UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

Inhibition of cortisol signaling and development of the hypothalamus-pituitary-interrenal axis (HPI-axis) during early ontogeny of Atlantic salmon (*Salmo salar L.)*

Therese Johanna Hovin Røtterud

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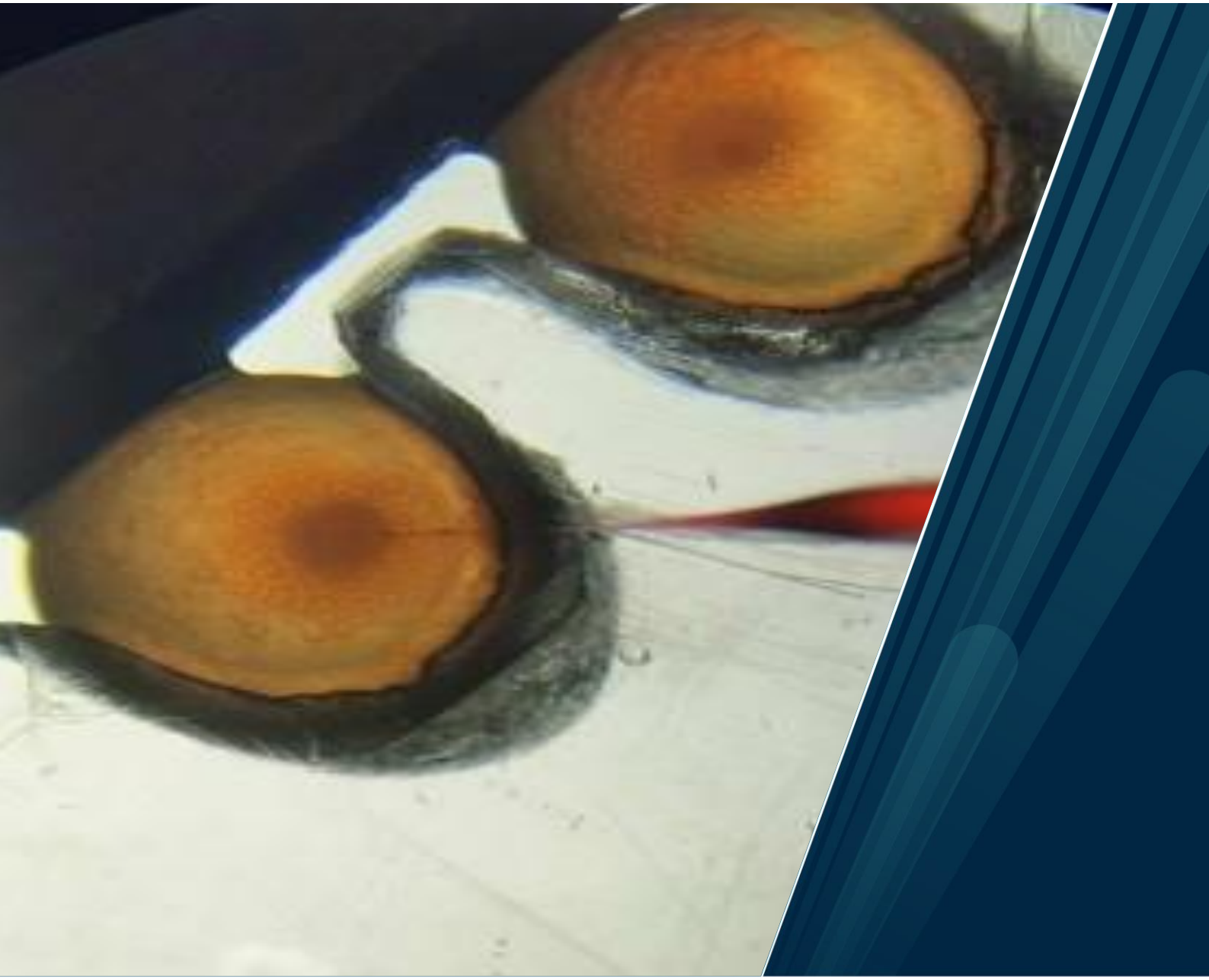

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Abstract

During early development much of the fish's physiological capacity and fitness is established. Still, our understanding of how the early development (endogenous and external) communicate with and influence embryonic development in fish is limited. This study investigated how signaling of maternal cortisol may influence early embryogenesis, with particular focus on the development of the hypothalamus-pituitary-interrenal (HPI) axis in Atlantic salmon (S*almo salar L.*). In this study maternal cortisol signaling was investigated through two approaches: 1. knockdown (KD) of glucocorticoid receptors (GR) using antisense oligonucleotides (GAPmers) and 2. inhibitors to block GRs and mineralocorticoid receptors (MR), both alone and in combination. Expression of genes critical to the HPI-axis: *gr1a, gr1b, gr2, pomca* and *pomcb* were measured using quantitative polymerase chain reactions (qPCR) and analyzed for possible effects on the timing of the HPI-axis. Embryo morphology was recorded from photos during important life stages: cell cleavage, mid-blastula, 50 % epiboly, 95 % epiboly, "eyed" stage and at hatching. GAPmer treatment resulted in significant reduction in *gr1b* expression during cell cleavage indicating successful KD of the GR1b receptor. From 50 % epiboly until hatch GAPmer treated embryos had generally lower gene expression than controls, significantly for *gr1a.* GAPmer injected embryos showed a delay or stop in development during gastrulation and appeared to be slightly larger in size, but with rounder and smaller yolk sacs at hatching. Embryos treated with inhibitors were smaller but had larger yolk sacs. Overall, gene expression matched the ontology based on other species. Use of GR and MR inhibitors resulted in few consistent effects except for the MR inhibitor which significantly elevated *pomca* expression from ca. 95 % epiboly until hatch. Results obtained in this study indicate that some important regulators of the HPI-axis may be affected by inhibited cortisol signaling during early development. Cortisol signaling may also influence early embryo morphology through effects of yolk allocation. To clarify if the observed changes result in altered HPI-axis functionality, further research is needed.

Abbreviations

18S	Sal_18S
actin	Beta actin (β-actin)
Ct	Cycle threshold
dd	Day degrees
EF1	Elongationfactor (Ef1-α)
GAPmers	antisense oligonucleotides
GC	Glucocorticoids
GR	Glucocorticoid receptor
HPI-axis	Hypothalamus-pituitary-interrenal axis
KD	Knockdown
MR	Mineralcorticoid receptor
mRNA	messenger ribonucleic acid
MZT	maternal to zygotic transition
POMC	Proopiomelanocortin
qPCR	Quantitative polymerase chain reaction

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1 Introduction

In recent years, the salmon aquaculture industry has expanded and developed (FAO, 2022). Although, awareness regarding the understanding of fish physiology and biology has increased and garnered more attention, we are far from understanding how the culture environment, from broodfish to the sea cage, may influence the physiology and production performance of the fish used in salmon aquaculture today. Still, for more than 10 years, the Norwegian salmon aquaculture industry have suffered annual losses of 16-20 % of the fish during the sea phase of the production cycle (Fiskeridirektoratet, 2023; Sommerset et al., 2024). High mortality and, consequently, reduced fish welfare is a result of different conditions for example pathogens and stressful handling, indicating that the "robustness" – the physiological capacity to maintain normal biological functions (Kitano, 2007) – of the fish is compromised, or inadequate, when exposed to the environmental conditions experienced under culture conditions. Similarly, losses during the hatchery/ freshwater phase of the production cycle reach to about 30 million individuals each year, excluding fish below 3 grams which makes up about 45 % of total mortality in this phase (Sommerset et al., 2024).

A thorough understanding of the entire lifecycle and how fish or fish species are influenced by environmental abiotic and biotic factors are crucial in obtaining good health and welfare under aquaculture conditions. In vertebrates, including fish, activation of the hypothalamus-pituitary-interrenal (HPI) axis and a systematic release of cortisol is a fundamental and well-conserved response to environmental perturbations, or stress (Nesan & Vijayan, 2013a) as well as under non-stress conditions (Bury & Sturm, 2007). A functional cortisol signaling pathway is established already at the earliest stages of development with cortisol (transferred from maternal plasma) and glucocorticoid receptors (GR), both as mRNAs and possibly proteins, being maternally deposited in the oocyte/ egg (Alsop & Vijayan, 2009; Nesan & Vijayan, 2013a). The influence of maternal cortisol on early development may have a substantial effect on embryonic and larval development however, has only been studied in a limited number of species, mostly the model zebrafish (*Danio rerio*) (Sopinka et al., 2017). The importance of maternal cortisol signaling in early development, especially in the establishment of the HPI-axis, but also for other endocrine systems that initiate their development during embryogenesis, requires further investigation, as it influences the foundation of the fish's physiology and biology.

Norway is the largest producer in the world of farmed Atlantic salmon (FAO, 2022), producing over 1500000 tons annually (Sommerset et al., 2024). In the industry, broodstock fish are stripped of eggs, which are fertilized with milt, and incubated until first exogenous feeding/ "start feeding". At approximately 330 day degrees (dd) (days from fertilization x water temperature) of development, at the "eyed stage", eggs are mechanically shocked, sorted, and shipped or transferred to hatcheries, where they are incubated until hatch. After hatch, yolk-sac larvae (alevins) absorb the yolk-sac, and initiate "start feeding" as fry, when most of the yolk sac has been consumed after ca. 850-900 dd. From fry, they develop to parr before completing smoltification becoming smolts (Sommerset et al., 2024). Smolts are then transferred to sea cages or to post-smolt facilities to grow until slaughter.

1.1 Stress response and Hypothalamus-pituitary-interrenal (HPI) axis.

In juvenile fish, the HPI-axis is activated as a response to stress to maintain homeostasis in teleost fish. The HPI-axis is regulated at the level of the hypothalamus, pituitary and interrenal tissue/cells in the head kidney, which work together in a biochemical cascade to produce cortisol (Sopinka et al., 2017). Firstly, a stressor is recognized by the hypothalamus that induces the release of corticotropin-releasing factor (CRF), also known as corticotropin-releasing hormone (CRH). Next, CRF moves to the pituitary gland through neurons. The distal cells in the pituitary gland then produce and secrete adrenocorticotropin hormone (ACTH), cleaved from the POMC protein encoded by the proopiomelanocortin gene (*pomc*) following translation (Nesan & Vijayan, 2016). ACTH is transported through the blood and binds to the receptors on interrenal cells, stimulating the synthesis and release of cortisol (Farrell, 2011). Cortisol modulates several physiological and metabolic functions by binding the ubiquitously expressed GRs, regulating processes during no-stress conditions and in the stress response. (Farrell, 2011).

In teleost fish, cortisol acts as the primary corticosteroid, binding to receptors, which work as transcription factors (Bury & Sturm, 2007). Cortisol binds to corticosteroid receptors (CS), including glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). When cortisol binds a receptor, a ligand-complex forms and the receptor is translocated to the nucleus, where the GR or MR recognize glucocorticoid response elements (GREs) placed upstream of target genes, which the receptor binds and enhance or inhibit gene transcription, changing gene expression (Bury & Sturm, 2007; Nesan & Vijayan, 2013a).

Glucocorticoid receptors (GR)

Teleosts have two GRs, GR1 and GR2, resulting from whole genomic duplication events 350 million years ago (MYA). More whole genome duplication events, including salmonids, have occurred since, at approximately 100 MYA, giving rise to gene duplications of GRs (Pasquier et al., 2016; Romero et al., 2020). In Atlantic salmon, there are identified two *gr1* genes (gr1a and gr1b) and two *gr2* genes (gr2a and gr2b) (Romero et al., 2020). Research has shown that the receptors have different affinities for cortisol, with GR2 having a significantly higher affinity for cortisol than GR1, indicating that GR1 receptors primarily function in the stress response when the cortisol levels rise, while GR2 aids in basal regulation under non-stress conditions (Bury & Sturm, 2007; Farrell, 2011). Cortisol influences many processes including growth, reproduction, osmoregulation and the immune system (Barton, 2002; Farrell, 2011).

Gene expression profiles during early ontogeny

In rainbow trout, unfertilized eggs contain maternal cortisol which is being metabolized towards the "eyed stage" (ca. 230-240 day degrees (dd) of development), where the storage becomes depleted. Shortly following hatching (ca. 350 dd), the cortisol levels increase towards start-feeding (Auperin & Geslin, 2008). In zebrafish, research has shown temporal changes in maternal *gr* expression, which decreases from fertilization towards the organogenesis (ca. 25 hpf) before it increases until hatching and stabilize approaching "start feeding" (Alsop & Vijayan, 2009). Additionally, it is shown in zebrafish that *pomc* linked to the HPI-axis is expressed from 24 hours post fertilization (hpf) and onwards (Hans-Martin & Matthias, 2007; Nesan & Vijayan, 2013a). Unpublished data on Atlantic salmon shows a corresponding cortisol profile. Furthermore, the gene expression profiles of *gr* and *pomc* in Atlantic salmon follows a similar trend to those observed in zebrafish. However, the decrease in gr expression occurs at the gastrula stage, while the pomc gene expression begins in the end of gastrulation (personal communication H. Tveiten: (Tveiten, 2022)).

1.2 Embryogenesis in Salmonids

An embryo is defined as "the early developing animal before it begins to look like the adult of the species" (Dye, 2012), in this thesis from the point of egg activation to start-feeding encompassing both the embryo and alevin period (Gorodilov, 1996). In this study, eggs were kept from fertilization to hatching during embryogenesis. Embryogenesis may be divided into

several stages of development advancing from fertilization: cleavage, gastrulation, and organogenesis (Danner, 2008).

Following the fertilization the cytoplasm gathers at the animal pole of the yolk and forms the blastodisc. If an egg is not fertilized but activated by water, it will also form a “blastodisc” known as a pseudocell, however development will stop at this stage (Danner, 2008). In the beginning, the embryos development is regulated by maternal factors in the egg, as the genome is not activated yet (Despic & Neugebauer, 2018). From the blastodisc more cells (blastomeres) are formed through meroblastic cell division in the cleavage stage (Danner, 2008). There are 11 synchronous cell cleavages until the early blastula (ca. 30 dd) (Gorodilov, 1996; Nagasawa et al., 2013). Cells will continue to divide and fill out the space of the blastodisc with smaller and smaller cells. During mid-late blastula (42-56 dd) the maternal-zygotic transition (MZT) begins (Jukam et al., 2017; Nagasawa et al., 2013; Pelegri, 2003). In this transition maternal RNA and factors are repressed and degraded by the help of micro RNAs, while the zygotic genome becomes activated (ZGA) and the embryo controls its own gene expression (Jukam et al., 2017; Lorenzo-Orts & Pauli, 2024).

Next in the development, the blastodisc will level out to cover the animal pole. Further, a thickened rim of blastomeres is formed, known as the germ ring. The germ ring overgrows and envelopes the egg yolk as the body is formed in a process known as epiboly (Danner, 2008). In the beginning of gastrulation (ca. 70 dd) (Musialak et al., 2023), at approximately 10 % epiboly, the axial structures of the embryo begin to form as cells migrate originating from the blastodisc ending in the terminal caudal bud. At 50 % epiboly (ca. 100 dd) involution begins and subsequently, the formation of endoderm and mesoderm occur (Gorodilov, 1996). Thereafter, tissues are formed from different specialized cells and make up the body (Danner, 2008). At 100 % epiboly the gastrulation has ended (ca. 135 dd) (Musialak et al., 2023). Within the span of gastrulation and parts of the organogenesis somitogenesis occur, formation of somatic pairs through a periodic division of the mesoderm axial bands (Gorodilov, 1996).

In the final complex stage of organogenesis (ca. 160 dd) (Nagasawa et al., 2013), vital organs develop. For instance, the kidney and the brain develops, the hindbrain, metencephalon and myelencephalon which gives rise to the medulla oblongata and cerebellum (Danner, 2008). In terms of the formation of the circulatory system, the heart begins to beat, pumping blood through the organs and the vascularized yolk. (ca. 160-297 dd) (Gorodilov, 1996; Musialak et al., 2023). The pituitary begins to develop at approximately 300 dd (Saga et al., 1993). The

hypothalamus, pituitary and kidney development during organogenesis is important for a properly functional HPI-axis. Additionally, research strongly suggests that the stress response and HPI-axis become functional right before start feeding even if all the components are developed in advance (Robinson et al., 2019; Terence et al., 1995).

1.3 Effects of cortisol signaling on embryonic development in Zebrafish

There are relatively few studies on Atlantic salmon (S*almo salar*) regarding signaling pathways or the influence of (maternal) cortisol during early development. Unlike the zebrafish, the Atlantic salmon is not a model fish but primarily serves as fish for consumption and has a more complicated lifecycle, being anadromous (Vøllestad & Halleraker, 2023), perhaps contributing to less research. Moreover, research regarding maternal cortisol in Atlantic salmon predominantly deals with elevated, or excess/pharmacological, cortisol levels (Eriksen et al., 2006). Additionally, Atlantic salmon has several GR genes compared to zebrafish which only has one (Nesan & Vijayan, 2013a; Romero et al., 2020).

Evidence from zebrafish reveals that maternal cortisol affects embryogenesis as it is a developmental regulator in fish (Nesan & Vijayan, 2013a), but may also affect stress reactivity, metabolism, and behavior in adult individuals. Through pharmacological, genetic, and environmental manipulation of maternal cortisol, scientists have found that cortisol changes the timing of organ development, the maturation of the HPI-axis, metabolism, fitness, and survival of larvae (Wilson et al., 2016). Results from previous studies indicate that the consequences depend on the type and dose (intensity/ concentration and duration) of the treatment. Often, higher the treatment dose, higher the significance (Wilson et al., 2016). To illustrate, when maternal cortisol bioavailability was inhibited in zebrafish, this resulted in deformed mesoderm structures and later increased the cortisol response in post-hatched larvae (Nesan & Vijayan, 2016). The study showed that the development and function, i.e. “programming”, of the HPI-axis is dependent on the proper signaling of maternal cortisol (Nesan & Vijayan, 2016). Evidence from Atlantic salmon report suggests that the stress during early development can cause permanent epigenetic effects like changes in DNA methylation (Robinson et al., 2019) but the mechanisms involved in this process is far from clear.

1.4 Experimental approaches

To investigate possible signaling of maternal cortisol in Atlantic salmon and its effect on early development, two different approaches were used:

1. Removal of GRs provided maternally as mRNAs using antisense oligonucleotides, called GAPmers (1.4.1)
2. Since GRs may also be maternally provided as proteins, GC inhibitors (GR and MR, alone and in combination) were also used (1.4.2)

Possible effects of GC inhibition on ontogenetic changes in morphology was recorded from photos of embryos at different stages of development, while expression of *gr1a, gr1b, gr2, pomca* and *pomcb* were used to analyze possible effects on the timing of HPI-axis development. Gene expression was measured using quantitative polymerase chain reaction also known as real-time qPCR or simply qPCR. B-actin (actin), elongation-factor 1 (EF1) and Sal_18S (18S) served as reference genes.

1.4.1 Antisense oligonucleotides - GAPmers

Antisense oligonucleotides (here, called GAPmers) were injected into Atlantic salmon eggs to knockdown (KD) maternal cortisol receptors (GR1a, GR1b and GR2) provided as mRNAs. GAPmers are single-stranded antisense oligonucleotides often consisting of 16 nucleotides. The GAPmers contain a central "gap" of 10 DNA molecules together with three RNA like locked nucleic acids (LNA) nucleotides in each flanking region to improve binding and stabilizing the molecule from degradation. In principle, injected GAPmers attach to a complementary sequence on the GR mRNA and form a RNA/DNA heteroduplex, recruiting RNase H to the complex. RNase H induces cleavage of the nucleotide strand, which is further degraded by exonucleases. After the degradation of a mRNA, the GAPmers attaches to another mRNA strand (QIAGEN). Different from other methods for manipulating gene expression, such as CRISPR Cas9, which induce DNA mutations, GAPmers target mature mRNA, disregarding the synthesis or processing of mRNA, making it a temporary method (Pauli et al., 2015).

1.4.2 Inhibitors

In addition to the GAPmer treatments, other groups of eggs were exposed to GC receptor inhibitors to prevent cortisol signaling in the developing embryo. The inhibitors used were eplerenone (E6657 (MR antagonist)) and mifepristone (M8046 (GR antagonist)) from Sigma

Aldrich, both acting as competitive antagonists. Antagonists binds (here reversibly) to receptors blocking other molecules inactivating the receptor (Brørs, 2018). Both inhibitors have been used previously in similar experiments with rainbow trout where eplerenone acts specifically with MRs (Kiilerich et al., 2015), while mifepristone binds GRs (Ferris et al., 2015).

1.5 Research question.

The works cited above strongly indicates that the HPI-axis in salmon develop during early ontogeny, and prior to start feeding. Researching the signaling of maternal cortisol in Atlantic salmon and its effect on embryogenesis and development on the HPI-axis by knockdown/ inhibition of the maternal cortisol receptors (GR/ MR) has not been previously done. Thus, this research could potentially contribute to understanding, the role of maternal cortisol and epigenetic mechanisms during embryogenesis. It may also serve as a foundation for further research. This thesis will focus on how reduced cortisol signaling during early ontogeny may alter expression of genes critical to the development and functionality of the HPI-axis. Such information may contribute to the understanding of how maternal cortisol regulates these genes, and possibly other genes related to early development in Atlantic salmon.

In the current study, attempts to reduce GC signaling was pursued through a combination of GR knockdown and GR and MR receptor antagonists, alone or in combination. Possible alterations in gene expression during development was monitored using quantitative polymerase chain reaction also known as real-time qPCR.

Subaims:

1) What are the influences of antisense oligonucleotide (GAPmer) treatment on GR gene expression during early development?
2) What are the influences of inhibited cortisol signaling during early ontogeny on expression of genes critical to the HPI-axis?
3) How do antisense oligonucleotide (GAPmer) and GR and MR-inhibitors influence embryonic phenotype?

