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URINAL SPERM MOTILITY AND PARASITE LOAD IN ANURAN AMPHIBIANS (ANURA)

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Parasite load effect on the spermatozoa motility of the moor frog (*Rana arvalis* Nilsson, 1842) native urinal sperm has been evaluated for the first time. Seven species of parasites were identified: Nematoda — *Oswaldocruzia filiformis* (Goeze, 1782), *Neoraillietnema praeputiale* (Skrjabin, 1916), *Cosmocerca ornata* (Dujardin, 1845), *Rhabdias bufonis* (Schrank, 1788); Trematoda — *Dolichosaccus rastellus* (Olsson, 1876), *Haplometra cylindracea* (Zeder, 1800); Opalinatea — *Opalina ranarum* (Dujardin, 1841). We found no impact of population specificity on sperm motility. The strong negative correlation between motile spermatozoa proportion and *O. ranarum* abundance was observed. For the purpose of selecting the optimal model of dependence pattern (linear-, logarithmic-, sigmoid- or threshold function), the Consistent Akaike Information Criterion (CAIC) was used. When the *O. ranarum* abundance was above the estimated threshold — 7.3 (95% CI 0.9 – 13.6) trophonts per probe, the expected probability to observe motile spermatozoa in the urinal sperm of moor frog decreased by 3.6 (95% CI for Risk Ratio: 1.9 – 6.8) times.

Keywords: *Rana arvalis*; parasites; protozoa; *Opalina ranarum*; urinal sperm; sperm motility; parasite load.

INTRODUCTION

Parasites have influence on various aspects of animal vital functions (Johnson et al., 2002). One of the poorly studied issues in contemporary parasitology is pathomorphological changes in reproductive organs and/or their products quality under the conditions of parasites infestation. Some protozoan parasites localized in fish and amphibian gonads caused decreased host fertility (Sitjà-Bobadilla, 2009; Kaur et al., 2015; Hartigan et al., 2013). Some authors (Zharkova et al., 2012) associate dystrophy of fish gonads with infestation by the crustacean *Ergasilus sieboldi* (Nordmann, 1832). Many species of Trematoda are assumed to have negative effects on reproductive potential of their hosts (Ginetsinskaya, 1968; Gorbushin, 2000; Ignatkin, 2007; Gangloff et al., 2008; Lajtner et al., 2008; Ivanov et al., 2009; Ganzha and Starunova, 2011; Nurtazin et al., 2012). Ovarian cysts formation and seminiferous epithelium dystrophy in laboratory rodents can be induced via intraperitoneal injection of metabolic products and somatic proteins of parasites (Kharitonova et al., 1982; Sivkova, 2010). Ascaridiasis causes changes of physiological and morphological characteristics of spermatozoa, semen hyperviscosity, and lower semen concentration in men of reproductive age (Yessilbaeva et al., 2013). Thus, parasites infestation can cause both direct (pathomorphological changes in host gonads) and indirect (intoxication by helminth metabolites) effects on animal reproduction. For example, trematodes *Codonocephalus urnigerus* (Rudolphi, 1819), concentrating mostly in amphibian gonads, in some cases lead to parasite castration (Ryzhikov et al., 1980) and suppression of behavioural reflexes, thereby decreasing host survivance (Ivanov et al., 2009; Ivanov et al., 2012; Nurtazin et al., 2012). However, sperm motility is one of the main determinants of animal fertility. This problem may be considered as a very poorly studied one for amphibians in contrast to mammals. The first model describing environmental activation of anuran sperm was introduced only in 2011. Obviously, the study of reproductive products

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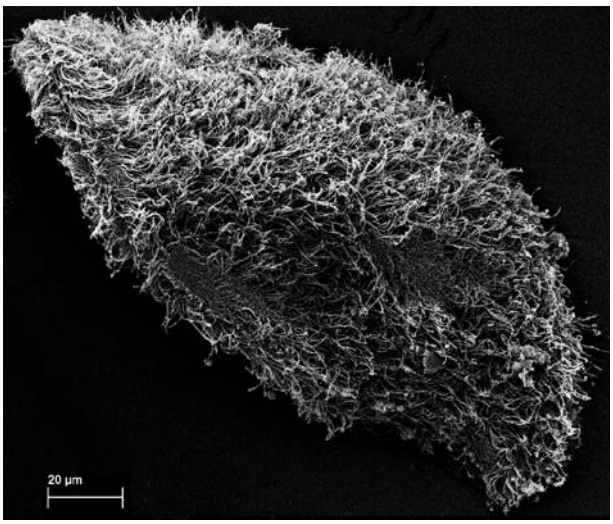

