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Short term cold storage and sperm concentration assessment of lumpfish (*Cyclopterus lumpus*. L) milt.

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Abstract

There is increased commercial interest in the production of lumpfish (*Cyclopterus lumpus*) as a biological control against sea lice infections in Atlantic salmon farming. To ensure a sustainable supply of lumpfish, reliable captive reproduction is required however, optimal husbandry and conditions for captive broodstock performance remain unknown. Artificial fertilisation of gametes remains the preferred management strategy for lumpfish, but this requires an effective milt management protocol. The present study tested several milt extender solutions for short term cold storage and validated sperm concentration assessment of lumpfish milt. Results demonstrated lumpfish sperm has a long motility survival time (motile for up to 3 hours) when compared to other marine teleosts. Importantly, all extenders used in the study were non-activating on dilution of the milt. Lumpfish milt was successfully stored at 4 °C in Modified Turbot Extender (MTE), Herring Ringers Solution and Aquaboost SpermCoat (Cryogenetics, Norway) for up to 14 days post stripping. MTE performed more effectively with regards to maintenance of sperm activation time in comparison to the other tested extenders. There was a significant positive correlation between sperm concentration identified through cell counts using a haemocytometer and both packed cell volume (spermatocrit) and measured optical density at 640 nm (r^2= 97.42 ± 2.14). This suggests both packed cell volume and spectrophotometric measurements can be effective methods for rapidly assessing sperm concentration in lumpfish. This study validated several options for quantifying sperm concentration and short-term cold storage of lumpfish milt that can be used for hatchery management in lumpfish aquaculture.

Key Words: Lumpfish, Cleaner fish, Milt extenders, Sperm motility, Sperm concentration, Spermatocrit.

1. Introduction

The Lumpfish (*Cyclopterus lumpus*) is a sub-Arctic species found on both sides of the North Atlantic (60 °E and 90 °W) (Davenport, 1985). They are commonly found along the Icelandic, Norwegian and British coastlines as well as the East coast of North America, between 41 °N and 70 °N (Davenport,1985). Lumpfish efficacy at delousing salmon was first reported by Imsland et al. (2014). Since then lumpfish have become an integral component of the strategy against sea lice in the salmon industry, opening a new aquaculture sector supplying juveniles for deployment in salmon farms (Treasurer, 2018). The current supply chain is reliant on wild caught broodstock to meet the increasing demand for juveniles however this capture tonnage is low in the context of the roe fishery, approximately 0.05 % of the 15,000 tonne annual harvest (Kennedy, 2018). In Norway alone, 30 million juvenile lumpfish were deployed in salmon farms in 2017 (Mortensen et al., 2020) with 1.9 million lumpfish in the UK in 2016 (Brooker et al., 2018) and worldwide, production is due to meet the forecast to exceed 50 million by 2020 (Powell et al., 2018). Given this increasing demand, there is currently a push for closing the life cycle of this species to enable captive breeding which would be considered more sustainable. This requires the development and validation of reliable hatchery production protocols including the effective management of gametes prior to artificial fertilisation which is the preferred production method. Collection of lumpfish milt is performed post-mortem, as stripping is difficult with at best very small volumes collected (Norberg et al., 2015). Because of this, males could be considered a limiting resource unless effective milt storage methods can be

validated to expand the functional window that male gametes would be available for use in a hatchery setting.

Both cold storage and cryopreservation can be used for milt preservation in aquaculture (Migaud et al., 2013). Cold storage requires storing milt diluted in extenders at low temperatures (typically 4 °C, (Gallego and Asturiano., 2019)) to reduce spermatozoa metabolism allowing them to be stored from 4 days in turbot, *Scophthalmus maximus* (Chereguini et al., 1997) to 56 days in cod, *Gadus morhua* (Degraaf and Berlinsky, 2004) without significant changes in milt quality (Chang, 2002). For longer term storage, between spawning seasons (Scott and Baynes, 1980) or for creating genetic storage banks (Gausen, 1993), cryopreservation is the only effective method, keeping milt diluted in a cryoprotectant solution at ultralow temperatures between -79 and -196 °C in liquid nitrogen. This method requires specific infrastructure to enable a precise freeze and thawing of the milt. Cryopreservation of lumpfish milt has been shown to be effective in a pilot study by Norberg et al. (2015), however the authors acknowledged that the protocol, while effective, still requires optimisation in several key areas.

Practically, cold storage of milt is the most useful technique available to support hatchery production by providing a low cost and technically simple solution to the challenge of male availability. It reduces the frequent collections from males, it enables transportation of milt to distant locations (Cabrita et al., 2008) and extends the functional window of availability to allow planned crosses of selected individuals (Jenkins-Keeran and Woods, 2002). For this reason, methods have been developed in a range of marine species like cod (DeGraaf and Berlinsky, 2004) and Atlantic halibut, *Hippoglossus hippoglossus* (Babiak et al., 2006). However, effectiveness of extenders can be very species specific due to the differences in the biochemical

composition of their seminal fluid (Beirao et al., 2019). The composition of the extender solution is an important factor impacting on storage time of the milt (Gallego and Asturiano., 2019). Some extenders are applicable to a range of species, such as Mounib's solution (Mounib, 1978). However, for more effective storage, species specific extender solutions have been developed to mimic their milt compositions, osmolarity, pH and dilution ratio (Beirao et al., 2019). Effective extenders such as Herring Ringers solutions (Pillai et al., 1994), and Modified Turbot Extender (Babiak et al., 2006), are easy to formulate in hatcheries and are easy to adapt to new species.

