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# Intensive smolt production is associated with deviating cardiac morphology in Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

High pre-slaughter mortality rates of Atlantic salmon (*Salmo salar* L.) are a reoccurring welfare issue and economic burden in aquaculture. Sudden death immediately prior to slaughter is particularly problematic given the considerable resources invested to reach this stage. Although the underlying causes of such mortality are largely unknown, cardiac deformities and diseases have become increasingly prevalent observations in deceased fish. The factors leading to this pathology remains to be revealed. Thus, we presently examined if intensive smolt production and concordant fast growth rates in young fish is associated with altered cardiac morphology at later production stages in Atlantic salmon. The observed alterations were subsequently related to mortality risk following de-lousing in a facility with a cardiomyopathy syndrome (CMS) outbreak.

We observed that intensive smolt production is indeed associated with slower growth rates at sea, distinct pathological cardiac morphological alterations, and expression of cardiac pathology markers. Moreover, the observed cardiac alterations co-occurred with CMS-related cardiac rupture at a different production facility. The present study demonstrates a clear link between pace of growth at early rearing stages and cardiac deformities later in life. Furthermore, these cardiac deformities are associated with cardiac rupture and mortality in individuals with CMS during delousing. We therefore believe that a slower pace of smolt production improves cardiac health and reduces the risk of mortality during CMS outbreaks.

## 1. Introduction

The biology of Atlantic salmon (*Salmo salar*) attracts growing research interest due to its complex life history and great economic importance for the aquaculture industry (Brocklebank and Raverty, 2002; Kongtorp et al., 2006; Kittilsen et al., 2009; Kittilsen et al., 2012; Vindas et al., 2016; Wessel et al., 2017). In aquaculture, massive efforts are devoted to developing efficient large-scale production while upholding sustainability and animal welfare. However, more than 50 million Atlantic salmon die annually during the seawater stage in Norwegian aquaculture alone (Grefsrud et al., 2018). This mortality results in annual economic losses exceeding 1.5 billion USD. During recent years loss of fish just prior to slaughter has emerged as an increasing cause of production losses (Grefsrud et al., 2018). Of particular concern, clear signs of compromised welfare and devastating production losses are reported during stressful interventions such as mechanical de-lousing at this stage in the production cycle (Grefsrud et al., 2018). Knowledge about the factors underlying this mortality is largely lacking and few studies have examined the pathophysiology causing this phenomenon. However, pre-slaughter mortality has been associated with cardiac deformities and failure (Brocklebank and Raverty, 2002; Poppe et al., 2007; Rodger and Mitchell, 2011). Indeed, heart diseases are an increasing challenge in farmed salmonids, predominated by deviating cardiac morphology and infectious heart diseases such as cardiomyopathy syndrome (CMS) (Poppe et al., 2002; Brun et al., 2003; Poppe et al., 2003). CMS is one of the leading causes of morbidity and mortality in the salmon aquaculture industry and has been estimated to affect up towards 20 % of all fish in affected sea cages (Garseth et al., 2018). Currently, there is no vaccine, cure or treatment for CMS and with rising prevalence, the economic burden of this disease may become unmanageable.

Cardiac function is closely related to cardiac morphology (*e.g.* shape

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of ventricle and composition of cardiac tissue), and the salmonid heart has previously been shown to remodel according to varying demands associated with environmental change or life stage (Farrell et al., 1990; Franklin and Davie, 1992; Clark and Rodnick, 1998; Johansen et al., 2011; Castro et al., 2013; Brijs et al., 2016a; Johansen et al., 2017; Nørstrud et al., 2018). A characteristic morphological trait associated with cardiac function in salmonids is the shape of the ventricle. This chamber resembles a pyramid in wild salmonids. Interestingly, ventricular shape tends to be more rounded with crooked (misaligned) outflow tracts (bulbus arteriosus) in farmed compared to wild fish, traits that appears to impair cardiac function and swimming performance (Poppe et al., 2003; Claireaux et al., 2005).

Intensive smolt production has been proposed as a possible cause for cardiac deformities in salmonids. A recent study examining a range of factors that may contribute to development of cardiac deformation and disease in rainbow trout (*Oncorhynchus mykiss*), found that accelerated growth rate in early rearing stages could be a major contributing factor for cardiovascular remodeling and disease in this species (Brijs et al., 2020). It remains to be examined whether the same is true for Atlantic salmon. Importantly, Leonard and McCormick (2001) found that smolt produced in the hatchery had smaller hearts compared to body mass than wild conspecifics. The consequences of transitioning to sea with a relatively smaller heart are unknown, but given the increased cardiac demand at this stage (*e.g.* thicker blood and increased blood flow to osmoregulatory organs) (Olson and Hoagland, 2008; Brijs et al., 2015; Brijs et al., 2016b), it is likely that a smaller heart is less able to meet the body's demands.

Consequences of deviating cardiac morphology has also not been directly investigated in Atlantic salmon. Thus, we are unable to predict how a farmed salmon will tolerate a stressful delousing procedure based on its cardiac morphology. Moreover, with common outbreaks of virus-associated diseases such as CMS, where only a fraction of the population develop disease and die from cardiac rupture (Garseth et al., 2018), we do not know if cardiac morphology is associated with susceptibility to disease and death.

In the present study, we compared morphological and molecular (*i.e.* molecular markers of cardiac remodeling) traits in hearts from Atlantic salmon reared with different smolt production protocols. More specifically, one group was produced over a period of ≈ 18 months (slow), and another group over a period of 11 months (fast). We found that fast smolt production is associated with slower growth rate at sea, distinct cardiac morphological deviations, and higher expression of the cardiac stress marker *atrial natriuretic peptide* (*anp*) and the angiogenesis marker *vascular endothelial growth factor* (*vegf*). In addition, the observed cardiac deformities were associated with increased mortality rates in fish undergoing mechanical delousing at a location with a CMS outbreak. The findings in the current study represent pioneering evidence for a link between growth conditions at early rearing stages and cardiac health later in life in Atlantic salmon. Furthermore, our findings underline the importance of maintaining good cardiac health in salmon that undergo frequent stressful interventions throughout their lifetime.

