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High Temperature is detrimental to captive Lumpfish (*Cyclopterus lumpus, L*) reproductive development.

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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 natural spawning productivity and gamete quality in captivity reared lumpfish. Sexually mature lumpfish (15 month old) were held on three temperature regimes (6ºC, 9ºC and 14ºC) from the onset of first spawning. Holding mature lumpfish at high temperatures (14 ºC) resulted in a notable reduction in natural 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 9ºC and 6ºC did not have a similar negative impact on gamete quality, however the natural spawning window for the 6ºC treatment was twice as long as the 9ºC treatment. These results indicate that holding temperature for lumpfish broodstock should not reach the 14ºC degree threshold, with a possible thermal optimum below 10ºC. The current findings are the first step in identifying optimal rearing conditions for captive Lumpfish broodstock.

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

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). Which 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 produce the roe (Kennedy *et al.* 2018). Following demonstration that the species can act as an effective biological control to sealice infestations in Atlantic salmon farms (Imsland, *et al.*, 2014) a new aquaculture sector has opened, targeting the supply of juveniles to salmon farms (Tresurer,1.1, 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 for moving towards closed life cycle management for this species would help to assure biosecurity, guarantee egg supply and open the possibility of genetic selection and improvement of the farmed stocks. The first step in closing the life cycle and securing reliable captive broodstock production is to define the environmental parameters that determine productivity and gamete quality.

The lumpfish reaches maturity in the wild after five to six years (Davenport, 1985; Haatuft, 2015b), however captive reared lumpfish have spawned after one to two years (Imsland, *et al.*, 2014; Powell *et al.*, 2017). Females spawn in shallow coastal

waters and have been known to migrate great distances to spawning sites (Mitamura et al., 2007). 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., 2007). 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 (2018) 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 being October to May.

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 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. as Precise histological definitions are key to interpreting the impact of management interventions on reproductive development for a given species in captivity e.g. common Snook (*Centropomus undecimalis*) (Rhody et al (2013)), Cod (*Gadus morhua*) (Kjesbu & Kryvi, 1989) and Bass (*Dicentrarchus labrax*) (Mayer, Shackley, & Ryland, 1988).

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, determination of spawning season, productivity and quality (Brooks, Tyler, & Sumpter, 1997). Imsland et al. (2018) suggest that timing of spawning can 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, however clearer definitions are required. In contrast, there is no information 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, temperatures manipulations can influence the pace of development and timing of subsequent spawning windows as demonstrated in the common Wolfish (*Anarhichas lupus*) (Tveiten & Johnsen, 1999) and to a lesser degree influence fecundity (Kraus, Müller, Trella, & Köster, 2000) as well as subsequent gamete quality, as demonstrated in Cod (Rideout, Burton, & Rose, 2000). During the spawning season itself temperature plays a stronger regulatory role, determining spawning windows (Kjesbu, 1994), ovulation cycles (Brown, Shields, & Bromage, 2006) and most importantly gamete quality (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 2007).

This current study was designed to explore the effects of temperature during the spawning season on broodstock performance in captive lumpfish. It aimed to define the environmental conditions which are required to assure reliable and good quality

captive spawning in the species. This study fundamentally aimed to describe the effects of rearing temperature on timing and productivity of spawning, as well as gamete quality. A secondary objective was to histologically describe oocyte development for the species. Such work would address knowledge gaps in the species while also providing clear guidance for optimal rearing of captive broodstock, which is key in realising the industries aspirations to work with closed life cycle management.

2. Materials & Methods

2.1 Animals and experimental design

All fish used in the experiment were captive stock reared from wild eggs caught by Skjerneset Fisk at Averøy, Norway and maintained at NOFIMA, AS, Sunndalsora. 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 summer. They were maintained on a low intensity, 24hr light photoperiod and the fish were fed on Skretting Silk (Skretting, Nutreco N.V, Netherlands) pellets at the appropriate size range during grow out. Individual morphometric (weight (±0.1g) and total length (± 1 mm)), gender and stage of maturity were recorded (*n.b.* all individuals had previously been tagged using a passive integrated transponder (PIT) tag) on the 1st of May 2017 when the stock (*n* = 513) were approximately 15 months old. Fish were randomly assigned to one of three treatment groups (*n* = 166 -169 per treatment) with the sex ratio being balanced 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 (combination of subjective assessment of the observed severity of swelling in females 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. Thereafter temperature ranges remained stable throughout the experimental period 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 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 image, free hydrated oocytes apparent on the dorsal region of the ovarian lobe. Following first spawning open regions filled with ovarian fluid become 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 natural spawning events with egg masses being removed and weight recorded (±0.1g).