The purpose of chilled storage is to allow farms to perform artificial fertilisation in a controlled manner using desirable males. Artificial fertilisation protocols must be standardised, and gamete quality assessed when crosses are made (Beirão et al., 2019). Simple and accurate methods for milt quantification are important in this context for two reasons. Firstly, it allows standardisation of egg to sperm ratios which have been shown to influence fertilisation success in many species including turbot (Suquet et al., 1995), and Atlantic Halibut (Tvedt and Benfey, 2001). Secondly, it enables the quantification of the total number of sperm being held in storage which allows farms to accurately plan the volume of eggs that can be fertilised (Cabrita et al., 2014). Absolute sperm counts using either a haemocytometer (Suquet et al., 1992) or Computer Aided Sperm Analysis (CASA) (Kime et al., 2001) are used to determine sperm concentration, with the former being the most common but time consuming, while the latter requires specialised microscopy capacity on farms. Alternative indirect estimation methods are possible based on the relationships between spermatocrit (packed cell volume) or spectrophotometric estimation of sperm concentration and haemocytometer cell counts (Tvedt and Benfey, 2001, Rideout et al., 2004). These methods are typically rapid to perform and utilise equipment commonly found in

commercial hatcheries. Assessments of sperm quality often relies on subjective assessments to quantify percentage sperm motility, or provide an evidence-based end point when sperm are deemed non-viable (Van der Horst et al., 1980; Jenkins-Keeran & Woods III, 2002) While these methods are less informative than CASA based assessments, they remain the most frequently used indicators of sperm quality in commercial fish hatcheries (Migaud et a., 2013; Valdebenito et al., 2015).

As for many other farmed fish species, artificial fertilisation will be the main production strategy used within commercial lumpfish hatcheries not least because this will enable selective breeding and the possibility of selective enhancement of captive stocks (Houston et al. 2020). Short-term storage of lumpfish milt and the lack of effective management of gametes during artificial fertilisation are two key knowledge gaps that need to be addressed in the optimisation of lumpfish hatchery management (Powel et al., 2017). Therefore, the purpose of this study was to test a range of extenders for cold storage of lumpfish milt and validate rapid and accurate methods for estimating sperm concentration both of which are basic requirements to improve artificial fertilisation protocols to be applied in commercial hatcheries.

2. Materials and Methods

2.1 Lumpfish broodstock

A total of 17 sexually mature males were sampled from a captive broodstock held at Otter Ferry Seafish Ltd, Argyll, Scotland. Prior to sampling fish were held on an altered temperature regime (from hatch, 9.4 °C ± 0.8 °C), as recommended by Pountney et al. (2020), with holding temperature not exceeding 10 °C to assure good gamete quality. Lighting was maintained at a low intensity 24 hr photoperiod for the entire grow out period and fish were fed ad libitum a commercial pelleted feed (Samaki Marine Pellet, World Feeds, James A Makie (agricultural), UK). Males initiated sexual maturation from 17 months post hatching in January 2019. Mean weight of males used in this study was 638.3 ± 188.4 g and mean total length was 228 ± 17 mm.

2.2. Sampling

Males were killed using an overdose of anaesthetic (MS222, Pharmaq, UK) followed by destruction of the brain. Post mortem, testes were dissected out, weighed (± 0.01 g) and gonadosomatic index (GSI) calculated, before testes were macerated and then placed into fine mesh to strain out milt which was gathered into a petri dish where the volume of milt (± 0.5 ml) was measured using a 1 ml syringe (Fisherbrand, Thermo Fisher Scientific, USA).

For each male, packed cell volume (spermatocrit) calculated as: ((length of cells/ length of cells and fluid) ×100) was measured in triplicate using non-heparinised haematocrit tubes (Bris, Modulohm A/S, Denmark) which had been centrifuged for 3 min at 4000 g using a Micro Haematocrit centrifuge (MSE,UK).

A 1:1000 dilution of milt was made using a commercial milt extender (SpermCoat, Cryogenetics, Norway) and three replicate counts were made in a haemocytometer (Hirschmann, Germany) using an Olympus microscope (Olympus optical, UK) to calculate sperm concentration (sperm per ml of milt). A minimum of 100 grids of 0.25 nl were counted to obtain the average cell count, which was calculated as *Sperm per ml = (((Total count/ 100) × 4) ×10^6).*

2.3 Cold storage experiment

Five different milt extender solutions, which had previously been reported as being effective in other marine species, were tested: Modified Turbot Extender (MTE)(Babiak et al., 2006), Herring Ringers Solution (HRS)(Pillai et al., 1994), Mounib's solution (Mounib, 1978), and Mounib's with a 1 % BSA inclusion, both of which have been previously tested for cryopreservation of lumpfish milt (Nordberg et al., 2015), and Spermcoat, a commercially available milt storage solution (Cryogenetics, Norway) (Table 1). Milt was obtained from 7 males and 1:5 stock dilution (based on commonly identified effective dilution ratios (Beirão et al., 2019)) was created for each extender solution (320 µl of extender and 80 µl of milt) in triplicate wells within 46 well, micro-well plates (Starstedt, USA) which were seam sealed and placed in a fridge (4 °C) between activation tests.

A standardised activation test was performed in triplicate for all samples in a temperature-controlled room (10 °C). A dilution of 1:1000 (milt: activating solution (seawater +1 % BSA)) was created (*n.b.* this equates to 1:200 milt and extender: activation solution) in a 2 ml eppendorf (Eppendorf, Germany). Activated spermatozoa samples were flooded into a haemocytometer well and swimming activity observed

under a microscope. Motility survival time (duration of sperm motility) was measured using a stopwatch and was defined as from the point of activation to the time at which linear movement of spermatozoa were observed to stop similar to the end point used in Jenkins-Keeran & Woods III (2002). Motility survival time tests were conducted every 7 days until milt was determined as non-activating at 21days, milt was re-tested at day 22 to confirm non activation.

2.4 Spectrophotometric assessment of sperm concentration

To validate the calculation of sperm concentration from optical density, milt was extracted from six males using the method described previously. Milt was then diluted 1:1 in MTE, in three separate aliquots per individual and held in cold storage (4 °C) prior to further manipulation. For each male nine serial dilutions using MTE were made in triplicate (1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10000). Three replicate counts were made using a haemocytometer to obtain the average number of sperm per ml for each dilution. A minimum of 100 grids of 0.25 nl were counted to obtain the average cell count for each dilution, which was calculated as sperm per ml using the following equation $Sperm\ per\ ml = (((Total\ count/\ 100) \times 4) \times 10^6)$. Absorbance was measured at 10 nm intervals between wavelengths ranging from 350 nm to 740 nm using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, USA) with a 1 cm path length cuvette (Fisher Scientific, USA) in triplicate for each milt and extender dilution.

2.5 Statistics

All data is expressed as mean ± standard error, unless stated otherwise. Statistical analysis was conducted using Minitab18 software. A General Liner Model (GLM) was conducted to test for effects of treatment on milt motility survival times, with a post-hoc pairwise Tukeys test used to assess differences between treatments and time.

