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2022-09-01

Pashay Ahi , E , Sinclair-Waters , M , Moustakas-Verho , J E , Jansouz , S & Primmer , C 2022 , ' Strong regulatory effects of vgll3 genotype on reproductive axis gene expression in juvenile male Atlantic salmon ' , General and Comparative Endocrinology , vol. 325 , 114055 . https://doi.org/10.1016/j.ygcen.2022.114055

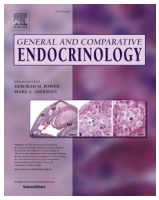
http://hdl.handle.net/10138/345106
https://doi.org/10.1016/j.ygcen.2022.114055

General and Comparative Endocrinology 325 (2022) 114055

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Short Communication

Strong regulatory effects of *vgll3* genotype on reproductive axis gene expression in juvenile male Atlantic salmon

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ARTICLE INFO

Keywords:
Gonadotropins
Gene expression
Atlantic salmon
Maturation timing
Vgll3

ABSTRACT

Age at maturity is a major contributor to the diversity of life history strategies in organisms. The process of maturation is influenced by both genetics and the environment, and includes changes in levels of sex hormones and behavior, but the specific factors leading to variation in maturation timing are poorly understood. *gnrh1* regulates the transcription of gonadotropin genes at pubertal onset in many species, but this gene is lacking in certain teleost species including Atlantic salmon (*Salmo salar*), which raises the possibility of the involvement of other important regulatory factors during this process. Earlier research has reported a strong association of alternative alleles of the *vgll3* gene with maturation timing in Atlantic salmon, suggesting it as a potential candidate regulating reproductive axis genes. Here, we investigated the expression of reproductive axis genes in one-year-old Atlantic salmon males with immature gonads and different *vgll3* genotypes during the spawning period. We detected strong *vgll3* genotype-dependent differential expression of reproductive axis genes (such as *fshb*, *lhb*, *amh* and *igf3*) tested in the pituitary, and testis. In addition, we observed differential expression of *jun* (*ap1*) and *nr5a1b* (*sf1*), potential upstream regulators of gonadotropins in the pituitary, as well as *axin2, id3*, *insl3*, *itch*, *ptgs2a* and *ptger4b*, the downstream targets of *amh* and *igf3* in the testis. Hereby, we provide evidence of strong *vgll3* genotype-dependent transcriptional regulation of reproductive axis genes prior to sexual maturation and suggest alternative models for distinct actions of *vgll3* genotypes on the related molecular processes.

1. Introduction

The age at which an individual reproduces, its age at maturity, is a critical time-point in an organism's life history as it affects fitness traits including survival, and reproductive success (Mobley et al., 2021). There is considerable variation in maturation age both within and among species, and maturation timing thus contributes to the remarkable variation observed in the life history strategies of organisms (Healy et al., 2019). The process of maturation is influenced by both genetics and the environment, and includes changes in levels of sex hormones and behaviour (Varlinskaya et al., 2013), but the specific factors leading to variation in maturation timing are not well understood (Mobley et al., 2021).

The onset of puberty is associated with the expression of gonadotropins, i.e. follicle-stimulating hormone (*fshb*) and luteinizing hormone (*lhb*) in the pituitary, and the level of gonadotropin release triggers sexual maturation (Lethimonier et al., 2004). The major upstream regulator of *fshb* and *lhb* is gonadotropin releasing hormone (GnRH), encoded by *gnrh1* and expressed in the hypothalamus (Whitlock et al., 2019). Genomes of fishes of the family Salmonidae, that includes Atlantic salmon (*Salmo salar*), lack *gnrh1* and the compensatory role of other members of the *gnrh* family, *gnrh2* and *gnrh3*, in controlling puberty has remained an ongoing area of debate (Muñoz-Cueto et al., 2020; Whitlock et al., 2019). Functional studies in zebrafish, which also lack *gnrh1*, showed potential compensatory role of *gnrh3* in regulation of gonadotropins (Muñoz-Cueto et al., 2020). However the presence of potential upstream regulators of gonadotropin release other than the *gnrh* family which can control the transcription of both *fshb* and *lhb* has also been proposed (Jin and Yang, 2014; Muñoz-Cueto et al., 2020). In Atlantic salmon, the vestigial-like family member 3 gene (*vgll3*) is strongly associated with maturation timing in the wild in both sexes but also exhibits sex-specific maturation effects (Barson et al., 2015;

