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Elsa Denker
University of Bergen
Marcus Gamst Johansen
UiT-The Arctic University of Norway
Tom Ole Nilsen
University of Bergen
Lauri Kapari
Akvaplan-niva
Birgitta Norberg
Norwegian Institute of Marine Research
Eva Andersson
Norwegian Institute of Marine Research
Thor Arne Hangstad
Akvaplan-niva
Karl-Erik Eilertsen
UiT-The Arctic University of Norway
Sigurd O. Stefansson
University of Bergen
John D. Stubblefield
University of Maryland Baltimore County
Yonathan Zohar
University of Maryland Baltimore County
Albert Kjartan Dagbjartarson Imsland
albert.imsland@uib.no

University of Bergen

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Towards controlling maturation and spawning in lumpfish (*Cyclopterus lumpus* L.): effect of GnRH analog-releasing implants on pituitary gene expression, gamete release and hatching success

Elsa Denker[1], Marcus Gamst Johansen[2], Tom Ole Nilsen[1], Lauri Kapari[3], Birgitta Norberg[4], Eva Andersson[4], Thor Arne Hangstad[3], Karl-Erik Eilertsen[2], Sigurd O. Stefansson[1], John D. Stubblefield[5], Yonathan Zohar[5] &Albert K. D. Imsland[1,6*]

[1] Department of Biological Sciences, University of Bergen, High Technology Centre, 5020 Bergen, Norway.

[2] The Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, UiT-The Arctic University of Norway, 9037 Tromsø, Norway.

[3] Akvaplan-niva, Framsenteret, 9296 Tromsø, Norway.

[4] Institute of Marine Research, Postbox 1870 Nordnes, 5817 Bergen, Norway.

[5] Institute of Marine and Environmental Technology, Department of Marine Biotechnology, University of Maryland Baltimore County, Baltimore, Maryland, USA.

[6] Akvaplan-niva Iceland Office, Akralind 6, 201 Kópavogur, Iceland. *e-mail: albert.imsland@uib.no, albert.imsland@akvaplan.niva.no

Abstract

The use of cleaner fish has become a popular alternative for chemical-free delousing of farmed Atlantic salmon, and common lumpfish (*Cyclopterus lumpus*) is extensively used as a cleaner fish in many Northern European countries. The supply of adult individuals has until recently only relied on wild-caught specimens, and efforts have now turned towards establishing breeding protocols that can produce large amounts of fully farmed lumpfish. One challenge encountered on the way to efficient and controlled spawning is the lack of synchronization of females. Implanting GnRH analog pellets into maturing adults has proved a successful method to induce maturation and gamete release in various fish species. Here we show that GnRH analog-treated lumpfish females are more homogenous as a group than sham-treated females, and tend to ovulate earlier, express higher levels of pituitary *lhb* and a lower 2-estradiol plasma concentration and produce a significantly higher amount of eggs. In males, GnRH analog treatment increases the volume of milt over time. In addition, we observed that after fertilization, eggs showed a significantly higher hatching rate. Our results suggest that the implantation of GnRH analog pellets is a good candidate to successfully induce ovulation and spawning in females at a large scale and improve egg hatching and larvae survival rates.

Keywords Lumpfish • spawning • GnRH • pituitary gene expression

Introduction

Sea lice (in particular *Lepeophtheirus salmonis*) represent one of the biggest challenges in Atlantic salmon aquaculture. The steady increase of populations resistant to conventional delousing chemicals, and the demand from the public and the farming industry to develop safe and sustainable yet effective methods have made the biological control of sea lice through the use of cleaner fish an alternative to pursue[1-2]. The common lumpfish (*Cyclopterus lumpus*) has become a species of choice in Northern Europe for its low temperature preference and is now extensively used as cleaner fish in Norway[3-6], Ireland[7], Scotland[8], Iceland[9] and the Faroese Islands[10]. There is however a need to systematize knowledge, and provide guidelines to optimize fish production and welfare, and cleaning efficiency.

To evolve away from an initial pure reliance on the capture of wild fish, the use of cleaner fish has gradually evolved towards farming, aiming at better biosecurity, control and sustainability. The first facility-produced juvenile lumpfish originated from *in vitro* fertilization and in-house incubation of larvae after stripping of wild-caught broodstock[3-6]. The gradual move towards a closed cycle and intensive cultivation was supported by the establishment of a breeding program for lumpfish in 2017[11-12]. As a successful breeding program relies on the capacity to produce juveniles on a year-round basis, it prerequires detailed basic knowledge about the reproductive and sexual maturation cycle of the species, to understand what parameters can promote and synchronize maturation and spawning in both sexes.

Photoperiod has been successfully used to alter the circannual phase of sexual cycles in broodstock and hence the spawning time in a variety of fish species, including lumpfish[13-14]. The next step was understanding the nature and timing of the downstream environment-driven basic mechanisms that regulate oogenesis and spermatogenesis and ultimately sexual maturation and spawning. In 2023, Andersson, Denker et al.[15] published a comprehensive overview on the sexual maturation cycle of lumpfish, describing the key molecular, physiological and endocrine changes associated with the annual reproductive cycle in both sexes. This study identified the endogenous modulation of the GnRH

(gonadotropin releasing hormone) signal through the regulation of GnRH receptor *gnrh2ba1* in the pituitary as one of the first identifiable signals towards maturation. Further research was therefore needed to determine whether provision of exogenous GnRH could be a method of choice to promote controlled, synchronized spawning in females, and optimize spermiation in males.

As in all vertebrates, reproductive functions in teleosts are controlled by the brain-pituitary-gonad axis. Specialized brain cells secrete decapeptides called GnRHs that have a hypophysiotropic effect, *i.e.* stimulate the release of gonadotropins by pituitary cells (Follicle stimulating hormone, FSH and Luteinizing hormone, LH) which in turn bind to receptors on the gonads, where they regulate germ cell maturation and steroid hormone production. GnRH analogs (GnRHa) have long been used in therapeutic approaches in humans and have been successfully used to stimulate and synchronize spawning in various teleosts since the 1980s[16], whether of natural or synthetic origin, as an acute or sustained treatment, sometimes in association with dopamine receptor antagonists (metoclopramide or domperidone).

Teleosts generally possess three types of GnRH[17-18] encoded by *gnrh1, -2* and *-3* genes, while mammals produce only the first two types. *gnrh1*-derived decapeptides vary slightly in sequence between species and generally appear to be the ones carrying the hypophysiotropic function, while the *gnrh2* (and *-3* in teleosts)- derived decapeptides are almost constant and seem more associated with regulatory function in the brain, in particular reproductive behaviours, except in fish species that have lost *gnrh1*, where *gnrh3* was shown to assume a hypophyiotrophic role (reviewed in[18-19]). Analogues of GnRH1 from mammals ("mGnRH", sometimes referred to as "LH-RH") and GnRH3 from teleosts (salmon form, "sGnRH") have both been shown to successfully induce and synchronize maturation in fish, either for commercial or conservation purposes[16,18]. [D-Ala6–Pro9-Net]-mGnRHa/LHRHa is a synthetic version of mGnRH displaying higher stability and lower metabolic degradation, and has been used successfully in many species, including salmonids[20-22], spariformes[23], moronidae[24], perciformes[25-26], gadiformes[27], pleuronectiformes[28-31] and cypriniformes[32-34]. The

development of implants as a method of delivery has allowed a lower handling amount (only one injection) and a longer-term effect (sustained release[16,18]).

The aim of the present study was to, develop, and use GnRHa implant technology for lumpfish to promote controlled, synchronized spawning in females, and optimize spermiation in males.

Results

Survival after implant injection

Out of the 24 females that were implanted in the first experiment, two (sham-treated) females died the week following the injections. Three other females (one GnRHa-treated and two sham-treated) had to be euthanized later during the experiment for animal welfare concerns, after observing eye wounds, buoyancy issues and appetite loss. The lethality was therefore 8.3% for GnRHa-treated and 33% for sham-treated individuals (Fig. 1A). Ultimately, 19 females (11 GnRHa-treated and 8 sham-treated) could be analyzed for maturation throughout this experiment. All the raw data for females are found in Table S1. Out of the 24 males at the beginning of the experiment, five (three GnRHa-treated and two sham-treated) died relatively early during the experiment, with no identifiable cause. One other individual (sham-treated) had to be euthanized as it was struggling to maintain an upright position, even with help. One individual (sham-treated) had to be removed from the experiment as it appeared during sampling that it was actually a female that had been misidentified for an immature male. Among the 23 males in the experiment, the lethality was therefore of 25% for GnRHa-treated and 27,3% for sham-treated individuals (Fig. 1B), and 17 males (9 GnRHa-treated and 8 sham-treated) were analyzed for maturation throughout this experiment. All the raw data for males are found in Table S2. In the second experiment, a single individual (male) died, representing 5%.

Effect on females

Effect on proportion and timing of maturation - first experiment

Out of 19 females analyzed for maturation, 81.8% (9/11) of GnRHa-treated and 75% of the controls (6/8) reached first spawning stage during the timeframe of the experiment (Fig. 1A). GnRHa-treated individuals reached first spawning on average 18.11 (±1.65) days after implant injection, varying between 10 and 27 days (Fig. 2A). On the other hand, it took on average 29,1. (±8,5.) days for sham-injected females to reach the same stage, with a variation between 8 and 57 days (Fig. 2A). A clear trend was thus observed towards a shorter average time to spawning for GnRHa-treated females. A

one-way ANOVA on this limited dataset did however not show a significant difference between these times (P = 0.255). Interestingly, the variance was significantly lower in the GnRHa-treated group (Levene's test, P <0.001) than in the sham-group, indicating a higher level of homogeneity in the GnRH-treated group than in the sham group.

Out of the 15 animals that reached the spawning stage, three females (two GnRHa-treated and one sham) spawned spontaneously before they could be stripped for controlled IVF, consequently they were not sampled then but were kept to observe the occurrence of a second batch (Fig. 2B). The time between batches seemed shorter in GnRHa treated females (7 to 9 days) than in the control group (14 days), The low numbers of fish made the data however not suitable for a statistical analysis. Most of the results presented below are therefore restricted to individuals at the time of their first batch, and the results for the few individuals sampled after their second batch in presented in Figures S1 and S2. The four individuals that did not reach maturity during the experiment still showed a very pale skin colour at the end of the experiment, with a flat and hard urogenital orifice.

Effect on animal weight - first experiment

The animals used in the experiment were selected to present a minimal variation in average initial body weight between the GnRHa-treated and the sham-treated fish. Consistently, the initial average weights of the animals that were sampled was 3030 (±305) g for GnRHa-treated females and 2979 (±68) g for sham-treated females (Fig 3A, P = 0.87, one-way ANOVA). When these animals were determined to be ready to spawn according to morphological criteria, their weights were 2833 (± 249) g and 2800 (±136) g for GnRHa-treated and sham-treated females, respectively (Fig. 3A, P = 0,9, one-way ANOVA). A repeated-measure ANOVA did not show any effect of time (P = 0.07) or group (P = 0.86), nor any interaction between those parameters (P = 0.92).

If we restrict the dataset to the animals which were sampled after the first batch (Fig 3B), their initial average weight was 3069 (±370) g for GnRHa-treated females and 3039 (±71) g for sham-treated females at injection, which was not significantly different (P = 0.94, one-way ANOVA), and 2920

(±285) g for the GnRH group and 2943 (±129) g for the sham group at sampling (P = 0,94, one-way ANOVA). No significance was found using a repeated-measure ANOV (P = 0.28 for time, P = 0.99 for group, and P= 0.81 for interaction between the two).

Effect on the quantity and quality of eggs - first experiment

When stripped, animals sampled at the first batch of eggs produced on average 0.56 (±0.03) l eggs and ovarian fluid for GnRHa-treated females, while sham-treated females produced 0.42 (±0.06) l (Fig. 3C, P = 0.045, one-way ANOVA), indicating that GnRHa-treated females produced 36% more roe and ovarian fluid than sham-treated females.