2 Materials and methods

2.1 Experimental set-up

Salmon eggs were provided by AquaGen (https://aquagen.no/), a commercial breeding company of Atlantic Salmon. All eggs originated from one female and milt from one male. Eggs and milt were delivered on 11 October. The eggs were kept in an incubation cabinet at NFH, distributed between separate compartments based on treatment and time of fertilization (Figure 1). The incubation cabinet was left running two days before the egg's arrival. Tap water mainly at 4 °C (average: 4 °C, range 3-7°C) (Appendix: Table 9) running 10-12 L/ min, was filtered through five filters: a 50 µm filter, two charcoal filters, a 25 µm and a 10 µm mechanical polypropylene filter. For the initial two weeks, a 5 µm filter was used instead of a 10 µm filter. Charcoal filters were used to remove any chlorine from the tap-water, as tap-water may occasionally be disinfected by chlorine, while the mechanical filters prevented particles from entering the incubation cabinet. Water flow went through four egg holding "shelfs", top to bottom.

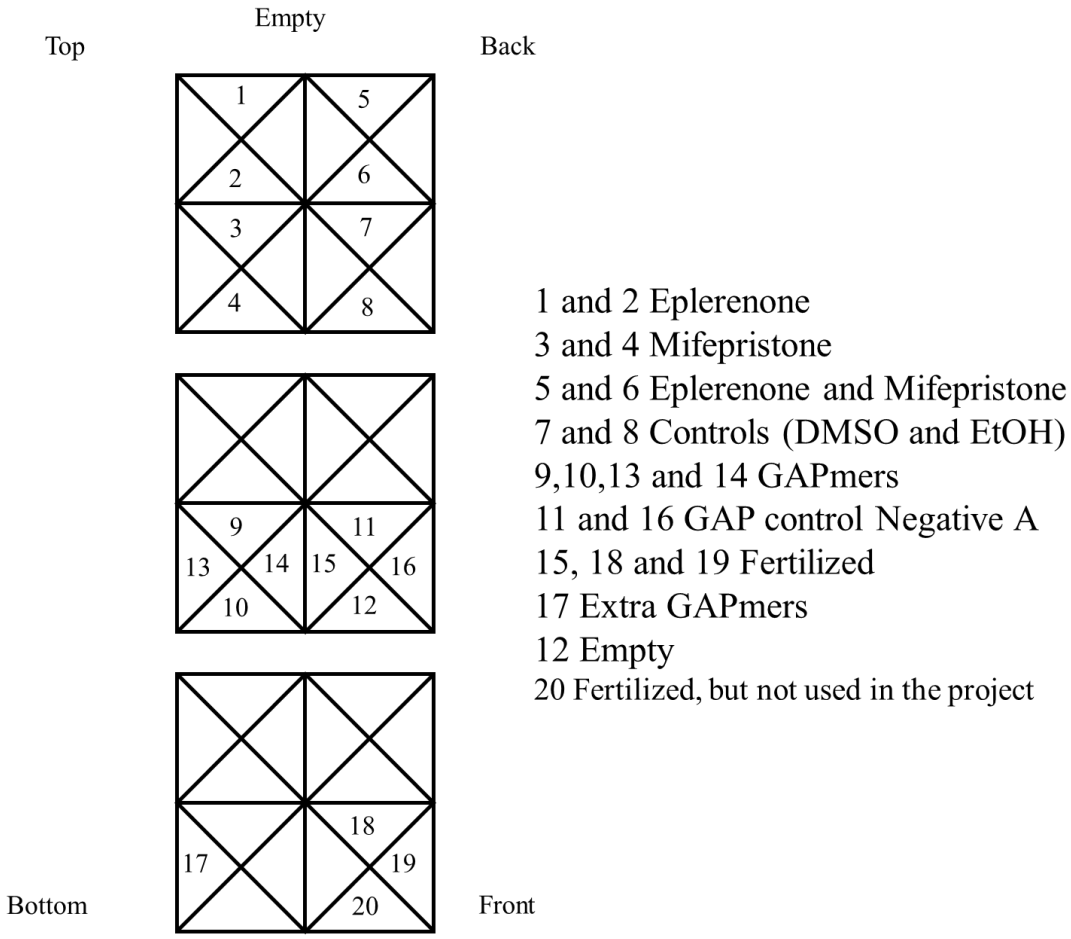

Figure 1 Setup in the incubation cabinet based in treatment and time of fertilization.

2.2 Inhibitors and GAPmers

To study the role of maternal cortisol signaling during salmon embryogenesis both GAPmers and inhibitors were utilized. GAPmers was used (Table 1) to KD GR1a, GR1b and GR2. Additional groups of eggs were incubated with the inhibitors, mifepristone (M8046, Sigma Aldrich) and eplerenone (E6657, Sigma Aldrich) inhibiting the GRs and MRs. Approximately 3000 eggs covered in ovarian fluid with a pH of ca. 8,2 and a temperature of ca. 3 °C were shipped overnight in a plastic bag from Kyrksæterøra, Møre og Romsdal. Eggs designated for microinjection were distributed into sip-lock plastic bags in single layer (approximately 120-140 eggs) and kept together with the milt in a temperature-controlled incubation cabinet at 3 °C until fertilization. All handling were completed in a cold room set to 7 °C before the eggs were transferred to the incubation cabinet at a water temperature of 7 °C (Appendix: Table 9).

Table 1 GAPmers designed and provided by Qiagen. Their catalogue number, product group and name, lot number and sequence.

Cat. No.	Product group	Product name	Lot number	Product sequence 5'-3'
339511 LG00834295-DDA	Antisense LNA™ GapmerR Standard (5)	SS_GR1AB_1_0	550596444	ACTCATTCAGGTCAGAC
339511 LG00834298-DDA	Antisense LNA™ GapmerR Standard (5)	SS_GR2_1_0	550596447	CGATGGCCTTTAGTAG
339515 LG00000002-DDA	Antisense LNA™ GapmerR Control (5)	Negative control A	40103305-1	AACACGTCTATACGC

Inhibitor incubation

In preparation for the egg arrival and treatments of the eggs, solutions: Cortland solution (for egg/ inhibitor incubation prior to fertilization), Activation fluid (standardized sperm activator which prevent egg activation), glutathione water (prevent egg hardening) and trout balanced salt solution (TBSS) (mimic intracellular ion composition and used for GAPmer dilution and injection) were made fresh 1-2 days prior to use by the lab engineer, see appendix for composition and preparation (Appendix: Preparation for egg arrival).

For this project, a total of 2985 eggs were used (Appendix: Table 13). Prior to incubation all eggs were washed three times in a Cortland salt solution (modified from (Kinkel et al., 2010; Perry et al., 1984)) with a pH of 8,5 and osmolality of 290 mOsmol to remove the viscous ovarian fluid. Eggs can be stored in Cortland salt solution to maintain egg quality for up to three days after stripping, being not different from storage in ovarian fluid (Moen, 2020).

2.2.1 Inhibitors and controls

The inhibitors used were mifepristone and eplerenone, which were dissolved and diluted for the incubation. Stock solutions of mifepristone was prepared with ethanol (10 mg/ml), while eplerenone was dissolved in dimethyl sulfoxide or DMSO (41641 Fluka) (4 mg/ml). Eight groups of approximately 200 unfertilized and washed eggs each, were placed into 600 ml beakers in a single layer and filled with 65 ml Cortland solution. Eplerenone was added in duplicates (groups 1 and 2), at a final concentration of 10^{-5} M and mifepristone was added to groups 3 and 4, creating a concentration of $1{,}18 \times 10^{-5}$ M. In groups 5 and 6, both eplerenone and mifepristone were added with the concentration of each inhibitor remaining the same. Groups 7 and 8, functioning as control groups received 67 µl of DMSO and 33 µl of ethanol. When all the inhibitors were added to their respective groups, the beakers were covered with aluminum foil and incubated for four hours at 7 °C. Following incubation, the eggs were fertilized.

Fertilization

After removal of the incubation media, eggs were fertilized using 50 mL activation fluid and 400 µl sperm (milt concentration ca. 40 x 10^6 per ml, sperm to egg ratio 200000:1). The activation fluid used for the sperm had a pH of 8 and an osmolality of 285 mOsm supporting high spermatozoan motility and fertilization without activating the cortical reaction and the swelling process (Kholodnyy et al., 2019). Use of activation fluid is industry standard. For fertilization, eggs were placed at the bottom of a 600 mL beaker. Into the eggs 400 µl sperm was added using a pipette and immediately after 50 mL of activation fluid. The contents were then mixed and left for two minutes. Next, the eggs were washed and placed in 300 mL filtered hatchery water in beakers covered with aluminum foil, which were left to swell undisturbed for two and a half hours. After the swelling, the eggs were transferred to the incubation cabinet. This method was repeated for all the inhibitor groups and controls. This method of fertilization was also used for the eggs being injected with GAPmers however, they were left in glutathione water while swelling and later before injection at 3 °C to prevent hardening of the chorion enabling the injection of eggs (Yoshizaki et al., 2005).

2.2.2 GAPmers

In this study a mixture of two different GAPmers was used (Table 1): One GAPmer was designed to KD both the *gr1* and *gr1b* mRNA while the second was designed to KD the *gr2* mRNA. The design was carried out by Quagen (Denmark) based on corresponding sequences retrieved from the salmon genome. The most likely GAPmers to induce KD of the GRs were chosen. See appendix for target sites (Appendix: Sequence 1, Sequence 2, Sequence 3). It is also important to note that the Atlantic salmon has two GR2-receptors and in this study only one GAPmer was used. As the student was initially not trained, egg injection was practiced beforehand to improve the execution of the project.

Injection tutorial

For practicing the injection method about 130 eggs for microinjection was fertilized as explained above. Here, only phenol red and TBSS were used as injecting agents. Phenol red ((1 % weight volume) in TBSS, pH 8) and TBSS were filtered through 0,2 µm filters (Acrodisc® Syringe Filter 0,2 µm Supor® Membrane Low Protein Binding Non-Pyrogenic) into Eppendorf tubes using a syringe.

A mixture of 9 µl of TBSS and 1 µl of phenol red were combined in an Eppendorf tube before being loaded into pre-made injection glass needles using a pipette equipped with a long thin pipette tip. The filled glass needle was then attached to the injector (pneumatic pico-pump), and the nitrogen opened. The eggs were placed in petri dishes with a mold, six at a time and oriented with the blastodisc/ first cell up. The petri dish containing the eggs was placed under the microscope, and the needle was adjusted (Figure 2). Eggs were injected at an angle of 20°.

GAPmers

TBSS was used to resuspend the GAPmers prior to the injection and should be compatible with the intracellular ion composition. Stock solutions (100 µM) were made by adding 50 µl sterile filtrated TBSS to 5 nmol of GR1AB, GR2 and a negative control GAP A, mixing well with a pipette. From the stock solutions, a "GAP mix" was made by combining the two different targeting GAPmers (5 µl each of GR1AB and GR2 into 40 µl sterile filtrated TBSS) to a concentration of 10 µM (total GAP concentration 20 µM). An injection needle was then filled with a mixture of 5 µl "GAP mix", 4 µl sterile filtrated TBSS and 1 µl phenol red giving a final concentration of 5 µM of the specific GAPmers and 0,1 % phenol red in the injected solution. To get the first blastodisc into an appropriate stage, that is, size or volume for injection the following morning, batches of eggs (ca. 130 eggs each) was fertilized in the evening and kept at 3 °C overnight before injections were carried out at 7 °C. Eggs or rather zygotes, were placed in a plastic mold and gently oriented with the blastodisc facing up and injected with 5-6 nl of the GAP solution described above (Figure 2). Injected eggs were placed in beakers with hatchery water and transferred to the incubation cabinet for further development. The same procedure as indicated above was used for the negative control GAP A. Eggs that were fertilized but not treated (only fertilized) were used as controls.

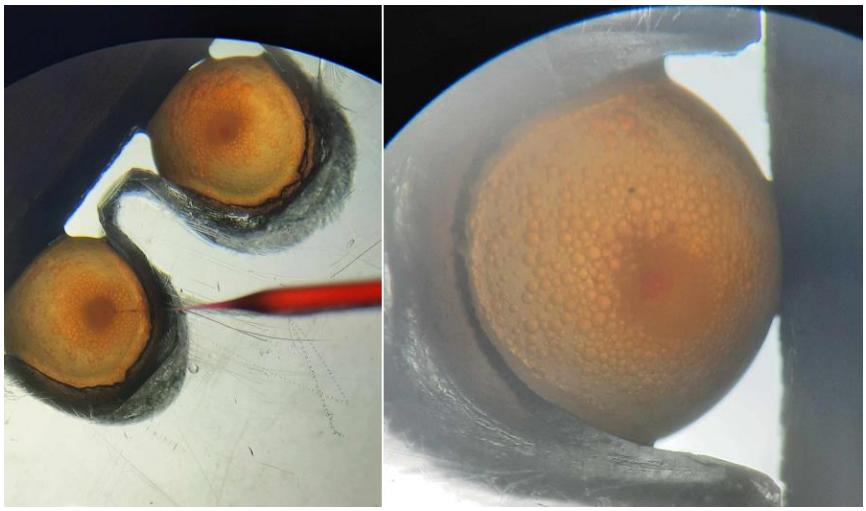

Figure 2 injection of GAPmers into Atlantic salmon eggs at the one-cell stage.

Sampling

Throughout the project, there were six samplings showed in Table 2. The sampling points were based on significant life events such as early cleavage, mid-blastula-transition, gastrulation and the assumed activation of HPI-axis gene expression.

Sampling method

One group at a time, 16 or 18 eggs were taken and placed in a petri dish filled with filtered hatchery water and the morphology was studied using a light microscope. 8 or 9 eggs from the petri dish were placed on a paper towel to remove water before transferring the eggs to a sampling tube with 5 mL RNA later (AM7021, Invitrogen) for the project. The eggs were placed in RNA later for one or two days in the fridge before they were either kept in the freezer at -20 °C or used in the extraction of RNA.

Table 2 An overview of the six samplings, showing both day degrees and developmental stage of the embryos.

Sampling	Day degrees	Developmental stage
1	14	Cell division
2	44-46	Midblastula
3	91-94	ca. 45% epiboly
4	118-119	ca. 95% epiboly
5	294-295	Early HPI-axis expression
6	423-426	Hatching

2.3 Gene expression studies

2.3.1 RNA-extraction

There are several methods for extracting RNA for instance, the conventional trizol method or commercially available Qiagen extraction kits.

Challenges in RNA-extraction

RNA extraction is the first step required for qPCR. In this study, RNA was isolated from whole salmon eggs undergoing different treatments. RNA extraction of whole salmon eggs presents several challenges. The chorion is thick, and the amount of yolk compared to cells is small, which contributes to disruptions of the sample (Bhat et al., 2023). This influences the accuracy of the gene expression data hence, the RNA extraction method used is important. For example, RNA-kits may not be used as egg yolk from the salmon eggs fastens to the filters. A recent study (Bhat et al., 2023) had an approach on isolating RNA from the cell clumps. Considering the methods different approaches were tested (Appendix: method testing). Following the method testing the best method suited for this study was the conventional trizol method (Appendix: Table 10).

2.3.2 The Trizol method

Eggs were stored in RNA-later until RNA-extraction. For RNA extraction, eggs were dried on paper towel to completely remove RNA later, pierced with a needle and transferred to a 2 ml eppendorf tube alongside a magnetic bead before homogenization. Needles were replaced in between groups and tweezers were cleaned using 70 % ethanol (EtOH). 1 ml trizol (15596026, Invitrogen) was added to each of the tubes and eggs were homogenized using a TissueLyzer II (Qiagen), at an oscillation frequency of 30 1/s (30 Hz) for 2 min and vortexed. If the eggs remained partially intact or whole after homogenization, this step was repeated for further 2 min.

The homogenized tissue was centrifuged at 4 °C for 10 min at 12000 rotations per min (rpm) separating the samples into supernatant and a solid residue of the egg. The supernatant of each sample was transferred into a new 1,5 ml tube using a pipette, changing tip for each sample, leaving the solid residue. 500 µl cooled chloroform was added to each supernatant (sample), which was then vortexed for 15 seconds and left for 5 min to incubate at room temperature. After incubation, samples were centrifuged for 15 min at 4 °C at 12000 rpm, resulting in three

different phases: an aqueous phase containing the RNA, an interphase of proteins and DNA and a bottom phase with residual of phenol as an organic phase. The aqueous phase without the inorganic phase were carefully transferred to a new eppendorf tube using a pipette.

To further get a better yield, the trizol and chloroform step was repeated on the aqueous phase, which was centrifuged at 4 °C for 15 minutes, the new aqueous phase was transferred to a DNA-Lo bind tube. Isopropanol (2-propanol) was added to the aqueous phase and mixed well. The tubes were stored at -20 °C for 1-2 hours incubation to precipitate the RNA. For a few early dd samples, the samples were left overnight with isopropanol for a better yield. After 1-2 hours, the samples were centrifuged at 4 °C for 10-15 min at 12000-14000 rpm. This step resulted in a supernatant (isopropanol) and a pellet containing the RNA for most of the samples. The isopropanol was removed, and the pellet was cleaned by adding 1 ml of 80 % ethanol before the pellets were centrifuged at 4 °C for 5 min at 12000 rpm. The ethanol was made using 40 ml absolute ethanol and 10 ml nuclease-free DPEC water.

Ethanol from the first wash was removed and 1 ml 80 % ethanol was added using a pipette before the samples were centrifuged at room temperature for 5 min at 12000 rpm. The ethanol used for cleaning was removed leaving pellets. The pellets were left to dry in the tubes for 35-50 min, depending on size and day degrees, until all the ethanol had evaporated. Once the pellets had dried, 20 µl (sampling 1-3) or 30 µl (sampling 4-6) nuclease-free water was added to each of the pellets using a pipette. Next, the samples were placed in a heating chamber at 55 °C for 10 min to dissolve the pellet (RNA). The samples were placed on ice before quality and concentration were measured in a Nanodrop spectrophotometer.

Nanodrop ®

After the RNA isolation, all samples were run through a NanoDrop ® ND-1000 spectrophotometer (Thermo Fisher Scientific) to measure the RNA concentration (ng/µl) and purity (260/280). The concentrations and purity of the samples are based on the absorption of wavelengths at 260 nm and 280 nm, where 260 nm was used for concentration, and the ratio of absorbance at 260 nm and 280 nm was used for purity, hence 260/ 280. The purity was used to determine the degree of contamination in the sample and asses if DNase treatment was needed to reduce contamination. All samples with a 260/ 280 value below 1,5 were regarded as contaminated samples. After the measurements, the RNA samples were stored in a -80 °C freezer until further processing.

2.3.3 DNase treatment

A DNase treatment was done using the TURBO DNA-free ™ Kit (AM1907, Invitrogen) according to the manufacturer protocol. All samples from samplings 1-3, some samples from sampling 4 in groups 1, 2, 3, 9 and 13, as well as some from sampling 6, encompassing all the groups were treated. The procedure involves a DNase enzyme degrading DNA from RNA samples with the help of a buffer.

The RNA samples selected for DNase treatment were thawed on the ice and short-spinned before the treatment. 10 % of RNA-sample volume of 10X TURBO DNase ™ buffer, here 2 µl (sampling 1-3) and 3 µl (sampling 4 and 6) was added to the samples along with 1 µl of TURBO DNase ™ enzyme. Reagents were added using a pipette equipped with filtered tips. Next, the samples were centrifuged and placed in a heating block at 37 °C for 25 min. Water was added to the heating block to establish an even temperature around the tubes. The DNase Inactivation buffer was thawed on ice and vortexed to a homogenous mass, and 10 % of the sample volume was added to each of the samples. The samples were incubated for 5 min and mixed every 1,5 min to resuspend the reagent to stop the enzyme reaction. Next, the samples were centrifuged for 5 min at 10000 g for 1,5 min, and the clean supernatants were transferred to new DNA-Lo bind tubes. Following the DNase treatment, the samples were measured using Nanodrop ® and placed in a -80 °C freezer.

2.3.4 cDNA synthesis

In qPCR, a segment of complementary DNA (cDNA) functions as a template which is exponentially amplified and quantified, thus the isolated RNA was utilized for cDNA synthesis. cDNA synthesis is a process where the enzyme reverse transcriptase (RT) binds RNA templates and synthesizes a complementary DNA strand. The RNA is degraded, and a DNA polymerase creates the second DNA strand, completing the cDNA (Campbell, 2018). Using High-Capacity RNA-to-cDNA™ Kit (4388950, Applied Biosystems) for all cDNA synthesis.

The High-Capacity RNA-to-cDNA™ Kit is made for up to 2 µg RNA reactions. 200 ng RNA was used as a fixed concentration for cDNA synthesis, due to the lower concentration of RNA in early dd samples. To achieve this in later dd, RNA samples were diluted using nuclease free water for a pipette volume. All volumes or dilutions were calculated based on nanodrop concentrations prior to the dilution, guaranteeing concentrations for the synthesis and a minimum pipette volume of 2 µl. The finished RNA dilution plates were sealed and placed in a -80 °C freezer until cDNA synthesis.

Before the cDNA synthesis, all volumes were calculated, ensuring that each well with a sample from the RNA dilution plates combined with nuclease-free water obtained a concentration of 200 ng RNA in a total volume of 9 µl. Each plate contained 2 control samples, one without reverse transcriptase enzyme (NoRT) and one without RNA to make sure that RNA samples are clean without DNA-contamination. To RNA samples in cDNA-plates, a buffer mix consisting of a 1:10 ratio of 20X RT Enzyme mix to 2X RT Buffer was added, except for the NoRT wells. Only 11 µl of 2X RT Buffer mix was added to the NoRT wells. The plate was then sealed using MicroAmp™ optical adhesive films and centrifuged. Next, the plates were placed straight into a thermal cycler (Thermal Cycler 2720, Applied Biosystems) running for 60 min at 37 °C, 5 min at 95 °C and then held at 4 °C. The cDNA plate was retrieved from the thermal cycler and diluted (tested and confirmed that 1:40 dilution). In a new plate, 195 µl nuclease-free water was added to each of the wells matching the cDNA plate set-up, and then 5 µl of cDNA, including the controls were added to the cDNA dilution plate with the water matching each sample and well creating a 1:40 dilution and the plate was resealed and frozen at -20 °C.

