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High temperature is detrimental to captive lumpfish (*Cyclopterus lumpus*, L) reproductive performance.

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Abstract

There is increased commercial interest in the production of Lumpfish (*Cyclopterus lumpus,* Linnaeus, 1758) as a biological control for sea lice infections in Atlantic salmon farming. To ensure sustainability, reliable captive breeding is required however, optimal husbandry conditions for broodstock performance remain unknown. The present study investigated the effects of holding temperature on spawning productivity and gamete quality in captivity reared lumpfish. Sexually mature lumpfish (15 month old) were held on three temperature regimes (6^{0} C, 9^{0} C and 14^{0} C) from the onset of first spawning. Holding mature lumpfish at high temperatures (14 ^{0} C) resulted in a notable reduction in spawning activity with a significant reduction in sperm density (50% reduction compared to pre-treatment levels) and furthermore resulted in the production of non-viable oocytes (0% to eyeing rate). Holding lumpfish at 9^{0} C and 6^{0} C did not have a similar negative impact on gamete quality, however the spawning season for the 6^{0} C treatment was twice as long as the 9^{0} C treatment. These results indicate that holding temperature for lumpfish broodstock should not reach the 14^{0} C degree threshold, with a possible thermal optimum below 10^{0} C. The current findings are the first step in identifying optimal rearing conditions for captive Lumpfish broodstock.

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Key Words: Lumpfish, Cleaner fish, Broodstock, Temperature, Vitellogenesis, Gamete quality.

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1. Introduction

The Lumpfish (*Cyclopterus lumpus,* Linnaeus, 1758), a pelagic/semi-demersal fish belonging to the family Cyclopteridae (Davenport, 1985), is considered a sub-Arctic species which is widely distributed on both sides of the North Atlantic (60°E and 90°W). It is commonly found along the coastlines of Iceland, Norway, the United Kingdom and the East coast of North America, between 41°N and 70°N (Davenport, 1985). Until recently, the main commercial focus for the species has been fisheries targeting females to harvest the roe (Kennedy et al. 2018). Following demonstration that the species can act as an effective biological control to sealice infections in Atlantic salmon (*Salmo salar*) farms (Imsland, et al., 2014), a new aquaculture sector has emerged, targeting the supply of juveniles to salmon farms (Treasurer, 2018). Being a new species to aquaculture, the current supply chain is reliant on wild caught broodstock to meet the egg demand for hatcheries. In Norway and the UK alone, 17.8 million juvenile lumpfish were deployed in salmon farms in 2016 (Brooker et al., 2018) with this number suggested to exceed 50 million by 2020 (Powell et al., 2017). While the annual harvest of mature brood fish to meet this demand is low in the context of the roe fishery, *circa* <0.05% of the ≈15000 tonne annual harvest (Kennedy et al. 2018), key advantages in moving towards closed life cycle management for this species include biosecurity, predictable egg supply and the potential to improve farmed stocks through genetic selection. The first step in closing the life cycle and securing reliable captive broodstock production is to define optimal environmental conditions for egg productivity and quality.

The lumpfish reaches maturity in the wild after five to six years (Davenport, 1985; Haauft, 2015); however, lumpfish can spawn after one to two years in captivity

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(Imsland, et al., 2014; Powell et al., 2017). Females spawn in shallow coastal waters and have been shown to migrate great distances to spawning sites (Mitamura et al., 2012). Males guard the egg masses prior to hatch and commonly maintain several egg batches from different females in a single location (Davenport, 1985). Female lumpfish remain within the spawning area for a short period of time, possibly moving to alternate spawning areas but then proceed to migrate offshore (Mitamura et al., 2012). There remains a lack of clarity in the natural spawning season in this species, Davenport (1985) described the lumpfish spawning season as occurring between April and July with no geographic reference. More recently, Kennedy (2014) noted that spawning season in Iceland was between January and March, which is the focal window for commercial exploitation. However, fishing for mature lumpfish in central Norway occurs between September and June (Pers. Com, Tor Otterlei Skjerneset Fisk) with the main catch window from October to May, with this fishery being the principle supplier of lumpfish eggs to Norwegian and UK based hatcheries. This is suggestive that reproductive entrainment in the species is not strictly controlled by photoperiod, inferring that temperature may play an important role in regulating reproduction. Interestingly, there are reports that across this broad spawning season there is notable variation in egg quality, with eggs derived from broodstock captured at low temperatures in winter/spring having the highest hatch rates compared to those caught at the start or end of the season when the water temperature was around 14-15 °C.

At present, there is limited published work on the reproductive physiology of lumpfish. Kennedy (2018) has reported oocyte development at the macroscopic level for the species, describing them as being a determinate batch spawning species with

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oocyte development taking up to 8 months. However, the authors acknowledge the limitations in their analysis due to the lack of histological descriptions of ovarian development. Precise histological definitions helped interpreting the impact of management interventions on reproductive development in many other marine fish species including European Seabass (*Dicentrarchus labrax*) (Mayer et al., 1988), Atlantic Cod (*Gadus morhua*) (Kjesbu & Kryvi, 1989) or Common Snook (*Centropomus undecimalis*) (Rhody et al., 2013) for example.

Rearing broodstock within optimal environmental conditions is essential for the reliable production of good quality gametes and subsequent offspring in any commercial hatchery (Migaud et al., 2013). In most marine species, it is apparent that both the seasonally changing day length as well as temperature play important roles in the regulation of reproductive development, timing of the spawning season, productivity and quality (Brooks, et al., 1997). Imsland et al. (2018) suggested that spawning could be influenced by photoperiod, with the simulation of long day to short day returning to long day photoperiod appearing to trigger maturation of lumpfish broodstock. In contrast, while there is anecdotal evidence suggestive of the impact of temperature on spawning in the species, there are no published studies with respect to the impact of temperature on reproductive development and gamete quality for lumpfish. Based on past work in temperate marine species there are two key regulatory aspects of temperature in the context of reproductive development and spawning. During gametogenesis, temperature manipulations can influence the pace of development and the timing of subsequent spawning seasons as demonstrated in the Common Wolfish (*Anarhichas lupus*) (Tveiten & Johnsen, 1999) and to a lesser degree influence fecundity (Kraus et al., 2000) as well as subsequent gamete

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quality, as demonstrated in Cod (Rideout et al., 2000). During the spawning season itself temperature plays a stronger regulatory role, determining the length of the spawning season (Kjesbu,1994; Tveiten et al., 2001), ovulation cycles (Brown et al., 2006) and most importantly gamete quality (Bobe & Labbe 2010; Migaud et al., 2013). While wild survey data suggest that lumpfish occupy a thermal range between 4°C and 15°C, mature adults tend to be associated with the lower end of this range (Mitamura et al., 2012).

This current study was designed to explore the effects of temperature during the spawning season on broodstock performance in captive lumpfish. This study aimed to describe the effects of rearing temperature on timing of spawning and egg productivity, and quality across a thermal range associated with wild mature lumpfish. A secondary objective was to histologically describe oocyte development for the species. Such work provides guidance for optimal rearing of captive broodstock, which is key in realising the industries aspirations to work with closed life cycle management.

