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Quality evaluation of sperm from livebearing fishes: Standardized assessment of sperm bundles (spermatozeugmata) from *Xenotoca eiseni* (Goodeidae)

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ABSTRACT

Standardized evaluation of sperm quality is essential for research, commercial-scale cryopreservation, and induced spawning. However, standardized methods for evaluation of sperm bundles (spermatozeugmata or spermatophores) have not been established. The purpose of the present study was to use Redtail Splitfin (*Xenotoca eiseni*) as a model for freshwater livebearing fishes to establish initial standardized methods to collect sperm bundles, and quantitatively and qualitatively evaluate quality-related attributes. No sperm or sperm bundles were able to be collected by stripping. Testes were removed, rinsed, weighed, placed in 50 μL of buffer solution on a glass slide, and crushed gently 3–5 times with angled spade-tip forceps. Sperm bundles were released into the buffer solution and collected with a pipette into 1.5-mL centrifuge tubes. To quantify size and shape, images of bundles were captured with a CCD camera connected to a microscope, and measured with computer software. There was no significant correlation between body wet weight and major bundle axis length ($P = 0.6759$), minor axis length ($P = 0.5658$), average axis length ($P = 0.5869$), aspect ratio ($P = 0.7839$), and observed area ($P = 0.5727$). The concentrations of sperm bundles, estimated with the three methods (Makler® counting chamber, a hemocytometer, and direct counting) were significantly different ($P < 0.0001$). Hemocytometers were suitable for estimation of bundles from *X. eiseni*. To evaluate activation of sperm, bundles were viewed with a microscope, and classified into one of five phases by evaluating morphology of the bundles and motion of sperm within the bundles as Phase 0 through Phase 4 that represented early through late activation stages. The frequencies and duration of each activation phase were used to evaluate dissociation of sperm bundles and motility capability of sperm within the bundles. Within 180 min of activation, all five phases were observed. Overall, this study for the first time established standardized methods to collect and evaluate quality-related attributes of sperm bundles. These standardized evaluations provide a basis for further modification, standardization, and generalization, which are useful in research on livebearing fishes involving male gametes, such as studies on cryopreservation, artificial insemination, and in development of germplasm repositories for imperiled species including goodeids.

1. Introduction

Standardized quality evaluation is essential for sperm cryopreservation and induced spawning [1]. In research to develop cryopreservation protocols for aquatic species, sperm quality is used to assess the effects of treatments in key steps such as extender choice, motility activation, cryoprotectant toxicity, cooling and thawing rates, and fertilization assays [2]. For applied and commercial-scale sperm cryopreservation, systematic quality evaluation has been established for humans [3], livestock [4], and has been proposed for aquatic species [5]. In aquatic species the most widely used parameter for the evaluation of sperm quality is percent motility, which can be an important indicator of fertilization success and can be monitored without the time- or sample-consuming observation of fertilization and embryo development [6]. For example, percent motility were correlated with fertilization

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yield of thawed sperm in Common Carp (*Cyprinus carpio*) [7]. Percent motility and motility duration can be estimated by direct observation (with naked eye) using a microscope or by computer-assisted sperm analysis (CASA) systems. However, standardization of evaluation approaches is usually overlooked, which can be problematic for the reproducibility of research findings and quality assurance of production [1]. Various procedures of motility evaluation from different sources make it difficult or impossible to directly compare research results, and insufficient checkpoints during commercial-scale cryopreservation make the quality of final products unpredictable. Sperm concentration is another important indicator of initial sperm quality upon collection, and it can significantly affect motility and the level of agglutination of thawed sperm and fertilization rates [8–10], but sperm concentration is often not adjusted or reported in standardized methods in publications regarding sperm cryopreservation for aquatic species.

Species that package sperm in bundles further challenge standardized evaluation of gamete quality. Bundles are formed by packing of numerous sperm cells into unencapsulated (spermatozeugmata) or encapsulated (spermatophores) clusters [11]. The formation of sperm bundles has been identified in invertebrates including nematodes [12], annelids [13], arthropods [14] and molluscs [15], and in vertebrates including amphibians [16], chondrichthyans [17], and teleosts [18]. The occurrence of sperm bundles is sporadic among vertebrates and usually accompanied with internal fertilization and viviparity in fishes [19]. Bundles are believed to facilitate the systematic transfer of sperm from male to female [18], however, they pose difficulty for standardized assessment of male gametes. For example, sperm from most externally fertilized fish do not form bundles (referred as 'free sperm'). Upon activation by suitable media, the percentage of motile free sperm can be estimated by counting. However, such methods cannot be applied to sperm within bundles, for example, to study the effects of physiochemical factors on activation of sperm within the bundles [20], cryopreservation of sperm bundles, or comparison of fertilization rates between free and bundle-form sperm. In addition to activation, the concentration of free sperm is usually measured with a hemocytometer or specialized counting chamber by identifying the number of sperm cells present in a unit volume, but these devices have not been reliably applied for use with sperm bundles. Sperm morphology is also used to assess sperm quality. For example, morphological examination of fish sperm is an useful tool for monitoring reproductive disruption caused by environmental pollution [21]. Bundle morphology can also be useful to indicate sperm quality, but to date, no standardized approaches have been established to evaluate quality-related attributes, such as activation, concentration, and morphology of sperm bundles from fishes.