Fig. 1. Scanning electron microscope image of *O. ranarum* (trophont) from *R. arvalis* Intestines (original photo).

quality in reference to parasites load in natural populations of different amphibian species is of great theoretical and practical interest.

The main purpose of our research was to assess the effect of parasite load on the sperm motility in the moor frog (*Rana arvalis* Nilsson, 1842).

MATERIAL AND METHODS

Study areas. The moor frogs were collected in three populations: 10 males in Shartash Park (Yekaterinburg, 56°51′40.9″ N 60°41′14.1″ E) — P_1, 14 — Verkhniye Sergi urban locality (Nizhneserginsky rayon, Sverdlovskaya oblast' 56°39′6.85″ N 59°33′0.72″ E) — P_2, and 9 from Stepnoye village (Kurgan oblast' 55°19′33.13″ N 67°46′74.02″ E) — P_3, in the spawning period (May) in 2014 and 2015.

Parasite load. Protozoa. The number of multinucleate trophonts (0.1 to 1 mm) of *O. ranarum* was counted in 10 randomly selected fields of view (Chernysheva et al., 2009) (Fig. 1). The protozoa counting considered only multinucleate trophonts, and did not account for cystic forms. Unlike gamonts and gamontocysts, the trophonts were always found in the infested adult animals (Hausmann et al., 2003). Expected number of gamonts (hundreds) resulting from trophonts divisions was a lot higher than the quantity of trophonts (Sharova, 2002).

Helminthes. A total of thirty three *R. arvalis* males were subjected to the full helminthological autopsy (Ivashkin et al., 1971). Species of helminthes were identified with the guides by K. Ryzhikov et al. (1980) and V. Sudarikov et al. (2002).

In order to qualify parasite load we estimated: prevalence — the percentage of infected hosts, median intensity — 50 percentile of sample distribution of parasites counts in infected hosts only; mean abundance — arithmetic mean of parasites number including both infected and uninfected hosts (Breev, 1976). Fisher's exact test, Mood's median test, and Bootstrap *t*-test (0.05 level of significance), implemented in Quantitative Parasitology software (Rózsa et al., 2000) were used.

Sperm motility. In order to obtain a sample of urinal sperm under laboratory conditions, a synthetic nanopeptid, an analog of gonadotropin releasing hormone LH-RH-luliberin — "Surfagon" was injected into the males' peritoneum (1.2 mg/g body weight). Next, 1 to 5 h after hormonal stimulation via gentle abdominal massage, a portion of urinal sperm was obtained. Sperm samples were taken successfully from 28 males. The sperm motility was evaluated by MMC-SK (Makler type) sperm counting chamber, which allows analyzing native undiluted semen. We counted the number of spermatozoa in random 10 squares of 0.01 × 0.01 mm grid that corresponds to 10^6 per 1 ml (= 10^3/µl) of semen. Spermatozoa with any kind of motion (progressive, *in situ*) were classified as motile.

Statistical modelling was performed via multimodel interference framework, a modern alternative to null hypothesis testing (Hilborn and Mangel, 1997; Burnham and Anderson, 2002). At the screening step, nonparametric Gamma correlation between the sperm motility and all variables of parasite load was evaluated. At the next step, non-parametric estimation of general pattern in dependency of motile sperm proportion on abundance of *O. ranarum* was done through locally weighted smoothers (LOWESS and splines), which adequacy was characterized by Pearson correlation coefficient between observed and predicted values. Then, a threshold of S-shaped curve was estimated using nonlinear estimation procedure (Levenberg – Marquardt algorithm). Pathological negative values in the predicted proportions (namely, in a lower bound of its confidence interval) when applying naive nonlinear least square algorithm for dependent variable measured in limited scale (0 – 1) were overcome via logit regression — a special kind of Generalized Linear Models (GLM) for binary response (McCullagh and Nelder, 1989). The following predictors were considered: habitat (3), number of *O. ranarum* (or its logarithm), and two dichotomies (1, 0) — indicating whether protozoa were found or not, and whether abundance of *O. ranarum* is above the threshold or not. Model selection was executed using the Consistent Akaike Information Criterion (Bozdogan, 1987):