Following temperature change, on four subsequent separate occasions (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 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 being initiated as well as individuals from the first two sample dates. This pool of individuals had a range of GSI's from 0.95% to 37.4% to capture the diversity of ovarian development. Fixed ovarian samples were embedded in paraffin with 5µm sections were 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 (PGod). Secondary Growth- Secondary growth includes three steps: early (SGe), late (SGl), and full-grown (SGfg). Oocyte maturation (OM) includes three steps: eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm), and preovulatory (OMpov). A minimum of 50 individual oocytes within a minimum of 10 individuals were identified for each developmental stage. Oocyte diameter was measured by digital image analysis where oocyte diameter was calculated as the average of two diameters perpendicular to each other measured through the nucleus.

2.3 Oocyte size distribution analysis

To measure the oocyte size distribution (OSD) a protocol similar to Kjesbu & Kryvi, (1989) was followed. Briefly, a digital image (Nikon 1, NIKON, Japan) was taken of

dissociated oocytes, with individual oocyte diameter (n =100 oocytes per individual) being measured by digital image analysis (Image Proplus, Media Cybernetics, USA). Population G1 oocyte diameter 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 taken from the two ovarian lobes within six independent females. Both G1 oocyte diameter and full oocyte size frequency distributions were compared 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 from pre-treatment sampled females. Briefly, for each individual, three weighed samples of ovarian tissue were preserved in 10% neutrally buffered formalin, a digital image (Nikon 1,NIKON,Japan) was taken of dissociated oocytes, with the total number of vitellogenic oocytes (oocytes > 370µm as determined by previous histological examination of oocyte development) being determined by digital image analysis (Image Proplus, Media Cybernetics, USA).

2.5 Egg 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 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 tested in triplicate against a pool of milt (n = 4 milt pools, representing high, medium, low temperature treatments and wild) which contained equal contributions of milt from the 6 individual males from each respective treatment group. To test individual milt sample viability, each of the six milt samples from the three treatments (n= 18 total milt samples) were tested in triplicate against a pool of eggs (n = 3 egg pools, representing high, medium and low temperature treatments) which contained equal contributions of eggs from the 6 individual females from each respective treatment group. For each test replicate, 1ml of eggs (*circa* 100 eggs) were wet fertilised with 600,000 sperm per egg in a petri dish using 20ml of 0.2µm-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 0.1mg per ml of streptomycin within the petri dish and these were maintained in a temperature controlled room at 8°C thereafter. Every two days water was exchanged within the petri dishes, with the eggs being maintained to the eyed stage and final eyeing measurements being conducted at 150-degree days at which point the proportion of eyed eggs was recorded.

2.6 Sperm density assessments

Sperm density was assessed just prior to temperature treatment being applied and at each sampling point thereafter. Gonads were excised from 6 sampled males at each time point, ground and sieved to produce milt. This milt was diluted 1:400 in a physiological solution (Munibs medium, 100mMKHCO3 and 125mMSucrose) in a cuvette and measured under 546nm in a spectrophotometer (SDM6, Cyrogenetics, Norway) values were given in sperm per ml.

2.7 Statistics

All statistical analysis was conducted using Minitab 18 software. A Kolemegov-Smirnov test for normality was performed on all data sets to assess for normality of distributions. A General Linear model was used to assess the effects of the treatment and time period for the milt density assessment and, the effects of treatment on egg quality. A Tukey's T test and ANOVA was used to assess the differences in gonadal development, oocyte histogram populations and G1 oocyte population differences. Significance was given at $p<0.05$ unless stated otherwise.

3. **Results**

3.1 Oocyte & ovarian development and fecundity estimates.

Oocyte development was typical of a marine teleost. Histological analysis confirmed the primary growth oocytes ranged in size between 82 µm and 216 µm (as defined by population mean diameters) dependent on developmental stage, while secondary growth oocytes ranged between 370 µm and 529 µm while oocyte maturation occurred in oocytes as they progressed from 624 µm to 1398 µm (Table 1, Figure 2). To aid the interpretation of subsequent oocyte size distribution analysis the following arbitrary thresholds were set: Primary growth oocytes ≤ 216µm; Secondary growth & oocyte maturation: ≥370 to ≤ 1616 (*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 discrete locations across the ovary of six pre-treatment females confirmed no difference in oocyte development across the gonad as a whole, when G1 oocytes and whole section oocyte distribution was compared across an individual's ovary (data not shown). When a minimum threshold of 370µm was applied (*i.e.* all oocytes in secondary growth or greater) the mean total fecundity was estimated to be 40440 ± 12434 oocytes per Kg body weight.

