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PRELIMINARY EVALUATION OF SEMINAL PLASMA PROTEINS AND IMMUNOREACTIVITY OF NERVE GROWTH FACTOR AS INDICATIVE OF AN OVULATION INDUCING FACTOR IN ODONTOCETES

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A B S T R A C T

In the seminal plasma of terrestrial mammalian species known as induced (e.g., camels) and spontaneous (e.g., cattle) ovulators, an ovulation-inducing factor (OIF) with a protein structure similar to beta-nerve growth factor (β-NGF) has been identified. Detection of an OIF/NGF in the seminal plasma of cetaceans would have both basic and applied implications in reproductive biology and conservation management programs. A preliminary evaluation was conducted to characterize the distribution and abundance of seminal plasma proteins in aquarium-based belugas and a Pacific white-sided and bottlenose dolphin. Initially, SDS-PAGE was used with 50 µg of total protein for separation; thereafter, Western immunoblot was used with anti-NGF. In addition to odontocete seminal plasma, a purified fraction of llama seminal plasma (100 ng protein) and an extract of mouse brain (20 µg total protein) were included as positive controls for NGF. Within the two belugas, visual inspection of the protein bands indicated similar distribution and intensity. However, among the belugas and Pacific white-sided and bottlenose dolphins there was more diversity than similarity in the distribution and abundance of seminal plasma proteins. While immunoreactivity of NGF was distinctly evident in the llama and mouse positive controls, there was no visual reactivity in any of the odontocete samples. These preliminary results provide novel information indicating more homogeneity within and heterogeneity among seminal plasma proteins of ondentocetes. Although NGF was not immunologically detected, future studies are required to address the apparent limitations of immuno-detection of NGF, especially if the post-translational form of β-NGF is in low abundance in the seminal plasma of belugas and Pacific white-sided and bottlenose dolphins.

Keywords: odontocetes, seminal plasma, ovulation-inducing factor, nerve growth factor.

INTRODUCTION

It is well accepted that seminal plasma facilitates delivery and provides a nutritive environment for sperm (Poiani 2006); however, recent studies in a wide variety of species have provided evidence that seminal plasma and its proteinaceous constituents play a much broader role in fertility (Druarta *et al.,* 2013, McGraw *et al.,* 2015, Bromfield 2016). Compared to multiple other mammalian species (Mogielnicka-Brzozowska and Kordan 2011), knowledge of the constituents of seminal plasma in cetaceans is nil. Apart from a single study (O'Brien *et al.,* 2008) that describes the clinical biochemistry of seminal plasma from belugas (*Delphinapterus leucas*), the proteinaceous constituency of seminal plasma in cetaceans is not known.

It remains equivocal if all cetaceans are spontaneous or induced ovulators or some proportion of both (i.e., facultative). Historically, bottlenose dolphins (*Tursiops truncatus*), *La Plata River dolphins* (*Pontoporia blainvillei*), and *belugas* were reported to be coitus-induced ovulators (Kleinenberg *et al.,* 1964, Harrison and Ridgeway 1971, Harrison *et al.,* 1972, Harrison 1977; Harrison *et al.,* 1981, Kirby and Ridgeway 1984, Yoshioka *et al.,* 1986, Schroeder 1990). Apart from the beluga, more recent studies have provided additional evidence that the bottlenose dolphin, common dolphin (*Delphinus delphis*),

false killer whale (*Pseudorca crassidens*), killer whale (*Orcinus orca*), Pacific white-sided dolphin (*Lagenorhynchus obliquidens*), Risso dolphin (*Grampus griseus*), and spotted dolphin (*Stenella attenuata*) are spontaneous ovulators (Benirshcke *et al.,* 1980, Sawyer-Steffan *et al.,* 1983, Kirby and Ridgeway 1984, Robeck *et al.,* 1993, Combelles 1995, Atkinson *et al.,* 1999, *Robeck et al.,* 2009). Recent longitudinal ultrasonic imaging and hormonal data have indicated that adult female belugas may be facultative-induced ovulators (Steinman *et al.,* 2012). In the presence of a male, belugas ovulated 85% of the time, whereas, in the absence of a breeding male, belugas spontaneously ovulated in 26% of estrous cycles. Combined, past and more recent data support the concept that the beluga may be a facultative-induced ovulator; however, additional evidence is required for confirmation.

