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Induced spermiation and sperm morphology in a dendrobatid frog, *Dendrobates auratus* (Amphibia, Anura, Dendrobatidae)

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Abstract. In recent decades an alarming worldwide decline of amphibian populations has been observed. Because of the worldwide amphibian decline, studies concerning the fundamentals for effective captive breeding programs are important. Frog sperm recovery, in general, is performed post-mortem after testis dissection. In this study typical dendrobatid sperm as well as irregularly shaped spermatozoa are described after gentle hormonal stimulation with human chorionic gonadotropin (hCG), cloaca lavage and light microscopic as well as fluorescent microscopic evaluation. Starting from this technique two different hCG stimulation protocols are tested to increase the obtained overall sperm number. Furthermore, two sperm media, isotonic phosphate-free amphibian saline (IPS) and *Xenopus* fertilization medium (XB), are compared regarding sperm motility. The typical spermatozoon of *Dendrobates auratus* consists of a filiform and curved head and a single flagellum without any accessory structures. Gained overall sperm number after double injection of 100 IU hCG at intervals of one hour is nearly ninefold higher in comparison to single injection of 100 IU hCG. In XB medium 10.5 % of the viable spermatozoa are motile, whereas 1.2 % of viable sperm cells in IPS are motile. These gentle techniques will be useful for breeding and conservation programs including sexing, spermatological examination, cryopreservation of spermatozoa, artificial fertilization and the build of gamete banks for endangered and/or fragile anurans.

Key words. hormonal stimulation, hCG, sperm recovery, spermatozoa, structure, motility, conservation.

Introduction

With 29 % of the species threatened with extinction, amphibian populations are declining dramatically worldwide (IUCN 2007). Therefore, the transfer of reproductive technologies useful for the preservation of endangered amphibians, primarily those originating from mammalian species, has to be promoted. The release of spermatozoa into the seminiferous tubules of mature anurans (spermiation) combined with the transport of sperm cells into the cloaca and the outside environment has been investigated primarily for studying the physiology of involved hormones. One application for this procedure was the diagnosis of human pregnancy using male toads (Galli-Mainini 1947). In frogs, a variety of different exogeneous substances as pituitary extracts (Easley et al. 1979, Minucci et al. 1989), gonadotropin-releasing hormone (GnRH) and luteinizing hormone releasing hormone (LHRH) (Easley et al. 1979, Minucci et al. 1989, Rowson et al. 2001, Iimori et al. 2005), luteinizing hormone (LH) (Burgos & Ladman 1955, Easley et al. 1979), follicle stimulating hormone (FSH) (Burgos & Ladman 1955, Easley et al. 1979, Iimori et al. 2005) and human chorionic gonadotropin (hCG) (Burgos & Ladman 1955, Chatterjee et al. 1971, Easley et al. 1979, Minucci et al. 1989, Iimori et al. 2005) have been tested in vivo over the decades to examine their potential to induce spermiation.

Studies on induced spermiation in dendrobatid frogs have yet to be conducted. In examinations investigating the sperm structure in the Dendrobatidae, this gentle instrument was ignored and euthanasia was preferred (Garda et al 2002, Aguiar-Jr et al. 2004). Also few reports are available concerning induced spermiation in frogs with the aim of amphibian conservation. In the endangered Wyoming Toad (*Bufo baxteri*) an average of

$1.9 \pm 0.9 \times 10^6$ sperm cells per ml were recovered after intraperitoneal LHRH treatment (Obringer et al. 2000).