2.3.5 Real-time qPCR

In qPCR the cDNA template is hybridized by specifically designed primers in which reverse transcriptase binds to and copies the template with the help of DNA-polymerase, creating double-stranded (ds) DNA. The ds DNA is quantified by using a marker, here Syber Green that binds and fluoresces once ds DNA is made, which allows for quantitative data in real-time. Primers for the genes: *actin, ef1, 18S, gr1a, gr1b, gr2, pomca* and *pomcb* were designed and tested in advance using the standard curve method by Dhivya Borra Thiyagarajan (Table 3). The gene expression is quantified, and relative concentration or gene expression is given as threshold cycles (Ct-values) based on the threshold. Ct-values represent the number of cycles the maker or fluorescence uses to pass a set threshold. The Ct-values will increase the lower abundance of qPCR products available.

Table 3 Primer sequences, accession number and efficiencies for the genes: actin, ef1, 18S, gr1a, gr1b, gr1, pomca and pomcb.

Gene	Accession number	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')	Efficiency
actin	BT059604	CAGCCCTCCTTCCTCGGTAT	CGTCACACTTCATGATGGAGTTG	103
ef1	AF321836	CGCCAACATGGGCTGG	TCACACCATTGGCGTTACCA	102
18S	AJ427629	TGTGCCGCTAGAGGTGAAATT	CGAACCTCCGACTTTCGTTCT	94
gr1a	>XM_045717356.1	ACCGCAGCAGAACCAACAG	TGGATCGATTCAAATCTGCAAT	81,7
gr1b	>XM_014136782.2	GGACTTGGGTGGATAGAGAATG	TCCAGAAGGGTCAGTTAGTTTG	84,5
gr2	>XM_014198677.2	TGTCCATGAGGACGGAGACA	CCAATGTACCCTTCCTGATCCA	84,5
pomca	AB462418	TGGAAGGGGGAGAGGGAG	CAGCGGAAGTGGTTCATCTTG	83,8
pomcb	AB462419	ACTAAGGTAGTCCCCAGAACCCTC	GCTACCCCAGCGGAAGTGA	81,9

qPCR was run for eight genes: *actin*, *ef1*, *18S*, *gr1a*, *gr1b*, *gr2*, *pomca* and *pomcb*. To prepare for the qPCR runs cDNA was aliquoted into qPCR plates following templates made in the software Design and Analysis Software 2.6.0. (Applied Biosystems). In qPCR, the samples are run in duplicates. For the housekeeping genes (HKG) *actin, ef1* and *18S*, a separate plate was made for NoRT, controls and three random samples from each cDNA dilution plate to ensure no DNA contamination in the RNA-samples.

qPCR plate with cDNA and primers was thawed on ice. Syber Green mastermix was placed in the fridge from the -20 °C freezer as needed. In cases where primers were used two or three days in a row, they were kept in the fridge at 4 °C or else frozen at -20 °C. Mastermix (Table 4) was made in eppendorf tubes. Working primer solutions were made as necessary. The primer stock was set to thaw while 950 µl of nuclease-free water was added to DNA-Lo-bind tubes. When the primer stock had thawed 50 µl of stock was added to the water and the tube was vortexed.

Table 4 Mastermix components and amounts (µL) for a qPCR-reaction.

Mastermix	Per reaction
Syber green	7,5 µL
Forward primer	1,25 µL
Reverse primer	1,25 µL
cDNA	5 µL

Using a multichannel pipette 10 µl mastermix was added to each well containing a sample. On each qPCR plate, two wells were filled with 5 µl nuclease-free water and mastermix as controls. The plate was sealed using a film, centrifuged, and placed in the Quant Studio 6 Pro (Applied Biosystems) and the program (Table 5) ran. Three additional qPCR plates were thawed, and prepared, during the first run. These plates were sealed using a film, centrifuged, and kept on ice or at 4 °C covered in aluminum foil. When the first run finished, plate two was sat to run, then three and then four. Each plate required about 50 minutes. If six or eight plates were run

in a day, the next two or four plates were prepared when there was about half an hour left of the last plate from the first batch.

Table 5 shows the PCR set-up/ program that was used for qPCR (Fast SYBR® Green Regaent).

°C	Seconds	Cycles	Phase
95	20	1	Hold
95	3	40	PCR
60	30		
95	15	1	Melt curve
60	60		
95	15		
60	15		

2.4 Processing of data

2.4.1 qPCR analysis

The results from qPCR were exported to a PC as eds files where thresholds were set for each gene, and Ct-values and melt curves inspected for contamination, removing unbelievable duplicates (difference ≥ 2)/ results and samples with abnormal melt curves. Furthermore, the files were exported and converted to Excel files for further analysis. The average was calculated for all the duplicates and then corrected based on the amplification efficiencies of the primers (Table 3) for each gene using F.1 (Lock et al., 2010).

$$Ct_{E=100\%} = log_2(E) \times Ct_E \quad \text{(F.1)}$$

The ΔCt-values (F.2) and their group standard deviation (std) were also calculated, the geometric mean of the reference genes (actin, EF1 and 18S) served as the internal control.

$$\Delta Ct_{sample} = Ct_{target} - Ct_{geometic\ mean\ of\ referance\ genes} \quad \text{(F.2)}$$

The ΔCt-values were further used to calculate the Z-value (F.3) to remove outliers within the treatment groups, $|Z| > 3$ were removed.

$$Z = \frac{x_{sample} - \overline{x_{group}}}{std} \quad \text{(F.3)}$$

Relative expression (fold change) was calculated following the $2^{-\Delta\Delta Ct}$-method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). ΔCt values were used to calculate ΔΔCt by subtracting the average of ΔCt controls from the ΔCt value of the samples. Fold change were then calculated for the individual samples using F.4 (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

$$Foldchange = 2^{-\Delta\Delta Ct} = 2^{-((Ct_{target} - Ct_{reference})_{test} - \overline{\Delta Ct}_{calibrator})} \quad \text{(F.4)}$$

Individual fold changes were later used to plot box plots for cell cleavage and mid-blastula. The graph from approximately 50 % epiboly until hatch was plotted using fold changes geometric mean with positive and negative standard deviation, which were calculated using F.4 and F.5 (Livak & Schmittgen, 2001).

$$\overline{Foldchange} + STD = 2^{-(\overline{-\Delta\Delta Ct} + STD)} \quad \text{(F.4)}$$

$$\overline{Foldchange} - STD = 2^{-(\overline{-\Delta\Delta Ct} - STD)} \quad \text{(F.5)}$$

2.4.2 Statistical analysis and presentation of data.

Organization and analysis of the Ct-values were performed in Microsoft Excel including calculation of Z-values, sample size ratios and variance ratios (Appendix: Table 11). Graphs and plots were made in Microsoft Excel and edited in Microsoft PowerPoint where labels were added. Statistical analyses were performed in Graphpad Prism version 10.2.2 (Graphpad Software Inc) and SPSS. To investigate whether there were any significant differences in relative gene expression between treatments at cell cleavage and at mid-blastula, a one-way ANOVA was run for all the genes ($p < 0,05$) with Šídák's multiple comparisons posthoc test. Two-way ANOVAs with Šídák's multiple comparisons posthoc tests ($p < 0,05$) were run for all target genes to inspect any significant differences in gene expression between treatments over time from approximately 50 % epiboly until hatching.

Parametric tests such as ANOVA is strongest when the data has a homogenous variance and the data normally distributed. Visual inspection (histograms) and normality tests performed on the residuals (D'Agostino-Pearson omnibus, Anderson-Darling, Shapiro-Wilk, and Kolmogorov Smirnov) concluding that the data is normally distributed enough (Knief & Forstmeier, 2021). In addition, Levene's test of equality of error variances and F test of the heteroskedasticity was performed to support the robustness of the ANOVAs (Appendix: Table 12).

Regarding the morphology photos were taken through a light microscope (SM168, Motic) at different magnifications 7,5-50X. In PowerPoint pictures were cropped to size and labeled. Photos of the embryos on millimeter paper were used to measure their sizes. Images were put into Imagemeasurement (www.imagemeasurement.online/select) where the photo was cropped, adjusted and a scale was set to 5 mm using the millimeter paper. Then lines were drawn from the head to the caudal fin or across the diameter of the eggs to measure.

During the project, dead and unfertilized eggs were recorded and removed. After hatching, all the embryos were counted and % unfertilized eggs/ mortality was calculated for each of the treatments (Table 6). Fertilization percentage decreased with time, indicating that it is most likely due to a relatively fast reduction of milt quality.

3 Results

For this study, the developmental stages of Atlantic salmon embryos were documented visually in pictures of embryos and larvae by utilizing a light microscope and qPCR was performed to record gene expression over time. Unfertilized eggs and mortality were recorded during the samplings and are represented in Table 6. Due to low degree of fertilization Negative GAP A was only sampled during cell division, mid-blastula and at approximately 50 % epiboly. Additionally, "only fertilized" eggs were only used for 50 % epiboly sampling since at this "stage" it was possible to select (by visual examination) embryos that were alive and developing. (Statistics analysis and plots were made including the only fertilized, Appendix: Sampling 1 and 2 with only fertilized eggs).

Table 6 Total embryo mortality/ unfertilized eggs (%) in the different treatments and variation from max and min over the course of the experiment. Time of fertilization of all eggs from day 1 (eggs arrival) to day 2.

	Average % unfertilized eggs	Max and min range (%)	Time of fertilization
Eplerenone	18,82	± 3,40	Fertilized 14.45 day 1
Mifepristone	15,74	± 2,21	Fertilized 14.45 day 1
Eplerenone and Mifepristone	20,06	± 2,53	Fertilized 14.45 day 1
Controls	22,82	± 3,08	Fertilized 14.45 day 1
GAPmers	73,53	+ 9,61	Fertilized 17.50 day 1
		- 17,97	
GAP Control Negative A	70,36	± 19,34	Fertilized 07.50 day 2
Only fertilized	91,74	+ 8,26	Fertilized 18.05 day 2
		- 15,30	Fertilized 07.50 day 2

3.1 Gene expression

Due to relatively low and changing overall expression during the earliest stages of development a large difference in reference gene expression (Ct-value average difference: 6) was recorded for the first two samplings compared to that of the rest of the experiment. Thus, treatment effects on relative gene expression were compared for early cleavage and mid-blastula separately, and within each sampling point only. From approximately 50 % epiboly until hatching, reference gene expression was stable and during this period treatment effects on temporal changes in gene expression was also compared.

3.1.1 GAPmer treatment

Early cleavage and mid-blastula

Figure 3 illustrates the relative gene expression during early cell cleavage of the genes *gr1a, gr1b, gr2, pomca,* and *pomcb* in embryos treated with negative control GAP A and GAPmers targeting the GR mRNAs. The GAPmer treatment did result in a statistically significant reduction in gene expression for *gr1b,* compared to both of the controls (Figure 3b). There was no other statistically significant effect on gene expression for any of the other genes investigated (Appendix: Table 14, Table 20).

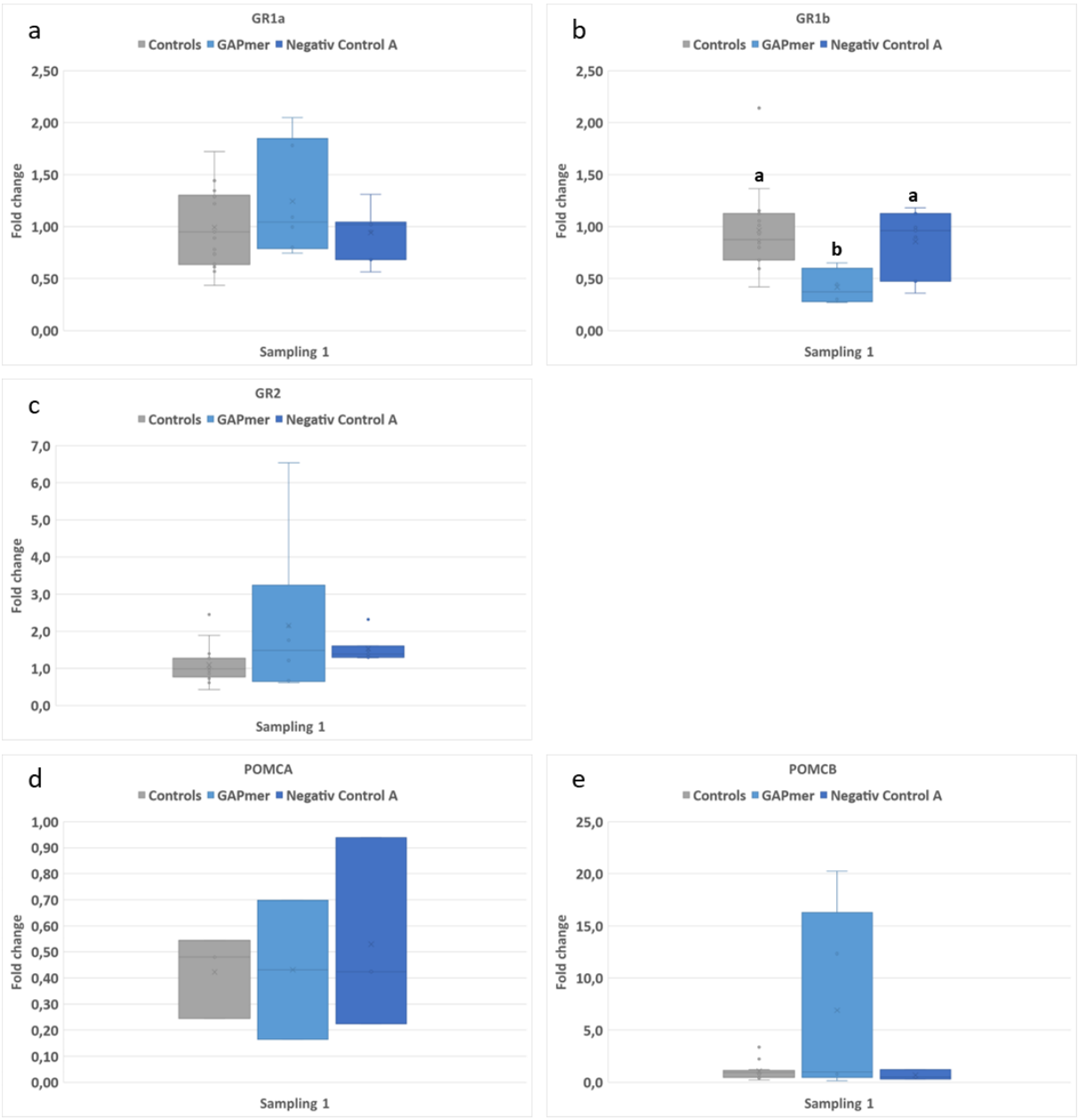

Figure 3 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control embryos and embryos injected with Negative control GAP A and GAPmers specifically targeting gr1a, gr1b and gr2 in Atlantic salmon at 14 day degrees of development. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". a and b represent significant differences between treatments (p<0,05). Omitted letters indicates no statistical difference in gene expression between treatments.

Figure 4 shows the relative expression of the genes *gr1a*, *gr1b*, *gr2*, *pomca*, and *pomcb* for controls and embryos injected with negative control GAP A and GAPmers at mid-blastula. For the genes *gr1a*, *pomca* and *pomcb* (Figure 4 a, d, e), there were significantly higher relative gene expressions in GAPmer treated eggs compared to the controls (Appendix: Table 15, Table 20). Furthermore, eggs treated with negative control GAP A had a significantly higher expression than the controls for *gr1a* and a significantly lower gene expression than the GAPmer treated embryos for *pomca.* Something to note is that the relative expression of *gr1a*

and *pomcb* have a relatively broad expression range in the GAPmer treated embryos. For *gr2* and *pomcb*, Negative Control A has a relatively large variation in gene expression.

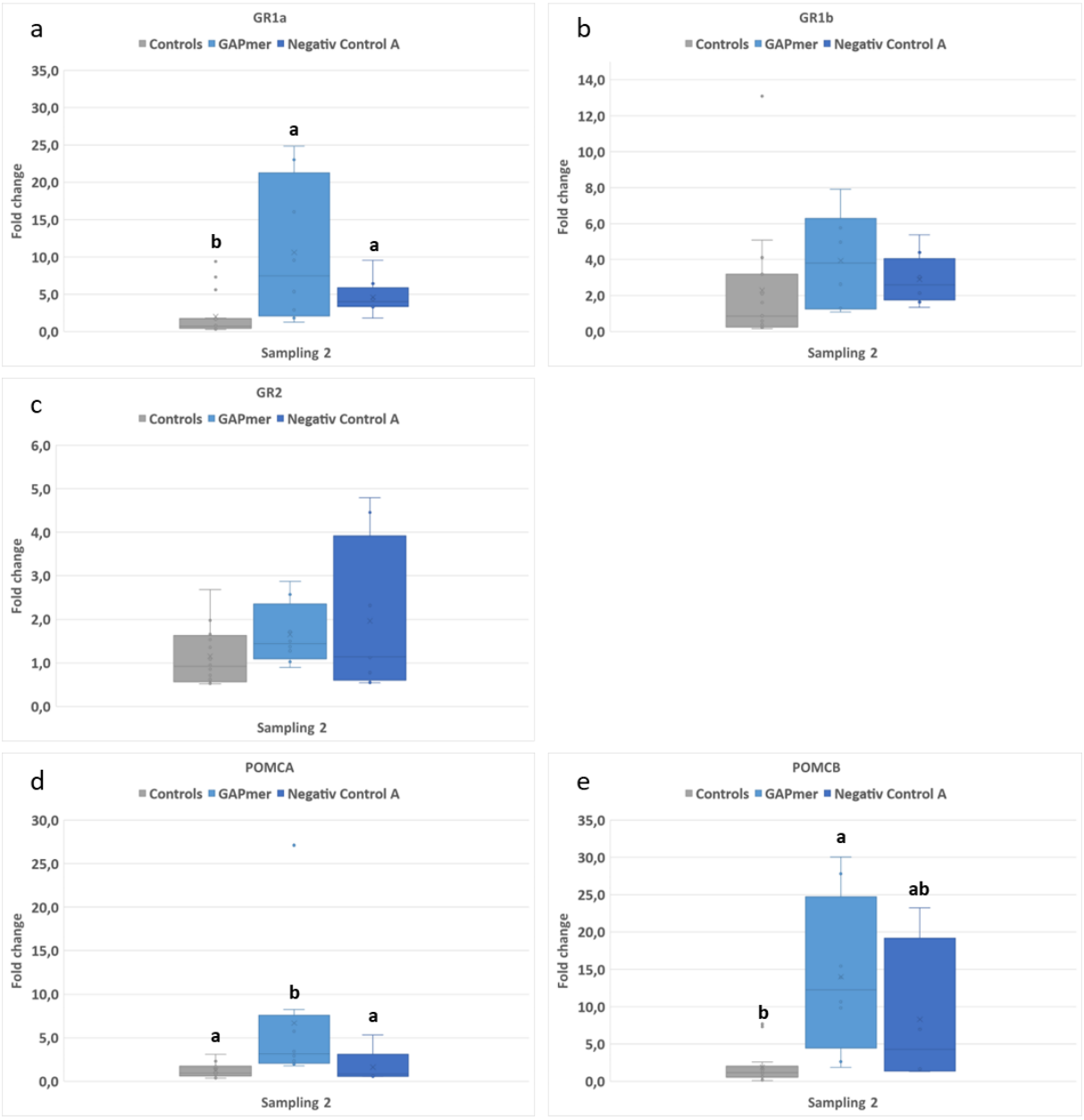

Figure 4 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs, negative control GAP A eggs and the eggs injected with GAPmers at 44-46 day degrees (sampling 2). These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". different lower case letters represent significant differences between treatments (p<0,05). Omitted letters indicates no statistical difference in gene expression between treatments.

Temporal changes in gene expression from 50 % epiboly (92 day degrees) to hatch.

Relative gene expression for *gr1a*, *gr1b*, *gr2*, *pomca*, and *pomcb* was examined both between treatments and over time (Figure 5). For *gr1a* (Figure 5a) and *gr1b* (Figure 5b) there is a relatively stable expression during epiboly before there is a significant increase in gene expression at 294-295 dd. For *gr1a* (Figure 5a) this increase was mitigated while it continues to increase significantly for *gr1b* (Figure 5b), from ca. 0,5 to slightly above 6 fold change at 423-426 dd. The expression of *gr2* had a relatively flat trend without any significant changes over time. At all samplings, the GAPmer treated embryos had a significantly lower *gr1a* expression compared to the control (Figure 5a) (Appendix: Table 16). Expression of *pomca* (Figure 5d) and *pomcb* (e) both have the same trend where it decreases significantly from ca. 50 % epiboly to 95 % epiboly before it increases significantly towards the "eyed" stage (295 dd). The increase continues towards hatching but is not significant, with fold change increasing 1-1,5. The expression of *gr2* has a relatively flat trend without any significant changes over time (Appendix: Table 16).