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) G1 oocyte diameters ranged from 708 ±4.6 to 2310 ± 7.6 indicating that all individuals were in the final stage of oocyte maturation or more advanced. The majority of individuals (92%) had a leading cohort in final oocyte maturation (OMgvm, OMpov)

(15%) or free hydrated oocytes (77%), with there being no apparent difference in overall oocyte size distribution or G1 oocyte diameter in relation to time or treatment. Assessment of oocyte diameter frequency histograms for each individual showed that, from the cold and mid temperature treatments, 50% and 50% respectively of individuals had a bimodal oocyte distribution as opposed to a unimodal distribution (Figure 4). Typically, this bimodal distribution included one population in the hydrated oocyte stage and one population in the Oocyte maturation stage (OMgvm, OMpov) stage. Within the high temperature treatment, the proportion of individuals with bimodal distribution was apparently lower at 18% of individuals sampled.

3.2 Temperature effects on female maturation and spawning

Stocks were not significantly different in weight, length, or maturation score before the start of the trial with an approximately equal sex ratio (as determined by ultrasound assessment at the beginning of the trial).

First spawning was recorded on 3rd June with the last ovulating female being recorded on 14th August in the 6°C treatment. Overall, the length of the natural 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 3). 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 noteably reduced at the high temperature (n = 3) (Figure 3). Furthermore, mean batch weight was comparable in the low (144 ± 81g) and medium temperature treatment (165 ± 105g) but signficiantly reduced (>50% reduction) in the high temperature treatment (65 ± 15g).

Using the ultrasound scores three groups of individuals were identified, females which have spawned (4.5 or 5 score) , females which exhibit progression towards maturation but did not spawn during the season (scores of 3-4), and non-maturing females (score of 1-2). In all treatments fish identified as not investing in maturation made up 20% of the experimental population. In the 6°C and 9°C treatments spawning individuals made up 80% of the total population. Wheras in the 14°C treatment group 12% of females exhibited spawning with the remaining 68% having exhibitied signs of maturation. At the point of tagging (31st march 2017), there was a significant difference in weight and length between those populations who invested in maturation and those which did not invest during the 2017 spawning season (Data not shown).

3.3 Temperature effects on egg quality

There was a significant treatment effect on egg quality; no egg batches (either as individuals or as pools) from the high temperature treatment reached the eyed stage of development (Table 2 & 3). 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 (31.85% ± 9.95%) with the exception of low temperature treatment eggs and milt pool where eyeing was significantly reduced by almost 90% in comparison to all other milt pool crosses (Table 2). With respect to the individual milt quality tests, as stated previously, no eggs derived 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 % (med temp milt vs med temp eggs) (Table 3).

3.4 Temperature effects on sperm density

Sperm density was assessed just prior to temperature treatment being applied and at four times thereafter, up to 13 weeks post temperature change. Prior to the initiation of temperature treatment sperm density was comparable between the populations (4.94-4.59 $\times 10^{12}$ 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 5). Furthermore, sperm density was also significantly lower in the high temperature treatment than that measured for the mid and low temperature treatments after one month on the temperature treatment (Figure 5). There was no significant impact of the low or medium temperature treatments on sperm density with respect pre-treatment levels.

4. **Discussion**

Closed life cycle management is an important milestone for the production of lumpfish for biological control of sealice. If this milestone is to be realised at commercial scale, it is essential to determine the optimal environmental conditions that will assure reliable production of good quality gametes. In numerous temperate and cold water marine species, photoperiod has been shown to influence the wider seasonality of spawning, while temperature has been shown to play a key role regulating the spawning windows, ovulatory cycles and gamete quality (Migaud et al., 2013). However sub-optimal holding temperatures can have a detrimental effect on broodstock performance with temperate and cold-water species being particularly affected by higher holding temperatures (Migaud et al., 2013). Thus determining optimal rearing conditions for captive broodstock is an important stage in broodstock management and securing a sustainable supply of juveniles. This current work aimed to determine the effects of holding temperature during the spawning window on the length of the spawning season and egg quality within captive reared lumpfish broodstock. The study demonstrated that higher holding temperature (*circa* 14 °C) has a significant negative effect on egg quality in lumpfish. This higher holding temperature also saw a significant reduction in milt density, although did not appear to reduce sperm viability. The natural spawning season was also inversely associated with holding temperatures. As such, this work provides definitions for thermal management of the species during the spawning window, which will play an important role in assuring reliable closed life cycle management for the species.

As some fish ovulated and spawned in all treatment groups at the onset of temperature treatments it is suggestive that all fish were at the same stage of the reproductive cycle

(late stage vitellogenesis). This is also backed by the ultrasound assessments conducted and OSD work. However, the lack of spawning activity in the higher temperature treatment suggests these final stages of gonadal development are temperature sensitive. This reflects the findings in other cold water marine species such as common wolfish (Tveiten,et al 2001), where higher temperature treatments displayed reduced spawning activity, although no cessation of this activity was observed in that study. The overall productivity of the spawning season for the higher temperature treatment was reduced in both spawning events and mass of eggs produced in both this study and the above mentioned in wolfish.