Due to annual variability within amphibian populations and missing long-term field studies, at the beginning the worldwide amphibian crisis was not easily detected (Blaustein et al. 1994). In 1997, the Declining Amphibian Population Task Force (DAPTF; Species Survival Commission of the IUCN) attested a significant decline in amphibian populations worldwide (Heyer 1997). The reasons for such losses are manifold and can be divided into two classes of hypotheses. Class I hypotheses (alien species, overexploitation, land use change) are well understood and have interfered with amphibians for more than a century. New and understudied threats such as global change, contaminants and emerging infectious diseases have been merged into the group of class II hypotheses (Collins & Storfer 2003). Eterovick (2005) reported exemplarily a dramatic change in the anuran assemblages at four sites in Brazil between 1971-76 and 1996-2000. Also the dendrobatid frog, *Ameerega flavopicta*, common between 1971-76, was completely missing during hers 1996-2000 study. Populations of other anurans (including the Dendrobatidae) will inevitably decline, therefore, working on assisted reproductive methods in small frogs may prove necessary to aid in the conservation of biodiversity in the tropics. The general aim of this study was to transfer the technique of induced sperm recovery in common frog species to the small and fragile dendrobatid species such as *Dendrobates auratus*.

Materials and methods

Animals

The online databases AmphibiaWeb (2008) and "Amphibian Species of the World" (Frost 2007) were inquired for the taxonomy used within the article. Male specimens of the Green Poison Frog (*Dendrobates auratus*; n = 12) purchased from a local breeder were used for the study described herein. The frogs were fertile, had an average weight of 2.7 g, and were housed in terraria together with female individuals prior to the experiments. Terraria were ventilated in the bottom and upper sections consisting of fine grids and were equipped with sufficient lighting. An easy to clean Plexiglas® plate formed the floor of the terraria. Frogs had access to refuges in plants and under half coconuts, as well as permanent access to water. Flightless *Drosophila melanogaster* fruit flies dusted with vitamin powder were provided every second day.

General experimental design for induced spermiation

Frogs were carefully immobilized in a wet gauze bandage and received one or two injections of human chorionic gonadotropin (100 IU hCG, Ovogest® 1500, Intervet, Unterschleissheim, Germany) each into the dorsal lymph sac at intervals of one hour. For injection purposes single-use fine dosage syringes with integrated needle (Omnican® F, 0.30 × 12 mm, Braun, Melsungen, Germany) were used. Sperm recovery was performed via cloacal lavages at different time intervals, depending on the selected stimulation protocol. Using single hCG injection cloacal lavages were carried out 15, 30, 45, 60, 90 and 120 min after the stimulation. In double stimulation experiments sperm cells were recovered 5, 15, 30, 45, 75, 90, 120 and 240 min after the first hCG injection. With a fine bulbous tip cannula (0.7 × 30 mm, Heiland, Hamburg, Germany) spermatozoa were washed out of the cloaca. Two media, isotonic phosphate-free amphibian saline (IPS, 111.3 mM NaCl, 3.3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $NaHCO_3$, 3.0 mM D-Glucose, pH 7.4 at 23°C, 220 mosmol/kg; Costanzo et al. 1998) and *Xenopus* fertilization medium (XB, 41.3 mM NaCl, 1.2 mM KCl, 0.3 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1.9 mM NaOH, 0.5 mM Na_2HPO_4, 2.5 mM HEPES, pH 7.8 at 20°C, 84 mosmol/kg; Hollinger & Corton 1980) were compared regarding sperm motility. Unless otherwise

stated, chemicals were obtained from Sigma-Aldrich, Taufkirchen, Germany. Between these treatments the frog was placed in a separate box for resting. To detect intracloacal pre-treatment spermatozoa, a control sample was attained immediately before the first hormone administration. All flushings were centrifuged for 5 min at 173 × g and 20 µl of the sediments were placed on non-coated microscopic glass slides and covered with a cover slip for light microscopic evaluation (BX60, Olympus, Hamburg, Germany; 800×). All animals rested between the experiments for seven to ten days. Statistics were performed using the program SAS® for Microsoft Windows (Version 9.1).

