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Spermatogenesis in the Siberian salamander, *Salamandrella keyserlingii* (Caudata: Hynobiidae)

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Abstract. Spermatogenic cycles of hynobiid salamanders are interesting for the study of male reproductive adaptations in amphibians living under different environmental conditions. In order to detect the main differences between spermatogenic cycles of hynobiids, we studied the spermatogenic cycle of *Salamandrella keyserlingii* from the suburbs of Tomsk (southeastern Western Siberia) and compared it with those in the literature of hynobiids from different regions of Asia. We histologically and histochemically examined the testes of males captured from April to September. In April, the testes of males entering breeding sites contained bundles of spermatozoa (Sz) and primary (Sg I) and secondary spermatogonia (Sg II). After spermiation and breeding, Sg II began to proliferate. Meiosis of spermatocytes occurred in late June through July. The spermiogenesis began in late July; spermatids and Sz appeared in August. In September, Sz, Sg I, and Sg II were found in testes, which was also when Sg II proliferated. There are two types of spermatogenic cycles in the studied salamanders. The first one includes one period of spermatogonial proliferation (SP) in the first half of the active season. The second type consists of two periods of SP, with one occurring at the beginning and the other at the end of the active season. To identify possible differences in hynobiid spermatogenic cycles, we tested the relation of the duration of active season (DAS), the duration of SP period in the first half of cycle (DSPP), and the number of SP periods per year (NSPPs), considering environmental (air) temperatures in these species' habitats. We could not find a direct relationship between NSPPs and air temperatures, but DAS and DSPP were correlated with temperature. We assume that two periods of SP can play the most apparent adaptive role in *S. keyserlingii* in a subarctic climate and in *Batrachuperus tibetanus* under mountain conditions.

Key words. Amphibia, germ cells morphology, microstructure of testes, reproductive adaptation, reproductive cycle, temperature.

Introduction

In salamanders, spermatogenesis occurs under endogenous (neuroendocrine) control and changes under exogenous (environmental) influences (Delsol et al. 1995, Uribe 2003, Brizzi & Corti 2006). In temperate regions, male reproductive cycles are clearly associated with seasonal changes in the environment. Data on the variation of salamander spermatogenic cycles under different environmental conditions relate to the species with internal fertilisation in the suborder Salamandroidea (Ifft 1942, Galgano 1943, Houck 1977, Chan 2003). For this group, the main factor influencing spermatogenesis is temperature, as shown under both environmental and experimental conditions (Ifft 1942, Galgano 1943, Fraile et al. 1989a, c, Paniagua et al. 1990). Low temperatures obstruct or prevent spermatogenesis, namely, the development from spermatogonia to early spermatids (Ifft 1942, Galgano 1943, Fraile et al. 1989a, c, Paniagua et al. 1990).

Among salamanders with external fertilisation (suborders Sirenoidea and Cryptobranchoidea), the family Hynobiidae is of great interest for such studies because of the following reasons: Hynobiids are widely distributed in Asia, where they inhabit plains and mountainous regions on the mainland and islands with different climatic conditions (Poyarkov 2010). Moreover, this group incorporates both lotic- and lentic-breeding species (Duellman & Trueb 1986).

Among hynobiids, males of *Hynobius lichenatus* (Makino 1931), *H. nigrescens* (Hasumi et al. 1990), *H. retardatus* (Iwasawa et al. 1992), *Batrachuperus tibetanus* (Wang

& Zhang 2004), and *Salamandrella keyserlingii* (Yartsev 2011, Bulakhova & Berman 2014, Yartsev & Kuranova 2015) exhibit annual reproductive cycles. In *H. nigrescens*, the completion of spermatogenesis occurs in September (Hasumi et al. 1990). In contrast, spermatozoa formation is completed already by August in the species *B. tibetanus* (Wang & Zhang, 2004), *H. retardatus* (Iwasawa et al. 1992), and *S. keyserlingii* (Yartsev 2011, Bulakhova & Berman 2014, Yartsev & Kuranova 2015). The described patterns of male gamete maturation occur under different climatic conditions, as the studied populations of hynobiids inhabit environmentally (climatically) distinct regions of Asia (see references mentioned). To understand differences in timing of male gamete maturation in hynobiids under different climatic conditions, comparisons of spermatogenic cycles and an analysis of their relations with climatic conditions are necessary (Yartsev & Kuranova 2015).

The study by Yartsev & Kuranova (2015) of seasonal dynamics of the male reproductive system in one *S. keyserlingii* population focused on external characteristics and smears taken from reproductive organs. In addition, we studied the spermatogenic cycle in the same population using histological observations of seasonal changes in the testes. Based on our examination and previous studies, we here compare spermatogenic cycles and identify types of spermatogenic cycles in hynobiid salamanders. Additionally, we tested the relationship between environmental (air) temperatures and spermatogenic cycles in hynobiids.

Materials and methods

We studied adult males (N = 13) collected from April through September 2005, 2009, and 2012 (Table 1) in the suburbs of Tomsk (southeastern Western Siberia, Russia: 56°26' N, 85°00' E; 150 m above sea level). We captured salamanders using trenches with pitfall traps on land and with a dip net in a breeding pond. In the laboratory, after anaesthesia and decapitation, we measured snout–vent length (SVL, as the distance from the tip of the snout to the anterior angle of the vent) to nearest 0.1 mm using digital slide callipers. We fixed all specimens in a 4% solution of formaldehyde. All histological procedures followed Exbrayat (2013). After fixation, we excised the left testes, dehydrated it in ethanol of increasing concentration, and cleared it in butanol. Following the embedding in paraffin, 5 μm transverse sections were sliced with a rotary microtome. We stained sections with modified azan (AM), alcian blue (AB) (pH = 2.5), and periodic acid-Schiff's (PAS) staining techniques. We observed preparations and took snapshots with an Axio Lab.A1 microscope with an AxioCam ERc 5s camera (Zeiss). We measured the maximum nuclear diameter of germ cells with the software AxioVision 4.9.1 (Zeiss) as an additional characteristic of spermatogenic stages.