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https://doi.org/10.1016/j.ygcen.2022.114055
Received 30 August 2021; Received in revised form 7 May 2022; Accepted 12 May 2022
Available online 14 May 2022

Czorlich et al., 2018). This association between *vgll3* and maturation probability has been subsequently validated in one year-old males in common garden settings (Debes et al., 2021; Sinclair-Waters et al., 2021; Verta et al., 2020). This makes *vgll3* a potential candidate regulating reproductive axis genes, however, the molecular details of the action of *vgll3* on reproductive axis genes has remained unexplored. It has already been suggested that *vgll3* might affect gametogenesis by regulating cell fate commitment genes (Kjærner-Semb et al., 2018; Kurko et al., 2020), and its repression in salmon testis has been associated with induced onset of puberty (Kjærner-Semb et al., 2018; Verta et al., 2020). A recent study demonstrated that expression of alternative isoforms of *vgll3* is linked with the alleles associated with variation in age at maturity in one-year-old salmon males (Verta et al., 2020). The *vgll3* alleles previously associated with later and earlier maturation, *vgll3**L and vgll3*E, respectively, display *cis*-regulatory expression differences and *vgll3**L expresses a rare transcript isoform in the testis at the pre-pubertal stage. However, the effects of alternative *vgll3* alleles on gene expression of other reproductive axis genes have yet to be investigated.

To further explore the role of *vgll3* in sexual maturation, we examined the expression of reproductive axis genes in phenotypically immature one year old Atlantic salmon males with different *vgll3* genotypes during the spawning period. We first investigated the expression levels of nine reproductive axis genes including in the brain, pituitary, and testis of one-year-old phenotypically immature male Atlantic salmon with different *vgll3* genotypes. Moreover, to elucidate the mechanism by which *vgll3* regulates the expression of reproductive axis genes, we tested the expression level of other potential upstream regulators of *fshb* and *lhb* in the pituitary (Jin and Yang, 2014), and downstream targets of anti-Müllerian hormone (*amh*) and insulin-like growth factor-3 (*igf3*) in the testis (Morais et al., 2017).

2. Materials and methods

2.1. Animal material and genotyping

Atlantic salmon used for this study were a subset of individuals used in Sinclair-Waters et al., 2021. Individuals used for this study were selected from the males of a single family (family "FM_T7" in dryad dataset https://doi.org/10.5061/dryad.kwh70rz3v), average length 13.5 cm, average mass 30.4 gm) resulting from crossing unrelated parents (Kymijoki origin, F1 hatchery generation) with heterozygous (*vgll3*EL*) genotypes, thus enabling assessment of expression patterns of all *vgll3* genotypes within a similar genetic background. Offspring *vgll3* genotypes were determined as described in Sinclair-Waters et al 2021. Phenotypically immature individuals were selected randomly until all three genotypes were equally represented. Sex and maturation status were determined phenotypically, by dissection of euthanized individuals and subsequent checking for the presence of female or male gonads as outlined in Verta et al. (2020). GSI was measured for a sub-set of fish whose maturation status had been subjectively categorized on a scale of 1 (little or no phenotypic signs of testes maturation) to 4 (large gonads leaking milt). The GSI for individuals in the immature category (1) ranged from 0.0 to 0.05 whereas the GSI for mature individuals (3 or 4) was 100 times higher 4.7–5.6. All the males used in the current study were classified as being stage 1 i.e. immature with slim testis and the term "phenotypically immature" used here refers to males with testes visually observed to belong to class '1'. Phenotype and genotype details of each individual in the family are reported in (Supplementary data).

2.2. Sample collection, RNA extraction and cDNA synthesis

Tissue sampling took place approximately one-year post-fertilization. Salmon were euthanized with anesthetic overdose (buffered tricaine methanesulfonate (MS-222)) and the brain, pituitary, and testes dissected immediately from phenotypically immature males (10 *vgll3*EE,* 16 *vgll3*EL*, and 8 *vgll3*LL* individuals) using fine forceps. Tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until extraction. Dissected tissues were carefully homogenized using the using a Bullet Blender (Next Advance). We extracted RNA from the sampled brain and testis using the NucleoSpin RNA extraction kit (Biotop), and pituitary using NucleoSpin RNA Clean-up XS (Biotop). The RNA pellets were dissolved in 50 µl of nuclease-free water (Ambion), and genomic DNA removed by incubation with DNaseI. The quality and quantity of RNA were assessed by NanoDrop 1000 v3.7 and Bioanalyzer (Agilent), respectively, and 500 ng of RNA per sample was used for cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen).