3. Results

3.1 Experimental animals and milt characteristics

The 17 males processed displayed a mean GSI of 3.5 ± 1.1 % and a mean volume of 5.1 ± 3.6 ml of milt was collected. Sperm concentration was $12.37\times10^9 \pm 2.41\times10^9$ sperm.ml^{-1}, with an average spermatocrit of 87.8 ± 5.7 %. A significant positive relationship between sperm concentration and spermatocrit ($p>0.001$, $r^2=91.8$) was observed (Figure 1). However, no relationship was observed between either sperm concentration or spermatocrit and the sampled males GSI or volume of milt recovered (data not shown). Similarly, there was no relationship between GSI and volume of milt recovered (data not shown).

3.2 Cold storage experiment

Preliminary testing confirmed that none of the five extenders activated sperm on contact. Following activation, fresh lumpfish spermatozoa remained active for 03:00 ± 00:20 (hh:mm) and no significant difference was found between fresh milt and milt diluted first in the extenders on the day of stripping (Figure 2). Following 7 days of cold storage, spermatozoa stored in Mounib's solutions (with and without BSA inclusion) were not motile following activation and activation time decreased significantly in milt diluted with all other extender solutions ($p>0.001$). Spermatozoa stored in MTE, HRS and Spermcoat displayed active swimming response following one week of storage, however they showed a 26.8 %, 50.5 % and a 54.4 % reduction in motility survival time, respectively. Motility survival time at 7 days was significantly higher in milt stored in MTE than for Spermcoat but not HRS. Following 14 days in the extenders the MTE, HRS and Spermcoat treatments were statistically comparable ($P = 0.064$) with all

showing a further significant reduction in motility survival time representing a 79.8 %, 98.8 % and 93.3 % reduction from the point of collection respectively. At 21 days, sperm could not be activated for any of the extender solutions.

3.3 Spectrophotometric assessment of sperm concentration

The spectrophotometric assessment of sperm concentration was performed using MTE, the extender which was shown previously to give the best storage performance. The absorbance spectrum for lumpfish milt diluted in MTE typically shows a steady decrease in optical density from 400 to 700 nm in wavelength (Figure 3). The linear relationship between sperm concentration (as measured by haemocytometer) and optical density, was tested for each male dilution curve at 10 nm intervals between 350 and 740 nm. All dilutions of milt in MTE at 1:20 and 1:50 milt to extender ratio produced measurements outside of the working range of the spectrophotometer and were therefore excluded. Dilution of milt in MTE at 1:10000 milt to extender ratio produced measurements outside the working range of the spectrophotometer at wavelengths greater than 660 nm, as a result the analysed wavelength range was restricted between 350 and 660nm (Figure 4). Within this range the 640 nm wavelength produced the highest average r^2 value (97.42%) of 6 male milt dilution curves, with the smallest deviation (± 2.14 (SEM)) between individual regressions (Figure 5).

4. Discussion

Reproductive management of captive lumpfish requires manipulation of gametes in order to improve stock management (Treasurer, 2018). At present, gamete collection requires sacrificing males which is a limiting factor for production (Powell et al., 2018). As a result, effective milt management is required. The present study aimed to test extender solutions for short term storage of lumpfish milt and provide a rapid and accurate test for sperm concentration which can be conducted in a farm setting.

This study assessed milt quality by measuring the motility window of sperm defined as the period during which sperm were able to move linearly (Jenkins-Keeran & Woods III, 2002). Motility survival time of lumpfish sperm (≈3 hours) is unusually high compared to many other marine teleosts such as Atlantic halibut (63 – 155 seconds, Tvedt and Benfey, 2001) and turbot (160 seconds, Suquet et al 1992). However, long motility survival times of sperm have been reported also in sterlet (*Acipenser ruthenus*) with sperm motility maintained for 5-6 hours (Dzyuba et al., 2012). Authors suggested this may be due to mixing of urea and seminal fluid upon release whereby sperm is activated at the point of release from the fish. The same may be true for lumpfish but there is no evidence yet available to support this. Importantly, the extended window of sperm motility does not always fully reflect differences in sperm quality as motility itself in terms of velocity matters and this does not always correlate with duration of motility (Valdebenito et al., 2015). Future studies should seek to more clearly define lumpfish sperm quality criteria utilising methods like CASA where possible.

Dilution of milt in extenders can effectively improve the lifetime of the milt over the spawning season. This can allow for more effective stock management; however, extender solutions effectiveness can vary significantly between species (Beirão et al.,

2019). In this study the efficacy of five different milt extenders, commonly used in other temperate marine species, was tested. Three extenders (*i.e.* Herring Ringers, Modified Turbot Extender and Spermcoat) significantly extended the life of captive lumpfish milt up to a minimum of 14 days post stripping. Despite the fact Mounib's solutions were shown to be effective cryopreservants (Norberg et al., 2017), the two Mounib's solutions tested in the present study did not appear to extend the window of viable milt availability. This may suggest that Mounib's solution does not match the composition of lumpfish seminal fluid for short-term cold storage. Osmolality of lumpfish milt has been reported at 463 mOsm/kg (Norberg et al. 2015) therefore it is possible that the additional hypo osmotic stress the spermatozoa will have experienced in the Mounib's solutions precluded it from being an effective extender solution, but has a lesser impact when utilised in cryopreservation. In addition, there was no significant difference between the motility survival times of milt diluted in MTE and HRS at any time point and while both solutions differ greatly in their chemical constituents they have similar osmolality and pH to that previously reported in lumpfish (Pillai et al., 1994, Vermeirssen et al., 2004; Norberg et al., 2015). In addition, differences in ion presence reflective of the formulation difference could account for changes in effectiveness between MTE and HRS, and the lack of effectiveness in Mounib's solution as seen in other species (Alavi et al., 2007). These compositional changes warrant further investigation in subsequent optimisation for Lumpfish. Spermcoat displayed significantly lower motility survival time compared with the MTE at 7 days, but not at any other time point. The recommended dilution ratio for Spermcoat was not used in this study (1:1) in order to maintain consistency with the 1:5 ratios with the other extenders. However it was still effective at storing milt to the expected 14 days according to the supplier. This study showed that MTE was the most effective extender

at 7 days post stripping, and still displayed a lower degradation in motility survival time (79.8%) compared to 93 – 100 % for all other treatments at 14 days. As such this study finds that there are three available milt extenders which can effectively store lumpfish milt for up to 14 days, MTE, HR and Spermcoat. Due to its lower degradation at 7 days, and 14 days this study continued to use MTE for the remainder of the work. Future work in the species should aim to optimise extender chemical composition for effective short term storage of lumpfish milt.

