

# Seasonal Dynamics of Male and Female Reproductive Systems in the Siberian Salamander, *Salamandrella keyserlingii* (Caudata, Hynobiidae)

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**Abstract** It is not well known how low temperatures, like a subarctic steppe–tundra climate, influence reproductive traits of ectothermic vertebrates. To begin answering this question, we studied male and female reproductive systems of *Salamandrella keyserlingii* inhabiting a Tomsk population (southeast of Western Siberia), Russia, in ecological and physiological terms. In males, before spermiation, the testicular size and weight in late April–early May were greatest of all. Spermiation occurred during breeding immigration in spring when mean air temperature was above 10°C, and at the same time rain fell. After spermiation, the testicular size and weight decreased sharply, and the diameter of the vasa deferentia increased. “Spawning” (i.e., simultaneous extrusion of sperm and oviposition) occurred from late April to late May, and this duration fluctuated in temperature and humidity. The testicular size and weight increased in summer. Sperm mass was detected in the testes by the smear method in April–September, except in June when single fragmented unrealized sperm was detected and in July when spermatids were detected. In females, ovarian weight was greatest in spring before ovulation. From late June, vitellogenesis began in ovarian follicles, in which mint green yolks accumulated. Melanin deposited in the surface of the ovary from July when oviducts were hypertrophying. In contrast, some large-sized females did not show any sexual maturity shortly before hibernation (although these females may be subadults). These results suggest that low temperatures in Siberia induce early timing of gamete maturation in females, but the females’ reproductive cycle might also be biennial. A reproductive cycle in males was annual with the completion of the gamete maturation process in August.

**Keywords** annual reproductive cycle, biennial reproductive cycle, breeding immigration, gamete maturation, subarctic climate

## 1. Introduction

Among terrestrial vertebrates, amphibians have a high diversity of reproductive forms, which are manifested in the modes of fertilization, breeding places, clutch characteristics, parental care, and reproductive cycles (Duellman and Trueb, 1986). Among the three recent groups of amphibians (i.e., anurans, urodeles, and

caecilians) there are various trends of terrestrialization in reproduction and two mode of fertilization – external and internal (Duellman and Trueb, 1986; Wake and Dickie, 1998). Most urodeles have internal fertilization, which is preceded by the use of spermatophores (Delsol, 1986; Duellman and Trueb, 1986). External fertilization occurs in the suborders Sirenoidea (Reinhard *et al.*, 2013; Sever *et al.*, 1996; Sever, 2013; Ultsch, 1973) and Cryptobranchioidea (Makino, 1934; Smith, 1907; Thorn, 1986).

Reproductive cycles of urodeles are diverse and have been studied in detail in groups with internal fertilization including Salamandridae (Degani *et al.*, 1997; Guarino

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*et al.*, 1992; Joly, 1986; Sharon *et al.*, 1997; Verrell *et al.*, 1986; Vilter, 1986), Plethodontidae (Angle, 1969; Highton, 1962; Houck, 1977; Wheeler *et al.*, 2013), and Ambystomatidae (Miltner and Armstrong, 1983; Norris *et al.*, 1985; Uribe *et al.*, 1994). Reproductive biology of Hynobiidae species has been studied to a lesser extent. Various aspects of reproductive cycles are more fully described in the genera *Hynobius* (Hasumi, 1996; Hasumi *et al.*, 1990; Iwasawa *et al.*, 1992; Makino, 1931, 1934; Matsubara, 1943; Whang *et al.*, 2009) and *Batrachuperus* (Jia and Zhang, 2000; Wang and Zhang, 2004, 2007; Wang *et al.*, 2005; Xu, 1995).

Many tropical and some cave species of salamanders have unseasonal cycles of reproduction (Chan, 2003; Hendricks and Kezer, 1958). Urodeles of temperate regions are characterized by a well-defined breeding season, associated with annual changes in environmental factors, mainly temperature and precipitation. As a rule, reproductive cycles have annual periodicity (Adams, 1940; Bizzi and Corti, 2006; Gaurino *et al.*, 1992; Miller and Robbins, 1954; Verrell *et al.*, 1986). However, females of some salamanders, mainly in mountain and northern populations, have biennial or triennial cycles of gamete maturation (Joly, 1986; Leclair *et al.*, 2008; Wheeler *et al.*, 2013). A longer period at maturity of females is associated with high energy expenditure for oogenesis compared with males' spermatogenesis (Wells, 2007).

In general, ectothermic vertebrates show three types of reproductive cycles for gamete maturation pattern: associated, dissociated, and continuous (Whittier and Crews, 1987). In 90% urodeles with internal fertilization and dissociated reproductive pattern, gonadal activity is low when mating occurs but increases at other times of the year (Houck and Woodley, 1995). In contrast, hynobiid salamanders with external fertilization have an associated reproductive pattern (Hasumi *et al.*, 1997). In this pattern, gonadal activity increases shortly before "spawning" (i.e., simultaneous extrusion of sperm and oviposition), and high levels of circulating sex hormones are associated with spawning (Hasumi *et al.*, 1993). Because of predictable seasonal changes in gonads and sex accessory structures, temperatures can play an important role in the initiation of reproductive cycles (Moore, 1987).

The Siberian salamander *Salamandrella keyserlingii* possesses the widest geographical range of any recent amphibian species (Kuzmin, 2012). The species range covers 12 million km<sup>2</sup> in Northern Palearctic. Significant part of the range is in the subarctic climate, and species

distribution extends beyond the Arctic Circle. Along with the data about the external fertilization (Ishchenko *et al.*, 1995a), there is a hypothesis that as adaptation to low temperatures in this species males and females have multiyear reproductive cycles and additional way of reproduction by internal fertilization (Kuranova and Saveliev, 2006; Saveliev *et al.*, 1991; Saveliev *et al.*, 1993). In males with a biennial reproductive cycle, in the first year, spermatogenesis is initiated from the proliferation of primary spermatogonia, through the formation of spermatocytes, and stops temporarily at the formation of spermatids before hibernation. These spermatids are then maintained in the testes until spring. In the second year, spermiogenesis begins from spring and ends to June with the formation of mature spermatozoa in early July. According to this hypothesis, males do not have mature sperm and cannot inseminate the eggs in spring. Mature sperm cells are formed in the middle of summer, when the internal fertilization is expected. During spring, immature, maturing, and mature follicles are present simultaneously in females. After spawning, the growth of follicles that were formed last year begins by the fall, and these follicles reach the size of mature eggs by the spring. This cycle is repeated annually. Using histological and immunohistochemical methods sperm is identified in the genital tracts of females (Kuranova and Saveliev, 2006; Saveliev *et al.*, 1993). In contrast to other hynobiids, this hypothesis follows data that male and female *S. keyserlingii* have multiyear reproductive cycles, internal fertilization, and dissociated patterns of reproduction. There is a contradiction between these data that need confirmation (Kuzmin, 2012) and data from other hynobiids examined (Hasumi *et al.*, 1990; Hasumi, 1996; Iwasawa *et al.*, 1992).

Our research questions were as follows: (1) Does every hynobiid show an associated reproductive pattern similar to that of other hynobiid species examined? (2) Is timing of gamete maturation in both sexes of *S. keyserlingii* from the zone of low temperatures (such as subarctic steppe–tundra climate) different to other hynobiids? (3) If it is different, are there signs of accelerated or decelerated gamete development, and which stage of reproductive cycles is influenced? (4) How do features of male and female reproductive cycles relate to the hypotheses of internal fertilization in this species? To address these questions, we determined the male and female reproductive cycles of *S. keyserlingii* under low-temperature climate conditions of southeast of Western Siberia.

