter and contained fewer
germs cells. Because we observed many degenerating primary pale spermatogonia, we
believe that the lower number of germ cells was a result of their degeneration at earlier
stages. In *R. lessonae* and *R. ridibunda* the number of primary spermatogonia (both pale
and dark) increases significantly at stages 36-40. At the same time in *R. es-
culenta* the loss of spermatogonia is observed, and formation of dark primary spermatogonia is rare.
Pale primary spermatogonia are the most common sort of germ cells in the hybrid testes
during the first year of life (up to after hibernation II). Their number slowly increases and
it seems that their further differentiation and meiosis starts after a pool, i.e. proper number
of spermatogonia with correctly eliminated chromosomes is formed.

The degeneration of pale primary spermatogonia is probably associated with the process
of genome elimination. Due to the hybridogenetic reproduction both sexes produce
gametes containing haploid sets of parental chromosomes (*lessonae* or *ridibunda*), while
the other chromosome set is eliminated before meiosis (for review see Berger, 1983, 1988;
Graf and Polls-Pelaz, 1989). Studies on oogenesis (Ogielska and Wagner, 1993; Ogielska,
1994) indicate that the genome is eliminated during interphase in form of nucleus-like
bodies (NLB) which bud off from the nucleus. The chromosome loss is gradual (Tunner
and Heppich, 1981; Tunner and Heppich-Tunner, 1991) and probably imprecise. Mitotic
divisions of oogonia with only partly rejected chromosomes are often abnormal and such
cells degenerate (Ogielska, 1994). Thus, oogonial proliferation is prolonged, probably
until the proper number of oogonia with correctly rejected chromosomes is established.
The same situation seems to occur during spermatogenesis. NLB were also observed
in developing testes of *R. esculenta* (Ogielska, 1994 and unpublished observations) and
the chromosome loss is gradual (Heppich et al., 1982). The phase of pale primary
spermatogonia proliferation is prolonged and meiosis is one year delayed, which strongly
resembles the situation during early ovary development, where the phase of oogonial
proliferation is also long and meiosis is delayed at least one year.
Further and more detailed studies on NLB formation and chromosome elimination during gametogenesis are necessary, and the results of the present and previous papers (Wagner and Ogielska, 1993; Ogielska, 1994) indicate that the crucial stages are 30-46, both in oogenesis and spermatogenesis. Chromosome elimination seems to occur only once in a lifetime. This hypothesis is additionally supported by previous results of Günther (1975) and Vinogradov et al. (1990) who found that spermatogonia of adult *R. esculenta* are composed of duplicated *ridibunda* genome.

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References


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