2.3. Candidate genes, designing primers and qPCR

We selected 9 candidate genes with a major role in regulating onset of reproduction, 5 genes upstream of *fshb* and *lhb*, and 6 genes downstream of *amh* and *igf3*, as well as 7 candidate reference genes (Supplementary data). Primer design was conducted as described by Ahi et al., 2019, using the Primer Express 3.0 (Applied Biosystems, CA, USA) and OligoAnalyzer 3.1 (Integrated DNA Technology) (Supplementary data) based on gene sequences retrieved from the annotated *Salmo salar* genome available in the Ensembl database, http://www.ensembl.org. The qPCR reactions were prepared as described by Ahi et al., 2019, using PowerUp SYBR Green Master Mix (Thermo Fischer Scientific), and were performed on a Bio-Rad CFX96 Touch Real Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The details of qPCR program and calculation of primer efficiencies are described by Lecaudey et al. (2019).

2.4. Analysis of gene expression data

We implemented two common algorithms to validate the most suitable reference gene(s); NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002). These algorithms use different analysis approaches to rank the most stably expressed reference genes. The geometric means of the Cq values of the top three ranked most stable reference genes across all individuals were used as normalization factors to calculate the expression levels of our target genes in pituitary (*Hprt1*, *Gapdh* and *Elf1a)* and testis (*Gapdh*, *Actb1* and *Hprt1*) (Table 1) using the following formula: $\Delta Cq_{\text{target}} = Cq_{\text{target}} - Cq_{\text{reference}}$. In brain, we used the expression of *Elf1a* as the normalization factor as it was already validated to be the most stable reference gene in this organ in Atlantic salmon (Olsvik et al., 2005). Within each tissue, a biological replicate with the lowest expression level for each gene (calibrator sample) was selected to calculate ΔΔCq values ($\Delta Cq_{\text{target}} - \Delta Cq_{\text{calibrator}}$). The relative expression quantities (RQ values) were calculated by $2^{-\Delta\Delta Cq}$, and their fold changes (logarithmic values of RQs) were used for statistical analysis (Pfaffl, 2001). The student *t*-test was applied for the direct comparisons of gene expression levels between the genotypes, followed by Benjamini-Hochberg correction for multiple comparisons.

3. Results

3.1. Expression pattern of reproductive axis genes

There were weak differences between Atlantic salmon individuals with different *vgll3* genotypes in brain expression levels of three *gnrh* family members. *gnrh2* expression was higher in *vgll3*EE* than in *vgll3*EL* and *vgll3*LL* individuals, but only significantly so compared to *vgll3*EL* (Fig. 1A). This suggests that the potential regulatory effects of *vgll3* on pituitary expression of gonadotropins may be partly mediated through differential regulation of *gnrh2*, but not *gnrh3a* and *gnrh3b*, which did not show any differences, at least at this developmental stage in males.

The most striking finding of this study was the remarkably higher expression levels of the gonadotropin encoding genes, *fshb* and *lhb*, in the pituitary of *vgll3*EE* individuals compared to the other two *vgll3*

Table 1
Expression stability ranking of reference genes in testis and pituitary of Atlantic salmon. Abbreviations: NE = Not expressed, SV = stability value, M = mean value of stability.

Pituitary				Testis			
geNorm		NormFinder		geNorm		NormFinder	
Ranking	M	Ranking	SV	Ranking	M	Ranking	SV
Hprt1	**0.780**	***Elf1a***	**0.216**	***Gapdh***	**1.021**	***Actb1***	**0.212**
Gapdh	**0.785**	***Gapdh***	**0.251**	***Actb1***	**1.043**	***Gapdh***	**0.349**
Elf1a	**0.788**	***Hprt1***	**0.266**	***Hprt1***	**1.107**	***Hprt1***	**0.390**
Rna18s	0.834	*Rna18s*	0.391	*Rps20*	1.117	*Rps20*	0.508
G6pd	0.940	*G6pd*	0.427	*Rna18s*	1.163	*Rna18s*	0.522
Rps20	1.050	*Rps20*	0.456	*Ef1a*	1.183	*Ef1a*	0.545
Actb1	1.666	*Actb1*	0.625	*G6pd*	NE	*G6pd*	NE