In terms of the impact of temperature treatment of gamete viability, clear effects were observed. There was a significant detrimental effect on egg quality in relation to the 14°C treatment, with all egg batches tested being non-viable. When considered in context with the significantly reduced natural spawning activity and the reduced proportion of observed spawning females it implies that this elevated temperature led to a rapid regression of oocyte development following the elevation of temperature. This follows a similar trend to that found in common wolfish where significant reductions in egg quality was present (Tveiten et al., 2001). They saw no effect on fertilisation rates (not assessed in this study), and developmental abnormalities and reduced egg survival was found later in development, data from the milt quality assessments suggests this could be a possible mechanism for reduced quality in this study. This effect appears to be similar in other temperate marine species, Van Der Meeren and Ivannikov (2006) also saw a slight decrease in fertilisation rates with higher temperature, with more significant egg mortality and reduction in egg

development in Cod. Higher holding temperatures were also detrimental to egg quality in Halibut (Brown et al., 2006).

In terms of the impact of temperature treatments on sperm, quality the impact of raised temperatures 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 and Cejko, 2019) and overall fertilisation success (Brown et al., 2006) as well as other parental factors (Ottesen and Babiak, 2007). There are very few studies which document sperm density in broodstock, however exposure to repeat stressors in Rainbow trout (*Oncorhynchus mykiss*) broodstock showed reduced sperm density (Alavi and Cosson, 2005). And temperature treatments in Common Wolfish saw reduction in spermatocrit values with increased temperature (Tveiten and Johnsen, 1999). For Wolfish this was suggested to be a seasonal effect where sperm production peaks but is produced throughout the year. At this stage, it is not thought to be the case for Lumpfish. Males spend more time in shallow coastal waters during the spawning season (Davenport, 1985; Goulet et al., 1986), potentially subjecting them to more extreme temperature fluctuations during maturation and spawning. This could suggest that they are able to function at a higher holding temperature than females, but still follow a colder water optimum, which appears to be the trend for this species.

This study found a significant difference in the low temperature treatment gamete cross (6°C females against a pool of 6°C milt). This suggests that there is a lower thermal limit, which can also be detrimental to egg quality. This can also be inferred

from Imsland et al (2019) where warmer (8°C) incubating eggs have lower mortality than cold (4°C) incubations. However wild data (Davenport, 1985; Mitamura et al., 2007) suggests that spawning occurs at lower temperatures and further work will be needed to confirm this.

In the present study there was an negative effect of the higher holding temperature on spawning window, number of natural spawning events and gamete quality but equally there was a negative impact of low temperature treatment on apparent embryo viability. Combined 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. The upper threshold of this limit at least is supported by anecdotal evidence from wild lumpfish fisheries where capture of mature lumpfish ceases when sea temperatures reach 14°C (Pers com. David Patterson OFSF), as well as published data which suggests lumpfish associate with colder waters in both Newfoundland (Stevenson and Baird, 1988) and the Barent sea (Kaltenberg and Benoit-Bird, 2013). In the latter study, 70% of adults were associated with 4-7°C water. Similarly, in captive studies such as those conducted by Nytro (Nytrø et al., 2014) and Hvas (Hvas, et al, 2018) there is a clear reduction in temperature preferences for optimal growth and survival in association with increasing size. Nytro (2014) displayed a clear reduction in optimal temperatures for growth with increased fish size. Fish at 120g-200g performed better at a temperature of 8.9°C, suggesting that broodstock at over 1Kg would continue to favour the colder holding temperatures. More work needs to be conducted to determine the optimal holding temperatures for maturing lumpfish, however this study provides an important ground work for further investigations.

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 helpful in interpreting ovarian development using methods like oocyte size distribution (Kjesbu and 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…".The current study addresses this knowledge gap by providing the relevant histological information to be able to define size classes for developmental stages for the species. 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 and Kryvi, 1989). The current work and histological data, reinforces the viteliogenic size ranges suggested in Kennedy, (2018) and provides a key which can be applied to future reproductive work in this species.

Davenport (1985) reported that lumpfish lay 2-3 egg batches over a period of two weeks. 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. As previously discussed the suboptimal elevated temperature driving ovarian regression

could explain the reduced abundance if bimodal oocyte development in this treatment. Notwithstanding this fact, these findings bring in to question as to whether lumpfish are truly a determinate batch spawning species.

Kennedy (Kennedy, 2018) suggested developmental size ranges for lumpfish where 1800µm is a threshold before hydration of oocytes, and that cohorts of oocytes are present between 1400 µm and 1600 µm at a “holding range” which makes up a potential second cohort. This fits with the current studies work where the final stages of maturation are present within the 1400 µm and 1600 µm ranges and free hydrated oocytes making up the G1 cohort. Within this study, the NSG of oocytes appeared to tail the secondary cohort of oocytes and in the 1400 to 1600 range once the second cohort of oocytes progressed.