For comparison of the spermatogenic cycles of hynobiids, we analysed the duration of active season (DAS), the number of spermatogonial proliferation periods per year (NSPPs), and the duration of the spermatogonial proliferation period in the first half of the cycle (DSPP) in the studied hynobiid species (Table 2). To examine the role of the ambient temperature in the spermatogenic cycle of hynobiids, we used monthly average (T_{mean}), maximum (T_{max}), and minimum (T_{min}) air temperatures (°C) at localities of the hynobiid species from the WorldClim database, version 1.4, which contains climatic parameters recorded from 1950 to 2000 (Hijmans et al. 2005). Climatic data were extracted with ArcGIS 9.3 (ESRI).

We performed statistical analysis with Statistica 8.0 (StatSoft). We tested differences in the nuclear diameter using Mann-Whitney U tests. We conducted the integrated assessment of the temperature factor in each location with the principal components analysis, PCA (Efimov & Kovaleva 2007). This method is widely used for analyses of climatic parameters (Litvinchuk et al. 2011, Roitberg et al. 2013). We tested the relations between the principal components (as an integrative temperature parameter) and spermatogenic cycle parameters (DAS and DSPP) by means of the Spearman rank correlation coefficient (Spearman's ρ).

Table 1. States, periods of capture, and snout–vent lengths (SVL) of studied males of *Salamandrella keyserlingii* of the Tomsk population.

State of males	Period of capture	SVL [mm]	Colour of testes
immigrating into the breeding pond	end of April 2009	65.3	yellow
		61.9	yellow
in the breeding pond	end of April 2009	56.6	yellow
emigrating from the breeding pond	May 2009	55.0	yellow
in terrestrial phase	June 2009	52.0	yellow
		52.2	yellow
		58.3	yellow
in terrestrial phase	July 2009	49.8	white
		59.8	white
in terrestrial phase	August 2005	53.2	yellow
		49.0	yellow
in terrestrial phase	September 2012	57.7	yellow
		58.9	yellow

Results

Testicular microstructure

The testes were elongated organs enveloped by the peritoneal epithelium and tunica albuginea. A testicular duct (= longitudinal collecting duct) passed along the medial surface of the testis, from which the efferent ducts extended (Fig. 1). The seminiferous lobules were elongated and perpendicular to the testicular duct (Fig. 1A). The interstitial (connective) tissue with blood vessels filled the space between the lobules. Each seminiferous lobule contained cysts formed by Sertoli cells. The germ cells at the same stages of spermatogenesis were located inside the cysts.

Table 2. Analysed data on spermatogenesis of hynobiid salamanders.

Species	Location	References	Acronym for the location in this study
Hynobius lichenatus	Vicinity of Sapporo, Hokkaido Island, Japan	Makino (1931)	Sapporo
Hynobius nigrescens	Iwamuro-mura, Niigata Prefecture, Honshu Island, Japan	Hasumi et al. (1990)	Iwamuro
Hynobius retardatus	Lake Komadome, Hokkaido Island, Japan	Iwasawa et al. (1992)	Komadome
Batrachuperus tibetanus	Qinling Mountain, China	Wang & Zhang (2004)	Qinling
Salamandrella keyserlingii	Mouth of Yana and Oira rivers, 70–100 km from Magadan on the north coast of the Okhotsk Sea, Russia	Bulakhova & Berman (2014)	Magadan
Salamandrella keyserlingii	Vicinity of Tomsk, western Siberia, Russia	this study	Tomsk

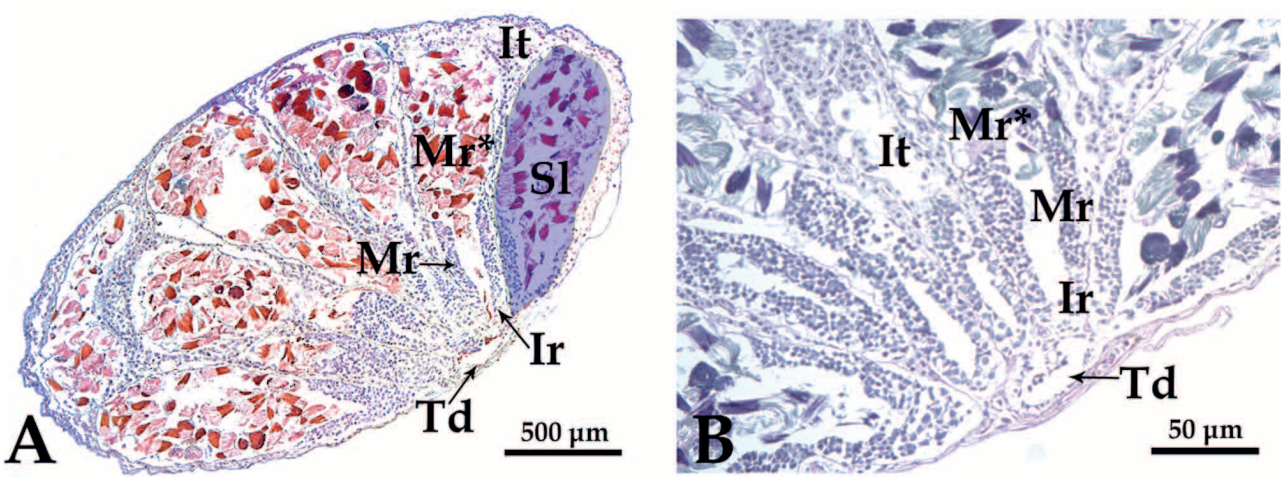

Figure 1. Testicular microstructure in *Salamandrella keyserlingii*: transverse sections of the testes collected in April. (A) General organisation of the testes; (B) high magnification of the median part of the testes showing zoning of the seminiferous lobule. Sections stained with AM in (A) and with PAS in (B). Abbreviations: Ir – immature region of the seminiferous lobule with primary spermatogonia; It – interstitial tissue; Mr – maturing region of the seminiferous lobule with secondary spermatogonia; Mr* – mature region of the seminiferous lobule with bundles of spermatozoa; Sl – seminiferous lobule; Td – testicular duct.