Experiment 1: Sperm morphology

In this first study the morphology of the spermatozoa describing the majority of the recovered sperm cells (typically shaped spermatozoa) was investigated using phase-contrast microscopy (800×). Twelve sperm samples from three differently? stimulated (100 IU hCG) animals were processed with a microscopic video system linked to the measurement unit (Kappa Messfadenkreuz MFK II, Gleichen, Germany). The measurements were repeated after the production and digitalization of video prints from a color video printer (Sony Mavigraph UP-1850 EPM). All digital images were processed using the program ImageJ (NIH). Segmented line measurements of the whole sperm cell and the head and tail regions were carried out. Additionally, surface area and perimeter of the sperm head were calculated.

Experiment 2: Membrane integrity

After morphological differentiation into the groups "typically shaped" (cells with filiform and curved head, slight or no cytoplasm drop and intact flagellum) and "abnormally shaped spermatozoa" (cells with major rest of cytoplasm drop, immature round-head, defective head or tail), the integrity of the cytoplasm membrane of recovered cells (n = 949) from four frogs was investigated using the dye propidium iodide (PI) that penetrates only strongly damaged membranes (Berthelsen 1981). Binding to the DNA of membrane defective spermatozoa and emitting red fluorescent light, sperm cells with an intact outer membrane were distinguishable from membrane-defective cells (Harrison & Vickers 1990). Cloaca lavages were centrifuged for 2 min at 173 × g before 1 µl PI solution (1 mg/ml; Sigma-Aldrich, Taufkirchen, Germany) was added to 199 µl sperm containing sediment. Following a second centrifugation (2 min at 173 × g) 20 µl of the sediments were deposited on non-coated microscopic glass slides and were covered with a cover slip. Sperm cells were evaluated by phase-contrast microscopy and fluorescent microscopy (excitation wavelength of 520-550 nm, emission wavelength of 610 nm; BX60 microscope, 800×, Olympus, Hamburg, Germany). Individual spermatozoa were classified firstly by their head surface structure in phase-contrast microscopy (smooth vs. protrusions) and subsequently by their PI staining behaviour. The correlation between the dying behaviour seen in fluorescent microscopy and the phase-contrast microscopic cell membrane morphology were calculated to estimate the viability of the spermatozoa in the following studies from phase-contrast microscopy.

Experiment 3: hCG dosage

Two dosages of hCG (100 IU vs. twice 100 IU; diluted with 50 µl sterile water each) were compared to induce spermiation in *Dendrobates auratus*. Male frogs were divided into two groups consisting of six animals each. The first group was tested with 100 IU hCG at least twice. A double stimulation with 100 IU hCG at intervals of one hour was performed at least twice with every male frog within the second group. Two different media (IPS, XB) were equally used in both groups. One hour after sperm collection, sediment samples

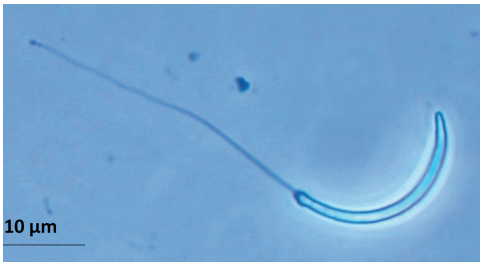

Fig. 1. Typical spermatozoon of *Dendrobates auratus*. Note the smooth and glossy surface of the cell membrane at the head. Phase-contrast microscopy, 800×.

from all points of time were processed seperately under the light microscope. All typically shaped spermatozoa (non-motile and motile cells were pooled to exclude medium effects on sperm motility) under the coverslip were counted. Differences in the spermiation response were determined using the Wilcoxon Rank-Sum Test (SAS 9.1).

Experiment 4: Sperm motility

To investigate possible effects on sperm motility the two different sperm media IPS and XB were compared. A group of eight male frogs was hormonally stimulated (twice 100 IU hCG). Cloaca flushings from all points of time were performed with either IPS or XB per experiment. Both media were used in every individual up to four times. Only typically shaped viable sperm cells demonstrating a pulsating flagellum were considered as motile, although in some cases altered cells showed a movement of the tail. Statistical analysis was performed using the Signed Rank Test (SAS 9.1).

