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Impaired leptin signaling causes subfertility in female zebrafish

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ABSTRACT

Reproduction is an energetically costly event across vertebrates and tightly linked to nutritional status and energy reserves. In mammals, the hormone leptin is considered as a link between energy homeostasis and reproduction. However, its role in fish reproduction is still unclear. In this study, we investigated the possible role of leptin in the regulation of reproduction in zebrafish, using a loss of function leptin receptor (*lepr*) strain. Impaired leptin signaling resulted in severe reproductive deficiencies in female zebrafish. *lepr* mutant females laid significantly fewer eggs, with low fertilization rates compared to wild-type females. Folliculogenesis was not affected, but oocyte maturation and ovulation were disrupted in *lepr* mutants. Interestingly, the expression of luteinizing hormone beta (*lhb*) in the pituitary was significantly lower in mutant females. Analysis of candidate genes in the ovaries and isolated fully grown follicles revealed differential expression of genes involved in steroidogenesis, oocyte maturation and ovulation in the mutants, which are known to be regulated by LH signaling. Moreover, subfertility in *lepr* mutants could be partially restored by administration of human chorionic gonadotropin. In conclusion, our results show that leptin deficiency does not affect early stages of follicular development, but leptin might be essential in later steps, such as in oocyte maturation and ovulation. To our knowledge, this is the first time that leptin is associated to reproductive deficiencies in zebrafish.

1. Introduction

Reproduction in fish, as in other vertebrates, is an energetically demanding process, coordinated by the brain–pituitary-gonad (BPG) axis. Ectothermic vertebrates, such as teleosts, can spend almost half of their energy reserves for maintaining a normal reproductive function and energy expenditure levels invested in reproduction are usually higher in females for the production of eggs and yolk, whereas in males, sperm production requires usually smaller energy amounts (Hayward and Gillooly, 2011).

The function of the BPG axis is controlled by several metabolic factors, which can act on all three levels of the axis, either directly or indirectly (Fernandez-Fernandez et al., 2006; Hill et al., 2008; Shahjahan et al., 2014). In mammals, one of these factors is the adipocyte-derived hormone leptin, a 16kD protein product encoded by the obese (*ob*) gene and produced primarily, but not exclusively, in the white adipose tissue (Zhang et al., 1994). Leptin mediates its signal through its receptor (*lepr*), which belongs to the class I cytokine receptor family (Tartaglia, 1997). Leptin is a key regulator of body weight, appetite and metabolism and is essential for the mammalian reproductive system. Absence of functional leptin prevents pubertal development and results in infertility, as seen in the leptin deficient *ob/ob* mouse (Chehab et al., 1996). Leptin has been identified in the majority of the vertebrate species, including teleosts, such as pufferfish (Kurokawa et al., 2005), common carp (Huising et al., 2006), medaka (Kurokawa and Murashita, 2009), zebrafish (Gorissen et al., 2009) and Atlantic salmon (Rønnestad et al., 2010). Even though mammalian and teleostean leptin genes have low levels of homology (Gorissen and Flik, 2014), the secondary and tertiary structures of the proteins, as well as gene synteny, seem to be highly conserved (Denver et al., 2011; Prokop et al., 2012; Londraville et al., 2014, 2017).

During the last decades, leptin research in teleosts mainly focused on its role in appetite, food intake and energy metabolism (Tian et al., 2015; Audira et al., 2018; Ahi et al., 2019), while information on its role in reproduction is still limited (Parker and Cheung, 2020). Several studies indicate that leptin might be important during later stages of the maturational process in fish. For example, plasma leptin levels were higher during and after spawning in both male and female ayu (*Plecoglossus altivelis*) (Nagasaka et al., 2006). In Arctic charr (*Salvelinus alpinus*), leptin mRNA in the liver increased seasonally, reaching a peak in

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autumn, when all fish were also sexually mature (Frøiland et al., 2010). In female chum salmon (*Oncorhynchus keta*), transcripts and plasma levels of leptin were associated with increased levels of sex steroids, stimulating sexual maturation, after transferring the fish from seawater to freshwater (Choi et al., 2014). Moreover, hepatic *lepa-1* transcripts were higher in mature, male Atlantic salmon parr (*Salmon salar*), compared to immature fish (Trombley and Schmitz, 2013). Hepatic *lepa* expression was also upregulated during pubertal transition in male but not in female chub mackerel (*Scomber japonicus)* (Ohga et al., 2015, 2017). A significant upregulation of *lepr* transcripts in the testis of male Atlantic salmon parr was also observed throughout mid to late spermatogenesis (Trombley et al., 2014). Moreover, both *lepa* paralogues were upregulated in Atlantic salmon parr hepatocyte cultures after treatment with testosterone, 11-ketotestosterone and estradiol (Trombley et al., 2015). In a recent study in the female chub mackerel leptin stimulation strongly induced gonadotropin secretion, promoted ovarian development and triggered pubertal onset in pre-pubertal females, suggesting that leptin is an important signal for the activation of the reproductive axis (Ohga et al., 2020).

So far little is known about the role of leptin in the regulation of the reproduction in zebrafish (*Danio rerio*). Zebrafish possesses duplicate leptin genes (*lepa* and *lepb*), which are mainly expressed in the liver and the gonads, respectively (Gorissen et al., 2009), and a single *lepr* gene, with strongest expression in the brain and the testes (Liu et al., 2010). In a study by Michel et al. (2016) adult zebrafish lacking a functional *lepr* exhibited normal fertility, with no effects of the genotype neither on the number of the fertilized eggs laid nor in the frequency of successful breedings. However, based on our laboratory observations, mating male and female *lepr* mutant zebrafish resulted in either none or few viable offsprings (data not shown).

In the present study, we aimed to give more insights into the role of leptin in the regulation of reproduction in adult zebrafish, using a loss of function *lepr* zebrafish strain. Our data show that the lack of leptin signaling causes impaired fertility in female zebrafish. We provide evidence that *lepr* mutant zebrafish have lower transcript levels of luteinizing hormone (LH), which results also in lower proportions of ovulated follicles. Genes involved in steroidogenesis, oocyte maturation and ovulation showed differential expression in *lepr* mutants compared to wild-type females suggesting that leptin plays an essential role in the last steps of oocyte maturation and ovulation in zebrafish.

2. Materials and methods

2.1. Animals

Lepr Sa12953 zebrafish were obtained from the European Zebrafish Resource Centre. The mutation was created by the Sanger Institute for the Zebrafish Mutation Project, replacing a thymine with an adenine on chromosome 6, resulting in a premature stop codon, which lead to a shortened polypeptide (Busch-Nentwich et al., 2013).

Zebrafish were kept at the Genome Engineering Zebrafish National Facility at Uppsala University in 3-Liter flow-through tanks, under an artificial photoperiod of 14:10 h light:dark conditions at 28.4 °C. Fish were fed three times, twice with commercial dry food (Zebrafeed, Sparos, Portugal) in the morning and in the afternoon and once with rotifers in the evening. Water parameters were monitored regularly: water temperature (^{o}C), pH and conductivity (μS/cm) on a daily basis, general (^{o}dGH) and carbonate hardness (^{o}dKH), together with the levels of ammonia (NH4, mg/l), nitrites (NO2, mg/l) and nitrates (NO3, mg/l) bi-weekly. All animal experiments were conducted in accordance with the guidelines and the approval of the Swedish Ethical Committee on Animal Research in Uppsala (permit C10/16).

2.2. Fertility determination

Eighteen couples of adult (4 months old) wild-type and mutant zebrafish were divided into three groups: six couples of a wild-type male and wild-type female (control group), six couples of a mutant male and a wild-type female and six couples of a wild-type male with a mutant female. The fish were first trained twice and then were mated for three times with an interval of eight days between each mating (breeding). In the evening the couples were transferred into breeding tanks and the spawning events were recorded the next morning; the eggs were collected and the fertilization rate ((number of fertilized eggs/total number of eggs) *100) was estimated. The survival of the embryos and larvae was checked on a daily basis (24 h post fertilization (hpf), 48 hpf, 72 hpf, 96 hpf, 120 hpf). Dead embryos and larvae were counted and removed daily and all surviving larvae were euthanized after 120 hpf.