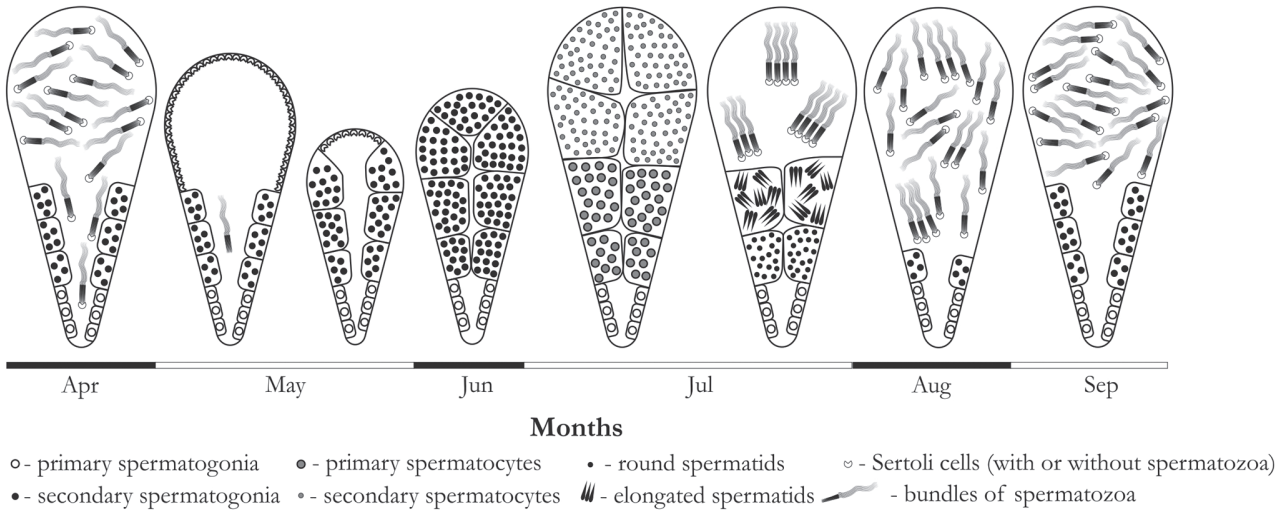

Figure 2. Seasonal changes in the seminiferous lobules during a spermatogenic cycle in the Tomsk population of *Salamandrella keyserlingii*. Teardrop shape – seminiferous lobule; irregular shapes inside it – cysts. Sertoli cells of the cysts are not shown.

The 'spermatogenic wave' along the caudo–cephalic axis of the testis was absent in all studied males. The seminiferous lobules were of the same type in all parts of the testes at every stage of the reproductive cycle (Fig. 2). However, there was a separate lobule consisting of several zones, which contained the cysts with germ cells at different stages (Figs 1, 2).

Seasonal variation in spermatogenic stages

The detailed histological states of testes throughout the season were as follows:

April: At the end of the month, each seminiferous lobule consisted of three regions (Fig. 1). Primary (I) spermatogonia were located in the immature region near the tes-