This study is the first to give an estimate for total fecundity in Lumpfish, which has been lacking to date. It has to be acknowledged that there was notable individual variation in the fecundity values leading to a large variance in the estimate i.e. 40440 ± 12434 oocytes per Kg body weight. OSD analysis of these individuals confirmed there was no apparent difference between bimodal and unimodal females. Davenport (1985) suggested that batch fecundity for most females would average 100,000 eggs possibly reaching 400,000 eggs per batch. The fish in this study were first time Spawners, and significantly smaller than most wild caught broodstock, upon which these numbers are based, the value determined from this study appears to corroborate for an average 4Kg wild broodstock (140,000 eggs per female).

At present, the management of lumpfish broodstock is in its infancy, with little published guidelines on best management practices. This study provides some detail on the required holding temperatures for effective broodstock spawning and suggest

a possible lower and upper temperature threshold. Although this study is limited in scope, it can provide a guideline for temperature management for captive broodstock. Future work on this Lumpfish should focus on optimising holding temperatures during early gametogenesis, this has been identified as important in determining egg quality in other species such as Halibut (Brown et al., 2006),and Common Wolfish (Tveiten and Johnsen, 1999). As well as a focus on reconditioning environmental conditions, identified as an important step in (Powell et al., 2017). The developmental key identified in this study will be able to inform future stock management strategies within the Lumpfish industry.

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Table 1: Oocyte size range in relation to developmental stage for lumpfish. Staging is in accordance with 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

631 Table 2: Proportion (%) of eyed embryos in individual egg batch assessments (n= 6
632 individuals per treatment) compared to pools of milt derived from treatment groups
633 and wild mature lumpfish (Each pool contained an equal contribution from 6 males).
634 Superscripts denote significant differences
635

	14 degree mil	**9 degree milt**	**6 degree milt**	**Wild milt**
14 degree eggs	0 ±0.0[a]	0 ±0.0[a]	0 ±0.0[a]	0 ±0.0[a]
9 degree eggs	33.3 ±29.6[b]	30.3 ±29.1[b]	41.8 ±27.9[b]	40.8 ±31.5[b]
6 degree eggs	33.3 ±33.7[b]	39.3 ±30.4[b]	4.3 ±7.0[a]	37.4 ±33.2[b]

636
637

Table 3, Proportion (%) of eyed embryos in individual milt assessments (n= 6 individuals per treatment) compared to pools of eggs derived from treatment groups (Each pool contained an equal contribution from 6 females). Superscripts denote significant differences.

	14 degree eggs	**9 degree eggs**	**6 degree eggs**
14 degree milt	0 ±0.0[a]	39.5 ±8.6[b]	30.9 ±13.0[b]
9 degree milt	0 ±0.0[a]	43.0 ±9.6[b]	30.2 ±17.8[b]
6 degree milt	0 ±0.0[a]	30.9 ±14.1[b]	22.1 ±15.8[b]

List of Figures

676 Figure 1

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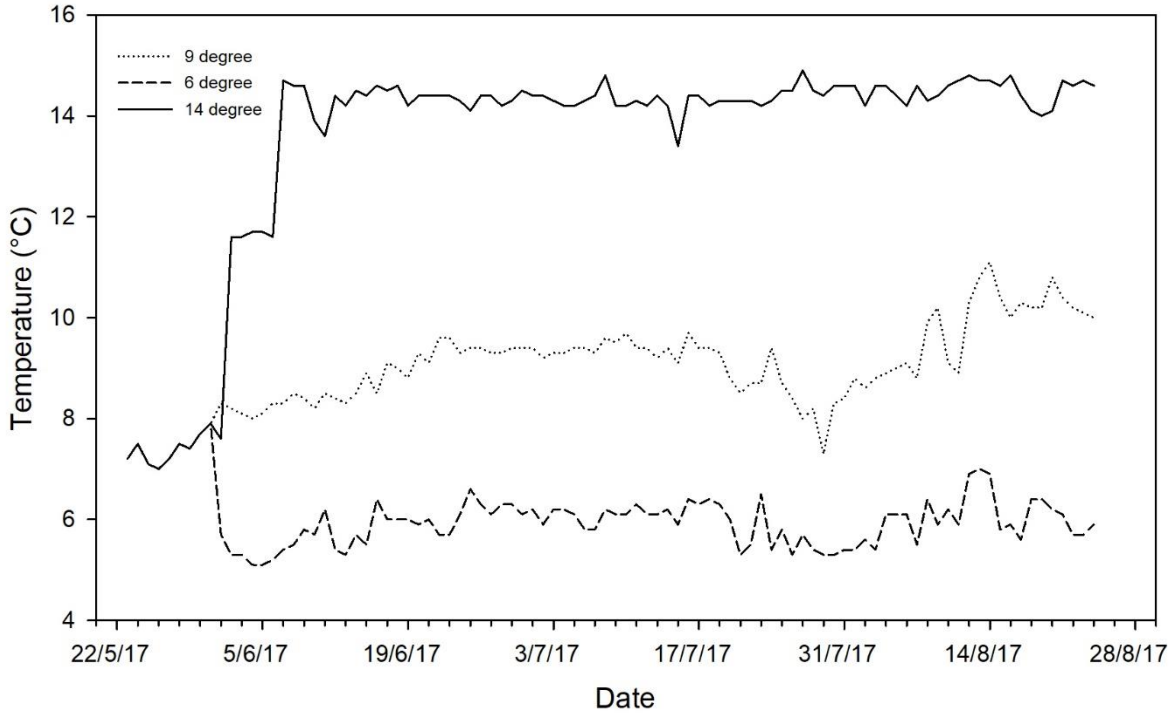