## 2. Material and methods

### 2.1. Experimental animals and sampling

#### 2.1.1. Fast and slow smolt production groups

To test the hypothesis that conditions under smolt production are related to deviating heart morphology later in life, two groups of Atlantic salmon reared at the same commercial sea farm (Korsnes, Ellingsen seafood AS, 68°19'53.3"N 14°56'53.6"E) were sampled in October 2019. The two groups originating from AquaGen (Trondheim, Norway) SHIELD roe were produced at the same hatchery (Silver Seed hatchery, Mølnarodden, Norway, 68°00'32.3"N 13°10'03.2"E), but with different smolt production protocols. One group of smolts were produced at (mean ± SD) 7.9 ± 3.2 °C over a period of 17.7 months (slow smolt). The other group was produced at (mean ± SD) 12.5 ± 1.8 °C over a period of 11 months (fast smolt). Both groups were kept on continuous light and fed a commercial diet (Skretting, Natura Olympic) from first introduction to feed till onset of smoltification. Smoltification was stimulated by SuperSmolt Feed Only (Stim AS, Leknes, Norway) and lasted 3-4 weeks for both groups. When fresh water ATPase (NKA1a) mRNA levels, condition factor, body mass, and smolt index indicated sea water tolerance, slow (n = 144 445) and fast (n = 1 363 593) groups were transferred to several sea water production units (2 for slow and 12 for fast, ≈ 75 000 fish in each) at the sea farm on Aug 13 (water temperature 13.7°C) and Oct 9 (water temperature 10.3°C), 2018, respectively. At transfer, the slow and fast groups had average body masses of 83.5 g and 59.5 g, respectively. Individual body weights were not measured at this time point. Instead, estimated average body weights were calculated according to standard practice at the hatchery. More precisely, estimated average body weights were based on : 1) body weight of each fish at vaccination (1-2 months prior to sea transfer when all fish were scanned in the vaccination machine Maskon VX-4, Skala, Stjørdal, Norway), 2) estimated growth based on the growth and feeding model AquaSim (Skretting) and 3) individual body weights of fish used for sea water tolerance analyses (n = 40-60 fish per group) in the month prior to sea transfer. Estimated average body weights were used for further calculations of specific growth rate.

At sampling, approximately one year after sea transfer (Oct 24, 2019) when the fast and slow groups were 23 and 32 months old, respectively, all fish (group n = 16) were captured by dip-netting in the sea cages and placed in water tubs containing 0.2mL $L^{-1}$ Benzocaine (Benzoak vet., ACD Pharmaceuticals AS, Leknes, Norway). For each fish, body mass (BM), fork length (FL), and condition factor (K) (calculated as $K = 100 \times (BM/FL^3)$) was measured. Standardized mass-specific growth rate (SGR) was calculated as $SGR(\Omega\ \%) = 100 \times ((BM_s^b - BM_{st}^b)/b \times d)$ where $BM_s$ is body mass at sampling, $BM_{st}$ is body mass at sea transfer, $b$ is the allometric mass exponent for the relationship between specific growth rate and body mass (estimated to be 0.31 for Atlantic salmon juveniles; Robertsen et al., 2013) and $d$ is number of days from sea transfer to sampling.

Intact hearts (consisting of atrium, ventricle and bulbus arteriosus, see *e.g.* Fig. 3C) were carefully dissected out, blotted dry of blood and weighed. The cardiosomatic index (CSI) was calculated as CSI(%) = 100 x (heart mass/BM). A subset of hearts (group n = 8) were fixated in neutral buffered 10% formalin solution (VWR International AS, Radnor, PA, USA). The bulbus and atrium were removed from the remaining hearts (group n = 8) before ventricles were weighed for calculation of relative ventricular mass (RVM). RVM was calculated as RVM(%) = 100 x (ventricle mass/BM). From these ventricles, three triangular tissue samples were cut off from the three "corners" of the ventricle when viewed from the dorsocranial projection and placed on RNAlater® solution (Ambion, Austin, TX, USA) at room temperature for 24 h before storing at -20 °C for later qPCR analysis.

The slow (two cages) and a proportion of the fast group (five cages) were slaughtered in December 2019 and April 2020, respectively. Producers kindly provided average sample weights per cage which was used for calculating average slaughter weights and growth rates up to slaughter.

#### 2.1.2. CMS-mortalities and survivors

To relate cardiac deformities to mortality risk, deceased and surviving individuals from a different fish cohort were sampled following mechanical delousing during a CMS outbreak at a commercial sea farm (Kråkholmen, 64°36'09.2"N 10°51'14.6"E). These fish also originated from AquaGen stock and were all sampled from one sea cage. All fish were produced at Åsen settefisk AS (63°36'08.8"N 10°57'18.2"E) for 8.5 months (from hatching to sea transfer) where they were kept at 10-14 °C under a continuous light scheme from first feed to smoltification. Smoltification was stimulated by Intro Tuning 40 40 A (Biomar AS, Brande, Denmark) and fish were transferred to sea in September 2018.

**Table 1**
Specific marker genes with primers used for quantitative real-time PCR

| Gene: | Primer pair | GeneBank accession number | Function/marker |
|---|---|---|---|
| *Hprt1* | F: CGTGGCTCTCTGCGTGCTCA<br>R: TGGAGCGGTCGCTGTTACGG | BT043501.1 | Housekeeping gene |
| *Ef1α* | F: CCCCTCCAGGACGTTTACAAA<br>R: CACACGGCCCACAGGTACA | BT059133.1 | Housekeeping gene |
| *S20* | F: GCAGACCTTATCCGTGGAGCTA<br>R: TGGTGATGCGCAGAGTCTTG | NM_001140843.1 | Housekeeping gene |
| *Ppia* | F: CTATCGGCGATTCCCCCGCC<br>R: AACCCCTTGTCCCCGGTGCA | NM_001146606 | Housekeeping gene/<br>Internal control gene |
| *Pcna* | F: TGAGCTCGTCGGGTATCTCT<br>R: CTCGAAGACTAGGGCGAGTG | BT056931.1 | Cardiomyocyte hyperplasia |
| *Anp* | F: GGCCCAATTGAAGAATCTACTG<br>R: GCACCCGGACATAGCTTTAC | NM_001123545.2 | Cardiac stress |
| *Bnp* | F: GCCGCCATAAGAGAGTTCCT<br>R: CATCTTGTCTTTGCATTGTGTTTGC | XM_014136528.1 | Cardiac stress |
| *Vegf* | F: AAACCACTGTGAGCCTTGCT<br>R: CTCCTTGGTTTGTCACATCTGC | XM_014169157.1 | Angiogenesis |

Sampling was conducted during mechanical delousing (December 2019), where mortalities were collected with a “dead fish collector”. Survivors were captured directly from the sea cage following delousing with a large dip net and sacrificed by a blow to the head. Body mass of all fish was measured before intact hearts were dissected and fixed in neutral buffered 10 % formalin solution until image analysis.

### 2.2. Magnetic resonance imaging (MRI) of fixed ventricles

Randomly selected fixed hearts (group n = 5) were rinsed in Dulbecco’s phosphate buffered saline (DPBS) with 0.5 mol/L Magnevist® (Bayer Schering Pharma, Berlin, Germany). A 1% agarose gel in DPBS with Magnevist® was used to mount the hearts in 50 ml plastic tubes. MRI was performed with a pre-clinical 9.4T MRI system (Agilent, Palo Alto, CA, USA) using a 35 mm quadrature driven birdcage radio frequency coil (Rapid Biomedical, Rimpar, Germany). A 3D gradient echo (FLASH) sequence was used for acquisition, with field of view 32 x 32 x 32 mm, yielding an isotropic resolution of 100 x 100 x 100 μm. Acqusition time was 3.42 hours per heart.