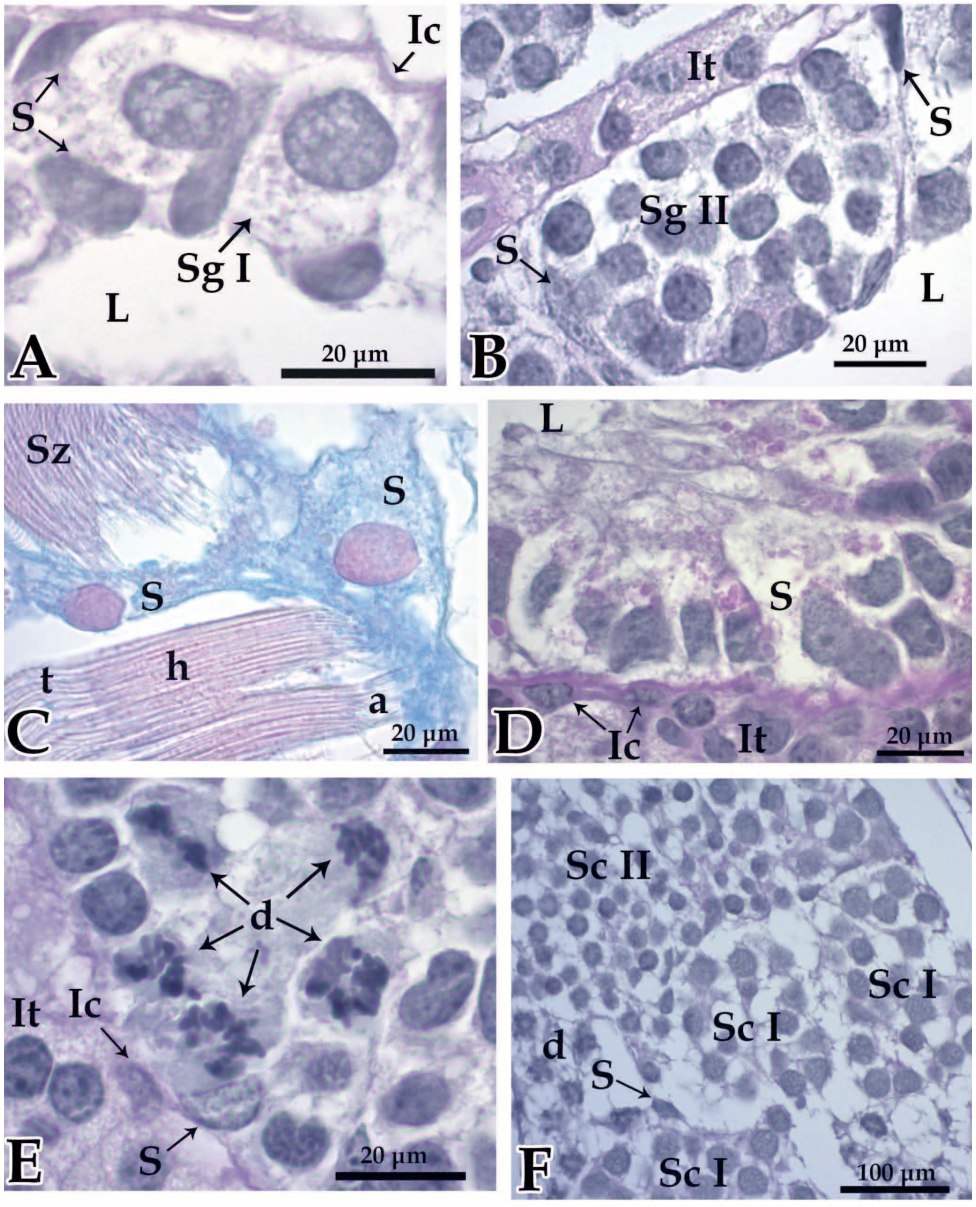

Figure 3. Spermatogenesis in *Salamandrella keyserlingii*: transverse sections of testes collected from April through July. (A) Primary spermatogonia in the immature region of the seminiferous lobule at the end of April; (B) cyst with secondary spermatogonia in the maturing region at the end of April; (C) Sertoli cells in contact with spermatozoa bundles, AB-positive staining of Sertoli cell cytoplasm, the matured region at the end of April; (D) fragment of the empty region in the second half of May, PAS-positive staining in interstitial and Sertoli cells; (E) mitosis of secondary spermatogonia in the maturing region in the second half of May; (F) meiosis of spermatocytes in the maturing region at the beginning of July. Sections stained with PAS in (A, B), and (E), with AB (pH = 2.5) in (C), and with AM in (F). Abbreviations: d – divisions; Ic – interstitial cells of lobular wall; L – lobular lumen; S – Sertoli cells; Sc I – primary spermatocytes; Sc II – secondary spermatocytes II; Sg I – primary spermatogonia; Sg II – secondary spermatogonia; other abbreviations as in Fig. 1.

Table 3. Nuclear diameters (μm) of germ cells at different stages of spermatogenesis.

Type of germ cells	N		Mean±SE Range	Cv, %
	Specimens	Nuclei		
Primary spermatogonia	4	60	13.82±0.20 10.52-18.00	11.36
Secondary spermatogonia	6	60	10.30±0.13 8.35-12.64	9.92
Primary spermatocytes	1	30	14.15±0.24 11.61-16.44	9.22
Secondary spermatocytes	1	30	9.25±0.15 7.44-10.63	8.63
Round spermatids	1	30	7.79±0.16 5.00-9.31	11.09

ticular duct (the proximal part of the lobule) (Figs 1, 2, 3A). This region was constantly present in the testicular lobules, but other regions changed throughout the active season (Fig. 2). Behind the lobules, there was the maturing region, containing the cyst with secondary (II) spermatogonia (Figs 1, 2, 3B). Nuclei of spermatogonia I were lighter in colour (Groat's hematoxylin and nuclear fast red staining) (Figs 3A, B) and larger in diameter than those of spermatogonia II (Mann-Whitney U test: $Z = 9.12$, $p < 0.001$; Table 3). Moreover, the nuclear diameter of spermatogonia I was more variable. The distal parts of lobules were formed by the mature regions, which consisted of spermatozoa bundles with Sertoli cells (Figs 1, 2, 3C). The basal parts of Sertoli cells had a large oval nuclei with one or two nucleoli each (Fig. 3C). The cytoplasm of these cells contacted the heads of the spermatozoa. Acidic mucopolysaccharides (AB-positive staining) were found in the cytoplasm of the Sertoli cells, probably indicating a preparation stage of spermiation (Fig. 3C). One of the examined males had bundles of spermatozoa in the lumen of the testicular duct.

May: Mature regions became empty (evacuated) regions as the result of completion of spermiation (Fig. 2). These regions were connected to the maturing regions. Fragments of unreleased spermatozoa were found inside some of the empty regions. After sperm release, Sertoli cells were located mainly at the boundary of empty regions (Fig. 3D). Their cytoplasm had become granular. Numerous processes elongated towards the lobular lumen were apparent in the apical part of the cells. PAS-positive regions were detected in the basal part, and PAS-positive granules were observed in the central and apical parts of the Sertoli cells. At this point, each nucleus was large and outfitted with fine granules and a well-noticeable nucleolus (rarely several nucleoli). The maturing region of each lobule contained resting cysts with spermatogonia II throughout the aquatic phase (Fig. 2). In the second half of May, when males entered land, the first spermatogonia II divisions of the current active season appeared (Fig. 3E).

June: The seminiferous lobules increased due to well-developed maturing regions as the result of spermatogonial proliferation (Fig. 2). Empty regions of lobules disappeared completely in late June, and formation of primary spermatocytes (I) was taking place. The nucleus increased in size during this process. The nuclear diameter of spermatocytes I was about 1.4 times as large as that of spermatogonia II (Mann-Whitney U test: $Z = 7.60$, $p < 0.001$; Table 3).