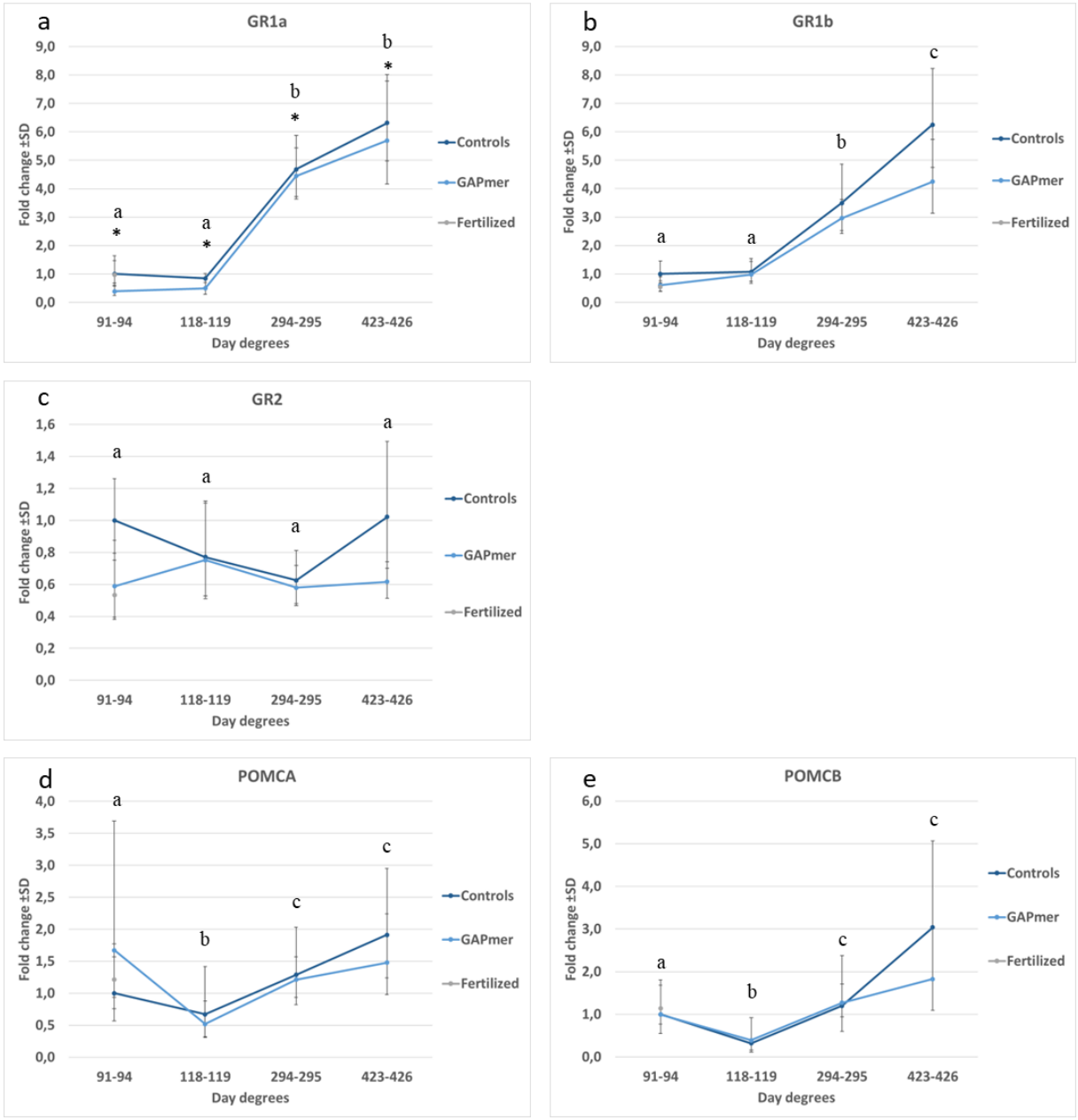

*Figure 5 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs and eggs injected with GAPmers over time (91-94 day degrees, 118-119 day degrees, 294-295 day degrees and 423-426 day degrees). Fold change for fertilized eggs is included in 91-94 day degrees. * shows differences between groups within samplings. Small letters indicate differences across samplings within all groups. * and small letters are omitted when no significant difference were found.*

3.1.2 Inhibitors

Early cleavage and mid-blastula

Figure 6 shows the relative expression of the genes *gr1a, gr1b, gr2, pomca*, and *pomcb* for non-treated controls, and inhibitor treated embryos at 14 dd. For *gr1a* (Figure 6a) the eplerenone treated eggs had a significantly lower gene expression compared to the controls, mifepristone and both eplerenone and mifepristone treated eggs. Expression of the other genes did not differ significantly between treatments (Appendix: Table 17, Table 20), being relatively stable amongst all treatments. Within the eplerenone and mifepristone treatments the *gr* genes showed a relatively large variance in gene expression (Figure 6 a, b, c).

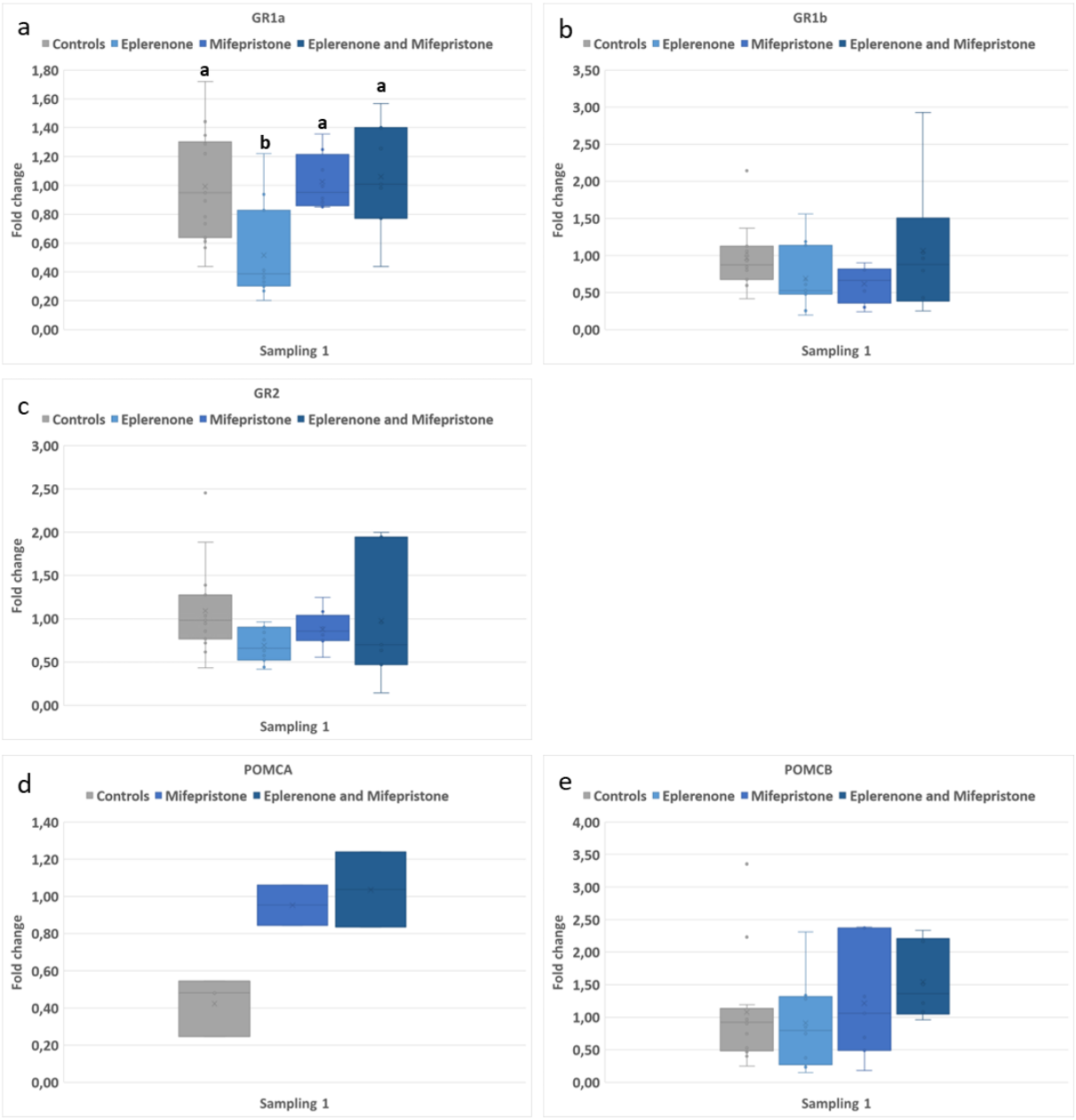

Figure 6 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for non-treated control eggs, eplerenone treated eggs, mifepristone treated eggs and eggs treated with both eplerenone and mifepristone at 14 day degrees. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". different lower case letters represent significant differences between treatments (p<0,05). Omitted letters indicates no statistical difference in gene expression between treatments.

Figure 7 presents the relative expression of the genes *gr1a*, *gr1b*, *gr2*, *pomca*, and *pomcb* for non-treated control eggs and inhibitor treated eggs during mid-blastula. There was a significantly higher expression between the eplerenone- and mifepristone treated eggs *gr2* (Figure 7c) (Appendix: Table 18, Table 20). The other genes investigated had no other significant differences in relative gene expression.

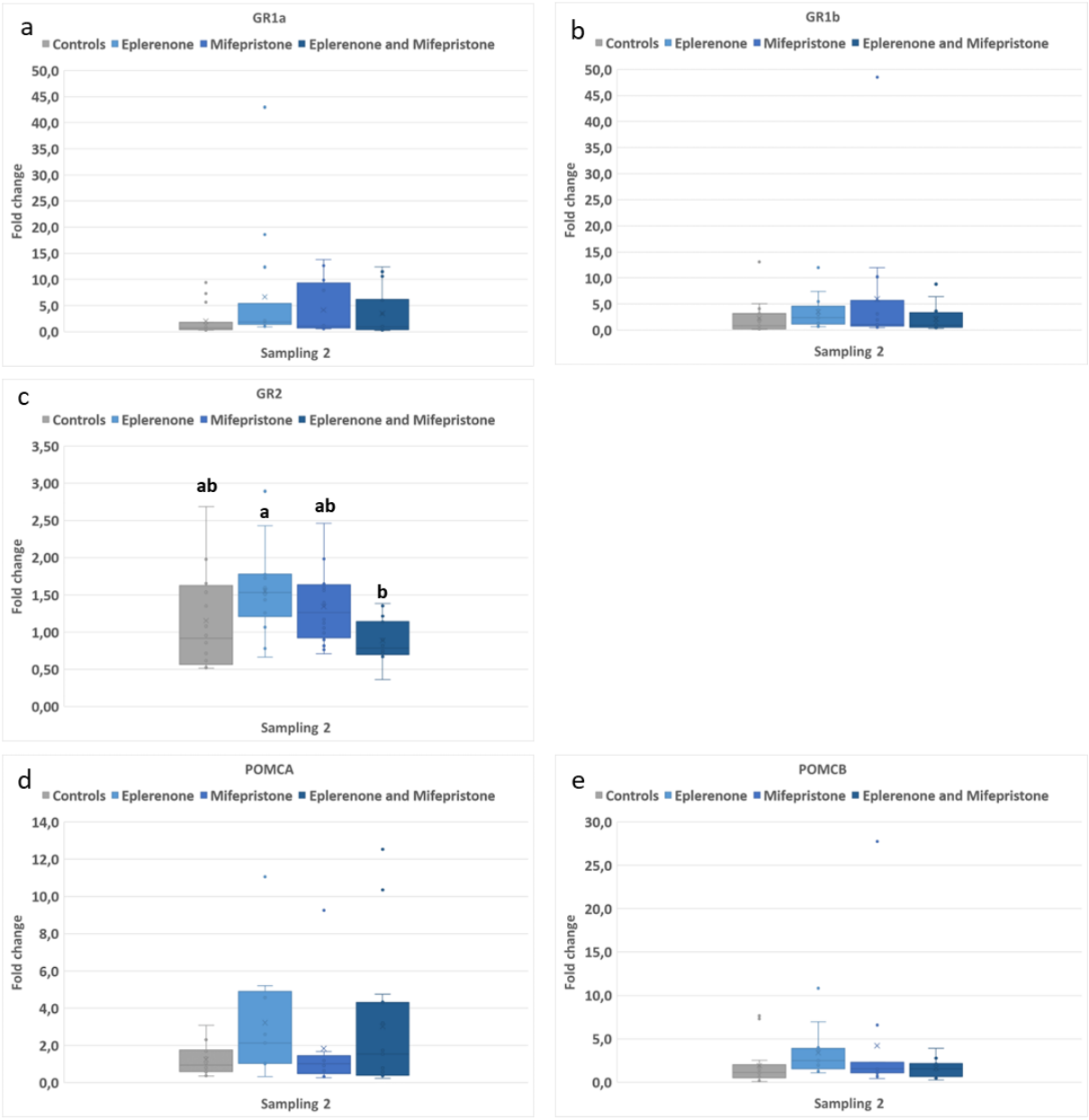

Figure 7 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for non-treated control eggs, eplerenone treated eggs, mifepristone treated eggs and eggs treated with both eplerenone and mifepristone at 44-46 day degrees. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". different lower case letters represent significant differences between treatments (p<0,05). Omitted letters indicates no statistical difference in gene expression between treatments.

Inhibitor influences on temporal changes in gene expression from 50 % epiboly (92 day degrees) to hatch

Relative gene expression for *gr1a*, *gr1b*, *gr2*, *pomca*, and *pomcb* was examined both between embryo treatments and over time (Figure 8) (Appendix: Table 19). Both *gr1a* (Figure 8a), *gr1b* (Figure 8b) and *pomca* (Figure 8d) appear to have similar trends. From approximately 50 % epiboly to approximately 95 % epiboly there is a relatively stable expression. After gastrulation there is a significant increase in expression towards the "eyed" stage. The expression of *gr1a* (Figure 8a) and *pomca* (Figure 8d) generally increases towards hatch, but not significantly. The g*r1b* (Figure 8b) expression on the other hand has a significant increase in gene expression towards hatching. Both *gr2* (Figure 8c) and *pomcb* (Figure 8e) gene expression significantly decreases from approximately 50 % epiboly to the end of gastrulation, where the expression stabilizes for *gr2* (Figure 8c) before it significantly increases towards hatching. From ca. 95 % epiboly the gene expression increases significantly towards hatching in for *gr1a, gr1b, pomca* and *pomcb*. The only fertilized eggs were significantly lower expressed than the controls in *gr2* (Figure 8c) at approximately 50 % epiboly. Additionally, *pomca* were significantly higher expressed in eplerenone treated eggs, than in eplerenone and mifepristone from approximately 95 % epiboly until hatching.

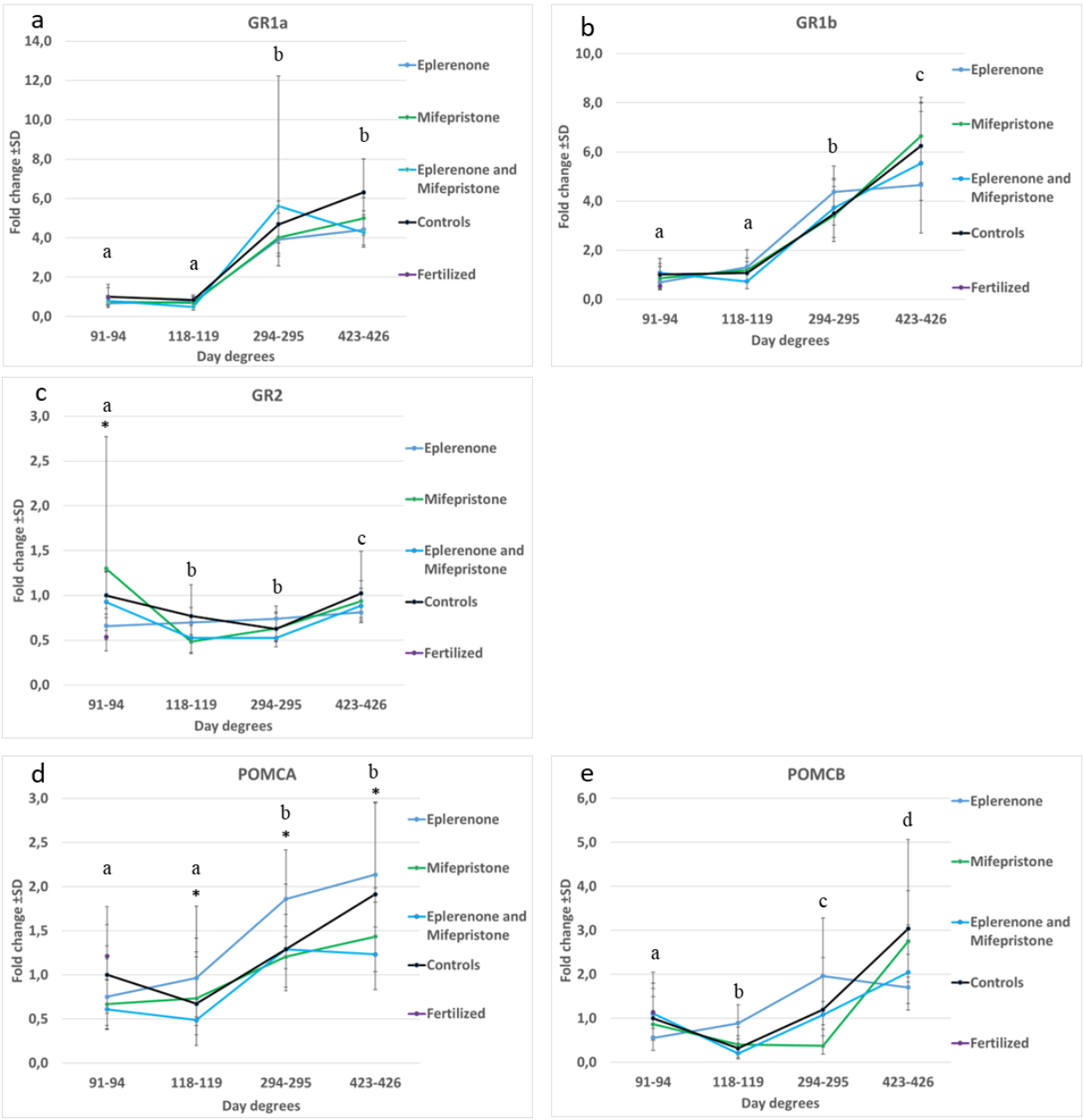

*Figure 8 Fold change (relative gene expression) of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs and eggs treated with the inhibitors eplerenone, mifepristone and eplerenone and mifepristone (91-94 day degrees, 118-119 day degrees, 294-295 day degrees and 423-426 day degrees). Fold change for fertilized eggs is included in 91-94 day degrees. * shows differences between groups within samplings. Small letters shows differences across samplings within groups. * and small letters are omitted when no significant difference were found.*

3.2 Morphology

The first sampling was conducted at 14 day degrees, ca. 32-64 cells (Musialak et al., 2023; Nagasawa et al., 2013). At sampling 1, the cytoplasm had gathered at the animal pole to form the blastodisc and cell division had begun. Figure 9 shows the eggs/ embryos at 14 day degrees. Cell division was not easily recorded through the microscope and to the untrained eye, all eggs appeared indistinguishable.

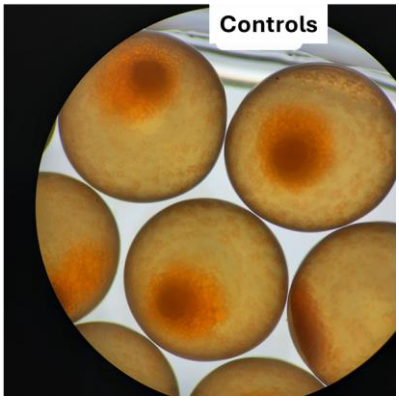

Figure 9 Atlantic salmon embryos at 14 day degrees of development (32-64 cellstage).

At sampling 2, the eggs were 44-46 day degrees, depending on the treatments. There are minimal visual differences and no visible effects of the treatments (Figure 10).

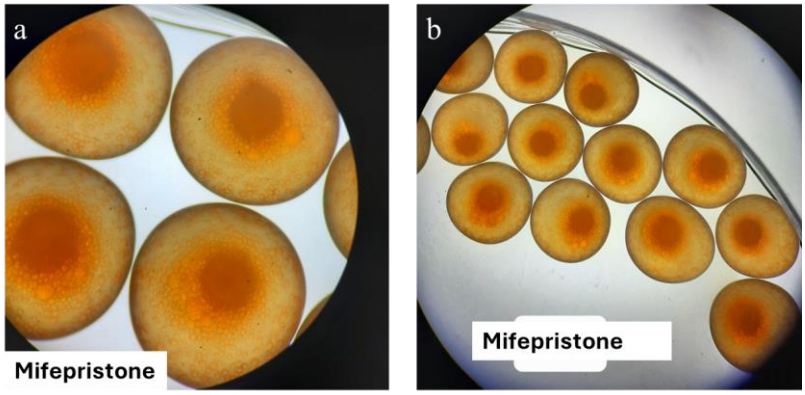

Figure 10 Atlantic salmon embryos at 44 day degrees of development at mid-blastula at different magnifications. a and b) shows eggs treated with mifepristone.

The third sampling, at 91-94 day degrees, embryos were at ca. 50 % epiboly (Figure 11). All groups treated with inhibitors (Figure 11b), appeared to have reached a similar stage of development. In contrast, the GAPmer treated eggs (Figure 11c) seemed to have a few embryos with delayed (ca. 20 % epiboly) or stopped development. Still, within the GAPmer treated embryos, embryos varied only slightly in the degree of epiboly.

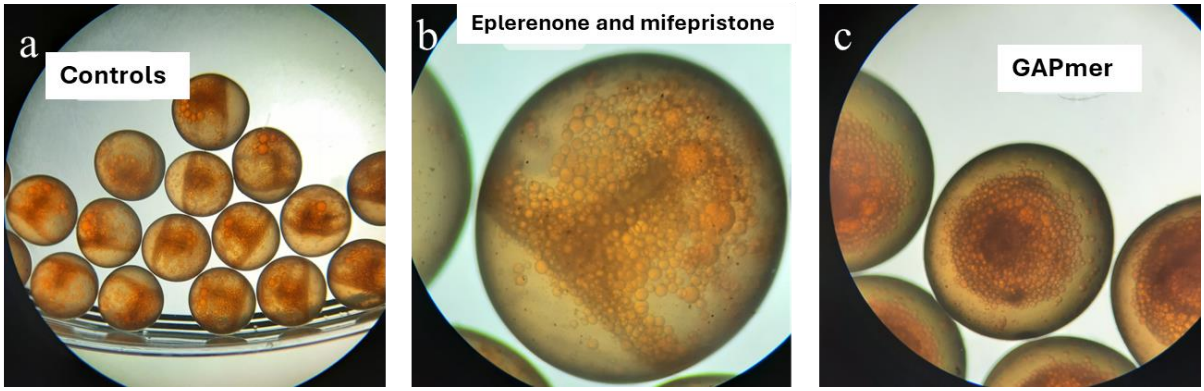

Figure 11 Atlantic salmon embryos at 91-94 day degrees at ca. 50 % epiboly. Pictures taken at different magnifications. a) shows eggs from controls. b) egg treated with eplerenone and mifepristone and c) shows eggs injected with GAPmers and delayed or stopped development.

Sampling 4 at 118-119 day degrees showed embryos at approximately 95 % epiboly (Figure 12). The GAPmer injected eggs (Figure 12 d-f) presented some eggs that had begun developing but stopped. Most eggs observed at approximately 50 % epiboly that began developing but stopped had turned white and were removed during this sampling (ca. 8 % of total mortality of GAPmer treated embryos). All other groups appeared to have the same development timing. Comparing eggs at ca. 50 % epiboly (Figure 11) to eggs at ca. 95 % epiboly (Figure 12), the embryo has become more evident and blastomere closure is observed.