2.3. Sampling

Two weeks after the last spawning event, all females were anesthetized by immersion in a 0.4 mg/ml tricaine solution (MS-222) and then euthanized by immersion in ice bath. The standard length (SL, cm) and total weight (W, mg) of each fish were measured and the Body Mass Index (BMI = W/SL^2, g/cm^2) was calculated. Zebrafish were decapitated, the tissues of interest (brain, pituitary, gonads and liver) were dissected and transferred into tubes with 200 μl of RNAlater (Ambion Inc, Austin Texas) at 4 °C for 24 h and then stored at −20 °C until further analysis. From female zebrafish one lobe of the ovarian sample was stored for histological analysis and the other one was stored for gene expression quantification.

2.4. Isolation of fully grown ovarian follicles

Additionally, 6–7 wild-type and mutant adult female zebrafish of the same age and after similar training, as described above, were used for the assay of the fully grown follicles. Briefly, after anesthetization and decapitation, as described above, the ovaries were carefully dissected and placed into a petri plate dish with 60% Leibovitz L-15 medium. Ovaries were mixed carefully with a plastic pipette and the follicles were released from the ovarian tissue. The follicles of different developmental stages were manually isolated, using fine forceps, grouped based on their size and the fully grown follicles were sampled. The staging system adopted for the ovarian follicles was based on the original definition of Selman et al. (1993), as modified by Wang and Ge (2004). The fully grown follicles (n = 50–62 per sample from each wild-type female, n = 13–46 per sample from each mutant female) were first washed twice with 500 μl 60% Leibovitz L-15 medium, then homogenized in 200 μl Trizol (Ambion) and stored in −80 °C until RNA extraction.

2.5. Intraperitoneal injection of lepr−/− adult female zebrafish

Prior to injection, the fish were fasted for 24 h. *lepr*−/− adult mutant females were anesthetized and quickly placed on a soft sponge soaked in facility water. Using a 5 μl Hamilton syringe (Sigma-Aldrich), 2 μl of 60% Leibovitz L-15 medium (control group, n = 3) and 2 μl of human chorionic gonadotropin (hCG) (10 IU/μL; Sigma-Aldrich) (hCG treated group, n = 3) were carefully injected into the midline between the pelvic fins. The fish were immediately transferred back to the tanks for recovery. Four hours after injection, the fish were euthanized, as described above, the ovaries were carefully dissected, placed into a petri plate dish with 60% Leibovitz L-15 medium and mixed carefully with a plastic pipette, until fully grown and/or ovulated follicles were released from the ovarian tissue. The ovulation rate for each fish was calculated as the number of ovulated follicles/(number of fully grown follicles + number of ovulated follicles).

Additionally, subfertile *lepr*−/− adult female zebrafish (n = 4) were i.p injected with hCG before mating. Prior to the experiment, these females were mated with wild-type males three times, but laid no or few eggs with low ovulation rate (control). The night before the experiment, the fish were transferred to 1-L breeding tanks. Each tank contained one

lepr mutant female and one wild-type male, separated overnight with a divider. The following morning the mutant females were anesthetized and carefully injected with 2 μl hCG (10 IU/μL), as described above. The fish were immediately returned to the breeding tanks for recovery for 30 min. The dividers separating males and females were removed and the fish were left to breed for 6 h. The spawning events were recorded, the laid eggs were collected and the fertilization rate was estimated. Spawning success was compared with or without i.p hCG treatment in the same fish.

2.6. Histological analysis

Ovarian samples stored for histological analysis were first fixed in 4% formaldehyde solution (VWR Chemicals) for 48 h and then placed in 70% ethanol solution (Histolab) until further analysis. The tissues were then transferred in a series of increasingly concentrated ethanol solutions, twice in 95% solution for 1 h and twice in 99% solution for 30 min. The tissues were later infiltrated overnight in plastic and embedded (Technovit 7100), before sectioning. The sections were finally washed and stained using Hematoxylin and Eosin (Sigma). The slides were observed under a Leica DFC550 microscope twice, in order to minimize any possible bias of the estimation. The staging system adopted for the ovarian follicles was based on the original definition of Selman et al. (1993), as modified by Wang and Ge (2004). The number of different stages of follicle development in a defined area were counted and presented as percentages.

2.7. RNA extraction and cDNA synthesis

The total RNA of the dissected tissues was extracted using Trizol, according to the manufacturer's protocol. Briefly, the tissues were removed from RNAlater and were homogenized in 200 μl Trizol, with a fine syringe needle. After homogenization, 40 μl of chloroform (Sigma-Aldrich) were added to each sample, followed by a 5 min' incubation in room temperature and a centrifugation at 12000 g/min for 20 min at 4 °C. The aqueous upper phase was carefully transferred into new RNAse-free tubes, in which 1 μl of glycoblue (Ambion) and 100 μl of ice cold (−20 °C) isopropyl alcohol (Sigma-Aldrich) were added directly. Samples were then incubated for 5 min in room temperature and centrifuged at 13000 g/min for 20 min at 4 °C. The supernatants were removed and the RNA pellets were washed twice with 200 μl of ice cold (−20 °C) ethanol 75% (VWR) by centrifugation at 9000 g/min for 5 min at 4 °C. The RNA pellets were then dried under a fume hood for 10 min and were finally solubilized in 10 μl of Nuclease-free water (Ambion).

The RNA samples were DNAse treated, in order to remove any genomic DNA contamination, using the Turbo DNA-free kit (Ambion), according to the manufacturer's instructions. The quantity and quality of the extracted RNA was finally measured spectrophotometrically using NanoDrop (Thermo-Fisher Scientific). cDNA synthesis was carried out by reverse transcription with 500 ng RNA input from each brain sample, 150 ng from each pituitary sample and 1000 ng from each ovarian, fully grown follicles and liver sample. In brief, 0.5 μL of random primers (50 ng/μl) and 0.5 μl dNTP (10 nM) were added to each RNA sample, followed by an incubation at 65 °C for 5 min and then the samples were kept on ice for 1 min. A total mix of 3.5 μl, containing 2 μl of 5X First-Strand Buffer, 0.5 μl of DTT (0.1 M), 0.5 μl of RNase OUT (40U/μl) and 0.5 μl of Superscript III RT (200U/μl) was prepared and added to each sample. The thermal profile of the reverse transcription was: 25 °C for 5 min, 50 °C for 50 min and 70 °C for 15 min. The final volume of 10 μl of cDNA was stored at −20 °C until gene expression quantification analysis.

2.8. Validation of candidate reference genes

Identification of reference gene(s) with stable expression in different experimental conditions, tissues and species is an essential step for the analysis of gene expression levels by qPCR (Kubista et al., 2006). Therefore, we selected 8 candidate genes based on previous studies in zebrafish, which were used as references genes in different experimental conditions and tissues (McCurley and Callard, 2008; Casadei et al., 2011; Xu et al., 2016) and we assessed their expression levels in the brain, pituitary, ovarian and liver samples from both wild-type and *lepr* mutant zebrafish. We used three different common algorithms to calculate the expression stabilities; BestKeeper, NormFinder and geNorm. BestKeeper ranks the candidate reference genes based on specific expression correlation analysis or so called BestKeeper index (r) across all samples (Pfaffl et al., 2004). NormFinder identifies the most stable genes by calculating expression stability values based on analyses of inter- and intra-group expression variations (Andersen et al., 2004). Finally, geNorm algorithm considers mean pairwise variations in a stepwise manner between each gene and the other candidates (M value) (Vandesompele et al., 2002). It is already known that the ranking results from each of these algorithms cannot be considered alone and it is recommended to select top ranked genes which are appeared to be consistent between all three algorithms (Ahi et al., 2013).

In the brain, we found the expression level of *g6pd* to be the most stable in almost all the three algorithms (Supplementary Table 1). In the pituitary, 3 out of the 8 candidates had very low expression, therefore we excluded them from the analyses and *ef1a* and *rplp0* appeared to be the most stably expressed genes (Supplementary Table 1). In the ovaries and the fully grown follicles, *ef1a* stands alone as the most stable gene across all the algorithms, while in the liver *ef1a* and *rpl13* were both suitable reference genes (Supplementary Table 1).