Egg diameter however was not significantly different between groups, with on average 2.11 (± 0.02) mm for GnRHa-treated females and 2.08 (±0.02) mm for controls (P = 0.33, one-way ANOVA, Fig. 3D). The average weight of the gonads after stripping was not significantly different, with 371 (±61) g for GnRHa-treated animals and 434 (±87) g for sham-treated animals (P = 0.56, one-way ANOVA, Fig. 3E).

Effect on fertilization and hatching success - first experiment

All the batches were fertilized using the same male and placed in duplicates in incubators. At 310 d°, the content of the incubators was categorized as unfertilized egg, unhatched larva or hatched larva (Fig. 4A). Among the first batches coming from GnRHa-treated females, the proportion of unfertilized eggs ranged from 0.1% to 28.5%, with an average of 4.6 (±3.99)%, while for sham-treated females, it varied from 0.1% to 100%, with an average of 20.95 (±19,77)% (Fig. 4A). In other words, the average fertilization rate for embryos issued from GnRHa-treated females was 95.36 (±3.99)%, while it was 79.05 (±19.77)% for embryos resulting from sham-treated females (Fig. 4B). This trend was, however, not statistically supported (P = 0,46, one-way ANOVA), due to the large variation in the sham group. The variance in the GnRHa-treated group was found to be significantly lower than in the sham-treated group (Levene's test, P = 0,038).

Between 63.3% and 98.7% of the content of incubators from GnRHa-treated individuals corresponded to larvae that had successfully hatched, with an average of 88.8 (±5.8) %, while successfully hatched larvae in incubators from sham-treated individuals represented from 0.0% to 96.8%, with an average of 56.9 (±16.8) % (Fig. 4A). This trend was, however, not significant ($P = 0.068$, one-way ANOVA). When restricted to the proportion of eggs that were fertilized, the hatching success for GnRHa incubators was on average 92.9% (Fig. 4C), against only 57.4% for sham incubators. This trend was, however, not statistically different ($P = 0{,}104$), due to the large variation in the sham group. Again, the variance in the GnRHa-treated group was significantly lower than in the sham-treated group (Levene's test, $P = 0.014$).

Effect on pituitary genes - first experiment

In GnRHa-treated animals, the average relative pituitary abundance of the GnRH receptor *gnrhr2ba1* transcript was not significantly different from that of sham-treated animals, with an average 0.9 (± 0.1) ratio to the sham group value ($P = 0.76$, Fig. 5A). The same was observed for *fshb*, the transcript encoding the β subunit of the gonadotropin FSH, with on average a 1.2 (±0.15) times higher relative abundance in the GnRH group compared to the sham group ($P = 0.59$, Fig. 5B). In contrast, *lhb*, the transcript for the β subunit of the gonadotropin LH, had a significantly higher expression in the GnRHa-treated group, with a 1.9 (±0.14) times higher average relative abundance compared to the sham group ($P = 0.01$, Fig. 5C).

Effect on circulating steroids - first experiment

The plasma concentration of 17β-estradiol (E2) in GnRHa-treated females was on average 0.7 (± 0.36) ng/ml, while it was on average 1.8 (±0.91) ng/ml in sham-treated animals, suggesting a higher level in the sham group (Fig. 5D). This difference was, however, not statistically supported ($P = 0{,}23$). Interestingly, however, the variance was lower in the GnRHa-treated group than in the sham-treated group (Levene's test, $P = 0.014$).

Lager scale experiment (second experiment)

To confirm the general results of experiment 1, a second experiment (experiment 2) was undertaken, with a higher number of individuals implanted. The results confirmed the trend towards a shorter time to spawning, with an average spawning time at 22.85 (±1.28) days after implantation in GnRHa-treated females, against 28.35 (±2.74), Supplementary Figure S3A. This trend was not significant, however the variance in the GnRHa-treated group was again found to be significantly smaller (Levene's test, $P = 0.017$). We did not observe a significant difference in the volume of roe and ovarian fluid produced by the GnRHa-treated individuals, with on average 0.36 (±0.03) l compared to the sham-treated individuals, with on average 0.35 (±0.01) ($P = 0{,}425$, Supplementary Figure S3B). Fertilization rate in the second experiment was slightly higher in the treated group (95 (±1)%) than in the control (88 (±5)%) (Figure S3C), however this trend was not significant ($P = 0.367$). Interestingly, the variance within the GnRHa group was significantly lower than in the sham (Levene's test, $P = 0.013$). The hatching rate did not present any statistical significance between groups ($P = 0.983$), with 69 (±4)% in the treated group *versus* 62 (±9)% in the control group (Supplementary Figure S3D). The variance was, however, again smaller in the former than in the latter (Levene's test, $P = 0.017$).

Effect on males

Effect on animal weight - first experiment

The average start weight of the 9 GnRHa-treated individuals was 1045 (±68) g and for the 8 sham-treated individuals 950 (±45) g (Fig. 6A). No statistical significance was detected for this difference ($P = 0.27$, one-way ANOVA). At sampling time, the GnRH individuals weighed 1014 (±64) g and the sham-treated 887 (±44) g (Fig. 6A), which represents a decrease of 3% and 7%, respectively. No statistical significance was detected between groups for this difference at this time point ($P = 0.13$). A repeated measures ANOVA showed that although there was a significant effect of time within

groups (P = 0.006), it was restricted to the sham group (Tukey post-hoc P = 0.045), and that no significant effect was found for the treatment group the individuals belonged to (P = 0.186).

Effect on gonad development and spermatocrit - first experiment

The gonad weight of GnRHa-treated individuals at sampling was on average 42 (±9) g, resulting in a gonado-somatic index of 4.5 (±0.97)%, while that of sham-treated animals was on average 47 (± 7) g, resulting in a gonado-somatic index of 5.3 (±0.80)% (Fig. 6B). Neither the difference in gonad weight nor in GSI was found to be statistically significant (P = 0,71 and 0.5 respectively, one-way ANOVA). At sampling, two GnRHa-treated and one sham-treated individuals had not developed the red body color characteristic of maturity. These individuals had under-developed gonads (gonado-somatic index < 1.5%). A third GnRHa-treated individual, which was red in color, also had a very low GSI. The maturation rate for GnRHa-treated individuals was thus of 66.7%, while it was 87.5% for the sham group (Fig. 6C).

Attempts to strip the males to obtain milt and measure the amount of sperm were unsuccessful. It was therefore decided to extract sperm by grinding the dissected gonads. For the individuals that had significantly developed gonads, the spermatocrit was on average 79.3 (± 4.2) % after GnRH treatment and 81.9 (±4.8) % after sham treatment (Fig. 6D). This was not found to be significantly different (P = 0.83, one-way ANOVA).

Effect on pituitary genes - first experiment

In GnRHa-treated males, the average relative abundance of *gnrhr2ba1* transcript in the pituitary was not significantly different from that of sham-treated animals, with an average 1.07 (±0.32) ratio to the sham group value (P = 0.87, one-way ANOVA, Fig. 7A). The same was observed for *fshb* and *lhb*, with an average relative abundance in the GnRH group respectively 0.70 (±0.20) and 0.84 (± 0.22) times that of the sham group (P = 0.27, one-way ANOVA, Fig. 7B and P = 0.57, one-way

ANOVA, Fig. 7C). No significant difference was detected if the dataset was restricted to the individuals that had significantly developed gonads.

Effect on circulating steroids - first experiment

The plasma concentration of 11-keto testosterone (11-KT) in GnRHa-treated males was on average 6.6 (±0.36) ng/ml, while it was on average 6.2 (±0.91) ng/ml in sham-treated animals. This difference was not statistically significant (P= 0.87, one-way ANOVA, Fig. 7D). No significant difference was detected if the dataset was restricted to the individuals that had significantly developed gonads.

Larger scale experiment (second experiment)

As no significant changes were observed in the first experiment, we decided to increase the GnRHa dose injected into males in experiment 2, from 50 µg kg^{-1} to 75 µg kg^{-1}. After 29 days the average milt volume collected from GnRHa-implanted was 28% higher than from sham-implanted individuals, with 1 (±1) ml and 0.22 (±0.22) ml, respectively (Fig. 8). However, this trend was not statistically supported (P = 0.19, one-way ANOVA). After 60 days, however, the values had increased to 10.5 (±1.82) ml and 5 (±2.38) ml, respectively, the GnRHa values thereby being 110% higher than the controls and reaching significance (Fig. 8, P = 0,04, one-way ANOVA).

Discussion

During the experiment, 11 fish died (four GnRH and seven sham), of which four were removed due to visible injuries. The reason for the mortality is likely handling and stress associated with implanting. Mature fish are more vulnerable to stress and infectious diseases during the reproductive period [38-39]. Lumpfish do not school, rather attach themselves to hard surfaces, even post-mortem, making detection of dead individuals more difficult. Of the four fish with visible injuries, three were females with eye injuries. We observed that fish with eye injuries did not complete ovulation despite the size and color indicating readiness to spawn. A reason may be that the eye injury prevented a normal response to photoperiod, and consequently lacking the signals which are necessary for final oocyte maturation and ovulation[11,13].

Here we show that the [D-Ala6 Pro9]NEt analog of mammalian GnRH1 can stimulate maturation and ovulation in lumpfish females. This compound has been shown to significantly increase the proportion of spawning females in a wide range of teleost species (for review see[17-18]), reaching up to 100% in southern flounder[31], in common bream or ruffe[26,32], in combination with metoclopramide), 90% in Atlantic salmon (and still up to 75% at adverse temperature[22]), 86.7% in silver perch[25], and 85,7% in Atlantic cod[27]. Our small-scale experiment shows that also in lumpfish, a higher proportion of females spawned after GnRHa treatment (81.8%) than after sham treatment (75%).

We also saw a clear trend towards a shorter time to reaching maturity (18.1 days in treated fish versus 29.2 in controls), which is a promising step towards synchronizing females. Furthermore, the variance in the GnRHa-treated group was significantly lower than in the sham-treated group, indicating a higher homogeneity between females after treatment. This reduced latency period has also been observed in Atlantic salmon and turbot[22,30]. For the few individuals we were able to follow until a second batch, the time between batches seemed shorter after GnRHa treatment (7 to 9 days) versus control (14 days), which is close to the reference value of 13 days observed for wild lumpfish by Fulton[40]. This has to be repeated on a larger number of individuals, but if confirmed, it would be

in line with findings on another batch spawner, turbot, in which shorter time between batches was observed after treatment[30].

The fact that four of our GnRHa-treated females did not reach maturity within the course of the experiment might mean they had not reached a critical size/stage where they are fully competent to respond to the signal. Similarly in summer flounder (*Paralichthys dentatus*) 7 of the 12 GnRHa-treated females did not spawn[29], and it was observed that only females with ovaries contain fully-grown follicles were responding to the treatment. We aimed at having a good representation of the diversity of the original population by picking individuals differing in size, colour and external signs of maturity, and the variation in response to the treatment is likely to be the result of this choice.

The average volume of roe in ovarian fluid spawned by GnRH-treated females was 36% higher than controls. The body weight and residual gonad weight of the individuals after spawning were otherwise not significantly different between groups, supporting the idea that the difference relied on the volume of eggs ovulated and spawned by the females. Our findings are further in line with studies in gilthead bream[41], turbot[30] and Eurasian carp[34] where they observed significantly higher spawn volume in treated group compared with controls (for reviews see[17-18,38]. Interestingly in turbot, while the mean batch volume increased, the total volume of eggs measured through the whole spawning season did not. In contrast, in cod, no differences were observed in the amount of eggs per body weight between groups [27].

Egg size was between 1.81 and 2.22 mm in diameter, which is within the size range previously reported for lumpfish[42-44] There was no significant difference in size between the groups, similar to what was observed in turbot[30].