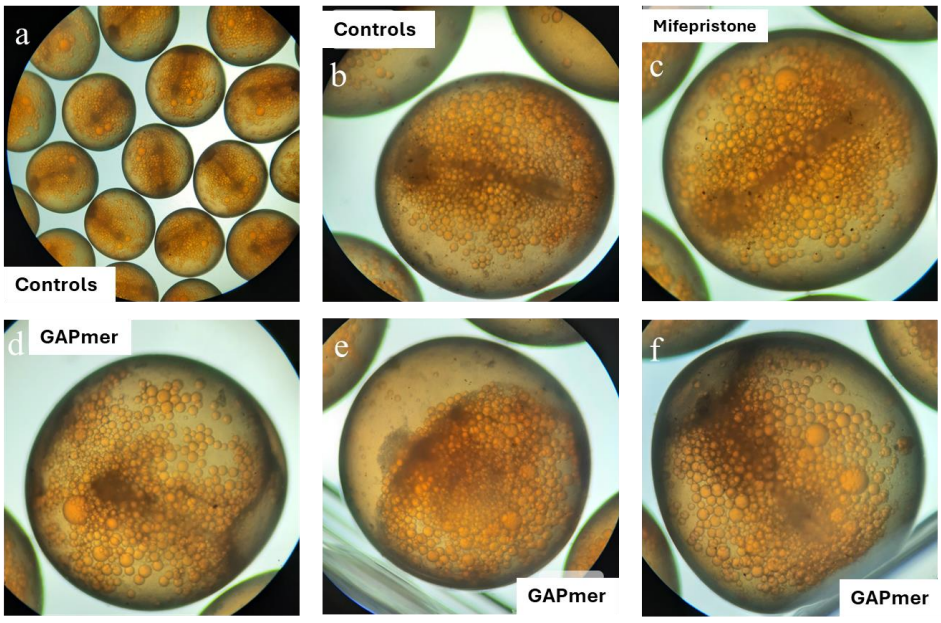

Figure 12 Atlantic salmon embryos at 118-119 day degrees form sampling 4 at different magnifications. a and b) shows controls, c) shows an egg treated with mifepristone, all at approximately 95 % epiboly. d-f) shows eggs injected with GAPmers at different stages of epiboly.

The fifth sampling was completed when the eggs were 294-295 day degrees (Figure 13). Features observed were a beating heart and the vitelline vein through the yolk, relatively large, pigmented eyes, formation of the pectoral fins and vascularization of the yolk sac. In addition, all treatments and controls appeared to be at the same stage in the organogenesis without any distinctive differences between the treatments.

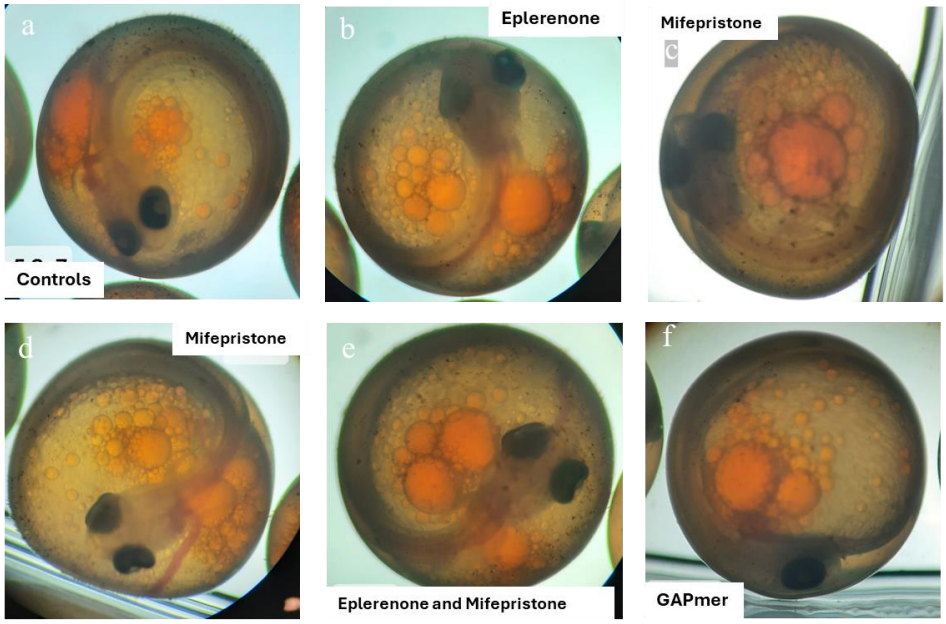

Figure 13 Atlantic salmon "eyed" embryos at 294-295 day degrees form sampling 5. a) controls, b) egg treated with eplerenone, c and d) eggs treated with mifepristone, e) eggs treated with eplerenone and mifepristone and f) shows an embryo treated with GAPmers.

The last sampling was conducted at 423-426 day degrees while the eggs were hatching (Figure 14 a-h). The appearance of the eggs was relatively similar across all the groups, with the body covering more of the egg's volume and wrapping around the yolk. Regarding the yolk-sac larvae, the most varying characteristic was the yolk-sac shape. The control yolk-sac larvae (Figure 14 a, b) appeared to have a mixture of elongated oval and jellybean shapes, also observed in the inhibitor groups (Figure 14 c-f) with more embryos having elongated yolk-sacs. The yolk-sac shape of GAPmer injected eggs (Figure 14 g, h) was dissimilar to the controls, the majority having a more spherical shape. In addition, to the yolk-sac shape some of the yolk-sac larvae treated with GAPmers had bent spines (Figure 14 h).

Egg diameter and yolk-sac larvae from head to caudal fin were measured in millimeter (mm) (Table 7) (Appendix: Table 21). The yolk-sac larvae in the control group had a similar size to the GAPmer-treated groups and eplerenone treated groups but were slightly larger than the

mifepristone and eplerenone and mifepristone treated groups. All eggs were relatively similar in size.

Table 7 The size of eggs in diameter and yolk-sac larvae from head to caudal fin in millimeter (mm) at 423-426 day-degrees.

	Controls		Eplerenone		Mifepristone		Eplerenone and mifepristone		GAPmer	
	Egg	Yolk-sac larvae	Egg	Yolk-sac larvae	Egg	Yolk-sac larvae	Egg	Yolk-sac larvae	Egg	Yolk-sac larvae
Average	5,84	17,71	6,25	17,65	5,79	17,33	6,09	16,68	5,68	17,98
Median	5,8	18	6,1	17,5	5,7	17,4	6,1	16,9	5,65	18
Max	6,4	19	7,6	19,6	6,2	18,8	6,6	18,8	5,9	19,7
Min	5,4	14,2	5,4	16,7	5,2	16	5,7	11,7	5,5	16,5

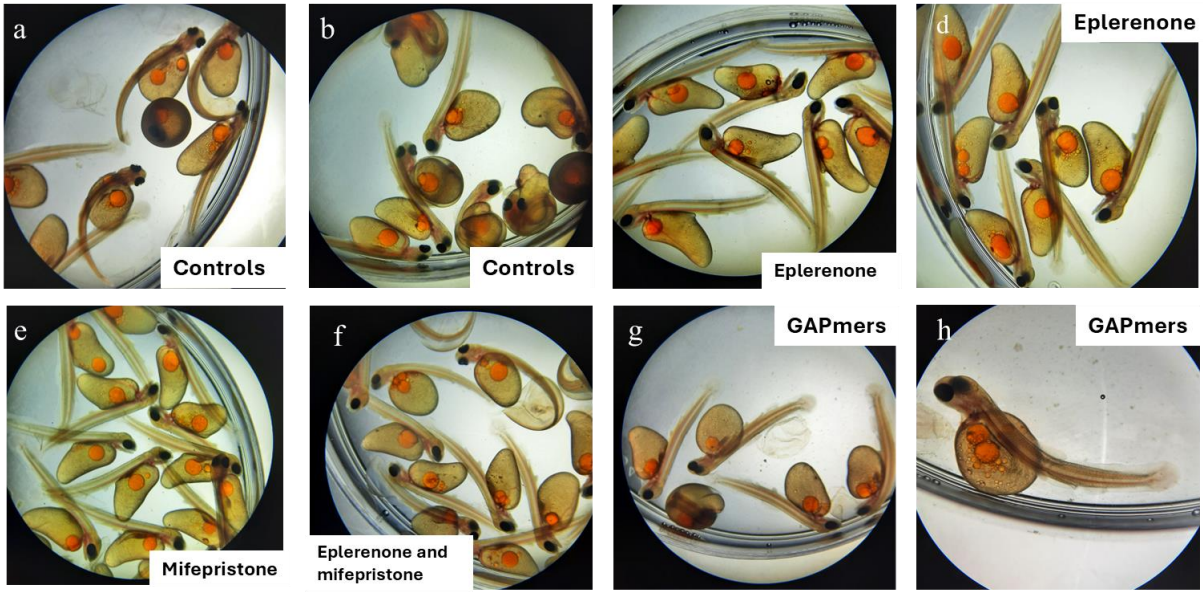

Figure 14 Yolk-sac larvae at 423-426 day degrees from sampling 6. a-c) shows the controls, d and e) shows yolk-sac larvae treated with eplerenone, f) shows a yolk-sac larvae treated with mifepristone, g-j) shows yolk-sac larvae treated with eplerenone and mifepristone, and k-o) shows yolk-sac larvae injected with GAPmers.

4 Discussion

The results suggest that the maternal cortisol signaling influences the early development, being able to influence the development and yolk-sac (energy) utilization of the developing embryo. This also highlights how cortisol works in many physiological systems (see below).

Following the completion of the laboratory procedures and processing of the results it is important to acknowledge a few details. There was a considerable percentage of eggs that never became fertilized, which affected this experiment (Table 6). The reason for this is most likely due to low-quality milt, which was supported by findings of another research group using milt from the same male, but egg from another female (Krasimir Slanchev AquaGen, personal communication). Here, spermatozoa motility declined substantially during the first day of post-shipment. Consequently, the number of eggs for the only fertilized and Negative Control A was not enough to complete all the samplings. Due to high percentage of unfertilized eggs in "only" fertilized eggs, they were removed from the results during sampling 1 and 2. Additionally, during cell cleavage and mid-blastula the live and unfertilized eggs were indistinguishable, and it is likely that there were dead eggs within the samples, influencing the results.

4.1 What are the influences of antisense oligonucleotide (GAPmer) treatment on HPI-axis gene expression during early development?

The gene expression data is split into cell cleavage stage, mid-blastula and from approximately 50 % epiboly to hatching, due to an inconsistency in the reference genes. Comparisons between treatments and time were done from 50 % epiboly to hatching as the reference genes stabilized after mid-blastula.

Cell cleavage and mid-blastula

During cell cleavage, only one significant difference was found in the expression of the different genes between the treatments. Gene expression of *gr1b* (Figure 3b) shows that the GAPmer treated embryos were significantly less expressed compared to the control and negative control GAP A. This indicate that the GAPmers did induce degradation of the *gr1b* transcript. In contrast the GAPmers does not appear to influence *gr1a* and *gr2*. The concentration of the GAPmers could have been too low. Alternatively, the GAPmers might not

have been able to access the target sequences, as mRNA forms secondary structure elements (Faure et al., 2016), that might block the binding site. Another possibility for the *gr2* gene is that the gene investigated could have been the other *gr2* gene, rather than the KD *gr2* gene, as that the Atlantic salmon has two *gr2* genes (Romero et al., 2020). The relatively small differences in expression for the other genes, regarding GAPmeres, negative GAP A and controls can be explained by natural variation. At this stage all gene expression is controlled by maternal factors (Jukam et al., 2017; Pelegri, 2003).

It is important to note in this study that the eggs should have been treated with different concentrations GAPmers over time as it is proven that the effects of ASO treatments are dose-dependent (Pikulkaew et al., 2011). Experiments performed on zebrafish using ASO (MOs) illustrated that the higher the dose, higher the degree of abnormalities and mortality. Some phenotypic changes in zebrafish observed in this study were delayed development, resulting in smaller larvae with underdeveloped eyes, brain and circulatory system, and some with bent spines and tails (Pikulkaew et al., 2011). In addition, GAPmers are custom designed, often by the supplier, and KD effects may vary considerably between targeted sequences within the same mRNA. Thus, to find the most KD efficient sequence towards a given mRNA, several GAPmers usually need to be tested but this was not within the scope of this project. Because of this there are a few questions to be asked which are important in the interpretation of the results. How efficient are the GAPmers chosen? Does it bind the target or randomly somewhere else? The concentrations of GAPmers used in this project was 5 µM which was sufficient to efficiently KD a germ plasm mRNA (Tveiten et al., 2022).

Mid-blastula is a developmental stage including the transition from maternal to embryonic gene expression (MZT), in a process where maternal mRNA is degraded and the embryonic genome becomes activated (Jukam et al., 2017; Pelegri, 2003). At this stage, there was a significantly higher expression of *pomcb* (Figure 4e), *pomca* (Figure 4d) and *gr1a* (Figure 4a) in the GAPmer treated embryos compared to that of the controls. For *pomcb* and *gr1a*, a similar difference was found between GAPmer and negative control GAP treated embryos and the controls (Figure 4 a, e). Apparent higher gene expression of GAPmers may be related to reduced or delayed capacity to degrade endogenous mRNA in non-fertilized/ non-developing embryos. Analyzing the samples, by random, less than half (44 % fertilization) of sampled GAPmer embryos were alive compared to almost four out of five in the controls (73-80 fertilized). Thus, less mRNA degradation due to non-developing embryos, may in part explain the apparent elevated

expression at this stage. This may also explain the negative control GAP A as only 20 % were fertilized of the eggs sampled for mid-blastula.

It is interesting to note that at the first sampling (embryo being ca. 32 cells), when most of the maternal mRNAs are likely to be intact (Despic & Neugebauer, 2018), the difference in gene expression recorded at mid-blastula was not observed. Another possible explanation is that the GAPmer through recruitment of the RNase H enzymes away from their regular tasks, preventing degradation of other maternal RNAs that's supposed to be degraded during the MZT. Another plausible explanation is that the GAPmer induced degradation of the *gr1b* transcript found at the first sampling, and the assumed subsequent reduction of the GR1b protein, may have influenced the clearance of maternal mRNAs in the GAPmer treated embryos. A combination of GAPmer inhibition and unfertilized eggs are also a possibility.

Regarding the *pomcb and pomca* genes, the Ct-values following the qPCR were high, varying between 34,4-39,6 and 33,9-39,7 with an average of 36,9 and 37,3 respectively. Additionally, several samples were undetermined, hence indicating that *pomca* and *pomcb* mRNA are not maternally deposited. This is in accordance with the literature where *pomc* is first observed after the beginning development of the pituitary (Nesan & Vijayan, 2013a), after 216 dd in Rainbow trout (*Oncorhynchus mykiss irideus*) (Saga et al., 1993). Alternatively, there might be an unspecific effect on the degradation of the *pomcb* RNA due to the GAPmers binding randomly or unspecific. It is also relevant to note that this too might be a result of unfertilized eggs. The *pomcb* expression in Negative Control A was also relatively high and where 90 % was unfertilized. Overall, there are relatively low visible effects of the treatments during cell cleavage and mid-blastula in the gene expression, but also in the morphology.

Ca. 50 % epiboly to hatching.

At ca. 50 % epiboly, when only live developing embryos (within all treatments) were sampled, a double control of only fertilized eggs and the (vehicle) controls, illustrated that only fertilized eggs (incubated without inhibitors and their vehicles pre-fertilization) have the same approximate expression as that of the controls GAPmer- and inhibitor treated embryos. This, suggest that unfertilized/ non-developing eggs may have affected the expression results especially at the mid-blastula stage, possibly masking treatment effects. This also strengthens the credibility of the controls.

The GAPmer treated embryos gene expression generally follows the same increasing trend as the controls and has an overall lower expression in all the genes, including *gr1a* and *gr1b*, but not for *gr2* (Figure 5). These result correlate with a study performed on zebrafish that were injected with antisense morpholino (Mo) targeting the GR gene (Wilson et al., 2016). This could indicate that knockdown of GR-signaling effect the expression of the GRs. If this may be a result of the initial degradation of gr1b, or a continued GAPmer effect also after initiation of gr expression from the embryonic genome is unclear. Within Atlantic salmon, it is not clear for how long GAPmers are effective, or how quickly they are degraded, or diluted, by the growing embryo. Tveiten et al. (2022) found a clear KD effect at 56 dd when targeting a germ plasm mRNA which is maternally supplied, but no KD- effect was detected when targeting the pigment gene (Edvardsen et al., 2014), where visible phenotype can be first observed at around 500 dd (Tveiten and Slanchev, unpublished). Thus, it is hard to decide if a KD effect beyond the earliest stages of development is possible to achieve, see however below.

The *gr2* trend appears to be slightly different having a stable expression. The gene expression of *pomca* and *pomcb* are relatively different between the GAPmer treated eggs and the controls, especially during hatching. As the GAPmers are designed to inhibit GR-signaling through degradation of mRNA, it is plausible that GR-signaling is important in the development of the pituitary regarding the timing and response. If there are GRs found in all levels of the HPI axis during early ontology as seen in juvenile fish (Farrell, 2011; Faught & Vijayan, 2018a), then inhibition of GR-signaling may lead to changes in development of the HPI-axis however, this is not confirmed and needs further research. The increase in gene expression of *pomc* may indicate the timing of development of the pituitary (Nesan & Vijayan, 2013a, 2016)

4.2 What are the influences of inhibited GR and MR signaling on HPI-axis gene expression related to maternal cortisol?

Cell cleavage and mid-blastula

Gene expression of *gr1a* (Figure 6a) showed that eplerenone were significantly less expressed than the controls, mifepristone treated eggs, as well as the eplerenone and mifepristone treated embryos. This may result from an early effect of the eplerenone on the MR in the eggs. If this is the case, the change in gene expression for embryos treated with eplerenone could indicate that MR-signaling may influence *gr1a* degradation in Atlantic salmon. It is hypothesized that MRs contribute to the development and regulation of HPI-axis however this is not proved

(Faught & Vijayan, 2018b). The apparent absence of effect in the other genes could also potentially indicate that maternal cortisol is not involved in the regulation of the investigated genes at this developmental stage or that the amount of GR protein in the embryo (maternal deposited as protein or translation of maternal mRNA).

The significantly higher *gr2* expression in eplerenone treated embryos during mid-blastula (Figure 7c) may also suggest a role for MR in *gr* mRNA stabilization or expression. Eplerenone and mifepristone treated embryos were also relatively lower expressed than the mifepristone treated embryos, perhaps indicating that the inhibitors affecting one another or that inhibition of both GRs and MR influence the expression of *gr2*. (Kiilerich et al., 2015) propose in rainbow trout, that MR-signaling can act as a repressor for GR-activity however the mechanisms regarding this are still unclear (Kiilerich et al., 2015). If this is the case using eplerenone to inhibit the MR could potentially increase the *gr2* expression. However, there is relatively scarce information regarding the relationship between MR- and GRs in fish (Kiilerich et al., 2015). It is plausible that the MR is maternally deposited both as mRNA transcripts but also as proteins, like GRs in zebrafish (Alsop & Vijayan, 2009).

50 % epiboly until hatch

Only *gr2* was differently expressed between the "only fertilized" eggs and the controls for the inhibitor treated embryos, suggesting that the vehicle control embryos may serve as adequate controls. Comparing the gene expression profiles of the embryos originating from control and inhibitor treated embryos, they revealed few differences. However, significant differences in *pomca* expression were, found, as eplerenone treated embryos were significantly higher than the eplerenone and mifepristone treated embryos from end of epiboly until hatch, but not from controls. This trend is also observed in a study performed on zebrafish, where *pomc* abundance was higher in MR knockout zebrafish embryos than GR knockout zebrafish embryos and wild-type controls (Faught & Vijayan, 2018b). As the eplerenone appeared to change the expression profile of *pomca* it can possibly indicate that it inhibited the cortisol signaling, through MR availability, which may contribute to regulation of the HPI-axis during early development (Faught & Vijayan, 2018b). In the same study (Faught & Vijayan, 2018b) *gr* transcripts were in higher abundance in the MR knockout mutants than the controls and GR knockout mutants at hatching. In this study it is there is an opposite trend, *gr* being slightly more expressed for in the controls and mifepristone treated embryos, which could indicate that the mechanisms

regulating HPI axis development may differ depending on the number of GRs (Sopinka et al., 2017). This should be researched further.

Similarly to the GAPmers different concentrations of inhibitors should have been tested as research has shown that the effects can be dose-dependent (Kiilerich et al., 2015).The ratio between cortisol and inhibitors are important, as the inhibitors are competitive, the concentration must be high enough to outcompete the cortisol without being toxic. The concentrations used in this project are based on literacy studies. The eplerenone concentration was set to 10^{-5} M based on (Kiilerich et al., 2015) study on rainbow trout. The mifepristone concentration used was $1{,}18x10^{-5}$ M, which is five times higher than in (Ferris et al., 2015), where there were relatively few observed effects. Admittedly, the mifepristone concentration was not entirely intentional. Equivalently, to the GAPmers it is difficult to decide how long the inhibitors are active. Overall, there were relatively few effects found in this study in regards to the inhibitors and gene expression.

4.3 How do antisense oligonucleotide (GAPmer) and GR and MR-inhibitors influence embryonic phenotype?

Phenotypes including morphology varied between the GAPmer treated embryos and the controls. At approximately 50 % epiboly (Figure 11) there were several GAPmer treated eggs appeared to have begun developing but stopped or developed at a slightly slower pace, representing approximately 8 % of the mortality in the GAPmer treated embryos. The phenotypic differences observed might stem from the changes in gene expression of *gr1a*, as it was significantly lower than the controls. A plausible cause might be abnormal mRNA degradation at mid-blastula stopping or stalling the development as it is essential for further development, as seen in zebrafish (Giraldez et al., 2006; Jukam et al., 2017; Nesan & Vijayan, 2013b). In zebrafish, both GR knockout and GR knockdown have resulted in fish with morphological deformities, elevated stress response and reduced cardiac function (Faught & Vijayan, 2018a), all of which could stem from timing of development, influenced by GR-signaling. No other treatment appeared to stall the development or change the morphology, which suggest that the other treatments may have been less effective at this stage or that it may be due to the GAPmers.