Sperm concentration reported for lumpfish in the current study are two orders of magnitude lower than those reported in Atlantic Halibut (2 - 6×10^{11} spermatozoa/ml) (Tvedt et al., 2001), however they appear to be in line with those reported in other marine teleosts such as sea bass *Dicentrarchus labrax* (4 – 6 $\times10^{10}$ spermatozoa/ml) (Fauvel et al., 1999) and Cod ($1.33\times10^{8} \pm 14.5\times10^{8}$ spermatozoa/ml) (DeGraaf and Berlinsky, 2004). Sperm concentrations measured in the current study support previously published data by the same authors (Pountney et al., 2020), however these appear to be subtly lower than previously reported data for wild caught fish ($31.44\times10^{9} \pm 8.35\times10^{9}$ sperm ml^{-1}) (Nordberg et al., 2015). Differences observed in sperm concentration could be explained by the methods used to collect milt; stripping in Norberg et al. (2015) compared to extraction of milt from macerated testis in the current study and that of Pountney et al. (2020). The current study also analysed spermatocrit in captive lumpfish and reported a packed cell volume (76% to 93.5%) which was more consistently at the higher limit than is reported in other temperate species in captivity such as Atlantic halibut (23-97 %, Tvedt et al., 2001), Atlantic cod (18-98.3 %, Rakitin et al., 1999) and common wolfish *Anarhichas lupus* (0.5-5.5 %, Tveiten and Johnson,. 1999). This could explain the difficulty in stripping male lumpfish and future work could focus on hormonal manipulations to increase milt production

and make stripping a viable option in lumpfish, as shown in Atlantic halibut (Vermeirssen et al., 2004).

Accurately assessing gamete quality is critical in broodstock management and optimising hatchery productivity (Gallego and Asturiano., 2019). There are several common methods for assessing sperm concentration in fish milt including cell counts using a haemocytometer, packed cell volume (spermatocrit), optical density measurements using a spectrophotometer and Computer Aided Sperm Analysis (CASA) (Kime et al., 2001). While CASA is the “gold standard” for sperm quality assessment, it is infrequently used in a hatchery setting due to the requirement for specialised equipment. While cell counts are precise and reliable, they are very time consuming and can be impractical in both a hatchery and lab setting, for example Suquet et al (1992) suggested that to reach an acceptable level of variation it could take 2 hours to assess one fish. Spermatocrit has been successfully used as an effective method of measuring sperm concentration in a range of species (Campbell et al., 1992; Suquet et al., 1995; Gallego et al., 2013) where there is a strong relationship between packed cell volume and sperm concentration. Equally spectrophotometry has been effectively used in several marine species as a reliable method for assessing sperm concentration (Fauvel et al., 2010; Rurangwa et al., 2004). In the present study, both spermatocrit and spectrophotometry were confirmed to be an accurate predictor of sperm concentration in the species. When working with raw milt samples, spermatocrit can be used as a rapid method for assessing sperm concentration in lumpfish rather than cell counts using a haemocytometer. However, if the hatchery intends to dilute the milt in an extender then spectrophotometric quantification of sperm concentration can be performed with an equally high level of precision. In the current study utilising MTE as the extender/diluent, the best

correlation between sperm concentration and measured optical density with the smallest individual variation was found when using a wavelength of 640 nm. However, Correlations remained strong (*i.e.* $r^2 > 95$ %) from 560-660 nm and at 540 nm. A wide range of wavelengths are used to assess sperm concentration's in other species, Fauvel et al (1999) assessed wavelengths in Sea Bass between 200 - 500 nm finding the best correlation at 260 nm. While Suquet et al (1992) recommends 420 nm as the optimal measurement for Turbot, having assessed relationships between 350 and 750nm. The high level of variation in absorbances is suggested to be due to compositional changes in the associated fluids rather than the sperm themselves (Suquet et al., 1992, Tvet et al., 2001). In terms of practical application of the method, based on experience during the study the authors would recommend a dilution of 1:500 (milt: MTE) to typically reach final sperm concentrations close to the centre of the linear relationship.

In conclusion, this study demonstrates that lumpfish milt can be effectively stored using extender solutions for up to two weeks. The most effective storage medium found in this study was the Modified Turbot Extender using a 1:5 milt to extender ratio. Sperm concentration can be estimated confidently either directly on fresh milt samples using spermatocrit (concentration $(\times 10^9) = 0.4076 \times Spermatocrit(\%) - 23.742$) or with milt samples diluted in MTE using optical density measured at 640 nm (concentration $(\times 10^9) = 3 \times 10^8 \times Optical\ density - 2 \times 10^6$) enabling more standardised and effective use of milt during artificial fertilisation. This work is an important step in generating reliable gamete handling protocols that will play a key role in advancing hatchery management and domestication of lumpfish.

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References

Alavi, S. M. H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., & Linhart, O. (2007). Semen of *Perca fluviatilis* L.: Sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology, 68(2), 276-283.

Babiak, I., Ottesen, O., Rudolfsen, G., & Johnsen, S. (2006). Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L.: I: Optimizing the protocol. Theriogenology, 66(9), 2025-2035.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck, T. R., & Cabrita, E. (2019). Sperm handling in aquatic animals for artificial reproduction. Theriogenology, 133, 161-178.

Brooker, A. J., Papadopoulou, A., Gutierrez, C., Rey, S., Davie, A., & Migaud, H. (2018). Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges. *Veterinary Record*, *183*.

Cabrita, E., Robles, V., & Herráez, P. (Eds.). (2008). Methods in reproductive aquaculture: marine and freshwater species. (219-236) CRC press.

Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., Herráez, M. P. & Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture, 432, 389-401.

Campbell, P. M., Pottinger, T. G., & Sumpter, J. P. (1992). Stress reduces the quality of gametes produced by rainbow trout. Biology of reproduction, 47(6), 1140-1150.

Chang, Y. J., Chang, Y. J., Lim, H. K., Lee, J. K., & Park, Y. J. (2002). Cold storage of milt from four species of flatfish. *Journal of Fisheries Science and Technology*, *5*(1), 64-74.

