Induction of gonadal maturation at different temperatures in burbot *Lota lota*

F. J. Schaefer*†, B. Hermelink*, P. Husmann*‡, W. Meeus§, J. Adriaen§ and S. Wuertz*†‖

Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Dept. Ecophysiology and Aquaculture, Müggelseedamm 310, 12587, Berlin, Germany, †Humboldt-Universität zu Berlin, Faculty of Life Sciences, Thaer Institute of Agricultural and Horticultural Sciences, Invalidenstraße 42, 10099, Berlin, Germany, ‡Freie Universität Berlin, Department of Biology, Chemistry and Pharmacy, Takustraße 3, 14195, Berlin, Germany and §Aqua-ERF, Odisee University College, Hospitaalstraat 23, 9100, Sint-Niklaas, Belgium

A rearing experiment was conducted to test whether temperature protocols that differed from a simulation of natural conditions might induce maturation after isothermal grow-out in burbot *Lota lota*. *Lota lota* were acclimated to two different temperature regimes: low temperature (LT), close to natural temperature at 4·0° C and elevated, high temperature (HT) at 8·5° C over 40 and 27 days respectively, with all fish then wintered for 47 days. Every second fish was treated with a gonadotropin-releasing hormone analogue. Maturational competence of oocytes was assessed with a germinal vesicle breakdown assay using a novel staining strategy. In both treatments, puberty and maturational progress occurred, characterised by an elevated gonado-somatic index and advanced gonadal stages (histological analysis). Progress of gonadal maturation was reflected by elevated plasma concentrations of testosterone and 11-ketosterone in males and 17β-oestradiol in females. Ovulation was not observed. Sperm could be activated equally across treatments. In general, LT was more effective than HT treatment, indicated by advanced gonadal stages, higher numbers of oocytes undergoing germinal vesicle breakdown *in vitro* and elevated sex steroid levels. Hormone treatment could improve effectiveness at HT. In conclusion, less drastic temperature regimes as previously reported in combination with hormone treatments seem sufficient to induce maturation in *L. lota* after isothermal grow-out.

© 2016 The Fisheries Society of the British Isles

Key words: broodstock management; oogenesis; puberty; reproduction; sex steroids; spermatogenesis.

INTRODUCTION

The introduction of new species to aquaculture is often impeded by the unreliable supply and quality of larvae and juveniles. In most species, sophisticated protocols are needed to induce maturation, support gonadal maturation and synchronise spawning in captive fish, *via* manipulation of environmental conditions in combination with hormonal therapy (Mylonas *et al.*, 2010). In temperate fish species, temperature and photoperiod control the reproductive cycle and even minor changes can affect reproductive success (Wang *et al.*, 2010; Hermelink *et al.*, 2011, 2013), indicating a great potential for optimisation.

‖Author to whom correspondence should be addressed. Tel.: +49 30 64181 614; email: wuertz@igb-berlin.de

Gonadal maturation in fishes is controlled by the hypothalamus-pituitary-gonad (HPG) axis and gonadotropin-releasing hormones (GnRH) regulate the differentiation of the gonad *via* the HPG axis, integrating environmental factors such as temperature or photoperiod at the level of the hypothalamus. In response to GnRH, hypophyseal gonadotropins (FSH, follicle stimulating hormone and LH, luteinising hormone) are secreted into the blood stream, regulating the synthesis of the respective sex steroids (T, testosterone, 11-KT, 11-ketosterone and E2, 17β-oestradiol then, during final maturation, MIS, the maturation inducing steroid) and thereby maturational progress. It is widely accepted that FSH predominantly regulates early gonadal development and induces maturation whereas LH dominates maturation from vitellogenesis onwards. Thus, activation of the HPG axis is commonly regarded as initiation of maturation and its progress can therefore be followed by measuring sex steroids in the blood (Taranger *et al.*, 2010).

Aquaculture broodstocks typically consist of wild-caught breeders that are adapted to a natural photothermal cycle. The simulation of appropriate seasonal changes is cost and labour intensive and optimisation of protocols to control reproduction in temperate fish species will promote their potential for aquaculture. This is particularly valid for an all-year round production in recirculating aquaculture systems (RAS), where full control of rearing conditions is technically feasible. Here, it is hypothesised that a drastic drop in temperature over a relatively short period of time, but not reaching the natural minimum, is sufficient to induce puberty and gonadal maturation in temperate fishes that have been isothermally reared through their entire ontogeny and are therefore not adapted to the natural temperature cycle. This hypothesis was tested in a species with a known adaptation to strong thermal gradients during reproduction, the burbot *Lota lota* (L. 1758). *Lota lota* has great potential for aquaculture diversification, but its commercial production is currently hampered by a shortage of fingerlings because reproductive management requires intensive cooling to extremely low temperatures.

Lota lota is a carnivorous coldwater species with a wide holarctic distribution and it is the only member of the cod family (Gadidae) that is adapted solely to fresh water (McPhail & Paragamian, 2000). Even though *L. lota* can reach sizes of over 1 m, most adults are 30–60 cm with a mass of 1–3 kg (McPhail & Paragamian, 2000). Age and size at sexual maturation vary geographically and between sexes, ranging from 3 to 7 years, with males usually maturing a year earlier than females (McPhail & Paragamian, 2000). Furthermore, gonadal maturation indicates a high percentage of non-spawning (*i.e.* infertile) individuals in various *L. lota* populations, suggesting a multiannual reproductive cycle, at least in some areas (Pulliainen *et al.*, 1992; Pulliainen & Korhonen, 1993, 1994).

Lota lota offer highly valued white flesh and therefore have attracted the attention of commercial aquaculture. In some regions (*e.g.* Scandinavia), the liver is considered a delicacy achieving high prices. Besides production for human consumption, farmed animals may protect wild stocks through conservation aquaculture, also allowing for the reintroduction of *L. lota* in areas where they have become extinct (Worthington *et al.*, 2010).

Lota lota is a winter-spawning species, often spawning under the ice cover from December to March, where decreasing temperatures during autumn and early winter induce gonadal maturation (McPhail & Paragamian, 2000). Existing protocols recommend a drastic drop of temperature below 6° C (Vught *et al.*, 2007) or even lower (Kouril *et al.*, 1985; Pokorny & Adámek, 1997; Lahnsteiner *et al.*, 2002; Jensen *et al.*,

2008) for the induction of puberty, final maturation and spawning. Zarski *et al.* (2010) developed an elaborate protocol based on observations under natural conditions that recommends a rapid temperature decrease from 6° to 1° C within days to achieve optimum spawning results and reproductive synchronisation. In addition to thermal induction, some studies have also applied hormone treatments to synchronise spawning (Lahnsteiner *et al.*, 2002; Jensen *et al.*, 2008; Zarski *et al.*, 2010). All of these protocols applying extremely low temperatures were established with wild-caught breeders.