At 118-119 dd when the embryo had reached about 95 % epiboly the GAPmer treated embryos also showed a different developmental pace compared to the other treatments and controls

(Figure 12). This can be a consequence of a stall in development due to possible previous abnormal mRNA degradation (Giraldez et al., 2006; Jukam et al., 2017). It may also indicate failure to complete gastrulation.

At hatch, the yolk-sac larvae differed between the treatments in appearance (Figure 14). The control yolk-sac larvae were overall slightly smaller in size with a relatively larger more elongated yolk-sac compared to the GAPmer treated embryos. The GAPmer treated embryos were slightly larger in size but had smaller and rounder yolk sacs perhaps indicating a faster mobilization/ allocation of the energy (Farrell, 2011; Sopinka et al., 2017) absorbed from the yolk sac leading to slightly increased growth. However, if this is the case it might present unknown future consequences, for example depletion of the yolk sac before "start feeding". In addition, a few of the yolk-sac larvae treated with GAPmers had bent spines as shown in (Figure 14h). These changes indicate that the GAPmers had an effect of some degree and illustrates how maternal cortisol signaling may be linked with or influence different developmental systems (Auperin & Geslin, 2008; Sopinka et al., 2017).

Embryos treated with both eplerenone, and mifepristone were slightly smaller in size, and their yolk-sacs had the approximate same size as the controls. A study performed on zebrafish indicated that inhibition of GR-signaling modulated insulin-growth-factor (IGF) availability leading to reduced body size (Nesan & Vijayan, 2013b). In comparison to the controls, the yolk-sacs of the eplerenone and mifepristone embryos appeared more elongated. Again, this may indicate that potential inhibition of maternal cortisol signaling influence metabolic processes (Auperin & Geslin, 2008; Farrell, 2011).

4.4 Influences in HPI-axis development

Results from this project did not provide conclusive evidence that GR- and MR- signaling is essential to the development of the HPI-axis. However, the results indicated that the GAPmers did have a KD effect on the GR1b receptor at 14 dd and that *gr1a* was significantly lower expressed from ca. 50 % epiboly until hatch. Consequently, GR- and MR-signaling could still have a functional role during early development. Treatment effects may have been observed later in life or not being apparent with the methods used to analyze the embryos under the current investigation. Also, confounded effects due to low fertilization may have occurred. The yolk-sac larvae was not sufficiently developed to elicit a stress response (in terms of cortisol release to plasma) when the experiment was terminated, and to investigate possible effects on

HPI-axis functionality would have been required (Auperin & Geslin, 2008; Sopinka et al., 2017). Additionally, the behavior of hatched larvae towards start-feeding would have been beneficial to study, as several studies has shown that modulation of GR-signaling and MR-signaling alter the behavioral (Faught & Vijayan, 2018a, 2018b). Another thing to note is that changes in expression as a result of the treatments is not necessarily limited to the HPI-axis (Sopinka et al., 2017).

4.5 Future research

In Zebrafish, blocking of GR-signaling lead to heighten stress response indicating the role of GR-signaling in the programming of the HPI-axis (Nesan & Vijayan, 2016; Wilson et al., 2016). It shows that changes during early development affect the fitness of adult fish. Most studies are performed on zebrafish that only has a single GR-gene, however salmonids and other teleost fish with several GR-genes might have systems that work differently (Sopinka et al., 2017). It is shown in Atlantic salmon that stress in early life development can cause epigenetic changes through methylation, which in turn affects adult fish (Moghadam et al., 2017).

Although there is relatively limited knowledge regarding maternal cortisol when it comes to deposition, regulation and metabolism in fish embryos, research has clearly shown that maternal cortisol can change phenotype and affect several developmental systems (Sopinka et al., 2017). The Atlantic salmon industry suffers losses through all phases of the production cycle. A large part of these losses is due to a physiological inferior fish, especially when it comes to coping with stressful handling operations during the production cycle (Sommerset et al., 2024). Therefore, more research is necessary to understand how stress and GR-signaling during early life may have an impact on how fish handle stress and cope with aquaculture conditions during later life. This may be especially important when it comes to female broodfish where stress, and cortisol transfer to the egg, may influence thousands of offsprings. Research investigating the effects of maternal cortisol signaling and how it may influence the entire lifecycle is therefore necessary to understand. In particular the role of GR signaling during development before the “eyed stage”, as much of the organ development happens here, and when, at the same time embryo cortisol content is high and changing (Auperin & Geslin, 2008). Early development creates the foundation for fish fitness and performance, thus understanding the early development may help improve fish health welfare and change the view of how the broodstock fish are handled and chosen.

5 Conclusion

There were no drastic effects observed due to the treatments. The GAPmer treated embryos appeared to have successfully knocked down (KD) the GR1b receptor resulting in a significantly lower expression during cell cleavage. From ca. 50 % epiboly until hatch the GAPmer treated embryos had reduced gene expressions compared to the controls in all genes, except in *pomca*. This decrease in gene expression was significant for *gr1a* between GAPmer treated embryos and controls. Regarding the inhibitor treated embryos, eplerenone treated embryos showed a lower *gr1a* expression during cell cleavage, but a slightly higher expression of *gr2* compared to eplerenone and mifepristone at mid-blastula. From ca. 95 % epiboly until hatch eplerenone treated embryos are significantly higher expressed compared to eplerenone and mifepristone treated embryos. Overall, the gene expression profiles of all the genes followed the expected ontology from 50% epiboly until hatch. In this study, the observed phenotypic changes were on the GAPmer treated embryos, where they displayed a delayed development leading to mortality and larger yolk sac larvae with slightly smaller and rounder yolk sacs compared to the controls. The results indicate that GR- and MR-signaling may affect important regulators of the HPI-axis during early development. There is a need for more research on maternal cortisol in early development and the influence it has on HPI-axis functionality and stress coping during later in life.

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7 Appendix

Preparation for egg arrival

Different solutions were made for the egg's arrival. Cortland solution, activation fluid, phenol red, glutathione water and trout balanced salt solution (TBSS) were prepared a couple of days in advance.

Table 8 Composition of the solutions used to wash and fertilize the eggs, as well as resuspend the GAPmers.

Buffer	Composition	Amount	pH	mOsm
Cortland solution	sodium chloride (NaCl)	ca. 35 g	8,5	290 ± 3
	potassium chloride (KCl)	1,9 g		
	magnesium sulphate ($MgSO_4$)	0,6 g		
	calcium chloride ($CaCl_2$)	1,66 g		
	glucose	5,38 g		
	HEPES	25,05 g		
	Milli-Q water	5L		
The activation fluid	sodium hydrogen carbonate ($NaHCO_3$)	5,04 g	8,00	280-285
	trizma base (Tris)	6,06 g		
	filtered water from the incubation cabinet	To make 1L		
Glutathione water	L-Gluth	1,536 g	7,8-8	
	filtered water from the incubation cabinet	5 L		
Trout balanced salt solution (TBSS)	sodium chloride (NaCl)	4,30 g	8	298
	potassium chloride (KCl)	0,115 g		
	magnesium sulphate ($MgSO_4$)	0,035 g		
	magnesium chloride ($MgCl_2$)	0,10 g		
	glucose	0,50 g		
	Milli-Q water	500 ml		

Cortland: KCl, $MgSO_4$, $CaCl_2$ and HEPES were added to 4,8 L of Milli-Q water in a large glass bottle with a magnet. Everything was combined using a magnet stirrer, and 30 g of NaCl was added. Osmolality at least three measurements with a mean of ± 3 of 290. Finished Cortland solutions were kept in the cold room.

TBSS: All the powders were mixed and added to a part of the water before the remaining water was added, giving a total volume of 500 mL. This solution was well mixed using a magnet stirrer and then sterile-filtered with a syringe into a glass bottle. All solutions were kept in autoclaved glass bottles or new 15 mL Falcon tubes and kept in the cold room.

Activation fluid: $NaHCO_3$ and Tris were mized with the water and pH and osmolaity adjusted. Glutathione water: L-Gluth was dissolved in filtered water from the hatching cabinet and pH adjusted.

The osmometer used to measure the osmolality is the Fiske 110 osmometer (Fiske®Associates) and the pH was measured using the pH7110 pH meter from inoLab®.

Table 9 Temperatures of the incubation water from fertilisation of the eggs until hatching.

Water temperature	**°C**
11-14/10/2023	7
15-21/10/2023	6
23-31/10/2023	5
01-10/11/2023	5
11-30/11/2023	4
01-31/12/2023	4
01.01.2024	4
02.01.2024	5
03-14/01/2024	4
15.01.2024	3

Sequence 1 sequence of the GR1a GAPmer and where in the genome the GAPmer bind and is most likely to induce knockdown.

Salmon GR1a Ssa04

>XM_045717356.1 PREDICTED: Salmo salar glucocorticoid receptor (LOC100380779), mRNA

GapmeR GR1AB 1_0 target on Salmon GR1a chr. 4 and GR1b on chr. 13 mRNA: GTCTGACCTGAATGAGT

gatcaagaa**ATG**GATCCAGGTGGACTGAAACACAGCATTAACAAGGACAAGGGCTTGGCTTTCGGTAAACTCTCAGA
GAGCGGTGTAGAAGGAAGCTTCTCGGGTGACGCAGGTGGTTCCAAGTCCACCACATCAACCTCCCTGATGCATC
TGCCAGGCTCCAGACCTCAGCCACCAGCCAGAGACTCCGCCAACGGACTGAATGTCACTACGACTCAGATGGA
GCTCTCCACAGGGGGACTCACTATAGAGGAGTCTGAAGTGAAGGTAATGGAGAAGGCCCTTAGAATGCAGCAG
CCACAGAAACCGCAGCAGAACCAACAGCTGTTTGAGAACTTTGCTCTGTTGGAGGCGAGTATTGCAGATTTGAA
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CAATACAATGGACATTCTGCAGGATTTGGACTTGCCTGGCTCTCT**GTCTGACCTGAATGAGT**TTTACGTGTCTGAT
GAGGCTGCTTTCCTTTCCTCCCTGTCAGTGGAGGATGTCCTGCTCGAGGACGGCAACATGGAAACCAAGCCCAT
AGACTGTAGCAATGGGGGAAACTGTACAAATGTGGACAGCGCGGACCAGCAGAAGCAGCTTCTGGAGCCAGGC
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GCTGCTCAGCACAGTACCAAAGGATGGATTGAAGAGCCAGGCTGTGTTTGATGAGATCCGCATGACCTACATAA
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GACCGTGAATCACTTCATAACAGTTGATTATTGTGAGATTGGAGGAATCAAATTCTACCAACGAAAAGAGATTAA
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AATGTCTGTAGTTCTGGTTGCTCTAGTTTCACTTCATGACCTCCTAGGTGATTATCAAATGTTTCTCCAGATAAACC
TGTTCATAAAATAAAAGCTTTTTCAATCATT

Sequence 2 sequence of the GR1b GAPmer and where in the genome the GAPmer bind and is most likely to induce knockdown.

Salmon GR1b Ssa13

>XM_014136782.2 Salmo salar glucocorticoid receptor (LOC106567492), transcript variant X1, mRNA

tcgccccatgtacattttatacggactgtgttgcgccattgaaatgattcatgttagtcacagtttgccgtgcttttgaagtttacacaattgtgccgaacttaactgtctctatataacgttgtta
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gtttggaaaactcccatttctgattaaggatcaagaa**ATG**GATAACCAGGACAAGAGCTTAGAGAGAGGTGAAGAGGGAAGCTTCTCG
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Sequence 3 sequence of the GR2 GAPmer and where in the genome the GAPmer bind and is most likely to induce knockdown.

Salmon GR2 Ssa 05

>XM_014198677.2 PREDICTED: Salmo salar glucocorticoid receptor-like (LOC106604224), transcript variant X1, mRNA

GapmeR GR2 1_0 target on Salmon GR2 chr. 5 mRNA: CTACTAAAGGCCATCG

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Method testing

Different techniques and methods for RNA extraction of salmon eggs were tested to determine which method to use in this project. Before the project began, older eggs (test eggs) stored in RNA-later at different day degrees were used. Based on (Bhat et al., 2023) test eggs at different day degrees were fixated in 5 % acetic acid for 5, 10 and 15 minutes. The objective of this fixation was to sturdy and stain the cell clumps into a darker shade. However, due to the RNA-later, this was not possible, making it difficult to isolate the cell clumps. In an attempt to counteract the consequences of RNA-later, eggs were placed in phosphate-buffered saline (PBS) for 15 minutes to dilute RNA-later before transferring the eggs to acetic acid for 5 minutes. This facilitated the isolation of cell clumps, however, cells became brittle in consistency and only worked on later day degrees. Thus, the decision that this method was unsustainable.

Due to the difficulty of older eggs stored in RNA-later, a new testing session was run with fresh eggs. Two days post fertilisation, fresh eggs were placed in acetic acid for 5 minutes, fixating the cells. The cells became darker in colour and firmer, enabling isolation. Cell clumps were then isolated using tweezers and placed in Eppendorf tubes, one tube per cell clump, containing 500 µl cooled PBS and centrifuged for 7 minutes at 12000 rpm. Next, the supernatant was removed using a pipette, and was repeated twice cleaning the cells as explained in (Bhat et al., 2023). Next, 200 µl RNA-later was added to three egg-cell clumps, which were stored at 4 °C. 200 µl trizol was added to another three egg-cell clumps and placed in a -20 °C freezer. Another three egg-cell clumps were directly taken through the trizol method (2.3.2). The following day, the egg-cell clumps stored in RNA-later and trizol overnight, as well as whole eggs left in RNA-later overnight from the same sampling, were taken through the trizol method. The cell clumps stored in RNA-later gave bad results. Cell clumps stored in trizol and just isolated cell clumps showed acceptable results however, after reviewing the method testing of RNA extraction, the trizol method of whole egg isolation would give the most coherent and best results for this project (Table 10).

Table 10 shows the concentration (ng/ µl) and purity (260/ 280) of the RNA-extractions performed during method testing. Eggs and cellclumps stored in RNA-later or trizol before trizol extraction. Additionally, eggs and cellclumps that had the RNA-extraction right after sampling.

Sample ID	ng/µl	A260	260/ 23(	260/28(	Date	Nr.	DD
egg group 6 RNA-later	172,60	4,315	1,14	1,68	14.10.2023	1	14
egg group 6 RNA-later	238,79	5,970	1,37	1,61	14.10.2023	2	14
egg group 6 RNA-later	294,87	7,372	4,30	1,65	14.10.2023	3	14
egg group 13 RNA-later	342,19	8,555	3,82	1,67	14.10.2023	4	14
egg group 13 RNA-later	198,01	4,950	1,05	1,59	14.10.2023	5	14
egg group 13 RNA-later	347,87	8,697	1,17	1,64	14.10.2023	6	14
cellclump group 6 RNA-later	2,09	0,052	0,07	1,51	14.10.2023	7	14
cellclump group 6 RNA-later	6,83	0,171	0,01	2,87	14.10.2023	8	14
cellclump group 6 RNA-later	3,17	0,079	0,01	2,22	14.10.2023	9	14
cellclump group 13	97,77	2,444	0,61	1,79	14.10.2023	10	14
cellclump group 13	19,68	0,492	0,24	1,72	14.10.2023	11	14
cellclump group 13	38,31	0,958	0,09	1,76	14.10.2023	12	14
egg group 1 RNA-later	339,84	8,496	2,20	1,91	15.10.2023	1	14
egg group 1 RNA-later	321,87	8,047	2,38	1,90	15.10.2023	2	14
egg group 1 RNA-later	305,47	7,637	1,56	1,94	15.10.2023	3	14
cellclump group 1 RNA-later	250,53	6,263	2,00	1,92	15.10.2023	4	14
cellclump group 1 RNA-later	114,32	2,858	1,75	1,87	15.10.2023	5	14
cellclump group 1 RNA-later	1,08	0,027	0,12	2,21	15.10.2023	6	14
cellclump Test egg	428,91	10,723	1,99	1,90	14.11.2023	1	ca.180
cellclump test egg	249,72	6,243	1,97	1,88	14.11.2023	2	ca.180
cellclump Test egg	363,94	9,098	2,36	1,92	14.11.2023	3	ca.180
egg isolation test egg	722,47	18,062	0,29	1,85	14.11.2023	4	ca.180
egg isolation test egg	1283,00	32,096	0,53	1,96	14.11.2023	5	ca.180
egg isolation test egg	564,35	14,109	1,75	1,92	14.11.2023	6	ca.180
cellclump RNA-later test eggs	339,84	8,496	2,20	1,91	15.11.2023	7	ca.180
cellclump RNA-later test eggs	321,87	8,047	2,38	1,90	15.11.2023	8	ca.180
cellclump RNA-later test eggs	305,47	7,637	1,56	1,94	15.11.2023	9	ca.180
cellclump trizol freeze test eggs	250,53	6,263	2,00	1,92	15.11.2023	10	ca.180
cellclump trizol freeze test eggs	114,32	2,858	1,75	1,87	15.11.2023	11	ca.180
cellclump trizol freeze test eggs	1,08	0,027	0,12	2,21	15.11.2023	12	ca.180

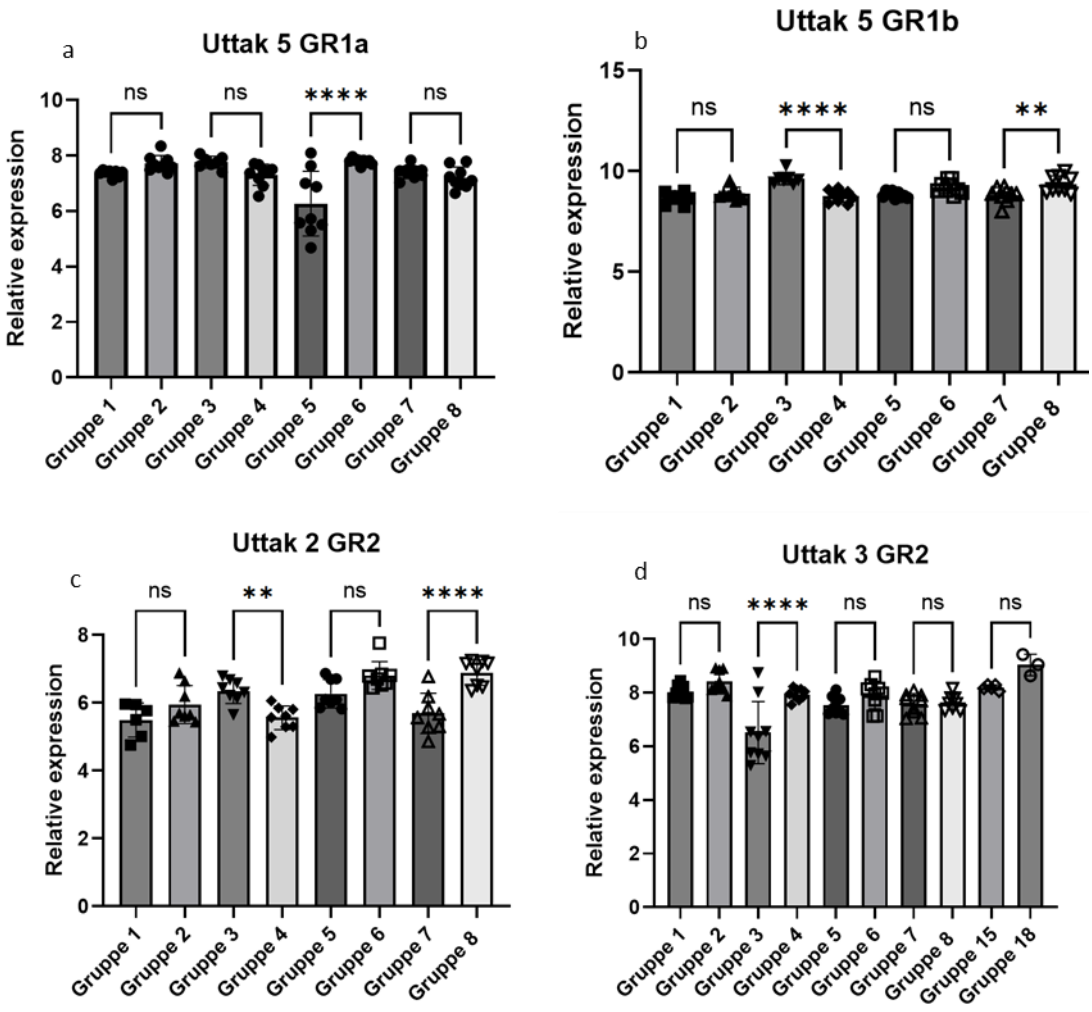

Figure 15 shows significant differences between duplicate groups that received the same treatment.

Table 11 Sample size ratio and variance of the genes for sampling 1, sampling 2, and from sampling 3 to 6 for both GAPmer treated embryos and inhibitor treated embryos and their controls.

Sampling	Treatments	GR1a	GR1b	GR2	POMCA	POMCB
		Sample size ratio				
1	Inhibitors	2,14	2,5	2,14	1,5	2
2	Inhibitors	1,14	1,23	1,14	1,78	1,27
3-6.	Inhibitors	2,25	2,25	2,25	2,25	2,25
1	GAPmers	2,6	3,75	2,5	1,5	4
2	GAPmers	2	2,5	2	3,2	3,5
3-6.	GAPmers	2,75	2,75	2,75	2,75	2,75
		Variance ratio				
1	Inhibitors	9,67	4,72	13,11	7,02	6,48
2	Inhibitors	1,65	2,51	2,59	3,39	3,28
3-6.	Inhibitors	17,78	9,58	24,26	24,15	19,47
1	GAPmers	2,13	1,42	17,34	5,67	9,11
2	GAPmers	5,57	8,44	4,53	1,82	1,76
3-6.	GAPmers	8,73	10,48	4,69	9,53	12,39

Table 12 Significance found in data variance and heteroskedasticity following an Levene's test in variance and an F test for heteroskedasticity in the software SPSS.