## 2. Materials and Methods

**2.1 Samples and measurements** We collected mature individuals in the suburb of Tomsk (southeast of Western Siberia, Russia: 56°28' N, 84°58' E) from April to September in 2005, 2009, 2010, and 2012 (Table 1). We captured animals using trenches with pitfall traps on land and a dip net in a breeding pond.

In the laboratory, after individuals were anesthetized with diethyl ether followed by decapitation, we measured snout–vent length (SVL: distance from the tip of the snout to the anterior angle of the vent) to within 0.1 mm using digital slide calipers and then weighed each individual (body mass: BM) to within 0.1 g using an electronic balance. After dissection, we weighed gonads (gonadal weight: Wg) to within 0.001 g using an electronic balance. For males, we registered the color of testes and measured their maximum length ( $L_{tes}$ , mm) and diameter ( $D_{tes}$ , mm) and the diameter of the middle section of the vasa deferentia (D.v.d, mm). For females, we measured the diameter of the pars convoluta of the oviduct (D.o.p.c, mm) and the maximum width of the ovisac (W.os, mm) and then described the predominant color of ovarian follicles. All measurements were performed separately for the right and left sides. For the detection of spermatids and spermatozoa, we prepared and stained smears from the testes and the vas deferens in males and the pars recta and the pars convoluta of the oviduct, the ovisac, and the surface of the ovary in females with the Romanovsky–Giemsa method (Romeys, 1954). We examined smears under a microscope Axiostar Plus (Carl Zeiss Microscopy) by scanning the entire surface of the glass at a magnification of 200×. We observed 56 smears from 28 males and 154 smears from 36 females. Snapshots of preparations were made by using a microscope Axio Lab.

A1 (Carl Zeiss Microscopy) with a camera AxioCam ERc 5s (Carl Zeiss Microscopy). After examination, all animals were fixed in 10% formalin (= 4% solution of formaldehyde) and deposited at the scientific collection of Department of Vertebrate Zoology and Ecology of Institute of Biology, Tomsk State University (DVZeE TSU).

We only used animals whose size was larger than the minimum size of males or females with signs of sexual maturity, for the analysis (groups M1 and M2 – for males, and F1, F2, and F7 – for females) (Table 1). We examined a total of 34 males and 67 females. They were divided into several groups according to the phases of the reproductive cycle, depending on the date of capture and key features of the reproductive system. We distinguished the following groups among males:

*M 1* – males immigrating to the breeding pond: light yellow testes and slightly swollen vasa deferentia ( $n = 5$ , the end of April–the beginning of May 2009/ the beginning of May 2010);

*M 2* – males located in the pond: yellow testes, but unlike M1, their vasa deferentia were greatly inflated ( $n = 5$ , the end of April–the beginning of May 2009/ the beginning of May 2010);

*M 3* – males emerging from the water: yellow testes and diminished vasa deferentia ( $n = 5$ , May 2009 and 2010);

*M 4* – males of terrestrial phase: yellow testes and diminished vasa deferentia ( $n = 5$ , June 2009 and 2010);

*M 5* – males of terrestrial phase: white testes and slightly swollen vasa deferentia ( $n = 10$ , July 2009 and 2010);

*M 6* – males of terrestrial phase: pale yellow testes and slightly swollen vasa deferentia ( $n = 4$ , August 2005/ September 2012).

**Table 1** Body size (snout–vent length: SVL), trapping time of year, and the number of individuals of *Salamandrella keyserlingii* at Tomsk, Russia.

SVL (mm)	Sex	April		May		June		July		August	September
		2009	2010	2009	2010	2009	2010	2009	2010	2005	2012
45–50	males									1	
	females										
50–55	males	1		1		1	1	2	1	1	
	females				1			2	1	2	
55–60	males	2		2	1			5	2		2
	females	4		3	4		3	5	1		
60–65	males	2		1	2	1	2				
	females	3		3	8	1	2	7	2		
65–70	males	1		1	1						
	females	5		1	5			3			
70–75	males										
	females				1						

By the condition of ovaries and oviducts, we divided females into the following groups:

*F 1* – females before spawning: the main part of an ovary consisted of large black follicles (their diameter was over 2 mm), and oviducts were greatly inflated ( $n = 25$ , the end of April–the beginning of May 2009/ the beginning of May 2010);

*F 2* – females after spawning: rather large shapeless ovary with small white follicles (up to 0.5 mm), and swollen oviducts ( $n = 7$ , May 2009/ May–June 2010);

*F 3* – females with a small compact ovary: the main part of which consisted of small transparent follicles (less than 0.5 mm), some follicles were white–opaque (about 0.5 mm), and oviducts were thin ( $n = 7$ , June 2009/ June–July 2010);

*F 4* – maturing females: compact ovary having many small follicles, but some separate follicles were large (0.5–0.7 mm) with white color or pale-green shade (especially in the individuals captured at the end of June and in July), and oviducts were slightly swollen ( $n = 15$ , April–July 2009/July 2010);

*F 5* – maturing females: large ovary, the diameter of follicles was larger than that of F4 (about 1 mm and more) with well-defined mint green color ( $n = 5$ , the end of June–July 2009/ July 2010).

*F 6* – females completing maturation: heterogeneous ovarian follicles [with various ratios of follicles from small white (less than 1 mm), through medium-sized green (1 mm and more), to large black (more than 1.5 mm)], and swollen oviducts ( $n = 6$ , July 2009 and 2010);

*F 7* – females completing maturation: ovary with a predominance of black follicles (more than 1.5 mm), and swollen oviducts ( $n = 2$ , August 2005).

To verify data on female reproductive cycle in the Tomsk population we also analyzed the reproductive state of females from the outskirts of Nizhneangarsk (south of Eastern Siberia, Russia: 55°47' N, 109°35' E) (the scientific collection of DVZeE TSU). We used females collected in ground shelters on a wetland shore of Lake Baikal in August 2009 and 2011. We were able to distinguish rather precisely these females as preparing for reproduction in the next spring. We measured SVL of the individuals in the same manner ( $n = 16$ ) and described the condition of their reproductive systems via phases chosen for the Tomsk population (see above) (Table 2).

**2.2 Statistical Analysis** We performed statistical analyses with Statistica 8.0 (StatSoft, Tulsa, Oakland, USA). All values were expressed by mean  $\pm$  standard error (SE) and range (minimum–maximum). We tested differences in the parameters of the male and female reproductive systems

**Table 2** Body size (SVL), trapping time of year, and the number of females of *Salamandrella keyserlingii* at Nizhneangarsk, Russia.

SVL (mm)	August	
	2009	2011
50–55	3	3
55–60		4
60–65	3	1
65–70		1
70–75		
>75		1

using Kruskal–Wallis test and in the SVL using Student's *t*-test and Mann–Whitney *U* test.