To test the hypothesis that temperature protocols that differed from a simulation of natural conditions might induce maturation after isothermal grow-out in *L. lota*, 25 month old fish were obtained that had been isothermally reared in RAS at constant mean temperatures of $18.7° \pm 1.0°$ C (mean $\pm$ S.D.). Two different temperature treatments, low temperature (LT) at $4.0° \pm 0.8°$ C (mean $\pm$ S.D.) and high temperature (HT) at $8.5° \pm 1.6°$ C (mean $\pm$ S.D.), were established to induce puberty and support maturation over 42 days. In both groups, 50% of all *L. lota* were randomly selected for treatment with GnRHa. After 5 days, gonadal maturation was assessed considering gonado-somatic index (I_G), histological determination of gonadal stages and blood plasma levels of the sex steroids 11-KT, E2 and T. In addition, sperm motility in males and the maturational competence of oocytes by germinal vesicle breakdown (GVBD) assay were analysed. As far as is known, this is also the first report on histological staging of gonads and a GVBD assay in *L. lota*.

MATERIAL AND METHODS

FISH REARING, TEMPERATURE AND HORMONE TREATMENT

Seventy-four immature *L. lota* with an age of 25 months, reared in RAS at constant temperatures of $18.7° \pm 1.0°$ C (mean $\pm$ S.D.), were used for the experiment. Mean $\pm$ S.D. body mass (M_B) was 440 ± 261 g. Twenty-one fish were sacrificed and dissected to characterize baseline values (stock group). The remaining fish were individually tagged with PIT tags (Trovan; www.trovan.com) and randomly distributed to six 500 l tanks arranged as RAS (5% daily water exchange; water turnover rate $\times 4$ h^{-1}) with a sedimenter, a trickling filter and a disinfection unit (UV light). Water temperature was adjusted for each tank using Titan 4000 cooling units (Aqua Medic; www.aqua-medic.de). Water temperature, oxygen (>7.2 mg l^{-1}), pH ($7.3-8.6$) were monitored daily. Other water variables (ammonia and nitrite) were checked at intervals and did not exceed critical values. Fish were kept at a photoperiod of 10L:14D and fed a commercial diet (EFICO Sigma 570°N 4,5, BioMar; www.biomar.com) with 54% crude protein, 18% crude fat and 19.1 MJ kg^{-1} gross energy at 0.5% of their M_B.

Experimental temperatures, LT at mean $\pm$ S.D. $4.0° \pm 0.8°$ C and HT at $8.5° \pm 1.6°$ C, were established over 40 and 27 days respectively with cooling starting 13 days earlier in the LT treatment. Subsequently, after 42 days at the respective temperature, half of the fish in each group received 4 μg kg^{-1} M_B Receptal [pGlu-His-Trp-Ser-Tyr-D-Ser(t-Bu)-Leu-Arg-Pro ethylamide, 4 μg ml^{-1}; Merck; www.merck-animal-health.com] by intra-muscular injection to induce final maturation (HT-I, LT-I). At the end of experiment all fish were killed and blood samples taken from the caudal vein with heparinised syringes. The M_B (to the nearest g) and total length (L_T; to the nearest 0.5 cm) were measured. For histological analysis, gonadal tissue was sampled. Gonado-somatic index (I_G) was calculated from the gonad mass (M_G; to the nearest mg) and M_B: $I_G = M_G M_B^{-1} * 100$.

SEX STEROID ANALYSIS

Full blood samples were centrifuged (4 min at 6000g, 4° C). Cell-free plasma was immediately shock frozen and stored at $-80°$ C. Hormone assays of E2, T and 11-KT were performed

according to Hermelink *et al.* (2011). Briefly, 100 μl plasma were extracted after mixing in 1 ml diethylether (Roth; www.carlroth.com) and 100 μl ultrapure water. All steroids were determined in duplicate.

SPERM ANALYSIS

Milt was obtained from dissected gonads and cooled on ice until analysis. For computer assisted sperm analysis (CASA; Microptics; www.micropticsl.com), < 1 μl of milt was transferred to the slide and flushed into the chamber with tap water to activate the sperm directly on the slide (Leja; www.leja.nl). CASA analysis was started 5 s after the end of the cell drift. Temperature was controlled at $15° \pm 0·1°$ C (mean $\pm$ S.D.) with a peltier temperature stage control (Linkam Scientific Instruments; www.linkam.co.uk). Prior to general sperm analysis, subsamples of three randomly selected individuals per group were analysed. Variables were recorded successively in time intervals of $0-5$, $5-10$ and $10-15$ s after activation. Mean overall motility showed no significant decline within the first 15 s after activation. Between 1060 and 3121 sperm cells were analysed per fish.

GONAD HISTOLOGY AND STAGING

Samples of gonadal tissue were fixated in glutaraldehyde (4% glutaraldehyde in 0·1 M phosphate buffer, pH 7·2) for 24 h, then washed three times in 0·1 M phosphate buffer (pH 7·2), twice for 1 h and final washing overnight. Samples were dehydrated in a dilution series (70, 96 and 100% ethanol) for 1 h each. Pre-infiltration was carried out with 1:1 solution of ethanol and embedding medium (Technovit 7100; Heraeus Kulzer; www.heraeus-kulzer.com) and overnight infiltration. After embedding, sections were cut to 2 μm, stained with haematoxylin-eosin and analysed with PALM Robo Imaging Software and a Zeiss AxioObserver microscope attached to a coupled charge device camera (Carl Zeiss MicroImaging; www.zeiss.com/microscopy). Staging of *L. lota* ovaries and testes was established according to general descriptions of oogenesis (Lubzens *et al.*, 2010; Breton & Berlinsky, 2014) and spermatogenesis (Schulz *et al.*, 2010) in teleosts, consistent with studies on cod *Gadus morhua* L. 1758 (Kjesbu & Kryvi, 1989; Burton *et al.*, 1997; Almeida *et al.*, 2008, 2009; Kjesbu *et al.*, 2011). For staging, an entire lobe was screened and scored according to the most advanced stages observed.

Forty female (stock group $n = 11$; HT $n = 20$; LT $n = 9$) and 32 male (stock group $n = 10$; HT $n = 11$; LT $n = 11$) *L. lota* gonads were grouped to their dominant germ-cell stage. In females, stages were early vitellogenesis (non vitellogenic and early vitellogenic follicles only), mid vitellogenesis (mid vitellogenic follicles dominating) and late vitellogenesis (late vitellogenic follicles dominating). In males, development was staged as early spermatogenesis (spermatogonia dominating), mid spermatogenesis (spermatocytes dominating) and late spermatogenesis (spermatids and spermatozoa dominating) by monitoring an entire lobe of the testis to consider central-peripheral gradients as reported for example in *G. morhua*.