2.9. Gene expression quantification

Specific primers for each target and reference gene were designed (Supplementary Table 2). Briefly, the gene's sequences were obtained from Blastn through a zebrafish database engine (zfin.org) and imported to the CLC Genomic Workbench software (CLC Bio, Denmark), whereas the exon/exon boundaries were tracked, using the annotated *Danio rerio* genome in the Ensembl database. The primers were designed by Primer Express 3.0 (Applied Biosystems, CA, USA) and their dimerization and secondary structure formation were evaluated using OligoAnalyzer 3.1 (Integrated DNA Technology).

Relative levels of gene expression in the tissues of interest were measured by quantitative polymerase chain reaction (qPCR) on a MxPro-3000 PCR machine (Stratagene, La Jolla, CA). For the qPCR assays, 1 μl of diluted cDNA of each sample was mixed with 7.5 μl of qPCR PowerUp SYBR Green Master mix, 0.3 μl of forward and reverse primers (10 μM) and 6.2 μl of RNA-free water in a total volume of 15 μl. Each biological replicate for each gene was tested in three technical replicates. The thermal profile of the qPCR assays was: 50 °C for 2 min (1 cycle), 95 °C for 2 min (1 cycle), 95 °C for 15 s and 60–64 °C (depending on the gene of interest) for 1 min (40 cycles). A dissociation step (60–95 °C) was performed at the end of the amplification step, in order to verify the specificity of each product. Standard curves were generated from pooled cDNA of random samples obtained from the reverse transcription and were tested in three technical replicates for each gene. The efficiencies of the qPCR assays were calculated from the slope of the standard curve by the MxPro™ QPCR software (Stratagene, La Jolla, CA, USA) and the R^2 values were in all cases higher than 0.990 (Supplementary Table 2).

2.10. Statistical analysis

Data are presented as mean ± standard error of the mean (SE). Comparisons among different groups were assessed with one-way ANOVA, followed by Tukey's honest significant difference (HSD) post hoc tests for multiple comparisons in any case needed. Percentages of the different stages of oocytes were arc-sin transformed prior to statistical analysis to fit test criteria. All statistical analysis was done, using

the IBM SPSS Statistics 24 software (Chicago, IL, USA). All graphs were made using GraphPad Prism version 7 (GraphPad Software Inc., CA, USA).

3. Results

3.1. *lepr* deficiency causes impaired fertility in females

Mutant females had lower spawning frequency (Fig. 1A), even though not significant and laid significantly fewer eggs, both in total ($p = 0.03$, Fig. 1B) and per spawning event ($p = 0.007$, Fig. 1C), comparing to the wild-type females of the control group. Among the eggs laid from the mutant females, there were many which had bad quality, looking completely dark, something which was not evident in the control group (data not shown). The fertilization rate in that group was also lower compared to the control group ($p = 0.001$, Fig. 1D). However, the survival of the fertilized embryos was not affected (Fig. 1E). Wild-type females mated with *lepr* mutant males did not show any differences in the measured fertility parameters compared to the control group (data not shown). Taken together, impaired leptin signaling had a clear effect on female fertility in zebrafish, while, in males, spermiation was not affected.

3.2. *lepr* deficiency has no effect on body size and weight in females

lepr mutant female zebrafish show a tendency to be longer than wild-type female zebrafish ($p = 0.06$, Fig. 2A), but no significant differences were found neither in the weight (Fig. 2B) nor in the body mass index (Fig. 2C) between the two genotypes.

3.3. Folliculogenesis is not impaired in *lepr* deficient female zebrafish

Histological analysis revealed that all different stages of follicles could be identified in the ovaries of both wild-type and *lepr* mutant zebrafish [primary growth (PG), pre-vitellogenic (PV), early vitellogenic (EV), middle vitellogenic (MV) and fully grown (FG)] (Fig. 3A–B). The ratios of EV and FG follicles were slightly, but not significantly higher in the ovaries of the *lepr* mutant fish compared to the control group (Fig. 3C) and no statistically significant differences were observed in the ratios of the different stages of follicular development between the two genotypes. During follicular growth, vitellogenins are synthesized in the liver under the control of estradiol, transported via the bloodstream to the ovaries and taken up by the growing oocytes. Expression of all known isoforms of the estrogen receptors (*esr1*, *esr2a*, *esr2b*) and two of the isoforms of the vitellogenins gene family (*vtg1*, *vtg2*) were measured in the liver. No significant differences in mRNA expression of these genes were observed between the two genotypes (Supplementary Table 3). Taken together, these results suggest that *lepr* deficiency did not affect neither vitellogenesis nor folliculogenesis in zebrafish.

3.4. *lepr* deficiency reduces *lhb* transcripts

The mRNA expression of the gonadotropic releasing hormone (GnRH) isoforms in the brain (*gnrh2*, *gnrh3*), as well as of their receptors and the follicular stimulating hormone (FSH) in the pituitary (*gnrhr2*, *gnrhr3*, *fshb*) did not show any significant differences between the wild-type and the *lepr* knockout females (Fig. 4). However, the transcripts of the luteinizing hormone (*lhb*) were significantly lower in the pituitary of the mutant females ($p = 0.017$, Fig. 4G). Both in the brain and in the pituitary, *lepr* mRNA expression did not differ between the two groups (Fig. 4C and H).

3.5. Differential gene expression in ovarian tissue and fully grown follicles

During the sampling of the fully grown follicles, we observed that the morphology of the ovaries from the *lepr* mutant females was more heterogeneous compared to those from wild-type females: ovaries of the *lepr* mutants were characterized by either complete or partial

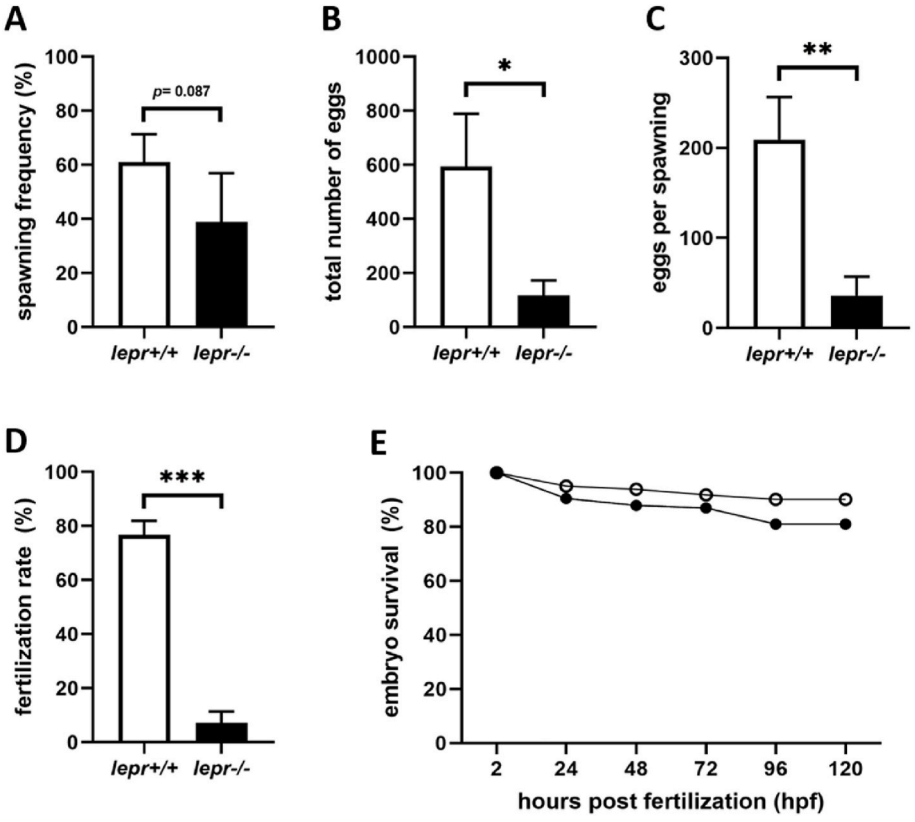

Fig. 1. Fertility assessment of wild-type and *lepr* mutant female zebrafish. Spawning frequency (A), total number of eggs spawned (B), number of eggs per spawning event (C), fertilization rate (D) and embryo survival (E) from couples of wild-type (*lepr*+/+) males and females (empty bars or circles) and wild-type (*lepr*+/+) males with mutant (*lepr*−/−) females (filled bars or circles). Data shown as means ± SE (n = 6). Significant differences between different groups are indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