Primarily, quantitative characteristics, which showed the state of male and female reproductive systems, were converted to more integral parameters. As an indicator of gonad mass, we calculated a gonadosomatic index (GSI) which is widely used in studies of reproductive cycles of different groups of animals (Andreone and Dore, 1992; Gosch and Fischer, 1989; Guarino *et al.*, 1992; Stéguert *et al.*, 2001). We calculated the GSI according to the following formula (Stéguert *et al.*, 2001):

$$GSI = \frac{W_g}{BM} \times 100 (\%).$$

Testis volume ( $V_{tes}$ ) can serve as a dimensional integral indicator of the testes, widely used in the studies of the reproductive biology of different vertebrate groups (Gosch and Fischer, 1989; van Wyk, 1990). We calculated this indicator according to the formula of ellipsoid volume:

$$V_{tes} = \frac{1}{6} \times \pi \times L_{tes} \times D_{tes}^2 \text{ (mm}^3\text{)}.$$

We averaged all paired measurements ( $V_{tes}$ , mm<sup>3</sup>; D.v.d, mm; D.o.p.c, mm; W.os, mm), taken from the right and left sides, for each animal. We tested the relationship of SVL varying with the characteristic of the reproductive system via Spearman's rank correlation coefficient (Spearman's  $\rho$ ). We did not use relative indices (i.e., primary values divided by SVL or transformed by other mathematical methods), because of marginally or no significant correlations between parameters and SVLs (see below).

**2.3 Climatic data** To analyze the impact of environmental factors on the course of reproduction we used climatic data from an open database of weather conditions from server “Weather of Russia” (<http://meteo.infospace.ru>), which was created by Space Monitoring Laboratory of Space Research Institute of Russian Academy of Sciences



(RAS). The data on daily air temperature ( $T_{\text{air}}$ , °C), relative air humidity (RH, %), and rainfall for April–June 2009 and 2010 were used (the results of 4 measurements per 24 hours). According to the initial values, we calculated and used for further analysis the average daily indicators. We considered only presence of precipitations or their absence during the studied period. We compared the weather conditions during the breeding season in 2009 and 2010 using the Mann–Whitney  $U$  test. We also used monthly air temperature from the Tomsk Meteorological Station (code 29430, 56°26' N, 84°58' E) (based on the 30-year period) and the Nizhneangarsk Meteorological Station (code 30433, 55°47' N, 109°33' E) (2000–2013). We calculated the yearly air temperature in Tomsk based on mean monthly temperatures and that in Nizhneangarsk using primarily daily measurements per 14 years, because monthly temperature data was absent for this locality. Additionally, we measured the air temperature ( $T_{\text{air}}$ , °C: at a height of 2 m), the water temperature ( $T_{\text{w}}$ , °C: at a depth of 0.4 m), and the surface soil temperature ( $T_{\text{soil}}$ , °C) at 06:00–08:00 p.m. (measurement time = 5 min) during each observation in the field.

**2.4 Nomenclature of the species of the genus *Salamandrella*** Different authors used two names for the species of the genus *Salamandrella* Dybowski, 1870 from the Far East: *S. schrenckii* (Strauch, 1870) (Berman *et al.*, 2005, 2009; Malyarchuk *et al.*, 2010; Matsui *et al.*, 2008) and *S. tridactyla* Nikolsky, 1905 (Kuzmin, 2008, 2012; Poyarkov and Kuzmin, 2008). Since the name *S. schrenckii* was considered as inappropriate (Frost, 2014; Kuzmin, 2008, 2012), in the present work we employed the name *S. tridactyla* for this species despite the name *S. schrenckii* used in the original publications on the reproductive biology (Bulakhova and Berman, 2012, 2014).

### 3. Results

**3.1 Phenology of the breeding period** In the suburbs of Tomsk, located in subarctic climate and characterized by mean yearly air temperature  $-0.5$  °C, breeding immigrations began in late April–early May (2009–2010) (Figure 1). In 2009, moving of males into the ponds occurred in the V–VI pentads of April, but immigration of females was delayed until the I pentad of May (Figure 1 A). Among the first arrived individuals, 80% were females. The percentage of females was also greater than that of males during all period of immigration. Exit of the males from the ponds occurred constantly throughout May, emigration of females was encountered only in late

May.

The dynamics of salamander breeding immigration and emigration in 2010 differed from those in 2009 (Figure 1 B). Breeding period in 2010 was longer than in 2009 and lasted from late April to early June. The first part of males immigrated to the pond at the end of April, and the other part arrived in the II–III pentads of May when males after the completion of reproduction started emigrating. Males left the pond before the II pentad of June inclusively, the main part of them emerged onto the land precisely at this time. The females started moving into the pond in late April–early May. This was indicated by the presence of individuals from F2 group simultaneously with females from F1 group in the catches of the second pentad of May. The main part of the females entered the pond for reproduction in the early IV pentad of May, and one of the last individuals from F1 group was captured in early June. The main part of males emigrated at the beginning of June.

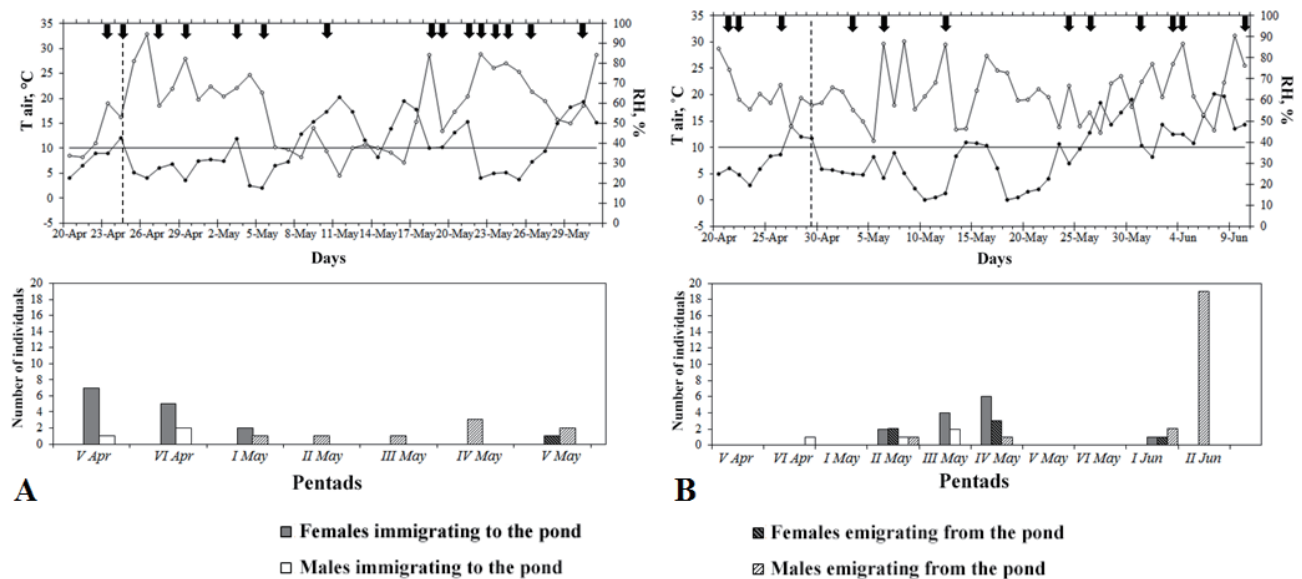
The first individuals appeared on 24 April 2009 ( $T_{\text{air}} = 11.1$  °C,  $T_{\text{s}} = 10.0$  °C,  $T_{\text{w}} = 1.5$  °C). At that time, about 35% of the surface of the pond was still under ice, but the eastern and southeastern shores were partially covered with snow. The immigration began after the transition of the average daily temperature of 10°C and after the rain. The average temperature in May was 11.33 °C (range = 0–19). In 2010, the first animals were noticed on 30 April ( $T_{\text{air}} = 12.0$  °C,  $T_{\text{s}} = 8.0$  °C,  $T_{\text{w}} = 2.8$  °C). The eastern and southeastern parts of the coast were under snow. At the same side of the pond around the perimeter stretched an ice line 3–4 meters wide. In 2010, the first spring rains fell on 20–21 April. Average daily temperatures in the V–early VI pentads of April were low, the transition through 10 °C occurred later, on 27 April, and remained at this level until 29 April. In a period of increased temperature, there was a reduction of relative air humidity to an average of 55.3% that limited the beginning of immigration. The appearance of the first animals became possible only after torrential rain in the night from 29–30 April (Figure 1 B). May was characterized by abundant rainfall and low temperatures: an average temperature was 7.2 °C (range =  $-3.3$  to 27.0), which was 2.6 °C lower than that in 2009 (Mann–Whitney  $U$  test,  $n = 248$ ,  $Z = 2.95$ ,  $P < 0.01$ ). The low temperatures of the V pentad of May interrupted exit of emigrants from the ponds, which began in the II pentad of May. Air humidity decreased by the end of May. Emigrations continued only in the beginning of June, when the air was warmer and wetter (Figure 1 B).

