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Acute and genomic effects of Thyroid Hormones on the Atlantic salmon (*Salmo salar*) heart

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Aquaculture

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by

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List of abbreviations

AP	action potential
adr_{b1}	b1-adrenergic receptor
bl	baseline
cDNA	complimentary DNA
CER	Ca^{+2} extrusion rate
CICR	Ca^{+2} induced Ca^{+2} release
CMS	cardiomyopathy syndrome
ECC	excitation contraction coupling
ef1a	elongation factor-1a
F	systolic/peak Ca^{+2} levels
F_0	diastolic/basal Ca^{+2} levels
GATA4	GATA binding protein 4
hprt1	hypoxanthine phosphoribosyltransferase 1
HSMI	heart and skeletal muscle inflammation
LTCC	L-type Ca^{+2} channels
MARK	mitogen-activated protein kinase
NCX	Na^+/Ca^{+2} exchanger
NCX_{rev}	Na^+/Ca^{+2} exchanger in reverse
NKX2-5c	cardiac homeobox transcription factor Nkx2-5c
PD	pancreas disease

PKB/AKT	protein kinase B
PLB	phospholamban
ppia	peptidylprolyl isomerase A
qPCR	real time quantitative polymerase chain reaction
RA	relative amplitude
RAS	recirculatory aquaculture system
RIN	RNA integrity number
RM	repeated measures
RVM	relative ventricular mass
RyR	ryanodine receptor
ryr1	ryanodine receptor 1
S20	ribosomal protein S20
SERCA2	sarco/endoplasmic reticulum Ca^{+2}-ATPase
SR	sarcoplasmic reticulum
SVC	salmon ventricular cardiomyocyte
T2	3,5-diiodothyronine
T3	L-triiodothyronine
T4	L-thyroxine
T50	time to 50% increase
TH	thyroid hormones
THR	time to half removal
TR	nuclear thyroid hormone receptor

TRa	nuclear thyroid hormone receptor-a
TRa1	nuclear thyroid hormone receptor-a1
TRa2	nuclear thyroid hormone receptor-a2
TRb	nuclear thyroid hormone receptor-b
TRb1	nuclear thyroid hormone receptor-b1
TTP	time to peak

Abstract

Farmed salmon suffer from a variety of heart pathologies that impair cardiac function and ultimately contribute to cardiac failure and mortality. Thyroid hormones (TH) have well-studied cardioprotective effects in mammals, through direct, genomic and non-genomic (acute) actions. TH enhance cardiac activity and promote physiological cardiac remodeling while they are an important regulator of Ca^{+2} transients of the cardiomyocyte. TH peak during critical events of salmon life cycle with increased cardiac demand (e.g., smoltification), but their role in cardiac physiology in Atlantic salmon (*Salmo salar*) remain largely unknown. The aim of the current study was to investigate whether TH have direct acute and genomic effects on Ca^{+2} dynamics in the salmon heart. To investigate acute effects, salmon ventricular cardiomyocytes were isolated using a standard enzymatic technique, while Ca^{+2} transients were recorded in the presence of different concentrations of the TH, 3,3',5-Triiodo-L-thyroinine (T3). To investigate genomic effects, whole hearts were incubated under different concentration of T3 and the expression of genes encoding proteins related to Ca^{+2} cycling and hypertrophy was quantified by qPCR. Stimulation with T3 acutely decreased the time of half removal and increased the Ca^{+2} extrusion rate of the Ca^{+2} transient. These findings are largely in line with the observed acute effects of T3 on mammalian cardiomyocytes and could help the heart to work effectively when the heart rate is elevated. Further investigation is required to understand the underlying physiological mechanisms. The study did not reveal any genomic effects of TH on the salmon heart. Although these findings partially agree with previous findings in other teleost fish but limiting factors of our study do not allow us to exclude genomic effects of TH on the salmon heart. Nevertheless, the results indicate that TH could have a cardioprotective role also in salmon, for example in contexts that increase cardiac demand, such as during smoltification.

1. Introduction

Aquaculture is an industry of great economic importance for the Norwegian economy and Atlantic salmon (*Salmo salar*) is the dominant farmed fish species in Norway (Figure 1). The production and creating value of salmon have steadily increased over the past decade. Salmon production was more than 13.5 million tonnes in 2019, creating a value of around 68 billion NOK (Fiskeridirektoratet, 2020). The industry could further expand as the worldwide food demand is expected to increase and the Norwegian economy is planned to shift to a post-oil era (Johansen et al., 2019). However, salmon farming is not risk-free. For example, huge fish mortality and events where farmed salmon escape represent serious obstacles to the sustainability of Norwegian aquaculture. More than 60 million fish were lost during production only in 2019, where mortality was the main reason, accounting for approximately 90% of losses (Fiskeridirektoratet, 2020). Apart from the economic impact of such huge losses, increased mortality is an indicator of impaired fish health and welfare (Noble et al., 2018).

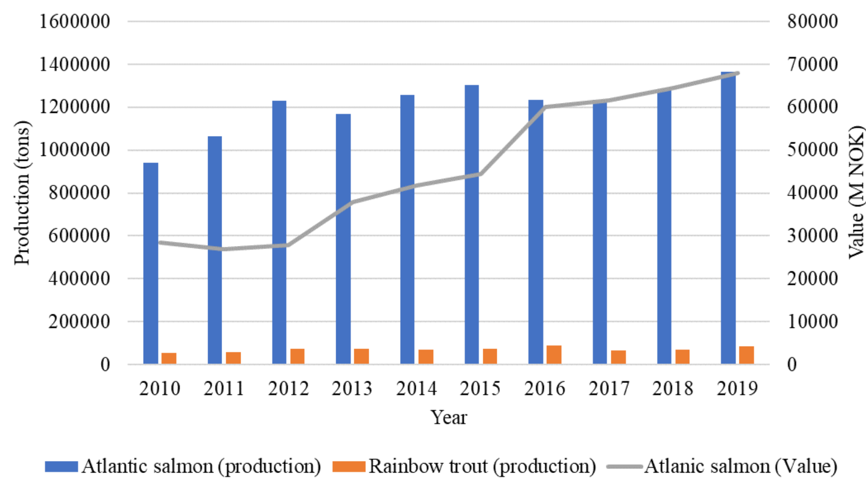

Figure 1. Atlantic salmon and Rainbow trout production (in tons) and Atlantic salmon creating value (in M NOK) between 2010-2019 in Norway. Both production and creating value of salmon have increased over the past decade (source: https://www.ssb.no/en).

Sudden death of seemingly healthy fish after stressful handling is often related to cardiac dysfunction (Hjeltnes et al., 2018). Indeed, several disorders and abnormalities of the cardiovascular system have been described over the years (Brocklebank & Raverty, 2002; Bruno et al., 2013; Dalum et al., 2017; Hjeltnes et al., 2018; Poppe et al., 2007; Poppe & Taksdal, 2000). Viral diseases, such as heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD) can affect the heart and lead to mortality. But infectious agents

are not always present or do not necessarily cause mortality (Bruno et al., 2013; Dalum et al., 2017; Hjeltnes et al., 2018; Poppe et al., 2021; Timmerhaus et al., 2012). Several other factors like nutrition and dietary conditions (Dessen et al., 2020; Martinez-Rubio et al., 2014), environmental and rearing conditions (Balseiro et al., 2018; Dessen et al., 2020; Frisk et al., 2020), interactions between the host and the virus (Timmerhaus et al., 2012; Wessel et al., 2020) and deviating heart morphology (Hjeltnes et al., 2018; Johansen et al., 2011; Poppe et al., 2003) are likely to affect cardiac health and influence mortality risk. Moreover, cardiac health is important for the general robustness of the fish (Brijs et al., 2016; Bruno et al., 2013; Johansen et al., 2017; Poppe et al., 2003). Despite the importance of cardiac health for current salmon farming, there are several knowledge gaps in our understanding of salmon heart physiology.

The heart is a vital organ for vertebrates and functions as the ¨pump¨ of the circulatory system, providing blood with oxygen and nutrients to other organs and tissues. Salmon, like other teleost fish, have a single closed circulation system and their heart (Figure 2) consists of four anatomically distinctive structures: the sinus venosus, the atrium, the ventricle and the bulbus arteriosus (Farrell & Jones, 1992). The ventricle, the main pump of the heart, consists of two circumferentially arranged layers of cardiac muscle, an outer layer (compact myocardium) and an inner layer (spongy myocardium) (Pieperhoff et al., 2009). The shape of the heart of wild Atlantic salmon is pyramidal, similar to that of other athletic fish species. This characteristic morphology assists with the increased cardiac demand during migration (Farrell & Jones, 1992). Farmed salmon usually has a more rounded heart with extensive fat deposits (Poppe et al., 2003).

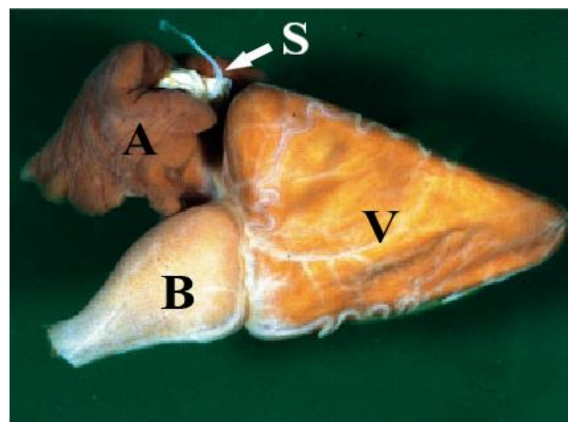

Figure 2. Wild Atlantic salmon heart. Venus blood flows into the atrium (A) through the sinus venosus (S). From there, the blood flows into the ventricle (V). The ventricle pumps the blood through the bulbus arteriosus (B) to the gills for oxygenation. After oxygenation, the blood is transferred to the other organs before it returns to the heart. Picture modified from (Poppe et al., 2003).

The salmonid heart demonstrates an extraordinary ability for plasticity and remodeling, allowing them to adapt to new environments with different cardiac demand (Gamperl & Farrell, 2004). Remodeling is a term used to describe the reorganization of already existing structures and includes changes in shape, size, tissue composition and genomic alterations (Cohn et al., 2000; Swynghedauw, 1999). Hypertrophy (increased cell mass) and hyperplasia (increased number of cells) are the two possible mechanisms of cardiac growth in fish (Gamperl & Farrell, 2004). In contrast, only hypertrophy is possible in mammals. A study on different sockeye salmon (*Oncorhynchus nerka*) populations showed that cardiac adaptation happens at a very local population level and it is dependent on the environmental conditions that each population encounter during river migration (Eliason et al., 2011). Genetic parameters are probably less involved as wild and farmed salmon showed similar cardiac performance when they raised under the same conditions (Dunmall & Schreer, 2003).