$$\mathrm{CAIC} = -2 \log L + K[\log n + 1].$$

Relative likelihood (weight) of each model:

$$w_i = \frac{\exp(-0.5\Delta_{\mathrm{CAIC}_i})}{\sum_i \exp(-0.5\Delta_{\mathrm{CAIC}_i})}$$

may be used as analog of Bayesian posterior probability in order to rank and compare (via evidence ratio, ER = = w_i/w) any competitive models. Statistical inference (here) was based on the optimal model since it had the maximal weight among others ($\Delta_{\mathrm{CAIC}} > 8$). This approach allows comparison of the model of interest with both the model corresponding to null hypothesis and a saturated (but biologically not interpretable) model, where the set of predictors are identifiers of every individual. To test an adequacy of logit-model and binary data, a dispersion parameter was estimated by the ratio of Pearson chi-squared statistic (or deviance) to the residual degrees of freedom $\varphi = \chi^2/rdf$ and $\varphi = \mathrm{Dev}/rdf$.

RESULTS

Parasite load. It was found that *R. arvalis* males were infested by 7 species of parasites of 3 taxa: Trematoda — 2, Nematoda — 4, Opalinatea — 1 (Table 1).

Protozoa. It was found that *R. arvalis* males were infested by only one species of Opalinatea taxon, *O. ranarum*. Individuals of *O. ranarum* were identified as trophonts and gamonts. It is worth mentioning that when no trophonts were identified in intestinal contents, gamonts were not found either. We did not find any interpopulation variance in male infection with protozoa.

Helminthes. Individuals of Trematoda and Nematoda were identified as marita (mature stage). The first rank in the parasite fauna of *R. arvalis* males (Table 1) took Nematoda in each of the three populations under study. Prevalence of nematode infestation was significantly higher in the moor frog populations 2 and 3 in contrast to 1 (by 2.5 and 2.2 times correspondingly). Maximum values of Nematoda prevalence were found in population 2, while maximum abundance and infection intensity indices — in population 3 (Table 1). Infestation analysis showed high 100% prevalence of multicellular helminthes (nematodes + trematodes) in populations 2 and 3, in comparison to only 40% prevalence in population 1. Maximum index values of multicellular parasites abundance and infection intensity were observed in population 3 (Table 1).

Sperm motility. As expected, prior to release into hypotonic aquatic environment, amphibian sperm motility depended on physiological condition of animals. The spermatozoa demonstrated two types of motion: progressive and *in situ*, the last one was the most frequent. Observed sperm count variability (1 — minimum, 63 — maximum × 10^3/μl) can be fitted by Gamma distribution (Fig. 2) with two parameters: $\alpha = 12.38$; $\beta = 1.81$. The semen samples were heterogeneous both in terms of concentration and sperm motility (Figs. 3, 4). The proportion