Chereguini, O., Cal, R. M., Dreanno, C., de Baulny, B. O., Suquet, M., & Maisse, G. (1997). Short-term storage and cryopreservation of turbot (*Scophthalmus maximus*) sperm. Aquatic Living Resources, 10(4), 251-255.

Davenport, J. (1985). Synopsis of biological data on the lumpsucker*, Cyclopterus lumpus (Linnaeus, 1758)* (No. 147). Food & Agriculture Org.

Dzyuba, B., Boryshpolets, S., Shaliutina, A., Rodina, M., Yamaner, G., Gela, D., Dzyuba, V & Linhart, O. (2012). Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet Acipenser ruthenus during sequential stripping. Aquaculture, 356, 272-278.

DeGraaf, J. D., & Berlinsky, D. L. (2004). Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. Aquaculture, 234(1-4), 527-540.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., & Suquet, M. (1999). Characteristics of sperm of captive seabass in relation to its fertilization potential. Journal of Fish Biology, 54(2), 356-369.

Fauvel, C., Suquet, M., & Cosson, J. (2010). Evaluation of fish sperm quality. Journal of Applied Ichthyology, 26(5), 636-643.

Gallego, V., Pérez, L., Asturiano, J. F., & Yoshida, M. (2013). Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture, 416, 238-243.

Gallego, V., & Asturiano, J. F. (2019). Fish sperm motility assessment as a tool for aquaculture research: a historical approach. Reviews in Aquaculture, 11(3), 697-724.

Gausen, D. (1993). The Norwegian gene bank programme for Atlantic salmon (*Salmo salar*). In Genetic conservation of salmonid fishes (pp. 181-187). Springer, Boston, MA.

Houston, R.D., Bean, T.P., Macqueen, D.J., Gundappa M.K., Jin Y.W., Jenkins T.L., Selly, S.L.C., Martin, S.A.M, Stevens, J.R., Santos, E.M., Davie A. & Robledo D. (2020) Harnessing genomics to fast-track genetic improvement in aquaculture. Nature Reviews Genetics. https://doi.org/10.1038/s41576-020-0227-y

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Nytrø, A. V., Foss, A., Vikingstad, E & Elvegård, T. A. (2014). Assessment of growth and sea lice infection levels in Atlantic salmon stocked in small-scale cages with lumpfish. *Aquaculture*, *433*, 137-142.

Jenkins-Keeran, K., & Woods III, L. C. (2002). An evaluation of extenders for the short-term storage of striped bass milt. North American Journal of Aquaculture, 64(4), 248-256.

Kennedy, J. (2018). Oocyte size distribution reveals ovary development strategy, number and relative size of egg batches in lumpfish (*Cyclopterus lumpus*). Polar Biology, *41*(6), 1091-1103.

Kime, D. E., Van Look, K. J. W., McAllister, B. G., Huyskens, G., Rurangwa, E., & Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 130(4), 425-433.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., and Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture 5, 1-30.

Mortensen, A., Johansen, R. B., Hansen, Ø. J., & Puvanendran, V. (2020). Temperature preference of juvenile lumpfish (*Cyclopterus lumpus*) originating from the southern and northern parts of Norway. Journal of Thermal Biology, 102562.

Mounib, M. S. (1978). Cryogenic preservation of fish and mammalian spermatozoa. Reproduction, 53(1), 13-18.

Norberg, G., Johannesen, A., & Arge, R. (2015). Cryopreservation of lumpfish *Cyclopterus lumpus* (Linnaeus, 1758) milt. PeerJ, *3*, e1003.

Pillai, M. C., Yanagimachi, R., & Cherr, G. N. (1994). In vivo and in vitro initiation of sperm motility using fresh and cryopreserved gametes from the Pacific herring, *Clupea pallasi*. Journal of Experimental Zoology, 269(1), 62-68.

Pountney, S. M., Lein, I., Migaud, H., & Davie, A. (2020). High temperature is detrimental to captive lumpfish (*Cyclopterus lumpus*, L) reproductive performance. Aquaculture, 522, 735121.

Powell, A., Treasurer, J. W., Pooley, C. L., Keay, A. J., Lloyd, R., Imsland, A. K., & Garcia de Leaniz, C. (2018). Use of lumpfish for sea-lice control in salmon farming: challenges and opportunities. Reviews in aquaculture, *10*(3), 683-702.

Rakitin, A., Ferguson, M. M., & Trippel, E. A. (1999). Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. Aquaculture, 170(3-4), 349-358.

Rideout, R. M., Trippel, E. A., & Litvak, M. K. (2004). Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. Journal of Fish Biology, 65(2), 319-332.

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture, 234(1-4), 1-28.

Scott, A. P., & Baynes, S. M. (1980). A review of the biology, handling and storage of salmonid spermatozoa. Journal of Fish Biology, 17(6), 707-739.

Suquet, M., Omnes, M. H., Normant, Y., & Fauvel, C. (1992). Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture, 101(1-2), 177-185.

Suquet, M., Billard, R., Cosson, J., Normant, Y., & Fauvel, C. (1995). Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture, 133(1), 83-90.

Treasurer, J. (2018). Cleaner Fish Biology and Aquaculture Applications . Sheffield: 5M Publishing. 122-146.

Tvedt, H. B., Benfey, T. J., Martin-Robichaud, D. J., & Power, J. (2001). The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture, 194(1-2), 191-200.

Tveiten, H., & Johnsen, H. K. (1999). Temperature experienced during vitellogenesis influences ovarian maturation and the timing of ovulation in common wolffish. Journal of Fish Biology, 55(4), 809-819.

Van der Horst, G., Dott, H. M., & Foster, G. C. (1980). Studies on the motility and cryopreservation of rainbow trout (Salmo gairdneri) spermatozoa. South African Journal of Zoology, 15(4), 275-279.

Vermeirssen, E. L., de Quero, C. M., Shields, R. J., Norberg, B., Kime, D. E., & Scott, A.P. (2004). Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotrophin-releasing hormone agonist implant. Aquaculture, 230(1-4), 547-567.

Valdebenito, I. I., Gallegos, P. C., & Effer, B. R. (2015). Gamete quality in fish: evaluation parameters and determining factors. Zygote, 23(2), 177-197.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., & Vilella, S. (2004). Adenosine triphosphate concentration and β-D-glucuronidase activity as indicators of sea bass semen quality. Biology of reproduction, 70(6), 1679-1684.