**3.2 Seasonal changes in male and female reproductive systems** In the Tomsk population of *S. keyserlingii* the

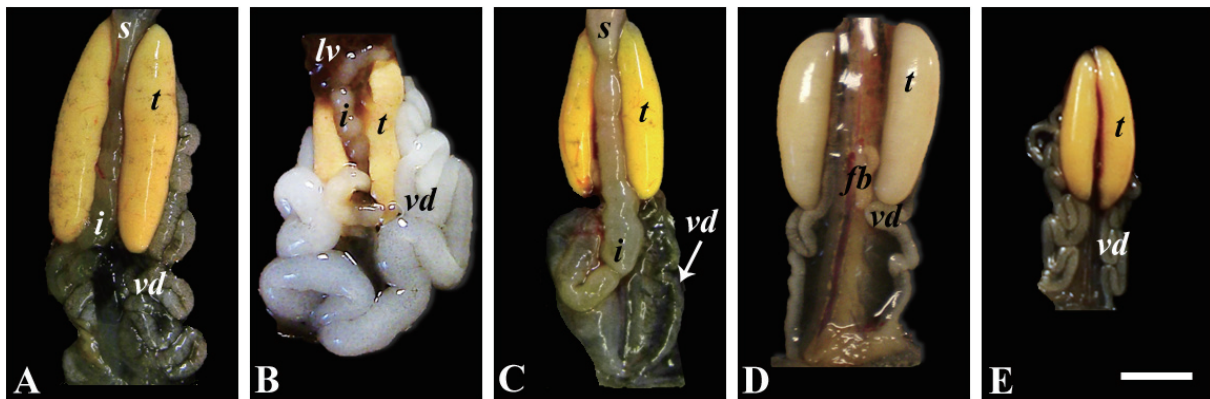
mean SVL of males examined was  $57.70 \pm 0.79$  mm (mean  $\pm$  SE) (range = 49.7–65.9,  $n = 34$ ), and the mean SVL of females was  $61.57 \pm 0.59$  mm (range = 52.2–72.1,  $n = 67$ ). The mean body size was greater in females than in males ( $t = -3.79$ ,  $P < 0.001$ ), as has been observed earlier in the Tomsk population (Kuranova and Yartsev, 2011) and populations from other parts of the species' range (Hasumi, 2010; Hasumi and Borkin, 2012). The mean SVL of females from Nizhneangarsk was  $58.96 \pm 1.61$  mm (range = 52.3–77.4,  $n = 16$ ), which was less than that in the Tomsk population (Mann–Whitney  $U$  test,  $Z = 2.24$ ,  $P < 0.05$ ).

**Males** GSI and  $V_{tes}$  were not correlated with SVL (Spearman's  $\rho = 0.15$  and  $0.19$  respectively,  $P > 0.05$ ).

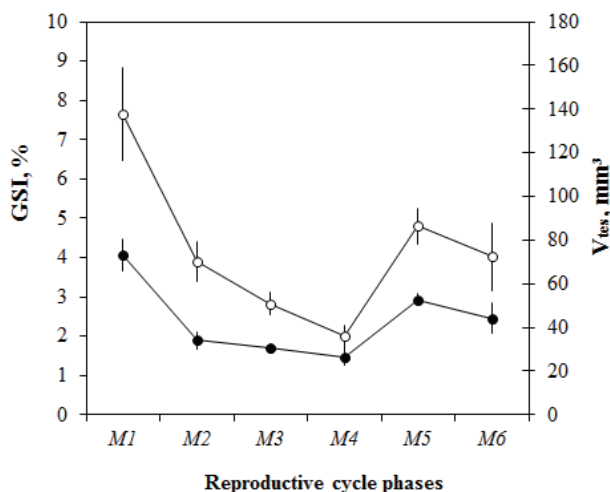
Positive correlation was detected between D.v.d. and SVL with a slight significance (Spearman's  $\rho = 0.35$ ,  $P < 0.05$ ). According to the analyzed characteristics selected groups of males (M1–M6) differed from each other (Kruskal–Wallis test; GSI:  $H = 24.54$ ,  $P < 0.001$ ;  $V_{tes}$ :  $H = 22.73$ ,  $P < 0.001$ ; D.v.d:  $H = 22.86$ ,  $P < 0.001$ ). In spring during the breeding season significant changes in indicators of the reproductive system were noted (Figures 2–4). The greatest GSI and  $V_{tes}$  were observed in males during breeding immigration (group M1: Figure 3). In males of group M2 these indicators were reduced, meanwhile the diameter of the vasa deferentia increased sharply (Figures 3 and 4). During emigration of males, all indicators were diminished, and this trend continued until the end of June



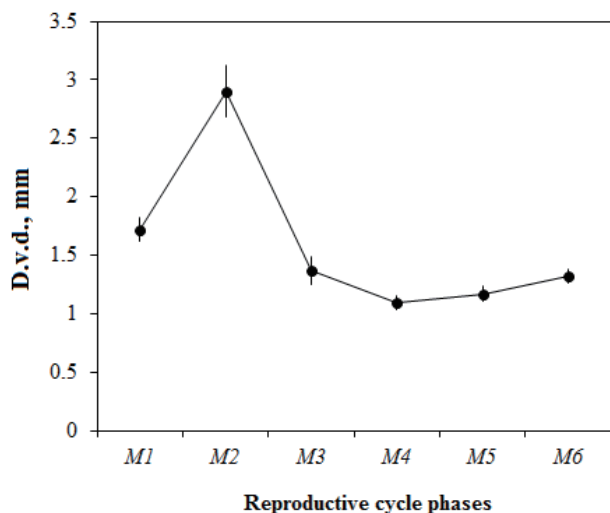
**Figure 1** Weather conditions and breeding immigration and emigration of male and female *S. keyserlingii* in 2009 (A) and 2010 (B). Solid circles denote air temperature (T<sub>air</sub>, °C); open circles, relative air humidity (RH, %); horizontal lines, the temperature level at 10 °C; arrows, presence of rainfall; and vertical dotted lines, the beginning of breeding immigration.