GERMINAL VESICLE BREAKDOWN ASSAY

Ovarian follicles were dissected from maturing females (late vitellogenic stage only). Since the MIS in *L. lota* is unknown, progesterone as precursor of two teleost MIS, 5α-dihydroprogesterone and 17,20β-dihydroxy-4-pregnen-3-one, was used here. Each follicle was assessed individually using 24 well plates as described by Semenkova *et al.* (2008). For scoring, triplicates of 10 ovarian follicles in 1 ml Leibovitz's L15 medium (pH 7·8; Life Technologies; www.thermofisher.com) with antibiotics (6 mg l^{-1} penicillin and 10 mg l^{-1} streptomycin; Pan Biotech; www.pan-biotech.co.uk) were incubated for 5 min with 5% ethanol as dissolvent control or 1 μM progesterone (Sigma-Aldrich; www.sigmaaldrich.com). Then, follicles were washed twice with Leibovitz's L15 medium. Since the germinal vesicle (GV) could not be observed directly in the opaque follicles, samples were incubated in formalin-ethanol medium and stained using DNA-specific fluorescent dyes. After evaluation of selected dyes [4′,6 diamidino-2-phenylindole dihydrochloride (DAPI), Sigma-Aldrich; SYBR Green, Qiagen; www.qiagen.com; 0·1% ethidium bromide, Roth], samples were incubated with DAPI

first in an ultrasound bath for 2 min, then for 4 h at 37° C to facilitate dye infiltration and counterstained with haematoxylin for 20 min. The GV migration was scored as central GV (no GV migration), migrating GV (migration of GV > 30% of oocyte diameter) or GVBD.

DATA ANALYSIS

Results are presented as mean ± s.d. of n samples. For the statistical analysis, data were tested for normality (Kolmogorov–Smirnov) and homogeneity of variances. For pairwise comparison, parametric t-test or non-parametric Mann–Whitney U-test was used. For multiple comparison (no normal distribution observed here), non-parametric Kruskal–Wallis and Dunn's multiple comparison tests were used. For all tests, level of significance was $P < 0.05$. Statistical analysis was performed with GraphPad Prism 4.03 (GraphPad Software; www.graphpad.com).

RESULTS

SOMATIC GROWTH AND I_G

Body mass for the stock group prior to the experiment was 440 ± 261 g. By the end of experiment, the fish had grown to 727 ± 173 g at LT and 691 ± 144 g at HT, with no significant size differences between temperature groups or hormone-treated *v.* untreated fish. At the end of the experiment, I_G had increased both at LT (female: 10.6 ± 7.4, Kruskal–Wallis test: $H = 12.27$, 2 d.f., $P < 0.01$, Dunn's multiple comparison: $P < 0.01$; male: 8.1 ± 4.1, Mann–Whitney U-test: $U = 6.0$, $P < 0.001$) and HT (female: 6.3 ± 5.5, Dunn's multiple comparison: $P < 0.05$; male data lost) compared with day 0 (female: 2.0 ± 1.1; male: 1.1 ± 2.0). The I_G of females at LT was higher than at HT (Dunn's multiple comparison: $P < 0.05$). Hormone treatment had no significant effect on I_G compared with non treated fish at the same temperature in females (HT 5.1 ± 4.2 *v.* HT-I 7.4 ± 6.5; LT 13.4 ± 7.3 *v.* LT-I 9.2 ± 7.7) and males (LT 8.2 ± 5.2 *v.* LT-I 7.9 ± 2.7).

GONADAL DEVELOPMENT IN FEMALE *L. LOTA*

Histological analysis of female gonads provided a first detailed documentation of oocyte development in *L. lota* (Fig. 1). Female gonads were analysed and grouped according to the dominant stage (Fig. 2). Within the stock group, early vitellogenic oocytes dominated (64%) and no mid vitellogenic oocytes were observed. LT resulted in an increase of late vitellogenic oocytes (78%) compared with HT (50%), but the amount of early vitellogenic oocytes was comparable (11·1 and 15·0% respectively). In both temperature treatments, additional hormone treatment revealed a complete absence of early vitellogenic oocytes compared with untreated fish (33% at HT, 17% at LT) and within the LT-I group all three females were late vitellogenic (55% at HT-I).