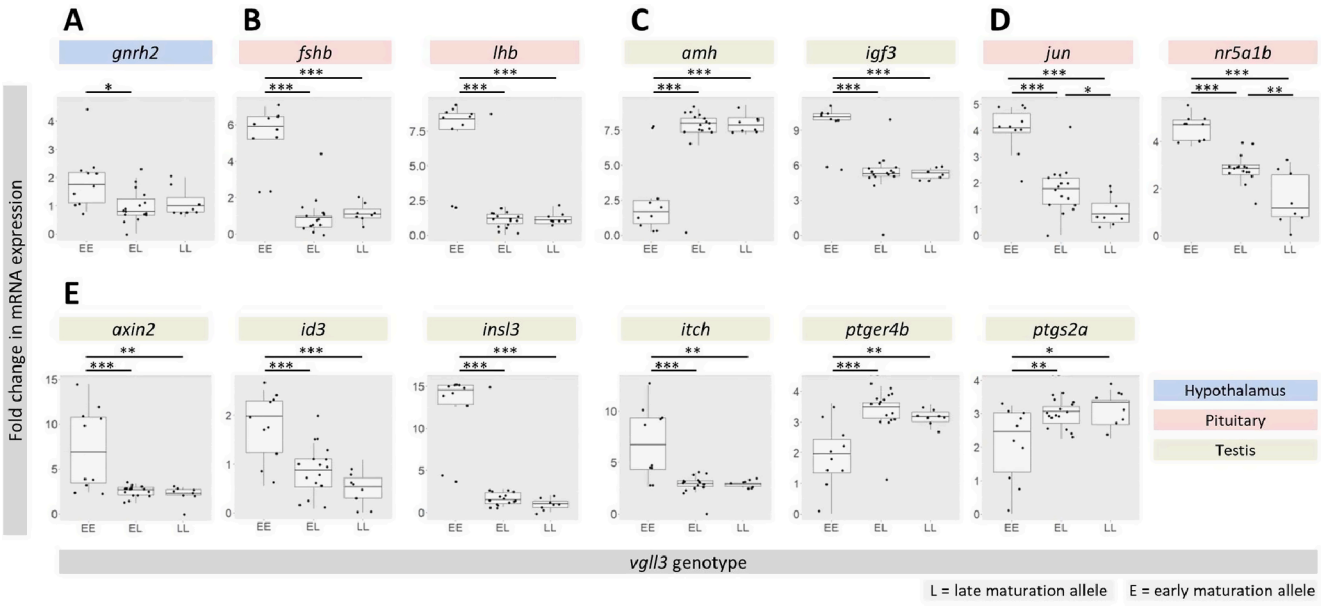

Fig. 1. Reproductive axis genes with significant differential expression in juvenile immature male Atlantic salmon with different *vgll3* genotypes. (A–C) Major reproductive axis genes with significant differences in expression levels in brain, pituitary and testis. **(D)** Differences in pituitary expression levels of transcription factors at upstream of *fshb* and *lhb*. **(E)** Testis expression levels of downstream targets of *amh* and *igf3*. Small dots indicate individual expression levels, the middle line represents the median, the box indicates the 25/75 percentiles and the whiskers the 5/95 percentiles for each plot. Asterisks above each plot indicate the level of significance for differential expression (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

genotypes (Fig. 1B). This indicates that the induction of gonadotropins has been initiated in the pituitary of *vgll3*EE* males during the reproductive season despite them being phenotypically immature.

In testis, we observed higher expression of *igf3* and lower expression of *amh* in *vgll3*EE* individuals compared to the two other genotypes (Fig. 1C). However, we did not find any differential expression in testis between the genotypes for the receptors of gonadotropins, *fshr* and *lhr* (Supplementary data).

3.2. Expression pattern of selected upstream and downstream genes

We tested the expression of five candidate upstream regulators which have been suggested to bind to promoters of both *fshb* and *lhb* across different groups of vertebrates (Jin and Yang, 2014). Differential expression between *vgll3* genotypes was detected for *jun* (*ap1*) and *nr5a1b* (*sf1*): the highest expression level was seen in *vgll3*EE* individuals, intermediate in *vgll3*EL* and the lowest expression level in *vgll3*LL* (Fig. 1D). *vgll3*-related expression differences were not detected for *pitx1* and *foxo1a* (Supplementary data), and the expression levels of *foxp3b* and *nr5a1a* were below the qPCR detection threshold. This suggests a potential direct regulatory link between the *vgll3* alleles and *jun* and *nr5a1b* in the pituitary of male salmon.