Livebearing has been documented in 54 extant families of fishes, including 40 families of chondrichthyans, one montypic family of coelacanths (*Latimeria*), and 13 families of teleosts [22]. Among these, species from 5 families inhabit freshwater, including 3 families within Cyprinodontiformes (Poeciliidae, Goodeidae, and Anablepidae), 1 family in Beloniformes (Hemiramphidae), and 1 family in Scorpaeniformes (Comephoridae) [23]. Livebearing fishes employ internal fertilization, and sperm from freshwater livebearing fishes are typically packed into spermatozeugmata [11]. Poeciliidae is the largest freshwater livebearing family, comprising more than 200 species with internal fertilization. Poeciliids are popular ornamental species, important cancer research models, and have been used for mosquito control [24]. Sperm from Poeciliidae has been used in studies addressing reproductive behavior [25], evolution [26], toxicology [27], and establishment of germplasm repositories [28]. Goodeidae, the second largest freshwater livebearing family (about 38 livebearing species), is considered to be one of the most at-risk fish groups in the world [29]. As of 2005, the conservation status of livebearing goodeids included 2 species categorized as extinct in the wild, 17 as critically endangered, 5 endangered, 2 threatened, 11 vulnerable, and only 3 at lower risk rankings [30]. Sperm from poeciliids and goodeids form sperm bundles and the mechanism by which the bundles are dissociated and sperm are activated in the female reproductive tract is not clear. Standardized quantitative or qualitative approaches are necessary to study the activation mechanism of sperm within bundles, improve artificial reproduction, and develop protocols of cryopreservation of sperm bundles for freshwater livebearing species. In the present study, the Redtail Splitfin (*Xenotoca eiseni*, Goodeidae) was used as a model for freshwater livebearing fishes to establish standardized methods to collect sperm bundles, and quantitatively and qualitatively evaluate quality-related attributes. The specific objectives were to: (1) establish and apply standardized methods to collect sperm bundles, (2) quantitatively evaluate their sizes and concentrations, and (3) classify activation patterns of sperm within bundles.

2. Materials and methods

2.1. Fish husbandry

Protocols for the use of animals in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA, USA). The *X. eiseni* used in this study were 2-y old and maintained at the Aquatic Germplasm and Genetic Resources Center (AGGRC) at the Louisiana State University Agricultural Center (Baton Rouge, LA). About 200 fish were cultured indoors at 22–26 °C with a 14 h:10 h (light:dark) photoperiod in four individual tanks within an 800-L recirculating system and fed twice daily with tropical flakes (Pentair Aquatic Eco-systems, FL, USA) and twice weekly with brine shrimp (Sally's Frozen Brine Shrimp™, San Francisco Bay Brand, CA, USA). Males were maintained at a 2:1 ratio with females in each tank until 2 d before experiments. Additional water quality parameters were monitored weekly and held within acceptable ranges including: pH (7.0–8.0), ammonia (0–1.0 mg/L), and nitrites (0–0.8 mg/L).

2.2. Collection of sperm bundles

Fish were anesthetized with 0.01% tricaine methanesulfonate (MS-222, Western Chemical, Inc. WA, USA) diluted with water from the fish tank. To eliminate MS-222 residues, the surface of fish was wiped with a paper towel and rinsed with buffer solution (NaCl solution at 300 mOsmol/kg buffered by 10 mM HEPES-NaOH at pH 7.0). The fish was wiped again and body wet weight was measured. Osmolalities of buffer solutions were measured with a freezing point osmometer (Model 5010 OSMETTE III ™, Precision Systems Inc., MA, USA) and pH was measured with a pH meter (EcoSense® pH100A, YSI Inc., OH, USA). To collect milt by stripping, fish were placed on their back on a sponge and squeezed gently, followed by milt being collected with a 10-μL capillary by mouth suction through a rubber tube. If no milt was collected, testes were removed by dissection. Testes were rinsed, weighed, placed in 50 μL of buffer solution on a glass slide, and crushed gently 3–5 times with angled spade-tip forceps. Sperm bundles were released into the buffer solution and collected with a pipette into 1.5-mL centrifuge tubes. Volumes of sperm bundle suspension were adjusted to 100 μL by addition of the buffer solution.

The gonadosomatic index (GSI) was calculated as: (testes weight/body wet weight) × 100%. Pearson correlation coefficient (r) with SAS (PROC CORR) (SAS version 9.4, SAS Institute, NC, USA) was used to evaluate the relationship between body wet weight