**Full title:** Reproductive biology of farmed lumpfish *Cyclopterus lumpus*: Gonadal development and associated profiles of selected endocrine factors

**Short title:** Reproductive biology of farmed lumpfish (*Cyclopterus lumpus*)

Frank Thomas Mlingi\(^{1,2}\)*, Velmurugu Puvanendran\(^3\), Erik Burgerhout\(^3\), Emanuele Guercini\(^1\), Maren Mommens\(^4\), Øyvind Johannes Hansen\(^3\), María Fernández Míguez\(^5\), Pablo Presa\(^5\), Helge Tveiten\(^6\), Jonna Tomkiewicz\(^7\), Elin Kjørvik\(^1\)

\(^1\)Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

\(^2\)Current address: Ode AS, Stadsbygd, Norway.

\(^3\)Department of Production Biology, Nofima AS, Tromsø, Norway.

\(^4\)Department of Breeding and Research and Development, AquaGen AS, Trondheim, Norway.

\(^5\)Laboratory of Marine Genetic Resources, CIM – Universidad de Vigo, Spain.

\(^6\)Norwegian College of Fisheries Science, UiT The Arctic University of Norway, Tromsø Norway.

\(^7\)Institute of Aquatic Resources, Technical University of Denmark, Kgs. Lyngby, Denmark.

\(^*\)Corresponding author

E-mail: frank.t.mlingi@ntnu.no (1) or frank.mlingi@ode.no (2) (FTM)
Abstract

The demand for lumpfish (*Cyclopterus lumpus*) as a cleaner fish for the Atlantic salmon industry to battle sea lice infestations results in an increased interest of juvenile production in aquaculture. For successful control of the lumpfish reproduction, it is crucial to adequately understand its reproductive biology. Female and male lumpfish were sampled four times during a 24-week experiment where gonadal development in relation to endocrine factors was studied. Histologically, we identified and described ten oogenic and four spermatogenic stages during gamete development. The female gamete stages were grouped into ovarian development stages to simplify categorization of ovarian development. The ovarian development and the associated endocrine factors were typical for fish with group-synchronous ovarian organization, and from our histological observations it is possible that lumpfish females release up to four batches of eggs in one spawning season. During early stages of testis development, spermatogenic cells were observed in cysts. The cystic spermatogenesis and the distribution of spermatogonial cysts through the germinal compartment indicated an unrestricted spermatogonial distribution type. Despite its variance, the expression of genes *gnrh2* and *gnrh3* in the brain of lumpfish decreased in females and increased in males during gonadal development. Gonadal development was also associated with increasing trends of gene expression in gonadotropin receptors, vitellogenin receptors, steroidogenic enzymes, and blood plasma concentrations of sex steroids. The genes *fshr*, *lhr*, *vtgr*, *cypl9a1* and the sex steroid E2 increased up to oocyte maturation and decreased at ovulation, while GSI, T and 11-KT increased up to ovulation. While *fshr* and *lhr* peaked at spermatocyte stage and decreased later, *cypl7a1*, T and 11-KT increased up to the spermatid stage and decreased at the spermatozoa stage in males. Our findings improve the current understanding on lumpfish reproductive biology and suggest future research directions.
Introduction

In order to control the incidence of sea lice (*Lepeophtheirus salmonis* Krøyer, 1837) in Atlantic salmon (*Salmo salar* L., 1758) cage aquaculture, the lumpfish (*Cyclopterus lumpus* L., 1758) is often preferred as a “cleaner fish” over ballan wrasse (*Labrus bergylta*, Ascanius, 1767) due to its continued activity at low temperature [1,2]. During the last decade, the demand for lumpfish juveniles in salmonid aquaculture has vastly increased. In Norway alone, the number of farmed lumpfish supplied as cleaner fish in the production of Atlantic salmon and rainbow trout increased from 10,000,000 individuals in 2015 to over 26,000,000 million individuals in 2021 [3]. However, commercial production of lumpfish juveniles still relies to a large extent on wild broodstock collection [2]. This caused pressure on its wild populations and thereafter this species was classified as Near Threatened in 2013 [4], i.e. likely, some spawning stocks are already overharvested. There is also a risk of transferring parasites and diseases from wild lumpfish to farmed salmon [2]. Thus, a farmed lumpfish broodstock is necessary to alleviate fishing pressure on wild populations, as well as to control its reproduction on a year-round basis.

In nature, lumpfish seems to spawn throughout most of the year, although pronounced spawning peaks in spring and summer are commonly referred to as its main spawning period [5–8]. The long spawning period occurs under widely different temperature and photoperiod conditions resulting in varying egg quality, e.g., eggs harvested in winter (which is not part of the spawning peak) and early spring generally exhibit higher hatching success than those harvested during warmer seasons [7]. Therefore, the availability of right-sized lumpfish juveniles for stocking into salmon cages is restricted to a specific period of the year, which is often uncoupled with both, the occurrence of lice infestations and the period of sea transfer of smolts [9].
A consistent and sustainable hatchery production of lumpfish has the potential to supply the year-round demand for juveniles of the suggested size. This can only be achieved by closing the breeding cycle under farm conditions [2], but little is known related to gonadal development, reproductive physiology and optimal environmental conditions for lumpfish reproduction. Therefore, studies aiming at improving basic knowledge and understanding of the reproductive biology of the lumpfish are necessary for maximizing its reproductive success [10]. Investigating the reproductive cycle of a species by defining the different stages of gonadal development, the corresponding endocrine changes, and the key triggers associated with the process of gametogenesis should contribute to improved protocols in aquaculture production and a more consistent supply for the ever-increasing demand of fish [10–13].

The endocrine system of the brain-pituitary-gonad (BPG) axis in vertebrates is the major signaling pathway for hormonal control of reproduction [14–16]. From the hypothalamus (brain), gonadotropin releasing hormone (GnRH) is released through neuronal fibers that project directly to gonadotropic cells in the pituitary gland, to stimulate synthesis and release of the gonadotropins (GTHs); follicle stimulating hormone (FSH) and luteinizing hormone (LH) [17]. The gonadotropins are the primary hormones regulating gametogenesis in a receptor mediated manner, stimulating synthesis of sex steroids in the gonads by the follicle cells in females and the Leydig cells in males [11,12,18]. In females, the steroid hormone 17β-estradiol (E2) stimulates the liver to produce vitellogenin (VTG), which is incorporated into the growing oocytes during vitellogenesis [12]. The VTG receptor (VTGR) is involved in VTG endocytosis, it normally increases with the onset of the lipid droplet stage in species with ooplasm lipid formation and remain elevated during early vitellogenesis [19,20]. Thus, gonads perform dual, interrelated functions, generation of gametes and steroidogenesis, steroidogenesis leads to accomplishment of gonadal development by mediating the effects of the gonadotropins [17,18]. All classes of steroids are synthesized de novo from the common
precursor cholesterol [21]. The enzymes responsible for steroid hormone biosynthesis from cholesterol are in the Cytochrome P450 (Cyp) family, they are membrane-bound proteins associated either with mitochondrial membranes (Cyp11a) or in the endoplasmic reticulum (Cyp17 and Cyp19) [22]. The estrogen 17β-Estradiol (E2) and, the androgens testosterone (T) and 11-ketotestosterone (11-KT) are among the most studied sex steroids in teleosts. While E2 is the most potent and prevalent estrogen and 11-KT is the main active androgen, all three are essential mediators of gametogenesis in both females and males [23].