We have previously shown that maturation in lumpfish females is associated with a gradual increase in relative expression of the GnRH receptor *gnrhr2ba1* in the pituitary, which is at its highest just before the peak in *lhb* relative expression and in plasma 2-estradiol that precede ovulation[15]. We proposed that the increased expression of this receptor enables a higher response to GnRH, which promotes a higher *lhb* transcription and therefore Lh production, and eventually spawning. In the

present study, the GnRHa-treated group did not present a significantly different *gnrhr2ba1* expression level than the sham-group, however it presented an almost two-fold higher level of *lhb* transcripts. This means that the increased concentration of circulating ligand did not affect the expression of its key receptor, so it is not through an earlier increase in *gnrhr2ba1* transcripts that GnRH induced a higher *lhb* expression. We therefore propose that the increased amount of ligand initiated an earlier response by bypassing the need for an increased receptor level, which produced a precocious increase in *lhb* transcript abundance and an earlier spawning. In addition to the minimum egg stage required, it is also possible that a threshold in *gnrhr2ba1* receptor expression is necessary for response, that could contribute to the non-responsiveness of the four females that did not mature.

Animals in the GnRHa-treated group also presented a trend towards lower levels of plasma E2. This could reflect a strong effect on ovulation, as E2 levels drop just before ovulation. The variance was significantly smaller in the treated group than in the sham group, which again might be a sign of less variability and a higher synchronicity between individuals.

The effect of GnRH on the downstream cascade depends on species, sex and reproductive status[45]. No significant effect of mGnRHa was found on the spermiation of summer flounder[29], in contrast to results on cod[27], common bream and ruffe (26-32 both in association with metoclopramide),

In the present study, we showed that in lumpfish, after implantation with 50 μg kg^{-1} GnRHa, most males had a bright red skin colour which is associated with maturation in males[14,42], however no significant differences in gonad weight nor spermatocrit were found between GnRHa and sham treated males. However, after implantation with 75 μg kg^{-1} GnRHa, males presented a significant increase in milt production after 60 days. While some authors have argued that a single treatment of males is only effective a couple of days after implantation and have recommended repeated injections or implantations, Mylonas and Zohar[24] showed an increased sperm production up to five weeks after implantation. Here we show that in lumpfish, an effect can be observed up to at least 60 days after implantation, but also that the effect, which is first a non-significant trend, increases and becomes significant over time, arguing that the effect is both lasting but also takes time to fully appear. By

comparison, fish were sampled after 13 and 27 days, respectively in the studies on cod and common bream cited above. Further studies will be necessary to confirm that the increased milt volume is due to increase sperm cell production and not only increased seminal liquid production.

In our previous study, we showed that male maturation was also associated with an increase in *gnrhr2ba1*, correlated with an increase of both pituitary *fshb* and *lhb* transcript abundance[15]. However, this did not occur at an early stage of maturation, and we suggested that *gnrhr2ba1* could be involved in the later maturation stages e.g. for secondary sexual character development. In the present study, we showed that treatment with 50 μg kg^{-1} GnRHa did not cause a significant effect on any of the pituitary genes analyzed (*gnrhr2ba1*, *lhb* and *fshb*), just a slight trend in increasing *lhb* and *fshb* levels in the pituitary, which is consistent with the idea that increasing GnRH signal does not have a significant inducing effect on early maturation processes. As our results after treatment with 75 μg kg^{-1} GnRHa showed a significant increase in milt volume over time, it would however be interesting to investigate pituitary gene expression in fish implanted with higher doses than 50 μg kg^{-1} or with a combination with other compounds.

Survival and hatching rates were high in both groups. Studies on Atlantic salmon showed the same survival until the eyed egg stage in mGnRHa treated and sham groups in cold water[22]. Similarly, no differences in egg survival were found between mGnRHa and sham treated groups in gilt-head seabream[17,38,41].

We did, however, observe a trend to a higher fertilization rate in the GnRHa group (95.4% vs 79.0% in controls), with lower variation. This is in contrast to studies on cod[27], where no significant difference was found with the control group, or in turbot[30], silver perch[25] and southern flounder[31], where lower fertilization rate was observed in GnRHa treated groups.

Finally, we observed a higher average hatching success (87.6% versus 59% in controls), while no significant difference was found in turbot[30] and a lower hatching rate was found in southern flounder[31].

In conclusion, present results show that GnRH analogue administration can successfully induce earlier, more synchronous maturation in lumpfish females, possibly through direct stimulation of Lh production in the pituitary, as proposed by Zohar et al.[45]. A similar effect was not observed in males, however the biggest challenge to date in lumpfish broodstock management has been the asynchronicity of females in the timing of their maturation. These first results suggest that this approach has the potential to overcome this challenge and be very beneficial for the field.

Materials and methods

Animal background and rearing conditions

Lumpfish broodstock fish used in this study are offspring from wild individuals caught outside Hekkingen, Northern Norway in November 2018, reared at the Akvaplan-NIVA FISK research and development facility in Kraknes, Norway. After hatching, animals were reared at 8 to 9°C, first in 90 l incubators, where they were start-fed using PTAqua Otohime B1/B2 (Marubeni Nisshin Feed Company, Tokyo, Japan), then at the fry stage (0,1 - 0,15 g) in 100 l shallow raceways where they were fed with Gemma diamond (Skretting, Stavanger, Norge). At an average weight of 270 g, animals were transferred into 14000 l tanks, kept at the natural inlet water temperature (3,6°C to 5,1°C), and fed with Vitalis Clean 7 broodfish feed (Skretting, Fontaine-les-Vervins, France) until and throughout the experiment.

GnRH-analog implant experiments

Two separate GnRH-analog-implanting experiments were performed in 2021 and 2025. In both cases, females and males approaching maturation, all first-time spawners, were randomly chosen from three different families, and all individually identified by a PIT tag. For each group, half of the individuals received a Reproboost® slow-release GnRH-analog implant (D-Ala^6, Pro^9 NEt, Center of Marine Biotechnology, Baltimore, USA, referred to as GnRHa in this article) dosed according to their body weight (see below), while the other half received an empty implant as a control (sham-injection).

Animals were treated two at a time, by pairs of same sex, weight, and visual level of maturity, one receiving GnRH, the other sham. After being anesthetized using 0,23 $g.l^{-1}$ MS222 (Finquel vet., Scan Aqua AS, Årnes, Norway) for approximately 4 min, animals were weighed and the implants injected with 12 G syringes dorsolaterally on the left side into muscle tissue, approximately 1 cm over the highest bony tubercle. In the first experiment, 12 females received 100 $\mu g.kg^{-1}$ while 12 males received 50 $\mu g.kg^{-1}$ with appropriate controls. In the second experiment, 20 females received 100 $\mu g.kg^{-1}$ and 20 males received 75 $\mu g.kg^{-1}$ with sham controls Animals were held for recovery together in 14000 l tanks for a week, before transferring all the males to a separate 1700 l tank to keep control of egg fertilization. An overview of the experiments and the downstream analyses can be found in Table 1.

Female maturity monitoring, in vitro fertilization and hatching success assessment

Fish were monitored daily and the maturity of females was assessed using the distention of the abdomen and the swelling in the urogenital region as criteria[35]. Females that were determined to be ready for spawning were stripped, and the volume of roe and ovarian liquid measured. Egg diameter was measured under a microscope, on 30 eggs for each batch. Two identical samples were prepared for *in vitro* fertilization, each containing 5 ml roe and 5 ml ovarian liquid.

All the egg batches were fertilized using milt from the same male. The male was anesthetized using 0,23 $g.l^{-1}$ MS222 and stripped. 20 µl milt were added to each roe sample for fertilization, carefully mixed before being transferred to upwelling sea water incubators (diameter 95 mm) with a mesh bottom at a flow of $100.min^{-1}$.

The content of the incubators was analyzed after 310 d°pf. Larvae were euthanized using 0,5 $g.l^{-1}$ MS222 and the number of unfertilized eggs, unhatched and hatched larvae was counted.

Tissue sampling, gonadosomatic index and spermatocrit measurements

Females were euthanized after having been stripped while males were sampled at the end of the experiment. In experiment 1, four individuals (three females and one male) had to be euthanized during the experiment for welfare reasons or were found dead and sampled immediately. Animals were anaesthetized in a lethal dose of 0,5 $g.l^{-1}$ MS222 and body weight was recorded. Blood was then drawn from the heart, plasma collected and stored at -80°C until steroid analysis. The fish were decapitated, and brain and pituitary were dissected out and kept in RNA later for gene expression analyses. Gonads were collected and weighed for gonadosomatic index calculation. For spermatocrit measurements, male gonads were ground over a mesh to separate gonadal tissue and sperm cells. The total liquid volume was measured, and 2 ml of the sample was centrifuged 3 min at 11300 g and the volume of cells was measured and calculated as a percentage of the total.

cDNA synthesis and qPCR analyses

Total RNA was extracted from pituitary tissues in RNA later using the RNeasy mini kit (Qiagen), and cDNA synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)18. qPCRs on our genes of interest (*gnrhr2ba1*, *lhb*, and *fshb*) and reference genes (*ef1-a* and *hprt*) were conducted as described in Andersson, Denker et al., 2023. qPCR was performed on 1:20 diluted cDNA in duplicates using iTaq Universal SybrGreen Supermix (BioRad) in 12,5 µl reactions with 200 nM primers. Reactions were loaded in 96-well plates, with each plate containing a common pool sample to allow inter-plate calibration. Plates were run in a CFX96 real-time PCR detection system (BioRad) with the following program: 2 min denaturation at 95 °C, 38 cycles with 15 s denaturation at 95°C and 25 s annealing/extension at 60°C or 62°C (depending on the target), followed by a melting curve analysis.

Relative gene expression was calculated according to Pfaffl's efficiency-corrected model[36] and results are presented as fold change in the treated group relative to the control group.

Steroid analysis

Plasma steroids were analyzed by Enzyme-Linked Immunosorbent Assays (ELISA) as described in[15]. Briefly, steroids were extracted from plasma by phase separation using ethyl acetate, vacuum drying and dissolution in a phosphate buffer (phosphate 0.1 M pH 7.4, 0.4 M NaCl, 1 mM EDTA). ELISA was performed using E2 antisera, acetylcholine esterase-labelled tracers and microplates precoated with monoclonal mouse antirabbit IgG supplied by Cayman Chemicals (USA). Antiserum against 11-KT was a kind gift from Dr David E. Kime. Standard steroids were purchased from Sigma Aldrich (Sigma reference standards).

Statistical analyses

Statistical analyses were performed using Jamovi 2.6.45 (https://www.jamovi.org). Data normality was tested using Shapiro-Wilk test and homogeneity of variances was tested using Levene's test. Results were tested using one-way ANOVA[37] or repeated-measures ANOVA when relevant. Significant differences between experimental groups were further analyzed using a Tukey or Games-Howell post hoc test depending on variance property, with a threshold of $p = 0,05$.

Ethic approval

The study was conducted according to the Animal Welfare Act (LOV- 2009-06-19-97). The present trial was approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by the authority by the following registration and clinical trial numbers FOTS 14987 (date of registration 1 March 2020) and 31189 (date of registration 1 February 2025).

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Author contributions E.D., M.G.J., S.O.S. and A.K.D.I. designed and conceived the study; E.D., M.G.J., T.O.N., L.K., T.A.H. and S.O.S. collected and quality controlled the samples. E.D., B.N., E.A., K.E.E., J.D.S. and Y.Z. performed the main experiments and analyses; E.D. interpreted the results and wrote the manuscript with input from A.K.D.I., S.O.S., J.D.S. and Y.Z. All authors reviewed the manuscript.

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Data availability Data will be made available on request.