In general, ovulation in mammals involves a neuroendocrine signaling mechanism between the reproductive organs and brain (Kauffman and Rissman 2006). Regardless of the mode of ovulation, hypothalamic GnRH secretion is necessary to stimulate the pre-ovulatory LH surge from the anterior pituitary gland that signals ovulation of one or more dominant ovarian follicles. In spontaneous ovulators (e.g., humans, rats, cattle), increased estrogen production by one or more pre-ovulatory follicles appears to be the primary stimulus that triggers GnRH secretion, whereas, in induced ovulators (e.g., camels, rabbits, ferrets), physical vagino-cervical contact associated with copulation during estrus is thought to be the primary trigger for GnRH secretion.

Traditionally, the stimulus for induced ovulation has been considered to be physical or tactile in nature (Jochle 1973); however, recent evidence indicates that the stimulus may be a biochemical constituent of semen (review Adams *et al.,* 2013, Allali *et al.,* 2017). The first direct evidence of semen-induced ovulation emerged more than 30 years ago from studies with Bactrian camels (*Camelus bactrianus*), which are known induced ovulators. It was demonstrated that, regardless of the route of conspecific administration, camel seminal plasma given intravaginal (Chen *et al.,* 1985; Xu *et al.,* 1985), intramuscular or intrauterine (Pan *et al.,* 1992) induced ovulations. More recent studies (Adams *et al.,* 2013) in other known induced ovulators have indicated that a single intramuscular dose of llama or alpaca seminal plasma induced ovulation in >90% of females of the respective species compared to 0% in those given saline (Adams *et al.,* 2005). Comparatively, intramuscular administration of seminal plasma from spontaneously ovulating cattle (Ratto *et al.,* 2006), horses and pigs (Bogle *et al.,* 2011) was also able to induce ovulation albeit at a lower rate in 29% of llamas with 0% ovulations in saline controls.

Biochemical isolation and purification of an ovulation-inducing factor (OIF) in llama seminal plasma resulted in the identification of a protein with a peptide sequence and structure (14 kDa) identical to beta nerve growth factor (β-NGF, Ratto *et al.,* 2012). The physiologicical effect of the purified OIF/NGF-like substance found in llama seminal plasma was evaluated using a single intramuscular injection in estrus-synchronized llamas. Results indicated a post-treatment pre-ovulatory LH surge, ovulation, and CL formation in 91% of the treated llamas compared to 0% of the saline controls (Ratto *et al.,* 2012).

While the nature and functional role of NGF associated with development, growth, and reproduction is well documented in humans and various laboratory (Wiesmann and de Vos 2001, Aloe *et al.,* 2013, Seidel *et al.,* 2013), domestic, and a few exotic terrestrial mammals (Adams *et al.,* 2013, Allali *et al.,* 2017), no published documentation was found on NGF in aquatic marine mammals. Detection, identification, and quantification of NGF as indicative of an OIF in the seminal plasma of odontocetes can provide additional evidence to clarify if the beluga is a facultative-induced ovulator. More broadly, detection of seminal plasma NGF can have basic and applied implications in reproductive management and conservation biology among cetaceans.

The present study was designed as a preliminary evaluation to characterize the basic distribution and abundance of seminal plasma proteins and immunoreactivity of NGF in several odontocetes - belugas, a Pacific white-sided dolphin, and a bottlenose dolphin.

MATERIALS AND METHODS

Odontocetes and management: The present study involved a retrospective analysis of archived sperm-free seminal plasma collected from two belugas, a Pacific white-sided dolphin, and a bottlenose dolphin during routine animal husbandry exams from 2014 to 2015 at SeaWorld Parks and Entertainment (San Diego, CA; San Antonio, TX; Orlando, FL, USA). The belugas were born August 8, 1992 (Beluga A, 22 yrs at the time of semen collection) and July 13, 2003 (Beluga B, 11 yrs), Pacific

white-sided dolphin born Sept 8, 2003 (11 yrs), and bottlenose dolphin born Aug 16, 1989 (26 yrs). Apart from the Pacific white-sided dolphin, the belugas and bottlenose dolphin are proven sires with live calves.