Various aspects of the reproductive biology and control of spawning in lumpfishes are known. These include the smooth lumpsucker (Aptocyclus ventricosus, Pallas, 1769), where seven maturity stages were described based on histological features of oogenesis. This species shows discontinuous oogenesis (determinate fecundity) and group-synchronous development of vitellogenic oocytes, releasing eggs only once during the spawning season [13]. However, the reproductive strategy of *A. ventricosus* likely differs from that of *C. lumpus* which is suggested to release eggs more than once during the spawning season [5,24]. Using photoperiodic manipulations on lumpfish, temporal shifts in spawning were observed in both females and males [9,25], but the dynamics of ovarian and testicular development were not described. Oocyte size progression has been described, but not including oocyte developmental stages [24]. Ovarian development was described after exposure to different temperatures during the spawning season, and three main stages were identified: primary growth, secondary growth, and oocyte maturation [7]. Changes in several endocrine factors during gonadal development in lumpfish females and males have been described recently [26]. However, endocrine factors such as gonadotropin releasing hormones, gonadotropin receptors, steroidogenic enzymes, vitellogenin receptor and testosterone, and their associations to the patterns of the standard gonadal development stages are yet to be understood.
In order to control species-specific reproductive processes under farming, the link between gonadal development and reproductive physiology needs to be well understood. For production of good quality gametes, reproduction in fishes is dependent on successful progression through all stages of gametogenesis [17]. Our aim in this study was to further improve current knowledge on gonadal development in lumpfish, with the objectives to (1) histologically characterize different gonadal development stages during oogenesis and spermatogenesis and to (2) describe the associated changes in gene expression levels of gonadotropin releasing hormones (GnRHS; GnRH2 and GnRH3), gonadotropin hormone receptors (GTHR; FSHR and LHR), vitellogenin receptor (VTGR), steroidogenic pathway enzymes (CyP17a1 and CyP19a1), gonadosomatic index (GSI) and blood plasma concentrations of testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2).
Materials and methods

Fish and sampling. The lumpfish used in this study were sampled over six months from a cultured 18 months old group expected to mature during that period. The fish were maintained in four 1500 L tanks (75 fish per tank and a 37:38 (0.97:1), male: female ratio) for 24 weeks (from 21 September 2017 to 8 March 2018) under a photoperiod and temperature experiment at the aquaculture station in Kårvika, Tromsø, Norway. A continuous photoperiod (L:D = 24:0 throughout) and a short to continuous photoperiod (L:D = 8:16 and later L:D = 24:0) were applied. The temperature ranged between 2.9 ºC and 9.9 ºC, higher in the beginning and lower towards the end of the experiment. Sampling was done four times and required fish sacrifice using an overdose of tricaine methane sulfonate (MS222; 165 mg L\(^{-1}\)). Body weight (BW ± 0.5 g) and total body length (BL ± 0.1 cm) were measured. Blood was collected from the caudal vein into vacutainer tubes (BD Vacutainer LH 68 IU (Lithium Heparin) 4.0 mL, BD, UK) and stored in ice during the sampling. Blood plasma was obtained by centrifuging at 5000 rpm for 10 minutes at 4 ºC and stored in cryotube vials (VWR Micro-centrifuge tubes with graduation 2.0 mL) at -20 ºC until further analyses. Gonads were excised and weighed (GW ± 0.5 g) to calculate GSI using the formula: GSI = (GW/BW) * 100. Gonadal tissue samples were cut and placed in histology cassettes (Simport Histonette Tissue Processing/Embedding Cassettes with Lid), fixed in 4 % buffered formalin, and stored at 4-5 ºC until embedding in paraffine and histological examination for gametogenesis.

Histology and oocyte size. Gonads from the sampled 71 females and 85 males were prepared for histological analysis. The gonadal tissues for histology were dehydrated in a tissue processor (TP 1020-1-1, Leica Microsystems Nussloch GmbH, Germany) and thereafter embedded in paraffin. Sections of 4 µm thickness were obtained using a microtome (RM 2255, Leica Biosystems, Nussloch GmbH, Germany) and stained with hematoxylin and eosin (H &
E). The stained sections were scanned at 40 X magnification and examined using a digital scanner (NanoZoomer, Photonics, Hamamatsu, Japan) and the scanner software (NDP, Hamamatsu, Photonics, Japan). One to three (depending on section size and gamete stage for ovary sections) and ten (for testis sections) images were taken per individual fish. The track map in the scanner software was applied to avoid overlapping of images taken on a single tissue.

ImageJ (version 1.53j; https://imagej.nih.gov/ij/) was used to observe and quantify gamete stages. The oogenic and spermatogenic stages were identified using the features described in Tables 1 and 2.
Table 1 Designated ovarian development stages using groups of oocyte stages.
<table>
<thead>
<tr>
<th>Ovarian development</th>
<th>Oocyte stages and features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary growth</td>
<td>Perinucleolar oocytes: multiple nucleoli are seen around the periphery of the nucleus. The cytoplasm stains mainly basophilic [28–30].</td>
</tr>
<tr>
<td>Secondary growth</td>
<td>Cortical alveolar oocytes: cortical alveoli are seen in the periphery of the oocyte. Oil droplet oocytes: oil droplets occur around the nucleus, and later become very prominent in the cytoplasm, which now stains light purple [28,29]. There is incorporation of yolk globules; primary yolk oocytes, yolk globules (which stain dark pink) appear and occupy the cytoplasm, there are few scattered yolk globules; secondary yolk oocytes, more yolk globules start to fill the cytoplasm; tertiary yolk oocytes, completion of yolk accumulation hence the cytoplasm is filled mainly with yolk globules (staining pink) which are also larger in size, and some have started coalescing [29,31].</td>
</tr>
<tr>
<td>Oocyte maturation</td>
<td>Germinal vesicle migration and breakdown (GVM and GVBD) have taken place [32,33]. Maturing oocytes: large yolk granules are heavily coalescing forming large globules which dominate the cytoplasm, there are also empty vacuoles in the oocyte periphery [29]. Matured oocytes: yolk globules coalesce further, homogenizing into one large globule in the ooplasm, lipid droplets are not visible [29,34].</td>
</tr>
<tr>
<td>Ovulation</td>
<td>Ovulating oocytes: thinning and formation of rapture in the follicular wall. Ovulated eggs: free from the follicular walls,</td>
</tr>
</tbody>
</table>
Table 2 Designated testicular development stages using spermatogenic stages.