Declarations

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

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Figures

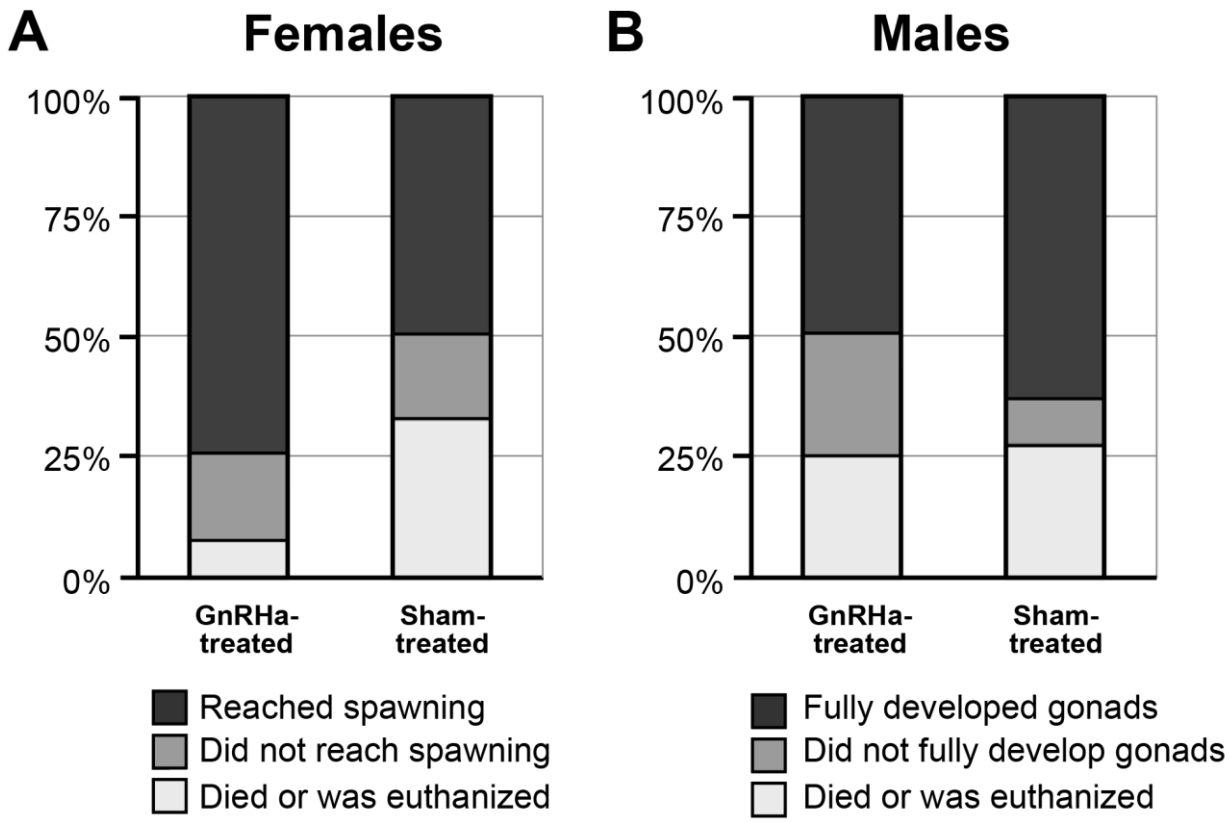

Fig. 1 Overall survival and maturation rate in females (A) and males (B) in first experiment.

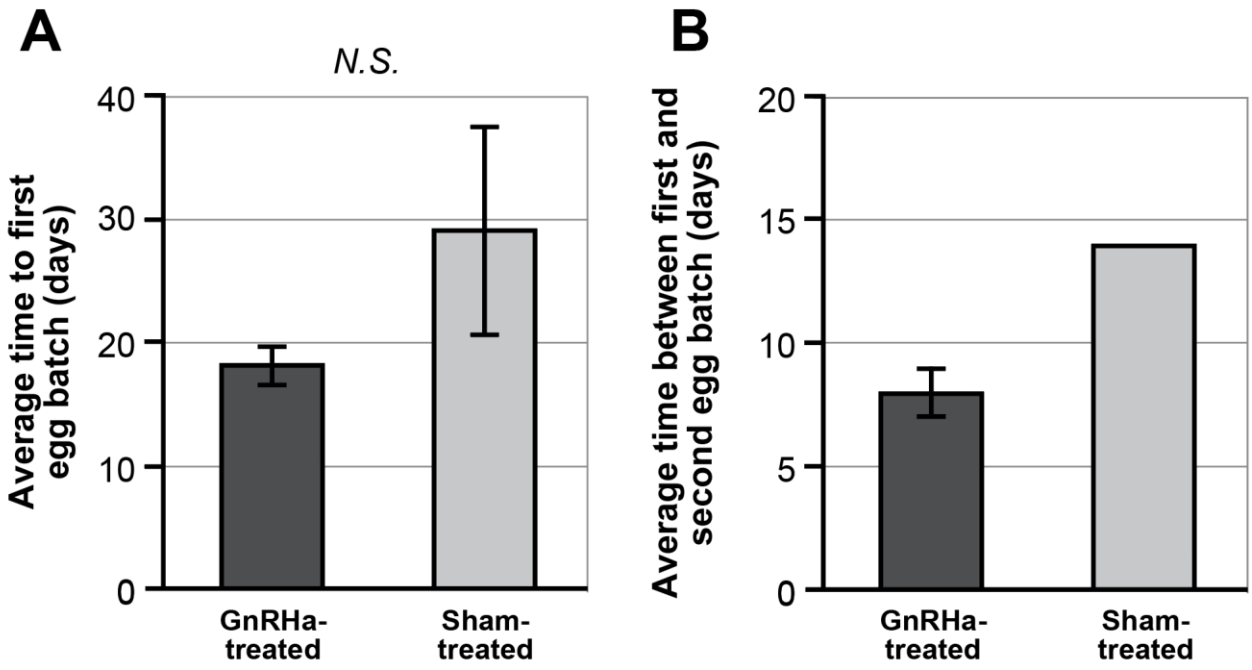

Fig. 2 Time to reach spawning in females in first experiment. **A.** Time to first egg batch. **B.** Time to second batch. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

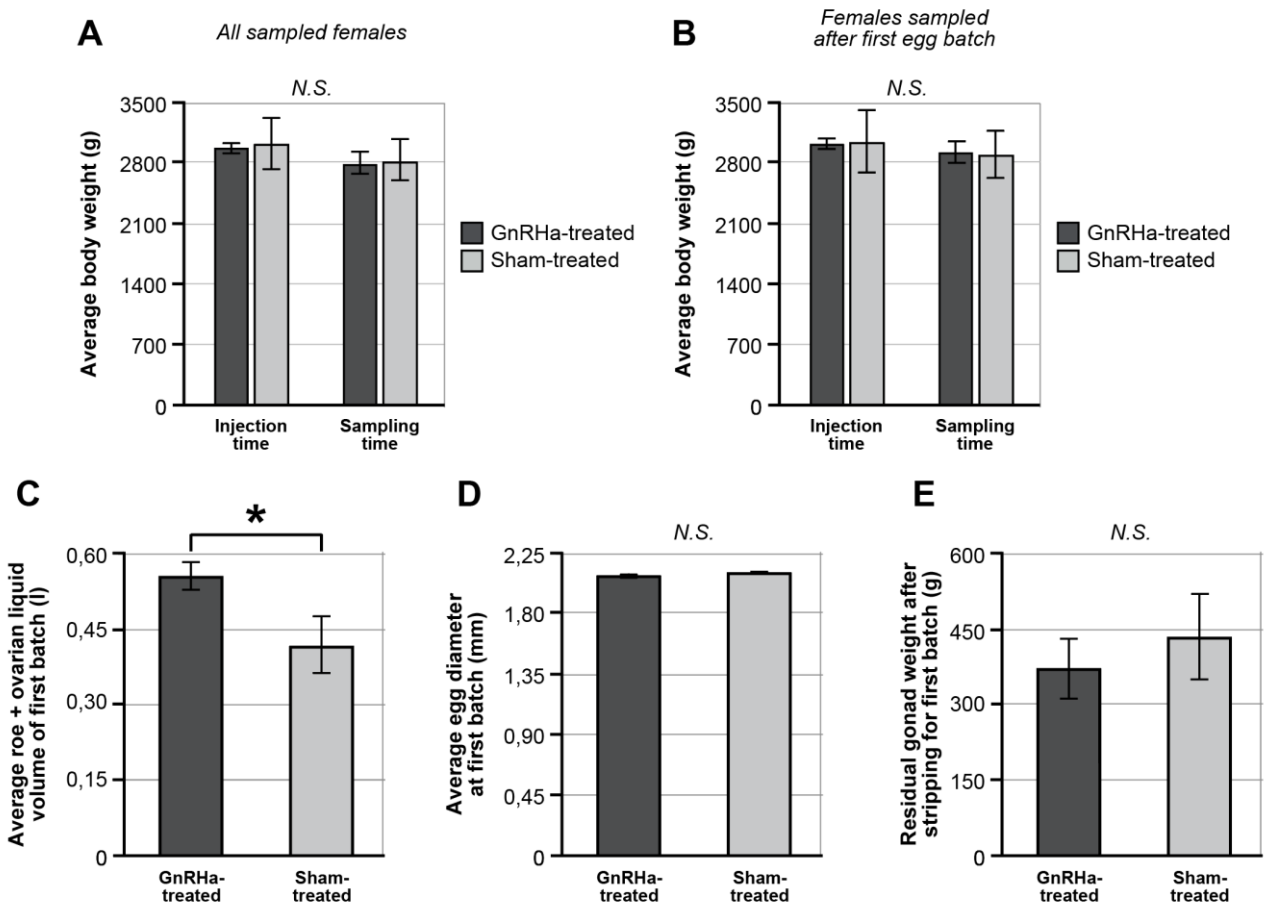

Fig. 3 Biological parameters at sampling in females, experiment 1. **A.** Average total body weight in all sampled females (first and second batch). **B.** Average total body weight in females sampled at the first batch. **C.** Average volume of roe and ovarian liquid collected at stripping (first batch). **D.** Average diameter of single eggs collected at stripping (first batch). **E.** Average residual gonad weight after stripping (first batch). Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