Table 1: Chemical composition of the four milt extender solutions prepared and tested in this experiment. No composition data is publicly available for commercial extender tested in this study (Spermcoat, Cryogenetics, Norway). All chemicals were acquired from Sigma Aldrich (Sigma, USA).

	Modified Turbot Extender	**Herring Ringers solution**	**Mounib's solution**	**Mounib's +BSA solutions**
NaCl	4.0908 gL^{-1}	12.0386 gL^{-1}	-	-
KCl	0.1118 gL^{-1}	0.5367 gL^{-1}	-	-
$CaCl_2$	0.2996 gL^{-1}	0.2331 gL^{-1}	-	-
$MgCl_2$	0.5807 gL^{-1}	0.3141 gL^{-1}	-	-
$NaHCO_3$	2.1002 gL^{-1}	0.0840 gL^{-1}	-	-
$KHCO_3$	-	-	1 gL^{-1}	1 gL^{-1}
BSA	10 mgL^{-1}	10 mgL^{-1}	-	10 mgL^{-1}
Sucrose	-	-	42.7875 gL^{-1}	42.7875 gL^{-1}
Glucose	36.032 gL^{-1}	-	-	-
pH	8.1[a]	7.8[a]	7.8[b]	7.8[b]
Osmolarity	400 mOsm/kg[a]	405 mOsm/kg[a]	310mOsm/kg[b]	310mOsm/kg[b]

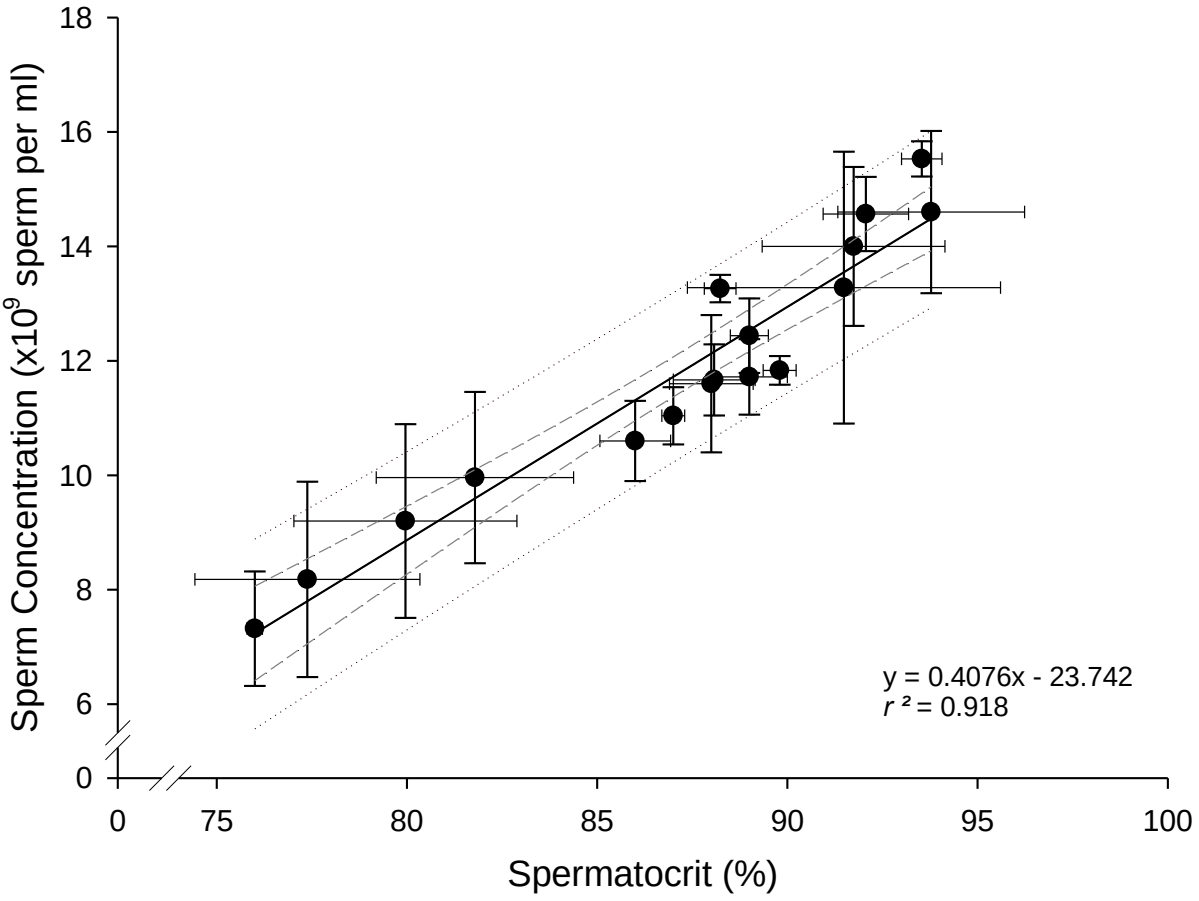
[a] Babiak et al., 2006, [b] Zilli et al., 2004