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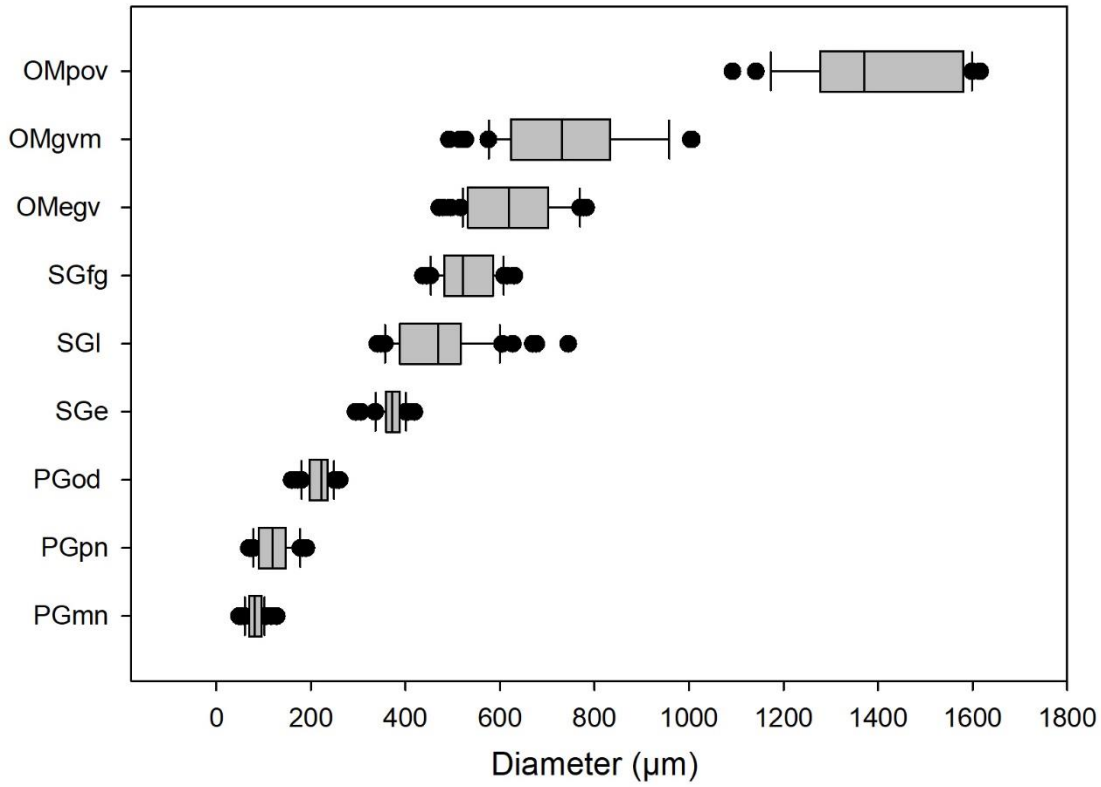
680 Figure 2