July: Testes were at different stages of maturation. In early July, the maturing regions of lobules contained spermatocytes at different stages of meiosis (Figs 2, 3F). The nuclear diameter of secondary spermatocytes (II) was about 0.7 times as large as that of spermatocytes I (Mann-Whitney U test: $Z = 6.65$, $p < 0.001$; Table 3). In the second half of July, active spermiogenesis occurred (Figs 2, 4A). Round spermatids represented the earliest stage of the spermiogenesis. The size of their nuclei was about 0.8 times as large as that of spermatocytes II (Mann-Whitney U test: $Z = 5.38$, $p < 0.001$; Table 3). The spermatids elongated during the course of spermiogenesis, the cysts disintegrated, and Sertoli cells began to contact with maturing spermatids (Fig. 4A). At this point, each lobule was divided into three zones: a small immature region (with spermatogonia I), a small new maturing region (with spermatogonia II formed earlier), and well-developed maturing regions (with spermatids at various stages). The presence of spermatogonia II in the proximal parts of the lobules indicated that spermatogonia I had proliferated in the preceding period.

August: At the end of this month, there were immature, new maturing, and matured regions in the lobules (Fig. 2). The matured region with Sertoli cells and spermatozoa bundles took up most of the space. The presence of sperm indicated the completion of spermatogenesis in this region of the lobule. The maturing region also contained spermatogonia II.

September: Groups of spermatozoa and Sertoli cells were now also found in the matured regions (Figs 2, 4B). Active divisions of spermatogonia II occurred in the cysts of new maturing regions (Fig. 4C). Maturing regions (with spermatogonia II) were more developed than in August.

Seasonal changes in the interstitial tissue

The interstitial tissue was well developed during the immigration of salamanders into the breeding pond, spermiation, and immediately after it. Some signs of physiological degradation in the interstitial tissue appeared after the males emerged from the water (Fig. 4D). In June, this tissue degenerated, forming thin strips along the periphery of the testes and between the lobules. This state persisted until the completion of spermiogenesis at the end of August. In September, the interstitial tissue was once more well developed.

The interstitial tissue also formed the cells of lobular walls (Figs 3A, D, E). They had a fibroblast-like morphology: the cytoplasm was stretched along the lobular border in the form of a narrow strip. These cells contained an oblong, often rod-shaped or triangular nucleus in the enlarged part. No clear-cut seasonal changes were observed in this group of cells. It was only after spermiation in the pond and immediately af-

Table 4. Factor loads based on correlations of air temperatures with the first principal component (PC_1).

Parameters of temperature	PC_1	Parameters of temperature	PC_1	Parameters of temperature	PC_1
T_{max} Jan	0.99	T_{min} Jan	0.98	T_{mean} Jan	0.99
T_{max} Feb	0.98	T_{min} Feb	0.96	T_{mean} Feb	0.98
T_{max} Mar	0.97	T_{min} Mar	0.98	T_{mean} Mar	0.98
T_{max} Apr	0.96	T_{min} Apr	0.98	T_{mean} Apr	0.97
T_{max} May	0.90	T_{min} May	0.96	T_{mean} May	0.94
T_{max} Jun	0.81	T_{min} Jun	0.94	T_{mean} Jun	0.89
T_{max} Jul	0.85	T_{min} Jul	0.98	T_{mean} Jul	0.93
T_{max} Aug	0.97	T_{min} Aug	0.95	T_{mean} Aug	0.97
T_{max} Sep	0.96	T_{min} Sep	0.95	T_{mean} Sep	0.96
T_{max} Oct	0.97	T_{min} Oct	0.99	T_{mean} Oct	0.99
T_{max} Nov	0.97	T_{min} Nov	0.98	T_{mean} Nov	0.98
T_{max} Dec	0.98	T_{min} Dec	0.97	T_{mean} Dec	0.98

ter the salamanders had gone onto land that boundary cells of empty regions had large nuclei, exhibited a striated shape, but they were broader in comparison with those found in other periods of active season. At this time, the cytoplasm of cells showed a PAS-positive reaction (Fig. 3D).

Table 5. Values of the first principal component (PC_1) for salamander localities (comp. Table 2).

Location	PC_1
Iwamuro	1.25
Qinling	0.76
Tomsk	-0.66
Komadome	-0.51
Sapporo	0.54
Magadan	-1.37

Spermatogenic cycles of hynobiids and temperature

The factor analysis identified four principal components (PC), which described 99.96% of the variability of air temperature in the studied locations. PC_1 had the highest factor load and described 91.78% of this variability (Table 4). This indicates that PC_1 was an integral characteristic that described temperature variability.

We rated all locations according to the value of PC_1 (Table 5). The first group comprised locations of cold regions ($PC_1 < 0$): Magadan, Tomsk, and Komadome. The second group included regions with a warmer climate ($PC_1 > 0$): Iwamuro, Qinling, and Sapporo.