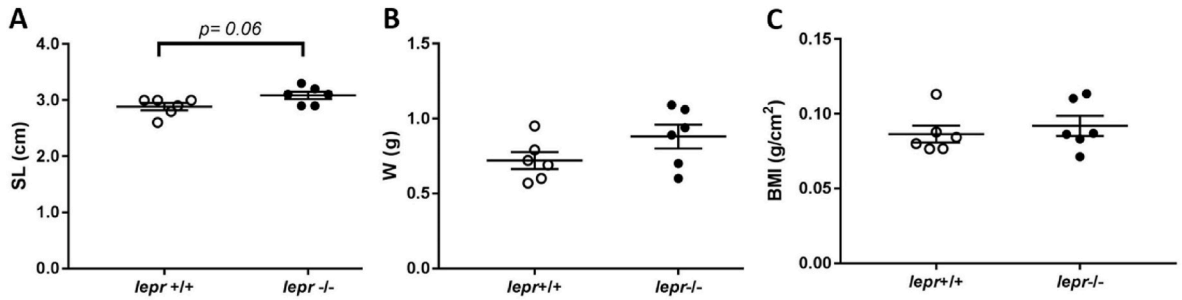

Fig. 2. Standard length (A), body weight (B) and body mass index (C) of *lepr*+/+ (empty circles) and *lepr*−/− (filled circles) adult female zebrafish. Data shown as means ± SE (n = 6).

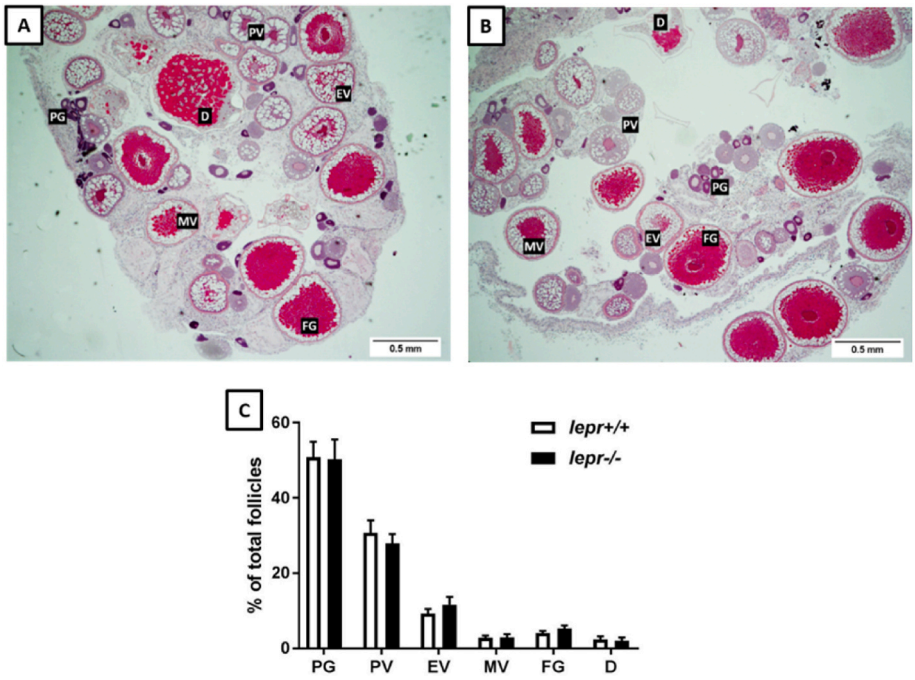

Fig. 3. Histology of the ovaries of *lepr*+/+ (A) and *lepr*−/− (B) adult female zebrafish. Ratios of different developmental stages of follicles (C) in the ovaries of *lepr*+/+ (empty bars) and *lepr*−/− (filled bars) adult female zebrafish. Data shown as mean ± SE (n = 6). Stages of follicular development: primary growth (PG), previtellogenic (PV), early vitellogenic (EV), middle vitellogenic (MV), full grown (FG), denatured (D).

anovulation (Fig. 5C–F), whereas in the wild-type ovaries ovulation occurred normally (Fig. 5A–B). Therefore, the fully grown follicles from the mutants were divided in 2 groups: those from fish which did not ovulate at all and those from fish which had partial ovulation. To further analyse the molecular mechanisms underlying the impaired fertility observed in *lepr* mutant females, expression profiles of a panel of genes involved in final oocyte maturation and ovulation, including genes of the leptin system, were assessed in entire gonads and isolated fully grown follicles in *lepr* mutant females and were compared to wild-type female zebrafish.

Gonadotropin receptors: *fshr* and *lhcgr* expression in the ovary did not differ between the *lepr* mutant and the wild-type zebrafish. Interestingly, the transcripts of *lhcgr* were significantly higher in fully grown follicles of mutant females which were laying eggs compared to those from mutant females which were not laying eggs at all ($p = 0.028$, Fig. 6A–B).

Steroid enzymes: *star* and *hsd3b1* transcripts were higher in the ovaries of *lepr* mutant females comparing to those of wild-type females ($p = 0.036$ and $p = 0.013$, respectively, Fig. 6C–D). *hsd3b1* expression was also significantly higher in fully grown follicles of mutant females, which were laying eggs compared to those from mutant females which were not laying eggs ($p = 0.03$, Fig. 6D). There were no differences in the transcripts of *cyp19a1a* in the ovaries, but its expression was higher in the fully grown follicles from mutant females not laying eggs, compared both from wild-types ($p < 0.001$, Fig. 6E) and from mutants which laid eggs ($p = 0.013$, Fig. 6E).

Progesterone membrane receptors: *mpra1* (*paqr7a*) expression in the ovary did not differ between the *lepr* mutant and the wild-type zebrafish. However, its expression was significantly higher in fully grown follicles of mutant females which were laying eggs compared to those from mutant females which were not laying eggs ($p = 0.045$, Fig. 6F). There were no differences in the transcripts of *mpra2* (*paqr7b*) either in ovaries or in the fully grown follicles between the two genotypes (Fig. 6G). *mprb* (*paqr8*) transcripts were higher, even though not significantly, in the ovaries from wild-type fish comparing to those from mutants ($p = 0.07$, Fig. 6H). *mprγ2* (*paqr5b*) transcripts were undetectable both in the ovaries and in the fully grown follicles in both genotypes.