GONADAL DEVELOPMENT IN MALE *L. LOTA*

Male gonadal samples were used for a first detailed documentation of spermatogenesis in *L. lota* (Fig. 3). Male gonads were analysed and grouped according to the dominant stages (Fig. 4). On day 0, only early spermatogenic gonads were observed. Both temperature treatments induced germ-cell development but revealed a higher abundance of late spermatogenic stages at LT (45% at HT incl. HT-I; 73% at LT
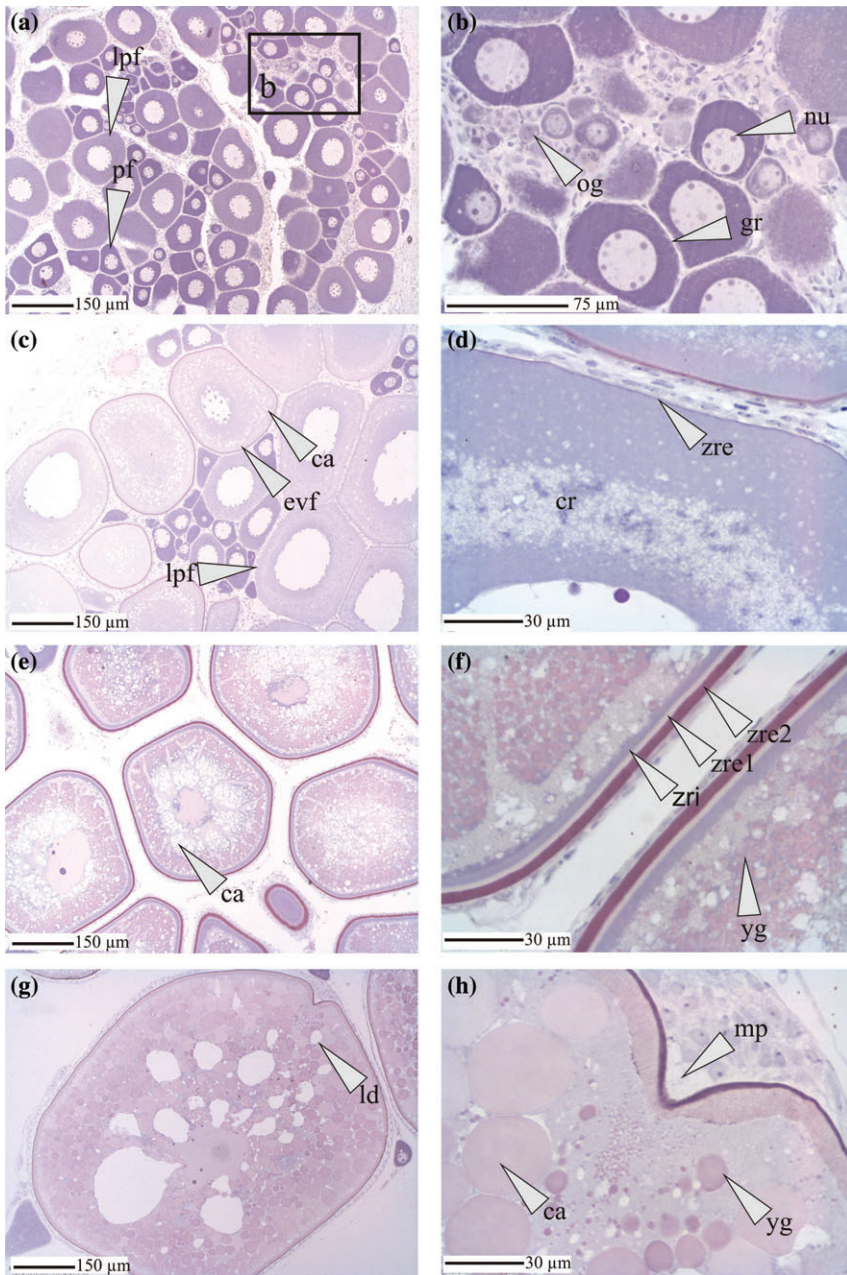
FIG. 1. Histological sections showing oogenesis in *Lota lota*. (a), (b) Previtellogenic gonad with oogonia (og) and previtellogenic follicles (pf), several at the perinucleolar stage [late perinucleolar follicle (lpf)] with multiple nucleoli (nu) and a monolayer of granulosa cells (gr) surrounding the oocyte. (c), (d) Late previtellogenic follicles with acidophilic cytoplasm, cortical alveoli (ca) and often a cytoplasmatic circumpolar ring (cr), as well as the zona radiata externa (zre) and early vitellogenic follicles (evf). (e), (f) Mid vitellogenic follicles with a stratified zre (two distinct layers zre1 and zre2) and a zona radiata interna (zri) and yolk globules (yg). (g), (h) Late vitellogenic follicles with large yg, ca close to the oocyte membrane, lipid droplets (ld) and a micropyle (mp).
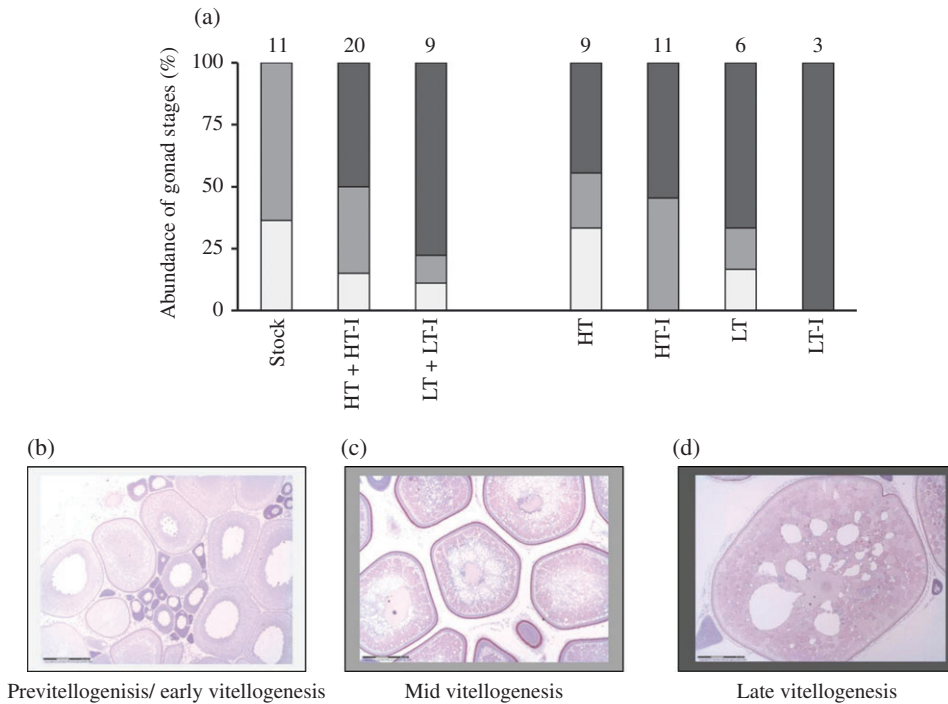
FIG. 2. (a) Per cent abundance of follicle stages in adult *Lota lota* before wintering (isothermally reared stock) and after temperature treatment in the high (HT, 8·5° C) and low temperature (LT, 4·0° C) group and additionally the gonadotropin-releasing hormones (LT-I and HT-I) treated subgroups. □, Previtellogenesis–early vitellogenesis; ▨, mid vitellogenesis; ■, late vitellogenesis. The number of samples is indicated at the top of each column. (b) Histological section showing previtellogenesis–early vitellogenesis in *L.lota*, (c) mid vitellogenesis and (d) late vitellogenesis.

incl. LT-I). Within the HT group, late spermatogenesis (71%) was only observed upon hormone treatment, whereas all untreated fish remained in mid spermatogenesis. In contrast, both treated and untreated fish at lower temperatures contained gonads in late spermatogenesis (83% at LT only, 60% at LT-I).

SPERM ANALYSIS

Percentage of motile sperm was $62·7 \pm 26·6\%$ for the HT group, $62·5 \pm 31·9\%$ for HT-I and $74·8 \pm 27·1\%$ for LT. Mean motility did not differ significantly between treatments, despite the slight increase in motile sperms at 4° C. All samples contained activated sperm.