**Figure 2** Seasonal changes in testes and vasa deferentia in males of *S. keyserlingii*: (A) late April, male immigrating to the pond (group M1); (B) late April, male captured from the pond (group M2); (C) mid-June (group M4); (D) July (group M5); (E) September (group M6). Scale: 5 mm. t: testis; vd: vas deferens; i: intestine; fb: fat body; lv: liver.



**Figure 3** Dynamics of gonadosomatic index (GSI, %: solid circle) and testis volume ( $V_{tes}$ : open circle) during the reproductive cycle of males (Tomsk: 2005, 2009, and 2010). Vertical bars represent  $\pm$  SE of the mean. The number of specimens in each group is 5 (M1), 4 (M2), 5 (M3), 5 (M4), 10 (M5), and 4 (M6).



**Figure 4** Dynamics of the diameter of vasa deferentia (D.v.d., mm) during the reproductive cycle of males (Tomsk: 2005, 2009, and 2010). Vertical bars represent  $\pm$  SE of the mean. The number of specimens in each group is 5 (M1), 5 (M2), 5 (M3), 5 (M4), 10 (M5), and 4 (M6).

(Figures 3–4, M3–4). In July, GSI and  $V_{tes}$  increased (M5), but their values were 28.1% and 37.2% less than those in spring (M1), respectively (Figure 3). At the end of activity period, these indicators insignificantly reduced compared to those of the previous phase (Figure 3). Diameter of the vas deferens of males varied slightly around 1.2 mm after they emerged from the pond until the cessation of the active season on land (Figure 4).

Mature spermatozoa were clearly distinguishable in the following parts: acrosome, head, tail with undulating

membrane, and end piece (Figure 5 A, B). Spermatozoa unlike spermatozoa had twisted heads and tails without undulating membrane (Figure 5 D).

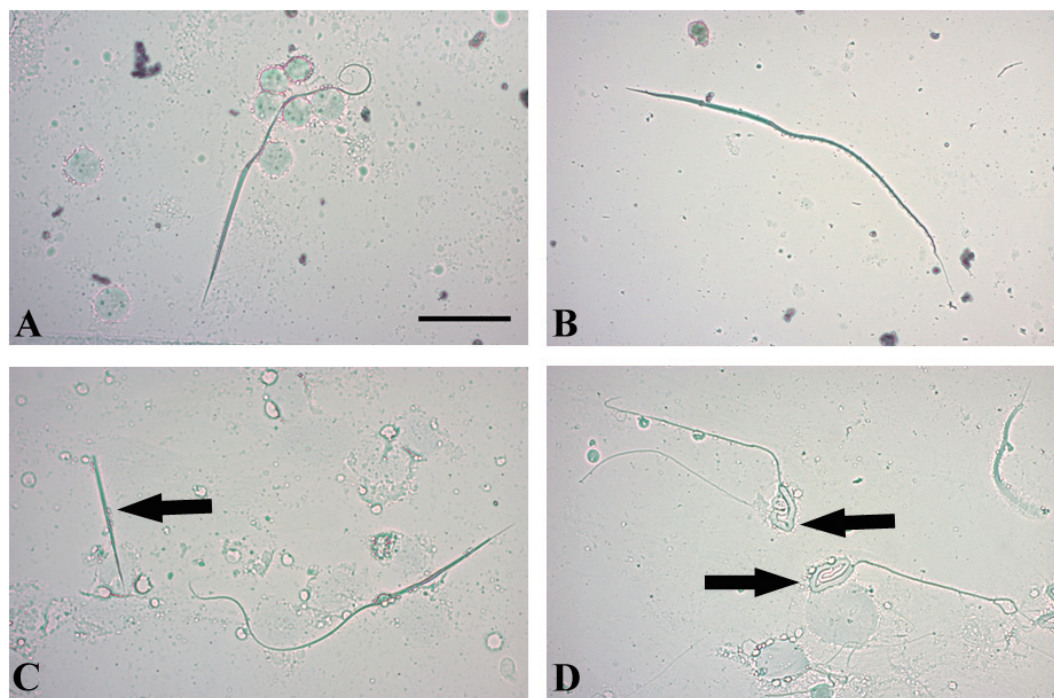
All males in April–early May (groups M1 and M2), which were immigrating toward the pond or were staying in the pond, possessed sperm in both the testes and the vasa deferentia (Figure 5A, B). In some males, which left the pond in May (M3) and were on land in May–June (M4), sporadic spermatozoa and their fragments were found in the testes and the vasa deferentia. In July (M5), half of the males had no spermatozoa or sperm in the testes and vasa deferentia, but in the other half spermatozoa were detected in the testes (Figure 5D). By the end of the active period (August–September), males (M6) had spermatozoa in the testes. At this time, sporadic spermatozoa were found in the vasa deferentia of one and only male, but sperm was absent in the vasa deferentia of other males.

**Females** From late April to early June, most of the captured females possessed mature follicles (F1), and several of these females were ready to lay a full clutch (Figure 6). Both the females that were clearly involved in reproduction and the females without showing “maturation signs” (i.e., yolk accumulation in ovarian follicles and hypertrophy of oviducts: F4) were captured in April when breeding activity was just started. The females having a yolk accumulation sign in the ovary appeared from June. From the second half of June females with lots of yolk in follicles (F5) were marked, as well as individuals without signs of vitellogenesis and hypertrophy of the oviducts (F3). In July, the females, which had an active accumulation of yolk (F4–F5) and the appearance of melanin in the follicles (F6), were dominated in the catches. In August, only individuals in the later stages of maturation were encountered (F6–F7).

In the Nizhneangarsk population from colder region with the mean yearly air temperature  $-1.72^{\circ}\text{C}$ , among the females, caught on land in August, group F6 was made up by 12.5% of individuals; F7 and F3, by 43.75%. This issue indicated that, along with individuals in the last stages of maturation (F6 and F7), the major part of females had not maturation signs in the reproductive system (F3).

GSI, D.o.p.c., and W.os. had slightly significant correlations with SVL (Spearman’s  $\rho = 0.39, 0.39$ , and  $0.47$  respectively,  $P < 0.05$ ). Among the females, significant interphase differences of GSI were detected (Kruskal–Wallis test;  $H = 52.53$ ,  $P < 0.001$ ) (Figure 7). The maximum GSI was observed in females before spawning, when her ovaries were filled with mature follicles – group F1 (Figure 8). Immediately after breeding, GSI of females





**Figure 5** Spermatozoa (A–C) and spermatids (D) in smears from reproductive system of males: (A) smear from testes at the end of April (group M2); (B) smear from vasa deferentia at the end of April (group M2); (C) smear from testes in June (group M3), arrow shows the fragment of spermatozoon's head; (D) smear from testes in July (group M5), arrows show curved spermatids' heads. Scale: 50  $\mu$ m.

reduced sharply – group F2. The lowest GSI was in the group F3 with bright ovary, in the second half of summer. Starting with the group F4, indicator increased. This trend persisted in individuals with pigmented ovaries, and GSI increased in females of groups F5 and F6 and reached the higher value in animals with black ovary in late August – F7 (Figure 8). GSI of this group was 69.7% less than that of F1 group (Figure 8).