Progesterone receptor membrane components: *pgrmc1* expression in the ovaries did not differ between the control and mutant groups (Fig. 6I). However, *pgrmc2* transcripts were higher in the ovaries from wild-type zebrafish than in those from *lepr* mutant zebrafish ($p = 0.016$,

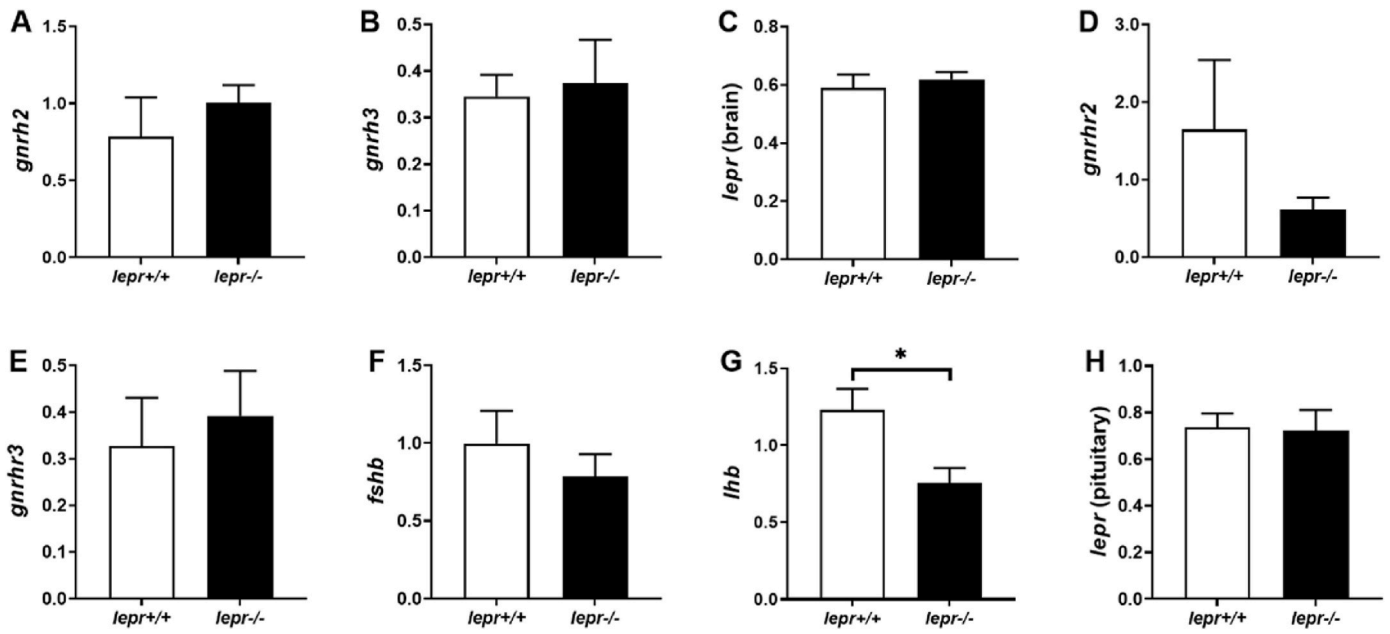

Fig. 4. Relative gene expression of *gnrh2* (A), *gnrh3* (B) and *lepr* (C) in the brain and of *gnrhr2* (D), *gnrhr3* (E), *fshb* (F), *lhb* (G) and *lepr* (H) in the pituitary of *lepr*+/+ (empty bars) and *lepr*−/− (filled bars) adult female zebrafish. Data shown as mean ± SE (n = 6). The expression levels of the target genes were normalized to the expression levels of *g6pd* in the brain samples and to the average of the expression levels of *ef1a* and *rplp0* in the pituitary samples. Significant differences between different groups are indicated by $*p < 0.05$.

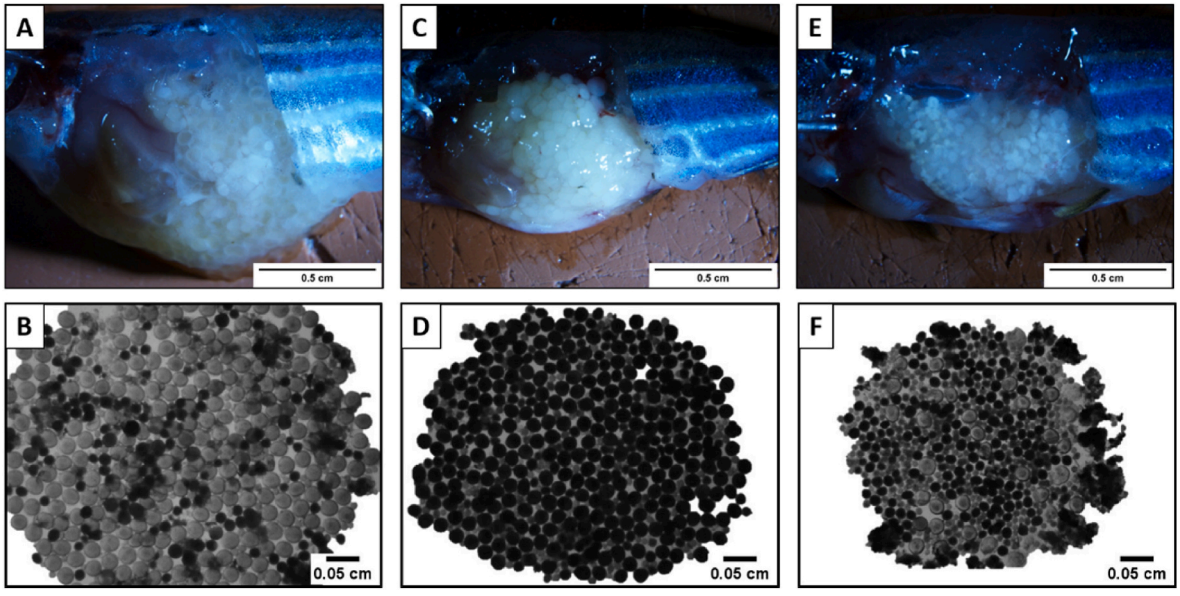

Fig. 5. Ovarian morphology of *lepr*+/+ and *lepr*−/− adult female zebrafish. Normal ovulation with mature ovulated oocytes in the ovary of *lepr*+/+ fish (A–B). Complete anovulation with no mature ovulated oocytes in the ovary of *lepr*−/− fish (C–D). Partial anovulation with few mature ovulated oocytes in the ovary of *lepr*−/− fish (E–F).

Fig. 6J). No significant differences for both genes were observed in the fully grown follicles between the wild-type fish and both mutant groups (Fig. 6I–J).

Progesterone nuclear receptor: The expression levels of *pgr* were almost 2-fold higher in the ovaries of the mutant zebrafish, comparing to the control group ($p = 0.001$, Fig. 6K). Moreover, significant higher transcripts were observed in fully grown follicles of mutant females which were laying eggs compared to those from mutant females which were not laying eggs ($p = 0.014$, Fig. 6K).

Prostaglandins and their receptors: None of the transcripts of the cyclooxygenases, the enzymes which catalyse the prostaglandin synthesis in zebrafish (*ptgs1*, *ptgs2a* and *ptgs2b*), were significantly different between the two genotypes (Fig. 6L–N). However, the transcripts of *ptger4b*, one of the prostaglandin receptors which is has been shown to be essential in ovulation in zebrafish, showed significantly higher levels in the ovaries of the mutant females ($p = 0.027$ Fig. 6O). No significant differences were observed for all the afore-mentioned genes in the fully grown follicles between the wild-type fish and both mutant groups (Fig. 6L–O).

Metalloproteinases: *mmp2* (Fig. 6P) and *mmp15a* (Fig. 6S) transcripts were upregulated in the *lepr* mutant ovaries, though the differences were only significant for *mmp15a* ($p = 0.05$). No differences in