TABLE 1. Species of Parasites and Infestation Statistics in *R. arvalis* Males

Parasites (localization)	Prevalence, % (CI)			Median intensity (CI) [min – max]			Mean abundance (CI)		
	P_1	P_2	P_3	P_1	P_2	P_3	P_1	P_2	P_3
Nematoda	40[a] (12 – 73)	100[b] (76 – 100)	88.9[b] (51 – 99)	5[a] [1 – 7]	5 (1 – 8)[b] [1 – 15]	27.5 (6 – 35)[a] [6 – 58]	1.8[a] (0.4 – 3.7)	5.1[b] (3.2 – 7.4)	23.1[c] (13.2 – 35.8)
Oswaldocruzia filiformis (Goeze, 1782)	40[a] (12 – 73)	64.3[a] (35 – 87)	88.9[b] (51 – 99)	4.5[a] [1 – 4]	5 (1 – 10)[a] [1 – 12]	19 (2 – 28)[b] [2 – 40]	1.6[a] (0.5 – 3.2)	3.43[a] (1.7 – 5.7)	16.8[b] (8.6 – 25.4)
Neoraillietnema praeputiale (Skrjabin, 1916)	20 (2 – 55)	—	—	1 [1]	—	—	0.2 (0 – 0.4)	—	—
Cosmocerca ornata (Dujardin, 1845)	—	57.1 (28 – 82)	—	—	1.5 (1 – 3) [1 – 3]	—	—	1 (0.4 – 1.6)	—
Rhabdias bufonis (Schrank, 1788)	—	28.6 (8 – 58)	55.6 (21 – 86)	—	2.5[a] [1 – 3]	10 (7 – 18)[b] [7 – 18]	—	0.6[a] (0.1 – 1.3)	6.3[b] (2.3 – 10.7)
Trematoda	30 (6 – 65)	14.3 (1 – 42)	33.3 (7 – 70)	2 [1 – 3]	5 [2 – 8]	6 [1 – 6]	0.6 (0 – 1.3)	0.7 (0 – 2.4	1.4 (0 – 3.3)
Haplometra cylindracea (Zeder, 1800)	30 (6 – 65)	14.3 (1 – 42)	—	2 [1 – 3]	5 [2 – 8]	—	0.6 (0.1 – 1.4)	0.7 (0 – 2.4)	—
Dolichosaccus rastellus (Olsson, 1876)	—	—	33.3 (7 – 70)	—	—	6 [1 – 6]	—	—	1.4 (0.1 – 3.3)
Helminths infestation	40[a] (12 – 73)	100[b] (76 – 100)	100[b] (66 – 100)	7 [1 – 9]	5 (1 – 10) [1 – 15]	27 (9 – 35) [6 – 58]	2.4[a] (0.3 – 4.9)	5.8[a] (3.6 – 8.4)	24.6[b] (15.3 – 37.2)
Opalinatea	50	28.6	33.3	5	11	10	3.1	4	3
Opalina ranarum (Dujardin, 1841)	(18 – 81)	(8 – 58)	(7 – 70)	[3 – 10]	[5 – 29]	[2 – 15]	(1.2 – 5.6)	(1 – 9.9)	(0.2 – 7.4)

Notes. CI, 95% confidence interval; [a], [b], [c], different superscripts marked a significant contrasts ($p < 0.05$).

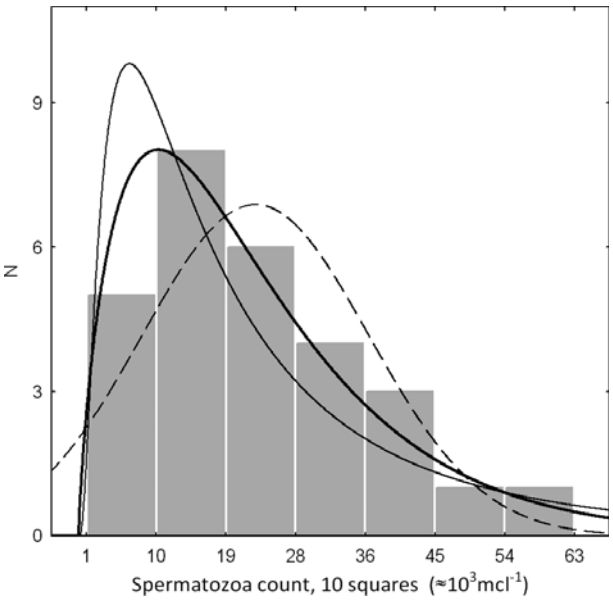

Fig. 2. Spermatozoa count in urinal sperm samples of 28 males *R. arvalis*. Distribution fitting: thick line, gamma; thin line, lognormal; dashed line, normal distribution. Gamma distribution (12.38; 1.81) has the best fit: $\chi^2(1_{adj}) = 0.83$, $p = 0.36$; K-S $d = 0.13$, p = n.s.