<table>
<thead>
<tr>
<th>Testicular development</th>
<th>Spermatogenic stages and features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatogonia</td>
<td>Shape ranges from roundish to elongate, contain light homogenous to dense heterogenous nuclei which may be round to oval. They occur singly or in cysts of a few germ cells [11,36].</td>
</tr>
<tr>
<td>Spermatocytes</td>
<td>They have clear chromatin in the nucleus, which may also appear denser with chromosomes as bold lines running from the periphery to the center of the nucleus [37].</td>
</tr>
<tr>
<td>Spermatids</td>
<td>They have small, round, and heterogenous nuclei. Thy are spherical in shape, smaller than spermatocytes, and have no flagella [38,39].</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>They have spherical nucleus containing highly condensed chromatin. They are seen with flagella. They are still held together by intercellular bridges or individualized (broken intercellular bridges) but not yet there are empty or deflated follicular layers which may appear arranged in convoluted strings [17,28,29,34].</td>
</tr>
</tbody>
</table>
A dot-grid sampling technique was used to estimate the relative composition of the various stages in the tissues, where the number of crosses were adjusted to match the ideal identification of gamete stages and sizes. For ovary sections, a grid of 252 crosses each covering an area of 3.2 mm\(^2\) while for testis sections, a grid of 112 crosses each covering an area of 0.0018 mm\(^2\) were selected and overlaid on the images using an ImageJ Grid plugin. A cross with its center on a tissue was qualified as a hit, where the total number of crosses hitting gametes of one stage was counted and multiplied by the area of each cross to calculate the area occupied by the stage. The total area of the section was obtained by adding up the areas of all stages identified and excluding somatic tissues and empty regions. Subsequently, the area fraction was estimated as follows:

\[
AF = \frac{\text{Area occupied by one gametic stage}}{\text{Total area (all gametic stages in the section)}}
\]

Where: \(AF = \text{Area fraction}\)

Then, the area fractions were converted to percentages as follows:

\[
AF\_\text{percentage} = \frac{\text{Gametic stage area fraction}}{\text{Total area fraction}} \times 100
\]

Using the area fractions, females and males were classified into different gonadal development stages. The gonadal development stage was assigned to a predominant stage, i.e., the stage with \(\geq 30\%\) of the total area fraction.

**Oocyte and nucleus size.** Using the ObjectJ plug-in (version 1.05i; [https://sils.fnwi.uva.nl/bcb/objectj/index.html](https://sils.fnwi.uva.nl/bcb/objectj/index.html)) marker tool on a scaled image, the largest and the smallest diameters of all oocytes sectioned through their nuclei were measured manually.
Nuclei diameters were also measured from perinucleolar oocytes, whose cytoplasms and nuclei were well distinguished, up to secondary yolk oocytes. Ratios of nucleus and oocyte diameters were obtained by dividing the nucleus diameters by the oocyte diameters (nucleus: oocyte diameter ratio). Later stages were excluded, because nuclei appearance may become obstructed by yolk granules or showed signs of disintegration. Similarly, diameters of the latest oocyte stages (oocyte maturation and ovulated eggs) were not measured, due to the lack of the nucleus as markers for maximum diameter measurements, in addition those stages are considerably affected by shrinkage compared to the earlier stages. In total, 3251 oocytes from all available histology sections were measured. These measurements were used to obtain oocyte size frequency distribution (OSFD) and leading cohort (LC) [24] diameters (the average diameter of oocytes representing the largest 10 % per stage). Further, by using the mean oocyte diameters, oocyte volumes were estimated assuming perfect spheres with the sphere volume formula $V = \frac{4}{3} \pi r^3$. To describe the relationship between oocyte and nucleus diameter, 425 oocytes with clearly visible and whole nuclei (perinucleolar, cortical alveoli, oil droplet, and primary and secondary yolk oocytes) were randomly selected, and the oocyte and nucleus diameters were measured.

**Sex steroid analyses.** Blood plasma concentrations of 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) were measured using radioimmunoassay (RIA), as previously described [40]. Assay characteristics and cross-reactivities of E2 and T antisera (isolated from New Zealand White (NZW) rabbits) were examined by Frantzen and co-workers [41]. Cross-reactivities of the 11-KT antiserum (isolated from NZW rabbits) were examined by Johnsen and co-workers [42]. In short, to extract free (non-conjugated) steroids, 300 µl blood plasma was mixed with 4 mL diethyl ether (DEE) in borosilicate glass tubes and subjected to vigorous shaking for four minutes. After 2-3 minute of phase separation, the aqueous phase was frozen on dry ice, and the organic phase with steroids was subsequently decanted into new glass tubes.
and placed in a water bath at 45 °C until the DEE was completely evaporated. The steroids were reconstituted by adding 900 µl of RIA-buffer. Tritiated E2, T, and 11-KT were from Perkin Elmer (Boston, MA 02118 USA, 800-762-4000). During RIA, 100 µl of the extracts in duplicates were mixed with the specific tracer (50 µl) and antiserum (200 µl). After an overnight incubation at 4 °C and subsequent charcoal extraction (of unbound steroids), the radioactivity of steroids bound to antisera was measured by scintillation counting on a Tri-Carb 2900TR (PerkinElmer Life and Analytical Sciences, Downers Grove, IL 60515). Dilution curves were established from pooled plasma and were parallel to the standard curve of the different steroid assays. For use in statistical calculations, values falling below the detection limits (0.2 ng/mL) would be assigned a value equal to half of the detection limit (0.1 ng/mL).

**Gene expression analysis.** The relative expression of genes involved in reproduction was investigated in brain and gonadal tissues using quantitative real-time PCR (qPCR) analyses. The RNA was extracted from 10 mg of tissue using Trizol (Tri-Reagent, SIGMA) and a pellet pestle homogenizer (Pellet pestle cordless motor, Kimble, SIGMA) following the manufacturer's instructions. After adding 200 µl chloroform and centrifuging at 10000 g x 15 min the supernatant was recovered into a fresh 1.5 ml tube. Five-hundred µl of ice-cold isopropanol was added and shaken briefly and the solution was kept at −20°C overnight to help precipitating the nucleic acids. The samples were centrifuged at 12000 g for 15 min and the supernatant was discarded. The pellet was washed for 10 min in 1 ml 75% ethanol and centrifuged at 12000 g for 5 min. Ethanol was discarded with a pipette and a syringe, the pellet was dried at room temperature and resuspended with nuclease-free water in a final volume of 20 µl. A final step was added to prevent DNA contamination by incubating extracts with 5 U of DNase I (Thermo Fisher) at 37 °C for 15 min, followed by enzyme inactivation with chloroform-isoamyl alcohol. RNA integrity and quantity were assessed with the Thermo Scientific NanoDrop Lite Spectrophotometer and visualized by electrophoresis in 2% agarose
Reverse transcription was performed from 500 ng of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) in a reaction volume of 20 μL using a thermocycler GeneAmp PCR System 9700 (Thermo Fisher Scientific).