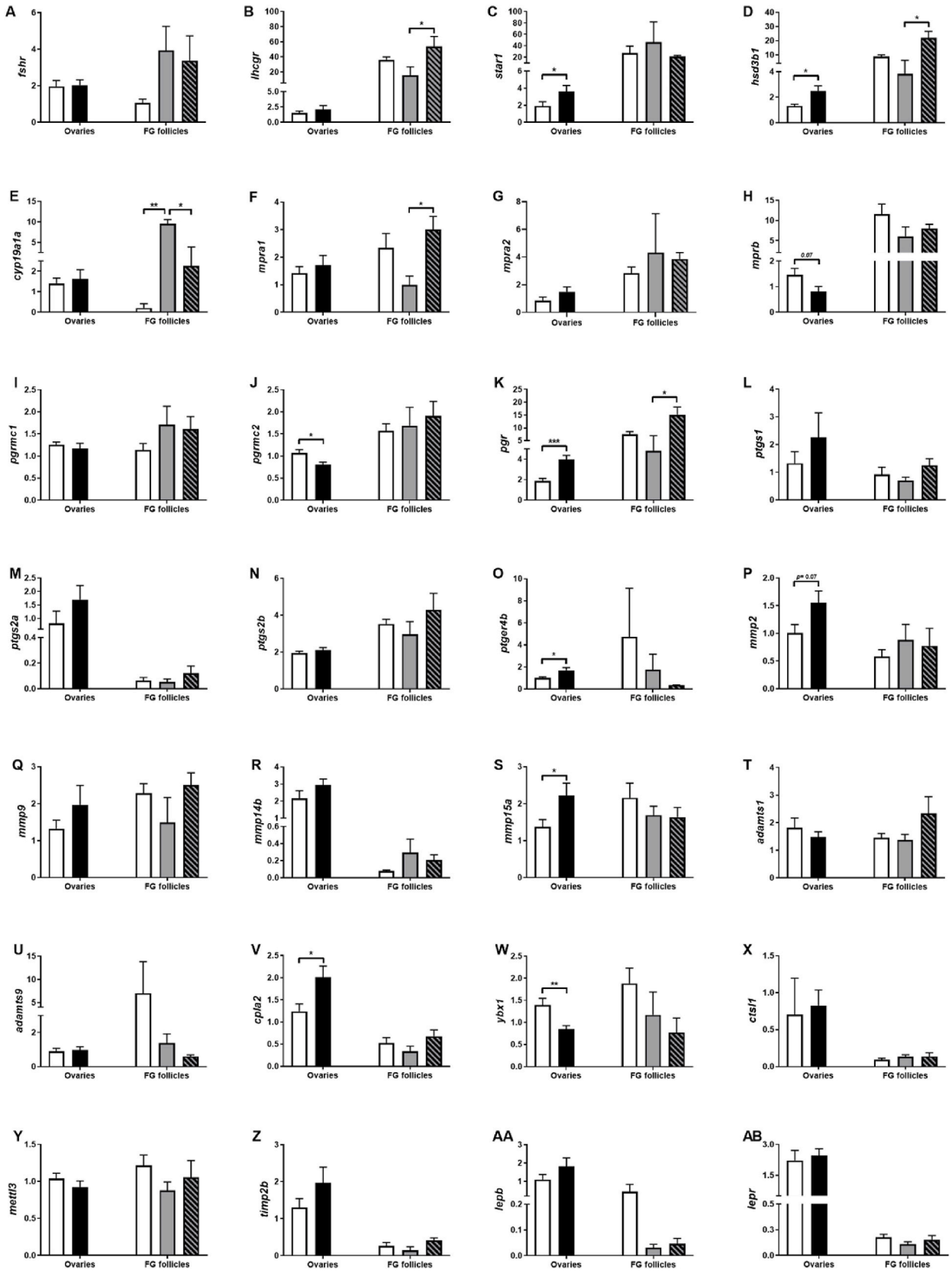

Fig. 6. Relative gene expression of genes involved in ovulation, steroidogenesis and genes of the leptin system in the ovaries and fully grown (FG) follicles of *lepr*+/+ and *lepr*−/− adult female zebrafish. Both for ovaries (n = 6) and for FG follicles (n = 3–6) data are shown as mean ± SE. The expression levels of the target genes were normalized to the expression levels of *ef1a*. Significant differences between different groups are indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. For the ovaries: empty bars indicate *lepr*+/+ and filled bars indicate *lepr*−/− ovaries. For the FG follicles: empty bars indicate follicles from *lepr*+/+ females, filled bars follicles from *lepr*−/− females which were not laying eggs and striped bars follicles from *lepr*−/− females which were laying few eggs.

expression of these genes were observed in the fully grown follicles. No differences were also found for the other measured metalloproteinases (*mmp9*, *mmp14b*, *adamts1*, *adamts9*) (Fig. 6Q–R, 6T-U).

Other genes involved in oocyte maturation and ovulation: The expression levels of *cpla2* were significantly higher in the ovaries from the mutant zebrafish, comparing to those from the control group ($p = 0.023$, Fig. 6V). On the contrary, *ybx1* expression was higher in the ovarian tissue from wild-type zebrafish compared to those from *lepr* mutant fish ($p = 0.006$, Fig. 6W). No significant differences were observed when these genes were tested in the fully grown follicle samples. *ctsl1*, *timp2b* and *mettl3* expression, both in the ovaries and in the fully grown follicles, did not differ between any groups (Fig. 6X–Z).

Genes of the leptin system: The transcripts of *lepr* and *lepb* were expressed both in the ovaries and fully grown follicles, but no differences in their expression were observed between the genotypes (Fig. 6AA-AB).

3.6. hCG administration partially restores subfertility in lerp−/− female zebrafish

In order to study if the lower levels of LH observed in the pituitary of *lepr* mutants could be one of the factors resulting in subfertility, *lepr*−/− female zebrafish were injected intraperitoneally with hCG, a LH-agonist. Indeed, significantly higher number of ovulated follicles ($p = 0.009$) were observed in the ovaries of the *lepr*−/− zebrafish after hCG administration (Fig. 7). Moreover, hCG administration resulted in increased spawning success by 25%, when *lepr*−/− females were mated with wild-type males (data not shown). These results provide further support to our initial hypothesis that the low ovulation rate might be caused by the reduced LH levels in the *lepr* mutants, whereas spawning might only be partly affected by LH signaling.

4. Discussion

In the present study we showed that the lack of leptin signaling had a strong inhibitory effect on the reproductive system of female zebrafish. In mammals, leptin has been established as a key factor in the regulation of pubertal onset and adult fertility (Barash et al., 1996; Cheung et al., 1997; Parker and Cheung, 2020). To our knowledge, this is the first study in fish, associating *lepr* deficiency with impaired fertility. Female *lepr* mutant zebrafish were spawning fewer eggs than wild-type females and the majority were not fertilized (Fig. 1), questioning therefore their quality. This phenotype is in contrast to the one described by Michel et al. (2016). Using a different *lepr* mutant zebrafish strain (sa1508), the authors showed that female *lepr* knockdown zebrafish were equally fertile and reproductively active as wild-type fish. Both strains have a point mutation on the chromosome 6, but differ in the position of the substitution, which leads to the stop codon (Ruzicka et al., 2019). In the sa12953 mutant used in the present study, the substitution leads to more truncated polypeptide compared to the sa1508 mutant and this could explain the different phenotypes. However, in the study of Michel et al. (2016), the authors presented only results on the reproductive productivity, without studying the effect of the mutation on the different levels of the reproductive axis, which could give further insights into the role of leptin in the regulation of reproduction in zebrafish.

One of the most important findings in our study was the lower *lhb* transcript levels in the pituitaries observed in the *lepr* mutant females (Fig. 4G). However, we did not observe any differences in the GnRH isoforms in the brain, as well as their receptors and the FSHb expression in the pituitary (Fig. 4). In teleost fish, LH is essential for normal reproductive function in females, stimulating oocyte maturation and ovulation (Levavi-Sivan et al., 2010). Indeed, LH-deficient female zebrafish (Chu et al., 2014; Shang et al., 2019; Zhang et al., 2015b) and medaka (Takahashi et al., 2016), even though they show normal gonadal growth, failed to spawn and were infertile. Similarly, in mammals, knockdown of the *lhb* gene disrupts folliculogenesis and decreases plasma estrogen levels in female mice, causing infertility (Ma et al., 2004). Thus, it could be speculated that there is a causal relationship between the low LH transcript levels and the impaired fertility observed in *lepr* mutant female zebrafish. This assumption is further supported by the findings that impaired ovulation in mutant females could be partially rescued by hCG administration (Fig. 7). So far, a regulatory connection between leptin and LH in teleost fish has not been shown *in vivo*. Previous *in vitro* studies indicated that mammalian recombinant leptin administration could stimulate LH release in pituitary cells in sea bass and rainbow trout (Peyon et al., 2001; Weil et al., 2003). In a recent study using female chub mackerel primary pituitary cell cultures,