Several environmental and physiological factors can promote cardiac growth and alter heart contractility in salmonids. Cardiac growth during cold acclimation, a well-studied adaptation mechanism in fish, compensates for the negative effect of decreasing temperature on contractile function (Vornanen et al., 2005). Anaerobic exercise increases cardiac mass and improves contractility similar to physiological cardiac growth (athlete's heart) in mammals (Castro et al., 2013). Moreover, hormones, like androgens during sexual maturation of rainbow trout (*Oncorhynchus mykiss*) (Bailey et al., 1997) and cortisol, after chronic stress (Johansen et al., 2011) can induce cardiac growth. Cortisol-induced cardiac growth though is pathological and related to impaired heart function (Johansen et al., 2017; Nørstrud et al., 2018).

Several studies indicate that the early life stages of salmonids are important for the morphology and function of the heart at later stages. Elevated temperatures during egg incubation can lead to heart malformation in Atlantic salmon (Poppe & Taksdal, 2000), while Atlantic salmon smolts hatched in hatcheries had smaller hearts compared to wild smolts (Leonard & McCormick, 2001). The rearing conditions during the early life stages of rainbow trout relate to the development of several heart disorders later (Brijs et al., 2020). Moreover, intensive farming conditions prior to smoltification can lead to increased prevalence of deviating hearts and reduced growth performance during the sea stage in Atlantic salmon (Frisk et al., 2020).

Smoltification (the parr to smolt transformation) is a crucial life stage transition in the salmon life cycle. Atlantic salmon is an anadromous fish that migrates from fresh water into the ocean and back into the river for spawning. During smoltification, several morphological, behavioral and physiological changes take place allowing the fish to adapt to the new sea-water environment. Changes include reorganization of organs, like the gills, gut and kidneys, increased activity of enzymes, like the Na^{+}/K^{+}-ATPase at the gills and increased cardiosomatic index (relative heart mass) (Hoar, 1988; McCormick, 2012). The plasma levels of several hormones, including Thyroid Hormones (TH), change dramatically during smoltification (Figure 3) (McCormick, 2012; McCormick et al., 2013).

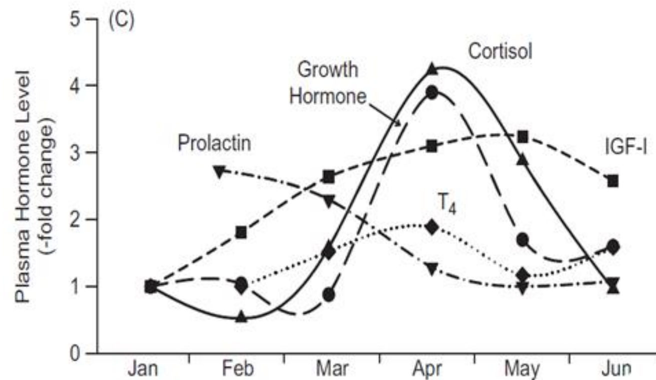

Figure 3. Changes in plasma levels of several hormones during smoltification including Thyroid hormone, L-thyroxine (T4). The plasma levels of T4 increase two folds during smoltification, while they return to previous levels after smoltification. Picture from (McCormick, 2012).

In mammals, TH are essential for the normal development and function of the heart, have a cardioprotective role, induce physiological cardiac growth and improve contractility. TH act through indirect and direct mechanisms on the heart, while the direct actions involve genomic and non-genomic (acute) actions. Genomic action involve the activation of nuclear thyroid hormone receptors (TR), while acute actions are independent of TR (Giammanco et al., 2020; Kahaly & Dillmann, 2005; Klein & Danzi, 2007; Yamakawa et al., 2021). The effects of TH on several aspects on salmonid and teleost fish biology (e.g., osmoregulation) have received research attention to a certain extend (Campinho, 2019; Høgåsen, 1998), but their effects on function and development of the salmon heart are largely unknown. TH actions and mechanisms seem to be quite universal and well preserved among species and taxa (Holzer wt al., 2017), while studies on salmonids and other teleost fish indicate that TH are possibly involved in heart function alterations

(Han et al., 2020; Little & Seebacher, 2014; Morin et al., 1993; Reddy & Lam, 1992; Tiitu & Vornanen, 2003).

Farming strategies and rearing conditions could alter the TH levels of the fish and improve (or hinder) production traits (Deal & Volkoff, 2020). Hatchery reared salmon demonstrated lower levels of TH during smoltification compared to wild conspecifics (McCormick & Björnsson, 1994). Although speculative, lower TH levels during the freshwater stage could be related to the observed reduced cardiac capacity of farmed salmon. Indeed, mutant zebrafish (*Danio rerio*) lacking nuclear thyroid hormone receptors-a (TRa) demonstrated deviating heart morphology and impaired contractility (Han et al., 2020). Interestingly, the deviating morphology of these mutant zebrafish shares some similarities with the most common morphological deviation of farmed salmon heart, as described by Poppe et al (2003). Therefore, studying the effects of TH on salmon heart could benefit the industry by providing knowledge on important physiological processes regulating cardiac development, health and performance.

Apart from the economic and welfare impact, studying the salmon heart can be useful from a comparative perspective. Indeed, teleost fish are excellent models for studying the evolution of the vertebrate heart (Pieperhoff et al., 2009), as well as for the study of heart physiology and pathology of other vertebrates (Han et al., 2020; Imbrogno et al, 2019).

1.1 Heart contractility

The function of the heart depends on dynamic and coordinated interaction of its constituent cells with the extracellular matrix (Banerjee et al., 2006). The heart consists of cardiomyocytes, fibroblasts, endothelial cells, connective and vascular tissue, while the ventricle, the main pump of the fish heart is dominated by cardiomyocytes (Shiels, 2017). Cardiomyocytes are the fundamental contractile units of the heart. The main function of the heart, pumping blood, is achieved through the synchronous contraction of cardiomyocytes. The process, known as excitation-contraction coupling (ECC) converts an autonomous electrical stimulus, the action potential (AP), into a mechanical action (contraction of the myofilaments). Several structures and proteins of the cardiomyocytes participate in the process by circulating Ca^{+2} (Ca^{+2} cycling) (Walker & Spinale, 1999).

1.1.1 ECC and Ca^{+2} cycling

In mammals, the ECC (Figure 4) starts when the AP triggers the opening of L-type Ca^{+2} channels (LTCCs), formations that are usually found in abundance on the T-tubules, and Ca^{+2} enters the cytosol (Ca^{+2} influx). Ca^{+2} can also enter the cytosol through the Na^{+}/ Ca^{+2} exchanger (NCX) running in reverse (NCX_{rev}). The Ca^{+2} influx induces release of Ca^{+2} stored in the sarcoplasmic reticulum (SR) through the ryanodine receptors (RyRs) in a Ca^{+2} induced Ca^{+2} release mode (CICR). This synchronized Ca^{+2} release, known as Ca^{+2} sparks generate the Ca^{+2} transient, which induces the contraction of the myofilaments. Removal of Ca^{+2} from the cytosol is the next step of the process and it is necessary for the relaxation of the cardiomyocyte. Sarco/endoplasmic reticulum Ca^{+2}-ATPase (SERCA2) and the NCX are the two proteins that simultaneously remove Ca^{+2} from the cytosol. SERCA2 returns Ca^{+2} into the SR, while NCX out of the cell. The amount of Ca^{+2} extruded by each route varies and it is species dependant (Shiels, 2017; Walker & Spinale, 1999).

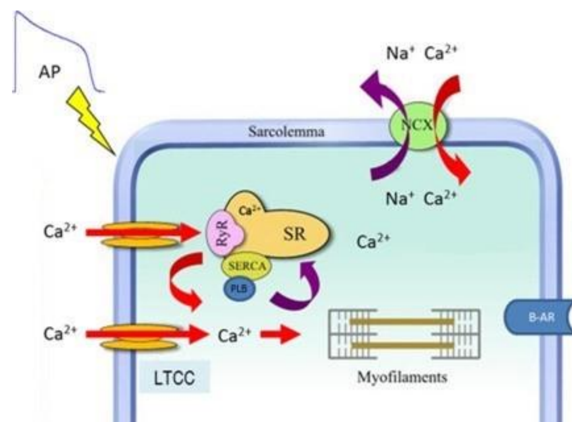

Figure 4. Excitation-contraction coupling of a cardiomyocyte. After electrical stimulation (AP), Ca^{+2} enters the cardiomyocyte through L-type Ca^{+2} channels (LTCC) or Na^{+}/ Ca^{+2} exchanger (NCX) in reverse. Incoming Ca^{+2} alone or in combination with Ca^{+2} released from sarcoplasmic reticulum (SR) through ryanodine receptors (RyR) generate the Ca^{+2} transient and the myofilaments contract. Ca^{+2} is extruded through either NCX or Sarco/endoplasmic reticulum Ca^{+2-}ATPase (SERCA2). Phospholamban (PLB), a protein co-localized to SERCA2, is an inhibitor of SERCA, while b-adrenergic receptor (B-AR) activity inhibits PLB. Picture from (Shiels, 2017).

The ECC of a fish cardiomyocyte shows some important differences compared to the mammalian. Ca^{+2} enters the cytosol primarily through the NCX_{rev} rather than through the LTCCs. The absence of T-tubules on fish cardiomyocytes and their characteristic morphology (long and thin cells with large surface to volume ratio) makes the Ca^{+2} transportation through the NCX_{rev} quick and efficient (Shiels, 2017). Therefore, extracellular Ca^{+2} entering the cytosol is enough to generate the Ca^{+2} transient. The CICR mode is a less used mechanism for most fish, even though fish can store great

amounts of Ca^{+2} in the SR (Shiels, 2017; Vornanen et al., 2002). Finally, Ca^{+2} removal is achieved mostly through NCX rather than SERCA2 (Hove-Madsen & Tort, 2001; Shiels, 2017).

Despite these differences in Ca^{+2} cycling, the ECC is not a fixed process neither in mammals nor in fish. The ECC shows great plasticity allowing fish to adapt to changing environments, that require different cardiac demands. The morphology of cardiomyocytes, expression of key proteins participating in the Ca^{+2} cycling and switching of different isoforms of proteins and enzymes involved in Ca^{+2} cycling is crucial for this regulation (Shiels et al., 2011; Shiels, 2017; Vornanen et al., 2002).