List of figures

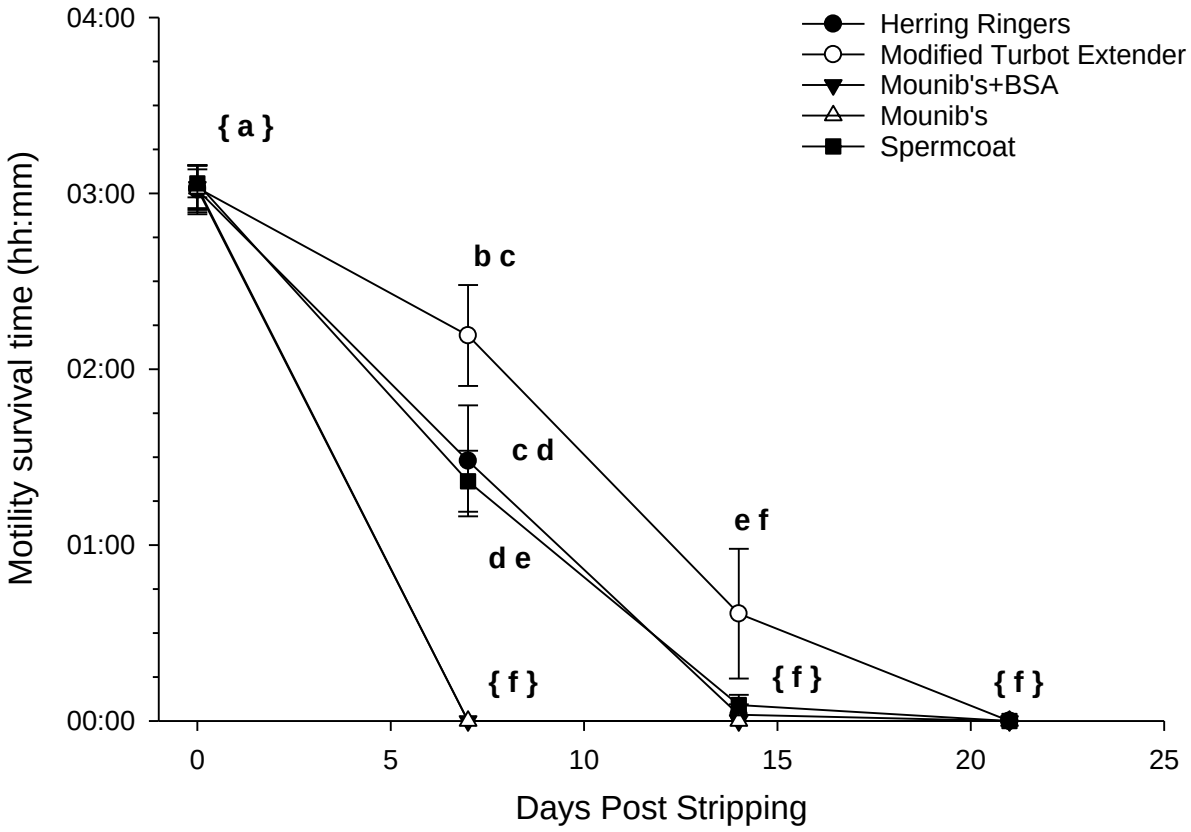
537 regression indicated by the dashed lines and the 95% prediction intervals for novel
538 values indicated by the dotted lines.

539

Figure 1


Sperm Concentration (x10^9 sperm per ml)
Spermatocrit (%)
y = 0.4076x - 23.742
r ² = 0.918
0
6
8
10
12
14
16
18
75
80
85
90
95
100

543 Figure 2



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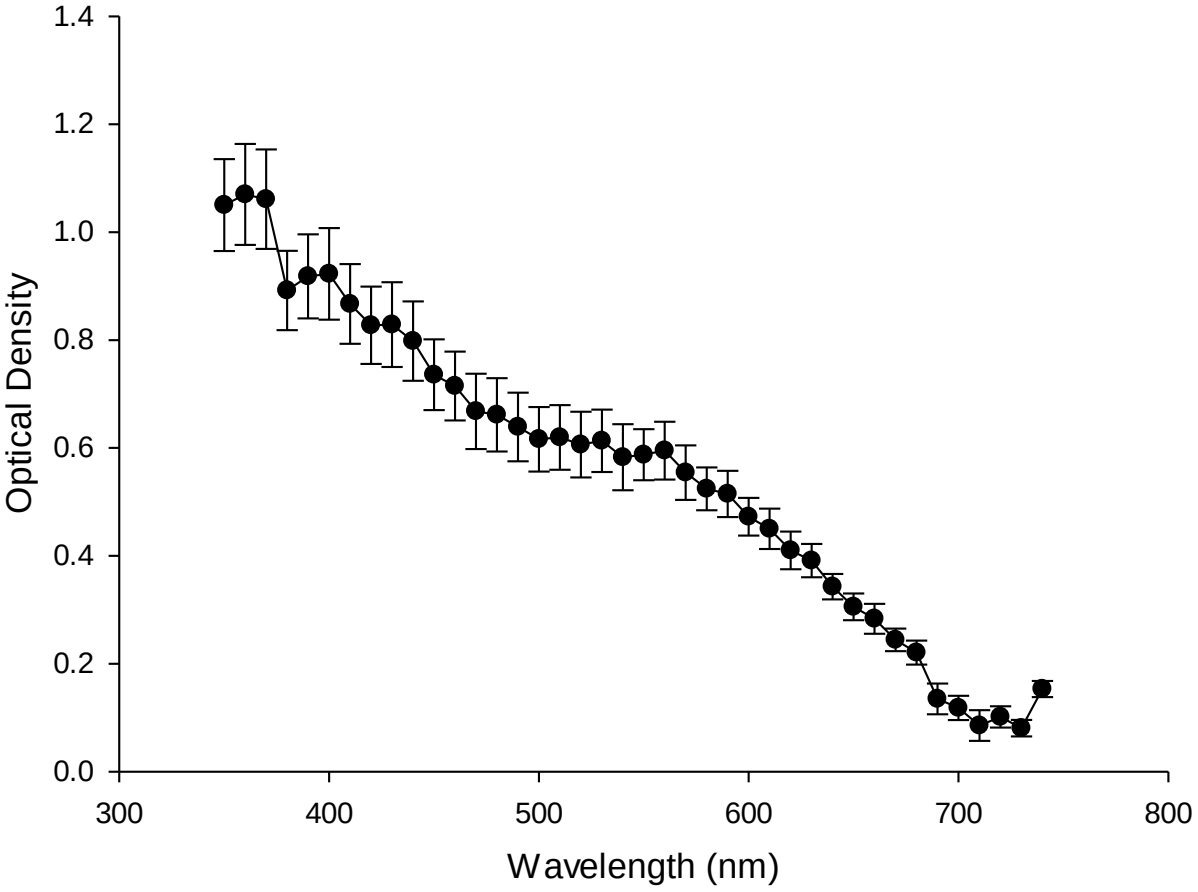
547 Figure 3