Animals were managed in compliance with the US Animal Welfare Act and by the Standards and Guidelines of the Alliance of Marine Mammal Parks and Aquariums. Practices and procedures associated with the study were reviewed and approved by SeaWorld's Animal Care and Use Committee. Feeding consisted of individual diets of frozen-thawed whole fish (herring, *Clupea harengus*; capelin, *Mallotus villosus;* Columbia River smelt, *Thaleichthys pacificus*) at approximately 4 to 5% of body weight per day.

Collection and processing of seminal plasma: Semen samples were collected from each animal using behavioral conditioning as previously reported (O'Brien *et al.,* 2008, Robeck and O'Brien 2004, Robeck *et al.,* 2009). Beluga samples were collected in Sept and Oct 2014, Pacific white-sided dolphin in Sept 2014, and bottlenose dolphin in June 2015. For Beluga B, semen samples were collected on Sept 25 and 27, and in the morning and afternoon on Sept 27 to initially assess changes in seminal plasma proteins within and between days. Once collected, ejaculates were held at ambient temperature (21^{o}C) and processed within 30 min. Only semen samples which had normal osmolarity (≤370 mOsm/kg for belugas, <350 mOsm/kg for Pacific white-sided and bottlenose dolphins), sperm concentration (>100 x 10^6 sperm/mL for belugas and >400 x 10^6 sperm/mL for dolphins), and percent progressively motile (>80%) and live (>90% intact plasma membrane) sperm were considered for further processing. Seminal plasma was isolated from spermatozoa by centrifugation (2000xg for 10 min at 11^{o}C). A seminal sample was observed under microscope (200x magnification) to confirm absence of spermatozoa. Thereafter, 2 mL aliquots were placed in cryotubes (Nunc, Rochester, NY, USA), flash frozen in liquid nitrogen, and stored at -80^{o}C until analysis.

Positive controls for NGF: Purified llama seminal plasma (Ratto *et al.,* 2012) and mouse brain extract (Katoh-Semba *et al.,* 1989, Katoh-Semba *et al.,* 1994) were used as positive controls for β-NGF. Purified llama seminal plasma was prepared as previously described (Ratto *et al.,* 2012) and a crude extract of mouse brain was prepared as follows. Briefly, the brain from a retired male breeder mouse (strain $B6C3F_1$) was collected after euthanasia via intraperitoneal injection of sodium pentobarbital (150 mg/kg) and exsanguinated via the descending aorta in accord with an approved IACUC protocol (NYU School of Medicine). After the skull was opened, the entire brain was placed in 5 mL of radioimmunoprecipitation assay (RIPA) buffer (100 mM sodium phosphate, pH 7.2, 10 mM each of EGTA and sodium fluoride, 12.5 mM EDTA, 1% each of sodium deoxycholate and Triton X-100, 0.1% sodium dodecylsulfate, and 200 Kallikrein units of aprotinin) containing a protease inhibitor cocktail (cOmplete Mini; Roche, Mannheim, Germany). Thereafter, the brain was homogenized with a tissuemizer powered hand homogenizer for 2 min in 30 sec pulses on ice followed by centrifugation at 8000xg for 10 min at 4°C. Supernatants were aliquoted and stored at -80°C until analysis.