SEX STEROIDS

There was no difference in females E2 plasma concentrations at LT (LT + LT-I: $14·7 \pm 17·4$ ng ml^{-1}; LT: $16·8 \pm 21·5$ ng ml^{-1}; LT-I: $11·1 \pm 9·8$ ng ml^{-1}), compared with the stock group ($3·6 \pm 6·9$ ng ml^{-1}) and to the HT treatments (HT + HT-I: $2·5 \pm 2·3$ ng ml^{-1}; HT: $2·6 \pm 2·5$ ng ml^{-1}; HT-I: $2·3 \pm 2·2$ ng ml^{-1}) (Fig. 5).
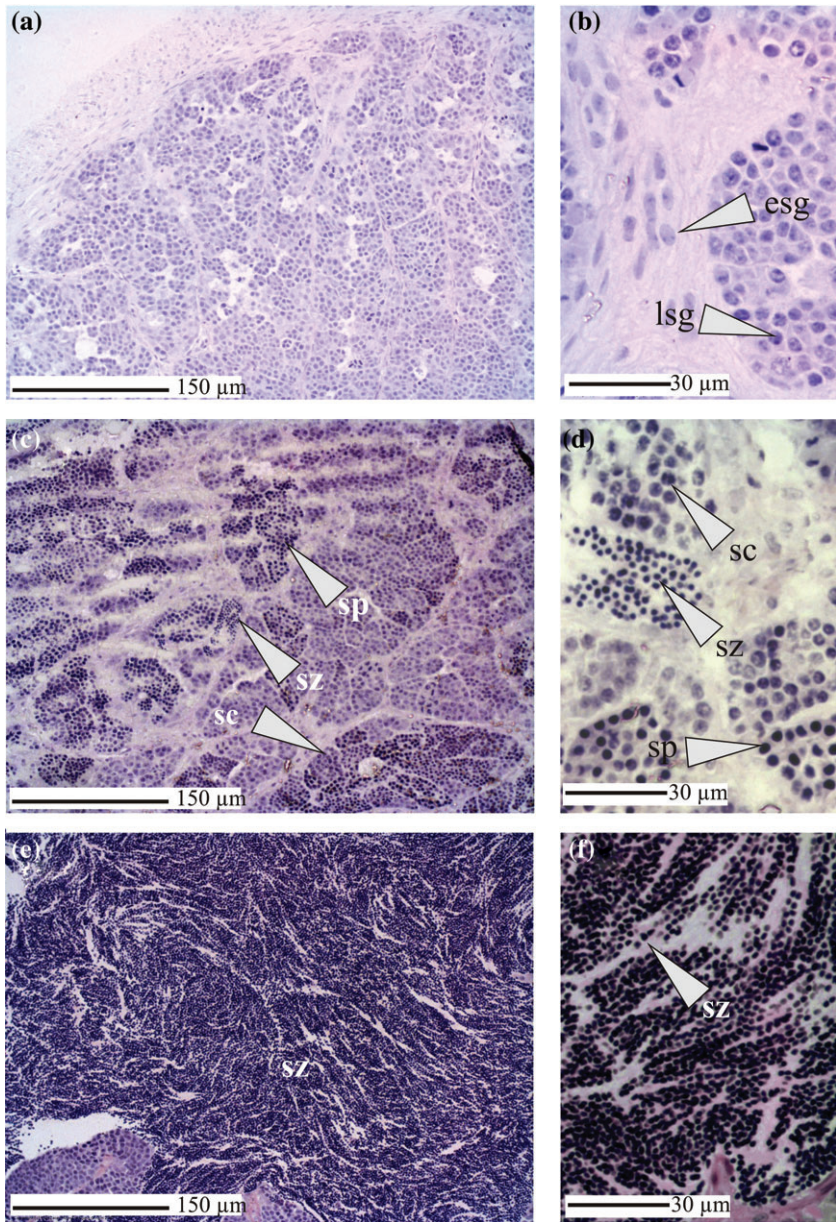
FIG. 3. Histological sections showing spermatogenesis in *Lota lota*. (a), (b) Early spermatogenesis with early (esg) and late (lsg) spermatogonia and few spermatocysts (not shown). (c), (d) Mid spermatogenesis with spermatocysts (sc), spermatids (sp) and some spermatozoa (sz)-elongated spermatids. (e), (f) Late spermatogenesis with mainly sz-elongated spermatids.
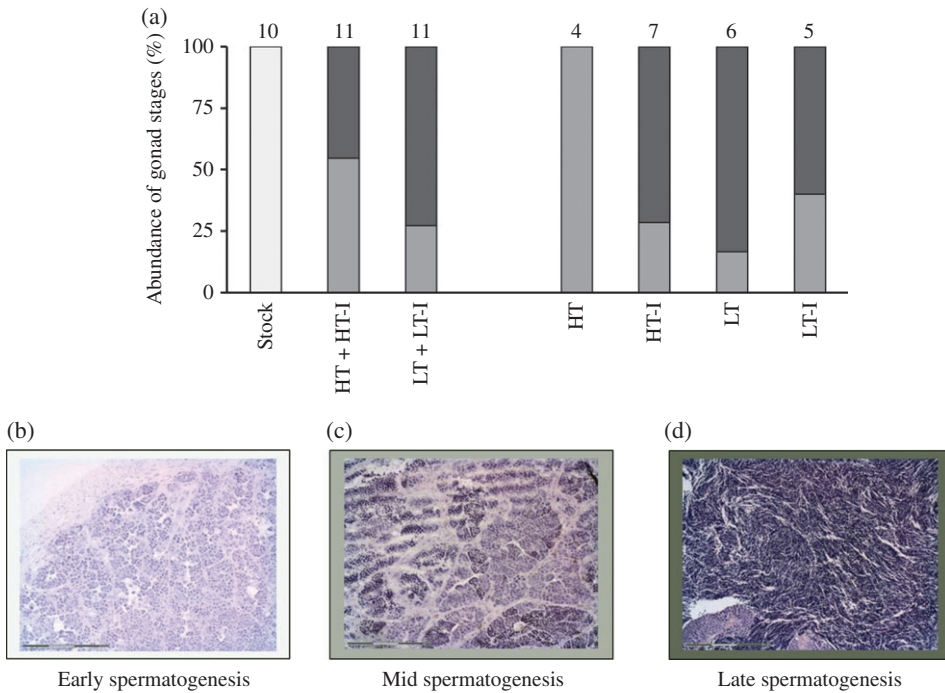
FIG. 4. (a) Relative abundance of testis stages in adult *Lota lota* before wintering (isothermally reared stock) and after temperature treatment in the high (HT, 8·5° C) and low temperature (LT, 4·0° C) group and additionally the gonadotropin-releasing hormones (LT-I and HT-I) treated subgroups. □, early spermatogenesis; ▨, mid spermatogenesis; ■, late spermatogenesis. The number of samples is indicated at the top of each column. (b) Histological section showing early spermatogenesis in *L.lota*, (c) mid spermatogenesis and (d) late spermatogenesis.