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2. Materials & Methods

2.1 Animals and experimental design

All fish used in the experiment were captive reared stock derived from gametes stripped from wild caught parents (3♀:5♂) caught by Skjerneset Fisk at Averøy, Norway and maintained at NOFIMA, AS, Sunndalsora from fertilisation. Prior to the start of the experiment, fish had been maintained at ambient temperature regimes for Sunndalsora, Norway ranging between 5°C in the winter to 13°C in the autumn. They were maintained on a low intensity, 24hr light photoperiod and fed on Skretting Silk (Skretting, Nutreco N.V, Netherlands) pellets at the appropriate size range during grow out. Individual morphometric (weight ±0.1 g and total length ±1 mm), gender and stage of maturity were recorded on the 1st of May 2017 when the stock (*n* = 513) were approximately 15 months old. At this point all fish were tagged with a passive integrated transponder (PIT) tag to enable individual traceability throughout the experiment. Fish were randomly assigned to one of three treatment groups (*n* = 166 -169 per treatment) with a balanced sex ratio at 1♂:2♀.

The three stocks were held in three 7000L tanks, fed to satiation using a commercial formulated feed (Silk 4.5mm, Skretting, Nutreco N.V, Netherlands) at 7.6 ± 0.3°C under 24hr low intensity lighting conditions. Following monitoring of the ovarian development of females (both external assessment of the female swelling and ultrasound scanning of a random selection of individuals), water temperatures were changed to experimental levels between the 1st and 7th of June at a rate of 1 ± 0.5 °C/day. At the time of applying the temperature treatments, stocks were not significantly different in weight, length, maturation score and sex ratio (Table 1). Thereafter, temperatures were kept constant throughout the experimental period (7th

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June – 26th August) being 5.9 ± 0.3 °C, 9.2 ± 0.7 °C and 14.3 ± 0.2 °C, for the low, medium and high temperature treatments respectively (Figure 1).

Following the alteration in temperature all fish were visually inspected every two weeks, when morphometrics were recorded and maturation was assessed both visually and using ultrasound imaging (6.5mHz, Log.Q book XP vet, GE medical systems, USA) of the body cavity. With respect to the ultrasound imaging, female ovarian development was classified using a subjective five point scale; 1.) Immature: Individual with no visible gonads, 2.) Immature: Small gonads, both ovarian lobes are apparent within the image, 3.) Maturing: Significantly enlarged gonads, single ovarian lobe fills the image, at later stages of development individual hydrating oocytes may become apparent within the ovarian tissue (classified 3.5), 4.) Spawning: Significantly enlarged gonads, single ovarian lobe fills the image, free hydrated oocytes apparent on the dorsal region of the ovarian lobe. Following first spawning, open regions filled with ovarian fluid became apparent within the lobe (classified 4.5), 5.) Spent: Ovarian lobes collapsed with small proportion of ovarian tissue left, no free eggs apparent. At the point of inspection if gametes were being freely released this was recorded. In addition, daily inspections of the tanks allowed the recording of spawning events with egg masses collected and weight recorded (±0.1 g).

Following temperature change, on four subsequent samplings (weeks 4, 6, 10 & 13 following temperature change), 17 individuals from each treatment, 10 females and 7 males were sacrificed, with females in the late maturing/spawning category being selected based on the maturation assessment. Following euthanasia, individual

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weight and total length were recorded and the gonads as well as livers were dissected and weighed. Samples of the ovaries were preserved in 10% buffered formalin for later image analysis of oocyte size and histological confirmation of oocyte development. For all males, milt samples were placed on ice for subsequent spectrophotometric assessment of sperm density.

2.2 Histological characterisation of oocyte development

In order to develop a histological scale of oocyte development for the species a total of 28 ovarian samples previously preserved in 10 % neutral buffered formalin were analysed. All fish came from the study population and included samples taken from prior to the study as well as individuals from the first two sampling dates. This pool of individuals had a range of GSI's from 0.93% to 37.4% to capture the diversity of ovarian development. Fixed ovarian samples were embedded in paraffin with 5 µm sections then mounted and stained using haematoxylin (Shannon) and Eosin. Slides were digitised using Axio Scan Z1 slide scanner (Zeiss, Oberkochen, Germany), and photographs were then analysed using digital image analysis (Image Pro Plus, Media Cybernetics, USA).

Oocyte development was classified in accordance with Rhody et al (2013) and were as follows: Primary Growth- The primary growth oocyte stage is characterised by 4 stages; one nucleolus (PGon); multiple nucleoli (PGmn); perinucleolar (PGpn); and oil droplets ending with the appearance of cortical alveoli (PGod). Secondary Growth (Vitellogenesis)- Secondary growth is characterised by active yolk accumulation in the oocytes with it being segregated into three steps: early (SGe), late (SGl), and full-grown (SGfg) in relation to the extent and size of accumulating yolk globules.

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Oocyte maturation (OM) includes three steps: eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm), and preovulatory (OMpov). A minimum of 50 individual oocytes from a minimum of 10 individuals were identified for each developmental stage. Oocyte diameter was measured by digital image analysis and calculated as the average of two diameters perpendicular to each other measured through the nucleus.

2.3 Oocyte size distribution analysis

Oocyte size distribution (OSD) was measured according to a protocol adapted from Kjesbu & Kryvi (1989). Briefly, a digital image (Nikon 1, NIKON, Japan) was taken of dissociated oocytes, with individual oocyte diameter (n =100 oocytes per individual) measured by digital image analysis (Image Proplus, Media Cybernetics, USA). Oocyte diameter of the leading cohort within the population was calculated based on the mean of the largest 10 oocytes following initial imaging and sorting of population size data (Thorsen & Kjesbu 2001). To confirm uniform ovarian development within the species prior to subsequent analysis, OSD was measured in 4 discrete samples (samples excised towards the posterior and anterior end of both ovarian lobes) within six independent females. Both the leading cohort oocyte diameter and oocyte size frequency distributions were compared between the four different lobe sections within each individual.

2.4 Fecundity estimates

The combined gravimetric and automated particle counting method (Murua et al., 2003) was used to estimate individual total fecundity from a random selection of 10 individuals (1614 ± 245.9 g, 275.1 ± 30.9 mm) from pre-treatment sampled females.

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Briefly, for each individual, three weighed samples of ovarian tissue were preserved in 10% neutrally buffered formalin, a digital image (Nikon 1, NIKON, Japan) of dissociated oocytes was taken, with the total number of viteliogenic oocytes (oocytes > 370 μm as determined by previous histological examination of oocyte development) determined by digital image analysis (Image Proplus, Media Cybernetics, USA).