We further tested the expression of five known downstream targets of *amh* and a downstream target of *igf3* in testis. The expression level of *vgll3*EE* individuals differed significantly from the other genotypes in all of these genes. *axin2*, a downstream target of *igf3*, showed a similar expression pattern to *igf3* with significantly higher expression in *vgll3*EE* individuals than the other genotypes (Fig. 1E). Among the *amh* downstream targets, two genes, *ptgs2a* and *ptger4b*, showed lower, and three genes, *id3*, *insl3* and *itch*, displayed higher expression levels in *vgll3*EE* individuals than the other two genotypes (Fig. 1E).

4. Discussion

Here, we report the differential expression of reproductive axis genes in the brain (*gnrh2*), pituitary (*fshb*, *lhb*, *jun*, and *nr5a1b*), and testis (*amh*, *igf3*, *axin2*, *id3*, *insl3*, *itch*, *ptger4b*, and *ptgs2a*) in phenotypically immature one year old Atlantic salmon males with different *vgll3* genotypes. We find significant differences in expression of these genes between *vgll3*EE* individuals compared to *vgll3*EL* and *vgll3*LL* individuals, suggesting that the regulation of these genes is *vgll3* allele-dependent.

The differential expression of *gnrh2* in the brain showed the weakest difference across genotypes, with other *gnrh* genes showing no

differences in expression. Although the receptor of *gnrh2* is also expressed in the pituitary of Atlantic salmon during maturation (Ciani et al., 2020), it is uncertain whether the weak changes in *gnrh2* expression can be responsible for the dramatic changes in expression of gonadotropins in this study, or whether other upstream player(s) are involved. However, dramatic changes in expression of gonadotropins may not necessarily lead to dramatic changes in their release. Further, it should be emphasized that *gnrh2* might bind to other gnrh receptors and expression changes of these receptors can be an interesting target of future studies. Therefore, we still cannot rule out the possibility of a regulatory connection between *vgll3* and *gnrh* genes in Atlantic salmon based solely on lack of strong expression differences, and future functional investigations are required to address this point.

We find higher expression of gonadotropins *fshb* and *lhb* in the pituitary of phenotypically immature *vgll3*EE* individuals compared to *vgll3*EL* and *vgll3*LL* individuals. Consistent with this, we find that each additional copy of the *vgll3*E* allele increased expression of *jun* and *nr5a1b*, candidate upstream regulators of *fshb* and *lhb* (Jin and Yang, 2014), in the pituitary of phenotypically immature individuals with *vgll3*EE* individuals having the highest, *vgll3*EL* individuals intermediate, and *vgll3*LL* individuals the lowest relative expression of these genes.

amh is a crucial factor in maintaining spermatogonia in an undifferentiated state (Pfennig et al., 2015), whereas upregulation of *igf3* stimulates spermatogonial proliferation and differentiation (Morais et al., 2017). Intriguingly, expression of *amh* was lower and *igf3* higher in the testis of phenotypically immature *vgll3*EE* individuals as compared to the testis of phenotypically immature *vgll3*EL* and *vgll3*LL* individuals. This is likely due to higher expression of *fshb* of *vgll3*EE* individuals, and potentially the essential higher level of release of Fsh from the pituitary (although not investigated here), since *fshb* induces *igf3* and inhibits *amh* expression, which can lead to the onset of maturation (Li et al., 2021). The inhibitory effects of *amh* on spermatogonial differentiation and the stimulatory effects of *igf3* may be mediated by differential regulation of their downstream targets, including *id3*, *insl3*, *itch*, *ptgs2a*, *ptger4b*, and *axin2* in testis.

Insulin-like peptide 3 gene, *inls3*, is expressed in Leydig cells in the testis, and it has been shown in zebrafish that *insl3* promotes spermatogonial differentiation and mediates the stimulatory effect of *fshb* on spermatogenesis (Assis et al., 2016). Moreover, Sertoli cell-derived amh inhibits spermatogonial differentiation partly through reducing the expression levels of *insl3* in Leydig cells in adult zebrafish (Skaar et al., 2011). Consistently, we found reduced expression of *insl3* in testis accompanied with increased expression level of *amh* in *vgll3*LL* individuals of phenotypically immature Atlantic salmon. This suggests *amh* dependent *insl3* inhibition as a potential molecular mechanism by which spermatogonial differentiation could be delayed in *vgll3*LL* individuals.