Total protein and separation (SDS-PAGE): Total protein concentrations in the cetacean seminal plasma samples and the purified llama sample were determined using the Bradford protein assay (Biorad; Hercules, CA, USA) with bovine serum albumin (Sigma, St. Louis, MO, USA) as standard. Total protein concentration of mouse brain extract was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli 1970) was used to initially separate odontocete seminal plasma proteins. Briefly, seminal plasma samples (50 ug of total protein) from each animal and time period were combined with 4x Laemmli buffer (final 1x) containing 20% β-mercaptoethanol, heated to 95°C for 5 min, and cooled to room temperature. For protein separation, individual samples were loaded into six separate lanes of the SDS-PAGE gel (12.5%). The gel was stained with Coomassie brilliant blue (50% methanol, 10% acetic acid, 40% water, 0.05% brilliant blue R250) for 1 h at room temperature and destained (staining buffer without R250) at room temperature overnight to visualize the protein bands. After destaining, the gel was imaged using a Gel Doc XR+ with Image Lab software (Biorad). A range of molecular weight markers (10 µL, Biorad) were used to estimate the molecular weights of the protein bands.

Western immunoblot analysis of NGF: Protein separation of odontocete seminal plasma samples including purified llama seminal plasma (100 ng) and an extract of mouse brain (20 µg of total protein) as positive controls was done by SDS-PAGE as described in the

previous section. Following gel separation, proteins were transferred to a nitrocellulose membrane using Towbin buffer and, thereafter, stained with Ponceau-S (0.1% Ponceau-S in 5% acetic acid) and imaged using the same process described in the previous section. Prior to immunoblot analysis, the membrane was destained in Tris-buffered saline and blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% Carnation® nonfat dry milk. After blocking, the membrane was incubated overnight at 4°C in blocking buffer containing 1:1000 dilution of rabbit anti-NGF (polyclonal NGF antibody [H-20]: sc-548; Santa Cruz Biotechnology, Dallas, TX, USA) directed against β-NGF. Subsequently, the blot was washed in TBST and incubated for 1 h at room temperature with gentle rocking in 1:5000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce Biotechnology) in blocking buffer. Lastly, the blot was rinsed in TBST and incubated in detection reagents as described by the manufacturer and exposed to film.

RESULTS

SDS-PAGE analysis of seminal plasma from four odontocetes with molecular weight distribution and abundance of various proteins is shown in Figure 1. Based on visual inspection of the gel, there are distinct and less distinct bands of proteins as indicated that appear similar within species (belugas) but different among species.

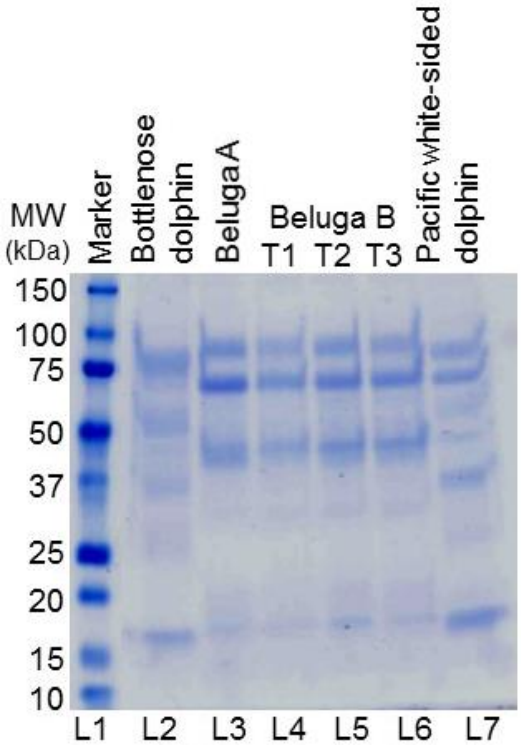

Figure 1. Protein separation of odontocete seminal plasma (50 ug total protein) with SDS-PAGE and Coomassie blue stain relative to a molecular weight (MW) marker in Lane 1. Lane 2 = Bottlenose dolphin; Lane 3 = Beluga A; Lane 4 = Beluga B (T1=collection Sept 25); Lane 5 = Beluga B (T2=collection Sept 27, morning hour); Lane 6 = Beluga B (T3=collection Sept 27, afternoon hour); Lane 7 = Pacific white-sided dolphin.