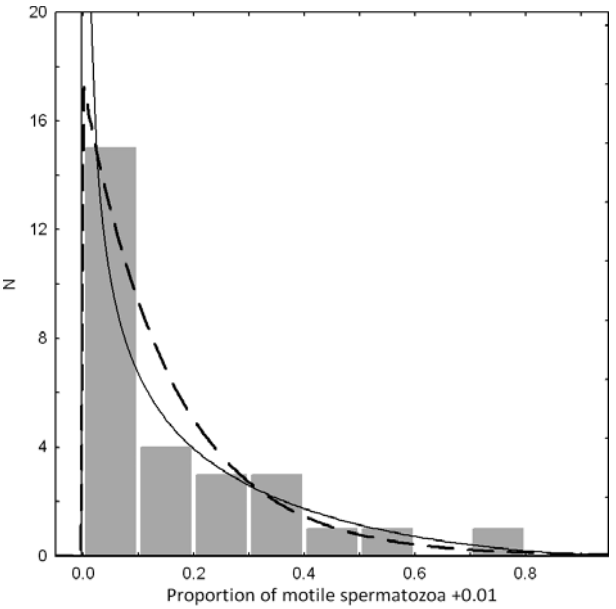

Fig. 3. Sperm motility (proportion of motile spermatozoa +0.01). Distribution fitting: thick line, beta (0.53, 2.70); dashed line, exponential (6.17); $\chi^2(1_{adj}) = 1.69$, $p = 0.19$.

of the motile spermatozoa correlated negatively with *O. ranarum* abundance ($\gamma = -0.79$, $Z = -2.83$, $p \leq 0.005$). Dependency of the motile spermatozoa ratio on the strengh of *O. ranarum* infestation (Fig. 5) can be described via nonparametric smoothing (LOWESS: $R = 0.42$, $p \leq 0.03$) or — parameterized as the S-shaped curve ($R = 0.37$, Table 2).

Model selection among simple logit regressions showed that the optimal model is the one describing the dependence of the motile spermatozoa proportion on *O. ranarum* abundance as a step function with a threshold at about 7 trophonts (in 10 fields of view) of *O. ranarum*. Other functions (Table 3) such as monotonic (linear — No. 2, logarithmic — No. 3) or dichotomy (whether *O. ranarum* was found or not — No. 5) gained lower sta-

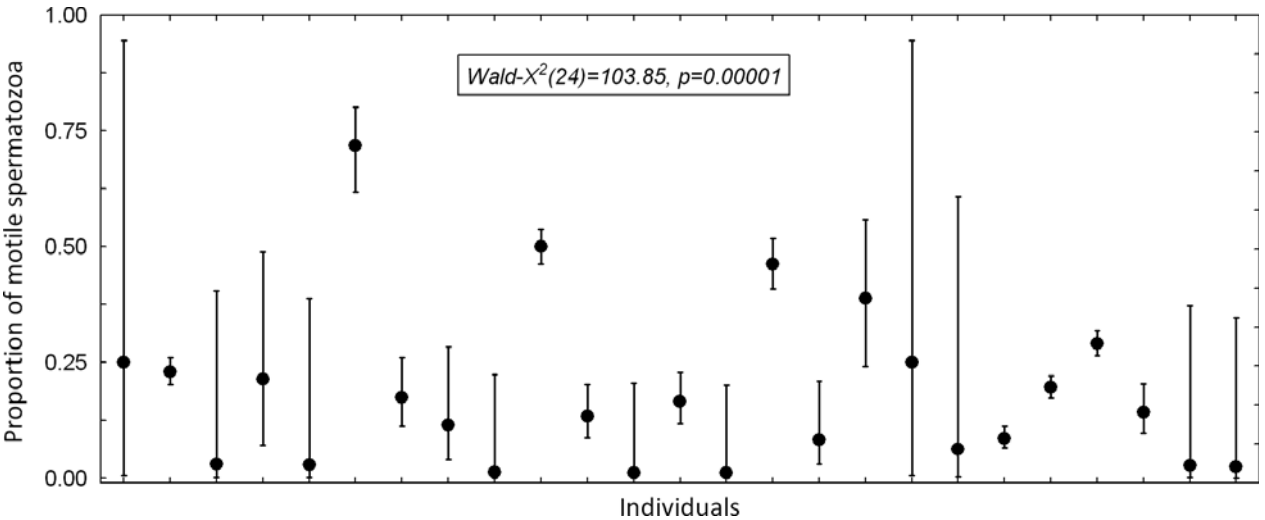

Fig. 4. Proportion (and CI, confidence interval) of motile spermatozoa in the urinal sperm of *R. arvalis* ($N_{males} = 25$). A wide CIs is a result of low total spermatozoa counts. Males (5), from which had not obtained any sperm samples or with 100% observed immotile sperm (3) were censured.