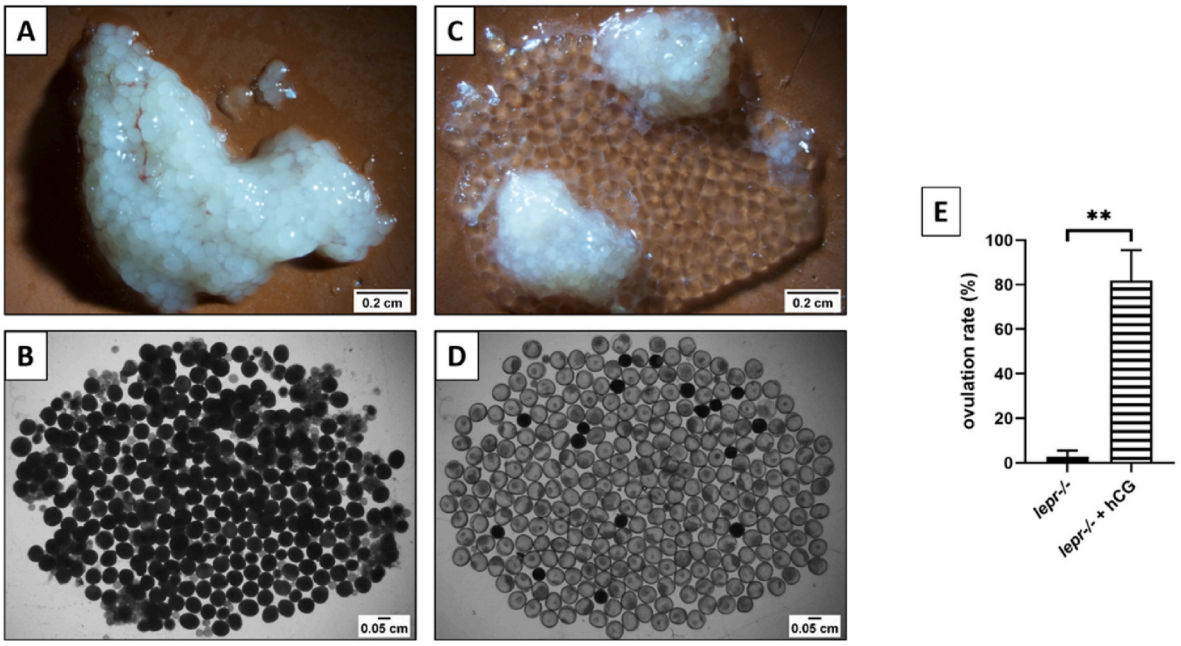

Fig. 7. hCG administration restores ovulation in *lepr*−/− female zebrafish. Ovarian morphology of *lepr*−/− adult female zebrafish before (A–B) and after (C–D) intraperitoneal administration of hCG. Comparison of the number of ovulated follicles from *lepr*−/− females before and after hCG administration (E). Data shown as mean ± SE (n = 3). Significant differences between different groups are indicated by **p < 0.01.

recombinant leptin administration induced the secretion of FSH and LH in pre-pubertal females, but the effect was almost absent during later stages of gonad development, whereas intra-cerebroventricular leptin administration upregulated the expression of *fshb* and *lhb* in the pituitary of sexually immature adult chub mackerel (Ohga et al., 2020). In mammals, even though it was already demonstrated that leptin can enhance LH levels in the bloodstream (Donato et al., 2011), the regulatory connection between leptin and LH got only recently more attention. *lepr* deficient female mice had lower LH serum levels and impaired estrogen synthesis than wild-types (Garris et al., 2005; Tu et al., 2018). Interestingly, a selected ablation of the *lepr* gene in the gonadotropes resulted in reduced fertility in females, while no effect was observed in males (Akhter et al., 2014). *lepr* mutant females had lower mRNA levels of activin, an important regulator of FSH in mammals (Bilezikjian and Vale, 2011), as well as lower GnRHR protein levels, but not mRNA expression (Akhter et al., 2014). Based on these results, Odle et al. (2018) suggested that leptin might act on the pituitary either on a transcriptional level for the regulation of activin or on a post-transcriptional level for the regulation of the GnRHR protein, controlling thus partly in both ways the mammalian female reproductive system. Even though we did not observe any differences in the GnRH system on transcriptional level (Fig. 4), it would be interesting to test possible post-transcriptional changes, as similar studies are not yet done in teleosts and the targets of leptin on the pituitary are currently unknown.

The histological analysis revealed that all the different follicular developmental stages were present in the ovaries of the *lepr* mutants, without differences between the ratios of each stage (Fig. 3), concluding that follicular development is not affected in the *lepr* mutant female zebrafish. In zebrafish, similarly to mammals (Kumar et al., 1997), follicular development is regulated via FSH signalling (Chu et al., 2015, Zhang et al., 2015a,b), but we did not see any significant differences in *fshb* (Fig. 4F) and *fshr* (Fig. 6A) expression. Similarly, to our results, oocyte growth was not affected in *lhb* deficient female zebrafish (Chu et al., 2014; Shang et al., 2019; Zhang et al., 2015b). In mammals, however, follicular development seems to be regulated by leptin: leptin administration enhanced folliculogenesis in mice (Barash et al., 1996) and rats (Elshafie et al., 2008), while folliculogenesis was impaired in the *ob/ob* mice (Hamm et al., 2004).

The surge of LH is responsible for oocyte maturation in almost all vertebrates. In fish, similarly to mammals, LH binds to its receptor (LHR) on the granulosa cells of the follicles, stimulating a cascade of events, leading to oocyte maturation and ovulation (Patiño and Sullivan, 2002). To understand the effect of impaired leptin signaling on these processes, we assessed a panel of genes involved in different paths of steroidogenesis, oocyte maturation and ovulation, both in whole ovarian tissue and in fully grown follicles.

Among the genes related to steroidogenesis, we found significant upregulation of both *star* and *hsd3b1* expression in the ovaries of the mutant fish (Fig. 6C–D), as well as *hsd3b1* upregulation in the fully grown follicles of the mutants which released eggs compared to those which did not (Fig. 6D). Shang et al. (2019) reported higher *hsd3b1* expression in fully grown follicles of *lhb* deficient zebrafish, but the expression of *star* was decreased. *star* is mainly expressed during vitellogenesis and *hsd3b1* during the primary growth stage in zebrafish, whereas both genes are almost undetectable in mature follicles (Ings and Van Der Kraak, 2006). Several studies show that *star* is under the control of LH in fish. In zebrafish, hCG treatment of vitellogenic follicles stimulated *star* mRNA expression (Ings and Van Der Kraak, 2006), while *lhb* deficiency resulted in lower *star* expression in fully grown follicles (Chu et al., 2014; Shang et al., 2019). In rainbow trout, incubation of vitellogenic follicles in the presence of LH had also stimulatory effect in the *star* transcripts (Nakamura et al., 2016). On the contrary, this does not seem to be the case for *hsd3b1*. In zebrafish, hCG treatment of vitellogenic follicles had no effect on *hsd3b1* mRNA expression (Ings and Van Der Kraak, 2006) and similar results were also reported by Nakamura et al. (2016), after incubation of vitellogenic follicles of rainbow trout in the presence of LH. Very low expression of the aromatase *cyp19a1a*, a key enzyme in estrogen synthesis, was also observed in fully grown follicles of wild-types. This could indicate a shift in the steroidogenic pathway from E2 to DHP synthesis, which occurs immediately prior to oocyte maturation (Nagahama and Yamashita, 2008). However, in mutants which do not lay eggs, *cyp19a1a* expression was dramatically higher (Fig. 6E), suggesting that in those fish the shift to DHP has not occurred, which could also explain their subfertility.

The action of DHP can be mediated by the activation of intracellular signaling pathways, involving non-genomic membrane receptors (Li and Ge, 2020), such as the progesterone membrane receptors (mPRs) and the progesterone receptor membrane components (Pgrmcs) (Zhu et al., 2003, 2008) In the present study, significantly lower *pgrmc2* (Fig. 6J) and lower, even though not significant, *mprb* expression (Fig. 6H) was observed in the ovaries from the mutant females, which could cause impaired fertility. Zebrafish lacking *mPRa1*, *mPRa2*, *mPRb*, and *mPRγ2* paralogs had impaired oocyte maturation, while both oocyte maturation and ovulation were affected in a zebrafish line lacking all seven mPRs genes (Wu et al., 2020). Moreover, subfertility and reduced progestin synthesis was also observed in *pgrmc1* and *pgrmc2* knockout female zebrafish (Wu et al., 2018, 2019), as well as in double knockout *pgrmc1/2* female zebrafish (Wu and Zhu, 2020). Lower expression levels in *pgrmc2* and *mprb* could also be a result of lower LH signalling, suggesting a possible regulatory link between those genes. In zebrafish, Yumnamcha et al. (2017) observed similar expression patterns between *lhb* and *mprb* during the circadian cycle, while in humans, *lhb* and *pgrmc2* were positively correlated in women with poor ovarian response (Vaitsopoulou et al., 2021).