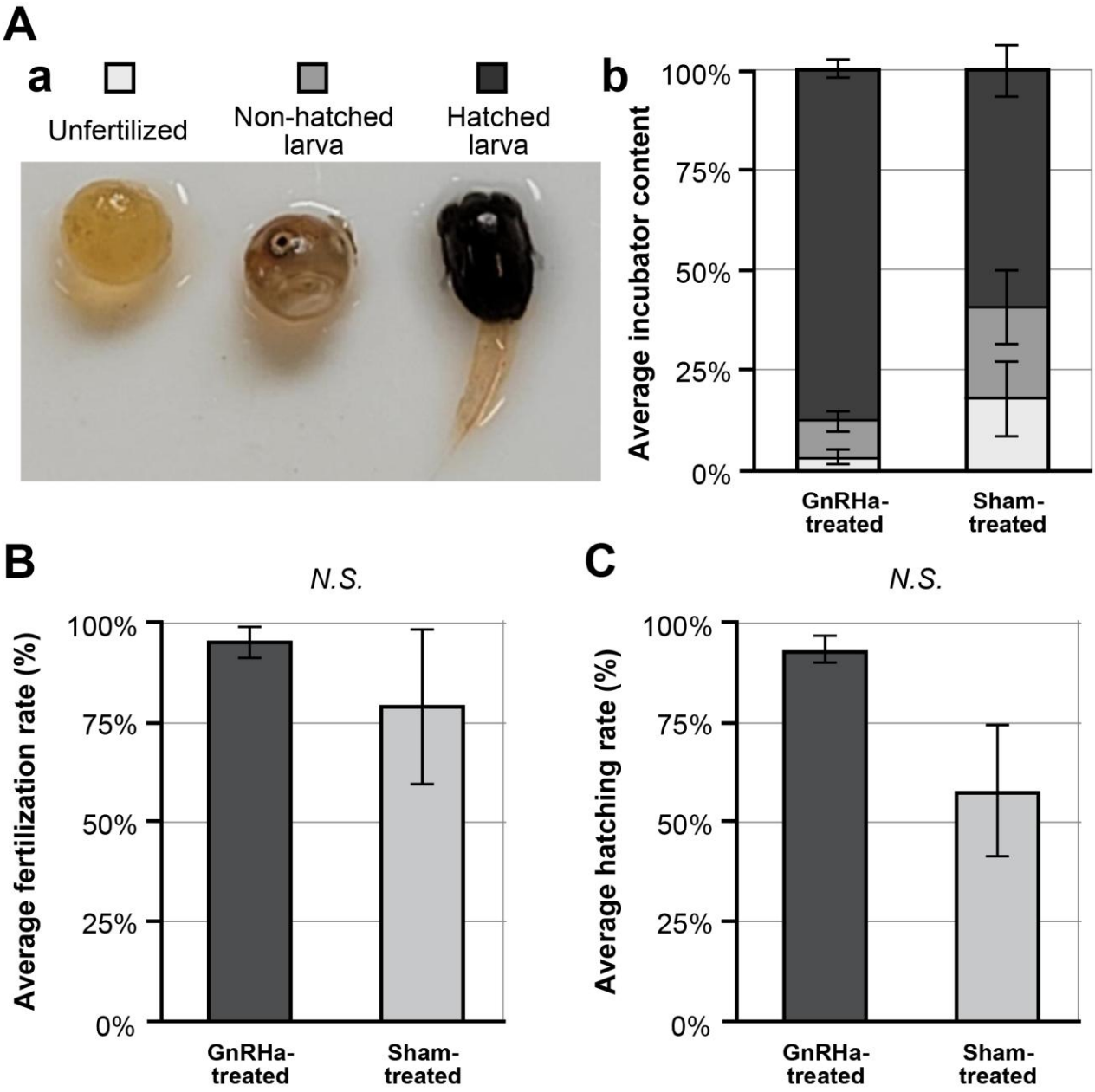

Fig. 4 Development and hatching success of embryos stemming from the first egg batches, experiment 1. **A.** Average incubator content. **a.** Representative pictures of the different categories (embryo status/stage) counted in this study. **b.** Distribution of the different categories in the GnRHa-treated and control groups. **B.** Average fertilization rate in different groups. **C.** Average hatching rate in the different groups. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

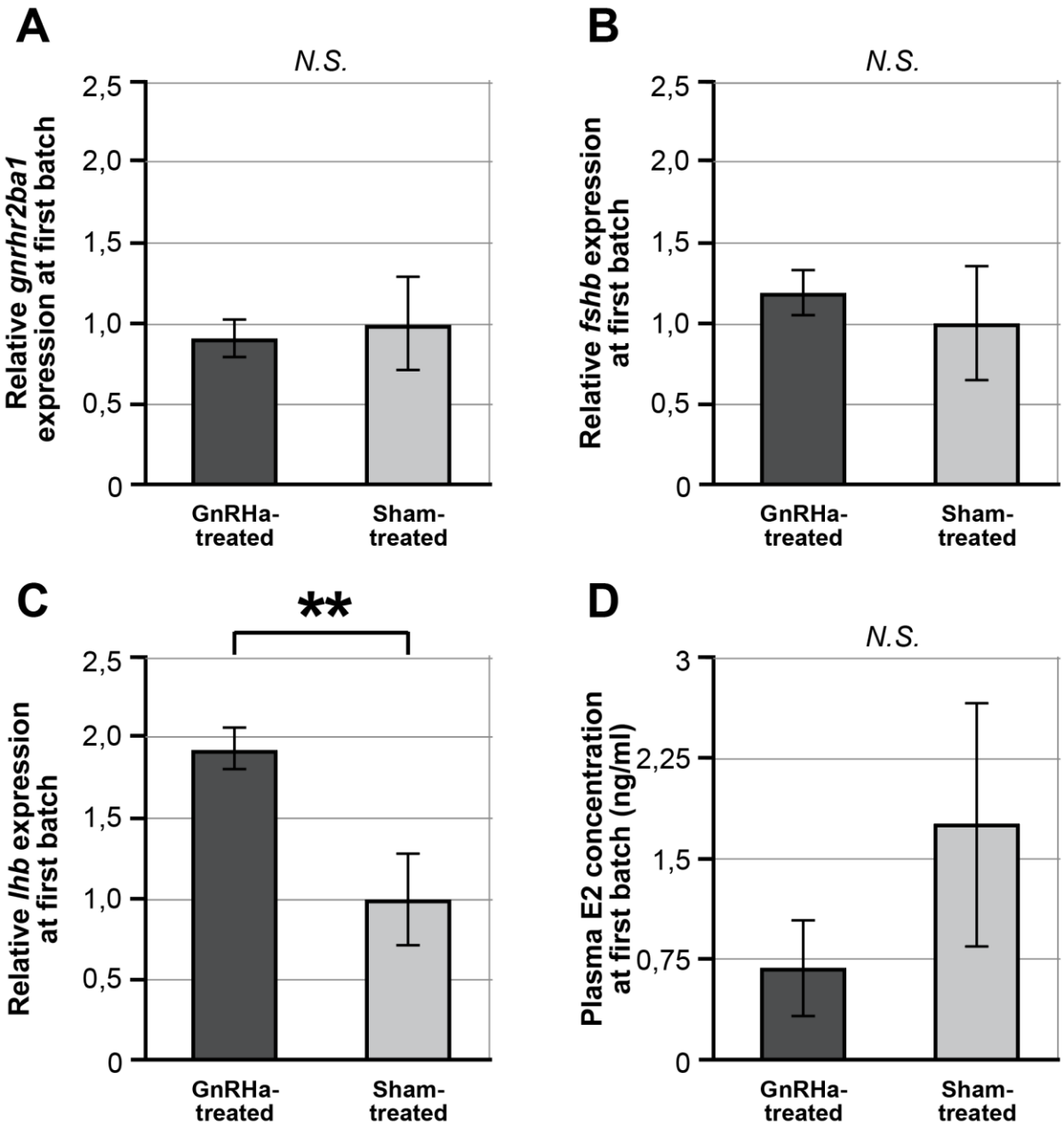

Fig. 5 Effect of GnRHa treatment on pituitary gene expression and circulating plasma steroids in females at the first batch. **A.** Relative expression of GnRH receptor *gnrhr2ba1* in pituitary tissue. **B.** Relative expression of FSH gonadotropin subunit *fshb* in pituitary tissue. **C.** Relative expression of LH gonadotropin subunit *lhb* in pituitary tissue. **D.** Average concentration of 2-estradiol (E2) in plasma. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

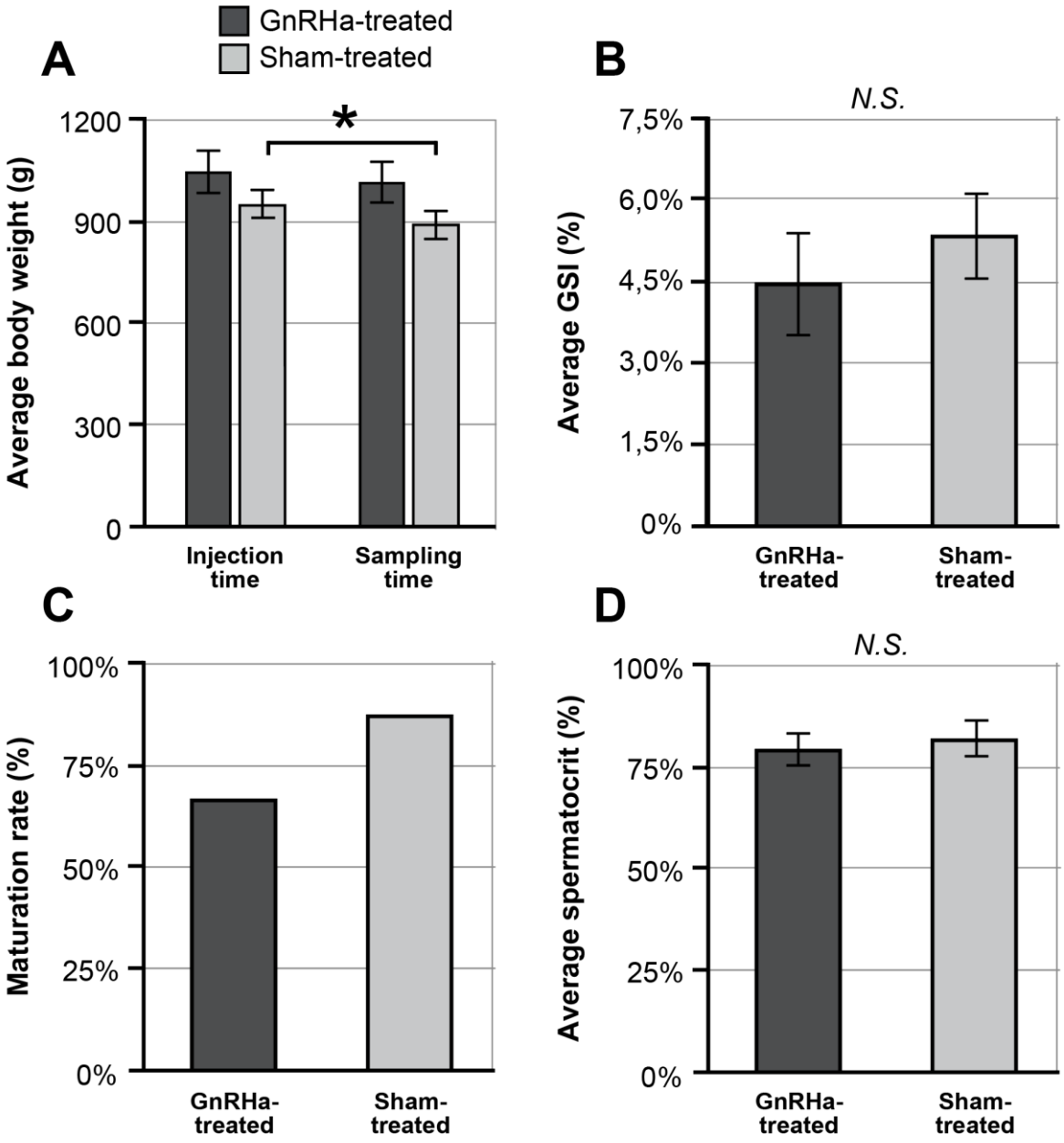

Fig. 6 Effect of GnRHa treatment on body on morpho-anatomical characters and maturation in males in the first experiment. **A.** Average total body weight at injection time and at sampling time. **B.** Average gonado-somatic index at sampling. **C.** Maturation rate. **D.** Average spermatocrit in males that presented significant gonadal maturation.