### 2.3. Imaging and analysis of cardiac morphology

All MRI recordings were manually segmented for visualization (Fig. 2C) and to enable volume estimations of the entire heart, ventricle, atrium, and bulbus. Three ventricular sections (15mm apart from the ventricular base) were selected and compact vs. spongious myocardial areas were determined. To gather information about thickness of the compact layer, these regions were binarized and distances analysed using the “Geometry to Distance Map” function in ImageJ. Due to large differences in ventricular size, distances were normalized to overall area of the compact layer. Irregularity of the compact myocardium was quantified as standard deviation of all distance measurements in each heart. Fixated hearts were photographed from the left lateral and ventrodorsal projection inside a Styrofoam box (Internal H: 24cm W: 21 cm L: 27 cm) lit by an internal LED-light (Northlight LED-lamp, Art. No. 36-6465). Photographs were captured with a Cannon EOS 4000D camera with an EF-S 18-55 III lens mounted on the Styrofoam box. Thus, all hearts were photographed from the same angle and under standardized light conditions. Ventricular height : width ratio was analysed from the ventrodorsal projection by dividing ventricle length (from apex to bulbus) by ventricle width (Fig. 3A) according to (Claireaux et al., 2005). Ventricular asymmetry and bulbus misalignment were quantified by analysing shoulder and bulbus angles from the left lateral projection. More specific, ventricular asymmetry is a measure of the angle between the ventricular vertical axis and the shoulder axis (Fig. 3B). The angle between the bulbular horizontal axis and the shoulder axis represents bulbus misalignment (Fig. 3 C). Ventricular coronaries project from the bulbus. Thus, the number of bulbular coronary collaterals is an indirect measure of ventricular vascularization. Due to the distinct contrast between the coronaries and the pale bulbus, counting of bulbular collaterals yields more precise results. Hence, the number of bulbular coronary collaterals were quantified as an indicator of overall cardiac vascularization. All photos and MRI captures were analysed using ImageJ (National Institutes of Health, USA).

### 2.4. Real-time qPCR

Ventricular tissue was thawed before the compact myocardium was separated from the spongious myocardium using a scalpel under a dissecting scope. RNA was then extracted from the compact tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The extracted RNA was treated for contaminating DNA with TURBO DNA-free kit by Invitrogen™ (Carlsbad, CA, USA). The quality and quantity of the RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), respectively. RNA quality was determined from RNA integrity numbers (RINs) calculated by the 2100 Bioanalyzer (range: 1–10). Samples with RIN values below 6 were excluded from further analysis ($n = 1$ in slow group). The RIN values for the rest of the tissue samples ranged from 7.6 to 8.2 with an average of 7.93, confirming good RNA quality. cDNA was synthesized from 1 μg total RNA by using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturers protocol. Previously published gene specific primer sequences for salmon *hypoxanthine phosphoribosyl transferase 1* (*hprt1*), *elongation factor 1α* (*ef1α*), *proliferating cell nuclear antigen* (*pcna*), *peptidylprolyl isomerase A* (*ppia*), and the *ribosomal protein S20* were used (Thörnqvist et al., 2015; Vindas et al., 2017). In addition, gene specific primer sequences for salmon *atrial natriuretic peptide* (*anp*), *B-type natriuretic peptide* (*bnp*) and *vascular endothelial growth factor* (*vegf*) were designed using the primer-BLAST tool provided by the National Center for Biotechnology Information (NCBI). A minimum of three primer pairs were designed at exon junctions for each gene and the primers showing the lowest crossing point values, single peak melting curve, and amplification of the right amplicon were chosen (Table 1). The quantitative real-time PCR (qPCR) products were also sequenced to verify that the primers amplified the correct cDNA sequence. Real time PCR was carried out using a Roche 96 Light cycler (Roche Diagnostics, Penzberg, Germany). The reference genes tested were *S20*, *hprt1*, *ppia* and *ef1α*. As *ppia* yielded the lowest cq-values and least variance between and within runs, this gene was selected as the internal control gene for calculation of relative gene expression (ΔΔCq).

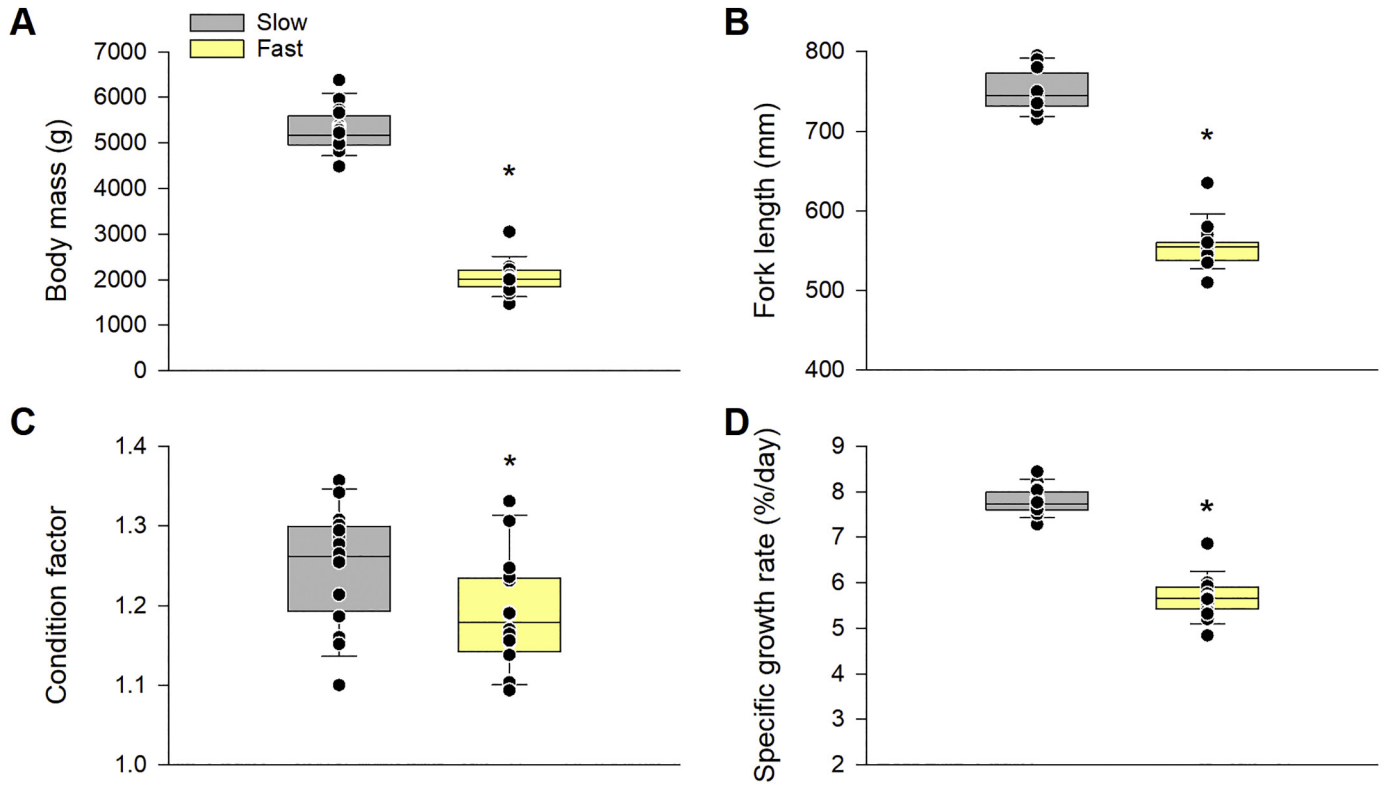


**Fig. 1.** Fast smolt production is associated with slower growth rate at sea. Body mass, fork length, and condition factor were all reduced after approximately one year of sea rearing in adult Atlantic salmon (*Salmo salar*) reared with a fast smolt production protocol (11 months at 13°C) compared to a slow protocol (18 months at 8°C, A-C). Similarly, specific growth rate was lower in fast produced smolts (D). n = 16 in each group. Data are presented as mean ± s.e.m. Statistical differences were tested by unpaired two-tailed *t*-tests. *indicates statistical difference < 0.05.