tistical support. For instance, threshold dependence had 58 times higher evidence ($ER_{1:2} = w_1/w_2$) than the linear function. When the quantity of trophonts was above the specified threshold, expected odds to find motile spermatozoa in *R. arvalis* urinal sperm declined by 5.9 (95% CI 2.7 – 12.9) times (Table 4). Statistical modeling revealed that data on sperm motility can be collapsed just into a two-way cross-tabulation table (2 × 2). The first input dichotomizes spermatozoa into motile or immotile and the second — splits the host across below/above specified threshold of *O. ranarum* abundance: 122/421/9/133. This collapsing allows us to estimate the Risk Ratio (RR). The latter is easier for interpretation than the Odds Ratio (OR). Thus, above the specified threshold, the probability to find motile sperm decreased by the average of 3.55 (95% CI RR: 1.85 – 6.80) times ($z = 3.81$, $p = 0.0001$). It is worth noting that no significant differences of average spermatozoa motility were found, neither associated with helminthes infestation nor habitat features $\chi^2(2) = 2.65$, $p = 0.27$ (Table 3).

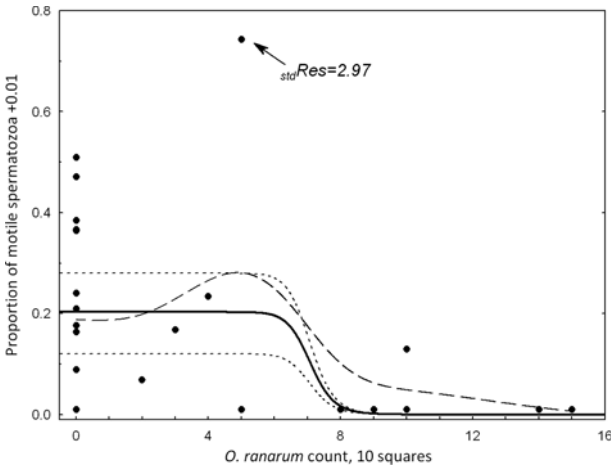

Fig. 5. Proportion of motile spermatozoa by *R. arvalis* males in relation to *O. ranarum* abundance. Long dashed line, LOWESS smoothing ($R = 0.42$, $p \leq 0.03$); thick line, logistic, $R = 0.37$ (short dashed line, 95% CI for upper asymptote, see Table 2); arrow, the only observation with an extreme standardized residual (not censured).

DISCUSSION

All the helminthes detected in *R. arvalis* males are found in two or more species of amphibians. Apparently, no helminthes found in this study had any significant impact on urinal sperm motility. The moor frogs got infected with new generation of helminthes after spawning, when they started active feeding (Kuzmin, 2012). Depending on helminth species, the infestation could take place via soil (*O. filiformis*, *R. bufonis*) (Ryzhikov et al., 1980; Kirin and Buchvarov, 2002), or water (*D. rastellus*, *H. cylindracea*, and *C. ornata*) (Ryzhikov et al., 1980; Kirillov and Kirillova, 2016).

The most frequently observed parasite in *R. arvalis* was *O. filiformis* (Kuranova, 1988) and the peak of its infestation was reached in June (Markov and Rogoza, 1953). Similar seasonal dynamics was described for *R. bufonis* (Aralhanova, 2010; Tarasovskaya, 2013). Some authors (Kuranova, 1988) showed that infestation by *H. cylindracea* (localized in lungs) and *D. rastellus* (localized in the intestine), increases during summer and reaches its maximum in August.

In the light of obtained results we can hypothesize that the sperm motility may be lowered in measurable quantity by high density of protozoa *O. ranarum* — obligate endosymbionts, usually described as commensals rather than parasites. *O. ranarum* life cycle is well synchronized with the reproductive cycle of its amphibian host (Hausmann et al., 2003).