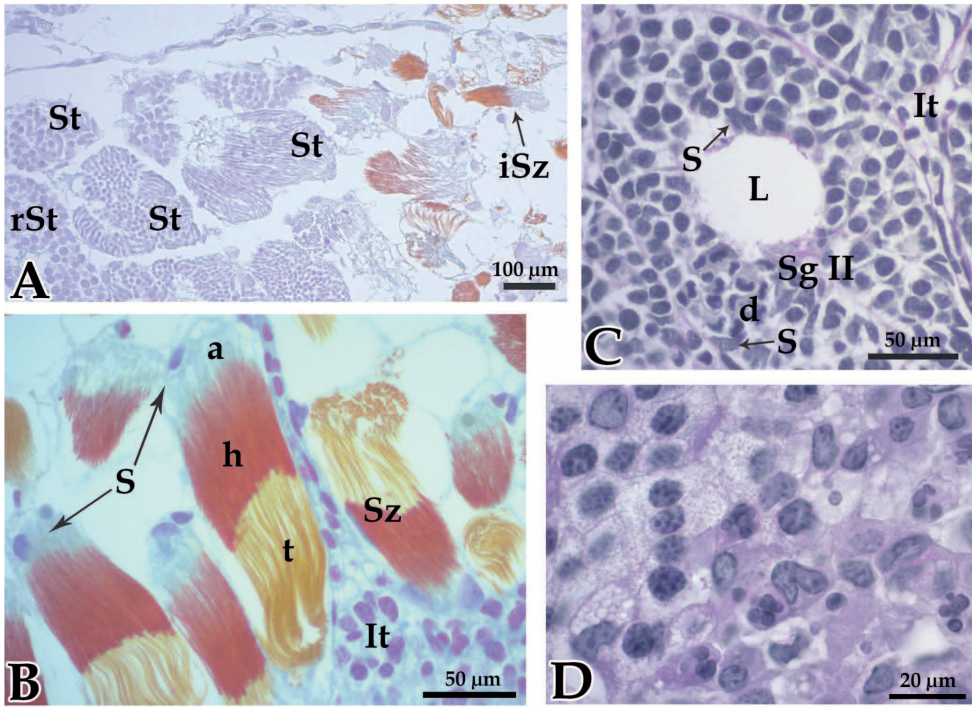

Figure 4. Spermatogenesis in *Salamandrella keyserlingii*: transverse sections of testes collected in May and from July through September. (A) Spermiogenesis in the maturing region of a seminiferous lobule at the end of July; (B) mature spermatozoa in the matured region in September; (C) the part of the maturing region with proliferation of secondary spermatogonia in September; (D) degenerative processes in interstitial tissue in the second half of May. Sections stained with AM in (A) and (B), and with PAS in (C) and (D). Abbreviations: a – spermatozoa acrosomes; h – head of spermatozoa; iSz – immature spermatozoa; rSt – round spermatids; St – spermatids of the other stages; Sz – spermatozoa; t – tails of spermatozoa; other abbreviations as in Figs 1 and 3.

Table 6. Characteristics of spermatogenic cycles in hynobiid salamanders. DAS – duration of active season (in months); NSPPs – number of spermatogonial proliferation periods; DSP – Duration of spermatogonial proliferation period of the first half of cycle (in months).

Species	Location (comp. Table 2)	DAS	NSPPs	DSP	References
Hynobius lichenatus	Sapporo	8	1 (end of April – beginning of July)	3	Makino (1931)
Hynobius nigrescens	Iwamuro	8	1 (April–June)	3	Hasumi et al. (1990)
Hynobius retardatus	Komadome	5	1 (beginning of May – end of June)	2	Iwasawa et al. (1992)
Batrachuperus tibetanus	Qinling	8	2 (1st: September–October; 2nd: April–May)	2	Wang & Zhang (2004)
Salamandrella keyserlingii	Magadan	4	1 (second half of May – end of June)	1.5	Bulakhova & Berman (2014)
Salamandrella keyserlingii	Tomsk	5	2 (1st: August–September; 2nd: middle of May–June)	1.5	this study

DAS, NSPPs, and DSPP varied between the studied hynobiids (Table 6). NSP in the studied species did not correspond to PC_1 values. In the warm climate ($PC_1 > 0$), there was a spermatogenic cycle with two periods of spermatogonial proliferation and, conversely, there was a spermatogenic cycle with one period of spermatogonial proliferation in the cold climate ($PC_1 < 0$) (Table 6). On the other hand, DAS and DSPP had strongly significant correlations with PC_1 (Spearman's $\rho = 0.93$ and 0.84, respectively $p < 0.05$).

Discussion

Testicular microstructure in *Salamandrella keyserlingii*

The cystic lobule type of the testes is common in salamanders (Roosen-Runge 1980, Gabaeva 1982, Delsol et al. 1995, Uribe 2003). The structural unit is the seminiferous lobule, which contains the cysts with gametes. The germ cells develop synchronously inside the cysts. Salamanders of the families Plethodontidae (Humphrey 1921, 1922, Burger 1936, Angle 1969, Houck 1977, Chan 2003, Siegel et al. 2014), Salamandridae (Champy 1913, Humphrey 1921, Adams 1940, Tso & Lofts 1977a, Verrell et al. 1986, Guarino et al. 1992), Proteidae (McGregor 1899, Pudney et al. 1983, Singh & Callard 1989), and Ambystomatidae (Miltner & Armstrong 1983, Uribe et al. 1994) possess a 'spermatogenic wave' that determines the zonal structuring of the testes. The seminiferous lobules with the germ cells at different stages are located along the caudo–cephalic axis of the testes. In hynobiid species, the microstructure of the testes deviates from this pattern in that there will be no caudo–cephalic zoning (Yamagiwa 1924, Makino 1931, Hasumi et al. 1990, Iwasawa et al. 1992, Wang & Zhang 2004, 2007, Bulakhova & Berman 2014, this study). All seminiferous lobules of one testicle exhibit the same state during each stage of reproductive cycle. However, in hynobiids, every single seminiferous lobule exhibits a zoned pattern (cf. Figs 1, 2). This phenomenon could be referred to as a 'lobular wave'. The microstructure of hynobiid testes is similar to that of *Cryptobranchus alleganiensis* of the family Cryptobranchidae (Ingersol et al. 1991). Among other salamanders, *Hydromantes italicus* (Plethodontidae) has the same zoning of the testicular seminiferous lobules as has *S. keyserlingii* (comp. Figs 1, 2 with Fig. 2 in Galgano 1958). Delsol et al. (1995) also noticed the similarity between the testicular microstructures of plethodontids and hynobiids and concluded that the testes of hynobiids have the most primitive structure among salamanders.