1.1.2 SERCA2

SERCA2 is an ATP dependant Ca^{+2} pump that decreases cytosolic Ca^{+2} by returning Ca^{+2} into the SR. SERCA2 activity is inhibited by phospholamban (PLB), a co-localized protein. Phosphorylation of PLB increases the activity of SERCA2, while dephosphorylation reduces it, making the phosphorylation status of PLB pivotal for Ca^{+2} cycling through SERCA2. For example, increased adrenergic activity increases cardiac output through phosphorylation of PLB. Despite the smaller contribution of SERCA2 to Ca^{+2} extrusion in fish, its role is crucial. The contribution of SERCA2 is increased in athletic fish (like salmon), in fish under stressful situations and plays an important role for cardiac function during thermal acclimation of the fish (Korajoki & Vornanen, 2013; Shiels, 2017; Vornanen, 2021). Moreover, enhanced cardiac performance is associated with increased Ca^{+2} extrusion through SERCA2 in fish and mammals (Castro et al., 2013; Periasamy et al., 2008; Shiels et al., 2011).

1.2 Thyroid hormones and heart function

Thyroid hormones, L-thyroxine (T4) and L-triiodothyronine (T3) are released by the thyroid gland. TH regulate the metabolism and affect almost every organ, tissue and cell including the cardiovascular system. T4 is considered the predominant form, while T3 is the most active. The synthesis and secretion of TH are regulated through the hypothalamus- pituitary- thyroid gland axis. TH are transported to the periphery mainly bound to transport proteins in the blood and are activated in target cells though deiodination of T4 to T3 by deiodinases (Giammanco et al., 2020; Kahaly & Dillmann, 2005; Klein & Danzi, 2007; Yamakawa et al., 2021). In salmon, T4 is synthesized and secreted by the thyroid follicles, while T3 derives mostly from peripheral deiodination of T4 (Høgåsen, 1998).

Transcription factors are proteins that bind to specific nuclear areas and regulate gene expresion (Latchman, 1997). Nuclear thyroid hormone receptors (TR) act like ligand-inducible transcription factors and the genomic effects of TH are directly mediated through binding on these receptors. Two main genes encoding nuclear thyroid hormone receptors-a (TRa) and nuclear thyroid hormone receptors-b (TRb) have been found in mammals, but several TRa and TRb isoforms with different binding capacity and functionality have been reported. While the two main isoforms, nuclear thyroid hormone receptors a1 (TRa1) and nuclear thyroid hormone receptors b1 (TRb1) are expressed in all tissues, their distribution among tissues varies (Giammanco et al., 2020; Kahaly & Dillmann, 2005; Yamakawa et al., 2021). TRa1 is the predominant TR in the mammalian heart (Gloss et al., 2001). In fish, several more TR isoforms have been found. Most likely they are all orthologous to either the mammalian TRa or TRb, but the functionality of many of these isoforms is poorly studied. Some fish TR isoforms seem to have a higher affinity for 3,5-diiodothryronine (T2), a catabolite of T3, rather than for T3 and T4 (Deal & Volkoff, 2020; Johnson & Lema, 2011; Kudo et al., 2018; Lazcano & Orozco, 2018; Mendoza et al., 2013). TR have been detected in many organs of salmonids, e.g brain, liver, kidneys (Harada et al., 2008; Jones et al., 2002; Kudo et al., 2018; Marchand et al., 2001) and studies from zebrafish have shown that TRa1 is probably the predominant TR in the teleost fish heart as well (Han et al., 2020), but the expression and function of TR isoforms in the salmon heart remains to be studied.

Apart from the genomic effects of TH, direct non-genomic (acute) effects have been observed. These actions are independent of binding to TR but can eventually lead to genomic alterations as well. The mechanism behind acute effects remains unclear, but possible pathways involve binding of TH on several cell membrane sites and activation of secondary messengers. For example, integrin αvβ3 is a cell membrane-binding site that activates intracellular kinases, such as protein kinase B (PKB/AKT) and the mitogen-activated protein kinase (MAPK). PKB/AKT and MARK effect the phosphorylation status of several intracellular proteins (Davis et al., 2008; Giammanco et al., 2020; Kahaly & Dillmann, 2005).

1.2.1 TH and the mammalian heart

The effects of TH on the mammalian cardiovascular system have been studied extensively and are well documented. TH have a cardioprotective role, are necessary for the normal development and function of the heart, promote physiological cardiac remodelling and improve contractility.

Moreover, thyroid gland and TH homeostasis disorders, are risk factors for several cardiovascular disorders, including heart failure, while hypothyroidism (reduced TH levels) leads to reduced cardiac size (Kahaly & Dillmann, 2005; Klein & Danzi, 2007; Yamakawa et al., 2021). In addition, TH are an important regulator of the Ca^{+2} transient and Ca^{+2} homeostasis of the cardiomyocyte (Wang et al., 2003; Zinman et al., 2006).

The effects of TH on the mammalian heart are indirect (e.g., decreased systemic vascular resistance) and direct through alterations in the expression of several cardiac protein encoding genes (Table 1). TH improves cardiomyocyte contractility by positively regulating the expression of SERCA2 and negatively regulating the expression of PLB. Moreover, TH upregulate the expression of b1-adrenrgic receptors (Klein & Ojamaa, 2001; Ojamaa, 2010; Yamakawa et al., 2021). TH promote cardiac growth through hypertrophy (Klein & Ojamaa, 2001; Ojamaa, 2010). GATA binding protein 4 (GATA4) and cardiac homeobox transcription factor Nkx2-5 (NKX2-5c) are two transcription factors expressed predominantly in the myocardium and they are related to hypertrophy. They regulate the expression of structural cardiac proteins and cardiac specific genes, like α-Myosin heavy chain and cardiac troponin I (Akazawa & Komuro, 2003). Both GATA4 and NKX2-5c are upregulated by TH (Ojamaa, 2010; Thurston et al., 2009). Cardiac remodelling can be either physiological or pathological, while the genomic profiles of physiological and pathological cardiac remodelling are distinct. The genomic alterations induced by TH are related to physiological cardiac remodelling and enhanced cardiac performance (Bernardo et al., 2010; Ojamaa, 2010).

Table 1. Regulation of cardiac protein encoding genes by TH. Several of the affected genes encode proteins involved in Ca^{+2} cycling. Modified from (Yamakawa et al., 2021).

	Positive regulation:	**Negative regulation:**
Myofilament	a-Myosin heavy chain	b-Myosin heavy chain
Ca^{+2} cycling proteins	SERCA2	PLB
Adrenergic receptor	b1-Adrenergic receptors	Adenylyl cyclase types V and VI
Membrane channels	Sodium/potassium ATPase	NCX
Voltage gated potassium channels	Kv1.5, Kv4.2, and Kv4.3	

Apart from the genomic actions, acute effects of TH have been observed in studies on isolated cardiomyocytes, intact hearts and individuals. TH affect several ion channels, including $Na^{+,}$ K^{+}

and Ca^{+2} channels leading to acute positive inotropic (increased force of contraction) and lusitropic (increased relaxation activity) effects. (Davis & Davis, 2002; Kahaly & Dillmann, 2005; Yamakawa et al., 2021). Moreover, acute administration of TH has been found to protect the cardiomyocyte from Ca^{+2} overload, a condition that can lead to cell death (Zinman et al., 2006).

1.2.2 TH and the salmon heart

Despite that TH have been studied extensively in mammals, their effects on the salmon heart, and fish heart in general, are poorly studied and largely unknown. Tilapia larvae (*Oreochromis mossambicus*) treated with T3 demonstrated increased hear rate, possibly through metabolic alterations and not through direct actions (Reddy & Lam, 1992). Increased activity of deiodinases in the heart during Atlantic salmon smoltification indicates that TH are possibly involved in changes in heart function through this period (Morin et al., 1993). A study on rainbow trout showed that, in contrast to mammals, hypothyroid fish had larger hearts and higher heart rate, than euthyroid (normal TH levels) and hyperthyroid (increased TH levels) fish. Moreover, TH status did not affect the contractile capacity and kinetics of the heart. The study concluded that the effects of TH on heart size and rate are probably due to indirect actions through metabolism (Tiitu & Vornanen, 2003). This study though focused only on phenotypic characteristics, like heart weight and rate, and did not investigate genomic differences among groups. A bigger heart could indicate pathological cardiac growth and further investigation on genomic level is required (Bernardo et al., 2010; Johansen et al., 2017; Nørstrud et al., 2018). Contrary to the study on rainbow trout, recent studies on zebrafish, indicate that TH could have direct actions on cardiac function. TH regulate cardiac performance during cold acclimation (Little & Seebacher, 2014), while mutant individuals missing TRa developed abnormally shaped hearts and weakened contractility (Han et al., 2020). Even though the actions of TH seem to be well preserved among species (Holzer et al., 2017), the effects of TH on heart development and function in fish are controversial and further clarification is needed.

Based on all the above we formed the hypothesis that TH improve contractility and promotes physiological cardiac remodeling in the Atlantic salmon heart through direct genomic and non-genomic (acute) actions. To investigate direct effects and to exclude any indirect actions, we conducted experiments *in vitro* on isolated salmon ventricular cardiomyocytes and *ex vivo* on intact hearts.

1.3 Aims of the studies

The main aim of the studies was to investigate 1. the direct acute and 2. genomic effects of TH on the Ca^{+2} dynamics of salmon ventricular cardiomyocytes (SVCs).

Study 1- Acute effects of TH on Ca^{+2} dynamics *in vitro* (Sub aims)

- Investigate if exposure to T3 has acute effects on Ca^{+2} dynamics of SVCs.
- Investigate if the effects are dose dependent.
- Investigate if the effects wash out after the exposure to T3 is terminated.

Study 2- Genomic effects on salmon heart ventricles *ex vivo* (Sub aims)

- Investigate if exposure to T3 alters the expression of genes encoding Ca^{+2} cycling proteins and transcription factors related to hypertrophy in the salmon heart ventricle.
- Investigate if the effects are dose dependent

2. Material and methods

2.1 Animals

Atlantic salmon smolt of both sexes, were obtained from the fish laboratory facilities at The Norwegian University of Life sciences (NMBU, ÅS campus, Norway). The fish were reared in fresh water recirculatory aquaculture system (RAS) at 13-15˚ C and constant 24h light, after hatching at 6-8˚ C. The fish were fed with a commercial diet appropriate for their size (Nutra XP, Nutra Sprint or Nutra RC, Skretting, Norway) from start feeding until harvesting. The *mean ±SD* body mass of the fish was 250 ±54.9 g for study 1 and 92 ±11.2 g for study 2.