	Levene's test of Equality of Error Variances				
	gr1a	*gr1b*	*gr2*	*pomca*	*pomcb*
Inhibitors (E, M, C, EM)	0,11	0,273	0,033	0,209	0,228
GAPmers (C, GAP, NGA)	0,442	0,693	0,028	0,334	0,003
Inhibitors (E, M, C, EM)	0,242	0,135	0,072	0,098	0,446
GAPmers (C, GAP, NGA)	0,052	0,002	0,032	0,785	0,746
Inhibitors (E, M, C, EM)	0	0	0	0	0
GAPmers (C, GAP, NGA)	0	0,082	0,029	0,005	0,006
	F Test for Heteroskedasticity				
	gr1a	*gr1b*	*gr2*	*pomca*	*pomcb*
	0,029	0,537	0,614	0,13	0,09
	0,679	0,849	0,373	0,457	0,001
	0,939	0,354	0,789	0,133	0,212
	0,423	0,006	0,645	0,488	0,483
	0,969	0	0	0	0
	0,001	0,011	0,651	0,485	0

Table 13 presents mortality or unfertilized eggs in the different treatment groups by percentage, as well as the number of unfertilized (dead) and alive eggs there were through the study.

	Group	Dead	Alive	Total	% Mortality/ unfertilized
Eplerenone	1	42	147	189	22,22
	2	31	170	201	15,42
Mifepristone	3	31	198	229	13,54
	4	35	160	195	17,95
Eplerenone and Mifepristone	5	34	160	194	17,53
	6	54	185	239	22,59
Controls	7	31	126	157	19,75
	8	43	123	166	25,90
GAPmers	9	143	29	172	83,14
	10	119	29	148	80,41
	13	100	80	180	55,56
	14	45	15	60	75,00
GAP Control Negative A	11	25	24	49	51,02
	16	148	17	165	89,70
Only fertilized	15	172	53	225	76,44
	18	245	3	248	98,79
	19	168	0	168	100

Table 14 p-values of a One-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 1 for the GAPmers. The table includes controls (C), eggs injected with Negative Control A (NGA) and eggs injected with GAPmers (GAP). Significant differences are coloured in blue (p≤0,05).

Sampling 1			
Gen	C vs. GAP	C vs. NGA	GAP vs. NGA
GR1a	0,55120	0,99970	0,61300
GR1b	0,00440	0,87820	0,03400
GR2	0,28790	0,27550	>0,9999
POMCA	0,99280	0,99680	0,96800
POMCB	0,45860	0,94480	0,41590

Table 15 p-values of a One-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 2 for the GAPmers. The table includes controls (C), eggs injected with Negative Control A (NGA) and eggs injected with GAPmers (GAP). Significant differences are coloured in blue (p≤0,05).

Sampling 2			
Gen	C vs. GAP	C vs. NGA	GAP vs. NGA
GR1a	0,00060	0,00940	0,77080
GR1b	0,11600	0,14420	0,99070
GR2	0,31510	0,51530	0,98800
POMCA	0,00090	0,99950	0,01420
POMCB	0,00070	0,11630	0,60350

Table 16 p-values of Two-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 3-6 for the GAPmers. The table includes controls (C), just-fertilized (F) eggs and eggs injected with GAPmers (GAP). Significant differences are coloured in blue (p≤0,05).

Sampling 3-6 GAP					
Gene	**GR1a**	**GR1b**	**GR2**	**POMCA**	**POMCB**
91-94:C vs. 91-94:GAP	<0,0001	0,0023	0,0012	>0,9999	>0,9999
91-94:C vs. 91-94:F	0,9998	>0,9999	0,0175	>0,9999	>0,9999
91-94:C vs. 118-119:C	>0,9999	0,0498	>0,9999	<0,0001	<0,0001
91-94:C vs. 118-119:GAP	0,0124	>0,9999	0,2543	0,0001	<0,0001
91-94:C vs. 294-295:C	<0,0001	<0,0001	0,1091	>0,9999	>0,9999
91-94:C vs. 294-295:GAP	<0,0001	<0,0001	0,0002	>0,9999	>0,9999
91-94:C vs. 423-426:C	<0,0001	<0,0001	>0,9999	0,7535	<0,0001
91-94:C vs. 423-426:GAP	<0,0001	<0,0001	0,9573	0,9887	0,0055
91-94:GAP vs. 91-94:F	0,003	>0,9999	0,9808	>0,9999	>0,9999
91-94:GAP vs. 118-119:C	0,0043	<0,0001	0,3392	0,0006	0,0003
91-94:GAP vs. 118-119:GAP	>0,9999	0,0498	>0,9999	<0,0001	<0,0001
91-94:GAP vs. 294-295:C	<0,0001	<0,0001	>0,9999	>0,9999	>0,9999
91-94:GAP vs. 294-295:GAP	<0,0001	<0,0001	0,1091	>0,9999	>0,9999
91-94:GAP vs. 423-426:C	<0,0001	<0,0001	0,0517	0,903	0,0004
91-94:GAP vs. 423-426:GAP	<0,0001	<0,0001	>0,9999	0,7535	<0,0001
91-94:F vs. 118-119:C	>0,9999	0,1425	0,0154	0,0648	0,0028
91-94:F vs. 118-119:GAP	0,0041	>0,9999	0,9734	0,0496	0,0016
91-94:F vs. 294-295:C	<0,0001	<0,0001	0,9829	>0,9999	>0,9999
91-94:F vs. 294-295:GAP	<0,0001	<0,0001	>0,9999	>0,9999	>0,9999
91-94:F vs. 423-426:C	<0,0001	<0,0001	0,0033	0,9851	0,1579
91-94:F vs. 423-426:GAP	<0,0001	<0,0001	0,7869	0,9943	0,2543
118-119:C vs. 118-119:GAP	<0,0001	0,0023	0,0012	>0,9999	>0,9999
118-119:C vs. 294-295:C	<0,0001	<0,0001	0,0793	<0,0001	<0,0001
118-119:C vs. 294-295:GAP	<0,0001	<0,0001	0,0002	0,0032	<0,0001
118-119:C vs. 423-426:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:C vs. 423-426:GAP	<0,0001	<0,0001	0,9562	<0,0001	<0,0001
118-119:GAP vs. 294-295:C	<0,0001	<0,0001	>0,9999	0,0001	<0,0001
118-119:GAP vs. 294-295:GAP	<0,0001	<0,0001	0,0793	<0,0001	<0,0001
118-119:GAP vs. 423-426:C	<0,0001	<0,0001	0,0301	<0,0001	<0,0001
118-119:GAP vs. 423-426:GAP	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
294-295:C vs. 294-295:GAP	<0,0001	0,0023	0,0012	>0,9999	>0,9999
294-295:C vs. 423-426:C	0,3047	0,0001	0,0153	0,7978	0,0057
294-295:C vs. 423-426:GAP	0,9996	0,9491	>0,9999	0,9815	0,0782
294-295:GAP vs. 423-426:C	<0,0001	<0,0001	<0,0001	0,9527	0,0371
294-295:GAP vs. 423-426:GAP	0,3047	0,0001	0,0153	0,7978	0,0057
423-426:C vs. 423-426:GAP	<0,0001	0,0023	0,0012	>0,9999	>0,9999

Table 17 p-values of a One-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 1 for the inhibitors. The table includes controls (C), eggs treated with eplerenone (E), mifepristone (M) and eplerenone and mifepristone (EM). Significant differences are coloured in blue (p≤0,05).

Sampling 1						
Gen	C vs. E	C vs. M	C vs. EM	E vs. M	E vs. EM	M vs. EM
GR1a	0,00060	0,99760	0,99960	0,00100	0,00230	>0,9999
GR1b	0,29570	0,31720	0,99800	>0,9999	0,86050	0,83380
GR2	0,22460	0,97530	0,66920	0,85820	0,99910	0,99110
POMCA		0,14690	0,11820			0,99640
POMCB	0,98000	>0,9999	0,67340	0,96550	0,33740	0,85750

Table 18 p-values of a One-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 2 for the inhibitors. The table includes controls (C) and eggs treated with eplerenone (E), mifepristone (M) and eplerenone and mifepristone (EM). Significant differences are coloured in blue (p≤0,05).

Sampling 2						
Gen	C vs. E	C vs. M	C vs. EM	E vs. M	E vs. EM	M vs. EM
GR1a	0,15440	0,70410	0,97490	0,91790	0,56330	0,99180
GR1b	0,22810	0,45730	0,98720	0,99830	0,60720	0,87470
GR2	0,10750	0,54990	0,82180	0,93470	0,00480	0,05040
POMCA	0,51530	>0,9999	0,87950	0,67080	0,98250	0,94860
POMCB	0,08870	0,56450	0,98740	0,94400	0,38790	0,94600

Table 19 p-values of Two-way ANOVAs with Sidak multiple comparison posthoc tests comparing sampling 3-6 for the inhibitors. The table includes controls (C), just-fertilized eggs (F) and eggs treated with eplerenone (E), mifepristone (M) and eplerenone and mifepristone (E and M). Significant differences are coloured in blue (p≤0,05).

Sampling 3-6 Inhibitor					
Gene	**GR1a**	**GR1b**	**GR2**	**POMCA**	**POMCB**
91-94:C vs. 91-94:E	0,0077	>0,9999	0,8233	>0,9999	>0,9999
91-94:C vs. 91-94:M	0,0837	>0,9999	>0,9999	0,9995	0,9787
91-94:C vs. 91-94:E and M	0,0171	>0,9999	0,124	0,0412	>0,9999
91-94:C vs. 91-94:F	>0,9999	>0,9999	0,0002	0,9967	>0,9999
91-94:C vs. 118-119:C	0,7152	0,9045	<0,0001	>0,9999	<0,0001
91-94:C vs. 118-119:E	0,0005	>0,9999	<0,0001	>0,9999	0,0133
91-94:C vs. 118-119:M	0,0034	>0,9999	<0,0001	0,9997	<0,0001
91-94:C vs. 118-119:E and M	0,0009	>0,9999	<0,0001	0,2355	<0,0001
91-94:C vs. 294-295:C	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
91-94:C vs. 294-295:E	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
91-94:C vs. 294-295:M	<0,0001	<0,0001	<0,0001	0,0216	>0,9999
91-94:C vs. 294-295:E and M	<0,0001	<0,0001	<0,0001	0,6655	>0,9999
91-94:C vs. 423-426:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:C vs. 423-426:E	<0,0001	<0,0001	0,9779	<0,0001	<0,0001
91-94:C vs. 423-426:M	<0,0001	<0,0001	>0,9999	<0,0001	0,0073
91-94:C vs. 423-426:E and M	<0,0001	<0,0001	0,5455	0,0159	0,0015
91-94:E vs. 91-94:M	>0,9999	>0,9999	>0,9999	0,0519	0,4313
91-94:E vs. 91-94:E and M	>0,9999	>0,9999	>0,9999	<0,0001	0,9017
91-94:E vs. 91-94:F	0,9995	>0,9999	0,0246	>0,9999	>0,9999
91-94:E vs. 118-119:C	>0,9999	0,9551	0,0916	>0,9999	0,0002
91-94:E vs. 118-119:E	0,7152	0,9045	<0,0001	>0,9999	<0,0001
91-94:E vs. 118-119:M	>0,9999	0,9738	0,0034	0,2521	<0,0001
91-94:E vs. 118-119:E and M	>0,9999	>0,9999	<0,0001	0,003	<0,0001
91-94:E vs. 294-295:C	<0,0001	<0,0001	0,2733	0,0073	>0,9999
91-94:E vs. 294-295:E	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
91-94:E vs. 294-295:M	<0,0001	<0,0001	0,016	0,6515	>0,9999
91-94:E vs. 294-295:E and M	<0,0001	<0,0001	<0,0001	>0,9999	>0,9999
91-94:E vs. 423-426:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:E vs. 423-426:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:E vs. 423-426:M	<0,0001	<0,0001	>0,9999	0,0153	0,0459
91-94:E vs. 423-426:E and M	<0,0001	<0,0001	>0,9999	0,6013	0,0112
91-94:M vs. 91-94:E and M	>0,9999	>0,9999	0,9961	>0,9999	>0,9999
91-94:M vs. 91-94:F	>0,9999	>0,9999	0,0027	0,4271	>0,9999
91-94:M vs. 118-119:C	>0,9999	>0,9999	0,003	>0,9999	0,435
91-94:M vs. 118-119:E	0,9592	>0,9999	<0,0001	0,9982	0,9139
91-94:M vs. 118-119:M	0,7152	0,9045	<0,0001	>0,9999	<0,0001
91-94:M vs. 118-119:E and M	0,9906	>0,9999	<0,0001	>0,9999	0,0077
91-94:M vs. 294-295:C	<0,0001	<0,0001	0,0128	<0,0001	0,7995
91-94:M vs. 294-295:E	<0,0001	<0,0001	<0,0001	<0,0001	0,3377
91-94:M vs. 294-295:M	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
91-94:M vs. 294-295:E and M	<0,0001	<0,0001	<0,0001	0,0086	>0,9999
91-94:M vs. 423-426:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:M vs. 423-426:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:M vs. 423-426:M	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:M vs. 423-426:E and M	<0,0001	<0,0001	0,9997	<0,0001	<0,0001
91-94:E and M vs. 91-94:F	>0,9999	>0,9999	0,0875	0,0433	>0,9999
91-94:E and M vs. 118-119:C	>0,9999	0,6702	0,4705	0,9969	0,1564
91-94:E and M vs. 118-119:E	0,9985	0,9965	0,0008	0,1735	0,5781
91-94:E and M vs. 118-119:M	>0,9999	0,7373	0,0326	>0,9999	0,0003
91-94:E and M vs. 118-119:E and M	0,7152	0,9045	<0,0001	>0,9999	<0,0001

91-94:E and M vs. 294-295:C	<0,0001	<0,0001	0,8484	<0,0001	0,9864
91-94:E and M vs. 294-295:E	<0,0001	<0,0001	0,0048	<0,0001	0,7138
91-94:E and M vs. 294-295:M	<0,0001	<0,0001	0,1313	<0,0001	>0,9999
91-94:E and M vs. 294-295:E and M	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
91-94:E and M vs. 423-426:C	<0,0001	<0,0001	0,9996	<0,0001	<0,0001
91-94:E and M vs. 423-426:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:E and M vs. 423-426:M	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:E and M vs. 423-426:E and M	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
91-94:F vs. 118-119:C	>0,9999	0,9874	>0,9999	0,9097	0,0975
91-94:F vs. 118-119:E	0,2795	>0,9999	>0,9999	>0,9999	0,2493
91-94:F vs. 118-119:M	0,5356	0,991	>0,9999	0,1712	0,0024
91-94:F vs. 118-119:E and M	0,3562	>0,9999	>0,9999	0,0121	0,0059
91-94:F vs. 294-295:C	<0,0001	<0,0001	0,9997	>0,9999	>0,9999
91-94:F vs. 294-295:E	<0,0001	<0,0001	>0,9999	0,9997	>0,9999
91-94:F vs. 294-295:M	<0,0001	<0,0001	>0,9999	>0,9999	>0,9999
91-94:F vs. 294-295:E and M	<0,0001	<0,0001	>0,9999	>0,9999	>0,9999
91-94:F vs. 423-426:C	<0,0001	<0,0001	0,0008	0,9993	0,4784
91-94:F vs. 423-426:E	<0,0001	<0,0001	0,0674	0,6566	0,2213
91-94:F vs. 423-426:M	<0,0001	<0,0001	0,0088	>0,9999	0,9998
91-94:F vs. 423-426:E and M	<0,0001	<0,0001	0,2131	>0,9999	0,994
118-119:C vs. 118-119:E	0,0077	>0,9999	0,8233	>0,9999	>0,9999
118-119:C vs. 118-119:M	0,0837	>0,9999	>0,9999	0,9995	0,9787
118-119:C vs. 118-119:E and M	0,0171	>0,9999	0,124	0,0412	>0,9999
118-119:C vs. 294-295:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:C vs. 294-295:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:C vs. 294-295:M	<0,0001	<0,0001	>0,9999	0,0022	0,0253
118-119:C vs. 294-295:E and M	<0,0001	<0,0001	0,9985	0,1725	0,0052
118-119:C vs. 423-426:C	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:C vs. 423-426:E	<0,0001	<0,0001	0,3664	<0,0001	<0,0001
118-119:C vs. 423-426:M	<0,0001	<0,0001	0,0212	<0,0001	<0,0001
118-119:C vs. 423-426:E and M	<0,0001	<0,0001	0,9154	0,0015	<0,0001
118-119:E vs. 118-119:M	>0,9999	>0,9999	>0,9999	0,0519	0,4313
118-119:E vs. 118-119:E and M	>0,9999	>0,9999	>0,9999	<0,0001	0,9017
118-119:E vs. 294-295:C	<0,0001	<0,0001	0,9901	0,0007	0,0002
118-119:E vs. 294-295:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:E vs. 294-295:M	<0,0001	<0,0001	>0,9999	0,1721	0,1303
118-119:E vs. 294-295:E and M	<0,0001	<0,0001	>0,9999	0,9966	0,0327
118-119:E vs. 423-426:C	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:E vs. 423-426:E	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:E vs. 423-426:M	<0,0001	<0,0001	<0,0001	0,0016	<0,0001
118-119:E vs. 423-426:E and M	<0,0001	<0,0001	0,0061	0,1481	<0,0001
118-119:M vs. 118-119:E and M	>0,9999	>0,9999	0,9961	>0,9999	>0,9999
118-119:M vs. 294-295:C	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:M vs. 294-295:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:M vs. 294-295:M	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:M vs. 294-295:E and M	<0,0001	<0,0001	>0,9999	0,0008	<0,0001
118-119:M vs. 423-426:C	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:M vs. 423-426:E	<0,0001	<0,0001	0,0201	<0,0001	<0,0001
118-119:M vs. 423-426:M	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:M vs. 423-426:E and M	<0,0001	<0,0001	0,1549	<0,0001	<0,0001

118-119:E and M vs. 294-295:C	<0,0001	<0,0001	0,6337	<0,0001	<0,0001
118-119:E and M vs. 294-295:E	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:E and M vs. 294-295:M	<0,0001	<0,0001	>0,9999	<0,0001	0,0002
118-119:E and M vs. 294-295:E and M	<0,0001	<0,0001	>0,9999	<0,0001	<0,0001
118-119:E and M vs. 423-426:C	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:E and M vs. 423-426:E	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:E and M vs. 423-426:M	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
118-119:E and M vs. 423-426:E and M	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
294-295:C vs. 294-295:E	0,0077	>0,9999	0,8233	>0,9999	>0,9999
294-295:C vs. 294-295:M	0,0837	>0,9999	>0,9999	0,9995	0,9787
294-295:C vs. 294-295:E and M	0,0171	>0,9999	0,124	0,0412	>0,9999
294-295:C vs. 423-426:C	>0,9999	<0,0001	<0,0001	>0,9999	<0,0001
294-295:C vs. 423-426:E	0,9991	0,0565	0,732	0,8833	<0,0001
294-295:C vs. 423-426:M	>0,9999	0,0076	0,076	>0,9999	0,2039
294-295:C vs. 423-426:E and M	>0,9999	0,2055	0,9981	>0,9999	0,0666
294-295:E vs. 294-295:M	>0,9999	>0,9999	>0,9999	0,0519	0,4313
294-295:E vs. 294-295:E and M	>0,9999	>0,9999	>0,9999	<0,0001	0,9017
294-295:E vs. 423-426:C	0,0267	0,0006	<0,0001	>0,9999	0,0047
294-295:E vs. 423-426:E	>0,9999	<0,0001	<0,0001	>0,9999	<0,0001
294-295:E vs. 423-426:M	>0,9999	0,0007	<0,0001	>0,9999	0,6753
294-295:E vs. 423-426:E and M	>0,9999	0,0315	0,0298	0,8485	0,3262
294-295:M vs. 294-295:E and M	>0,9999	>0,9999	0,9961	>0,9999	>0,9999
294-295:M vs. 423-426:C	0,121	0,006	<0,0001	0,7571	<0,0001
294-295:M vs. 423-426:E	>0,9999	0,0528	0,0796	0,0262	<0,0001
294-295:M vs. 423-426:M	>0,9999	<0,0001	<0,0001	>0,9999	<0,0001
294-295:M vs. 423-426:E and M	>0,9999	0,1926	0,4527	>0,9999	0,0001
294-295:E and M vs. 423-426:C	0,0392	<0,0001	<0,0001	0,0226	<0,0001
294-295:E and M vs. 423-426:E	>0,9999	0,0011	0,0002	0,0001	<0,0001
294-295:E and M vs. 423-426:M	>0,9999	0,0001	<0,0001	0,8737	0,0021
294-295:E and M vs. 423-426:E and M	>0,9999	<0,0001	<0,0001	>0,9999	<0,0001
423-426:C vs. 423-426:E	0,0077	>0,9999	0,8233	>0,9999	>0,9999
423-426:C vs. 423-426:M	0,0837	>0,9999	>0,9999	0,9995	0,9787
423-426:C vs. 423-426:E and M	0,0171	>0,9999	0,124	0,0412	>0,9999
423-426:E vs. 423-426:M	>0,9999	>0,9999	>0,9999	0,0519	0,4313
423-426:E vs. 423-426:E and M	>0,9999	>0,9999	>0,9999	<0,0001	0,9017
423-426:M vs. 423-426:E and M	>0,9999	>0,9999	0,9961	>0,9999	>0,9999

Table 20 Standard deviation of relative gene expression for different treatments during sampling 1 and 2, calculated using $\overline{Foldchange} \mp STD = 2^{-(\overline{-\Delta\Delta Ct} \mp STD)}$.