In males, blood plasma concentrations of androgens (11-KT, T) were elevated in all treatments compared with the stock group (11-KT: $2·5 \pm 3·5$ ng ml^{-1}; T: $2·8 \pm 4·07$ ng ml^{-1}). Significant differences could be observed at LT for both 11-KT (LT + LT-I: $34·2 \pm 24·9$ ng ml^{-1} Kruskal–Wallis test: $H = 16·2$, 2 d.f., $P < 0·001$, Dunn's multiple comparison: $P < 0·001$; LT: $36·5 \pm 31·7$ ng ml^{-1} Kruskal–Wallis test: $H = 16·9$, 4 d.f., $P < 0·01$, Dunn's multiple comparison: $P < 0·05$; LT-I: $31·9 \pm 18·6$ ng ml^{-1} Dunn's multiple comparison: $P < 0·01$) and T (LT + LT-I: $35·5 \pm 21·3$ ng ml^{-1} Kruskal–Wallis test: $H = 18·3$, 2 d.f., $P < 0·001$, Dunn's multiple comparison: $P < 0·001$; LT: $39·7 \pm 25·2$ ng ml^{-1} Kruskal–Wallis test: $H = 18·5$, 4 d.f., $P < 0·01$, Dunn's multiple comparison: $P < 0·01$; LT-I: $31·4 \pm 18·1$ ng ml^{-1} Dunn's multiple comparison: $P < 0·01$), whereas there were no significant differences in 11-KT (HT + HT-I: $14·5 \pm 14·1$ ng ml^{-1}; HT: $10·2 \pm 2·1$ ng ml^{-1}; HT-I: $16·9 \pm 17·7$ ng ml^{-1}) or T levels at HT (HT + HT-I: $11·5 \pm 5·4$ ng ml^{-1}; HT: $14·0 \pm 5·5$ ng ml^{-1}; HT-I: $10·1 \pm 5·2$ ng ml^{-1}).

When grouped according to the gonadal stage of each individual (pre, early and mid vitellogenesis or spermatogenesis respectively), female sex steroid levels did not differ among developmental stages, from pre (E2: $2·4 \pm 2·5$ ng ml^{-1}), over early (E2: $3·5 \pm 6·0$ ng ml^{-1}) to mid vitellogenesis (E2: $8·6 \pm 13·5$ ng ml^{-1}) (Fig. 6). In males, levels of 11-KT and T were significantly lower during pre
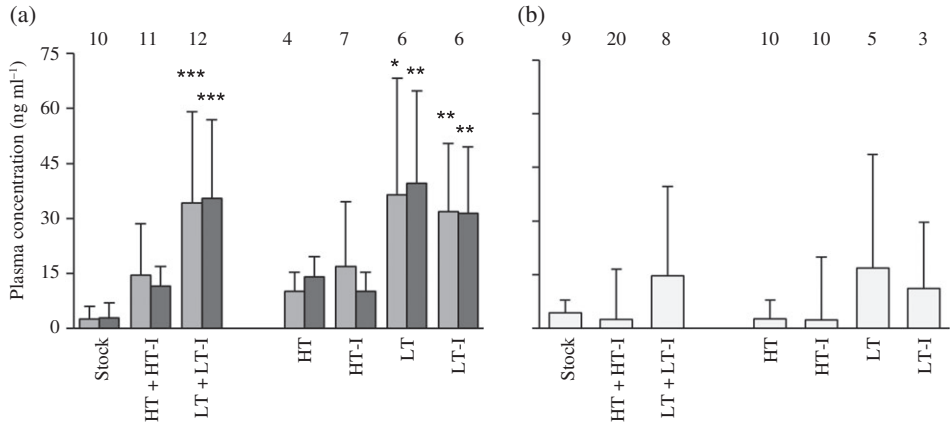
(a)

(b)

FIG. 5. Blood plasma concentrations of sex steroids (mean ± S.D.) in *Lota lota* before wintering (isothermally reared stock) and after temperature treatment in the high (HT, 8·5° C) and low temperature (LT, 4·0° C) group and additionally the gonadotropin-releasing hormones (LT-I and HT-I) treated subgroups. (a) Androgens 11-ketosterone (□), testosterone (■) in males and (b) 17β-oestradiol (□) in females. Significant differences against the stock group are indicated by asterisks (Dunn's multiple comparison; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). The number of samples is indicated at the top of each column.

spermatogenesis (11-KT: 2.5 ± 3.5 ng ml^{-1} Kruskal–Wallis test: $H = 15.9$, 2 d.f., $P < 0.001$; T: 2.8 ± 4.1 ng ml^{-1} Kruskal–Wallis test: $H = 16.8$, 2 d.f., $P < 0.001$) compared with early (11-KT: 27.1 ± 29.1 ng ml^{-1} Dunn's multiple comparison: $P < 0.01$; T: 22.6 ± 21.0 ng ml^{-1} Dunn's multiple comparison: $P < 0.01$) and mid spermatogenesis (11-KT: 25.1 ± 17.4 ng ml^{-1} Dunn's multiple comparison: $P < 0.001$; T: 26.9 ± 19.1 ng ml^{-1} Dunn's multiple comparison: $P < 0.001$). There were no differences in 11-KT or T levels between early and mid spermatogenesis.
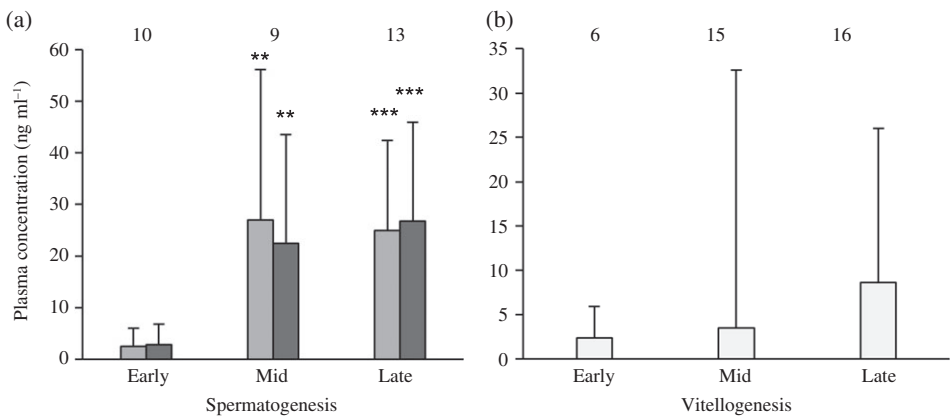
(a)

(b)