2.2 Salmon ventricular cardiomyocyte (SVC) isolation

Fish for SVC isolation were harvested approximately at the same time of the day each time (9-9:10 AM). Fish were euthanized by a sharp blow to the head before decapitation. The use of chemical anaesthetics was avoided, as it has been previously reported that this may affect the results on cardiac contractility (Farrar & Rodnick, 2004). SVCs were isolated using a standard enzymatic technique for rainbow trout as described by Laasmaa et al (2016). First, the heart was carefully excised and placed in ice-cold Ca^{+2}-free isolation solution (in mM: 100 NaCl, 10 KCl, 1.2 KH2PO4, 4 MgSO4, 50 Taurine, 20 Glucose, 10 HEPES and pH 6.9 with 1M NaOH). All chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA), unless it is stated differently. The blood was pumped out by gently massaging the heart. After that, the heart was cannulated through the bulbus arteriosus and mounted on a Langendorff perfusion system (Figure 5) with a clip and a thread. The heart was perfused at room temperature (22˚ C) with isolation solution for 8-10 min with a flow rate of ~1.5 ml/min. Then, 0.65 mg/ml trypsin (Sigma-Aldrich), 1 mg/ml collagenase type II (Sigma-Aldrich) and 1 mg/ml bovine serum albumin (Sigma-Aldrich) were added to the isolation solution. The perfusion continued until the heart was digested (heart was pale and soft) which lasted for approximately 20-24 min, depending on the size of the heart. The external part of the heart was kept moist during the isolation by pouring isolation solution with a Pasteur pipette every 1-2 min. Following digestion, the ventricle was placed in a small beaker with ice cold isolation solution and the bulbus arteriosus and the atrium were removed. The ventricle was cut into 4-5 pieces and a cut-off Pasteur pipette was used to gently triturate ventricular chunks to release cells. The suspension was filtered through a nylon mesh (200 μm) and the SVCs were allowed to sediment. After sedimentation, the SVCs were washed twice with ice cold isolation

solution, by removing the supernatant and resuspending the cells in new ice-cold isolation solution. The isolated SVCs were kept on ice until use (up to 8 hours).

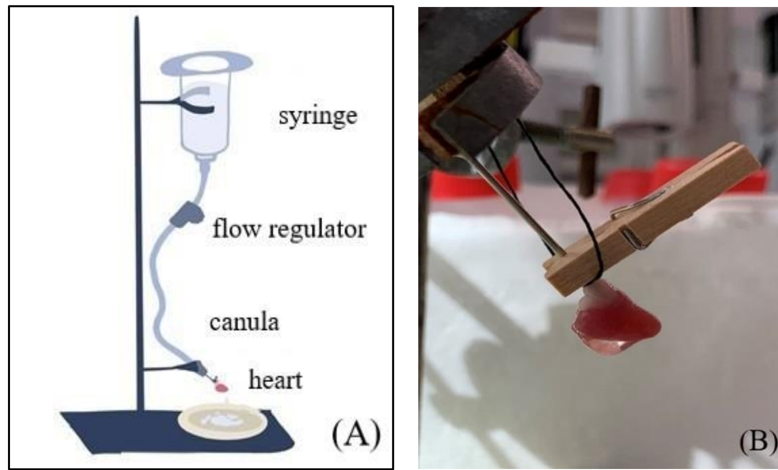

Figure 5. (A) The perfusion system consisted of a 20 ml syringe, an iv-infusion set, a metallic cannula (18G) and a stand. The height between the syringe and the canula was approximately 50 cm. (B) The heart was stabilised on the cannula with a clip and a thread. The canula was placed to a 45˚ angle. Figure (A) from Vilde A. Engdal.

2.3 Ca^{+2} imaging

Ca^{+2} transients were recorded as described by Tazmini et al (2020) using an Axiovert 100M microscope (Carl Zeiss, Jena, Germany) with a 40X water emersion objective (Figure 6). Isolated SVCs were loaded with 20 µM fluo-4 AM (Invitrogen, Waltham, MA, USA) for 10 min at room temperature. Loaded SVCs were then plated on a glass cover slip and allowed to settle for 5-10 mins. After sedimentation, SVCs were perfused with ringers solution (containing in mM: 130 NaCl, 5.4 KCl, 1.5 MgSO4*7H2O, 0.4 NaH2PO4, 10 HEPES, 10 Glucose, 2 CaCl2 and pH 7.6 with 1M NaOH) at room temperature (22°C) and with a flow rate of approximately 4 ml/min. The perfusion solution has been used in electrophysiology experiments with rainbow trout cardiomyocytes by Laasmaa et al (2016). Contractions were elicited by two platinum electrodes at a voltage 20% above the stimulation threshold at a frequency of 0.5 Hz, using a pulsar 6bp as stimulator (FHC, Bowdoin, ME, USA). Fluo-4 was excited with a PE-300 white LED illumination unit (CoolLED, Andover, UK) at 488 nm and emitted light above 510 nm was detected by a PTI D-104 microscope photometer (Horiba Scientific, Kyoto, Japan). Data was recorded and stored in Clampex10.4 software (Molecular Devices, San Jose, CA, USA). T3 (Sigma-Aldrich) was dissolved in DMSO and diluted to a final concentration of 5 µg/ml. Aliquots of the above solution were kept in the freezer (-20˚C) and were added to the desired concentration just before use.

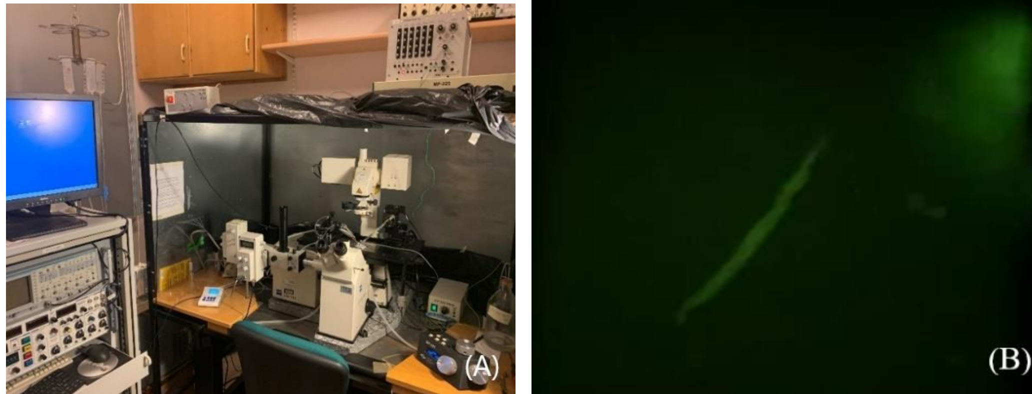

Figure 6. (A) The microscope set up. (B) A Characteristic thin and long salmon ventricular cardiomyocyte loaded with fluorescent dye. Only thin and long salmon ventricular cardiomyocytes with no spontaneous activity were included in the study.

Only viable SVCs (n = 20) with no spontaneous activity were included in the study. Initial baseline recordings for 30-60 s were followed by increasing concentrations of T3 (0.03, 0.3, 3, 30 mg/ml) in incrementing intervals of 2 minutes. T3 concentrations used in the study were determined based on previous studies assessing plasma levels in smolt (Eales et al., 1993; Ebbesson et al., 2008; McCormick et al., 2013). A TTL system allowed rapid switch between concentrations. Finally, to examine if the effects of T3 can be washed out, SVCs were re-perfused with ringers solution without T3 for 1-1.5 minutes. Total recording duration was ≤10 minutes for each cell.

Ca^{+2} transient characteristics were quantified by determining diastolic/basal Ca^{+2} levels (F0), systolic/peak Ca^{+2} levels (F), relative amplitude (RA) (F/F0), time to 50% increase (T50), time to peak (TTP), time to half removal (THR), slope of decline (tau) and Ca^{+2} extrusion rate (CER) (1000*1/tau). All measurements were corrected for background emission by recording and subtracting background fluorescence from each cell. All measures were quantified using Clampfit 10.4 software (Molecular Devices, San Jose, CA, USA).

2.4 Heart incubation

The fish were euthanised as previously described and their hearts were carefully removed. First, the hearts (n = 32) were placed in ringers solution (same as the Ca^{+2} imaging perfusion ringers solution) and gently massaged to remove the blood. Hearts were randomly assigned to one of four different treatment groups by placing them in 50 ml tubes filled with 15 ml perfusion ringers solution with 0, 0.3, 3 or 30 ng/ml T3 (n = 8). Hearts were incubated at 15° C (rearing temperature) for 2 hours in a water bath. Visual observation of the heart beating was used as an indicator of

heart viability, while a preliminary study of 5 hearts confirmed that isolated hearts can survive for at least 2h in the ringers solution. At the end of the incubation period the atrium and bulbus arteriosus were carefully removed before the ventricle was placed in RNAlater^{TM} stabilization solution (Invitrogen, Waltham, MA, USA) and stored at 4º C overnight. The next day, ventricles were weighed, and stored at -80º C until further use.

2.5 RNA extraction

RNA was extracted from ventricular cardiac muscle using an RNeasy® plus universal mini kit (Qiagen, Germantown, MD, USA). First the whole ventricles were placed in 2 ml tubes with two stainless steel 5 mm beads (Qiagen) without defrosting. Ventricles weighing more than 50 mg were cut in half and processed in different tubes. Then, 900 µl QIAzol lysis reagent (Qiagen) was added to each tube and the tissue lysed and homogenized using a precellys evolution tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France) at 5500 RPM for 20s twice. The RNA was extracted from the homogenized lysates according to the manufacturer's instructions. For more details on the used protocol please see (Appendix A). The RNA was eluted with 40 µl RNase-free water and stored at -80˚ C.

The quantity of eluted RNA was determined with an epoch microplate spectrometer (Biotek instruments, Winooski, VT, USA). The quality of eluted RNA was determined based on RNA integrity numbers (RIN) calculated by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RIN values range from 0 to 10, where RIN values above 8 indicate excellent RNA quality and RIN values between 6 and 8 indicate good RNA quality. The RIN values were calculated for 50% of the samples where the samples with the lowest and highest RNA concentration were chosen. RIN values ranged from 7.5 to 9.5 with an average of 9.1. For more details on RNA quantity and quality please see (Appendix B).