2.5 Gamete quality assessment

Following approximately four weeks of temperature treatments, a gamete quality assessment study was performed. Eggs were stripped from six females from each of the three treatments, with females being selected based on ultrasound assessment of ovarian development to assure they were ready to be stripped and had not previously spawned. For each individual, the egg batch volume was recorded and then eggs were held in chilled storage covered in ovarian fluid prior to the quality test. Males (6 from each treatment) were killed using an overdose of MS222 and the whole testis was dissected, ground, and mixed in a 1:1 ratio with a commercial sperm extender solution (Sperm Coat™, Cryogenetics, Norway). Sperm density was assessed by spectrophotometry and activation in response to seawater was confirmed in an aliquot under a light field microscope. In addition, milt from wild mature male lumpfish (n = 3) was obtained from Skjerneset Fisk at Averøy and processed in the same manner. The gamete quality test was performed in a manner to allow both individual egg viability as well as individual milt viability to be tested. To test individual egg batch viability, each of the six egg batches from the three treatments (n= 18 total egg batches) were fertilised in triplicate by four different pools of milt from the high, medium, low temperature treatments and wild) which contained

equal contributions of milt from 6 individual males under each treatment. To test individual milt sample viability, each of the six individual milt samples from the three treatments (n= 18 total milt samples) were used to fertilise eggs, in triplicate, from the high, medium and low temperature treatments with equal contributions of eggs from the 6 individual females from each respective treatment group. For each replicate test, 1 ml of eggs (*circa* 100 eggs) were wet fertilised with 300,000 sperm per egg in a petri dish using 20 ml of 0.2 µm-filtered seawater. Following fertilisation 20ml of water was exchanged with the further addition of penicillin/streptomycin (Sigma,USA) at 100 units per ml of penicillin and 0.1 mg per ml of streptomycin within the petri dish and these were maintained in a temperature controlled room at 8°C thereafter. Water was exchanged every two days within the petri dishes, with the eggs incubated until 150 °C days at which point the proportion of eyed eggs was recorded.

2.6 Sperm density assessments

Sperm density was assessed just prior to the temperature treatments and at each sampling point thereafter. Gonads were excised from six sampled males at each time point, ground and sieved to collect milt. Milt samples were diluted 1:400 in a physiological salt solution (9 gL^{-1} NaCl in deionized water) in a cuvette and analysed by spectrophotometry at 546 nm (SDM6, Cyrogenetics, Norway) with data presented as sperm per ml.

2.7 Statistics

All statistical analyses were conducted using Minitab 18 software. A Kolmogorov-Smirnov test for normality was performed on all data sets to assess for normality of

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distributions. A General Linear model was used to test the effects of the treatments and time on milt density and egg quality. An ANOVA with a post hoc Tukey's T test was used to assess the differences in gonadal development, oocyte histogram populations and leading cohort oocyte population differences. Significance was set at $p<0.05$.

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3. **Results**

3.1 Oocyte development, size distribution and fecundity estimates.

Histological analysis confirmed that oocyte development was typical for a marine teleost with the primary growth oocytes ranging in size between 82 μm and 216 μm (as defined by population mean diameters), while secondary growth oocytes ranged between 370 μm and 529 μm and oocyte maturation occurred in oocytes between 624 μm and 1398 μm (Table 2, Figure 2). Typical histological sections are displayed in figure 3. To aid the interpretation of subsequent oocyte size distribution analysis the following arbitrary thresholds were set: Primary growth oocytes ≤ 216 μm; Secondary growth & oocyte maturation: ≥370 μm and ≤ 1616 μm (*n.b.* as oocyte development is continuous in this phase there was no clear segregation that could be applied based on size alone) and hydrated oocytes ≥ 1616 μm. OSD analysis of four independent samples extracted from six pre-treatment females confirmed synchronous development in all, with no difference in total oocyte distribution or the leading cohort oocyte diameter between four independent gonad sections within individuals (data not shown). When a minimum threshold of 370 μm was applied (*i.e.* all oocytes in secondary growth or greater), the mean relative fecundity in ten pre-treatment females was estimated to be 40,440 ± 12,434 oocytes per Kg body weight with no apparent difference between females.

All females sacrificed during the study were selected based on ultrasound screening to ensure there were no immature females nor previously spawned or spent individuals. These samples do not inform on treatment effects but rather provide a snap shot of oocyte development in final oocyte maturation. For all female samples (n= 90), the leading cohort oocyte diameters ranged from 708 ± 4.6 μm to 2310 ± 7.6

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μm indicating that all individuals were in the later stages of oocyte maturation or with ovulated oocytes. The majority of individuals (92%) had a leading cohort in final oocyte maturation (OMgvm, OMpov) (15%) or free hydrated oocytes (77%) and no apparent differences in overall oocyte size distribution or the leading cohort oocyte diameter in relation to time or treatment were observed.

Assessment of oocyte diameter frequency histograms for each individual showed that, from the low and medium temperature treatments, 20 out of 40 (50%) of individuals presented a bimodal oocyte distribution as opposed to a unimodal distribution (Figure 4). Typically, this bimodal distribution included one population at the hydrated oocyte stage and one population at the oocyte maturation stage (OMgvm, OMpov) stage. Within the high temperature treatment, the proportion of individuals with a bimodal oocyte distribution was apparently reduced being recorded in 7 out of 40 sampled individuals (i.e. 18% of individuals sampled).

3.2 Temperature effects on female maturation and spawning

First spawning was recorded on 3rd June with the last ovulating female recorded on 14th August in the 6°C treatment. Overall, the length of the spawning season appeared to be inversely related to holding temperature lasting 11, 28 and 72 days for the high, medium and low temperature treatments respectively (Figure 5). Total productivity in terms of the number of naturally spawned batches was comparable in the low and medium temperature treatments (n = 25 and 20 respectively) but notably reduced in the high temperature treatment (n = 3) (Figure 5). Furthermore, mean batch weight was comparable in the low (144 ± 81 g) and medium temperature

treatment (165 ± 105 g) but significantly reduced (>50% reduction) in the high temperature treatment (65 ± 15 g).

In each treatment there was a core population not sacrificed during the length of the study (n = 48, 31 and 45 in the high, medium and low temperature treatments, respectively). Based on the repeat assessment of individuals with ultrasound three groups could be distinguished: females, which have spawned (*i.e.* attained a score of 4.5 or 5 during the study), females, which were progressing towards final maturation but did not spawn during the season (*i.e.* did not pass scores of 3-4), and non-maturing females (*i.e.* did not pass scores of 1-2). In all treatments, 16.4± 4.5% (n = 9,6 and 5 for high, medium and low treatments respectively) of fish were not maturing. In the low and medium temperature treatments, 84.7± 5.8% of females (n = 40 and 25 respectively) were spawning individuals compared to only 12.5% (n = 6) in the high temperature treatment, with 68.7% (n = 33) maturing but not spawning. No such "mature but not spawning" individuals were observed in the low or medium treatments. At the point of tagging (3 months prior to start of spawning), non-maturing females were significantly smaller (454.3 ± 15 g, 242.4 ± 3.1 mm) compared to maturing females (879.4 ± 51.89 g, 279.5 ± 5.8 mm) irrespective of treatments. However, no significant difference in size was observed between females that spawned or not in the high temperature treatment.