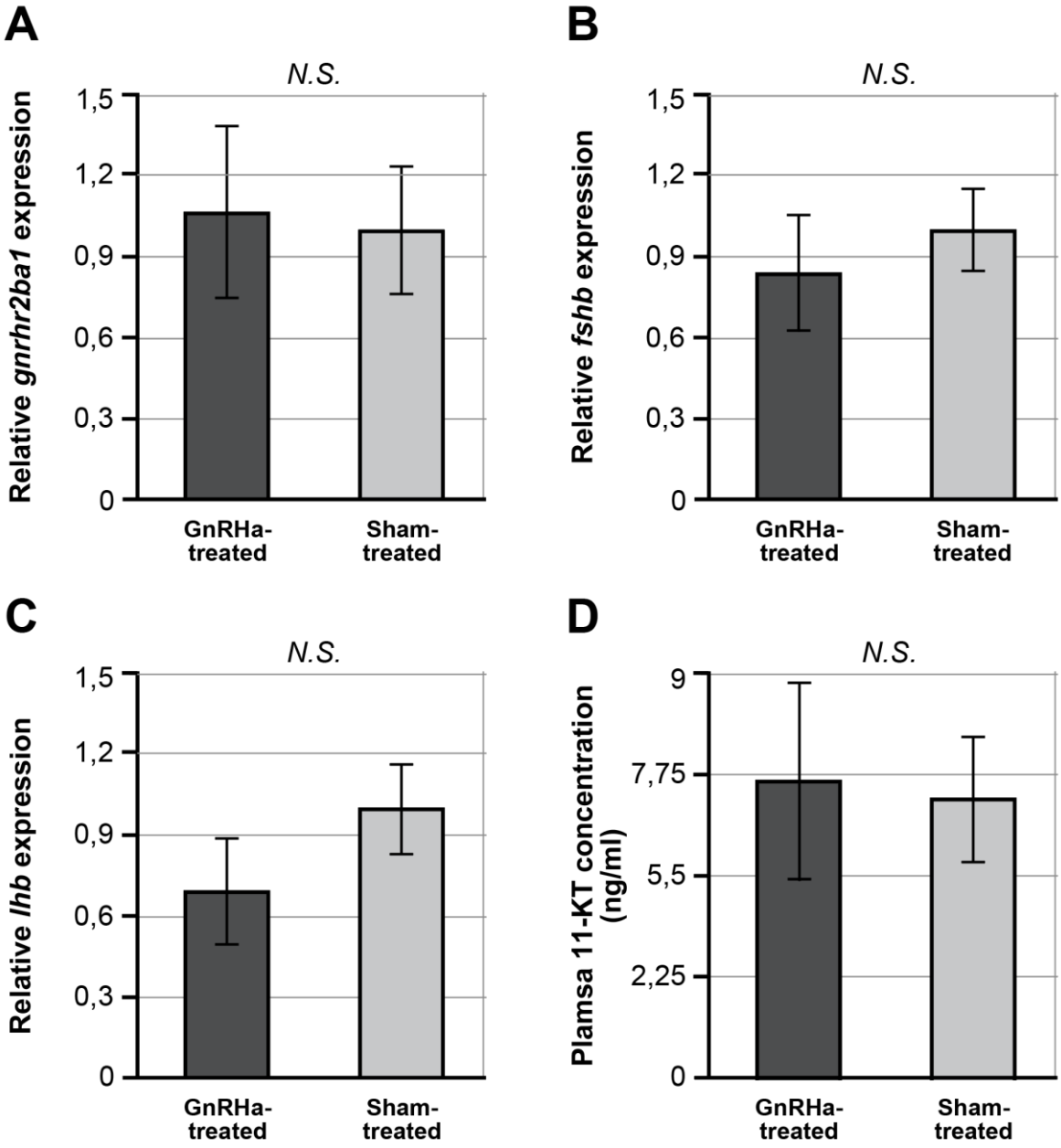

Fig. 7 Effect of GnRHa treatment on pituitary gene expression and circulating plasma steroids in males. **A.** Relative expression of GnRH receptor *gnrhr2ba1* in pituitary tissue. **B.** Relative expression of FSH gonadotropin subunit *fshb* in pituitary tissue. **C.** Relative expression of LH gonadotropin subunit *lhb* in pituitary tissue. **B.** Average concentration of 11-keto testosterone (11-KT) in plasma. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

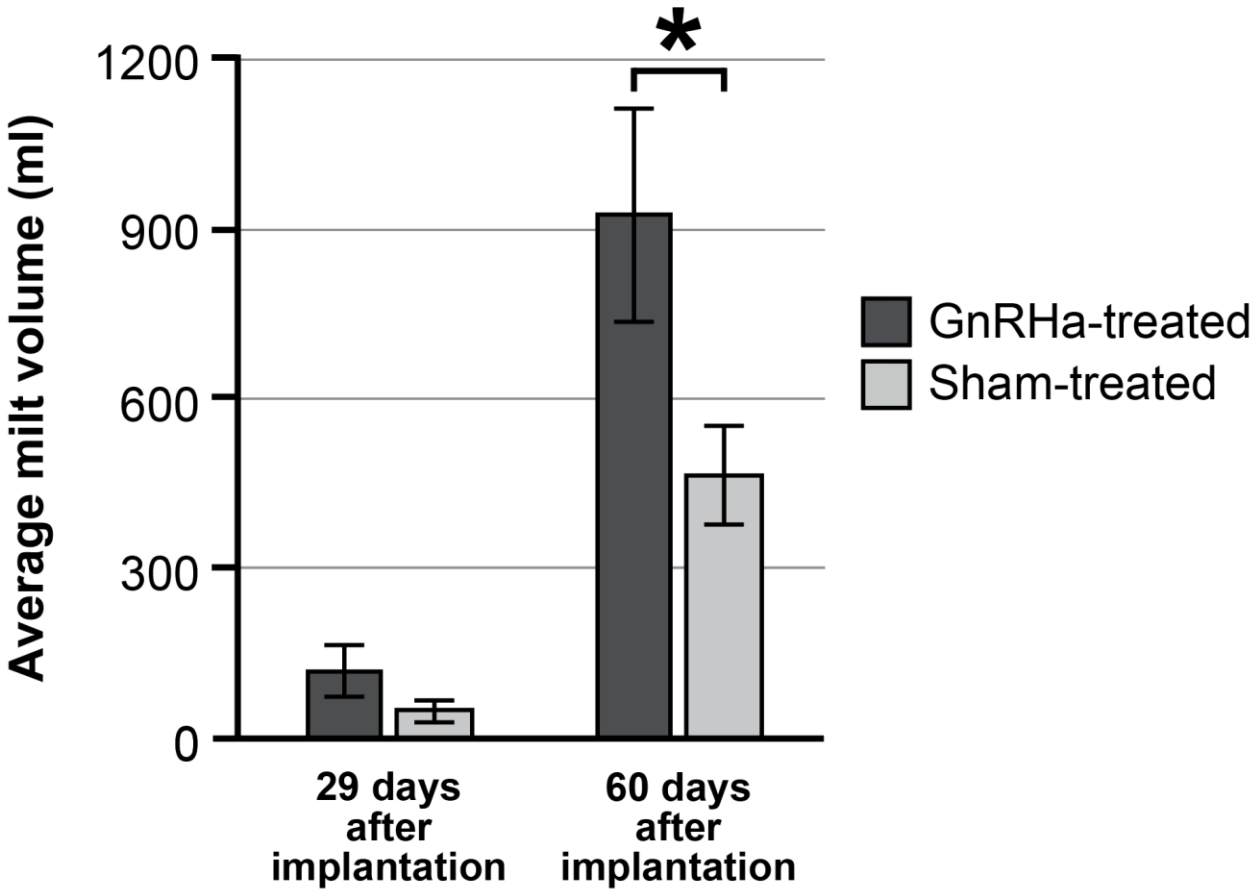

Fig. 8 Effect of GnRHa treatment on milt production in males in the second experiment. Average milt volume. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

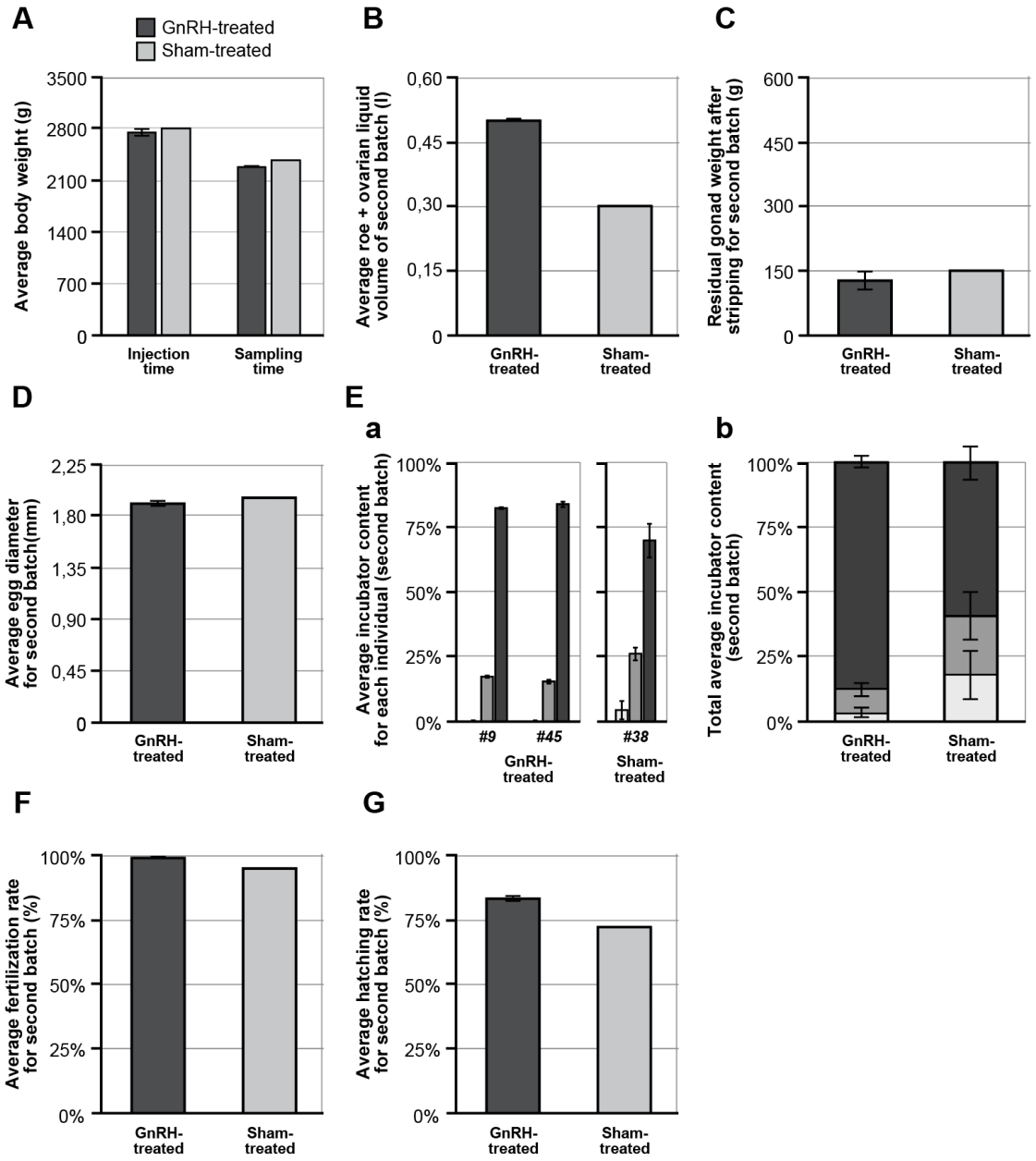

Fig. S1 Data for animals sampled after second batch, experiment 1. **A.** Average total body weight. **B.** Average volume of roe and ovarian liquid collected at stripping (first batch). **C.** Average residual gonad weight after stripping. **D.** Average diameter of single eggs collected at stripping (first batch). **E.** Average incubator content. **a.** Detailed incubator content. **b.** Distribution of the different categories in the GnRHa-treated and control groups. **F.** Average fertilization rate in different groups. **G.** Average hatching rate in the different groups. Results are presented as average +/- standard error of the mean.