Western immunoblot analysis of NGF in odontocete seminal plasma relative to purified llama seminal plasma and an extract of mouse brain as positive controls is shown in Figure 2. Of the 3 timed seminal plasma samples from Beluga B, only the sample collected on the morning of Sept 27 was used since there appeared to be no visible differences in the number or intensity of the protein bands within and between days (Figure 1). While there were multiple protein bands associated with the extract of mouse brain (Figure 2, Panel a, Lane 4) the protein band expected at about 14kDa for purified llama OIF/NGF was not distinctly visible (Figure 2, Panel a, Lane 2). Apparently, the amount of highly purified llama OIF/NGF protein (100 ng) loaded on the gel was below the

sensitivity level for the Ponceau-S stain. Nonetheless, subsequent immunoblotting with anti-NGF resulted in distinct bands of expected molecular weights for the llama and mouse positive control samples but not for any of the odontocete seminal plasma samples (Figure 2, Panel b, Lanes 2 and 4, respectively).

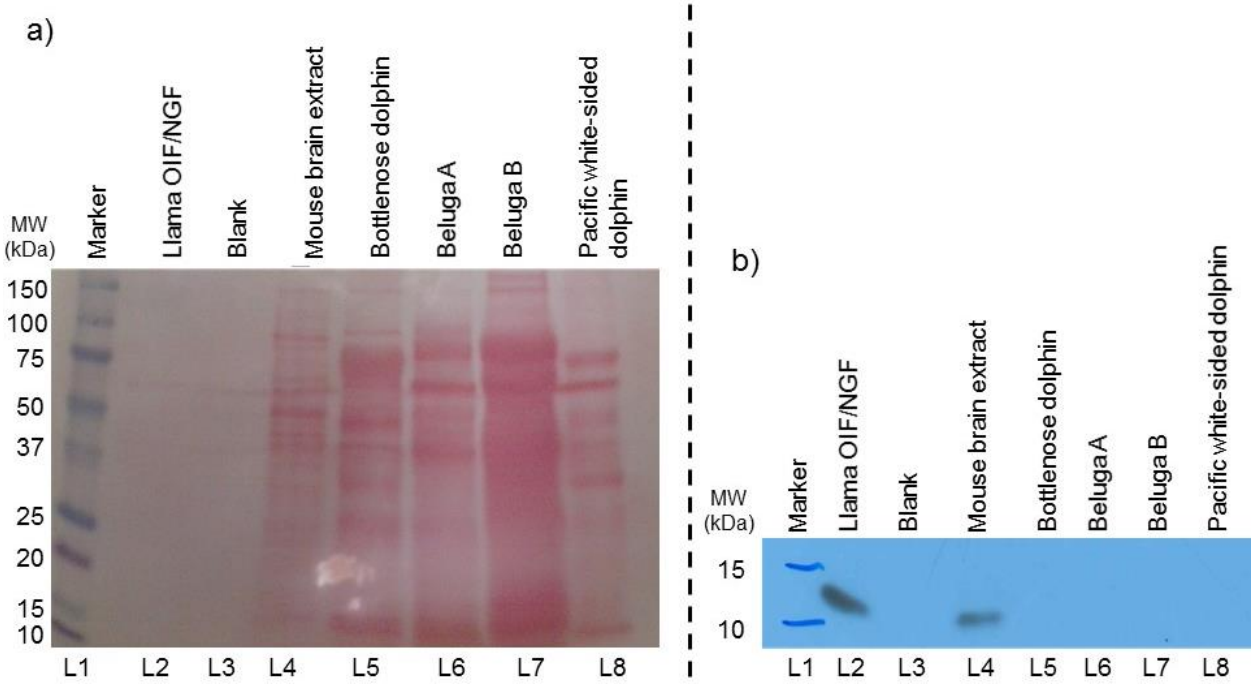

Figure 2. Western immunoblot analysis of seminal plasma (50 ug total protein) in odontocetes relative to purified llama seminal plasma ovulation-inducing factor (OIF/NGF, 100 ng) in Lane 2 and an extract of mouse brain (20 ug total protein) in Lane 4 as positive controls for β-NGF. Protein separation followed by transfer to a nitrocellulose membrane and stained with Ponceau-S stain (a) and immunoblot with rabbit anti-NGF at 1:1000 dilution followed by goat anti-rabbit antibody conjugated to horseradish peroxidase at 1:5000 dilution (b). Beluga B in Lane 7 involved seminal plasma collected Sept 27, morning hour.