3.3 Temperature effects on gamete quality

There was a significant effect of the temperature treatment on egg quality with no egg batches (either as individuals or as pools) from the high temperature treatment reaching the eyed stage of development (Table 3 & 4). With respect to the individual egg batch test, there was a high level of variation within and between individual

females. However, eyeing rate was comparable in all treatment groups (overall mean eyeing rate = 31.9 ± 10.0%) with the exception of low temperature eggs and milt pool, where eyeing was significantly reduced by almost 90% in comparison to all other milt pool crosses (Table 3). With respect to the individual milt quality tests, as stated previously, no eggs from the high temperature treatment were viable, thereafter eyeing rate in all other tests were statistically comparable ranging from 22.1 ± 15.8 % (low temp. milt vs. low temp. eggs) to 43.0 ± 9.6 % (medium temp. milt vs. medium temp. eggs) (Table 4).

3.4 Temperature effects on sperm density

Prior to the initiation of temperature treatment, there was no difference in sperm density between populations (11.9 ±1.5 $\times 10^{9}$ sperm.ml^{-1}). Following four weeks of thermal treatment, and for the remainder of the study thereafter, sperm density was significantly reduced (*circa* 50% of the pre-treatment value) in the high temperature treatment compared to the pre-treatment level (Figure 6). Furthermore, sperm density was also significantly lower in the high temperature treatment compared to that measured for the medium and low temperature treatments after one month of exposure to the temperature treatments (Figure 6). There was no significant impact of the low or medium temperature treatments on sperm density with respect pre-treatment levels during the study.

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4. **Discussion**

Closed life cycle management is an important milestone for the production of lumpfish and biological control of sealice. If this milestone is to be realised at the commercial scale, it is essential to determine the optimal environmental conditions that will assure reliable production of good quality gametes. To this end, this study is the first to consider the impacts of temperature during the spawning season on its subsequent length and gamete quality within captive reared lumpfish broodstock. The work demonstrated that a higher holding temperature (*circa* 14 °C) significantly reduced spawning activity and had a significant negative effect on egg quality in lumpfish. This higher holding temperature also saw a significant reduction in milt density, although sperm viability appeared not to be impacted.

Despite the continuous photoperiod the study population demonstrated a clear synchrony in reproductive development with <16% of all fish not maturing (as assessed by ultrasound examination) in the study. Temperature treatments were applied once it was apparent that the study population were ready to spawn with the intention to assess thermal impacts during the spawning season itself not on gametogenesis *per see*. To this end, there was a stricking inverse relationship between temperature and length of the subsequent spawning season. The cessation of spawning within 11 days in the high temperature treament in conjunction with the 50% reduction in egg production and the presence of over $^{2}/_{3}$ of maturing females showing developmental regression during the study period, all indicate that final oocyte maturation and spawning are temperature sensitive in the species. This agrees with findings in other temperate species such as Arctic Charr (*Salvelinus alpinus* L.) (Gillet, 1991), Common Wolfish (Tveiten et al., 2001), Pollack (*Pollachius*

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pollachius) (Suquet et al., 2005) or Atlantic halibut (*Hippoglossus hippoglossus* L.) (Brown et al., 2006), where elevated temperatures during the spawning season inhibited subsequent spawning performance and, in the case of European seabass, was associated with gonadal regression (Carillo et al., 1995).

Following four weeks of temperature treatment there were clear effects observed on gamete viability. The high temperature had a significant detrimental effect on egg quality with all egg batches tested being non-viable. When considered in context with the significantly reduced spawning activity and the reduced proportion of observed spawning females, it implies that this elevated temperature suppressed spawning and led to oocyte regression in ovarian development following the elevation of temperature. The change from viable to non-viable eggs in the range of 5 °C is quite abrupt thought it could be assumed that there will be a proportional response to elevated temperature and egg viability between the 9°C and the 14 °C conditions tested. Such a graded response to increasing temperature has been reported in other species like Common Wolfish (Tveiten et al., 2001), Atlantic cod (Van Der Meeren & Ivannikov, 2006) and Atlantic halibut (Brown et al., 2006).

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The impact of temperature treatments on sperm quality was not as evident. While the high temperature significantly reduced milt density over the treatment period, there was no negative impact on measured viability when sperm density was standardised during the gamete quality assessment. Environmental factors such as temperature have been shown to have an effect on milt volume produced (Kowalski & Cejko, 2019) and overall fertilisation success (Brown et al., 2006) There are very few studies which document sperm density in marine fish broodstock. In Common

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Wolfish, elevated temperatures during gametogenesis was shown to reduce spermatocrit (and by inference sperm density) (Tveiten & Johnsen., 1999), while exposure to repeat stressors in Rainbow trout (*Oncorhynchus mykiss*) broodstock equally reduced sperm density (Alavi & Cosson., 2005). Given that male lumpfish spend more time in shallow coastal waters during the spawning season compared to females (Davenport, 1985; Goulet et al., 1986; Mitamura et al., 2012), it could explain their need to have a greater temperature tolerance.

It was surprising to observe a significant reduction in embryo viability in the low temperature treatment cross (6°C individual egg batches against a pool of 6°C milt). This suggests that there is also a lower thermal limit for the species, which is supported indirectly by the embryo viability results of Imsland et al. (2019a). The fact that the impact in the current study was only significant in the gamete quality test using the individual egg batches (where individual variability is controlled for) as opposed to the egg pools (where a reduction is apparent though not significant) implies again the effect is mediated primarily by the oocytes themselves.

Ultimately these data suggest an optimal thermal window (>6°C and <14 °C) for holding lumpfish during the spawning season to assure reliable and good quality productivity. There is anecdotal evidence from wild lumpfish fisheries suggesting that the capture of mature lumpfish ceases when sea temperatures reach 14°C (Pers com. David Patterson, Otter Ferry Seafish Ltd). Furthermore, in Mid-Norway mature fish are caught almost year round at very different temperatures (5-7°C during winter/spring and up to 15°C during the autumn), however autumn caught broodfish produce much poorer eggs than winter/spring caught broodfish. Combined, this

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information points towards a narrow thermal window required to assure optimal egg quality in the species that needs to be incorporated in future captive broodstock holding.

In addition to defining thermal windows for optimal broodstock performance, this study has provided data on important elements of basic reproductive physiology for the species. While oocyte development typically follows a common cellular development process, species-specific definitions of size at stage of development are very important in interpreting ovarian development using methods like oocyte size distribution (Kjesbu & Kryvi, 1989). Such detail is lacking for lumpfish with Kennedy (2018) acknowledging that interpretation of oocyte size distribution in the species was "…hindered by the lack of histological examination of the ovaries…". Classification of oocyte development is variable throughout the literature, meaning that direct comparisons between species can be difficult (Brown-Peterson et al., 2011; Rhody et al., 2013). However, size ranges for primary, secondary and oocyte maturation were comparable to those published in cod (Kryvi, 1989, Kjesbu). The current work and histological data reinforce the viteliogenic size ranges suggested in Kennedy (2018) and provides a scale that can be applied in future reproductive studies in this species.