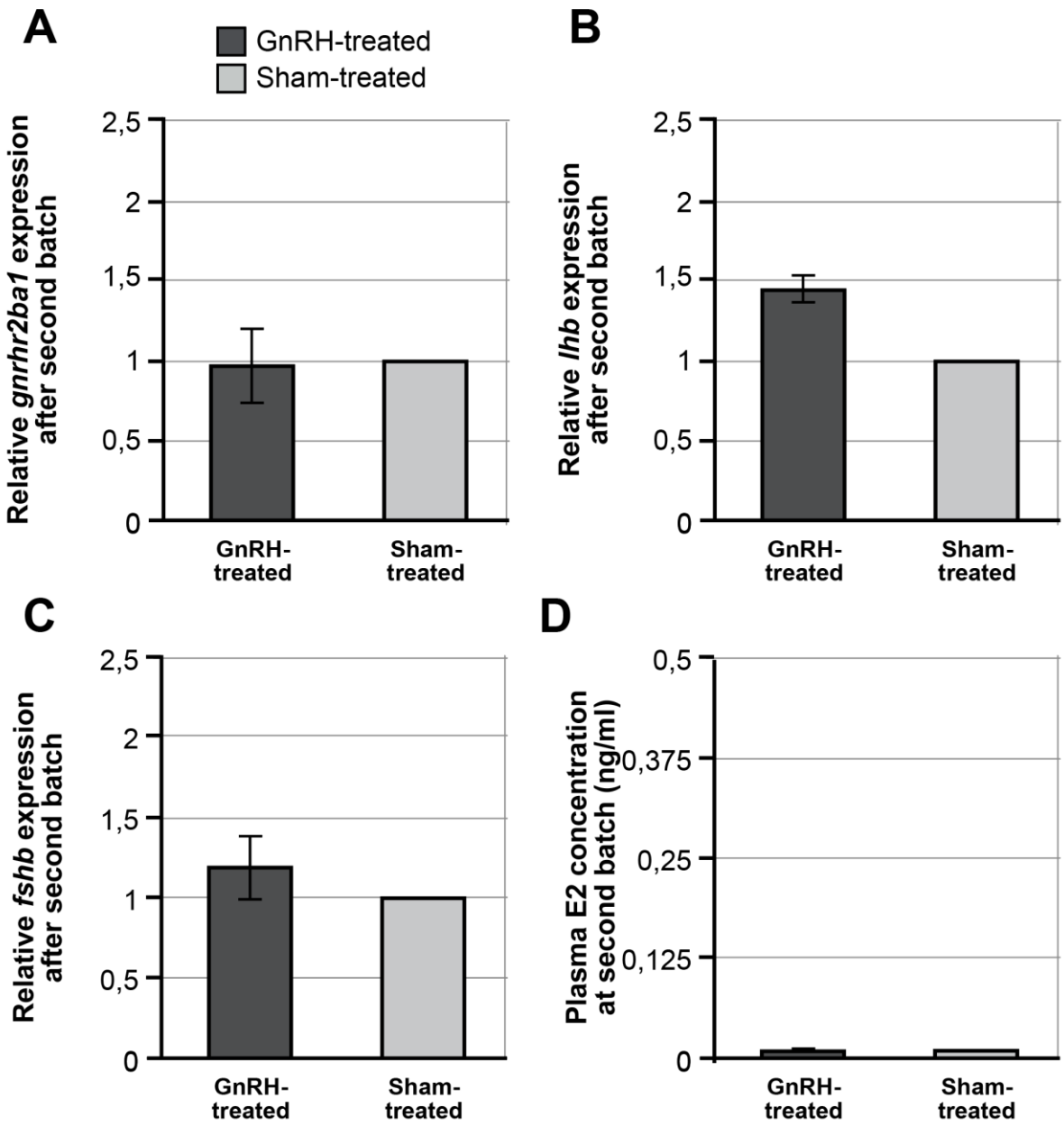

Fig. S2 Effect of GnRHa treatment on pituitary gene expression and circulating plasma steroids in females at the second batch, experiment 1. **A.** Relative expression of GnRH receptor *gnrhr2ba1* in pituitary tissue. **B.** Relative expression of FSH gonadotropin subunit *fshb* in pituitary tissue. **C.** Relative expression of LH gonadotropin subunit *lhb* in pituitary tissue. **D.** Average concentration of 2-estradiol (E2) in plasma. Results are presented as average +/- standard error of the mean. Statistical significance is represented by an asterisk ($p<0,05$) or two asterisks ($p<0,01$). N.S. = non-significant.

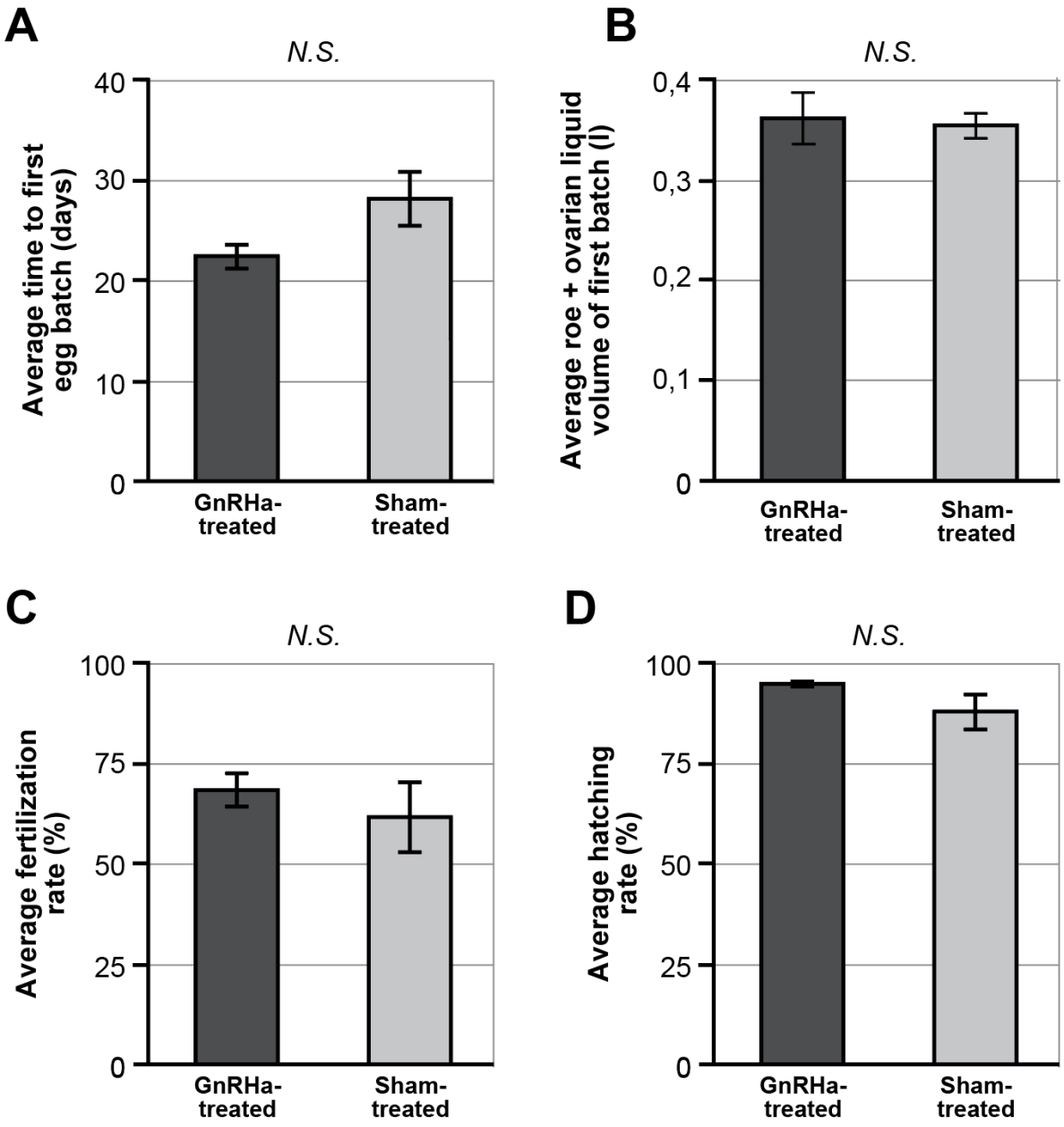

Fig. S3 Effect of GnRHa treatment on females from experiment 2. A**.** Average time to first egg batch. **B.** Average volume of roe and ovarian liquid collected at stripping. **C.** Average fertilization rate in corresponding incubators. **D.** Average hatching rate in corresponding incubators

Tables

		First experiment		Second experiment	
		Females	**Males**	**Females**	**Males**
Number of injected individuals	**GnRHa**	12	12	20	20
	Sham	12	12	20	20
Average initial weight		2746 ± 112 g	982 ± 39 g	2175 ± 47 g	932 ± 34 g
Dose injected		100 µg kg^{-1}	50 µg kg^{-1}	100 µg kg^{-1}	75 µg kg^{-1}
Maturation monitoring		•	•	•	•
Females	**Number of batches followed**	2		1	
	Measurement of volume of roe + ovarian liquid	•		•	
	Egg diameter measurement	•			
	Gonadosomatic index	•			
Males	**Spermatocrit measurement**		•		
	Gonadosomatic index		•		
	Milt volume measurement				•
Plasma collection		•	•		
Steroid analysis		•	•		
Pituitary tissue collection and qPCR analysis		•	•		
In vitro fertilzation and embryo incubation		•	•	•	•
Calculation of fertilization, survival and hatching rate in the incubators		•	•	•	•

Table 1. Main parameters for experiments 1 and 2 and differences in the downstream set of analyses

Experiment	Treatment	Initial parameters			Status		Time to maturation		Parameters at sampling				Fertilization and hatching rates								Relative mRNA abundance in the pituitary						Plasma [E2] (ng/ml)
													First incubator duplicate				Second incubator duplicate				*gnrhr2ba1*		*lhb*		*fshb*		
		Injection date	Body weight (g)	GnRHa dose (µg)	Status	Sampling date	Time to first batch (days)	Additional time to second batch (days)	Body weight (g)	Residual gonad weight (g)	Volume roe + ovarian liquid (l)	Average roe size (mm)	Unfertilized	Unhatched	Hatched	Total	Unfertilized	Unhatched	Hatched	Total	Relative to HKG	Relative to average of sham group (same batch)	Relative to HKG	Relative to average of sham group (same batch)	Relative to HKG	Relative to average of sham group (same batch)	
1	GnRHa	15.03.21	2932	300	Euthanized	16.04.21			*2800*	*33*											###		*9,655*		*8,489*		*0,63*
		15.03.21	2686	275	Did not mature - not sampled																						
		15.03.21	2352	225																							
									Values for first batch																		
		15.03.21	2904	300	**Matured - stripped and sampled at first batch**	25.03.21	10		3100	405	0,6	2,11	58	12	92	162	45	19	148	212	0,485	1,253	60,594	1,987	19,736	1,404	0,12
		15.03.21	2968	300		28.03.21	13		3000	407	0,5	2,14	4	7	528	539	2	15	509	526	0,495	1,278	54,636	1,792	19,954	1,420	0,57
		15.03.21	3034	300		01.04.21	17		3000	452	0,6	2,08	8	142	350	500	13	143	361	517	0,334	0,863	62,696	2,056	20,027	1,425	0,01
		15.03.21	3014	300		03.04.21	19		2700	193	0,6	1,97	4	11	666	681	1	9	658	668	0,167	0,431	69,568	2,281	14,984	1,066	0,01
		15.03.21	3326	325		04.04.21	20		3300	448	0,6	2,11	4	9	529	542	1	10	521	532	0,445	1,149	56,005	1,837	12,496	0,889	1,77
		15.03.21	2784	275		06.04.21	22		2300	113	0,6	2,05	4	8	554	566	1	2	612	615	0,240	0,620	37,846	1,241	7,004	0,498	0,01
		15.03.21	3242	325		11.04.21	27		3200	582	0,4	2,08	0	15	486	501	1	20	477	498	0,301	0,777	71,329	2,339	22,785	1,621	2,3
									Values for second batch																		
		15.03.21	2818	300	**Matured / spawned in tank - stripped and sampled at second batch**	07.04.21	16	7	2300	149	0,5	1,94	1	104	517	622	0	103	472	575	0,325	1,204	41,087	1,355	13,250	0,996	0,01
		15.03.21	2718	275		12.04.21	19	9	2300	105	0,5	1,9	3	79	400	482	1	74	432	507	0,198	0,733	46,639	1,538	18,579	1,396	0,01
	Sham	15.03.21	1710	0	Found dead in tank	20.03.21																					
		15.03.21	2362	0		21.03.21																					
		15.03.21	2136	0	Euthanized - sampled	16.04.21			*2100*	*22*											*0,040*		*2,827*		*0,993*		*0,01*
		15.03.21	2686	0		30.03.21			*2500*	*600*											*0,452*		####		####		*0,18*
		15.03.21	2160	0	Did not mature - not sampled																						
		15.03.21	1892	0																							
									Values for first batch																		
		15.03.21	3010	0	**Matured - sampled at first batch**	27.03.21	12		3000	441	0,5	2,16	9	170	399	578	13	117	421	551	0,437	1,129	34,239	1,123	16,005	1,139	0,15
		15.03.21	3770	0		05.04.21	21		3800	485	0,6	2,11	5	21	560	586	2	9	551	562	0,723	1,867	50,857	1,668	17,879	1,272	3,65
		15.03.21	2694	0		08.04.21	24		2900	361	0,4	2,1	1	127	366	494	0	117	349	466	0,340	0,878	23,355	0,766	6,844	0,487	
		15.03.21	3954	0		07.05.21	53		2900	709	0,3	2,07	337	0	0	337	405	0	0	405	0,003	0,008	0,043	0,001	0,014	0,001	2,99
		15.03.21	1918	0		11.05.21	57		2000	174	0,3	2,09	4	81	369	454	12	349	0	361	0,433	1,118	43,975	1,442	29,518	2,101	0,26
									Values for second batch																		
		15.03.21	2834	0	**Matured / spawned in tank - stripped and sampled at second batch**	06.04.21	8	14	2400	152	0,3	1,97	48	172	374	594	3	116	378	497	0,270	1,000	30,323	1,000	13,304	1,000	0,01