Experiment 5: Proportion of recovered spermatozoa

Viable motile and non-motile spermatozoa with typical morphology, as well as cells with altered conformation were analysed. These aberrant sperm cells were divided into four groups: (1) major rest of cytoplasm drop, (2) immature round-head, (3) defective head with faulty membrane integrity, and (4) defective tail. With equal usage of both sperm media (excluding medium effects on sperm motility) for every individual frog, data of 30 successful double stimulation experiments (total number 15011 spermatozoa) were pooled for analysis (Signed Rank Test; SAS 9.1).

Results

Experiment 1: Sperm morphology

Spermatozoa of *Dendrobates auratus* consisted of a filiform, curved head and a single flagellum. The heads possessed in some cas-

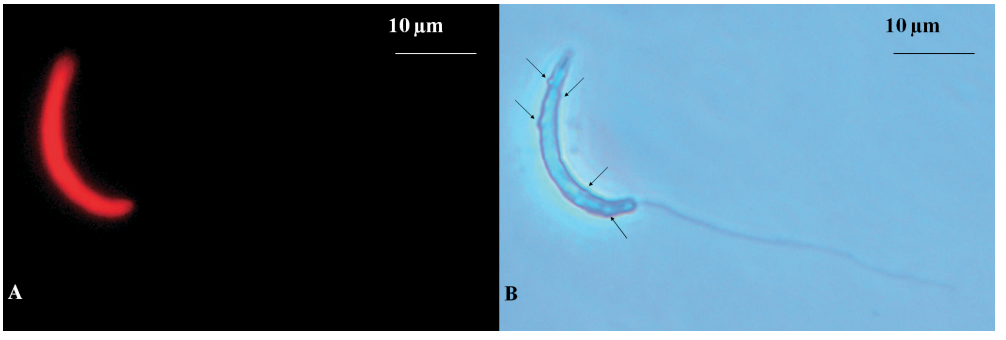

Fig. 2. Dead spermatozoon of *Dendrobates auratus.* A: Sperm head with defective cell membrane, PI dyed (fluorescent microscopy, 800×). B: Cytoplasm membrane at the head with numerous protrusions (→) (light microscopy, 800×).

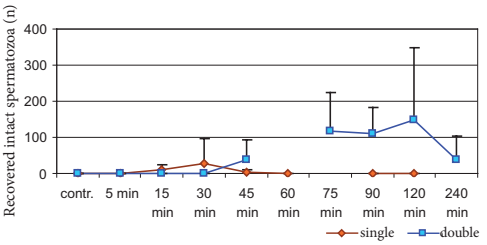

Fig. 3. Differences in the distribution of the spermiation responses (only membrane-intact spermatozoa with typical morphology, non-motile and motile sperm cells were pooled) after single and double hCG stimulation. IPS and XB medium was used equally in both groups.

es a slight cytoplasm drop, which was clearly distinguishable from large cytoplasm drops belonging to cells grouped within irregular shaped spermatozoa. The surface of the cell membrane at the head region appeared consistently smooth and glossy in phase-contrast microscopy. A midpiece was not visible. No undulating membrane was present at the tail (Fig. 1). Typically shaped sperm cells with intact cytoplasm membrane (n = 39) from three different frogs were selected for measurement purposes. The complete spermatozoon of *Dendrobates auratus* had an average length of 56.1 ± 5.5 µm, whereas 21.1 ± 2.7 µm was allotted to the head and 35.0 ± 4.2 µm to the tail. The head had a width of 2.0 ± 0.2 µm and the surface area of the headpiece was calculated with 36.0 ± 5.7 µm². The perimeter of the sperm head averaged 44.0 ± 5.9 µm.

Experiment 2: Membrane integrity

All dead sperm cells (n = 78) with DNA-bound propidium iodide (PI) emitting red light in fluorescent microscopy had been previously detected in phase-contrast microscopy. The membrane showed in these cells numerous bullous protrusions in the head area which were distributed equably (Fig. 2). For the vast majority (99.2 %) of spermatozoa ranked membrane intact (n = 864) in phase-contrast microscopy this observation was verified by the PI staining behaviour. As a result of this examination, viable spermatozoa and membrane-defect sperm cells mentioned below were differentiated using phase-contrast light microscopy.