2.6 Complementary DNA (cDNA) synthesis

The cDNA was synthesized from 3 µg/µl total RNA using an iScriptTM cDNA synthesis kit (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. Each reaction contained 3 µg/µl total RNA, 12 µl 5x iScript reaction mix, 3 µl reverse transcriptase and nuclease-free water up to a total volume of 60 µl. The reaction mix was kept on ice, until it was incubated in a simpliAmp thermal cycler (ThermoFischer scientific, Waltham, MA, USA). The incubation conditions were as follow:

- Priming: 25º C for 5 min
- Reverse transcription: 46º C for 20 min
- Reverse transcriptase inactivation: 95º C for 1 min
- Hold: 4º C until storage at -20º C

2.7 Real-time quantitative PCR (qPCR)

qPCR

The qPCR was run on a Roche96 light cycler (Roche Diagnostics, Penzberg, Germany) based on previously published protocols (Frisk et al., 2020; Vindas et al., 2017). Each reaction contained 5μl Power SYBR® Green PCR master mix (AppliedBiosystems, Waltham, MA, USA), 1 μl nuclease-free water, 5 μM F primer, 5 μM R primer and 2 μl 1:20 diluted cDNA sample. Samples were run on duplicates on 96 well plates (BIOplastics, Landgraaf, The Netherlands). The running conditions were:

- Preincubation: 95º C for 600 s
- 3 step amplification: 95 º C for 10 s; 60 º C for 10 s; 72 º C for 10 s — × 40 cycles
- High resolution melting: 95º C for 60 s; 40º C for 60 s; 65º C for 1 s; 97º C for 1 s
- Cooling: 37 º C for 30 s

The relative expression (ΔCp) of the targeted gene was calculated by the formula:

$$\Delta Cp = E_{targ}^{Cq_{targ}} / E_{ref}^{Cq_{ref}}$$

E_{targ} is the efficiency of the primers for the targeted gene, Cq_{targ} is the mean Cq value for the two duplicates of qPCR reaction of the sample for the targeted gene, E_{ref} is the efficiency of the primers for the reference gene and Cq_{ref} is the mean Cq value for the duplicates of the qPCR reaction of the sample for the reference gene. The *s20* was chosen as a reference gene, between four different genes (*hprt1, ef1α, ppia* and *s20*), since it showed lower Cq values, better primer efficiency, and

less variation among dilutions. The efficiency of the primers was determined in the beginning based on dilution carves.

Primer design

Gene-specific primers (Table 2) were adopted form previously published work or designed using the primer blast software from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). More specifically, primers for genes encoding ryanodine receptor 1 (*ryr1*) and cardiac homeobox transcription factor Nkx2-5 (*nkx2-5c*) were adopted from (Castro et al., 2013) and primers for Sarco/endoplasmic reticulum Ca^{+2}-ATPase (*serca2*) from (Korajoki & Vornanen, 2012). Three different pairs of primers were designed at exon junctions for genes encoding phospholamban (*plb*), GATA binding protein 4 (*gata4*) and b1-adrenergic receptor (adr_{b1}). The pair of primers with the lowest Cq value in PCR, single peak melting curve and amplification of the right sequence was chosen for each gene. The qPCR products were sequenced to verify that the primers detected the right sequence (Appendix C). Primers for the reference gene ribosomal protein S20 (*s20*) were adopted from previous work (Vindas et al., 2017). The primers for *serca2* and *s20* were synthesized by Invitrogen. All the other primers were synthesized by Eurofins genomics.

Table 2. Targeted genes and primer sequences used for the qPCR.

Gene:	Function:	Primer pair:	Accession number:
serca2	Ca^{+2} cycling protein	F-GTGCAATGCCCTTAACAGCCT R-ACGGGCAGTGGCTCCACATA	XM_014160409.2
plb	SERCA2 inhibitor	F-ATAAGGTGCAGCACACGATG R-ACAACACGATGATGTAGATGAGCA	XM_01420513.1
ryr1	Ca^{+2} cycling protein	F-CTCTACCGGGTGGTCTTTGA R-ACCTGCTCTTGTTGGTCTCG	XM_01496271.1
adr_{b1}	b1-adrenergic receptor	F-GGGCATTATCATGGGCACCT R-CGCAGCTCCTTGTAGAAGATGT	XM_014172005.1
gata4	Hypertrophy transcription factor	F-CCATTCGACAGCTCCGTCC R-CACTCTTCGAGATGCAGACAGC	XM_014205425.1
nkx2-5c	Hypertrophy transcription factor	F-CCCAGTACGTCCACACCCTT R-GGAGGTCGGTAAGGCACAGT	XM_014198814.1
s20	Reference gene	F-GCAGACCTTATCCGTGGAGCTA R-TGGTGATGCGCAGAGTCTTG	NM_001140843.1

2.8 Statistical analysis

Results are expressed as *mean* ± *s.e.m.* All statistical analysis were performed using SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA). The data from the acute reaction studies were analyzed using a one-way repeated measures (RM) ANOVA followed by all pairwise multiple comparisons (Tukey test), when relevant. The data for Ca^{+2} extrusion rate was log_{10}-transformed prior to analysis to achieve normalization. All the data on relative gene expression were analyzed using a non-parametric (Kruskal-Wallis) one-way ANOVA on ranks, except for *nkx2-5c*, which was analyzed with an one-way ANOVA, since it achieved normality. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Study 1 - Acute effects of TH on Ca^{+2} dynamics

To examine the effects of acute TH administration on Ca^{+2} cycling, Ca^{+2} transients were recorded in 20 SVCs (*n=20*) for 3 fish. Ca^{+2} transients were recorded from SVCs exposed to tenfold increasing concentrations of T3 after initial recording of baseline (bl) conditions. To investigate if the effects could wash off, the SVCs were re-perfused with ringers' solution without T3 (0 ng/ml T3) at the end of each recording. The results are summarized in (Table 3).

Table 3. Summary of measurements of Ca^{+2} transient characteristics, relative amplitude (RA), time to 50% increase (T50), time to peak (TTP), time to half removal (THR) and Ca^{+2} extrusion rate (CER), after acute exposure of isolated salmon ventricular cardiomyocytes (*n=20*) to tenfold increasing concentrations of T3, presented in *mean (±s.e.m.)*. Significant differences ($P < 0.05$) are indicated by letters. a: *vs* baseline, b: *vs* 0.03 ng/ml T3, c: *vs* 0.3 ng/ml T3. For more details on statistical analysis please see (Appendix D).

Characteristic	baseline	0.03 ng/ml	0.3 ng/ml	3 ng/ml	30 ng/ml	0 ng/ml
RA (F/F_0)	1.51±0.09	1.53±0.1	1.42±0.08	1.43±0.08	1.43±0.08	1.51±0.12
T50 (ms)	74±2.6	77.6±2.3	78.8±3	77.1±3.6	77.9±4.1	77.4±3.3
TTP (ms)	191.9±6.3	200±5.9	191.1±6.8	190.1±5.6	190.5±6.9	189.1±5.7
THR (ms)	426.9±24.5	415.6±20.7	393.7±20.3[a,b]	373.5±16.6[a,b]	372±16.5[a,b]	364.4±15[a]
CER (s^{-1})	3.4±0.3	3.68±0.33	3.89±0.32[a,b]	4.47±0.36[a,b,c]	4.72±0.41[a,b,c]	4.74±0.34[a]

Exposure to T3 concentrations ≥ 0.3 ng/ml decreased time to half removal (THR) ($P < 0.001$) and increased Ca^{+2} extrusion rate (CER) ($P < 0.001$) in SVCs (Figure 7). For example, 3 ng/ml T3 decreased THR by 12.5 % and increased CER by 31.5 %. On the other hand, Ca^{+2} transient time to 50 % increase (T50) ($P = 0.744$) and time to peak (TTP) ($P = 0.307$) were not significantly altered by T3 exposure. One way RM ANOVA revealed a general effect of the treatment on relative amplitude (RA) ($P = 0.015$), but the Tukey post hoc multiple comparisons test did not reveal any significant differences between treatment groups. Thus, the study did not reveal any acute inotropic effect of T3 on SVCs.

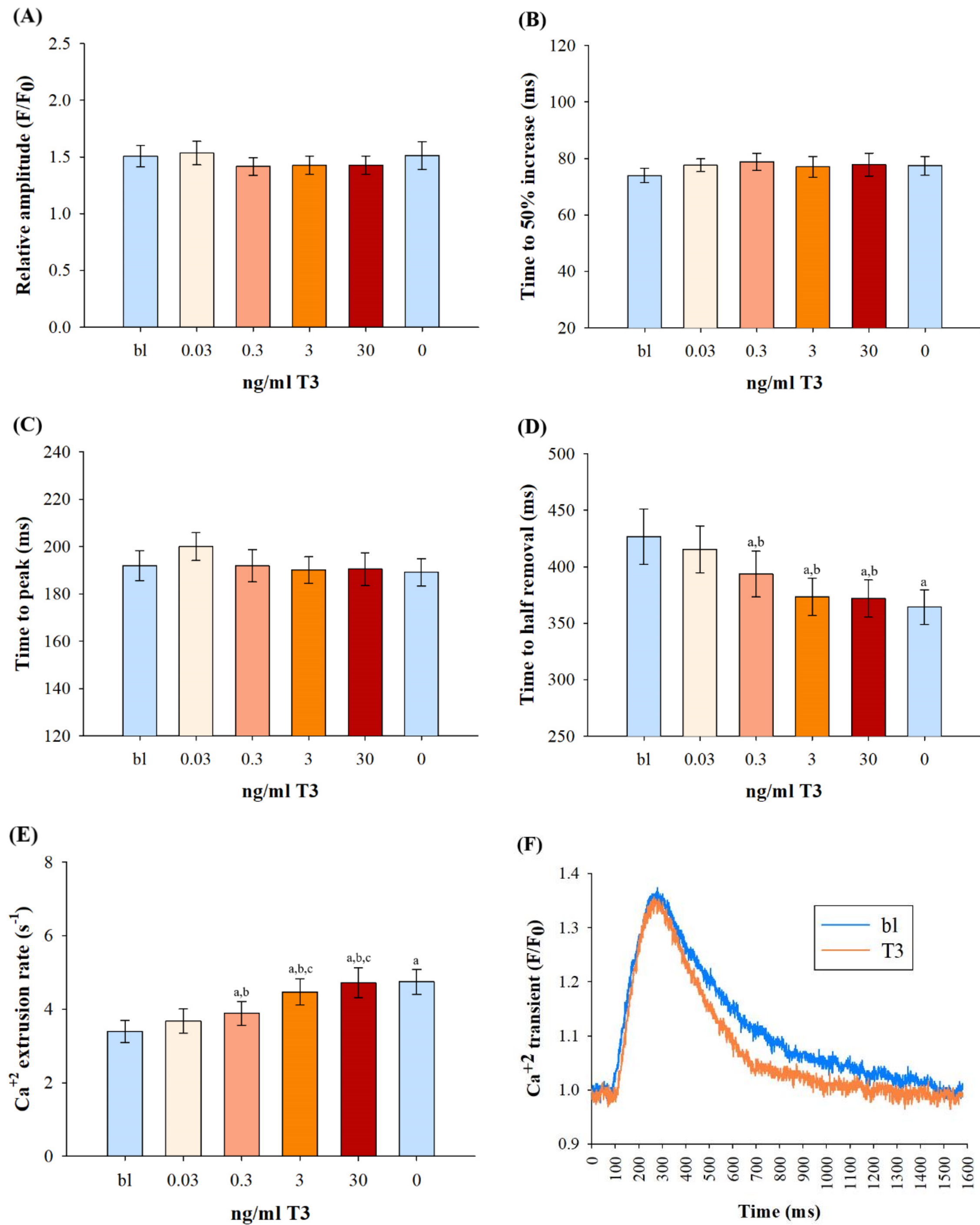

Figure 7. (A) Relative amplitude, (B) Time to 50% increase, (C) time to peak, (D) time to half removal and (E) Ca^{+2} extrusion rate of Ca^{+2} transient after acute exposure of isolated salmon ventricular cardiomyocytes *(n=20)* to tenfold increasing concentrations of T3 (*mean ± s.e.m)*. Statistical analysis was performed by one-way repeated measures ANOVA followed by Turkey test, when relevant. Significant differences ($P < 0.05$) are indicated by letters. a: vs baseline (bl), b: vs 0.03 ng/ml T3, c: vs 0.3 ng/ml T3. The data for Ca^{+2} extrusion rate was $\log_{10}$-transformed prior to the analysis. (F) Representative Ca^{+2} transients from isolated salmon ventricular cardiomyocytes before (blue) and after (orange) exposure to 3 ng/ml T3.