The morphology of the male germ cells in *S. keyserlingii* is similar to that found in other salamander species (cf. Champy 1913, Grassé 1986, Uribe 2003). The variation of the nuclear size of germ cells in *S. keyserlingii* is similar to that in *Salamandra salamandra* (Schindelmeiser et al. 1983) and *Triturus marmoratus* (Fraile et al. 1992), both members of the family Salamandridae. The karyometric dynamics of germ cells are associated with the quantitative change of nuclear DNA during spermatogenesis (Fraile et al. 1992).

In many salamanders, the lobules empty after spermiation and transform into glandular tissue (Champy 1913, Humphrey 1921, Miller & Robbins 1954, Tso & Lofts 1977a, Verrell et al. 1986, Fraile et al. 1990, Guarino et al. 1992). The development of this glandular tissue coincides with the formation of secondary sexual characteristics. The steroid synthesis in the cells of this region occurs immediately after sperm release (Picheral 1968, Tso & Lofts 1977a, b, Imai & Tanaka 1978, Pudney et al. 1983, Fraile et al. 1989b, Guarino et al. 1992). During the formation of glandular tissue, the interstitial cells change their shape from the typical elongated fibroblast-like shape into a cubic one. In *S. keyserlingii*, we found that active physiological processes also take place in interstitial and Sertoli cells of the empty regions of lobules. However, the changes were not very clear-cut, and these regions differed from the glandular tissues of other salamanders. Hasumi et al. (1990) also concluded that in male *H. nigrescens*, 'empty lobules' were not similar to the glandular tissue. We observed the maximum development of interstitial tissue in testes of *S. keyserlingii* just after spermiation and following its degradation during the spermatogonial proliferation stage. Applying a quantitative analysis, Wang et al. (2005) described the same changes in interstitial tissue in the testes of *B. tibetanus*.

Spermatogenic cycles of hynobiids

In the studied species of hynobiids, the formation of spermatocytes, their meiosis, and spermiogenesis occur in summer (Makino 1931, Hasumi et al. 1990, Iwasawa et al. 1992, Bulakhova & Berman 2014, this study), which is also true for many other amphibian species living in temperate climates (Delsol et al. 1995). Spermatogenesis will be completed in September in *H. nigrescens* (Hasumi et al. 1990), and already in August in *S. keyserlingii*, *B. tibetanus*, *H. lichenatus*, and *H. retardatus* (Makino 1931, Iwasawa et al. 1992, Bulakhova & Berman 2014, this study). Direct observations in winter indicate no testicular activity during hibernation in *H. lichenatus* (Makino 1931) and *H. nigrescens* (Hasumi et al. 1990). Experimental data confirm that low temperatures prevent the proliferative activity of germ cells (Paniagua et al. 1990). However, testicular activity does not completely cease in *Lissotriton italicus* (spermatogonial proliferation) (Guarino et al. 1992) and *Taricha torosa* (spermiogenesis) (Miller & Robbins 1954), while a state of 'hemistasis' is observed in *Salamandrina terdigitata* (Brizzi et al. 1985, cited by Guarino et al. 1992).

The most important distinctions of spermatogenic cycles in hynobiids concern spermatogonial proliferation (Table 6). In *H. nigrescens* (Hasumi et al. 1990) and *H. retardatus* (Iwasawa et al. 1992), there is only one annual period of spermatogonial proliferation (from spring to early summer). In spring and autumn, the seminiferous lobules of these species contained only spermatozoa and primary spermatogonia. Makino (1931) describe the same seasonal states of the testes for *H. lichenatus*. In contrast, Wang & Zhang (2004) observed two periods of spermatogonial divisions in *B. tibetanus*: the first occured from September through November and the second from April through May. In this species, the seminiferous lobules contained spermatozoa (mature region), secondary spermatogonia (maturing region), and primary spermatogonia (immature region) in spring and autumn. A similar phenomenon was observed in the Tomsk population of *S. keyserlingii* (this study), but Bulakhova & Berman (2014) described only one period of spermatogonial proliferation for the Okhotsk population of *S. keyserlingii*: from the end of spermiation to when the salamanders go onto land (May to June). However, in males of the conspecific Okhotsk population (Bulakhova & Berman 2014: Fig. 5), the states of testes are histologically similar to those observed in the Tomsk population (this study). The maturing regions with secondary spermatogonia were well developed both in spring (before spermiation) and autumn in both populations. This suggests that the presence of only one period of spermatogonial proliferation in the Okhotsk population (Bulakhova & Berman 2014) might be questionable. In males from Ekaterinburg (Middle Urals) and Irkutsk (Eastern Siberia), well-developed cysts with remnants of secondary spermatogonia were observed in the testes just after spermiation (April and May, respectively) (Lepeshkin 1916). Thus, there are two types of spermatogenic cycles in the family Hynobiidae (Fig. 5). The first type is present in the species *H. nigrescens*, *H. retardatus*, and *H. lichenatus* (Fig. 5A), and the second one in *B. tibetanus* and *S. keyserlingii* (Fig. 4B).

Two types of spermatogenic cycles define different periods of spermatogenesis in all studied hynobiid species.