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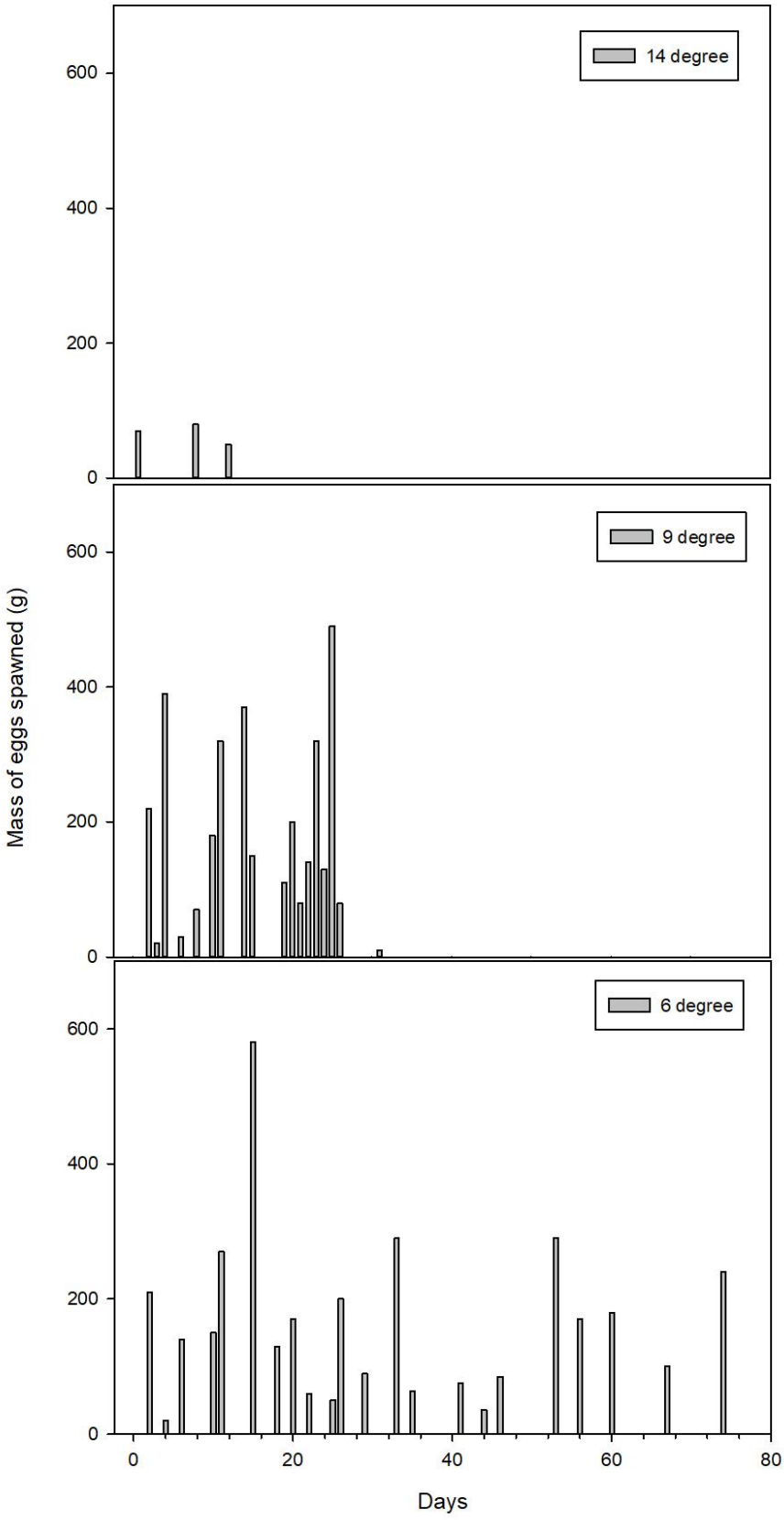