The qPCR primers for the target genes were designed using NCBI primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The efficiency (%) for each primer set was determined using a two-fold standard dilution of cDNA from pooled tissue samples. The qPCR was run in duplicate using a QuantStudio 5 system (Applied Biosystems, Thermo Fisher Scientific), and the 20 μl reactions consisted of 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 0.6 μl 10 μm forward and reverse primers, 7 μl 1:40 diluted cDNA, and nuclease-free water. The cycling profile was 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. A non-template (water) control was included to rule out non-specific contamination and a melting curve analysis was performed to verify the measurement of a single specific product. QuantStudio Design & Analysis Software (Applied Biosystems, Thermo Fisher Scientific) was used for data collection and analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [43] and the geometric mean expression of the reference genes ellα and rps20.

**Statistical analyses**

All data visualizations and statistical analyses were conducted using the free and open-source integrated development environment (IDE); RStudio (version 1.3.1093) for R (version 4.1.3), with help from the packages: tidyverse and emmeans [44,45]. All data are presented as Mean ± Standard Error of the Mean (Mean ± SEM) if not stated otherwise. To analyze the differences in GSI, oocyte sizes, gene expression levels and blood plasma concentrations of sex steroids across development stages, one-way analysis of variance (one-way ANOVA) and Kruskal-
Wallis tests were fitted to normal and non-normal data, respectively. Normality and homoscedasticity were checked using Shapiro-Wilk and Levene tests, respectively. Multiple pairwise comparisons for normal data were accomplished using the emmeans procedure based on adjusted Tukey tests. For non-normal data, the pairwise Wilcoxon rank sum test was used. For all tests, the significance level was set to $P \leq 0.05$.

Results

Gonad morphology and gametogenesis

Ovarian development. Based on histological observations, the ovarian tissue consisted of ovigerous lamellae with oocytes in various stages of development, surrounded by follicle cells and a vascularized support tissue, as exemplified by the developing ovarian tissue from two females (Fig 1). Overall, nine (9) oocyte stages were identified: 1 perinucleolar; 2 cortical
alveolar; 3 oil droplet; 4 primary yolk; 5 secondary yolk; 6 tertiary yolk; 7 maturing; 8 matured; 9 ovulating oocytes, in addition to ovulated eggs and post-ovulatory follicles (Fig 2). These oocyte and egg stages were grouped to establish four ovarian development stages (a more detailed description is presented in Error! Reference source not found. where: 1.) primary growth (PG): perinucleolar (pn) oocytes; 2.) secondary growth (SG): cortical alveoli (ca), lipid droplet (ld), primary yolk (py), secondary yolk (sy), and tertiary yolk (ty) oocytes; 3.) oocyte maturation (OM): maturing (mat) and matured (matd) oocytes; and 4.) ovulation (OV): ovulating oocytes and ovulated eggs. Initiation of oocyte maturation was observed as the germinal vesicle breakdown and yolk coalescence. Ovulating oocytes and ovulated eggs were categorized as the ovulation stage; the ovulating oocytes were still in the ovigerous lamellae with thinning and rupturing follicles (Fig 2), while ovulated eggs were already expelled from the lamellae (implying that they are already shed into the lumen).

Fig 1 Ovary sections from two lumpfish females. These samples taken at 17 weeks from the start of the experiment show more than one ovarian development stage in a female at the same time. Primary growth (pg), Secondary growth (sg), Oocyte maturation (om). The ovigerous lamellae (ol) are also shown.

Fig 2 Morphologies of the different oogenic stages in a lumpfish ovary. Perinucleolar (pn), cortical alveolar (ca), oil droplet (od), primary yolk (py), secondary yolk (sy), tertiary yolk (ty), maturating (mat), matured (matd), ovulation (ov), post-ovulatory follicle (pof).

Up to four different vitellogenic-maturing stages were observed in six (6) % of all females examined as three main secondary growth stages in addition to the oocyte maturation. In 16 % of all females examined, at least two of the main secondary growth stages (primary, secondary, and tertiary yolk oocytes) were observed besides the oocyte maturation stage.
Macroscopically, two distinct oocyte and egg batches could be distinguished in the ovary of a mature female (Fig 3), and the left ovary was smaller than the right ovary in that female.

**Fig 3 Photograph of a lumpfish female laid on its right side.** Two ovaries are shown (black arrows), developing oocytes and ovulated eggs (white arrow and black text highlighted in yellow) are shown in one ovary.

**Testis development.** For the lumpfish male gonads, the testicular tissue consisted of germ cells and vascularized interstitial support tissue (Figs 4 and 5). Four spermatogenic cell stages were identified: spermatogonia (sg), spermatocytes (sc), spermatids (st), and spermatozoa (sz) (Fig 4). In 27% of the 85 males examined, all four spermatogenic cell stages were present, and 76% contained spermatozoa in addition to other stages. Distribution of spermatogonia and spermatozoa in the testis is shown in the transverse section (Fig 5), where spermatogonia was distributed in an unrestricted manner in the testis.

**Fig 4 Internal morphology of a lumpfish testis.** Spermatogenic stages and vascularized interstitial tissues (It), with blood vessels/cells (Bv) are shown. Spermatogonia (Sg), spermatocytes (Sc) and spermatids (St) appear in cysts (Cy) while spermatozoa (Sz) are free. Scale bars = 100 µm.

**Fig 5 Organization of the germinal compartment in a lumpfish testis section.** Spermatocysts with spermatogonia appear throughout the testis lobe.

**Oocyte size**

**Oocyte and nucleus diameters:** The oocyte diameters, nucleus diameters and nucleus: oocyte diameter ratios changed with the recruitment to more developed oocyte stages (Fig 6). In perinucleolar oocytes the mean oocyte diameter was $0.22 \pm 6.1 \times 10^{-3}$ mm and it increased significantly to $0.79 \pm 0.01$ mm in secondary yolk oocytes. The mean nucleus diameter
increased significantly from $0.1 \pm 2.3 \times 10^{-3}$ mm in perinucleolar oocytes to $0.18 \pm 3.2 \times 10^{-3}$ mm in primary yolk oocytes, followed by an insignificant change to $0.15 \pm 0.01$ mm in secondary yolk oocytes. The mean nucleus: oocyte diameter ratios on the other hand decreased significantly from $0.5 \pm 6.9 \times 10^{-3}$ (perinucleolar oocytes) to $0.2 \pm 0.02$ (secondary yolk oocytes) (Fig 6). For the ovarian development stages, the mean oocyte diameters were $0.22 \pm 6.1 \times 10^{-3}$ mm (Primary growth), $0.74 \pm 1.5 \times 10^{-2}$ (Secondary growth) and $1.44 \pm 2.4 \times 10^{-2}$ (Oocyte maturation).

**Fig 6 Changes in oocyte diameter and volume, and nucleus: oocyte diameter ratio with oocyte stages.** The solid line shows the average oocyte diameters, the blue dotted line shows the average nucleus: oocyte diameter ratios and, the black dashed line shows the average oocyte volumes. Different letters on top of error bars between stages indicate significant differences in the oocyte dimensions between oocyte development stages.