Another feasible scenario for inhibition of spermatogonial differentiation in *vgll3*LL* individuals could be the increased expression of *ptgs2a* and *ptger4b*, which are known to be transcriptionally induced by *amh* in fish testis (Morais et al., 2017). *ptgs2a* encodes a key enzyme involved in production of a prostaglandin (PGE_2), which reduces the mitotic activity of differentiating spermatogonia, and thereby inhibits spermatogenesis (Crespo et al., 2020). The inhibition of PGE_2 on the development of spermatogonia seems to be mediated through a receptor encoded by *ptger4b* expressed mainly by testicular somatic cells (Crespo et al., 2020). Taken together, we suggest that in salmon with the *vgll3*L* allele, sexual maturation may be delayed through an *amh* dependent mechanism of inhibition of spermatogenesis (summarized in Fig. 2A).

The gene expression results were more complicated to interpret in the phenotypically immature *vgll3*EE* salmon, since both gonadotropins

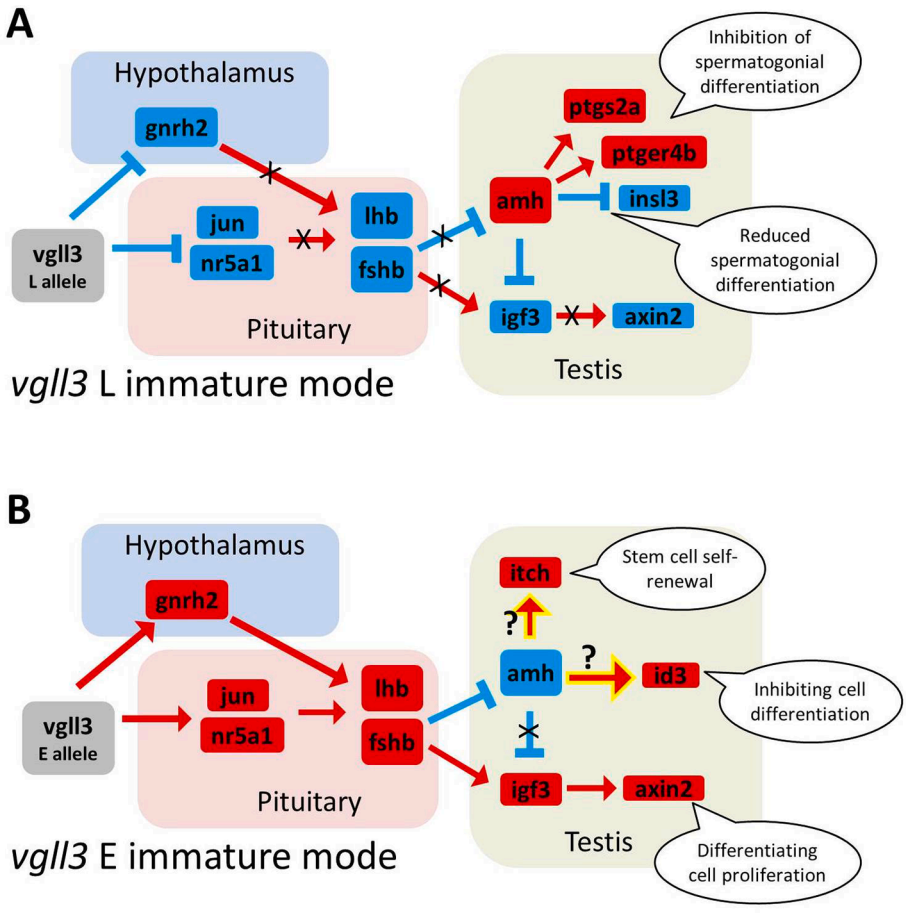

Fig. 2. Models for alternative modes of potential *vgll3*-allele dependent regulatory interactions between the differentially expressed genes along the reproductive axis in premature male Atlantic salmon. Red arrows indicate transcriptional induction, blue blocked lines represent transcriptional inhibition and black crosses display expected inhibition of transcriptional regulatory connections, whereas question marks on the highlighted arrows indicate unexpected regulatory outcomes via currently unknown molecular mechanism(s). We hypothesize that the *vgll3**L allele promotes the premature state in the testis via inhibition of spermatogonia differentiation, whereas the premature state in *vgll3*EE* individuals results from cell fate commitment prevention and thus the maintenance of pluripotency in the testis via an unknown *amh* independent mechanism.