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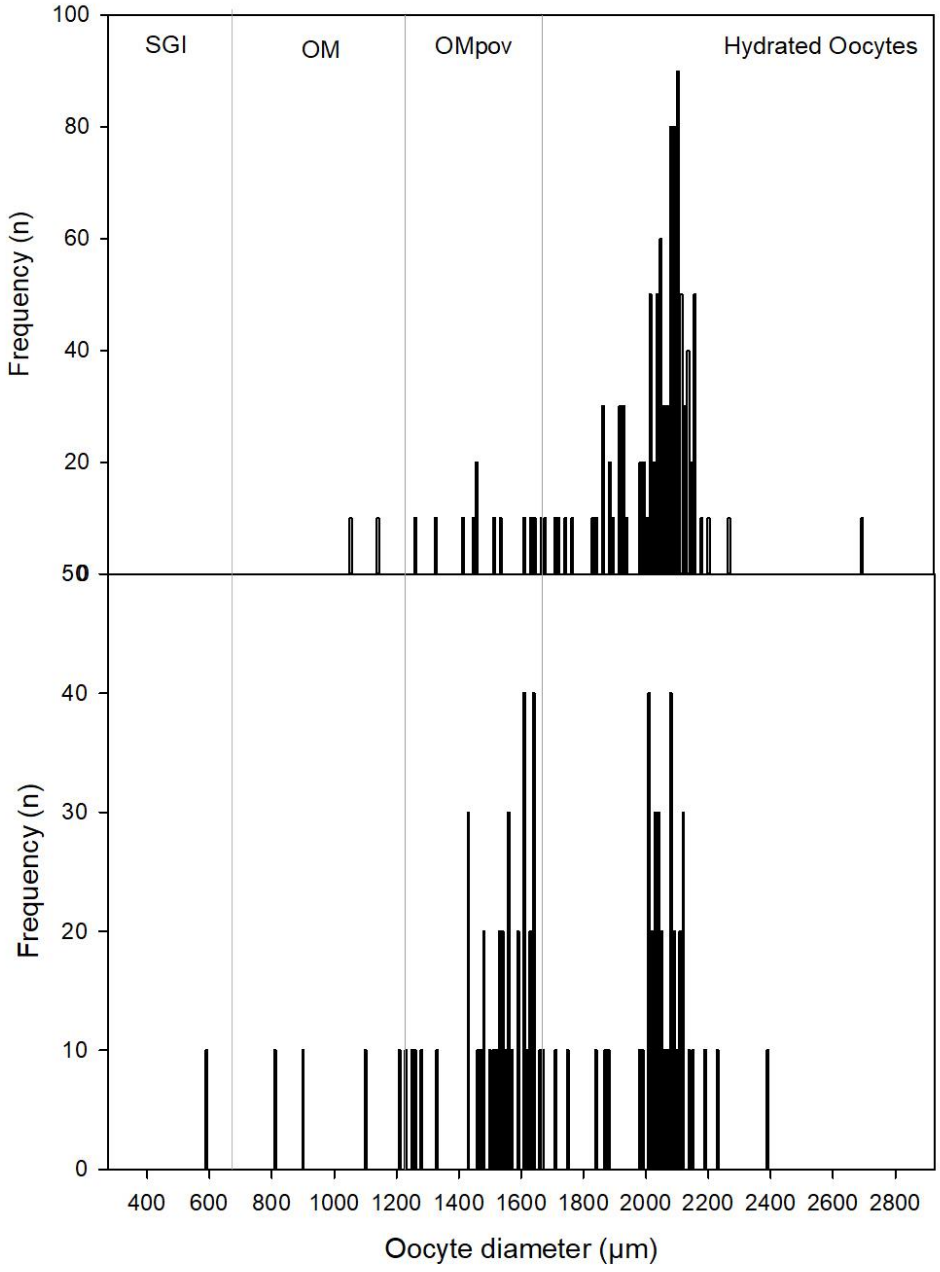

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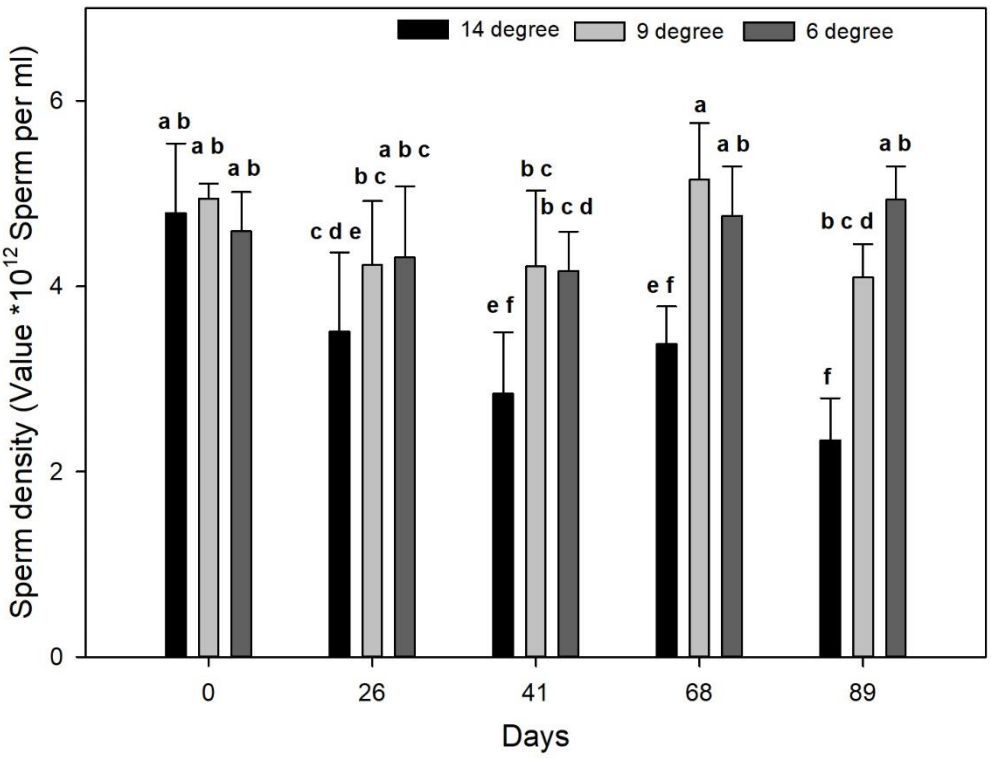
688 Figure 5



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