**Oocyte size frequency distribution (OSFD):** The OSFD in the different development stages were multimodal and right skewed (Fig 7). There were several size groups within each development stage as well as an overlap in sizes between the different stages. The oocyte maturation stage had the widest size range and with a large overlap in size range also with the secondary growth stages. Apart from their whole population mean diameters, the primary growth, secondary growth, and oocyte maturation stages had mean LC diameters of $0.37$ mm, $1.07$ mm, and $2.28$ mm, respectively. There were very few secondary growth oocytes larger than $1.5$ mm. Further, OSFDs from six individual females also showed overlaps between secondary growth and oocyte maturation in addition to the existence of more than one oocyte stage per female (Fig 8).
**Fig 7 Diameter frequency distribution of lumpfish oocytes (3251 oocytes).** The oocytes are grouped according to ovarian development stages: Primary growth, Secondary growth, and Oocyte maturation. Values in brackets indicate mean leading cohort (LC) diameters.

**Fig 8 Diameter frequency distribution of oocytes from six individual lumpfish females.** The frequency distributions are grouped according to development stages. Total number of oocytes measured = 131.

**Endocrine factors in relation to gonadal growth and development**

**Females:** The mean relative gene expressions of both gonadotropin releasing hormones *gnrh2* and *gnrh3* changed insignificantly during ovarian development, but putatively showed a declining trend (Fig 9 A and B). Mean gene expression of receptors of the follicle stimulating hormone *fshr* and of the luteinizing hormone *lhr* fluctuated in parallel (Fig 9 C and D), they peaked at oocyte maturation and decreased at ovulation, and the mean expression levels at oocyte maturation were significantly higher than at primary and secondary growth. Similar trends were observed for the expression of the vitellogenin receptor *vtgr* and the P450 Aromatase enzyme *cyp19a1* (Fig 9 E and F), which peaked significantly at oocyte maturation after which they decreased (significantly for *vtgr*).

**Fig 9 Gene expression levels of selected endocrine factors in relation to ovarian development stages.** Gonadotropin-releasing hormone II (*gnrh2*, A); gonadotropin-releasing hormone III (*gnrh3*, B); Follicle stimulating hormone receptor (*fshr*, C); Leuteinizing hormone receptor (*lhr*, D); vitellogenin receptor (*vtgr*, E); and cytochrome P450 19a1 aromatase (*cyp19a1*, F). Different letters on top of error bars indicate significant differences in expression levels between the development stages.

GSI and blood plasma concentrations of the sex steroid hormones testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) increased significantly with the recruitment
into more advanced development stages (Fig 10). Mean GSI increased from primary growth to its peak at ovulation, significant differences were found between all development stages. Mean blood plasma concentrations of T and 11-KT increased through all stages from primary growth to ovulation, and the oocyte maturation and ovulation demonstrated significantly higher blood plasma concentrations of both steroids than in primary and secondary growth stages. The mean blood plasma concentration of E2 increased to its peak at oocyte maturation and decreased at ovulation. E2 was significantly lower at primary growth than the rest of the stages, and at secondary growth it was significantly lower than at oocyte maturation only.

**Fig 10 Ovary growth and plasma concentrations of sex steroids in relation to ovarian development stages.** Gonadosomatic index (GSI, A), and plasma concentrations (ng/ml) of testosterone (T, B), 17β-Estradiol (E2, C) and 11-ketotestosterone (11-KT, D) grouped according to ovarian development stages. Different letters on top of error bars indicate significant differences between the development stages.

**Males:** Compared to females, values related to the endocrine factors of the testis development stages in the male lumpfish were characterized by large individual variations. The mean expression levels of *gnrh2* and *gnrh3* in males increased with the increase of more developed stages in the testis, although without significant differences between any of the stages. The mean expression levels of *fshr* and *lhr* peaked at spermatocyte stage, while *cyp17a1* peaked at the spermatid stage (Fig 11). Individual variation in gene expression levels was considerable and no significant differences were found between the different development stages. The mean GSI and blood plasma concentrations of T and 11-KT increased significantly to the spermatid stage and decreased slightly to the spermatozoa stage (Fig 12).

**Fig 11 Gene expression levels of selected endocrine factors in relation to testicular development stages.** Gonadotropin-releasing hormone II (*gnrh2*, A); gonadotropin-releasing
hormone III (gnrh3, B); Follicle stimulating hormone receptor (fshr, C); Leuteinizing hormone receptor (lhr, D); and cytochrome P450 17a1 (cyp17a1, E), grouped according to testicular development stages. Different letters on top of error bars indicate significant differences in expression levels between the development stages.

**Fig 12 Testis growth and mean plasma concentrations of androgens in relation to testicular development stages.** Gonadosomatic index (GSI, A), and plasma concentrations (ng/ml) of testosterone (T, B) and 11-ketotestosterone (11-KT, C) grouped according to testicular development stages. Different letters on top of error bars indicate significant differences between the development stages.

**Discussion**

The oogenesis and spermatogenesis in lumpfish resembled these processes in other teleosts [46]. Gametogenesis in lumpfish was accompanied with changes in endocrine factors linked to gonadal development; the common pattern of endocrine factors was an increase with the successive recruitment of advanced stages and a decrease later. The exceptions to such patterns were demonstrated by brain gnrh2 and gnrh3 expressions that decreased in females
but increased in males, and T and 11-KT that increased throughout ovarian development. Despite large variation in some of the endocrine factors especially in males, the patterns could be related to their physiological roles during gonadal development.

Characterization of gonadal development in lumpfish

Females

The oocyte development in ovigerous lamellae is a common ovarian organization in most teleosts [17,28]. The primary growth stage of lumpfish comprised of perinucleolar oocytes which increase in cell and nucleus sizes and synthesize organelles and molecules for later stages [28]. The secondary growth stage comprises the vitellogenic process [12,33], and here the starting point in lumpfish females was the formation of cortical alveoli and visible lipid droplets, followed by the three main vitellogenic stages: primary yolk, secondary yolk, and tertiary yolk oocytes. The presence of a lipid droplet in the lumpfish oocytes categorizes the future eggs into the energetic category Type II implying that the offspring acquire half of their energy from amino acids and half from lipids [47]. The lipid droplets in lumpfish occurred simultaneously with cortical alveoli, although the common order seems to be the appearance of cortical alveoli first [19]. During the secondary growth stage, oocyte growth is associated mainly with the synthesis and incorporation of nutritional reserves and maternal RNA needed for the development of the embryo [12,28]. At oocyte maturation, we observed the germinal vesicle breakdown and yolk coalescence, which is when the incorporation of yolk materials is stopped and the first meiotic division advances to metaphase of the second meiotic division; along with germinal vesicle breakdown, yolk coalescence and oocyte hydration [12,17,48]. Whereas ovulating oocytes were still in the thinning follicles of the ovigerous lamellae, ovulated eggs were already expelled from the observed ruptured follicles lamellae, implying their release into the lumen as described by Davenport [5].
We observed three main vitellogenic oocyte stages (primary, secondary, and tertiary yolk oocytes) present simultaneously with the oocyte maturation stage or with ovulated eggs in the ovaries of lumpfish females, which is typical of a group-synchronous ovarian organization [49,50] with a batch-spawning pattern [51]. Thus, the lumpfish females, we analyzed would possibly have released four batches in one spawning season. Similarly, two to four batches were suggested elsewhere [5,52], on the other hand, a maximum of two batches has also been suggested [24,26]. Apart from possible differences in the methodologies used, the timing and location of sampling during the season could have led to the different conclusions in number of batches. Multiple batch spawning may be regarded as a bet-hedging strategy by the fish to endure environmental stochasticity where a greater number of egg batches is spawned in unpredictable rather than in predictable environments, as explored by the Atlantic cod [53,54]. Thus, to adequately understand the reproduction potential of lumpfish under farm conditions, the total fecundity and number of egg batches necessitate further studies.