3.2 Study 2- Genomic effects on salmon heart ventricles

To investigate genomic effects of T3 on the salmon heart suspected target genes encoding proteins involved in Ca^{+2} cycling and hypertrophy were quantified by qPCR. This was done on ventricular tissue after incubation of the intact fish heart ($N = 32$) in the presence of 4 different concentrations of T3 ($n = 8$).

Relative expression of Ca^{+2} cycling protein genes

Exposure with T3 did not affect relative expression levels of *serca2* ($P = 0.939$), *plb* ($P = 0.767$), *ryr1* ($P = 0.395$) or adr_{b1} ($P = 0.828$) (Figure 8).

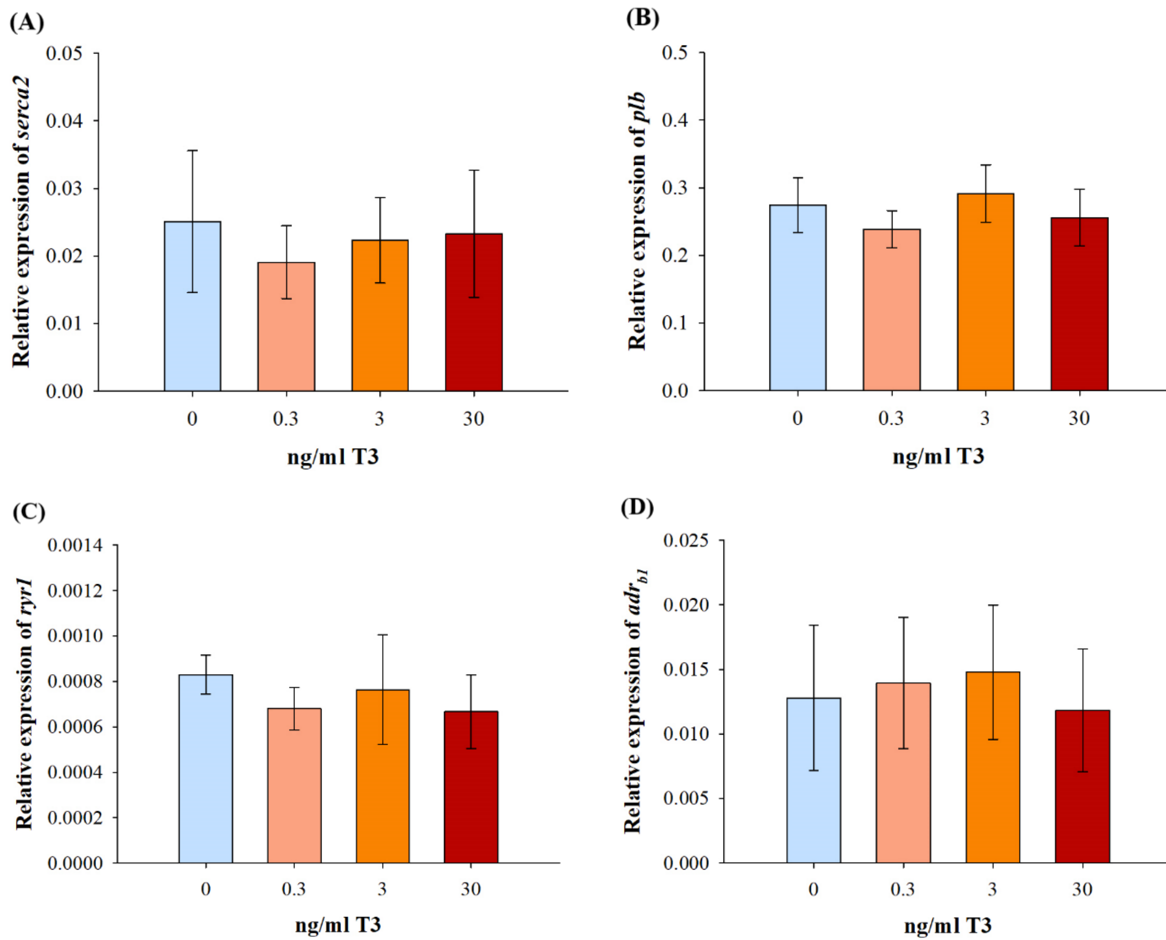

Figure 8. Relative expression (*mean ± s.e.m.)* of the Ca^{+2} cycling proteins (A) *serca2,* (B) *plb*, (C) *ryr1* and (D) adr_{b1}. Statistical analysis was performed by Kruskal-Wallis ANOVA on Ranks. No significant differences ($P < 0.05$) were observed.

serca2 inhibition by plb

The relative expression of *serca2* and relative expression of *plb* ratio (*serca2/plb*) was calculated to investigate if *plb* inhibits the expression of *serca2*. No significant changes were observed on *serca2/plb* (*P=0.998*) among the treatment groups (Figure 9).

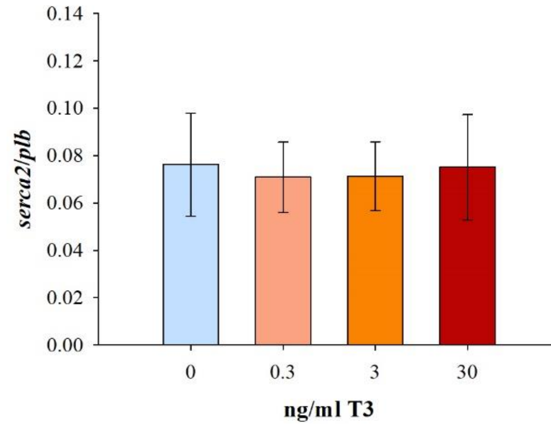

Figure 9. *serca2/plb* (*mean ± s.e.m.)*. Statistical analysis was performed by by Kruskal-Wallis ANOVA on Ranks. No significant differences (P < 0.05) were observed.

Relative expression of cardiac hypertrophy transcription factors genes

Exposure with T3 did not affect relative expression levels of hypertrophy transcription factors *gata4* (*P = 0.988*) or *nkx2-5c* (*P = 0.981*) (Figure 10).

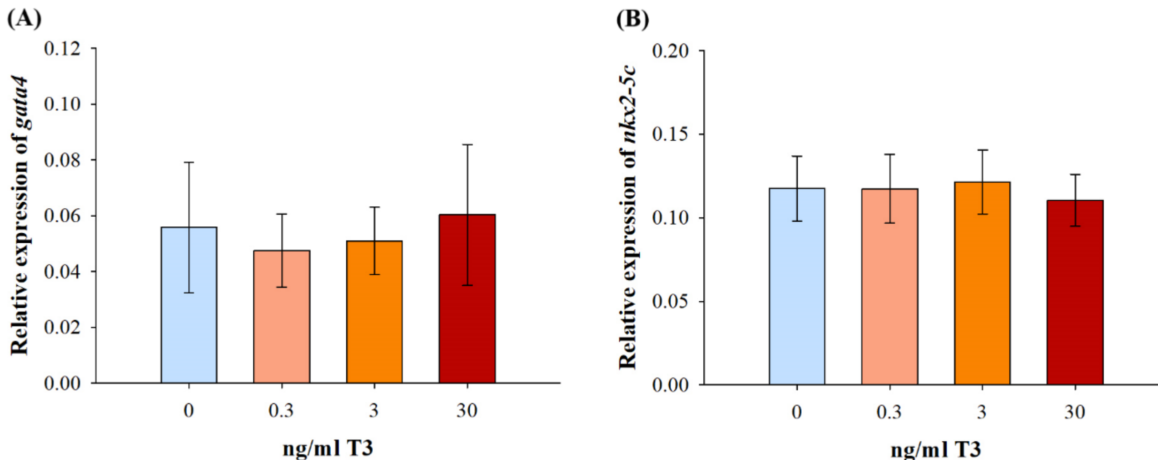

Figure 10. Relative expression (*mean ± s.e.m.)* of hypertrophy related transcription factor (A) *gata4* and (B) *nkx2-5c.* Statistical analysis was performed by by Kruskal-Wallis ANOVA on Ranks for *gata4* and one way ANOVA for *nkx2-5c*. No significant differences (P < 0.05) were observed.

4. Discussion

To our knowledge, this is the first study to investigate direct effects of TH on Atlantic salmon cardiac muscle. We find that T3 results in reduced time to half removal (THR) and increased Ca^{+2} extrusion rate (CER) of the Ca^{+2} transient in SVCs. This finding is largely in line with effects of T3 on the mammalian heart, where TH is an important regulator of the Ca^{+2} transient and has positive lusitropic (increased relaxation activity) and inotropic (increased force of contraction) effects. Papillary muscle preparations from the left ventricle of TH-treated rats demonstrate a 22.1% reduction in time from peak to 80% fall of the Ca^{+2} transient compared to preparations from hypothyroid rats, which means increased Ca^{+2} extrusion and relaxation activity (Bing et al., 1994). In addition, the TH treated mammalian cardiomyocytes show increased Ca^{+2} transient amplitude indicating a positive inotropic effect of the hormones. In our study, we did not observe a similar inotropic effect. There were also no obvious dose-dependent effects of T3 under the tested concentrations, and the effects did not wash off following 1.5 min of T3 removal. Finally, our study was not able to reveal any genomic effects of T3 on the salmon heart.

4.1 Acute effects

Our results show that administration of T3 decreases the THR and increases the CER of the Ca^{+2} transient. The immediate (within 2 minutes) response to T3 indicates that TH regulate Ca^{+2} handling in SVC in an acute manner (Dillmann, 2002). Research on acute effects of TH in fish in general is limited. However, acute effects of TH have been observed in zebrafish, where acute T4 administration increases the amplitude of Na^{+} currents on embryonic neurons (Yonkers & Ribera, 2009). Much more evidence of acute effects on Ca^{+2} handling and on cardiac muscle in general exist in mammals. For example, acute administration of both T4 and T3 increases Ca^{+2} extrusion in neonatal rat cardiomyocytes overloaded with Ca^{+2}. Within minutes after administration of the hormones, Ca^{+2} levels return to physiological levels (Zinman et al., 2006).