Diameter of the pars convoluta of oviducts and width of the ovisac also undergo significant seasonal changes (Kruskal–Wallis test; D.o.p.c.:  $H = 50.22$ ,  $P < 0.001$ ; W.os.:  $H = 46.42$ ,  $P < 0.001$ ) (Figures 7 and 9). The maximum values of both parameters were observed in females of the group F1. After spawning, they began to decrease in the group F2 and reached a minimum in females of the group F3. Diameters of the pars convoluta of oviducts and width of the ovisac began to increase with the appearance of pigmentation in the ovarian follicles (F4–F7), and the indicators reached the second maximum at the end of the active season – group F7. In females of the group F7, width of the ovisac and diameter of the pars convoluta of oviduct were 14.7% and 27.9% less than the values observed in spawning females (F1), respectively (Figure 9).

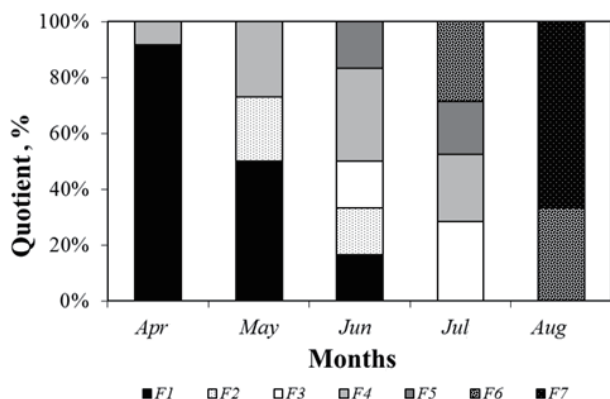
Microscopy of smears from the pars recta and the pars convoluta of oviduct, the ovisac, and the surface of the

ovary of all examined females showed no sperm and its fragments.

#### 4. Discussion

Breeding of the Siberian salamander in the suburbs of Tomsk occurred from late April to late May. Males and females began to immigrate at the same time, which agrees with the published data (Basarukin and Borkin, 1984; Hasumi and Kanda, 2007). Duration of the breeding period was determined by the weather conditions and varied by years. In 2010, when during the breeding season air temperatures were low, time of male and female stay in the pond was maximum – from late April to early June. Similar dependences were obtained while studying the breeding dynamics: there were shorter durations of breeding season in years with higher temperatures (Ishchenko *et al.*, 1995a). In the Extreme North, males emigrate from a pond only in late June (Alfimov and Berman, 2010; Bulakhova and Berman, 2014). In the mountain population of *S. tridactyla*, Kuranova *et al.* (2011) also observed the long staying of males in the breeding ponds. This phenomenon is associated with the formation of optimal temperature and feeding conditions for salamanders in ponds in comparison with the land in the northern areas and mountainous regions (Alfimov and





**Figure 6** Seasonal changes in the ratio of different reproductive groups of females (F1–F7).

Berman, 2010; Kuranova *et al.*, 2011).

Temperature, humidity, and rains are the main factors, which determine the breeding migrations of amphibians (Duellman and Trueb, 1986; Kusano and Inoue, 2008). Our observations showed that the periods of high humidity with air temperatures near or above 10 °C were the most optimal for the moving of salamanders in and out of the pond. Rains are the factor activated the breeding migration. Maslova (2006) demonstrated the influence of rainfall on the breeding activity in *S. tridactyla* populations from the forest lowland habitat of the Nature Reserve “Kedrovaya Pad” (south of the Far East, Russia). In 1998, the extremely dry year, the fresh clutches of salamanders appeared only in mid-June after heavy rains (Maslova, 2006). In the population of *S. keyserlingii* from the outskirts of lowland swamps in Japan, Hasumi and Kanda (2007) detected that the quantity of immigrating salamanders was in the positive relation with air temperature in the period since 00:00 a.m. to 04:00 a.m. and precipitations in the day, before the immigrations.

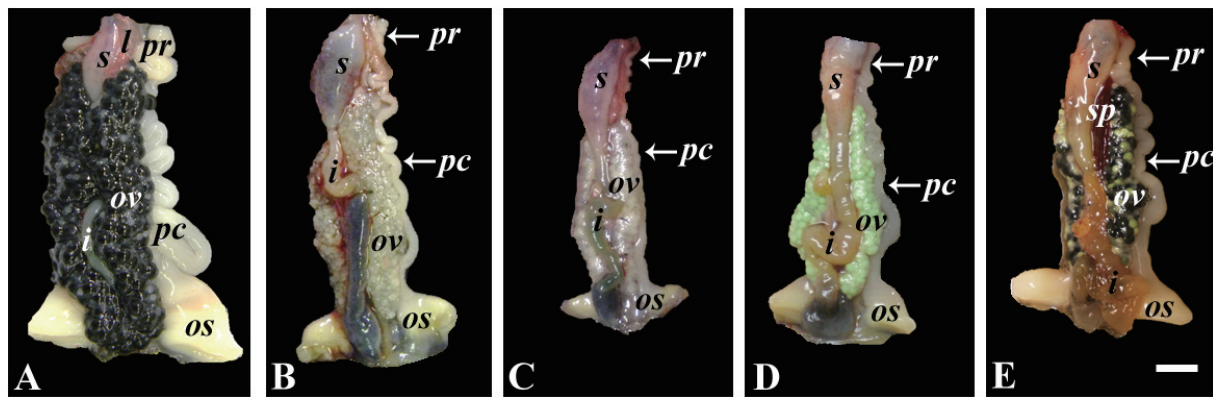
**4.1 Male reproductive cycle** In *H. nigrescens*, “spermiation” (i.e., sperm release from the testes into the vasa deferentia) is just beginning in males shortly after entering the pond (Hasumi *et al.*, 1990). On the other hand, in *H. retardatus*, spermiation is slightly beginning in some males during breeding immigration (Iwasawa *et al.*, 1992). In the Tomsk population of *S. keyserlingii*, spermiation was beginning in some males during breeding immigration and other males after entering the pond (this study). The absence of mature spermatozoa in actively breeding males was indicated previously in the Tomsk population (Saveliev *et al.*, 1993). Probably it was due to the fact, that in this histological study, the individuals emigrating from the breeding pond had been used. Thus,

the spermiation in studied hynobiid species takes place in spring in a rather short period, whereas for salamandrids it begins in fall, although it’s peak is in spring (Guarino *et al.*, 1992); for plethodontids, from fall to spring (Wheeler *et al.*, 2013). However, in any species of the family Hynobiidae, it is still unclear whether or not males during spermiation experience a stimulus from some body of water on land even if spermiation occurs before the males enter the water (Hasumi, 2015).

In *H. nigrescens* under a warmer climate (breeding season is March), spermatogenesis begins in April (after the males emerged onto land), spermiogenesis begins in August, and many bundles of spermatozoa are discernible in the testes in September (i.e., gamete maturation process needs approximately six months: Hasumi *et al.*, 1990). When compared with this gamete maturation process, in males of *S. keyserlingii* studied herein (breeding season was April–May) mature spermatozoa were produced by August. The dynamics of the male reproductive system in the Tomsk population of this species is similar to that of the coastal tundra of the Sea of Okhotsk, despite the strong climatic differences between regions: spermatozoa appeared in the second half of August (Bulakhova and Berman, 2014). We had not determined the beginning of the spermatogenic cycle (as well as the full duration) in the Tomsk population of *S. keyserlingii* by the smear method. However, we can conclude that an early timing of gamete development completion (one month earlier than that of *H. nigrescens*) occurs for annual reproduction under a subarctic steppe–tundra (cooler) climate. In contrast to decelerated gamete maturation of *H. nigrescens*, spermatogenesis seems to be mostly completed by August in *B. tibetanus* (Wang and Zhang, 2004) under a warm climate (Tang and Fang, 2006), and in *H. retardatus* under a cool climate (Iwasawa *et al.*, 1992). The special study is needed for detecting the cause of this difference.