2	GnRHa	05.03.25	2096	225	Matured - sampled at first batch	21.03.25	16				0,25		33	10	619	662	102	30	567	699									
		05.03.25	2102	225		21.03.25	16				0,34		195	56	375	626	296	45	310	651									
		05.03.25	2194	225		21.03.25	16				0,30		79	8	417	504	101	7	479	587									
		05.03.25	2482	250		24.03.25	19				0,37		128	14	428	570	167	17	471	655									
		05.03.25	2246	225		24.03.25	19				0,65		147	42	532	721	110	27	415	552									
		05.03.25	2196	225		24.03.25	19				0,30		41	10	404	455	46	9	506	561									
		05.03.25	2316	250		26.03.25	21				0,40		332	17	318	667	304	26	401	731									
		05.03.25	2442	250		26.03.25	21				0,40		308	19	325	652	282	22	308	612									
		05.03.25	2548	275		26.03.25	21				0,60		289	26	226	541	266	21	248	535									
		05.03.25	2320	250		26.03.25	21				0,30		213	33	406	652	196	28	394	618									
		05.03.25	2074	225		27.03.25	22				0,35		51	10	588	649	45	9	579	633									
		05.03.25	2114	225		27.03.25	22				0,35		134	12	491	637	136	15	453	604									
		05.03.25	1988	200		28.03.25	23				0,33		396	6	247	649	254	1	331	586									
		05.03.25	1810	200		28.03.25	23				0,30		335	29	360	724	370	16	350	736									
		05.03.25	1974	200		28.03.25	23				0,30																		
		05.03.25	1750	200		28.03.25	23				0,26		254	19	425	698	411	14	242	667									
		05.03.25	2344	250		07.04.25	33				0,36																		
		05.03.25	2394	250	Matured / spawned in tank	06.04.25	32				0,35																		
		05.03.25	2240	250		06.04.25	32				0,27																		
		05.03.25	2098	225		09.04.25	35				0,50																		
	Sham	05.03.25	1872	0	Matured - sampled at first batch	21.03.25	16				0,35		80	15	391	486	147	20	419	586									
		05.03.25	1970	0		21.03.25	16				0,27		111	17	448	576	128	10	485	623									
		05.03.25	2842	0		21.03.25	16				0,5		92	5	474	571	89	2	417	508									
		05.03.25	2230	0		21.03.25	16				0,23		146	7	416	569	208	11	555	774									
		05.03.25	2522	0		24.03.25	19				0,36		75	90	442	607	76	103	453	632									
		05.03.25	1640	0		26.03.25	21				0,33		126	35	399	560	120	27	614	761									
		05.03.25	2290	0		27.03.25	22				0,35		276	3	30	309	278	3	20	301									
		05.03.25	2080	0		28.03.25	23				0,35		336	3	5	344	298	16	1	315									
		05.03.25	2048	0		28.03.25	23				0,34																		
		05.03.25	2022	0		31.03.25	26				0,40		353	6	222	581	228	2	397	627									
		05.03.25	1758	0		31.03.25	26				0,30		505	0	8	513	468	1	6	475									
		05.03.25	2020	0		31.03.25	26				0,38		133	11	494	638	580	9	127	716									
		05.03.25	2410	0		02.04.25	28				0,40		48	19	617	684	36	9	568	613									
		05.03.25	2322	0		04.04.25	30				0,42		154	21	529	704	154	57	489	700									
		05.03.25	2214	0		07.04.25	33				0,41		34	84	389	507	54	98	459	611									
		05.03.25	2996	0		08.04.25	34				0,32																		
		05.03.25	1932	0		12.04.25	38				0,36																		
		05.03.25	1982	0		13.04.25	39				0,30																		

		05.03.25	2200	0		28.04.25	54				0,35																	
		05.03.25	1782	0		05.05.25	61				0,38																	

Table S1. Raw data for females

Initial parameters			Status		Parameters at sampling						Relative mRNA abundance in the pituitary						Plasma 11-KT concentration (ng/ml)
											gnrhr2ba1		*lhb*		*fshb*		
Injection date	Body weight (g)	GnRH analog dose (µg)	Status	Sampling date	Red color	Body weight (g)	Gonad weight (g)	Spermatocrit (%)			Relative to HKG	Relative to average of sham group	Relative to HKG	Relative to average of sham group	Relative to HKG	Relative to average of sham group	
15.03.21	968	50	Found dead in tank - sampled	04.05.21		942	41,5				0,43		34,87		27,19		*0,29*
15.03.21	1182	50		22.04.21		1201	49,5				0,30		50,12		25,40		*0,24*
15.03.21	916	50		09.04.21		830	2,4				0,27		33,31		25,30		*0,05*
15.03.21	1008	50	Matured - sampled	06.05.21	yes	973	57,5	16,9			0,99	2,91	101,41	2,00	48,16	1,95	9,11
15.03.21	984	50		06.05.21	yes	939	70,3	13,4			0,31	0,91	50,18	0,99	16,22	0,66	16,6
15.03.21	718	50		05.05.21	yes	699	51,3	13,6			0,54	1,59	63,53	1,25	22,21	0,90	10,99
15.03.21	1308	75		05.05.21	yes	1159	67,1	17,3			0,72	2,13	59,76	1,18	26,48	1,07	4,56
15.03.21	1320	75		05.05.21	yes	1227	64,3	19,1			0,31	0,90	49,18	0,97	12,33	0,50	10,98
15.03.21	802	50		06.05.21	yes	749	45,9	16,3			0,25	0,75	49,47	0,97	21,82	0,88	
15.03.21	1024	50	Did not mature - sampled	06.05.21		1124	2				0,03	0,07	2,79	0,05	2,68	0,11	0,43
15.03.21	1176	50		05.05.21	yes	1196	8,9				0,08	0,25	6,99	0,14	3,91	0,16	0,29
15.03.21	1062	50		05.05.21		1063	13,2				0,03	0,10	1,17	0,02	0,71	0,03	0,05
15.03.21	650	0	Found dead in tank - sampled	30.04.21		637	19,8				0,16		11,16		5,78		*0,05*
15.03.21	802	0		11.04.21		802	12				0,55		23,96		21,05		*0,05*
15.03.21	732	0	Euthanized - sampled	03.04.21		733	25				0,12		22,48		7,62		0,18
15.03.21	1110	0	Matured - sampled	05.05.21	yes	1013	65,2	15,5			0,19	0,57	54,81	1,08	34,26	1,39	5,59
15.03.21	946	0		05.05.21	yes	901	54,6	16,5			0,78	2,29	77,09	1,52	22,11	0,90	13,99
15.03.21	1016	0		05.05.21	yes	925	42,1	22,0			0,51	1,49	65,95	1,30	20,75	0,84	7,05
15.03.21	1064	0		05.05.21	yes	953	54,7	17,4			0,33	0,98	47,60	0,94	23,08	0,94	7,08
15.03.21	1024	0		05.05.21	yes	1010	58,7	17,2			0,32	0,96	48,75	0,96	34,00	1,38	7,76
15.03.21	788	0		06.05.21	yes	715	43,7	16,4			0,14	0,43	65,42	1,29	41,75	1,69	3,62
15.03.21	774	0		05.05.21	yes	679	52,7	12,9			0,40	1,17	44,95	0,88	18,36	0,74	4,23
15.03.21	876	0	Did not mature - sampled	06.05.21		899	1,4				0,04	0,12	2,08	0,04	3,14	0,13	0,28
15.03.21	1326	0	*Actually female - excluded*	05.05.21													
				Sampling date 1	Sampling date 2	Red color level				Volume milt 1 (ml)	Volume milt 2 (ml)							
05.03.25	1022	75		03.04.25	10.05.25	3				7	10							
05.03.25	1182	100		03.04.25	10.05.25	3				4,5	23							
05.03.25	1312	125		03.04.25	10.05.25	3				1,5	21							
05.03.25	942	100		03.04.25	10.05.25	3				0,9	9							
05.03.25	826	75		03.04.25	10.05.25	3				0,4	14							
05.03.25	858	75		03.04.25	10.05.25	3				0,35	12							

05.03.25	992	75		03.04.25	10.05.25	2				0,4	10							
05.03.25	1210	100		03.04.25	10.05.25	2				0,22	8							
05.03.25	808	100		03.04.25	10.05.25	3				0,2	1,9							
05.03.25	1238	100		03.04.25	10.05.25	3				0,04	4							
05.03.25	1178	100		03.04.25	10.05.25	2				0,04	2,5							
05.03.25	908	75		03.04.25	10.05.25	2				0,04	3							
05.03.25	1322	125		03.04.25	10.05.25	*dead 28.04.25*				0,04	*dead 28.04.25*							
05.03.25	803	75		03.04.25	10.05.25	2				0,02	0,5							
05.03.25	1000	100		03.04.25	10.05.25	2				0	0,06							
05.03.25	1306	125		03.04.25	10.05.25	1				0	4							
05.03.25	850	100		03.04.25	10.05.25	1				0	0,02							
05.03.25	968	75		03.04.25	10.05.25	2				0	0,06							
05.03.25	620	50		03.04.25	10.05.25	1				0	1,2							
05.03.25	1036	100		03.04.25	10.05.25	3				0	8							
05.03.25	860	0		03.04.25	10.05.25	3				2	3,9							
05.03.25	698	0		03.04.25	10.05.25	3				1,2	12							
05.03.25	710	0		03.04.25	10.05.25	3				0,35	2							
05.03.25	612	0		03.04.25	10.05.25	2				0,2	2							
05.03.25	748	0		03.04.25	10.05.25	2				0,16	8,3							
05.03.25	1316	0		03.04.25	10.05.25	2				0,14	3,8							
05.03.25	878	0		03.04.25	10.05.25	3				0,12	4							
05.03.25	884	0		03.04.25	10.05.25	3				0,1	3,1							
05.03.25	1120	0		03.04.25	10.05.25	1				0,08	3,5							
05.03.25	1056	0		03.04.25	10.05.25	2				0,04	1,5							
05.03.25	984	0		03.04.25	10.05.25	3				0,02	4,6							
05.03.25	878	0		03.04.25	10.05.25	3				0,02	3,3							
05.03.25	968	0		03.04.25	10.05.25	1				0	0,04							
05.03.25	1234	0		03.04.25	10.05.25	2				0	5,2							
05.03.25	860	0		03.04.25	10.05.25	2				0	1,2							
05.03.25	795	0		03.04.25	10.05.25	2				0	2,7							
05.03.25	1072	0		03.04.25	10.05.25	2				0	1,5							
05.03.25	942	0		03.04.25	10.05.25	1				0	0,1							
05.03.25	1122	0		03.04.25	10.05.25	2				0	1,5							
05.03.25	684	0		03.04.25	10.05.25	3				0	1,3							

Table S2. Raw data for males