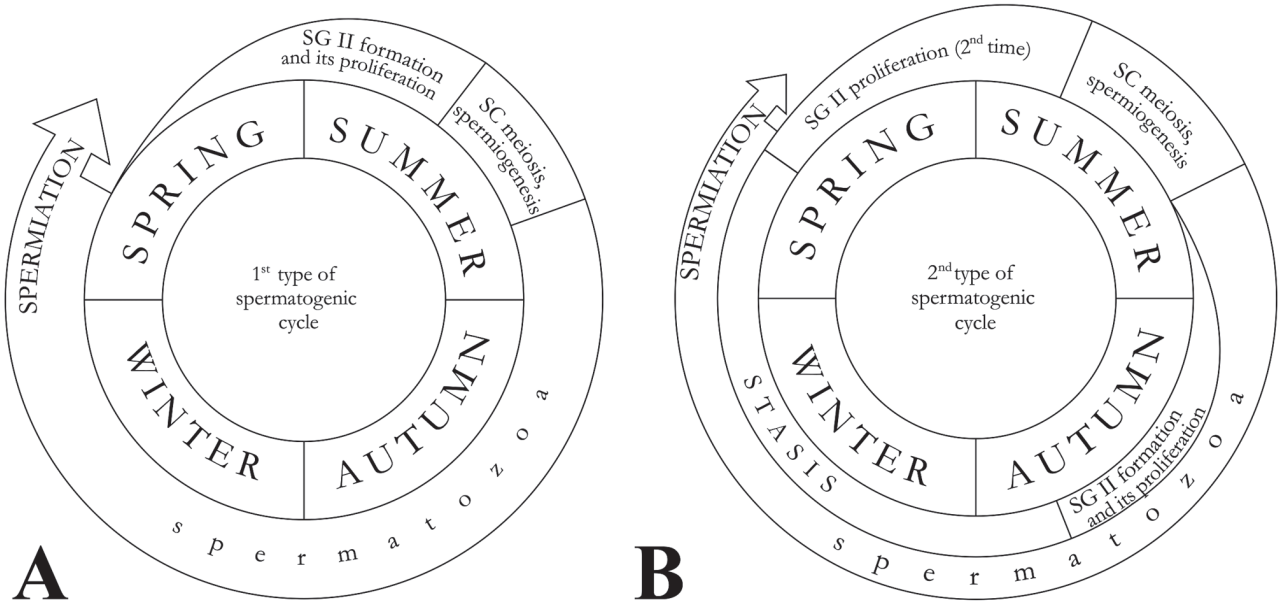

Figure 5. Types of spermatogenic cycles in hynobiid salamanders. (A) The first type includes one period of secondary spermatogonia proliferation only in the first half of the active season as described for *Hynobius lichenatus*, *H. nigrescens*, and *H. retardatus*; (B) the second type includes two periods of secondary spermatogonia proliferation at some points in the first and second halves of the active season as occurring in *Batrachuperus tibetanus* and *Salamandrella keyserlingii*.

According to the general periodisation of spermatogenesis in animals, it starts with the division of primary spermatogonia and continues to the formation of spermatozoa (Roosen-Runge 1980). From this perspective, the period of spermatogenesis approximately corresponds to the active season of *H. nigrescens*, *H. retardatus*, and *H. lichenatus* (Makino 1931, Hasumi et al. 1990, Iwasawa et al. 1992). In contrast, the spermatogenic cycles clearly decelerate in *B. tibetanus* and *S. keyserlingii*: they begin in late summer or early autumn (when divisions of spermatogonia I have formed spermatogonia II) and end only in August of the following year. Spermatogenesis in these species probably includes a long period of stasis in winter (Wang & Zhang 2004, this study).

These types of spermatogenic cycles are not associated with the air temperatures of regions (Tables 5+6). The Iwamuro population of *H. nigrescens* (Hasumi et al. 1990), the Sapporo population of *H. lichenatus* (Makino 1931), and the Qinling population of *B. tibetanus* (Wang & Zhang 2004) all inhabit relatively warm regions and exhibit the longest DAS (Tables 5+6). The Komadome population of *H. retardatus* (Iwasawa et al. 1992) and the Tomsk population of *S. keyserlingii* (this study) instead exhibit a moderately long DAS under cool and cold climatic conditions, respectively (Tables 5+6). The Okhotsk population of *S. keyserlingii* has the shortest DAS and lives in a cold climate (Bulakhova & Berman 2014) (Tables 5+6). Thus, species with different types of spermatogenic cycles inhabit regions with similar temperature conditions. However, there was a strong correlation between ambient temperatures and DSPP in these species (this study), indicating an influence of air temperatures on spermatogonial divisions. We assume that the two periods of spermatogonial division in *S. keyserlingii* are adaptive 'enough' to evolve spermatogenesis in the subarctic climate of Western Siberia: spermatogonial proliferation occurs under more unstable temperature conditions (May and June). Cold weather may return in May, which will then result in an extended breeding season that continues until early June (Yartsev 2011, Yartsev & Kuranova 2015). Irrespective of the warm climate in the Qinling Mountains, *B. tibetanus* is a typical lentic form, breeding in streams with water temperature from 6 to 13°C (Rafaeilli 2014). In both species, two periods of spermatogonial proliferations separated by a hibernation phase could be an adaptation aiming at supporting the formation of the necessary amount of sperm, because the NSPPs directly affect the productivity of spermatogenesis (Roosen-Range 1980). Secondary spermatogonia could represent a 'waiting stage' of spermatogenesis in these species. Galgano (1936) noted in *Rana* kl. *esculenta*, and Ifft (1942) in *Notophthalmus viridescens*, that the primordial germ cells and spermatogonia were insensitive or less sensitive to low temperatures in comparison with later stages of spermatogenesis.

The presence of the first cycle type in the Komadome population of *H. retardatus* (Iwasawa et al. 1992) indicates the possibility of this cycle type version existing in a cool-climate species. In this regard, we consider that air temperature might be one of the factors influencing the formation of these spermatogenic cycle types. The second cycle type could be linked to a strategy of forming sperm in larger amounts. The latter may to some extent depend either on the fecundity of females or the degree of competition for fertilising clutches between males. On the other hand, the genera *Salamandrella* and *Batrachuperus* are phylogenetically closer to each other according to molecular data than to the genus *Hynobius* (Zhag et al. 2006, Poyarkov 2010). Future studies of spermatogenic cycles in different species of the family Hynobiidae will be very interesting in this context.

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