Experiment 3: hCG dosage

In 11 out of 15 experiments (73.3 %) with frogs stimulated with a single injection of 100 IU hCG, sperm cells were obtained. Thirty-one out of 32 sperm collection attempts (96.9 %) were successful when double stimulation with 100 IU hCG each was performed. All pre-treatment samples (0 min) were aspermic. An average number of 42.6 ± 73.0 viable sperm cells were collected after single hCG stimulation (2.1 ± 3.7 × 10^3 cells per ml) between 15 min and 120 min, whereas an average number of 382.6 ± 409.8 viable sperm cells (19.1 ± 20.5 × 10^3 cells per ml) were found during experiments with double

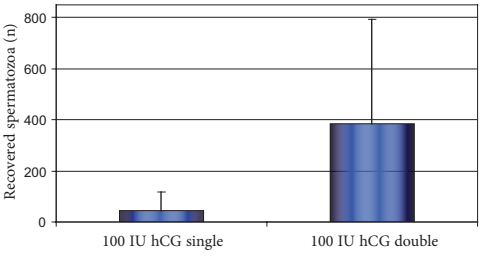

Fig. 4. Number of recovered viable (motile and non-motile) spermatozoa of *Dendrobates auratus* after hCG single and double stimulation (* P<0.05).

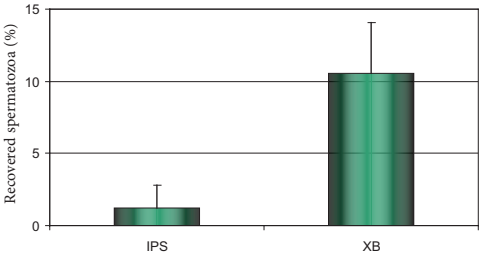

Fig. 5. Percentage of recovered motile sperm cells with typical morphology using either IPS of XB medium after hCG double stimulation (*** P<0.001).

hCG administration between 5 min and 240 min (Fig. 3). Both individual and intraindividual variations in the spermiation response were noticeable. Four single stimulated frogs showed no or low sperm release, whereas two frogs reacted with moderate spermiation. After double stimulation in two individuals low and moderate and in four individuals high spermiation responses were detectable. Table 1 displays the individual numbers of released sperm cells of single and double stimulated frogs.

The average distribution of spermatozoa during the recovery process after single hCG administration showed a low single peak 30 min following the stimulation. The first samples were positive at 15 min and in some cases few cells were observed at 90 min and later. In frogs receiving a double stimulation, the sperm response began 30 min following the first injection, increased until 75 min (117.3 ± 107.6 sperm cells) for a first peak before decreasing again. After 2 hours, the maximum sperm release was noticeable (147.9 ± 201.1 viable spermatozoa). Analogous to the averaged calculations, the individual spermiation responses showed a biphasic characteristic in 18 out of 32 successful experiments with double stimulation. This stimulation method was more effective ($P<0.05$) than the single injection of 100 IU hCG inducing spermiation in *D. auratus* (Fig. 4).

Experiment 4: Sperm motility

Using IPS medium in 9 out of 19 experiments no motile spermatozoa with typical morphology were counted during the whole recovery procedure. On average, 5 motile (maximum 39) sperm cells were recovered. Gained average motile sperm percentages ranged in individual frogs from 0 to 4.8 % ($\bar{x}$=1.2 %). In contrast, in 16 out of 17 experiments with XB medium, motile spermatozoa were found with a mean of 39 (maximum 198). Percentages of motile spermatozoa ranged in individuals from 8.1 to 16.7 % ($\bar{x}$=10.5 %) (Fig. 5). The XB medium provided a significantly higher number ($P<0.05$) and percentage ($P<0.001$) of motile spermatozoa coming from cloaca flushings of hormonally stimulated dendrobatid frogs than the IPS medium. No significant correlation could be observed in the distribution of non-motile cells in the two different media.