DISCUSSION

Although preliminary, the novelty of the present results indicated multiple bands of proteins of different molecular weights and intensity in the seminal plasma from four odontocetes (belugas, a Pacific white-sided dolphin, and a bottlenose dolphin). Within the two belugas, including different days and times within a day, the distribution and abundance of the seminal plasma proteins appeared visually similar. However, when visually compared among the three species, there were some distinctive differences. Compared to immunoreactivity of β-NGF in llama seminal plasma and mouse brain extract as positive controls, β-NGF was not detected in the seminal plasma samples from the belugas or Pacific white-sided and bottlenose dolphins.

In mammals, seminal plasma is considered the fluid portion of semen without spermatozoa (Mogielnicka-Brzozowska and Kordan 2011, Juyena and Stelletta 2012). While the only source of sperm is the testes, seminal plasma can originate from several sources, including the rete testes, epididymides, accessary sex glands (i.e., ampullary, seminal vesicles, prostate, bulbourethral) as well as exudate of blood plasma (Mann and Lutwak-Mann 1981). In cetaceans, the only major accessary sex gland identified is the prostate (Rommel *et al.,* 2007). Hence, for the odontocetes herein, expected sources of seminal plasma proteins were the rete testes, epididymides, prostate, and blood exudate.

In the present study, SDS-PAGE results characterizing the distribution and abundance of seminal plasma proteins among the four odontocetes indicated similarities and differences among the belugas and Pacific white-sided and bottlenose dolphins. Visually, distribution and abundance of distinct protein bands were observed

between 90-95kDa and 65-70kDa in both the belugas and white-sided dolphin but not in the bottlenose dolphin. Comparatively, only a single distinct protein band was observed at about 80kDa in the bottlenose. The next level of distinct protein bands were observed at about 48kDa in the belugas but not in either dolphin species. While apparently absent or indistinct in the beluga whales, prominent protein bands were observed at 38 and 17kDa in both the Pacific white-sided and bottlenose dolphins. Other less distinct protein bands were observed with some apparent variability among species but, because they were faintly visible, no attempt was made to further characterize them due to speculation. While some noticeable similarities in distribution and abundance of seminal plasma proteins were observed between the beluga and Pacific white-sided dolphin, and Pacific white-sided and bottlenose dolphins, the bottlenose seemed most divergent from the beluga.

Comparison of seminal plasma proteins among several terrestrial mammalian species (boar, bull, ram, buck, stallion, alpaca, camel) using proteomics resulted in considerable divergence among species with only three common proteins (Druart *et al.,* 2013). While genetic diversity likely contributes to the degree of heterogeneity of seminal plasma proteins among species as reported among three different breeds of sheep (Carvajal-Serna1 *et al.,* 2018), other factors such as seasonality reported in rams (Domínguez *et al.,* 2008), buffalo bulls (Sharma *et al.,* 2014), and stallions (Abou-Ahmed *et al.,* 1993) can also have an effect on the proteinaceous constituency of seminal plasma. In the present study, perhaps the degree of heterogeneity of seminal plasma proteins among odontocetes may have been due, in part, to season since the beluga (Steinman *et al.,* 2012) and Pacific white-sided dolphin (Robeck *et al.,* 2009) are seasonally polyestrous. Nonetheless, while the present results require clarification, these preliminary observations in odontocetes appear to support the variability of seminal plasma proteins among species.