had much higher expression levels in pituitary than the other *vgll3* genotypes as well as higher expression of *igf3* and lower expression of *amh* in testis. These expression patterns are reminiscent of the mature state (Morais et al., 2017); however, all these individuals, sampled during the spawning period, were immature. This indicates that although these molecular changes were consistent with promotion of maturation, it appears they were not sufficient for inducing maturation in these individuals in time for the spawning season. Previous studies showed that male Atlantic salmon entering maturation can show transcriptional changes along the reproductive axis while their testis are still phenotypically immature (Crespo et al., 2019; Schulz et al., 2019). Therefore, although the males in this study had visually immature testis, the transcriptional changes along the reproductive axis could indicate entrance to maturation already at molecular level (Crespo et al., 2019; Skaftnesmo et al., 2017). We find this unlikely in this experiment however as other research with families created using the same salmon broodstock, conducted in the same facilities and in similar conditions resulted in clearly phenotypically distinguishable mature vs immature individuals, with a significant association between mature parr maturation and the *vgll3*E* allele (Debes et al., 2021; Sinclair-Waters et al., 2021). This suggests that males able to mature during the spawning season in this experiment should already have done so. Below we provide a scenario that may explain this.

In *vgll3*EE* fish, the increased expression of gonadotropins was accompanied by increased expression of *igf3*, a growth factor with gonad specific expression in teleost fish, which is induced by *fshb* and mediates its effects on testis (Li et al., 2021). Moreover, the increased expression of *axin2*, a downstream target of Igf3 involved in proliferation of undifferentiated spermatogonia (Safian et al., 2018), indicates functional activity of Igf3 in the testis of *vgll3*EE* fish. In contrast, the reduced expression of *amh* was not accompanied by similar changes in two of its downstream effectors, *itch* and *id3*, in *vgll3*EE* fish testis. This might explain why these individuals exhibited no visually mature testes, since both *itch* and *id3* are known to inhibit cell fate commitment by controlling the maintenance of self-renewal, pluripotency and the undifferentiated state of progenitor cells (Morais et al., 2017) (summarized in Fig. 2B) although it should be recognized that alternative options exist. Why the reduced expression of *amh* did not result in decreased expression of *itch* and *id3* in *vgll3*EE* fish thus requires further investigations. This might be due to the presence of other unknown molecular gatekeepers acting independently, as well as other potential regulatory scenarios affecting this process, such as problems with the release of growth factors, expression of binding proteins neutralizing growth factors and insufficient expression of growth factor receptors. To summarize, the *late vgll3* allele appears to promote the premature state in the testis via inhibition of spermatogonia differentiation, whereas the premature state in *vgll3*EE* individuals might be a result of cell fate commitment prevention and thus the maintenance of pluripotency in the testis via an unknown *amh* independent mechanism (Fig. 2). Therefore, the effects of the *early vgll3* allele alone on testicular gene expression pattern appeared to be insufficient to trigger testis maturation at this stage in juvenile Atlantic salmon.

5. Funding source

Funding was provided by Academy of Finland (grant numbers 307593, 302,873 and 327255), the European Research Council under the European Articles Union's Horizon 2020 research and innovation program (grant no. 742312) and a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship.

Ethical approval

Animal experimentation followed European Union Directive 2010/63/EU under license ESAVI/42575/2019 granted by the Animal Experiment Board in Finland (ELLA).

Declaration of Competing Interest

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.

Data availability.

Supplementary file containing raw expression data and primers information.

Acknowledgements

We thank the funders of this project; Academy of Finland (grant numbers 307593, 302873 and 327255), the European Research Council under the European Articles Union's Horizon 2020 research and innovation program (grant no. 742312) and a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship. We acknowledge Jaakko Erkinaro and staff at the Natural Resources Institute Finland (Luke) hatchery in Laukaa and members of the Evolution, Conservation and Genomics research group for their help in coordinating and collecting gametes for crosses. We thank Jukka-Pekka Verta and Iikki Donner for valuable discussions. We also thank Nikolai Piavchenko for help with fish husbandry as well as Iikki Donner, Seija Tillanen and Annukka Ruokolainen for laboratory assistance and several anonymous reviewers for constructive comments on earlier manuscript versions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2022.114055.

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