Understanding the reproductive strategy of a given species is important in defining how to manage the species. While not the main focus of the study, results indicate that lumpfish should be considered a determinate batch spawning species. In both the histological examination and a wider OSD survey there was no evidence of continuous recruitment of oocytes and as such the potential annual fecundity should

be considered fixed prior to the start of the spawning season. The current estimate of potential annual fecundity at 40,440 ± 12,434 oocytes per Kg body weight is the first accurate estimates for the species. Davenport (1985) suggested that batch fecundity for most females would average 100,000 eggs possibly reaching 400,000 eggs per batch and estimations by Hedeholm et al. (2017) suggested a range between 51,000 to 208,000, although both authors failed to correct for individual size. If the current estimate is extrapolated to a 4 kg weight, which was typical of the Davenport (1985) study, then absolute fecundities of 160,000 ± 48,000 oocytes is in alignment with their realised fecundity estimates. It also became apparent from the OSD that individuals display both unimodal and bimodal OSD. This did not apparently influence total fecundity estimates but it does suggest that the species can be batch spawning in nature. Davenport (1985) reported that lumpfish lay 2-3 egg batches over a period of two weeks and Kennedy (2018) supported this with oocyte size histograms displaying two clear cohorts; however, this was only present in some females with the assumption that unimodal females had spawned previously. During the current study, the proportion of individuals with bimodal oocyte development ranged from 18% to 50% with the lower abundance being evident in the high temperature treatment. The precise drivers of the extent of batch spawning in lumpfish needs further investigation. The current study brings into question whether all individuals are truly batch spawning, which of course has impact on the captive management and the estimates of spawning performance in commercial hatcheries that needs closer consideration. A final important definition for setting up broodstock populations is size at which animals are capable of spawning. The demonstration that sibling females < 500 g did not mature during the course of the current study supports Imsland et al. (2014) who suggested that females began to mature at

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around 450 g (non-spawning individuals mean for the current study was 454.3 g). This requires further investigation as such definitions play an important role in defining production cycles, generation times and predict egg productivity from captive broodstocks.

At present, the management of lumpfish broodstock is in its infancy, with little published guidelines on best management practices. This study provides the first definitions for broodstock thermal management. Future work must build on these definitions to include the optimisation of holding temperatures during early gametogenesis, which has been demonstrated to be a key determinant of egg quality in other species such as Halibut (Brown et al., 2006), and Common Wolfish (Tveiten & Johnsen, 1999). If such work is combined with ongoing work on the photoperiod entrainment of spawning in the species (*e.g.* Imsland et al. 2019b) it is realistic to consider that reliable and effective environmental management of lumpfish broodstock will be a commercial reality soon.

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Acknowledgments

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Table 1: Summary of descriptive data for lumpfish used in the present study.

Treatment	Mortality (%)	Number of males	Number of females	Average weight (start) female (g)	Average weight (start) male (g)
High	5%	58	111	1224.7 ±353	744.7±201
Medium	10%	56	110	1208.9 ±448	659.4±165
Low	1%	56	112	1306.4 ±398	653.3±289

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Table 2: Oocyte size range in relation to developmental stage for lumpfish according to Rhody et al. (2013). Values represent mean ± SD of a minimum of 10 individuals in which 50 oocytes were measured for a given developmental stage.

Stage	Step	Diameter (µm)	Number of fish
Primary growth (PG)	**Multiple nucleoli (PGmn)**	82.2 ± 7.6	11
	Perinucleolar (PGpn)	119.9 ± 29.2	12
	Oil droplets (PGod)	216.5 ± 15.2	14
Secondary growth (SG)	**Early (SGe)**	370.4 ± 6.2	10
	Late (SGl)	467.8 ± 60.2	10
	Final (SGfg)	528.5 ± 39.4	10
Oocyte maturation (OM)	**Eccentric germinal vesicle (OMegv)**	623.7 ± 39.0	12
	Germinal vesicle migration (OMgvm)	740.5 ± 88.9	15
	Preovulatory (OMpov)	1398.2 ± 87.3	15

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Table 3: Proportion (%) of eyed embryos in individual egg batch assessments (n= 6 individuals per treatment) compared to pools of milt derived from high, medium and low temperature treatment groups and wild mature lumpfish (each pool contained an equal contribution from 6 males). Superscripts denote significant differences.

	High temp. milt	Medium temp. milt	Low temp. milt	Wild milt
High temp. eggs	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}
Medium temp. eggs	33.3 ± 29.6^{b}	30.3 ± 29.1^{b}	41.8 ± 27.9^{b}	40.8 ± 31.5^{b}
Low temp. eggs	33.3 ± 33.7^{b}	39.3 ± 30.4^{b}	4.3 ± 7.0^{a}	37.4 ± 33.2^{b}

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Table 4, Proportion (%) of eyed embryos in individual milt assessments (n= 6 individuals per treatment) compared to pools of eggs derived from high, medium and low temperature treatment groups (each pool contained an equal contribution from 6 females). Superscripts denote significant differences.

	High temp. eggs	**Medium temp. eggs**	**Low temp. eggs**
High temp. milt	0 ± 0.0^{a}	39.5 ± 8.6^{b}	30.9 ± 13.0^{b}
Medium temp. milt	0 ± 0.0^{a}	43.0 ± 9.6^{b}	30.2 ± 17.8^{b}
Low temp. milt	0 ± 0.0^{a}	30.9 ± 14.1^{b}	22.1 ± 15.8^{b}

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List of Figures

Figure 1: Daily temperature (°C) recorded within the three treatment groups.

Figure 2: Box and whisker plot displaying the diversity in oocyte size (*n*= >10 individuals per stage) observed within developmental stages (Primary (multiple nuclei (PGmn), perinucleolar (PGpn) and oil droplet (PGod)), Secondary (early (SGe), late (SGl) and final (SGf)) and Oocyte Maturation (eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm) and pre-ovulatory (OMpov))). Box represents 25th and 75th percentiles while whiskers represent 5th and 95% percentiles, with mean value is indicated as the vertical line within the box. Individual values out with these range are indicated as •.

Figure 3: Typical examples of histological sections of Primary (multiple nuclei (PGmn), perinucleolar (PGpn) and oil droplet (PGod)), Secondary (early (SGe), late (SGl) and final (SGf)) and Oocyte Maturation (eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm) and pre-ovulatory (OMpov)) oocytes of lumpfish with scale bar shown.

Figure 4: Oocyte size distribution histograms showing typical example of females with either unimodal (top) or bimodal (bottom) development (*n.b.* Unimodal individual is from low temp treatment sampled 11th July, Bimodal individual is from medium temp treatment sampled 28th June). Boundaries of stages of development for hydrated oocytes, OMpov, Oocyte maturation (OM), and Late Secondary Growth phase (SGl) are indicated by vertical lines.

Figure 5: Frequency of natural spawning events during the study period for each treatment group. Bars represent total mass of eggs released during that day (g) for the respective treatment. Multiple discrete spawning events in a given day are indicated by proportional dual shading.