Our data on the dynamics of the male reproductive system of the Tomsk population of *S. keyserlingii* supported an annual reproductive cycle (also see Yartsev and Kuranova, 2010; Yartsev *et al.*, 2013). This annual reproductive cycle is also described for the Okhotsk population of this species (Bulakhova and Berman, 2014) and species of the genera *Hynobius* (Hasumi *et al.*, 1990; Iwasawa *et al.*, 1992) and *Batrachuperus* (Wang and Zhang, 2004, 2007).

**4.2 Female reproductive cycle** In females of *S. keyserlingii* studied herein, qualitative and quantitative changes of ovaries were similar to those in females of *H. nigrescens* (Hasumi, 1996). Among urodeles



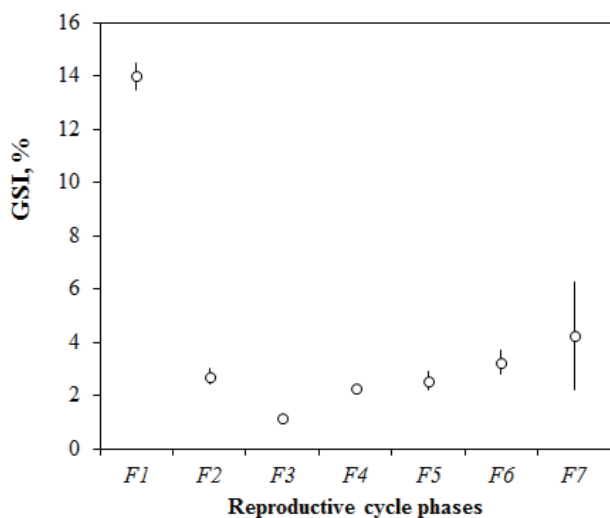
**Figure 7** Seasonal changes in ovaries, oviducts, and ovisacs in females of *S. keyserlingii*: (A) the end of April, female during immigration into the pond (group F1); (B) May, female after spawning (group F2); (C) the end of April (group F3); (D) the beginning of July (group F5), female with ovarian follicles colored mint green; (E) the end of July (group F6). Scale: 5 mm. *ov*: ovary; *pr*: pars recta of the oviduct; *pc*: pars convolute of the oviduct; *os*: ovisac (=homologous uterus); *s*: stomach; *i*: intestine; *sp*: spleen; *l*: lung.

mint green color of ovarian follicles in the process of vitellogenesis is known only in *H. nigrescens* and *H. hidamontanus* (Hasumi, 1996), because amphibians generally deposit cream-yellow eggs with or without melanin (Duellman and Trueb, 1986). Females of *S. keyserlingii* also had mint green coloration in their ovarian follicles during maturation (this study). Hasumi (1996) suggests that the ovarian eggs colored mint green function as camouflage during aquatic development in the algae-laden ponds. Mature ovarian follicles have dark-brown color in *H. nigrescens* (Hasumi, 1996) and black color in *S. keyserlingii* (this study). In contrast to these species, the laid eggs have yellowish color in the genus *Batrachuperus* (Ebrachimi *et al.*, 2004). The cause of this phenomenon is the absence of melanin deposition during oogenesis, as described in *B. pinchonii* (Jia, Zang, 2000). In amphibians, the lack of melanin deposition in the surface of the ovarian eggs occurs generally in lotic-breeding species (e.g., Duellman and Trueb, 1986).

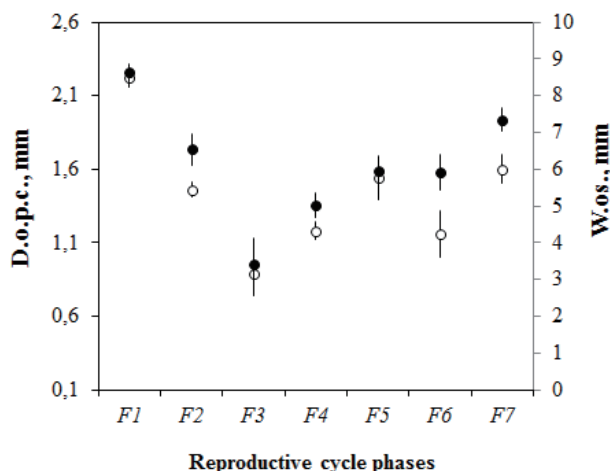
Our results particularly support the hypothesis of a biennial cycle of females (Kuranova and Saveliev, 2006; Saveliev *et al.*, 1993; Yartsev and Kuranova, 2010). In the second half of summer except the individuals with signs of maturation of the reproductive system, animals with light ovaries and thin oviducts (group F3) were recorded in the Tomsk and Nizhneangarsk populations of *S. keyserlingii* (this study). Moreover, in the collection of Zoological Museum of Institute of Plant and Animal Ecology UrB RAS, there is the female of this species, which was caught on 7 September 2008 in the suburbs of Ekaterinburg (No. 364541, Coll. V.L. Vershinin). Its SVL (i.e., distance from the tip of the snout to the anterior angle of the vent) was 60.4 mm, and its reproductive condition was the same as that of the group F3, which

were determined by us: ovary with white small follicles and oviducts in the form of thin tubules. This indicates the inability of this female to participate in breeding the next year after being caught. The size of this female corresponds to that one of mature individuals: according to skeletochronological data of a Ural population, it falls into the class of individuals at 4 years of age (SVL: range = 52.3–61.8 mm) (Ishchenko *et al.*, 1995b). Then, these females (group F3) are a group of individuals participating in breeding in the year of trapping, and restoring the reproductive system after it, as it was shown by histological examination of females (Saveliev *et al.*, 1993; Kuranova and Saveliev, 2006). Groups F1–3 reflect the dynamics of the reproductive system of the females bred in the current season, and F4–7 are the females which were not involved in reproduction this year but maturing reproductive systems for the next year. The observations in the Upper Kolyma with general catch of animals in the reproductive period (with continuous drift fence) show that all females, which are active in this period, do not always take part in reproduction. Near the pond with 32 clutches, 41 females were caught (Ishchenko *et al.*, 1995a).

Comparison of our results with the annual female reproductive cycle of *H. nigrescens* (Hasumi, 1996) at the first view may correspond to the same in females of *S. keyserlingii*. Nevertheless, there are the following differences in the patterns of female gamete maturation between these species. In *H. nigrescens*, vitellogenesis starts in August, melanin deposition begins in September, and the ovarian eggs have a dark-brown color in October (Hasumi, 1996). In contrast to these data, all these ovary changes in the Tomsk population of *S. keyserlingii* occurred about 1.5–2 months earlier.



**Figure 8** Dynamics of gonadosomatic index (GSI, %) during the female reproductive cycle (Tomsk: 2005, 2009, and 2010). Vertical bars represent  $\pm$  SE of the mean. The number of specimens in each group is 25 (F1), 15 (F2), 9 (F3), 10 (F4), 6 (F5), and 2 (F6).