The results of Western immunoblot analysis indicated β-NGF was not detected in the seminal plasma of belugas and Pacific white-sided and bottlenose dolphins. While the NGF antibody was visibly reactive with purified llama seminal plasma and an extract of mouse brain as positive controls, no distinctive immunoreaction was observed for any of the odontocete seminal plasma samples. Immuno-reactivity of OIF/NGF in the control samples concurs with that previously reported for purified llama seminal plasma (Ratto *et al.,* 2012) and mouse brain extract (Katoh-Semba *et al.,* 1989, Katoh-Semba *et al.,* 1994). In a comparative study across species (Druart *et al.,* 2013), β-NGF was detected in the seminal plasma from the ram, stallion, alpaca, and camel using gel-based proteomics (2D LC-MS/MS). However, in the same study, seminal plasma NGF immuno-reactivity was not visibly detected in the ram and stallion using Western immunoblot analysis. The authors did not discuss the basis for the discrepancy between techniques. However, considering that mature, bioactive NGF descends from proteolitic cleavage of a precursor pro-NGF form (Fahnestock *et al.,* 2004a,b), the antibody generated against the post-translational modified form may have been either of poor specificity or protein abundance was below the level of sensitivity for immuno-detection. In the preset study, the rabbit polyclonal NGF antibody (H-20): sc-548, which is directed against β-NGF, was the same as that used to detect seminal plasma NGF in previous studies (Ratto *et al.,* 2012; Druart *et al.,* 2013). Although the basis for not detecting NGF in the seminal plasma of ondentocetes is unknown, future evaluation of ondentocete seminal plasma should consider a broad-based or non-targeted approach. Proteomic analysis casts a wide net that can potentially reveal post-translational changes of the NGF protein and reveal more changes than previously anticipated even in low abundance.

As reviewed (Adams *et al.,* 2013, Allali *et al.,* 2017), OIF/NGF was detected in the seminal plasma of known induced ovulators (e.g., alpaca, llama, camel) as well as known spontaneous ovulators (e.g., cattle, sheep, horse, pig; Ratto *et al.,* 2006, Bogle *et al.,* 2011,Druart *et al.,* 2013). In this regard, administration of seminal plasma from spontaneous vs induced ovulators to llamas, induced ovulation although at a reduced frequency (29% vs >90%) in spontaneous ovulators (Ratto *et al.,* 2006, Bogle *et al.,* 2011). Furthermore, β-NGF was detected in the seminal plasma of both spontaneous and induced ovulators via proteomic analysis. (Druart *et al.,* 2013). Hence, in the present study it was unexpected not to detect β-NGF in the belugas as proposed facultative-induced ovulators (Steinman *et al.,* 2012), and, perhaps to a lesser degree, in dolphins as spontaneous ovulators (Benirshcke *et al.,* 1980, Sawyer-Steffan *et al.,* 1983, Kirby and Ridgeway 1984, Combelles 1995, Atkinson *et al.,* 1999, Robeck *et al.,* 2005). The present results did not provide additional evidence to support the beluga as a facultative-induced ovulator. Technical inconsistencies

(i.e., analytical vs immunological methods; Druart *et al.,* 2013) and potential confounding biological factors (e.g., seasonality; Robeck *et al.,* 2009, Steinman *et al.,* 2012) indicate a need for future studies to utilize alternative approaches (e.g., gel-free proteomics) with appropriate collection times to detect, identify, and quantitate seminal plasma proteins in potentially low abundance in belugas and other odontocetes. The practical implications of characterizing the proteome of seminal plasma proteins has enormous value for selecting a suite of potential biomarkers that can be used to improve semen preservation (Jonakova *et al.,* 2010, Mogielnicka-Brzozowska and Kordan 2011) or enhance clinical diagnosis/prognosis of reproductive health (Drabovich *et al.,* 2014) for conservation purposes in cetaceans.

CONCLUSION

The novelty of these preliminary results involving seminal plasma in belugas and Pacific white-sided and bottlenose dolphins using SDS-PAGE provides a rational foundation for more comprehensive studies to evaluate the apparent diversity of proteins in the seminal plasma among odontocetes for basic and applied purposes. Although Western immunoblot analysis failed to detectβ β-NGF in belugas and Pacific white-sided and bottlenose dolphins as indicative of an OIF, the results are not conclusive that the beluga is not a facultative-induced ovulator. Future studies are required to address the apparent limitations of immuno-detection of NGF, especially if the post-translational form of β-NGF is in low abundance in the seminal plasma of odontocetes and, perhaps, other cetaceans.

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