The action of DHP can also be directly mediated by the activation of the nuclear progesterone receptor (pgr), an important factor for LH-dependent ovulation in mammals (Akison and Robker, 2012; Kim et al., 2009; Gal et al., 2016). In zebrafish, *pgr* is expressed in the follicular layer cells, which surround the oocytes (Hanna et al., 2010) and *pgr* mutant females are infertile, due to their failure to ovulate (Zhu et al., 2015; Tang et al., 2016), suggesting a conserved role of pgr in ovulation in fish as well. In the present study, the transcripts of *pgr* were significantly higher in the ovaries of the mutant females (Fig. 6K). One explanation could be that the upregulation of the *pgr* transcripts might act as a downstream compensatory response to the lower pituitary levels of *lhb* (Trudeau, 2018). Another explanation could be that this upregulation could also work as a compensatory response to the lack of leptin signaling. Recent data in mice showed that short-term leptin administration can upregulate *pgr* transcripts in the uterine tissue of ovariectomized mice, while long-term administration did not have such an effect (Shetty et al., 2020; Shetty and Suresh, 2021). However, a direct link between leptin and pgr is still unknown both in mammals and fish.

Higher expression levels in the ovaries of the mutant females were also seen for the prostaglandin E2 receptor EP4b (*ptger4b*) (Fig. 6O) and the cytosolic phospholipase A2 (*cpla2*) (Fig. 6V). Both genes are upregulated as the time when ovulation approaches and are involved in ovulation in teleosts, including zebrafish (Lister and Van Der Kraak, 2009; Fujimori et al., 2012; Tang et al., 2018, Baker and Van der Kraak, 2019). Moreover, in zebrafish, *ptger4b* and *cpla2* expression are dependent by *pgr*, under the regulation LH-signaling (Tang et al., 2016) and this could explain their upregulation in the mutant ovaries too. The link between LH, *pgr* and *ptger4b* seems also to be conserved among teleosts, as *in vitro* incubation of pre-ovulatory follicles with recombinant leptin induced the mRNA expression of both *pgr* and *ptger4b* transcripts in medaka (Hagiwara et al., 2014).

The matrix metalloproteinases (mmps) play also an important role not only in mammalian ovulation (Hägglund et al., 1999), but also in medaka (Ogiwara et al., 2005). Among mmps, only a significant upregulation of *mmp15a* was seen in the ovaries of the mutant group (Fig. 6S). As far, as to our knowledge, there are currently no studies linking mmps neither to leptin nor to LH in teleosts. However, in

Table 1
A summary of the main findings of the present study. Impaired leptin signaling resulted in significant upregulation (↑) or downregulation (↓) of genes related to steroidogenesis, oocyte maturation and ovulation in the pituitary, the ovaries and the fully grown follicles of *lepr*−/− females comparing to wild-types.

Tissue	Gene	Effect	Role
Pituitary	*lhb*	↓	oocyte maturation and ovulation
Ovary	*star*	↑	steroidogenesis
	hsd3b1		
	pgrmc2	↓	oocyte maturation – actions of DHP
	pgr	↑	
	ptger4b	↑	
	mmp15a	↑	ovulation – follicular rupture
	cpla2		
	ybx1	↓	egg quality
Fully grown follicles	*cyp19a1a*	↑	steroidogenesis

mammals, the link between LH and mmps looks controversial. mRNA expression of *mmp1* and *mmp9* were elevated in response to LH exposure *in vitro* in monkey granulosa cells (Duffy and Stouffer, 2003), while in primary mouse granulosa cells LH treatment had no effect in any of the mRNAs of the mmps studied, including *mmp2* and *mmp9* (Light and Hammes, 2015).

In addition, all isoforms of the cyclooxygenases (*ptgs1*, *ptgs2a* and *ptgs2b*), the enzymes which catalyse the synthesis of prostaglandins, as well as two types of disintegrins and metallopeptidases with thrombospondin (*adamts1* and *adamts9*), were also analysed, since they were suggested as possible regulators of ovulation in zebrafish (Tang et al., 2017; Liu et al., 2018,2020). However, no significant differences in their expression were observed between the two genotypes (Fig. 6T–U). Lastly, *ybx1*, the gene encoding the Y-box binding proteins (Zaucker et al., 2018), is essential for embryonic development (Kumari et al., 2013; Yang et al., 2019, Huang et al., 2021) and oocyte maturation in zebrafish, ensuring a good egg quality (Sun et al., 2018). In the present study, *ybx1* transcripts in *lepr* mutant ovaries were significantly lower than the control group (Fig. 6W), supporting thus the speculations for lower egg quality within the mutant group.

To summarize, genes related to steroidogenesis and ovulation and known to be under the regulation of LH in fish were either up- (*cpla2*, *hsd3b1*, *pgr*, *ptger4b*, *star*) or down-regulated (*mprb* and *pgrmc2*) in the ovaries of *lepr* mutant females (Table 1). However, no differences in the expression of those genes were seen in fully grown follicles between the two genotypes. In zebrafish, which are asynchronous spawners (Selman et al., 1993), gene expression in the entire gonads reflects changes related to several developmental stages, depending on the number of follicles from each stage in the gonads at the time of sampling. Therefore, targeted gene expression analysis of those genes in each developmental stage of follicular development in zebrafish might unmask those differences hidden behind the entire gonadal tissue.

5. Conclusions

In conclusion, our study showed that impaired leptin signaling resulted in severe reproductive deficiencies in female zebrafish. The findings that *lepr* mutant females had lower *lhb* expression compared to wild-type females argue that leptin could play a role in regulating LH at the pituitary level, as also seen in other vertebrates. This assumption is further supported by the findings that impaired ovulation in mutant females could be rescued by administration of hCG. Lower LH levels, as a result of impaired leptin signaling, would lead to a similar phenotype as observed in the *lhb* knockdown zebrafish (Chu et al., 2014; Zhang et al., 2015b; Shang et al., 2019), in which folliculogenesis is normal, but oocytes fail to mature and ovulate from the ovaries. In addition, several genes related to steroidogenesis, oocyte maturation and ovulation, which are under the regulation of LH in teleosts, were differentially expressed in the gonads of *lepr* mutant females. To our knowledge, this is the first *in vivo* study, linking leptin to reproductive deficiencies in a teleost species. However, our understanding of the role of leptin in fish reproduction is still limited. Considering the fact that both leptin and its receptor are expressed at the gonad level, the leptin system could also be directly involved in regulation of ovulation. So far, direct effects of impaired leptin signaling at the gonad level in teleosts remain unknown. A comprehensive transcriptome analysis of the different stages of follicular development from wild-type and *lepr* mutant zebrafish could provide more insights into possible mechanisms, resulting in this interesting phenotype.

Author contributions

All authors contributed to the study. Emmanouil Tsakoumis and Monika Schmitz conceived and designed the experiments. Emmanouil Tsakoumis and Ehsan Pashay Ahi collected and analysed the data. Emmanouil Tsakoumis and Monika Schmitz wrote the manuscript. All authors read, commented and approved the final manuscript.

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CRediT authorship contribution statement

Emmanouil Tsakoumis: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, preparation All authors read, commented and approved the final manuscript. **Ehsan Pashay Ahi:** Investigation, Writing – review & editing, All authors read, commented and approved the final manuscript. **Monika Schmitz:** Conceptualization, Writing – review & editing, Supervision, All authors read, commented and approved the final manuscript.

Declaration of competing interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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