Figure 6: Mean sperm density ($\times 10^9$ sperm.ml^{-1}) ± SD for males (n = 7 per sample) maintained under either low (6 °C), medium (9°C) or high (14°C) temperature treatments. Superscripts denote significant differences.

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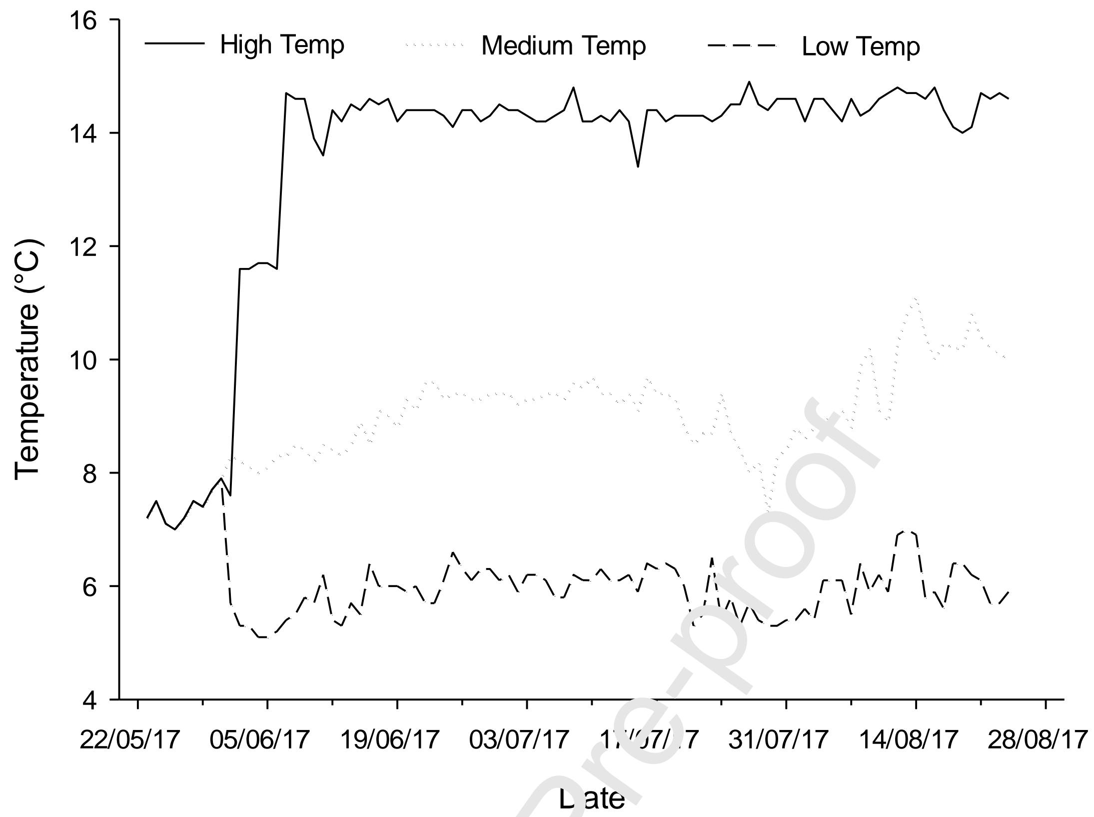

Figure 1

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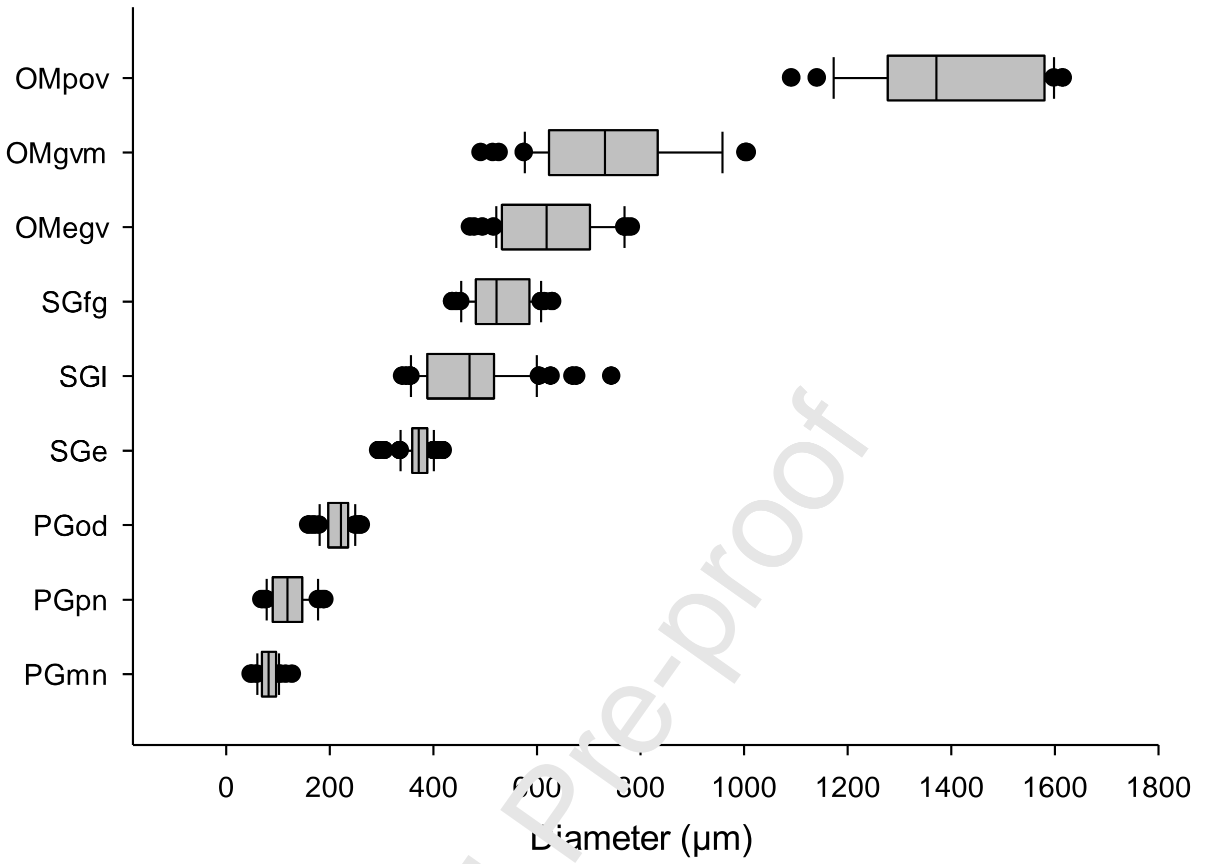