### 2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) or SigmaPlot (Systat Software Inc, Chicago, Il, USA). Differences between groups (slow vs. fast and mortalities vs. survivors) were tested by unpaired two-tailed *t*-tests and data are presented as group mean ± s.e.m. or as normalized values to mean of the slow group (for mRNA expression levels). Following tests for normal distribution, linear regression analyses were performed using either Pearsons Correlation or Spearman Rank Correlation in SigmaPlot. Statistical outliers were removed by Grubbs' method (alpha = 0.05) and differences were considered significant for $p < 0.05$.

## 3. Results

### 3.1. Lower growth rate at sea in fast smolt

Despite a comparable time period (14.5 months for slow vs. 13 months for fast) at sea for both groups, body mass (BM), fork length and condition factor were markedly lower in the fast group (Fig 1A-C). This smaller size was related to a markedly lower (27 %) mass-specific growth rate (Ω %) (Fig. 1D). Note, that average growth rates for the entire cohort of the two groups were 6.79 ± 0.01 and 5.42 ± 0.02 %/day at slaughter, which is comparable to the subsets reported at sampling (Fig. 1D)

### 3.2. Larger hearts in fast smolt

The CSI (Fig. 2A) and RVM (Fig. 2B) were both higher in the fast compared to the slow group. Representative captures of MRI-scanned hearts from slow and fast smolt groups are shown in Fig. 2C. Due to higher ventricular volume, overall cardiac volume (% body mass) was significantly larger in fast smolt (Fig. 2D). However, when quantifying cardiac chamber volumes as fraction of whole heart volume, it appeared that the bulbus was relatively smaller in fast smolt (Fig. 2E).

### 3.3. Signs of pathological cardiac remodeling in fast smolt

Ventricular height : width ratios were lower in fast compared to slow smolt, indicative of rounder ventricles in the fast group (Fig. 3A). No significant difference was observed in ventricular asymmetry between the two groups (Fig. 3B). Bulbus misalignment, on the other hand, was higher in fast smolt (Fig. 3C). Despite smaller relative bulbus size in this group (Fig. 2E), the total number of bulbular coronaries (*i.e.* number of blood vessels on the bulbus arteriosus) were higher (Fig. 3D). Lastly, the proportion of compact myocardium was also higher in fast smolt, suggestive of hypertrophic growth of this tissue. In addition, compact irregularity (*i.e.* varying thickness) tended to be higher in this group (Fig. 3E).

The natriuretic peptides *anp* and *bnp* are diagnostic markers of cardiac pathology in mammals and salmonids (Volpe et al., 2014; Johansen et al., 2017). The mRNA abundance of *anp* was significantly higher in ventricular tissue from fast compared to slow smolt (Fig. 4A). Similarly, *bnp* levels tended to be elevated (P = 0.10, Fig. 4B). To evaluate whether the observed ventricular enlargement (Fig. 2 and 3) was caused by cell proliferation (increased number of cardiomyocytes) or hypertrophy (larger cardiomyocytes), mRNA levels of the cell proliferation marker PCNA was quantified. Expression levels were similar between groups (Fig. 4C), indicating that the observed ventricular enlargement in fast smolt was caused by cardiomyocyte hypertrophy. In accordance with a higher number of bulbular coronary collaterals in fast smolts (Fig. 3D), mRNA levels of the angiogenesis (growth of blood vessels) marker *vegf* were higher in this group (Fig. 4D). Taken together,