Experiment 5: Proportion of recovered spermatozoa

Due to the complexity of the used classification for spermatozoa, morphology and PI staining behaviour are summarized in Figure 6. The sperm cell population consisting of viable but non-motile cells amounted to

Tab. 1. Individual numbers of recovered viable sperm cells (motile and non-motile were pooled) from single and double stimulated frogs per experiment. Note the individual and intraindividual variation of the spermiation response.

	100 IU hCG single						100 IU hCG double					
frog	1	2	3	4	5	6	7	8	9	10	11	12
viable sperm cells	60	10	12	0	2	3	11	1073	0	1164	251	6
per experiment	37	5	356	0	0	3	413	159	32	1802	30	35
	83			0	1		522	205	3	874		
							209	35	34	1274		
							874	149	801	1464		
							416	1133	562	434		
							340	99	4			
								50				
$\bar{x}$	60	7.5	184	0	1	3	397.9	362.9	205.1	1168.7	140.5	20.5

73.5 % and 5.8 % were motile. With 79.3 %, most sperm cells had a typical morphology and were viable. All other cells were divided into four groups of abnormally shaped sperm cells. A large remainder of cytoplasm at the head region was found in 5.1 % (18.6 ± 14.5) of the sperm, 1.6 % (5.9 ± 3.8) of the cells showed a rounded and non-elongated headpiece, 4.9 % (22.8 ± 20.8) had a defective cell membrane (membrane protrusions, rarely entire breaks of the head) and in 9.2 % (34.2 ± 24.7) of all cells, tail alterations were visible (breaks or rarely elongations). Figure 7 gives an overview of the constitution of sperm sub-populations together with photographs of representative cells.

Discussion

This study was designed to offer the ability to manipulate spermiation in future assisted breeding programs for endangered dendrobatid species. Using the non-endangered Green Poison Frog (*Dendrobates auratus*) as a research model, a gentle sperm collection method was established for the potential adoption in other small tropical frogs, in particular endangered dendrobatid frogs such as the closely-related *Dendrobates tinctorius,* listed as vulnerable, the endangered *Oophaga speciosa* or the critically endangered *Oophaga lehmanni* (IUCN 2007). The subcutaneous application of hCG into the dorsal lymph system was previously performed only in large and common laboratory frogs belonging to other families other than the Dendrobatidae, such as *Rana pipiens* (Burgos & Ladman 1955), *Rana catesbeiana* (Easley et al. 1979) and *Rana esculenta* (Minucci et al. 1989) representing the family Ranidae. In toads, the family Bufonidae, more invasive intraperitoneal injections of hCG were done in *Bufo melanostictus* (Chatterjee et al. 1971) and *Bufo marinus* (Iimori et al. 2005) to characterize the sperm release. A ventral dermal administration of LHRH in *Bufo americanus* and *Bufo valliceps* resulted in the release of sperm containing urine (Rowson et al. 2001), but similar effects of hCG have not yet been demonstrated.

Low numbers of recovered spermatozoa and low percentages of motile sperm cells in *D. auratus* compared to other amphibian species are noticeable, although in myobatrachid frogs similar percentages of motile sperm cells were observed (Edwards et al. 2004),and sperm suspensions were obtained from macerated testes. Despite little or no forward progressive motility and a low general motility, the authors of this study detected high rates of fertilization in *Limnodynastes tasmaniensis*. Although no data exist on released sperm numbers under natural mating conditions, low concentration is likely due to the special terrestrial fertilization mode of dendrobatid species. A high sperm number placed directly on only few laid eggs not only means waste of resources but increases the risk of polyspermy. From this it follows that released numbers of spermatozoa from frog species fertilizing high numbers of eggs in waters cannot be compared to the condition found in the Dendrobatidae.