Researching *R. arvalis* males during the breeding season, we observed *O. ranarum* at the stage of adult trophonts and elongated (intermediate) forms of trophonts that can be found at any time. Thus, the peak of helminth infestation in *R. arvalis* is reached during active feeding on land after reproduction; whereas increased abundance of protozoan parasites (*O. ranarum*) is observed during spawning period.

Spermatozoa in amphibian gonads are immotile until their release into a hypotonic environment, when the osmotic shock activates the sperm (Kouba et al., 2003; Roth and Obringer, 2003). Amphibian urinal spermatozoa are considered partially activated by urine contact. Therefore, the motility we observed in the amphibian native sperm is generally considered to be caused by osmotic pressure of the urine. The observation that non-progressively motile sperm found in the majority of cases is in good agreement with the considered sperm activation mechanisms (Brien et al., 2011).

Negative correlation between the proportion of motile spermatozoa and *O. ranarum* abundance may be explained by a number of reasons (Fig. 6). There are known cases of decreased sperm motility in cattle and humans caused by influence of protozoan parasites at certain stages directly to spermatozoa. For example, us-

TABLE 2. Parameter Estimates (via nonlinear least square) Regression for Proportion of Motile Spermatozoa of *R. arvalis* on Abundance of *O. ranarum*: $y = a/(1+(x/k)^{10})$

	b	*se*	*t*(23)	$p \leq$	95% CI	
a	0.19	0.04	4.77	0.001	0.11	0.27
k	7.28	3.09	2.36	0.03	0.93	13.63

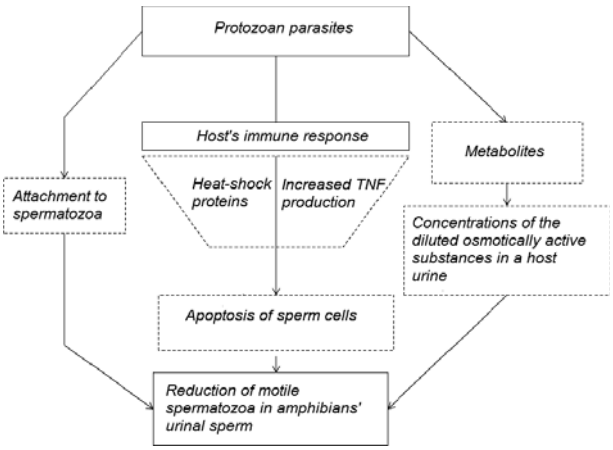

Fig. 6. Possible mechanisms of the reduction of sperm motility in urinal sperm in amphibians caused by parasites protozoa: TNF, tumor necrosis factor.

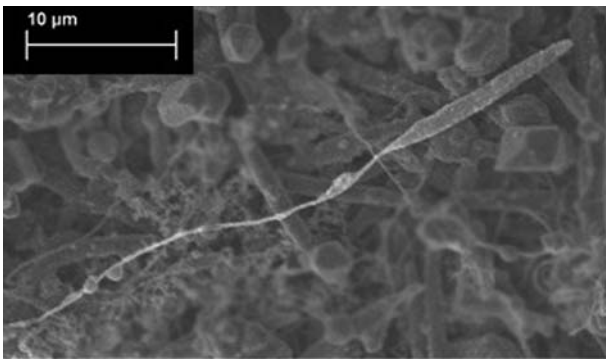

Fig. 7. Scanning electron microscope image of *R. arvalis* spermatozoon (original photo).

ing the scanning electron microscope, Benchimol et al. (2008) identified Trichomonads attached (*Trichomonas foetus* Riedmüller, 1928, *Trichomonas vaginalis* Donne, 1836) to a spermatozoon head or tail. Cases describing phagocytosis of spermatozoa also exist. The size of the spermatozoa head was about 15 – 20 µm (Fig. 7). *O. ranarum* trophonts varied from 100 to 700 µm, cysts and small cells resulting from palintomical division — 30 – 90 µm (Hausmann et al., 2003). In reference with the above, we could not exclude completely the potential attachment of cysts and small cells to the semen. Carefully screening sperm samples found no spermatozoa with protozoan attached.