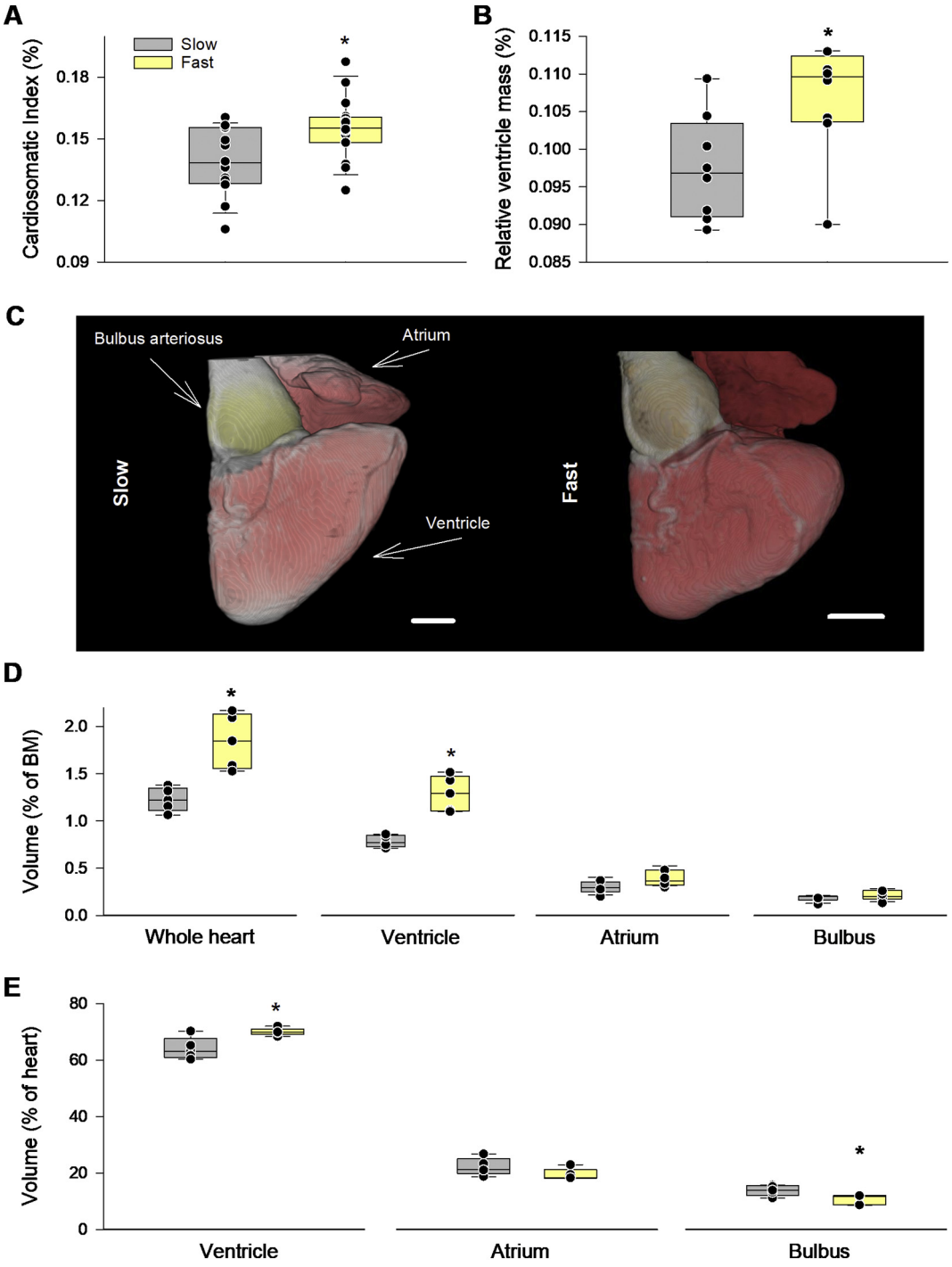


**Fig. 2.** Fast smolt production yields increased heart size. (A) Cardiosomatic index ($n$ = 16 pr. group) and (B) relative ventricular mass ($n$ = 8 pr. group) of adult Atlantic salmon (*Salmo salar*) were increased in individuals that underwent fast (11 months at 13°C) compared to slow smolt production (18 months at 8°C) after approximately one year of sea rearing. (C) Representative 3D reconstructions of MRI captures in hearts from slow and fast smolt groups. The larger overall heart size in fast smolts was due to increased ventricular volume (D and E). n = 5 pr. group. Data are presented as mean ± s.e.m. Statistical differences were tested by unpaired two-tailed *t*-tests. *indicates statistical difference < 0.05.

these results indicate that fast smolt production induces maladaptive cardiac remodeling and pathophysiological hypertrophy.

### 3.4. Relative heart weight predicts cardiac health

Regression analyses were performed to evaluate if easily accessible measures such as CSI, bulbus misalignment, ventricular asymmetry and relative ventricle mass reflect the more complex and thorough measures of heart morphology also applied in this study. First, as expected CSI predicted heart size (cardiac volume) assessed from MRI recordings (Fig. 5A). Second, heart size correlated with both ventricular roundness and bulbus misalignment (Fig. 5B and C). Third, bulbus alignment correlated positively with thickening of the ventricular compact layer (hypertrophy, Fig. 5D) and ventricular asymmetry was negatively correlated with irregularity of this tissue (Fig. 5E). Finally, an important association between relative ventricle mass and ANP gene expression levels was observed (Fig. 5F). Thus, our results indicate that simple measures involving a weighing scale and a camera can be applied to

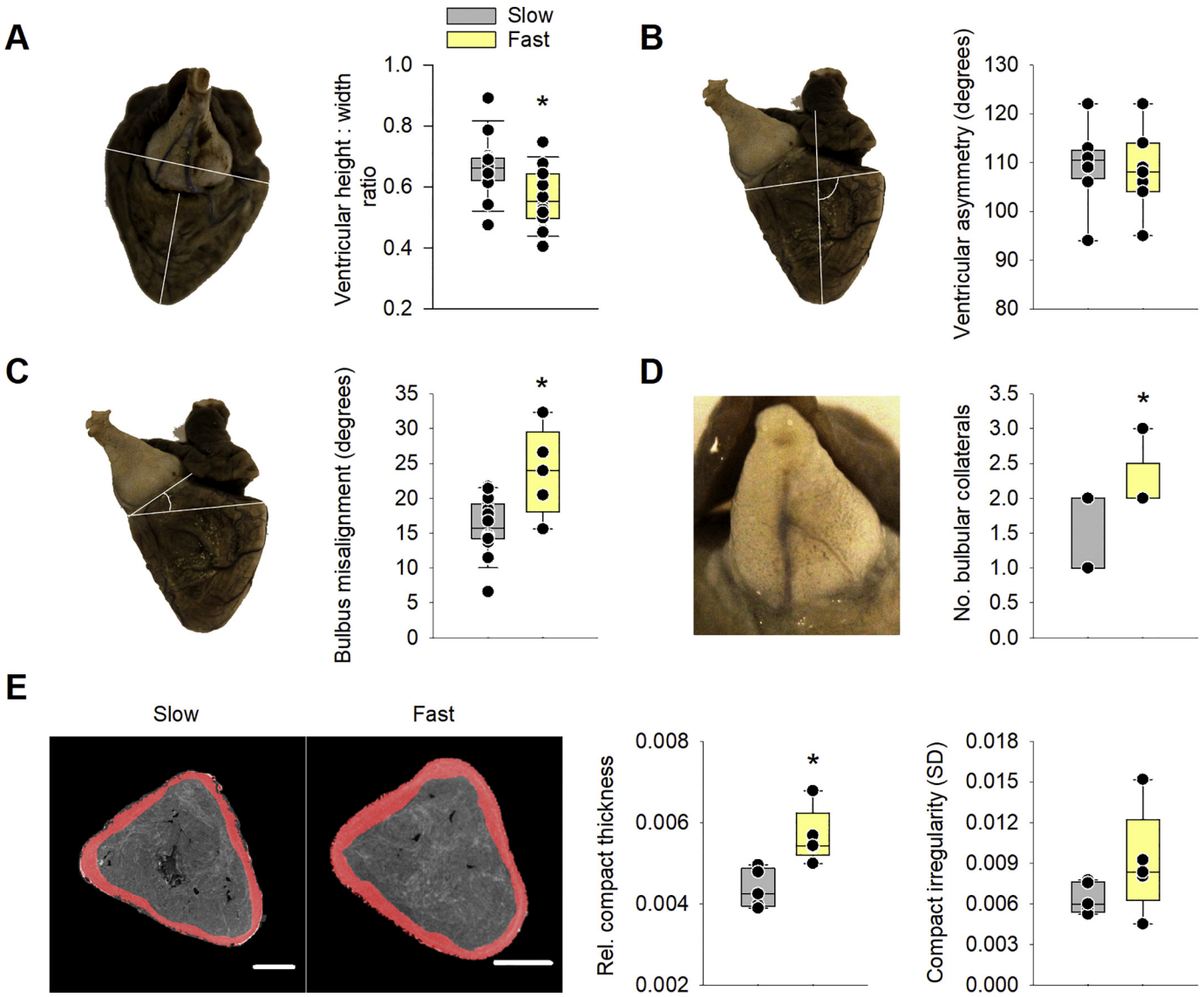


**Fig. 3.** Fast smolt production is associated with rounded ventricles, bulbus misalignment and thicker compactum. Fish produced under fast (11 months at 13°C) compared to slow smolt production conditions (18 months at 8°C) had reduced ventricular height : width ratios indicative of rounder ventricles, whereas ventricular symmetry was similar between groups (A and B) after approximately one year of sea rearing. The bulbus was misaligned with the ventricle and more coronary arteries were observed on this chamber (C and D, $n = 8$ pr. group). Concomitant to these alterations, thickness of the compact myocardium was also increased in the fast group (E, $n = 5$ pr. group). Data are presented as mean ± s.e.m. Statistical differences were tested by unpaired two-tailed *t*-tests. * indicates statistical difference < 0.05.

predict cardiac health and remodeling.