Ca^{+2} handling and contractility

Increased CER allows the cardiomyocyte to relax quicker, since cytosolic Ca^{+2} levels regulate the force and duration of contraction (Bers, 2000). Therefore, our findings are in agreement with studies where acute T3 administration increases the relaxation activity. Isolated normal porcine cardiomyocytes stimulated with T3 demonstrated a 30% increase on shortening velocity, which indicates increased relaxation activity of the cardiomyocyte (Walker et al., 1994). Moreover,

lusitropic effects after acute administration of T3 have been observed in conscious dogs with pacing-induced cardiomyopathy (Jamali et al., 1997).

Acute effects of TH on the mammalian heart are strongly associated with positive inotropic effects. Studies on adult cat atrial cardiomyocytes showed that T3 increases the amplitude of the Ca^{+2} transient within 5 minutes after exposure to the hormone (Wang et al., 2003). Moreover, positive inotropic effects have been observed on isolated porcine cardiomyocytes *in vitro* (Walker et al., 1994), intact rat hearts *ex vivo* (Segal et al., 1996) and in dogs *in vivo* (Jamali et al., 1997). Our study did not indicate any inotropic effects of T3 on SVCs since there were no differences in the relative amplitude (RA) of the Ca^{+2} transient among the different treatments.

The reason behind this discrepancy could be due to differences between mammalian and fish Ca^{+2} cycling physiology. While in mammals, incoming extracellular Ca^{+2} induces the Ca^{+2} transient by releasing the Ca^{+2} stored in the SR through RyRs (CICR mode), in fish CICR mode is less used and the incoming extracellular Ca^{+2} is enough to induce the Ca^{+2} transient. However, several other factors can mask the acute effects of TH. For example, age or endogenous levels of TH have been reported to alter the observed acute effects (Carr & Kranias, 2002). Neonatal mammalian cardiomyocytes are considered naive to TH exposure and preferred for acute studies (Zinman et al., 2006), but adult mammalian cardiomyocytes have been used as well (Wang et al., 2003).

Dose-dependent fashion and action duration

A study on porcine cardiomyocytes suggests that the acute effects of TH are dose dependent in mammals, while the effects plateau around the physiological levels of T3 (Walker et al., 1994). In our study, the initial effects on CER appeared when SVCs were exposed to 0.3 ng/ml and increased further when T3 increased to 3 ng/ml. CER did not increase further when T3 was increased to 30 ng/ml. Similarly, the first effects on THR were observed when the cells were exposed to 0.3 ng/ml. However, exposure to higher T3 concentrations did not result to any further decrease of THR. Our data do not demonstrate clearly that TH acts in a dose-dependent fashion as clearly demonstrated by Walker et al (1994). A wider range of concentrations were tested in their study, and it is possible that including a wider range of relevant concentrations could have resulted in more convincing dose-dependency in our study.

The effects of T3 did not wash off for at least 1.5 min after the final exposure to T3. Neither the THR nor the CER returned to the baseline levels after T3 exposure was terminated. This is

somehow expected, as acute effects of TH have been reported to last from a few minutes (Segal, 1990; Wang et al., 2003) to even up to 24 hours (Jamali et al., 1997). We cannot determine the exact duration of the observed effects with the used experiment design.

Mechanisms of action

In our study, we did not investigate possible mechanisms underlying the observed effects of T3 on THR and CER. We do, however, hypothesize that the observed effects may be mediated through increased SERCA2 and/or NCX activity, since these represent the two main pathways for Ca^{+2} removal. SERCA2, an ATP dependant Ca^{+2} pump, decreases cytosolic Ca^{+2} by returning Ca^{+2} into the sarcoplasmic reticulum (SR), while NCX removes it out of the cell. In mammals, TH acutely regulate SERCA2 activity (Davis & Davis, 2002). Phospholamban (PLB), an inhibitor of SERCA2, and the phosphorylation status of PLB is pivotal for SERCA2 activity (Carr & Kranias, 2002).

Even though, the literature suggests that SERCA2 is a likely mediator of the observed acute effects of TH on Ca^{+2} handling, the role of NCX cannot be excluded. To determine the role of SERCA and NCX a first step would be to perform similar electrophysiology experiments with isolated SVCs and acute administration of caffeine. Caffeine releases the Ca^{+2} of the SR and diminishes the removal of Ca^{+2} through SERCA2. Caffeine has been used to determine the way that Ca^{+2} is extruded in mammalian cardiomyocytes (Wang et al., 2003). If the observed effects are mediated through SERCA2, then they should not be present in caffeine-incubated SVCs. Accessing the protein levels and phosphorylation status of SERCA2 and PLB before and after T3 administration will be the next step to determine the role of PLB. Finally, studies on mammals indicate that TH could mediate the observed effects through b1-adrnergic stimulation (Tielens et al., 1996; Zinman et al., 2006). Experiments with b-adrenergic receptor blockers have been performed in mammals to determine if the observed actions are dependent or independent of adrenergic stimulations (Zinman et al., 2006).

Cardioprotective role of TH on salmon

Ca^{+2} levels regulate the force and duration of contraction allowing the heart to work effectively (Bers, 2000). When the heart rate is elevated, the time between each cardiac cycle is reduced allowing less time for diastolic filling of the ventricle and increased Ca^{+2} extrusion is required otherwise the heart might collapse. Positive lusitropic effects can help the heart to function

effectively under these conditions (Davis & Davis, 1993; Little & Seebacher, 2014; Vornanen, 2021). The evidence from mammalian studies indicate that the observed effects can be safely extrapolated to tissue or organ function level and therefore, our results suggest that increased TH levels could have an acute cardioprotective role in salmon and allow the heart to work effectively when the heart rate is elevated. Experiments on isolated intact salmon hearts or heart strips acutely stimulated with T3 could further confirm the above hypothesis. However, as mentioned earlier, the effects of TH can differ between individuals of different age or thyroid status and the observed effects might differ between the several developmental stages of salmon.

4.2 Genomic effects

Ca^{+2} handling and contractility

Genomic effects of TH on the contractile properties of the mammalian heart have received considerably more attention than acute effects. Indeed, nuclear TH receptors act as ligand-inducible transcription factors that bind to DNA and regulate the expression of several cardiac proteins. Our study did not reveal any genomic effects of T3 on expression of genes related to contractility. This contrasts with mammalian studies, where TH-induced upregulation of SERCA2 and downregulation of PLB are well documented (Adamson et al., 2004; Carr & Kranias, 2002; Klein & Ojamaa, 2001).

It is possible that TH may not have similar effects on the salmon heart as they have on the mammalian heart, though the effects of TH on the fish heart are controversial. A study on rainbow trout daily injected intraperitoneally with T3 for 7 days (hyperthyroid), methazole for 14 days (hypothyroid), hormone vehicle for 7 days (sham) or not injected at all (control) did not show differences in atrial muscle contraction kinetics among the groups (Tiitu & Vornanen, 2003). The study, however, did not access the kinetics of ventricular cardiac muscle and did not investigate any alterations on transcriptomic level. Studies on zebrafish, evaluating genomic effects of TH in various tissues, showed that TH do not regulate the expression of *serca2* and *plb* in cardiac muscle, even though they affect gene expression in skeletal muscle (Little & Seebacher, 2013, 2014). More precisely, no significant differences in cardiac expression of *serca2* and *plb* were observed between cold acclimated hypothyroid fish (maintained in water with propylthiouracil, a TH production inhibitor, and treated daily with iopanoic acid, a deiodinase inhibitor), TH treated fish (maintained in water with propylthiouracil and supplemented with T3 or T2 daily) and control fish

(maintained in water without propylthiouracil and not supplemented with anything else). In contrast, a recent study, using CRISPR/Cas9-mediated genetically modified zebrafish lacking genes encoding TRa1 and TRa2 (*thraa 8-bp insertion* or *thrab 1-bp insertion* mutations), showed that TH alter the expression of contractile proteins (Han et al., 2020). The mutant fish demonstrated lower relative RNA expression of *serca2* compared to the wild-type (control) and lower protein abundance of SERCA2 as well, confirming the transcriptomic differences. Several other contractile protein genes showed lower relative expression in the mutant fish as well.

Another possible reason for the discrepancy between our study on isolated salmon hearts and mammalian studies is the incubation time and exposure method. The incubation time is an important factor for genomic effects to appear and depending on the genes of interest, genomic effects of TH in the mammalian heart are observed following a few hours to several days (Davis et al., 2008). It is possible that the incubation time used in our study (2 hours) was insufficient for the transcriptomic alterations to appear. Moreover, cultured cardiomyocytes are preferred to intact hearts in mammalian studies. For example in these studies investigating transcriptomic alterations of TH in mammalian heart, isolated rat ventricle cardiomyocytes were cultured and incubated in the presence of T3 for at least 24h (Adamson et al., 2004; Iordanidou et al., 2010). Cardiomyocyte cultures not only allow longer incubations periods, but also hormone and oxygen are evenly distributed to all cardiomyocytes. Even though the visually observed heartbeat indicated that the hearts were viable during our study, hypoxic conditions might have occurred. Oxygen monitoring should have been included in the experimental design or alternatively an assessment of hypoxia indicators, like the salmon cardiac peptide (Arjamaa et al., 2014).

Moreover, the genomic effects of TH are mediated when the hormone is binding to TR. Several isoforms of TRa and TRb with different functionality and binding affinity exist, while some of them, like nuclear thyroid hormone receptor -a2 (TRa2), have zero affinity to TH (Giammanco et al., 2020; Lazar et al., 1988). Of note, several more isoforms have been observed in fish and their functionality remains unknown, while their expression can change during the different developmental stages (Jones et al., 2002; Kudo et al., 2018; Lazcano & Orozco, 2018). TRa1 is the predominant TR in the mammalian heart (Gloss et al., 2001) and in the zebrafish heart (Han et al., 2020). We can assume that the same applies to salmon as well, though information on the expression of TR on the salmon heart are missing. In addition, some fish TR isoforms seem to

have a higher affinity for T2, rather than for T3 and T4 (Lazcano & Orozco, 2018; Mendoza et al., 2013). T2 is a catabolite of T3 and has recently received more attention in mammals as well, as its metabolic effects are similar to that of T3 (Giammanco et al., 2020). Since there are no known target genes of TR in salmon that we could use as a positive control, we cannot be certain that our treatment worked. Therefore, considering all the above reasons, we cannot exclude any possible genomic affects yet.