The next point to consider is that every parasite during its lifecycle produces metabolites, modifies the chemical composition of host biological fluids and influences the concentration of osmotically active substances in animal urine (Fig. 6). *O. ranarum* is osmotrophic and absorbs nutrients all over its surface. Nutrients uptake and metabolites excreting by the protozoan are continuous processes. From this point of view, changes in urine chemical composition induced by the parasite invasion may be viewed as a probable agent that modified the osmotic shock for spermatozoa and, therefore, affected their motility.

Protozoan parasites metabolites may affect indirectly via apoptosis increased in the maturating spermatozoa

TABLE 3. Model Selection Results Among Simple Logit Regressions for Motile Spermatozoa Proportion in Urinary Sperm of *R. arvalis*

No.	Predictors, X_i	k	–2LL	CAIC	Δ_{CAIC}	w
1	Number of *O. ranarum* above threshold	2	588.91	603.88	0	0.983
2	Number of *O. ranarum*	2	597.04	612.02	8.14	0.017
3	log(number *O. ranarum*)	2	604.81	619.79	15.91	3×10^{-4}
4	$^{\#}H_0$ (independence)	1	615.57	623.06	19.18	7×10^{-5}
5	*O. ranarum* (0; 1)	2	609.14	624.12	20.24	4×10^{-5}
6	Population	3	612.96	635.42	31.54	1×10^{-7}
7	Individual^{Sat}	25	496.34^{min}	683.53	79.65	5×10^{-18}

Notes. $n = 685$; $^{\#}$, null hypothesis; ^{Sat}, saturated model, the number of parameters equals the number of individuals (25). The effect of «population» LR(2) = 615.57 – 612.96 = 2.62, (ns). Formally, models Nos. 5 and 7 are "statistically significant" by likelihood ratio test LR = $-2LL(m_{H0}) - 2LL(m_i)$ but using the CAIC they got extremely low support by Evidence Ratio (Lindley's paradox).

TABLE 4. Parameter Estimates of the Best Logit Regression Model (No. 1 in Table 3, $\Delta_{CAIC} > 8$)

	b	Q_{asc}	Q-Wald	$p <$	OR = e^b	95% CI	
b_0	–1.31	0.10	172.96	0.001	0.27	0.22	0.33
Number *O. ranarum* >7	–1.77	0.40	19.54	0.001	5.87^{-1}	12.85^{-1}	2.68^{-1}
Scale, $\varphi^{0.5}$	0.93						

(Fig. 6). It was shown that the proportion of spermatozoa with the signs of apoptosis and the fertility decline had correlation between them (Ploskonos, 2013). Apoptosis was induced by the increased tumor necrosis factor (TNF) production in the host organism. This was a protective immune response aimed to induce programmed cell death in parasite's tissues. Highly resistant cell proteins, called heat-shock proteins or HSPs (Lindquist, 1986), being involved in apoptotic processes, may also cause apoptosis activation (Bekish et al., 2005). Synthesis of many HSPs can be found in all animal taxa including amphibians. It is commonly known that protozoan parasites are able to activate the synthesis of heat-shock proteins in a host (Ishikawa et al., 1997; Davis-Hayman et al., 2000). As a result, it may increase the number of apoptosis-prone spermatozoa with impaired motility (Bekish et al., 2005).

CONCLUSIONS

Seven species of parasites have been found in *R. arvalis* males. 6 of them were multicellular (*O. filiformis*, *N. praeputiale*, *C. ornata*, *R. bufonis*, *D. rastellus*, *H. cylindracea*) and the only one was protozoa (*O. ranarum*). The nonlinear dependency of the motile spermatozoa proportion on the *O. ranarum* invasion strength was shown. When the abundance of *O. ranarum* exceeded a certain threshold (about 7 trophonts per 10 fields of view), the probability of detecting motile spermatozoa in the native urinal sperm decreased by 3.6 times.

It is of importance to point out when amphibian spermatozoa were released into the hypotonic aquatic environment, motility characteristics could change after osmotic shock. Therefore, the reduction in sperm motility found in *R. arvalis* males as a result of the protozoan invasion could not be clearly interpreted as an ultimate sign of impaired fertility and requires additional research.

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