FIG. 6. Blood plasma concentrations of sex steroids (mean ± S.D.) in *Lota lota* grouped according to the respective stages of gonad development. (a) 11-ketosterone (□), testosterone (■) in early, mid and late spermatogenesis and (b) 17β-oestradiol (□) in early, mid and late vitellogenesis. Significant differences against the early group are indicated by asterisks (Dunn's multiple comparison; $^{**}P < 0.01$; $^{***}P < 0.001$). The number of samples is indicated at the top of each column.
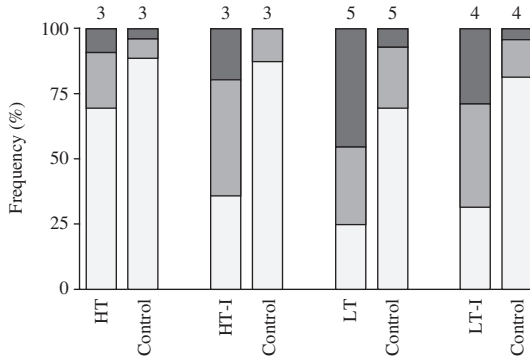
FIG. 7. Germinal vesicle breakdown (GVBD, ■) assay after progesterone treatment (5 min, 1 μM progesterone) assessing migration of the nucleus–germinal vesicle [central GV (□), migration of GV (▨) > 30% of oocyte diameter] and GVBD ($n = 30$ fish^{-1}) of respective female *Lota lota* after temperature treatment in the high (HT, 8·5° C) and low temperature (LT, 4·0° C) group, and additionally the gonadotropin-releasing hormones (LT-I and HT-I) treated subgroups compared with the solvent controls (5 min, 5% ethanol). The number of samples is indicated at the top of each column.

GERMINAL VESICLE BREAKDOWN

Upon *in vitro* progesterone exposure, percentage of oocytes with migrating GV or GVBD increased compared with the controls (Fig. 7). Number of oocytes with GVBD (9·2%) and migrating GV (21·3%) were lowest at HT, but were increased at HT-I fish (migrating GV: 44·4%; GVBD: 19·8%). LT contained 45·3% of oocytes with completed GVBD and 29·9% with migrating GV. The combination of LT and hormone treatment (LT-I) did not lead to higher number of GVBD (migrating GV: 39·7%; GVBD completed: 28·9%).

DISCUSSION

Here, both temperature treatments induced maturation, as indicated by an activation of the HPG axis (significant only for the androgens in males), as well as vitellogenic stages of the gonad in females and late spermatogenic stages in males. Furthermore, a significantly elevated I_G was observed. The LT treatment, however, led to more advanced maturation, higher competence of oocytes to undergo GVBD and, on average, higher I_G compared with HT. To some extent, the lesser maturation progress at HT compared with LT could be compensated by hormone treatment.

Within the present study, I_G was slightly lower than reported by other authors in *L. lota*, particularly in males. Brylinska *et al.* (2002) reported average I_G of 5·6 in females and 12·5 in males and Cott *et al.* (2013) observed a peak in I_G during late winter at around 10–12 in males and only 8–10 in females. Still, fish in the present study were first maturing fish and differences are within the expected range.

Despite the indicated competence of oocytes to undergo GVBD, the absence of ovulated eggs in either treatment hindered an analysis of the full reproductive cycle. Therefore, conclusive statements about reproductive success of the experiment in females are precluded. In contrast, sperm were obtained that could subsequently be activated.

Principal sex steroids clearly indicated activation of the HPG axis and maturation in males (T, 11-KT), whereas elevations were not significant in females (E2). Mustonen *et al.* (2002) reported plasma oestradiol of 13 ng ml^{-1} in maturing females, 7 ng ml^{-1} during spawning and 5 ng ml^{-1} post-spawning. In males, no 11-KT was determined but testosterone revealed 12 ng ml^{-1} during maturation, 4 ng ml^{-1} at spawning and <1 ng ml^{-1} post-spawning (Mustonen *et al.*, 2002). These steroid levels differed from the results of the present study in regard to male 11-KT, which was higher at LT. As expected, this pattern suggests a strong correlation of sex steroid levels and maturation progress.

The hormone treatment did not affect steroid levels in either treatment compared with untreated fish at the same temperature. In contrast to HT, only minor effects of hormone treatment on gonadal maturation and *in vitro* GVBD were observed at LT. Sperm could also be activated in untreated fish at HT, suggesting maturation at HT in males irrespective of hormone therapy. A similar pattern was observed in HT female gonadal maturation, where hormone treated individuals showed on average further developed gonads. These findings suggest that, at least to some extent, compensation of the smaller temperature gradient at higher temperatures by GnRHa therapy is feasible.

In general, the study emphasises sex-specific differences in appropriate temperature protocols in *L. lota*, with males being more adapted towards reproduction at higher temperatures. It was shown by Jensen *et al.* (2008) that spawning upon segregation of male and female *L. lota* within the same recirculation system occurs, suggesting that at least physical contact may not be necessary for spawning. These findings may allow for a sex-specific adaptation of rearing protocols in *L. lota* reproduction. On the other hand, results clearly indicate the potential of GnRH therapy, particularly with regard to elevated temperatures.

In this study, less drastic temperature adjustments than have previously been recommended were sufficient to induce gonadal maturation in a RAS-hatched and reared *L. lota* broodstock. Furthermore, LT was clearly more effective than HT, but it was possible to compensate for these differences through the application of hormones at HT. In a future study, gamete and larvae quality of the respective protocol need to be studied in detail. In addition, ovulated eggs were not observed and a successful reproduction consequently has to be carried out in the future.

In conclusion, the application of higher temperatures in reproductive management of *L. lota* is a feasible strategy to facilitate farming of this species. Generally, experiments as conducted here will contribute to optimising reproduction protocols for RAS-hatched broodstocks of temperate species adapted to higher temperature after isothermal grow-out. Aquaculture of such species would greatly benefit from the establishment of less cost-intensive protocols.

References

Almeida, F. F., Kristoffersen, C., Taranger, G. L. & Schulz, R. W. (2008). Spermatogenesis in Atlantic cod (*Gadus morhua*): a novel model of cystic germ cell development. *Biology of Reproduction* **78,** 27–34. doi: 10.1095/biolreprod.107.06366

Almeida, F. F., Taranger, G. L., Norberg, B., Karlsen, Ø., Bogerd, J. & Schulz, R. W. (2009). Photoperiod-modulated testis maturation in Atlantic cod (*Gadus morhua*, L.) *Biology of Reproduction* **80,** 631–640. doi: 10.1095/biolreprod.108.071340

Breton, T. S. & Berlinsky, D. L. (2014). Characterizing ovarian gene expression during oocyte growth in Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology D* **9,** 1–10. doi: 10.1016/j.cbd.2013.11.001

Brylinska, M., Chybowski, L. & Boguszewski, A. (2002). Reproductive biology of burbot, *Lota lota lota*, in Lake Hancza, Poland. *Folia Zoologica* **51,** 141–148.