The oocyte size showed the steepest increase during the transition from secondary growth to oocyte maturation, which was also observed by Kennedy and Pountney and co-workers [7,24]. This increase during the transition to the most advanced stages is due to the accumulation of maternal products [17,55], inclusion of yolk granules [17], and hydration [17,50,55]. The leading cohort diameters of primary growth, secondary growth and oocyte maturation in the present study were smaller than those reported for stages 2, 3 and 4 which were described for wild populations of lumpfish by Kennedy [24]. Such difference is possibly due to the formalin preservation and embedding in the present study. Further, the oocyte size distribution in our study appears to be largely different, possibly due to the absence of histological assessment in the other study [24], thus leading to potentially unaccounted continuity in oocyte development as revealed by the overlaps in the oocyte sizes, we observed
in our study. Since oocyte development is continuous, clear distinctions of oocyte stages based on size is difficult as acknowledged by Pountney and co-workers [7], who also reported overlaps in oocyte sizes between stages. The three development categories we have described (primary growth; secondary growth and oocyte maturation) were also used by Pountney and co-workers [7], although the maximum oocyte diameters per stage were larger in our study. We suggest that caution be taken when using oocyte size to estimate the ovarian development stages in lumpfish [24], as oocyte size differences can occur between populations and may be related to genotype, fish size, or the environment in which the females are exposed to [49,56,57]. Although fish size can also be positively correlated with oocyte/egg size in temperate marine fishes [58], this may not be supported by the differences in fish weight between our study and that of Pountney and co-workers [7], as the average start weight in our study was smaller (ca 900 g) than in the other where it ranged between 1208.9 and 1306.4 g [7]. The decrease in nucleus: oocyte diameter ratios from perinucleolar to secondary yolk oocytes may be due to the intensities of activities in the nucleus and cytoplasm at different stages of development, where in the earlier stages there are nucleolar proliferation; growth; fragmentation and the eventual intense nucleolar transcriptional activity [59,60]. In the later stages, there are proliferation of membranous organelles and accumulation of ribonucleoproteins and yolk materials in the cytoplasm, thus the oocyte diameter increases faster compared to the nucleus diameter [60,61].

**Males**

In lumpfish males, the observed cystic spermatogenesis is typical for fish and amphibians [39]. The survival of germ cells relies on their association with Sertoli cells leading to a Sertoli/germ cell unit which begins as a single spermatogonium enveloped by a few Sertoli cells, hence synchronously developing germ cells within the spermatogenic cysts are making up the germinal epithelium [16,18,62]. The spatial arrangement of germ cells in a lumpfish testis
resembled the unrestricted spermatogonial distribution type [11] because the spermatogonia was spread along the germinal compartment of the testis [39]. The production of new spermatogenic cysts has also been observed in both peripheral and central areas of the lumpfish testis [26]. The blood vessels, we observed, were harbored in the interstitial epithelium, in which Leydig cells, macrophages, mast cells and lymphatic vessels are present [11,26], and the demarcation between the germinal and interstitial compartments is a basement membrane [63]. Spermatogenic cells similar to those we found in lumpfish are highly conserved in fish spermatogenesis that has three major phases, proliferative/spermatogonial phase, meiotic/spermatocytary phase and spermiogenic/differentiation phase [11,64].

Molecular changes during gonadal development

Gonadotropin releasing hormones

With respect to the release of gonadotropins and gonadal development, we found declining tendencies although insignificant of the mean gnrh2 and gnrh3 expressions in lumpfish females and increasing tendencies in males, which may question the physiological roles of GnRH2 and GnRH3 during gonadal development and reproduction. In fishes with two GnRH forms, the levels of one GnRH form correlate with gonadal development, while in those with three GnRH forms one, two or all three GnRHs can correlate with gonadal development [65]. Andersson and co-workers [26] found an increase of the GnRH2 receptor gnrh2ba1 to mid hydration (oocyte maturation) followed by a decrease later in lumpfish females, which is different from our gnrh2 results. Such difference could be addressed through further analysis of the gnrh2 and gnrh2ba1 expressions in relation to the feeding behavior and apoptosis prior to and during spawning. In support of this, increases of gnrh2 though insignificant, and atresia were detected in fasted compared to fed Zebrafish [66]. On the other hand, gnrhl and gnrh3 decreased from primary growth to final oocyte maturation in blue gourami (Trichogaster trichopterus) [67].
oocytes, which corresponds to our current findings. However, we acknowledge that there could be expression differences in different tissues and \textit{gnrh} expressions in the oocytes could be destined for a different role for example apoptosis [12]. Otherwise, the patterns of different GnRH forms and their gene expressions in the brain and pituitary have generally correlated positively with gonadal development in turbot [68,69], Atlantic cod [70] and striped bass [71]. These patterns imply that the neurohormones play critical physiological roles in the regulation of gonadal development and reproductive behavior in the studied species. As the primary regulators of reproduction, future studies are necessary to further understand the relationships of the GnRHs with the ovarian development in lumpfish. Studying other neurohormones and neurotransmitters such as NPY (Neuropeptide Y) and GABA (\textit{\gamma}-amino-butyric acid) [17] that have been shown to regulate GTH secretion and reproduction could also improve our understanding of the brain control of ovarian development and reproduction in lumpfish.

It is likely that the expressions of \textit{gnrh}s and \textit{gthr}s are sexually dimorphic in lumpfish as evidenced by higher expression levels of \textit{gnrh2}, \textit{gnrh3}, \textit{fshr} and \textit{lhr} in males compared to females. Additionally, the opposite changes of the \textit{gnrh2} and \textit{gnrh3} in males to that of females may suggest that there is a differential neurohormonal regulation according to sex [72]. The increase in \textit{gnrh} expression during testicular development suggests that the GnRHs trigger reproduction and directly regulate testicular development in lumpfish males. Andersson and co-workers [26] also found an increase in expression of the GnRH2 receptor gene \textit{gnrh2ba1} to its highest level in most developed males and a decrease at completion of spawning in the same species, similar to what is found in the brain of turbot [68] and blue gourami [73].
Gonadotropin receptors