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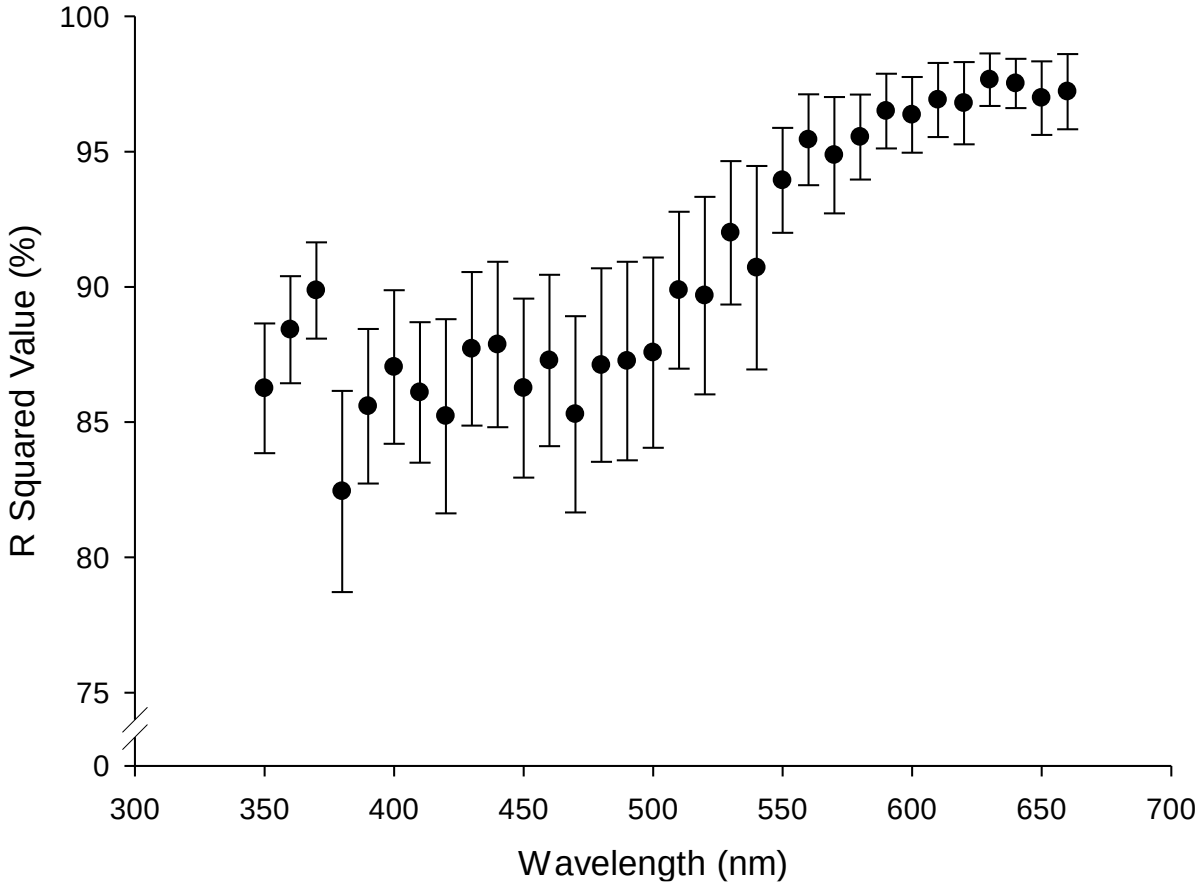
550 Figure 4



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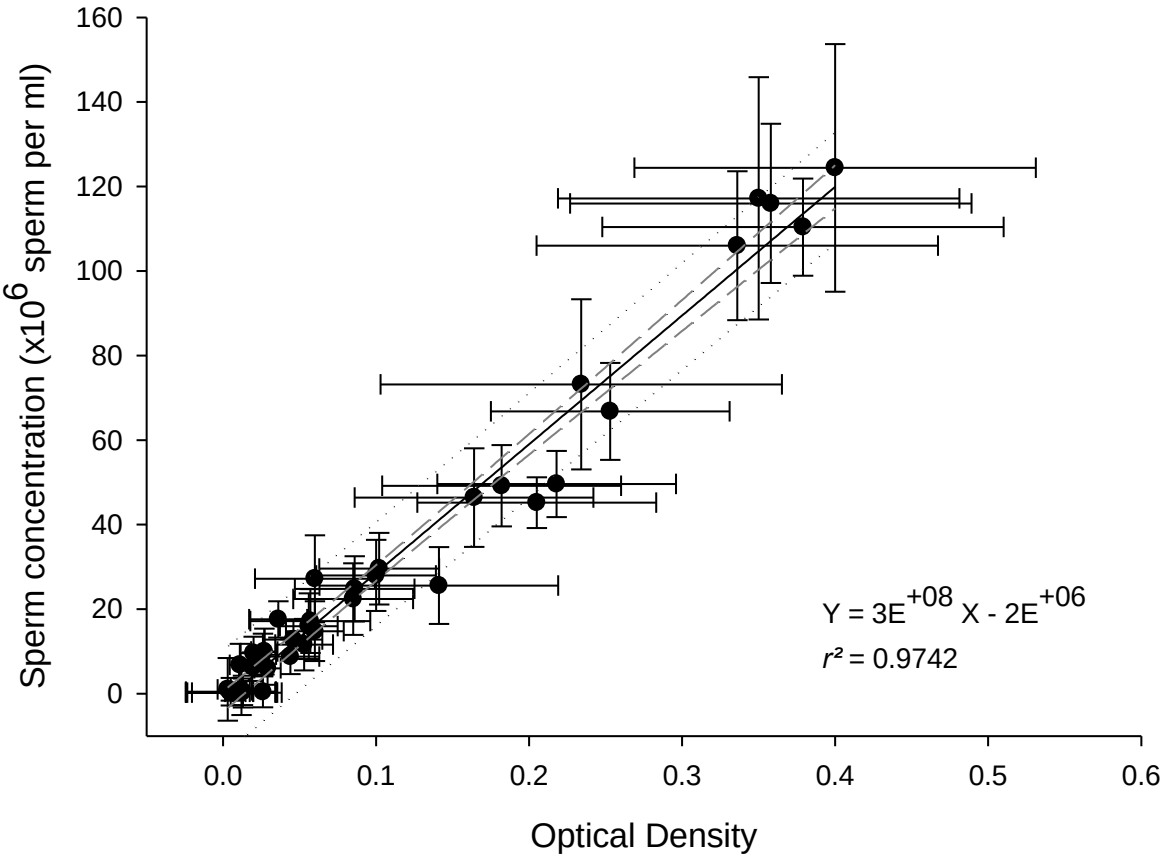
553 Figure 5



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