### 3.5. Ventricle shape predicts risk of mortality during a CMS-outbreak

To evaluate if the morphological traits presented in this study are associated with mortality rate, the same traits were analysed in hearts from deceased and surviving fish following mechanical delousing at a location with CMS-outbreak. 17 of 20 dead fish presented with cardiac rupture, a clinical manifestation of CMS (Bruno and Poppe, 1996). In contrast, none of the surviving fish (survivors) showed clinical signs of CMS. There was no significant difference in CSI between the two groups (Fig. 6A) but notice that CSI was equal to that of the fast smolt (Fig. 2A and 5A) in both survivors and deceased specimens. Dead fish had more rounded ventricles (Fig. 6B) whereas bulbus misalignment was similar between the two groups (Fig. 6C). Ventricular asymmetry was increased in deceased fish (Fig. 6D). Thus, it appears that large salmon hearts with round and asymmetric ventricles predispose for fatalities due to cardiac rupture.

## 4. Discussion

By comparing hearts from Atlantic salmon reared at the same sea farm and produced at the same hatchery but employing different smolt production protocols, we found that fast smolt production is associated with decreased growth rates at sea and distinct cardio-morphological deviations compared to smolt produced at slower rates. Fast smolts had higher relative heart and ventricle sizes, rounder ventricles and more misaligned bulbuses compared to slow smolts. These hearts also presented with larger proportions of compact myocardium and unaltered expression of *pcna*, both indicative of hypertrophic growth. Combined, our findings are indicative of pathological cardiac hypertrophy and remodeling in fast smolts. Furthermore, these morphological indications of cardiac pathology were accompanied by higher expression of molecular pathology markers. Several of the observed morphological

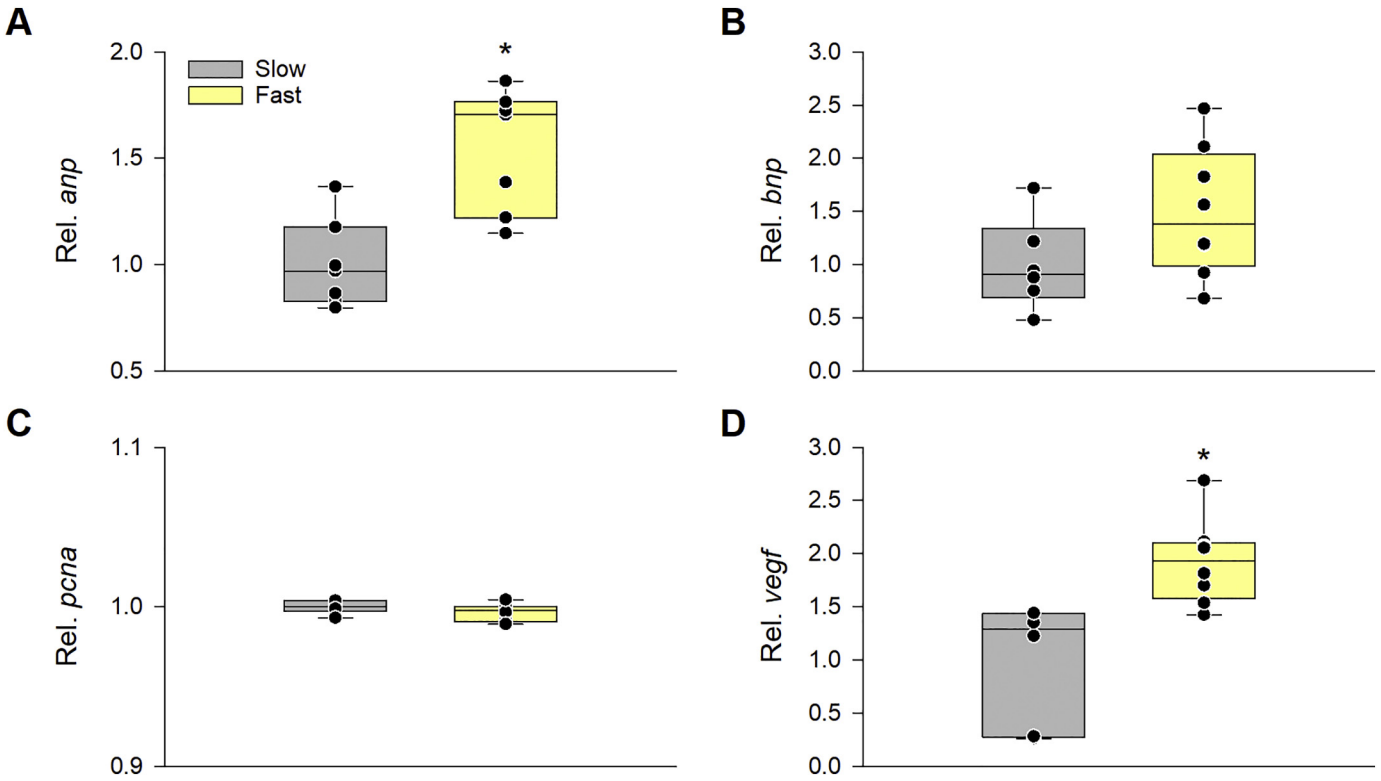


**Fig. 4.** Evidence of pathological remodeling in fast smolt production. Relative (rel.) transcript (mRNA) levels (relative to the control gene *peptidylprolyl isomerase*; *ppia*) of (A) *atrial natriuretic peptide* (*anp*), (B) *B-type natriuretic peptide* (*bnp*), (C) *proliferating cell nuclear antigen* (*pcna*) and (D) *vascular endothelial growth factor* (*vegf*) in compact ventricular tissue of Atlantic salmon (*Salmo salar*) reared at either a slow (18 months at 8°C) or fast (11 months at 13°C) smolt production protocols ($n_{slow}$ = 7, $n_{fast}$ = 8). Data are presented as mean ± s.e.m. Statistical differences were tested by unpaired two-tailed *t*-tests. * indicates statistical difference < 0.05.

deviations were also associated with increased risk of mortality following mechanical delousing during a CMS outbreak. Taken together, our data provide pioneering evidence for a link between smolt production protocols and cardiac health later in life, and demonstrate adverse consequences of cardio-morphological deviations in farmed Atlantic salmon.