Sampling	Treatment	Standard deviation	GR1a	GR1b	GR2	POMCA	POMVB
1	Controls	STD +	0,38	0,32	0,53	0,21	0,73
		STD -	-0,38	-0,39	-0,35	-0,14	-0,59
	Eplerenone	STD +	0,33	0,51	0,23		1,02
		STD -	-0,19	-0,27	-0,17		-0,40
	Mifepristone	STD +	0,20	0,36	0,24	0,17	1,35
		STD -	-0,17	-0,22	-0,19	-0,14	-0,54
	Eplerenone and Mifepristone	STD +	0,53	1,04	1,07	0,33	0,65
		STD -	-0,35	-0,45	-0,43	-0,25	-0,45
	Negative control GAP A	STD +	0,30	0,44	0,36	0,47	0,55
		STD -	-0,23	-0,29	-0,29	-0,23	-0,28
	GAPmers	STD +	0,60	0,19	2,12	0,60	13,07
		STD -	-0,39	-0,13	-0,88	-0,22	-1,72
2	Controls	STD +	2,08	2,89	0,72	1,00	2,43
		STD -	-0,68	-0,73	-0,42	-0,50	-0,71
	Eplerenone	STD +	6,31	3,44	0,70	3,85	2,62
		STD -	-1,97	-1,44	-0,47	-1,32	-1,32
	Mifepristone	STD +	4,85	5,85	0,56	1,84	3,90
		STD -	-1,33	-1,51	-0,39	-0,65	-1,26
	Eplerenone and Mifepristone	STD +	4,52	2,30	0,34	3,80	1,54
		STD -	-1,07	-0,83	-0,24	-1,06	-0,70
	Negative control GAP A	STD +	2,63	3,90	0,78	6,34	17,32
		STD -	-4,44	-1,72	-0,51	-2,54	-6,29
	GAPmers	STD +	14,15	3,90	0,78	6,34	17,32
		STD -	-4,44	-1,72	-0,51	-2,54	-6,29

Table 21 Size of eggs in diameter and yolk-sac larvae from head to caudal fin in millimeter (mm) at 423-426 day-degrees at hatch.

Gr.1 og 2		Gr.3 og 4		Gr.5 og 6		Gr.7 og 8		Gr.13, 10, 14	
Yolk-sac larvae	Egg	Yolk-sac larvae	Egg	Yolk-sac larvae	Egg	Yolk-sac larva	Egg	Yolk-sac	Egg
18,2	5,7	18,2	6,1	16,9	6,6	17,5	5,9	16,7	5,5
19,1	6,1	17,5	5,5	15,7	6,2	19	6	18,3	5,9
18,7	5,9	17,6	6,2	16,9	5,7	19	5,8	17,6	5,6
17,4	5,4	18,3	6,1	18,2	5,9	18,5	5,9	18	5,6
17,3	5,7	18,2	5,7	17	6,5	18	5,9	18,5	5,5
17,3	6,2	17,9	5,7	17,6	5,9	18,2	6	17,3	5,8
17,4	5,6	17,5	5,9	14,4	6	17,2	6	17,6	5,8
17,4	5,8	17,9	6,1	17,5	6	18,6	6	17,6	5,7
17,9	5,9	17,3		17,6	6,2	17,2	6,2	17,9	
17,5	5,8	18		17,4	5,7	18,7	5,8	18	
18	6,2	17,5		17,8	5,9	17,5	5,8	17,9	
17	6	17,1		17,4	6,2	17,6	5,5	16,5	
17,8	5,7	16,6		17,5	6	17,9	5,5	17,9	
19	6,2	16,5		17,2	6,1	18	6,1	17,7	
18,4		16,5		16,2		18	5,8	18	
17,9		17		15,6			6	18	
17,5		16,7		15,8			5,4	17,2	
17,3		17,4		15		17,3	5,8	17	
17,7		17		16,3		16,1	6	18,6	
18,8		16,5		15,1		17,7	5,6	18	
16,8		18		15,4		14,2	5,7	18	
17,4		17,9		14,7		18	6	19,6	
18	6,1	16,9		16,4	6,1	18,1	5,6	18	
19	6,2	17,2		17,5	6,1	18	5,7	19,7	
18,1	6,1	18		17,1	6,2	16,6	5,7	18,1	
17	6,1	16		16,8	6,4	18,2	5,5	19,3	
18,2	6	17,5		17,2	6,3		5,8	18,2	
17,6	7,3	17,8		17,3	6,3		6	18,3	
17,5	7,2	16,1		16,4	5,9		5,6		
17,7	6,9	17		18,5	5,8		6,1		
17,1	7,3	16,6		17,2	6,1		6,4		
17,2	7,2	16,5		16,7	6,2		5,9		
18,3	7,6	16,9		16,6	6,2		5,7		
18		18,1		16	5,9				
17,5		17,8		17,2	5,9				
17,8		17,7		17	6,3				
17,5		18,2		17,1	5,9				
17,1		17,5		16,9					
17,7		18,8		17,4					
17		18,1		17,2					
17,4		17,5		16,9					
17,9		17,2		16,7					
17,2		17,4	6,1	16,3					
17,2		17,6	6,1	17,1					
17,5		18	5,5	17,1					
19,6		17,6	5,9	17,3					
17,3		17,3	5,7	17,8					
18		17,8	5,9	16,1					
16,9		17,4	5,7	18,4					
17		17,2	5,7	17,7					
18,3		17,1	5,8	17					
17,3		17,5	5,6	15,6					
17		16,9	5,7	15					
17,3		16,8	5,9	17,4					
17,4		16,6	6	17,6					
18,1		16,3	5,7	16					
17,3		17,8	5,5	18,2					
16,7		17	5,5	16,2					
17,5		17,4	5,2	16,5					
17,5		17,3		11,7					
16,9		17,5		15,3					
16,9		17,4		16,6					
18,1		17,4		16,9					
17,5		17,3		16,5					
		16,3		14,8					
		17		15,4					
		17,2		18,4					
		17,2		16,4					
		17,3		18,8					
		17,1		17					
				15,7					

Sampling 1 and 2 with only fertilized eggs

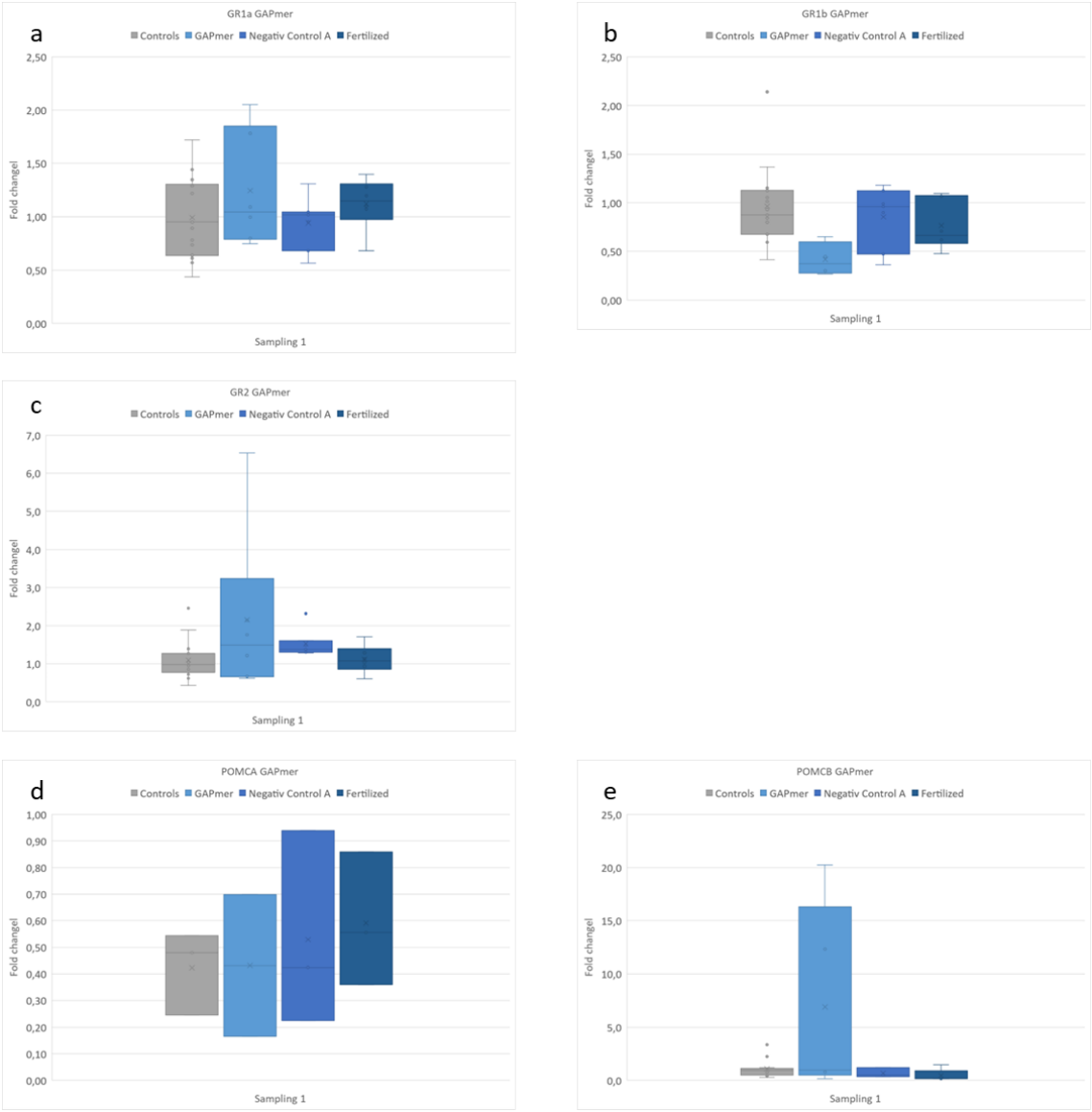

Figure 16 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs, fertilized eggs, Negative control GAP A eggs and the eggs injected with GAPmers at 14 day degrees. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers".

Table 22 p-values of a One-Way ANOVAs with the Sidak posthoc tests comparing sampling 1 for the GAPmers. The table includes controls (C), just-fertilized (F) eggs, eggs injected with Negative Control A (NGA) and eggs injected with GAPmers (GAP). There were no significant differences based on the Sidak post hoc test (p<0,001).

Sampling 1 GAP						
Gen	C vs. GAP	C vs. NGA	C vs. F	GAP vs. NGA	GAP vs. F	NGA vs. F
GR1a	0,75790	>0,9999	0,91830	0,81740	>0,9999	0,93760
GR1b	0,00520	0,98230	0,87050	0,04920	0,12040	0,99950
GR2	0,43070	0,41270	>0,9999	>0,9999	0,77340	0,77930
POMCA	>0,9999	>0,9999	0,99110	0,99810	0,96270	0,99910
POMCB	0,67020	0,99630	0,44640	0,61860	0,08180	0,96300

Figure 17 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs, fertilized eggs, Negative control GAP A eggs and the eggs injected with GAPmers at 44-46 day degrees, sampling 2. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". Significant differences between groups are marked with a ∗★●▲ (p-value ≤ 0,001). b) fertilization has an outlier of 48,33and e) fertilization has an outlier of 61,743 which, were removed to create identical scales in the box-plot.

Table 23 p-values of a One-Way ANOVAs with the Sidak posthoc tests comparing sampling 2 for the GAPmers. The table includes controls (C), just-fertilized (F) eggs, eggs injected with Negative Control A (NGA) and eggs injected with GAPmers (GAP). Significant differences are coloured in blue (p≤0,001)

Sampling 2 GAP						
Gen	C vs. GAP	C vs. NGA	C vs. F	GAP vs. NGA	GAP vs. F	NGA vs. F
GR1a	0,00020	0,00600	<0,0001	0,91510	0,99900	0,68490
GR1b	0,16440	0,20780	<0,0001	0,99990	0,22550	0,08110
GR2	0,43950	0,69710	0,00300	0,99980	0,38580	0,21740
POMCA	0,00160	>0,9999	0,00010	0,02820	0,84370	0,00310
POMCB	0,00030	0,13800	<0,0001	0,77860	0,80050	0,18980

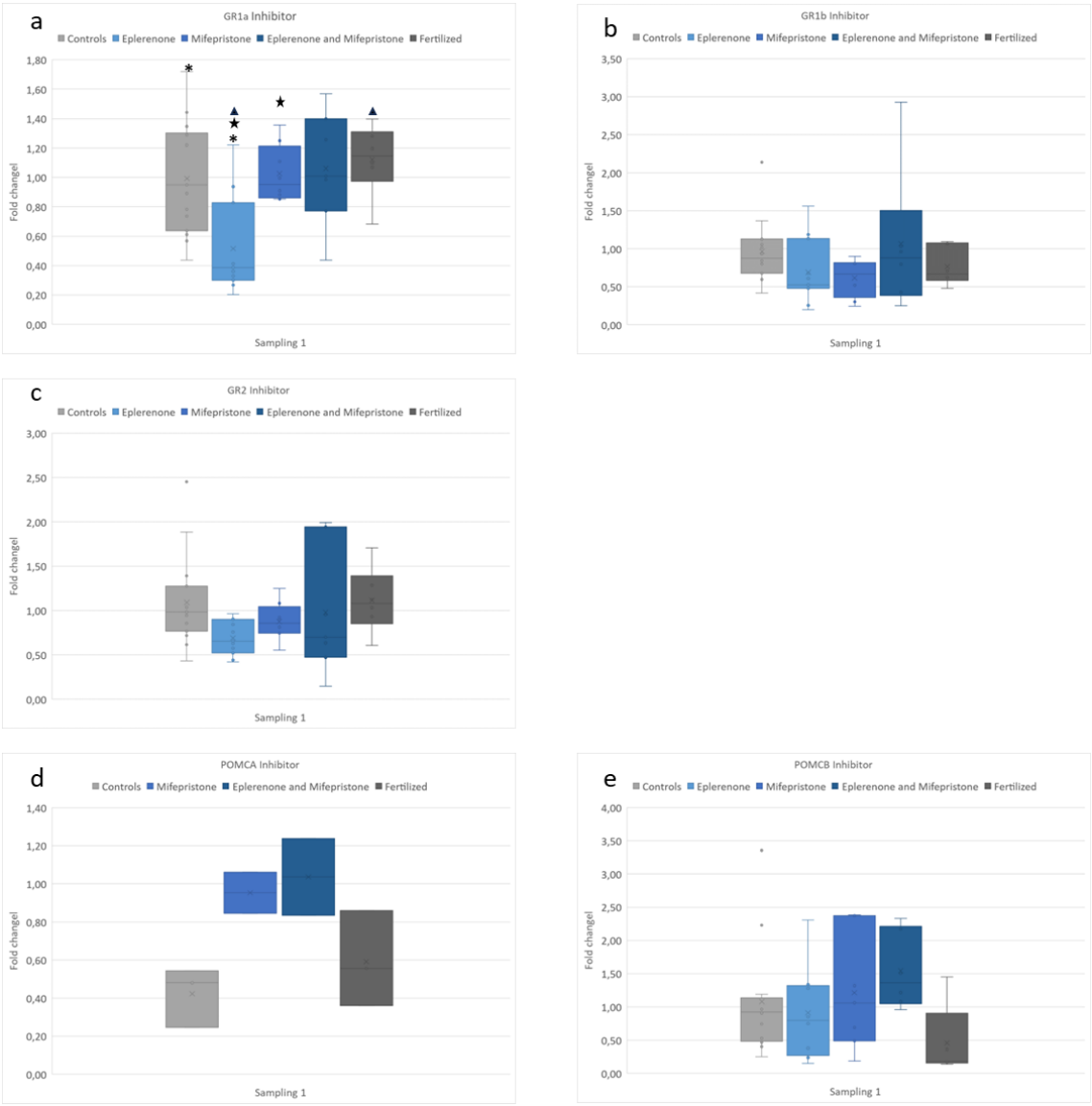

Figure 18 Relative gene expression of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs, fertilized eggs, eplerenone eggs, mifepristone eggs and the eggs treated with both eplerenone and mifepristone at salmpling 1, 14 day degrees. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". Significant differences between groups are marked with a ∗★●▲ (p-value ≤ 0,001).

Table 24 p-values of a One-Way ANOVAs with the Sidak posthoc tests comparing sampling 1 for the inhibitors. The table includes controls (C), just-fertilized eggs (F) and eggs treated with eplerenone (E), mifepristone (M) and eplerenone and mifepristone (EM). Significant differences are coloured in blue (p≤0,001).

Sampling 1 Inhibitor										
Gen	C vs. E	C vs. M	C vs. EM	C vs. F	E vs. M	E vs. EM	E vs. F	M vs. EM	M vs. F	EM vs. F
GR1a	0,00050	>0,9999	>0,9999	0,99390	0,00080	0,00200	0,00080	>0,9999	>0,9999	>0,9999
GR1b	0,37940	0,40720	>0,9999	0,99640	>0,9999	0,95030	0,99420	0,93430	0,98920	>0,9999
GR2	0,30120	0,99730	0,81460	>0,9999	0,95260	>0,9999	0,44560	0,99950	0,99340	0,83030
POMCA		0,24630	0,19190	0,90550				>0,9999	0,67830	0,56500
POMCB	0,99890	>0,9999	0,86570	0,17440	0,99710	0,53290	0,61040	0,96790	0,20640	0,02330

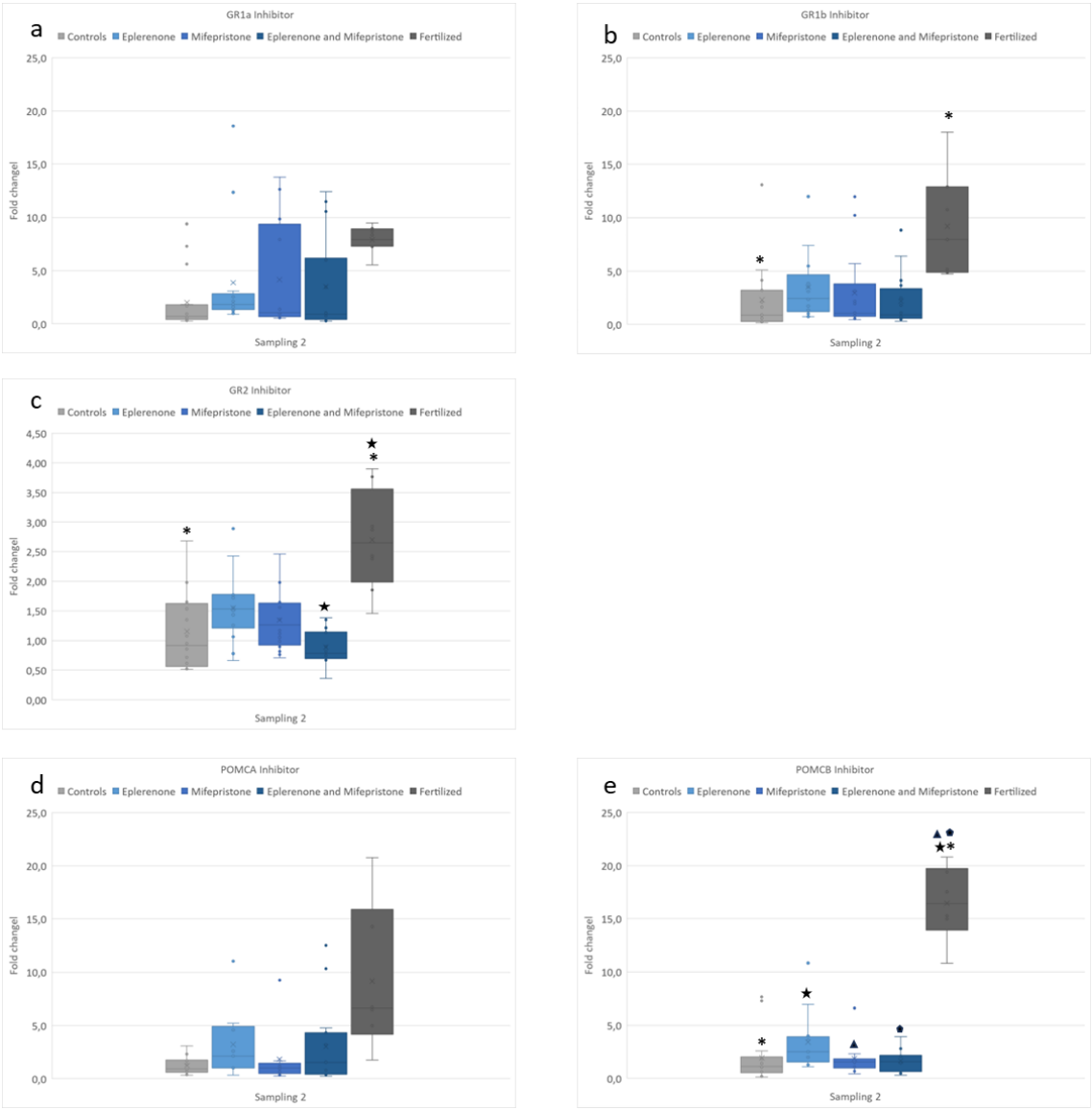

Figure 19 Relative gene expression) of a) gr1a, b) gr1b, c) gr2, d) pomca and e) pomcb for control eggs, fertilized eggs, eplerenone eggs, mifepristone eggs and the eggs treated with both eplerenone and mifepristone at sampling 2. These box plots show the median, minimum, and maximum value of the fold changes. Interquartile ranges of the data are also shown. Points situated outside of the data are "outliers". Significant differences between groups are marked with a ∗★●▲ (p-value ≤ 0,001). a) eplerenone has an outlier of 42,961, b) fertilization and mifepristone has two outliers of 48,330 and 48,479 respectively, e) fertilization and mifepristone has two outliers of 61,743 and 27,734 respectively, which, were removed to create identical scales in the box-plot for comparison.

Table 25 shows p-values of a One-Way ANOVAs with the Sidak posthoc tests, comparing sampling 2 for the inhibitors. The table includes controls (C), just-fertilized eggs (F) and eggs treated with eplerenone (E), mifepristone (M) and eplerenone and mifepristone (EM). Significant differences are coloured in blue (p≤0,001).

Sampling 2 Inhibitor										
Gen	C vs. E	C vs. M	C vs. EM	C vs. F	E vs. M	E vs. EM	E vs. F	M vs. EM	M vs. F	EM vs. F
GR1a	0,18070	0,82420	0,99660	0,00190	0,97680	0,68200	0,47530	0,99950	0,06540	0,01480
GR1b	0,30510	0,59400	0,99910	0,00020	>0,9999	0,75540	0,07910	0,96100	0,02100	0,00100
GR2	0,15150	0,71100	0,93570	<0,0001	0,98760	0,00590	0,02480	0,06960	0,00160	<0,0001
POMCA	0,68060	>0,9999	0,96720	0,00220	0,82970	0,99870	0,21510	0,99200	0,00730	0,02670
POMCB	0,10720	0,69890	0,99900	<0,0001	0,98860	0,49510	0,00060	0,98930	<0,0001	<0,0001