The genes encoding the gonadotropin receptors \(fshr\) and \(lhr\), the vitellogenin receptor \(vtgr\), and the aromatase enzyme \(cyp19a1\) developed similarly to the sex steroids T, E2 and 11-KT, and demonstrated a contrasting pattern with those of the \(gnrh\)s in the females. The parallel trends demonstrated by \(fshr\) and \(lhr\) imply that the development of primary and secondary growth oocytes when the dominant batch is at oocyte maturation, is associated with both \(fshr\) and \(lhr\) peaking at oocyte maturation. The gene \(fshr\) is associated with vitellogenesis due to its highest peak at this stage, while \(lhr\) has been linked to oocyte maturation and/or ovulation as it showed highest peak at the fully grown oocyte stage in tilapia and zebrafish [74,75]. Our findings may indicate that FSH and LH are synthesized simultaneously to support different oocyte batches at the same time [76] causing them to increase in parallel at oocyte maturation. However, due to different batches developing at different times and speeds in the same ovary, there is a possibility that while different stages of oocytes are exposed to distinct levels of gonadotropins, the signaling is controlled by varying expressions of their receptors [77]. For example, Andersson and co-workers [26], found that while \(fshb\) increased (not significantly) from early vitellogenesis (secondary growth) to late vitellogenesis (secondary growth) and then decreased, \(lhb\) peaked at mid hydration (oocyte maturation) and decreased later significantly. With focus on seasonal changes, \(fshr\) expression increased moderately during gonadal growth while that of \(lhr\) showed a high peak at spawning in Atlantic cod [77]. Furthermore, despite their distinct biological roles that are universal, the gonadotropin receptors are probably differentially expressed between fish species with similar ovarian organizations and spawning strategies. Differences in gonadal stage-based peak expression levels of the studied endocrine factors in this study from others could also be associated with different methods used in determination of the gonadal development stages in the multiple batch spawners, but also the possible bet-
hedging strategies of multiple batch spawners (hence spawning different numbers of egg
batches) as discussed before.

In male fish as in other vertebrates, LH stimulates LH receptors (LH-R) in Leydig cells
which produce sex steroids while FSH stimulates the FSH receptor (FSH-R) in Sertoli cells
which support the development and survival of germ cells [16]. We found in the lumpfish males
that both \textit{fshr} and \textit{lhr} expressions showed an increase (not significant) to the spermatocyte
stage and a decrease during later stages. Varying profiles are found in different fish species: In
the Atlantic salmon parr \textit{fshr} increased from early spermatogenesis and peaked at
spermiogenesis, there was no further increase at spermiation while \textit{lhr} increased and peaked at
spermiation [78]. In the rainbow trout, both \textit{fshr} and \textit{lhr} expression levels were initially low,
peaked at spermiogenesis and decreased at post-spermiation [79]. In the Atlantic salmon grilse,
\textit{fshr} and \textit{lhcgr} (Luteinizing hormone chorionic gonadotropin receptor) were on the contrary
higher in fish with spermatogonia, they decreased in fish with spermatocytes/spermatids but
increased slightly when spermatozoa were present [80]. The parallel profiles of \textit{fshr} and \textit{lhr}
may indicate that the receptors respond by binding FSH and LH which function simultaneously
in the way that FSH regulates Sertoli cell activities and LH regulates Leydig cell sex steroid
production, in addition to the possible cross-activation of their receptors [11,81]. However,
based on the study by Andersson and co-workers, \textit{fshb} and \textit{lhb} expressions increased to their
highest levels in ripe males and decreased at completion of spawning. We speculate that this
may imply that gonadotropins may not express temporal variations in concert with their
receptors as discussed before for the females.

The vitellogenic receptor (\textit{vtgr}) and steroidogenic enzymes

The pattern displayed by \textit{vtgr} in lumpfish ovaries may also be associated with the multiple
batch spawning of the species, where the highest expression at oocyte maturation is due to
enrichment of different generations of secondary growth oocytes. Interestingly, vtgr has shown different patterns in various species, i.e., it has been shown to increase from the onset of lipid droplet stage remaining elevated at vitellogenesis or decrease from its highest expression level at pre-vitellogenesis [19,82]. CyP19a1 is responsible for the aromatization of androgens (testosterone) to estrogens (17β-estradiol) [83], thus the expression pattern displayed in lumpfish ovaries explains the fluctuation in the blood plasma concentrations of E2 which also peaks at oocyte maturation. Similarly, in the Atlantic cod (Gadus morhua), cyp19a1 expression levels were found to vary concomitantly with E2 blood plasma concentrations, and they exhibited a positive correlation [42].

Since CyP17a1 is crucial in the biosynthesis of sex steroids [84], the slight elevation in males of cyp17a1 at the same stage (spermatid stage) as the blood plasma concentrations of T and 11-KT (discussed later) could indicate an increased activity of this enzyme during synthesis of the androgens.

**Changes in GSI and sex steroids during gonadal development**

**Females**

During ovarian development in lumpfish, GSI and the mean blood plasma concentrations of T, 11-KT and E2 increased with the recruitment of more developed stages. At ovulation the mean blood plasma concentration of E2 showed a decrease whereas those of T and 11-KT, and GSI peaked. The increase in GSI is associated with the high investment in reproduction by females [12], and it decreases after/during spawning [85]. However, we did not monitor spawning in our study, and we could not observe a decrease in GSI. Due to the multiple batch spawning [51] in lumpfish, recruitments to vitellogenesis and final maturation could be taking place simultaneously and so do the distinct sex steroid requirements to support the different existing oocyte batches. As suggested for the gonadotropin receptors, the recruitment of new clusters
of oocytes into vitellogenesis reflected in the increasingly high blood plasma E2 levels when
the predominant oocytes are at oocyte maturation. Therefore, the high T, 11-KT and E2 blood
plasma concentrations persist during the spawning period to promote development and growth
of less advanced germ cells [86]. In agreement to our findings, Andersson and co-workers [26]
reported an increase in E2 up to mid hydration (oocyte maturation) and a decrease later in
lumpfish females. Similarly, in the Atlantic cod (Gadus morhua) blood plasma concentrations
of E2 correlated with GSI and were highest in spawning females [86]. The steroid T continued
to persist in peak levels during oocyte maturation and ovulation because its production in the
thecal cells as a precursor for E2 is not decreased contrary to the production of E2 [18]. The
increase in 11-KT during ovarian development associated with batch spawning in lumpfish
could imply that 11-KT supports several primary growth oocyte batches which later develop
through vitellogenesis and maturation. In eels, 11-KT has been shown to play roles in
development and growth of primary growth oocytes, and hence ensuring entry into
vitellogenesis [87–90]. Due to release of eggs in batches during the spawning period, there
could also be fluctuations in E2, 11-KT and T blood plasma concentrations in lumpfish
however, due to the design of our experiment we could not confirm this thus further studies are
necessary. For example, in the Atlantic halibut (Hippoglossus hippoglossus) and European
seabass (Dicentrarchus labrax) blood plasma concentrations of E2 and T exhibited fluctuations
that were associated with recruitment of successive batches of eggs during the spawning period
[91,92].