### 4.1. Intensive smolt production is associated with cardiac morphological and molecular indications of pathology

The described link between smolt production protocol and cardiac morphology is entirely novel. We observed that hearts from the slow and fast smolt groups are distinct with regards to heart size, ventricular shape, composition of ventricular tissue and alignment of the bulbus arteriosus. Although increased relative ventricular mass usually indicates physiological hypertrophy and increased cardiac function (Graham and Farrell, 1989; Franklin and Davie, 1992), a recent study showed that cardiac hypertrophy in salmonids can indeed be pathological and result in reduced pumping capacity of the heart (Johansen et al., 2017). Several factors indicate that the ventricular deviations occurring in fast smolt represent maladaptive hypertrophic growth and remodeling. First, a rounder ventricle is related to impaired cardiac pumping and physical swimming performance (Claireaux et al., 2005). Second, fish in this group have a crooked outflow tract (misaligned bulbus). This trait is likely associated with increased cardiac workload since a larger angle at the ventrio-bulbular junction increases ventricular resistance (Poppe et al., 2003). In mammals, increased cardiac resistance elevates workload and induce compensatory, yet pathological heart growth (Shimizu and Minamino, 2016). Indeed, the cardiac deviations observed in fast smolt were associated with increased RVM and thicker ventricular compactum. In further support of this notion, bulbus misalignment correlated positively with both heart volume and proportion of compact tissue. Third, unchanged expression levels of the proliferation marker *pcna*, suggests that the thicker compactum in fast smolt was a result of hypertrophy (cardiomyocyte growth) rather than cell proliferation, further indicating pathological remodeling in this group (Johansen et al., 2017). The increased amount of compact tissue was accompanied by elevated expression of the angiogenesis marker *vegf* and increased vascularization. In mammals, coronary angiogenesis is essential to maintain vascularization and perfusion of the heart during physiological and pathological hypertrophy (Gogiraju et al., 2019). In the present study, increased vascularization likely serves to support a thicker layer of compact tissue in fast smolts. Fourth, pathological cardiac remodeling is associated with increased expression of several specific molecular pathology markers in mammals, including *anp* and *bnp* (Volpe et al., 2014). Assuming a similar role for natriuretic peptides in salmonids, the observed increase in *anp* mRNA levels along with a strong tendency towards *bnp* upregulation in fast smolt, is yet another indication that maladaptive remodeling occurs in this group. The positive correlation between *anp* and RVM further support this statement. Of note, the ventricular phenotype observed in fast smolt resembles that of cortisol-induced ventricular remodeling in rainbow trout. This cortisol-induced remodeling is associated with increased RVM and compact myocardium, but impaired cardiac – and physical (*i.e.* swimming) performance and an increased expression of both *anp* and *bnp* (Johansen et al., 2017; Nørstrud et al., 2018).

### 4.2. Intensive smolt production results in slower growth rates at sea

Although slow smolt production involves slower growth in the hatchery, it appears to accelerate growth at sea. Our data indicate that a fast smolt production protocol is associated with more vulnerable hearts later in life. Supposing, that the observed cardiac morphology truly reflects pathological remodeling and impaired cardiac function in

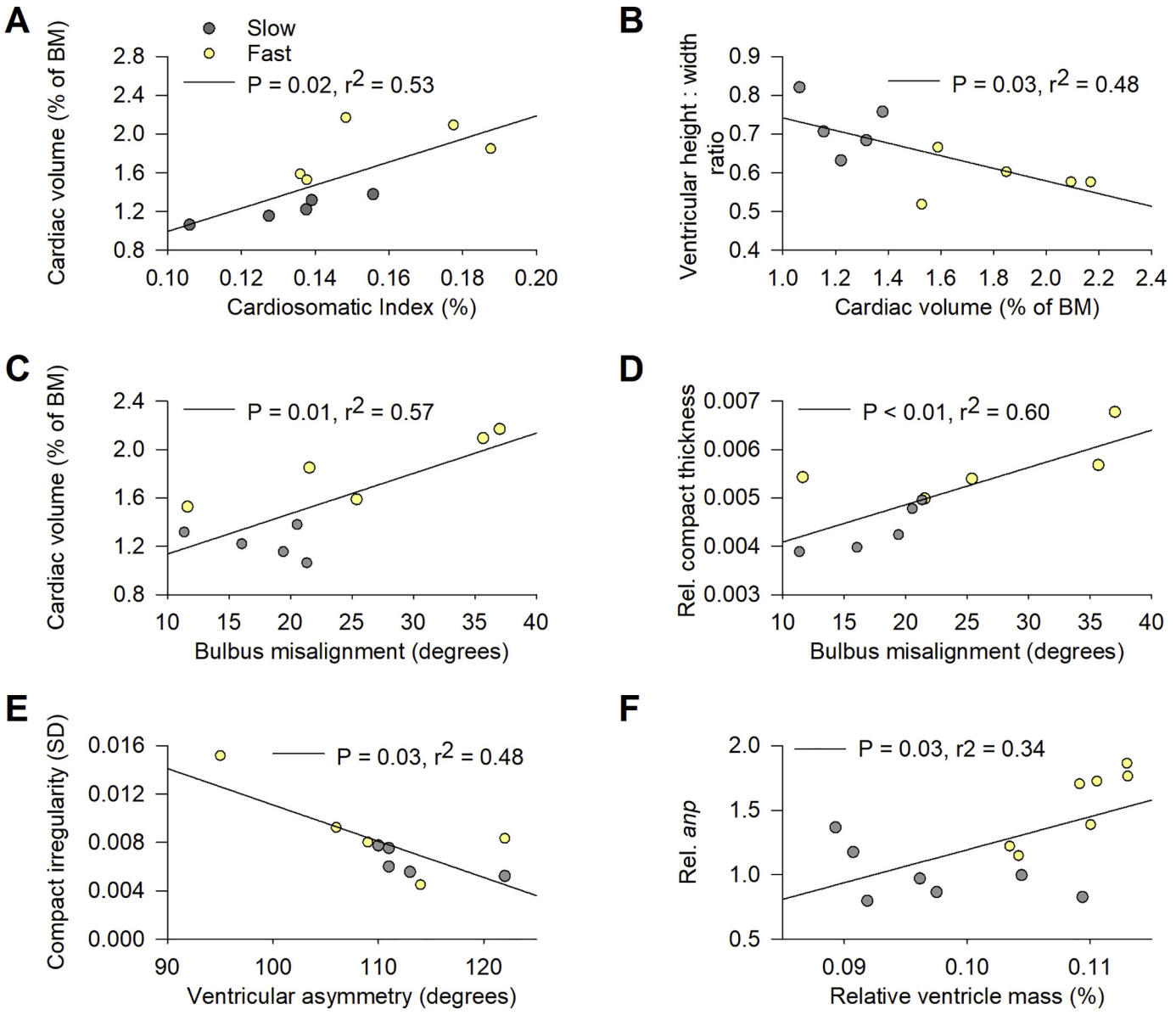


**Fig. 5.** Heart weight and shape predict thickness of compact myocardium and *anp* levels. Statistically significant correlations were observed between (A) cardiosomatic index and heart volume, (B) heart volume and ventricular height : width ratio, (C) bulbus misalignment and heart volume, (D) bulbus misalignment and relative (rel.) compact thickness, (E) Ventricular asymmetry and compact irregularity (*i.e.* varying thickness of compact tissue), and (F) relative ventricular mass and rel ANP mRNA levels in Atlantic salmon (*Salmo salar*) reared and produced at either a slow (18 months at 8°C, grey dots) or fast (11 months at 13°C, black dots) smolt production protocol. Data is presented as individual datapoints and relationships were tested by linear regression analysis.

fast smolt, it is tempting to hypothesize that reduced cardiac capacity in this group is responsible for the restricted growth at sea. Further research linking ventricular morphology and vessel misalignment to cardiovascular performance is, however, required to verify this. Thus, since fast smolt production is associated with slower growth at sea and cardiac deviations, promotion of fast growth at sea rather than in the hatchery might be a promising strategy to develop efficient large-scale production while upholding cardiac health and welfare.