**Figure 9** Dynamics of the diameter of pars convoluta of oviduct (D.o.p.c: open circle) and the width of ovisac (W.os: solid circle) during the female reproductive cycle (Tomsk: 2009 and 2010). Vertical bars represent  $\pm$  SE of the mean. The number of specimens in each group is 25 (F1), 16 (F2), 9 (F3), 10 (F4), 6 (F5), and 2 (F6).

Is this phenomenon associated with acceleration or deceleration pattern of gamete maturation (e.g., biennial reproductive cycle) in *S. keyserlingii*? To address this question we analyzed principal female reproductive characteristics in *S. keyserlingii* and *H. nigrescens*. In the Iwamuro population (Niigata Prefecture, Japan) of *H. nigrescens*, mean size of full clutch is 100.6 eggs, mean SVL of females is 82.44 mm, and mean ovarian egg size is about 2 mm (Hasumi, 1996, 2001a). In contrast, in the Tomsk population of *S. keyserlingii*, mean number of 145 eggs was counted per full clutch (our unpublished

data), mean female SVL was 61.6 mm (this study), and mean size of eggs was about 2 mm (our unpublished data). Also, in *H. nigrescens*, the previtellogenic phase lasts four months from April to July, the vitellogenic phase lasts four months from August to November, and the complete follicle maturation occurs during four months of hibernation from December to March (Hasumi, 1996). If assume annual female reproductive cycle in the Tomsk population of *S. keyserlingii*, females have only two months for previtellogenesis, three months of period activity for vitellogenesis, and longer hibernation period for complete of follicle maturation. This assertion may be correct, if the groups F3 and partially F4 include subadult females, which have reached the sizes of mature individuals. Nevertheless, in conditions of southeast part of Western Siberia (short season of activity and low temperature of subarctic climate) females of *S. keyserlingii*, having smaller body size, should generate larger (1.5 times) clutch size with same egg size than females of *H. nigrescens*, inhabiting in a warmer climate. We suggest that one season of activity is not enough for a full cycle of female gamete maturation in the Tomsk population of *S. keyserlingii*: one season is needed for the restoration of the female genital system and the formation of previtellogenic follicles, and thus vitellogenesis, melanin deposition, and postvitellogenesis occur during the next season. A similar pattern is observed in *O. fischeri*, when the individuals, which did not participate in reproduction in a current season, appeared in the early period of activity (Serbinova and Solkin, 1995). The following assumptions can be pushed forward: (1) two peaks of reproduction (in spring and fall), as in *O. japonicus*; (2) not an annual reproduction of mature individuals; and (3) sexually immature animals that reach adult size (Serbinova and Solkin, 1995).

**4.3 Mode of fertilization** The absence of spermatozoa in the female genital tracts, described in this study, disagrees with previously published data for *S. keyserlingii* (Kuranova and Saveliev, 2006; Saveliev *et al.*, 1993) and *S. tridactyla* (Bulakhova and Berman, 2012). Probably the methodology used by us does not allow identifying spermatozoa in the female genital tracts, although Bulakhova and Berman (2012) observed spermatozoa on smears from the ovisac of *S. tridactyla* females by phase-contrast microscopy.

Regardless of the patterns of male and female reproductive cycles of *S. keyserlingii* (i.e., annual or biennial), mature reproductive products of both sexes (i.e., spermatozoa and ova) were formed only in spring. In spawning, these reproductive products were extruded

simultaneously from the sexes after spermiation and ovulation. This suggests the possibility of breeding only in spring. Moreover, a number of other characteristics of morphology and behavior confirm this. The secondary sexual characteristics, which are of primary importance in urodeles during the breeding season, occur in the Siberian salamander in the aquatic phase of the seasonal cycle (Basarukin and Borkin, 1984; Hasumi, 2001b; Kuranova and Yartsev, 2011; Sytina *et al.*, 1987). Spawning in hynobiid salamanders, including several behavioral actions such as amplexus, midwifing, the formation of a mating ball, and scramble competition (Hasumi, 2015), occurs during spring also in *S. keyserlingii* (Basarukin and Borkin, 1984; Nakabayashi *et al.*, 1986; Sytina *et al.*, 1987). The sperm structure of *S. keyserlingii* corresponds to a general sperm pattern of the suborder Cryptobranchoidea with external fertilization (Baker, 1963; Guo *et al.*, 2010; Kim *et al.*, 1995; Kuramoto, 1995; Makino, 1934; Qin *et al.*, 2005; Smith, 1912; Wang and Zhang, 2007; Zheng *et al.*, 2005). Selmi *et al.* (1997), comparing data of sperm morphology of different groups of urodeles, found that the species of the suborder Cryptobranchoidea differ from urodeles with internal fertilization by two synapomorphic characters: (1) absence of mitochondria in spermatozoa tail, and (2) cylindrical shape of the tail axial filament. Moreover, Hynobiidae and Cryptobranchidae have a similar type of cloacal glands (Hasumi, 1996; Hasumi *et al.*, 1990; Sever, 1991a, b). In hynobiids, rather simple cloacal glands, which differ slightly between males and females, probably play a role in participating in spawning behavior by the secretion of sexual chemical signals (Hasumi, 1996; Hasumi *et al.*, 1990; Sever, 1991b). Cloacal glands of *S. keyserlingii* have not yet been studied to date. If we assume for this species that internal fertilization occurs in the second half of summer on land, male cloacal glands must have a complex structure, like in the other families of urodeles with internal fertilization by means of spermatophores (e.g., Ambystomatidae, Amphiumidae, Dicamptodontidae, Plethodontidae, Proteidae, Salamandridae: Sever, 1991a, b).

## 5. Conclusions

In *S. keyserlingii*, complete gamete maturation in males occurred in late summer, while full maturation of ovarian follicles in females was completed only in the following spring. However, in both sexes, mature gonads needed for spawning were available only in spring. This demonstrates that *S. keyserlingii* has an

associated reproductive pattern, as has been shown in *H. nigrescens* (Hasumi, 1996; Hasumi *et al.*, 1990, 1993, 1997). Both the timing of sperm maturation and the annual reproductive cycle of male *S. keyserlingii* were the same as those in other hynobiid species examined (Hasumi *et al.*, 1990; Iwasawa *et al.*, 1992; Wang and Zhang, 2004). In contrast, female *S. keyserlingii* had early timing of ovarian follicle maturation in comparison with *H. nigrescens* (Hasumi, 1996). We think that this phenomenon is possible only if the female reproductive cycle is decelerated and biennial under a subarctic climate because the expenses for oogenesis in females of urodeles are higher than for spermatogenesis in males (Wells, 2007). To unambiguously answer the question on the pattern of the female reproductive cycle, it is necessary to observe the animals captured throughout the year or more, as has been conducted in *H. nigrescens* (Hasumi, 1996). Although terrestrial internal fertilization is hypothesized (Kuranova and Saveliev, 2006; Saveliev *et al.*, 1991, 1993), all the reproductive traits (e.g., lack of sperm in the vasa deferentia even after the completion of spermiogenesis, sperm morphology, simultaneous existence of sperm in male genital tracts and ova in female genital tracts, spawning behavior) determined in the present study suggest that *S. keyserlingii* adopts the external mode of fertilization only, as in other hynobiids examined (Hasumi, 1994; Makino, 1931; Thorn, 1968).

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