**Males**

The testicular development of lumpfish was characterized by increasing GSI, T and 11-
KT up to spermatid stage and a slight decrease towards the spermatozoa stage. The increase in
GSI is due to germ cell proliferation during spermatogenesis and the decrease is due to
completion of spermatogenesis and release of sperm during spawning as observed in the
Atlantic cod [93]. It could also be due to the reduction in Sertoli cell number and cyst volume during spermiogenesis; through apoptosis about 30-40% of germ cells are lost and during metamorphosis to spermatozoa, there are residual bodies discarded by spermatids; all this cellular debris is removed and recycled by Sertoli cells [11]. This eventually results in the observed recurrent growth-shrinkage cycles of testis weight and volume in many fish species [11]. Similarly, Andersson and co-workers [26] reported an increase in GSI of lumpfish males to the most mature stages and a decrease at the completion of spawning. The development of the androgens in lumpfish follows the common pattern during spermatogenesis, T and 11-KT increase up to and decrease at spermiation [11,94]. In agreement with our findings, 11-KT increased towards the most mature stages and decreased at the completion of spawning as reported by Andersson and co-workers [26]. Similar patterns are observed in both Atlantic cod [86] and Atlantic halibut [95], although the decreases in the androgens associated with spawning period were not as notable as those in the other two species. Androgens are said to be effective in supporting either the whole spermatogenesis or at least some stages, including roles such as initiation of puberty and spermiation, and suppression of Anti-Mullerian hormone that inhibits spermatogonial differentiation [11,96]. In lumpfish the blood plasma concentrations of T were generally higher than those of 11-KT, which appears to differ from those in the males of Atlantic halibut [95], Senegalese sole [97], Pacific halibut [98] and winter flounder [99] in which blood plasma concentrations of 11-KT were in higher quantities. This could in part be due to the activities of the enzymes 11β-hydroxylase (11βH) and 11β-hydroxysteroid dehydrogenase (11βHSD) that directly regulate 11-KT biosynthesis, where high 11-KT levels are associated with high testis enzyme expression [100]. On the other hand, T could also be a potent androgen in lumpfish due to its high blood plasma concentrations as suggested for the Japanese sardine (Sardinops melanosticus) [101,102]. However, the parallel changes of T and 11-KT throughout the testis development still imply that both steroids play
important androgenic roles during testis development in lumpfish. For 11-KT, the final peak could also be related to androgen functions outside the testis such as complete development of secondary sexual characteristics and male reproductive behavior [16,80,103].

The sexual dimorphism in the blood plasma androgen levels observed in our study support the knowledge that, in teleosts, blood plasma concentrations of 11-KT are higher in males [103]. While the blood plasma concentrations of 11-KT in lumpfish males are comparable to those found in Atlantic cod [86] and Atlantic halibut [95], the blood plasma concentrations of T are higher. The apparent presence of 11-KT in blood plasma of females, which could be a result of T cross-binding [101], warrants the need for further verification. The trends of the studied endocrine factors are also associated with feedback and feed-forward mechanisms where sex steroids exert feedback effects on the brain-pituitary axis eventually regulating the release of gonadotropins [17]. The decreasing trends of gnrhs could imply negative feedback at the brain level by the sex steroids. There could also be positive and negative feedbacks up to oocyte maturation and ovulation, respectively exerted by the sex steroids on the release of FSH and LH as reflected by the profiles of the gene expression levels of their receptors fshr and lhr. Feedback and feed-forward mechanisms have been suggested in other fish species: In Atlantic cod E2 and T stimulated GnRH2 which was suggested to have a possible feed-forward effect at the pituitary level [104]. In tilapia low doses of E2 were associated with possible positive feedback on FSH release while increasing levels were associated with possible negative feedback on FSH and LH release. Additionally, an increase in GnRH3 was associated with a possible positive feed-forward effect on FSH release during vitellogenesis and a negative feed-forward effect on LH release at maturation [105].
Conclusion

We describe oogenesis and spermatogenesis of lumpfish by distinguishing ten and four gamete stages, respectively. In females, four ovarian developmental stages are described based on those oocyte stages. The lumpfish ovary organization was typical for a group-synchronous type. In addition to the conserved nature of the process, lumpfish demonstrated cystic spermatogenesis, with an unrestricted spermatogonial distribution. While changes in the temporal gene expression of males indicate that GnRH2 and GnRH3 play physiological roles at the pituitary level, the opposite pattern in females warrants further investigation. At the gonadal level, changes in gene expression levels for the gonadotropin receptors FSH-R and LH-R, the vitellogenic receptor VTG-R and, the steroidogenic enzyme genes cyp17a1 and cyp19a1 indicate that they have important physiological roles during gonadal development. The blood plasma concentrations of sex steroids, GSI, and gonadal development in lumpfish were positively associated as expected. The season-round change in sex steroids of females resembled those of fish with group-synchronous ovarian organization.

Acknowledgments

The authors want to thank the following people/institute for their contributions to the completion of this study:
Ivar Nevermo of the Aquaculture Research Station in Kårvika, Norway for taking care of the fish during the experiment.

Senior Engineer Tora Bardal of the Department of Biology, Norwegian University of Science and Technology, for providing support in terms of resources, teaching and advice during tissue processing, imaging, and assessment of histology images.

Dr. Montse Pérez of the Sustainable Marine Aquaculture and Biotechnology Research Group (AquaCOV), Instituto Español de Oceanografía (CSIC-IEO), Centro Oceanográfico de Vigo, Spain, for participating in planification and gene amplification trials.

The Norwegian University of Science and Technology (NTNU) for funding Frank Thomas Mlingi’s Ph.D.

References


18. Nagahama Y. Endocrine regulation of gametogenesis in fish. International Journal of


morphological changes accompanying gonadal maturation. Aquaculture. 2023;566.
doi:10.1016/j.aquaculture.2022.739162


doi:10.1016/j.aqrep.2016.05.001


doi:10.1007/bf00003428


34. Macchi GJ, Barrera-Oro E. Histological study on the ovarian development of mackerel icefish (Champsocephalus gunnari) from the south Georgia islands. CCAMLR Science.


58. Christopher Chambers R. Maternal influences on variation in egg sizes in temperate


101. Matsuyama M, Adachi S, Nagahama Y, Kitajima C, Matsuura S. Testicular...


Supporting information

S1 Table Composition of RIA buffer made to 1 L of distilled water.

S2 Table Data on individual fish used for the study.
Fig 2
Figure 6: Graph showing the changes in oocyte diameter (mm) and volume (mm$^3$) across different stages of oocyte development. The x-axis represents the oocyte stages, including Perinucleolar, Cortical alveoli, Oil droplet, Primary yolk, Secondary yolk, Tertiary yolk, Maturing, and Matured. The y-axis on the left represents the oocyte diameter and volume, while the y-axis on the right represents the diameter ratio (nucleus: oocyte). Different letters (a, b, c, d, e, f, g, h) indicate significant differences between stages.
Fig 7

- **Primary growth**
  - (0.37 mm)

- **Secondary growth**
  - (1.07 mm)

- **Oocyte maturation**
  - (2.28 mm)
Fig 10
**Fig 12**

A: Gonadsomatic index (%) (not shown)

B: Testosterone (ng/ml) (not shown)

11-Ketotestosterone (ng/ml) (not shown)

Testicular development:
- Spermatogonia
- Spermatocytes
- Spermatids
- Spermatozoa