Hypertrophy and cardiac remodelling

TH-induced cardiac growth in mammals has been studied extensively and it is well documented (Ojamaa, 2011). To investigate if the same applies to salmon, the gene expresion of cardiac hypertrophy markers GATA4 and NKX2-5c was measured. GATA4 and NKX2-5c are transcription factors related to hypertrophy that are predominantly expressed in heart (Akazawa & Komuro, 2003) and have been found to be regulated by TH (Klein & Ojamaa, 2001; Lee et al., 2010; Thurston et al., 2009). In mammals, TH-induced cardiac growth, combined with alterations in the expression of contractile proteins promotes a physiological cardiac remodelling (Bernardo et al., 2010).

Our study did not show any differences in the gene expression of *gata4* and *nkx2-5c* among the treatments. Taking the previously mentioned methodological limitations into consideration this suggests that TH does not promote cardiac growth in salmon. This is somewhat in line with previous studies. TH-treated rainbow trout show no differences in heart size compared to sham and control fish (Tiitu & Vornanen, 2003). Moreover, the effects observed in rainbow trout are opposite to what is observed in mammals. Hypothyroid fish showed a 20% and 35% increase in relative ventricular mass (RVM) compared to sham and control fish, respectively. The authors suggested that the observed increase in RVM with lower TH levels stem from indirect actions, like changes in metabolism. Also, in line with our study no differences in *gata4* and *nkx2-5c* mRNA expression were observed between wild type and zebrafish lacking genes encoding TRa (Han et al., 2020). The mutant fish, however, showed increased atrium size and had rounder ventricles.

4.3 Future perspectives

The genomic effects of TH on the fish heart are controversial and our study was aiming to shed more light on those effects. Our results suggest that TH does not alter the expression of the contractility related proteins and hypertrophy transcription factors genes. Considering the

methodological limitations previously discussed, we can however not exclude any possible genomic effects on salmon heart yet. Since cardiac dysfunction is a problem with serious economic and fish welfare implications for current salmon farming, further investigations are required to elucidate a possible role for TH on the salmon heart development. Future projects should first and foremost determine the expression of TR in the salmon heart at different developmental stages before proceeding to any further experiments. Molecular methods, like PCR and immunohistochemistry could help towards this direction. Since incubation time and exposure method are crucial for the genomic effects to appear, similar conditions to mammalian studies could be achieved with the use of recently established protocols for salmon cardiac primary cultures (Noguera et al., 2017). In addition, genetically modified salmon lacking genes encoding TR, like the mutant zebrafish used by Han et al (2020), can be proven useful on studying genomic effects and investigating the functionality of TR isoforms in the salmon heart. Moreover, our study focused on a limited number of genes based on mammal bibliography. RNA sequencing might provide a deeper understanding on the actions of TH on the salmon heart.

Regarding the acute effects, our results indicate that the observed effects on SVCs are largely in line with findings in mammals. As discussed earlier, further electrophysiology experiments, (caffeine administration) and experiments assessing protein levels for SERCA2 and PLB before and after T3 exposure could reveal the underlying mechanisms. Also, it would be interesting to acutely expose intact salmon hearts mounted on a Langendorff perfusion apparatus or cardiac strips to T3 and confirm that the observed alterations on Ca^{+2} transient apply to organ or tissue level as well.

4.4 Conclusion

Heart pathologies and cardiac dysfunction are serious economic and welfare problems for current salmon farming. TH have a well-established cardioprotective role in mammals but research on their effects on the salmon heart (or on the teleost fish heart in general) is lacking. This first study on the direct effects of TH on salmon cardiomyocytes showed that TH acts on SVCs in an acute manner, while these effects are largely in line with findings from mammals. Future electrophysiology experiments could help to shed more light on the underlying mechanisms of the observed acute effects. Direct genomic effects of TH on fish heart are controversial and the study was aiming to reveal some possible genomic effects and bring some clarity. The study did not reveal any genomic effects, but it identified possible flaws in the experimental design and set the directions for future projects. Nevertheless, the study indicates that TH could have a cardioprotective role also in salmon in situations where the heart rate is elevated e.g., during smoltification.

Appendices

Appendix A. Quick-Start Protocol for RNeasy® plus universal mini kit (Qiagen)

1. Disrupt and homogenize ≤50 mg tissue (or ≤100 mg brain or adipose tissue) in a suitably sized vessel containing 900 µl QIAzol Lysis Reagent using the TissueRuptor®, TissueLyser LT or TissueLyser II.

2. Incubate the homogenate at room temperature (15–25°C) for 5 min.

3. Add 100 µl gDNA Eliminator Solution. Securely cap the tube containing the homogenate and shake it vigorously for 15 s.

4. Add 180 µl chloroform and shake vigorously for 15 s.

5. Incubate sample at room temperature for 2–3 min.

6. Centrifuge at 12,000 x g for 15 min at 4°C.

7. Transfer the upper aqueous phase to a new tube. Be careful to avoid the interphase. Add 1 volume (usually 600 µl) of 70% ethanol and vortex. Do not centrifuge. Proceed immediately to step 8.

8. Transfer up to 700 µl of the sample to an RNeasy Mini spin column in a 2 ml collection tube (supplied). Close the lid, centrifuge at room temperature for 15 s at ≥8000 x g and discard flow-through.

9. Using the same collection tube, repeat step 9 using the remainder of the sample. Discard the flow-through.

10. Add 700 µl of Buffer RWT to the RNeasy Spin column. Close the lid, centrifuge for 15 s at ≥8000 x g and discard flow-through.

11. Add 500 µl Buffer RPE to the RNeasy Spin column. Close the lid, centrifuge for 15 s at ≥8000 x g and discard flow-through.

12. Add 500 µl Buffer RPE to the RNeasy Spin column. Close the lid, centrifuge for 15 s at ≥8000 x g and discard flow-through.

Optional: To further dry the membrane, place the RNeasy Mini Spin column in new 2 ml tube, close the lid, and centrifuge at full speed for 1 min.

13. Place RNeasy Spin column in a new 1.5 ml. Add 30–50 µl RNase-free water, close the lid and centrifuge for 1 min at ≥8000 x g.

Optional for RNeasy Plus Universal Mini users: Repeat step 13 using another volume of RNase-free water or using the eluate from step 13 (if high RNA concentration is required). Reuse the collection tube from step 13.

Appendix B. RNA quantity and quality

Table 4. RNA yield from ventricular cardiac muscle and RIN values for each sample. The quantity (in ng/µl) was determined with an epoch microplate spectrometer (Biotek instruments) and the quality (RIN values) with a 2100 Bioanalyzer (Agilent Technologies). RIN values range from 0 to 10, where RIN values above 8 indicate excellent RNA quality and RIN values between 6 and 8 indicate good RNA quality. Rin values were calculated for 50% of the samples.

Sample	ng/µl	RIN	Sample	ng/µl	RIN
04	2892.37	n/a	01	2866.25	9.5
08	2676.35	9.2	06	2970.26	n/a
09	2438.96	9.3	11	3031.45	9.3
17	2032.67	9	13	2435.82	9.3
21	2909.12	n/a	16	1755.03	n/a
22	2899.96	n/a	23	2765.24	n/a
29	1413.30	7.5	26	2349.48	n/a
32	2975.47	n/a	28	2389.04	n/a
05	2529.12	9.4	02	3000.22	9.1
07	2226.21	9.4	03	2923.16	n/a
12	2707.28	n/a	10	2668.79	9.4
15	2843.39	n/a	14	2886.34	n/a
20	2301.84	9.2	18	2995.97	9.1
25	2862.82	n/a	19	3039.47	9.3
30	1937.23	8.5	24	1644.26	n/a
31	1790.04	9.1	27	1950.83	n/a

Appendix C. Sequencing results

Table 5. Sequenced PCR product for each targeted gene. The sequencing was performed by Eurofins genomics.

Gene	PCR Product
serca2	CATCCTGGCTGCTGGGGGCCATCTGCCTCTCCA
plb	GCCTCCCCAGGCCAGGCAGAA
ryr1	TCATCCCTGGTTTGGGCCATCATCCCAGGGTCTGATCATTGACGCCTTCGGAGAGCTCCGAGAACCAACAAGAGCAGGTAAAGAACCTCAAGCAAGGGCAATCTCGGATATCTAGTCAGAATTCCATTCGGCTGTAAGGGATGTGATGGCTGTGAGATTGTGTGTTTCCTACAGGTCTGATCATTGACGCCTTCGGAGAGCTCCGAGACCAACAAGAGCAGGTAA
adr_{b1}	TTACCGCCTGCTCAACTGGCTGGGCTACATCAATTACTGGCCTCAACCCCATCATCTACTGCAGGAGCCCTGAGTTCCGCAC
gata4	CCTAACAGCTGTATTGATAGACTTCAGCTGAGGGGCGTGAGTGTGTGAACTGTGGAGCGATGTCCACCCCACTGTGGGAGACGGGATGGTACGGGCCACTACCTGTGTAACGCCTGTGGACTTTACCACAAGGTGAACGGTATAAACAGACCCCTCATCAAACCCCAGAGACGGCTGTCTGCTCT
nkx2-5c	GCACAAGTGTGCTGCTTCCAGGTTTAATAT

Appendix D. Statistical analysis

Table 6. Summary of the one-way repeated measures ANOVA statistical analysis for the acute reaction experiment. F and P (overall analysis) for relative amplitude (RA), time to 50% increase (T50), time to peak (TTP), time to half removal (THR) and Ca^{+2} extrusion rate (CER) are shown in first and second row respectively. P-values from Turkey test are shown for each comparison. Turkey tests performed only when the overall analysis showed significant differences. Significant differences ($P<0.05$) are shown in bold. Data for CER was $\log_{10}$-transformed prior to analysis to achieve normality.

	RA	T50	TTP	THR	CER
F	2.986	0.542	1.218	13.388	21.282
P	**0.015**	0.744	0.307	**<0.001**	**<0.001**
0.03 vs bl	0.995	n/a	n/a	0.868	0.545
0.3 vs bl	0.457	n/a	n/a	**0.016**	**0.037**
3 vs bl	0.607	n/a	n/a	**<0.001**	**<0.001**
30 vs bl	0.623	n/a	n/a	**<0.001**	**<0.001**
0 vs bl	0.919	n/a	n/a	**<0.001**	**<0.001**
0.3 vs 0.03	0.187	n/a	n/a	0.257	0.764
3 vs 0.03	0.290	n/a	n/a	**<0.001**	**<0.001**
30 vs 0.03	0.302	n/a	n/a	**<0.001**	**<0.001**
3 vs 0.3	1.000	n/a	n/a	0.347	**0.029**
30 vs 0.3	1.000	n/a	n/a	0.267	**<0.001**
30 vs 3	1.000	n/a	n/a	1.000	0.860
0 vs 30	0.124	n/a	n/a	0.913	0.951

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