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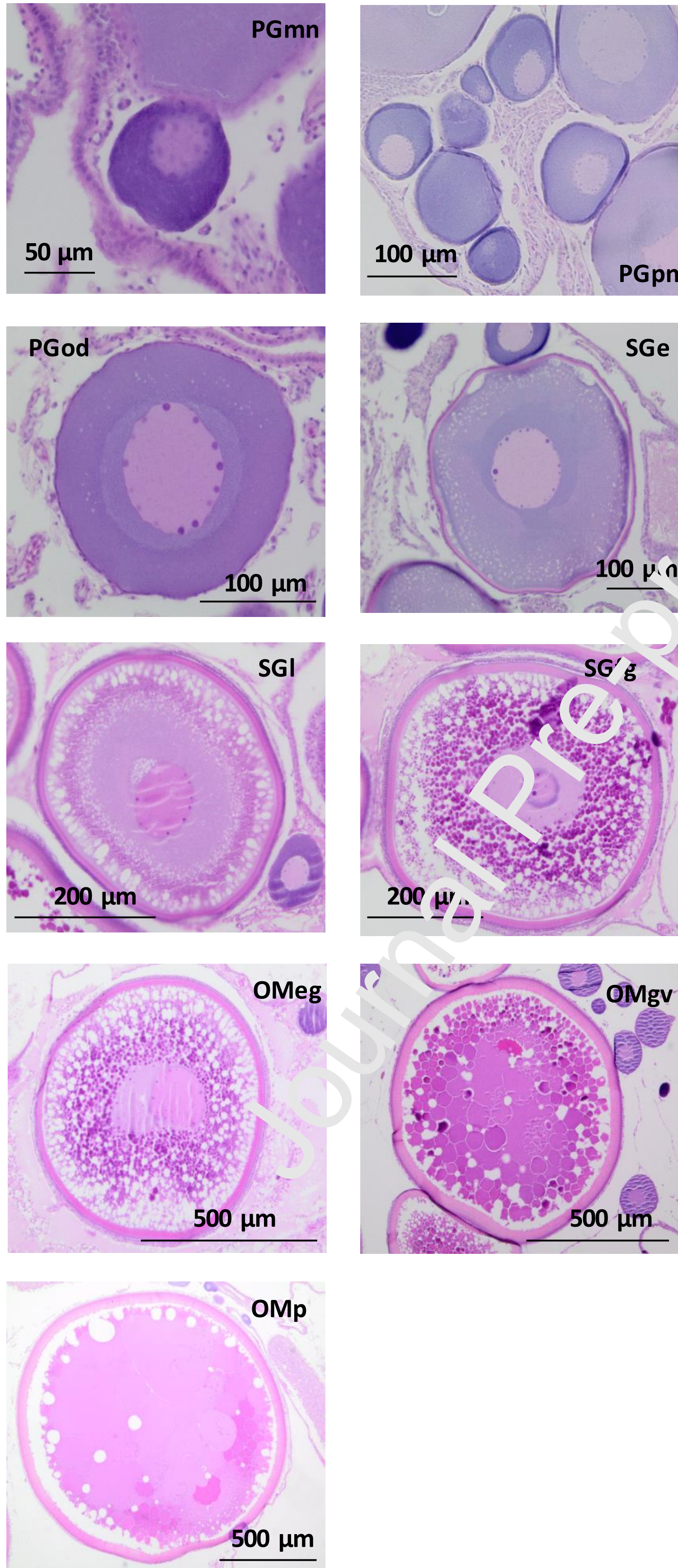

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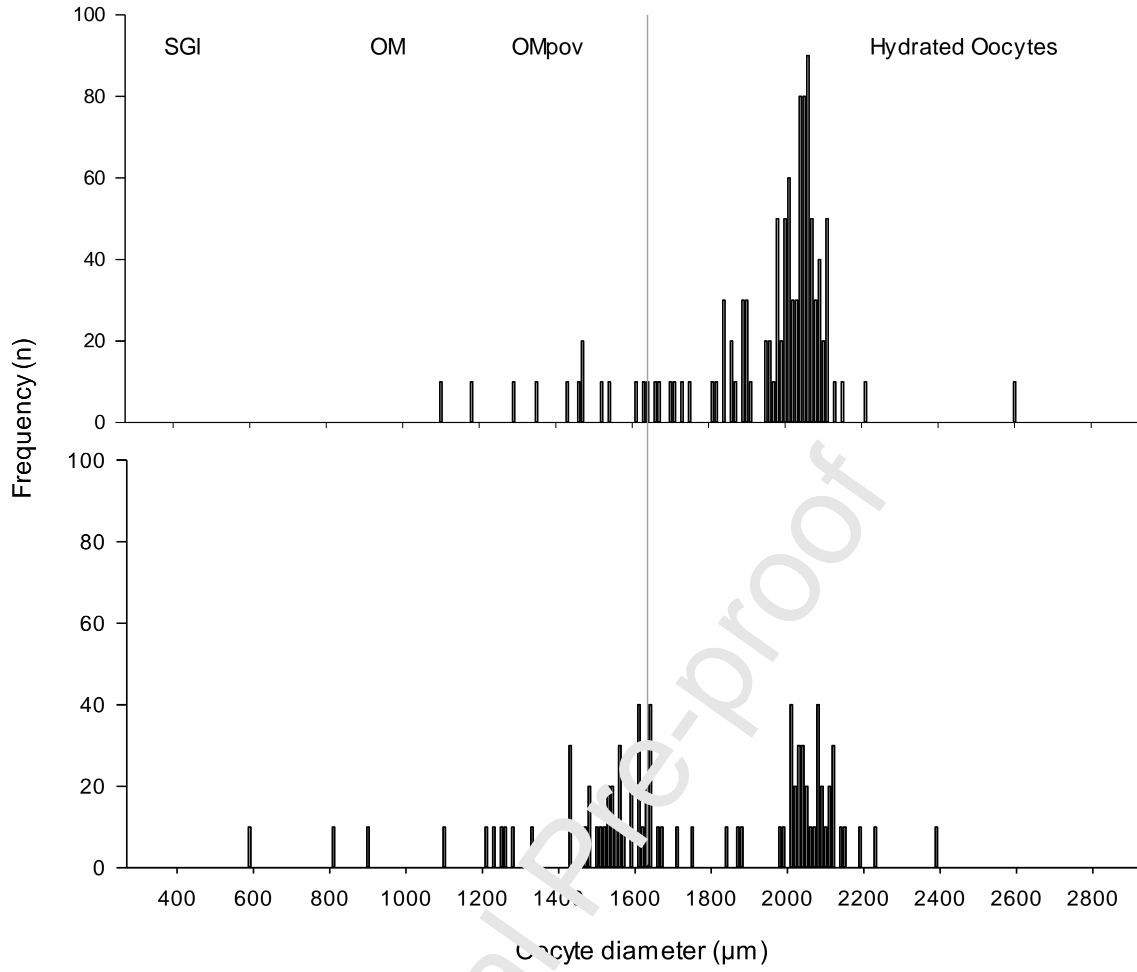