Both sperm media sufficiently supported the integrity of the outer cell membrane. No significant differences in the appearance of morphologically altered spermatozoa were observed. Higher sperm motility in the XB medium without energy source means a rapid loss of cellular energy and should not take place before contacting the egg jelly coat. This medium is adequate to study movement characteristics of sperm cells and with the addition of an energy source it may be useful in fertilization experiments. The correlation of PI staining behaviour and the occurrence of specific cytoplasm membrane alterations seen in phase-contrast microscopy is obvious but should be verified. Particularly early and small defects on the outer membrane might not be noticeable when using phase-contrast microscopy alone.

The external morphology of the spermatozoa of *Dendrobates auratus* is similar to that of *Ameerega trivittata* and *Ameerega*

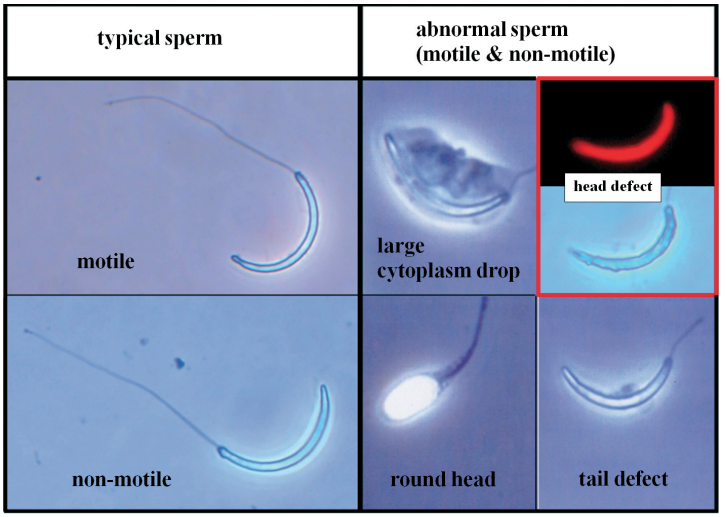

Fig. 6. Composition of arranged groups of spermatozoa including accordant morphology in phase-contrast microscopy and additional PI staining behaviour in head defective cells.

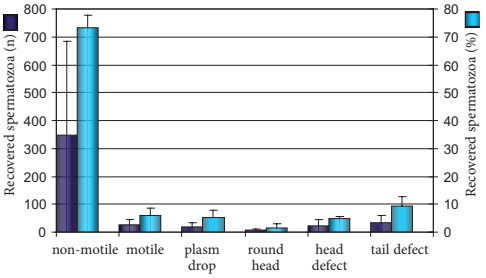

Fig. 7. Distribution of typically shaped (non-motile and motile) and abnormally shaped spermatozoa in recovered cloaca flushings (data from all moments of recovery were pooled). Absolute numbers and percentage of sperm cells were gained with equal usage of both sperm media for every individual frog after hCG stimulation (100 IU twice).

hahneli (Aguiar-Jr. et al. 2004) and *Ameerega flavopicta* (Garda et al. 2002), except for the absence of accessory tail structures such as an undulating membrane. It is also similar to the spermatozoa of *Allobates femoralis* and *Colostethus sp.* (Aguiar-Jr. et al. 2003) except for the presence of two flagella with accessory tail structures in the latter two species. In *Rana* and *Xenopus* spermatozoa (Bernardini et al. 1986, Lee & Jamieson 1993) as well as in *D. auratus* (Lipke et al. 2007) one flagellum composed of an axoneme is present.

Although a non-invasive hormone stimulation method is preferable in assisted breeding programs for endangered anurans, the hCG double injection is effective in inducing spermiation and never resulted in negative consequences for the animals during the study. The rapid decline in frog populations worldwide shows the necessity of research in the field of amphibian reproduction. Results of this project could be useful in breeding programs including assisted mating, evaluating male fertility, artificial fertilization and cryopreservation of gametes and embryos as well as in veterinary practise for sexing in species lacking external sex dimorphism.

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