### *4.3. CMS-related cardiac rupture and mortality is related to deviating cardiac morphology*

Poppe et al. (2003) proposed that deviating cardiac morphology contributes to mortality in association with stressful aquaculture operations such as mechanical delousing. In agreement with this notion, we observed that CMS-related cardiac rupture and mortality was associated with rounder and less symmetric ventricles. Interestingly, hearts in the fast smolt group also presented with rounder ventricles, suggesting that fast smolt production predisposes fish for maladaptive cardiac morphology that promote disease progression and death during CMS. Although further studies are required, the present results strongly suggest that a slower pace of smolt production reduces the risk of mortality during CMS outbreaks.

A number of the parameters presently measured correlated well with each other, and measures of heart size (cardiac volume and RVM) were able to predict ventricular roundness, bulbus misalignment and expression of the cardiac disease marker *anp*. Additionally, bulbus alignment predicted thickness of the ventricular compact layer whereas, ventricular symmetry correlated with the regularity of this tissue. Regularity of the fish compactum has hitherto not been described in the literature. However, an irregular compactum leads to weaker contraction in affected areas of the mammalian heart (Frisk et al., 2016). Since the compact layer is the force-generating tissue of the salmonid ventricle (Pieperhoff et al., 2009), similar consequences of

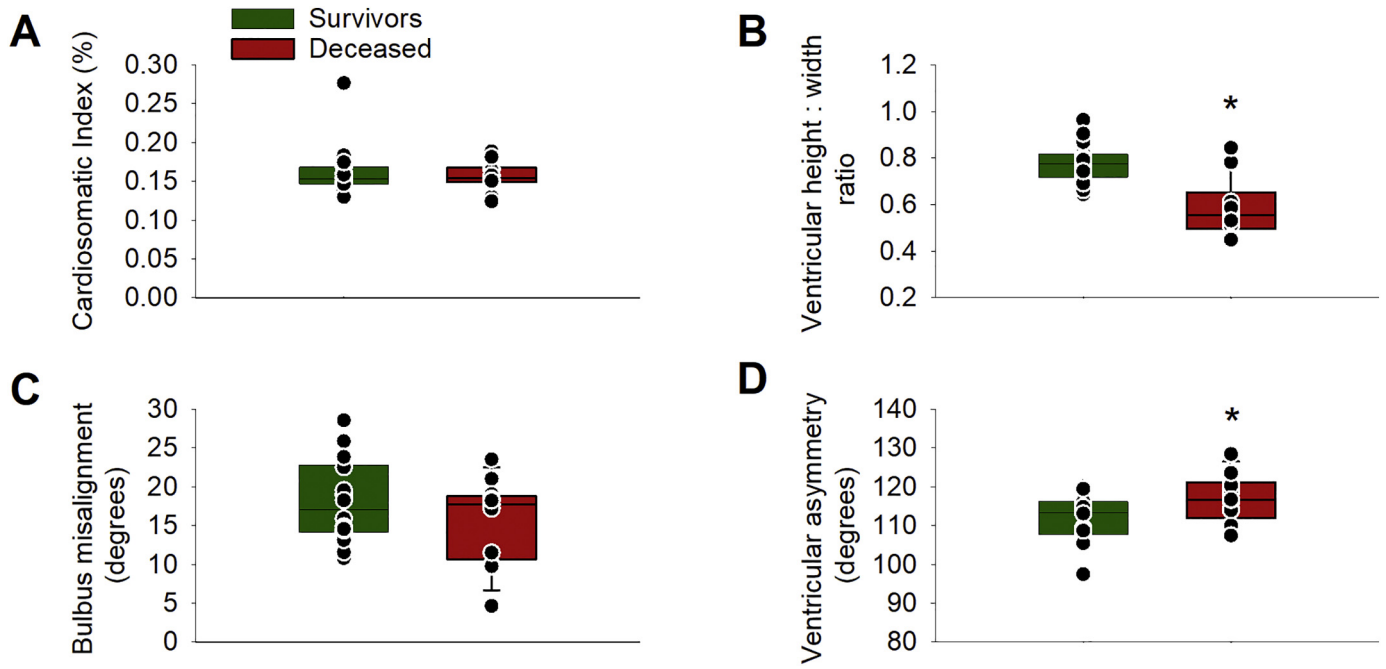


**Fig. 6.** Morbidity is associated with ventricular morphology. Cardiac weight and morphology were measured in surviving and deceased Atlantic salmon (*Salmo salar*) following mechanical de-lousing during a cardiomyopathy syndrome outbreak ($n_{survivors}$ = 20, $n_{deceased}$ = 17). (A) Cardiosomatic index was similar between survivors and deceased individuals. However, ventricles were significantly rounder in the deceased group indicated by reduced ventricular heighth : width ratio (B). Bulbus alignment was similar between groups although, ventricles from deceased fish were less symmetric (C and D). Data are presented as mean ± s.e.m. Statistical differences were tested by unpaired two-tailed *t*-tests. * indicates statistical difference < 0.05.

irregular compacta would be expected. Currently, diagnostic tools in the aquaculture industry are limited to histology and PCR, which are costly, time consuming, and yield restricted information on organ performance. Thus, readily available tools to execute precise risk assessments prior to de-lousing and other stressful interventions are lacking. In the short term, the correlations identified here offers important perspectives in this regard. We show that morphological traits like ventricular roundness, asymmetry and CSI predicts cardiac health and mortality risk. Intriguingly, these parameters can be assessed with a simple weighing scale and a camera; tools that are readily available and easily implemented.

## 5. Conclusion

A common strategy to increase efficiency of production in salmon aquaculture is to reduce rearing time and costs by accelerating smolt production in the hatchery. However, our present results indicate that this strategy compromises cardiac health and welfare. Promoting fast growth at sea rather than in the hatchery appears to be a more promising strategy for development of efficient large-scale production. In conclusion, increased rearing time in the hatchery improves cardiac health and reduces production losses.

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## Author contributions

I.B.J. and Ø. Ø.: Conceptualization, I.B.J.: Project administration, I.B.J.: Funding acquisition, I.B.J, M. H., M. A.V., Ø.Ø.: Investigation, data collection., L.Z.: Methodology, MRI, M.H.: Methodology, ELISA and qPCR, M.F. and I.B.J.: Formal analysis and image analysis, M.F.: visualization, I.B.J. and M.F.: writing, original draft, I.B.J., M.F., M.H., M.A.V., L.Z., and Ø.Ø.: writing, review & editing.

## Declaration of Competing Interest

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

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