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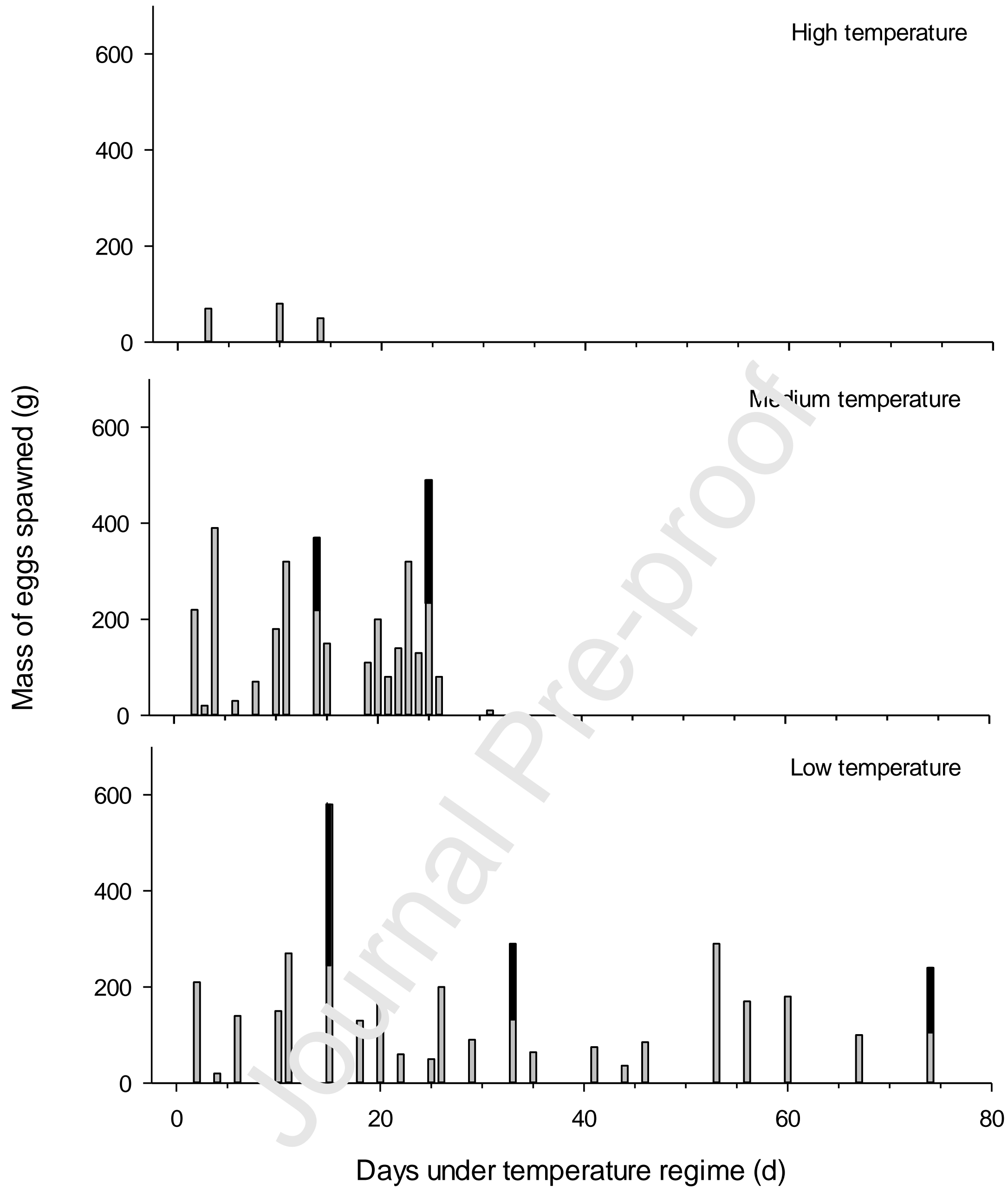

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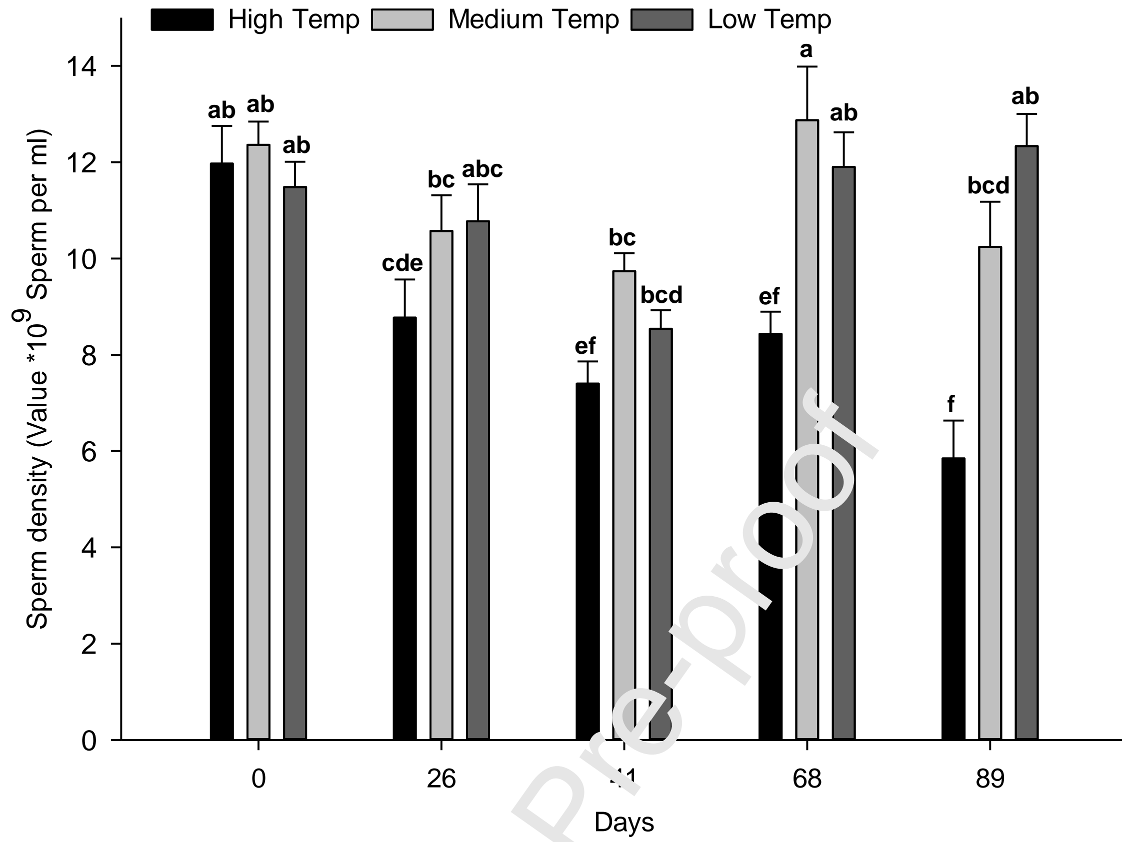

Figure 6

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- High temperature (14°C) is detrimental for egg quality in captive broodstock
- Poor embryo viability produced in low temperatures (6°C) suggests a lower thermal limit
- Milt density was reduced over the spawning season at high temperatures (14°C)
- Oocyte size ranges for developmental stages from primary to hydrated oocytes have been defined

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