Burton, M. P. M., Penney, R. M. & Biddiscombe, S. (1997). Time course of gametogenesis in Northwest Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 122–131. doi: 10.1139/f96-167

Cott, P. A., Johnston, T. A. & Gunn, J. M. (2013). Sexual dimorphism in an under-ice spawning fish: the burbot (*Lota lota*). *Canadian Journal of Zoology* **91,** 732–740. doi: 10.1139/cjz-2013-0083

Hermelink, B., Wuertz, S., Trubiroha, A., Rennert, B., Kloas, W. & Schulz, C. (2011). Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. *General and Comparative Endocrinology* **172,** 282–292. doi: 10.1016/j.ygcen.2011.03.013

Hermelink, B., Wuertz, S., Rennert, B., Kloas, W. & Schulz, C. (2013). Temperature control of pikeperch (*Sander lucioperca*) maturation in recirculating aquaculture systems – induction of puberty and course of gametogenesis. *Aquaculture* **400,** 36–45. doi: 10.1016/j.aquaculture.2013.02.026

Jensen, N. R., Williams, S. R., Ireland, S. C., Siple, J. T., Neufeld, M. D. & Cain, K. D. (2008). Preliminary captive burbot spawning observations. In American Fisheries Society Symposium *59:* Burbot – Ecology, Management, and Culture (Paragamian, V. L. & Bennett, D. H., eds), p. 155. Bethesda, MD: American Fisheries Society.

Kjesbu, O. S. & Kryvi, H. (1989). Oogenesis in cod, *Gadus morhua* L., studied by light and electron microscopy. *Journal of Fish Biology* **34,** 735–746. doi: 10.1111/j.1095-8649.1989.tb03353.x

Kjesbu, O. S., Thorsen, A. & Fonn, M. (2011). Quantification of primary and secondary oocyte production in Atlantic cod by simple oocyte packing density theory. *Marine and Coastal Fisheries* **3,** 92–105. doi: 10.1080/19425120.2011.555714

Kouril, J., Linhart, O., Dubsky, K. & Kavasnicka, P. (1985). The fertility of female and male burbot (*Lota lota* L.) reproduced by stripping. *Vodnany Research Institute of Fishery and Hydrobiology* **14,** 75–79.

Lahnsteiner, F., Mansour, N. & Weismann, T. (2002). The cryopreservation of spermatozoa of the burbot, *Lota lota* (Gadidae, Teleostei). *Cryobiology* **45,** 195–203. doi: 10.1016/s0011-2240(02)00140-2

Lubzens, E., Young, G., Bobe, J. & Cerda, J. (2010). Oogenesis in teleosts: how fish eggs are formed. *General and Comparative Endocrinology* **165,** 367–389. doi: 10.1016/j.ygcen.2009.05.022

McPhail, J. D. & Paragamian, V. L. (2000). Burbot biology and life history. In American Fisheries Society Symposium *59:* Burbot – Ecology, Management, and Culture (Paragamian, V. L. & Bennett, D. H., eds), pp. 11–23. Bethesda, MD: American Fisheries Society.

Mustonen, A. M., Nieminen, P. & Hyvarinen, H. (2002). Leptin, ghrelin and energy metabolism of the spawning burbot (*Lota lota*, L.) *Journal of Experimental Zoology* **293,** 119–126. doi: 10.1002/jez.10142

Mylonas, C. C., Fostier, A. & Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *General and Comparative Endocrinology* **165,** 516–534. doi: 10.1016/j.ygcen.2009.03.007

Pokorny, J. & Adámek, Z. (1997). Artificial spawning and fingerling culture in burbot. *Vodnany Research Institute of Fishery and Hydrobiology* **53,** 1–10. English translation.

Pulliainen, E. P. & Korhonen, K. (1993). Does the burbot, *Lota lota*, have rest years between normal spawning seasons? *Journal of Fish Biology* **43,** 355–362. doi: 10.1111/j.1095-8649.1993.tb00570.x

Pulliainen, E. P. & Korhonen, K. (1994). Sagittal otolith growth patterns in regularly and irregularly spawning burbot, *Lota lota*, in northern Finland. *Environmental Biology of Fishes* **40,** 149–157. doi: 10.1007/bf00002541

Pulliainen, E. P., Korhonen, K., Kankaanranta, L. & Mäki, K. (1992). Non-spawning burbot on the northern coast of the Bothnian Bay. *Ambio* **21,** 170–175.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., Legac, F., Chiarini-Garcia, H., Nobrega, R. H. & Miura, T. (2010). Spermatogenesis in fish. *General and Comparative Endocrinology* **165,** 390–411. doi: 10.1016/j.ygcen.2009.02.013

Semenkova, T., Bayunova, L., Wuertz, S., Kirschbaum, F. & Barannikova, I. (2008). Effect of C21 steroids on germinal vesicle break down in sturgeon follicles *in vitro*. *Cybium* **32,** 268–269.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F. A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E. & Hausen, T. (2010). Control of puberty in farmed fish. *General and Comparative Endocrinology* **165,** 483–515. doi: 10.1016/j.ygcen.2009.05.004

Vught, I., Harzevilli, A., Auwerx, J., De Charleroy, D., Paragamian, V. & Bennet, D. (2007). Aspects of reproduction and larviculture of burbot under hatchery conditions. In *American Fisheries Society Symposium 59:* Burbot – Ecology, Management, and Culture (Paragamian, V. L. & Bennett, D. H., eds), p. 167. Bethesda, MD: American Fisheries Society.

Wang, N., Teletchea, F., Kestemont, P., Milla, S. & Fontaine, P. (2010). Photothermal control of the reproductive cycle in temperate fishes. *Reviews in Aquaculture* **2,** 209–222. doi: 10.1111/j.1753-5131.2010.01037.x

Worthington, T., Kemp, P., Osborne, P. E., Howes, C. & Easton, K. (2010). Former distribution and decline of the burbot (*Lota lota*) in the UK. *Aquatic Conservation – Marine and Freshwater Ecosystems* **20,** 371–377. doi: 10.1002/aqc.1113

Zarski, D., Kucharczyk, D., Sasinowski, W., Targonska, K. & Mamcarz, A. (2010). The influence of temperature on successful reproductions of Burbot, *Lota lota* (L.) under hatchery conditions. *Polish Journal of Natural Sciences* **25,** 93–105. doi: 10.2478/v10020-010-0007-9