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RESEARCH ARTICLE

Growth hormone transgenesis in coho salmon disrupts muscle immune function impacting cross-talk with growth systems

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ABSTRACT

Suppression of growth during infection may aid resource allocation towards effective immune function. Past work supporting this hypothesis in salmonid fish revealed an immune-responsive regulation of the insulin-like growth factor (IGF) system – an endocrine pathway downstream of growth hormone (GH). Skeletal muscle is the main target for growth and energetic storage in fish, yet little is known about how its growth is regulated during an immune response. We addressed this knowledge gap by characterising muscle immune responses in size-matched coho salmon (*Oncorhynchus kisutch*) achieving different growth rates. We compared a wild-type strain with two GH transgenic groups from the same genetic background achieving either maximal or suppressed growth – a design separating GH's direct effects from its influence on growth rate and nutritional state. Fish were sampled 30 h post-injection with phosphate-buffered saline (control) or mimics of bacterial or viral infection. We quantified mRNA expression levels for genes from the GH, GH receptor, IGF hormone, IGF1 receptor and IGF-binding protein families, along with immune genes involved in inflammatory or antiviral responses and muscle growth status marker genes. We demonstrate dampened immune function in GH transgenics compared with wild-type. The muscle of GH transgenics achieving rapid growth showed no detectable antiviral response, coupled with evidence of a constitutive inflammatory state. GH and IGF system gene expression was strongly altered by GH transgenesis and fast growth, both for baseline expression and responses to immune stimulation. Thus, GH transgenesis strongly disrupts muscle immune status and normal GH and IGF system expression responses to immune stimulation.

KEY WORDS: Growth, Immunity, Growth-immune cross-talk, Skeletal muscle, Growth hormone, Insulin-like growth factor, Transgenesis, *Oncorhynchus kisutch*

INTRODUCTION

Skeletal muscle is the most important target for growth investment and energy storage in teleost fish, representing more than half of body mass in salmonid species. This tissue is remobilised regularly during life, e.g. upon maturation or during fasting, with the resultant resources available for allocation to other physiological systems. The regulation of skeletal muscle mass represents a dynamic balance between protein synthesis and degradation pathways, controlled by growth hormone (GH) and insulin-like growth factors (IGFs) (Johnston et al., 2011; Fuentes et al., 2013). GH is the master growth regulator in vertebrates, acting on target tissues via its receptor GHR, or indirectly by stimulating hepatic production of IGFs (Fuentes et al., 2013). The IGF system comprises IGF-I and IGF-II hormones, which promote growth through IGF-1R signalling pathways (Jones and Clemmons, 1995; Wood et al., 2005), including in skeletal muscle (Johnston et al., 2011). The action of IGFs is modulated in the extracellular environment by a family of IGF-binding proteins (IGFBPs) that influence IGF availability to IGF-1R (Firth and Baxter, 2002; Garcia de la serrana and Macqueen, 2018). Genes from the GH and IGF pathways have been expanded by genome duplication events in teleost evolutionary history (Ocampo Daza et al., 2011; Macqueen et al., 2013; Lappin et al., 2016; Alzaid et al., 2016a; Robertson et al., 2017), including a salmonid-specific event that occurred 88–103 mya (Macqueen and Johnston, 2014).

Many past studies of teleosts have investigated the *in vivo* regulation of GH and IGF pathway genes under distinct nutritional states, highlighting a key role for these systems in the growth and remodelling of skeletal muscle. For example, Atlantic salmon (*Salmo salar* L.) fed after a period of fasting showed upregulation of *IGF-I* and *IGFBP-4*, whereas *IGF-II*, *IGF1R* and *IGFBP-2* were downregulated (Bower et al., 2008). Reciprocally, muscle *IGF-I* transcript levels were downregulated in Atlantic salmon (Breves et al., 2016), yellowtail (*Seriola quinqueradiata*) (Fukada et al., 2012) and tilapia (*Oreochromis mossambicus*) (Fox et al., 2010) by fasting, whereas *GHR* transcripts were induced by fasting in tilapia (Fox et al., 2010). Despite such progress, to the best of our knowledge, the regulation of GH and IGF pathway genes during an immune response in teleost skeletal muscle remains uncharacterised.

It has long been recognised that growth rate and immune function are highly interrelated in multicellular organisms, and that energetic allocation into growth must be traded off against immune function and disease resistance (reviewed in Arendt, 1997). When considering the underlying molecular mechanisms, past work provides evidence for cross-talk between the GH and IGF pathways and immune function (Heemskerk et al., 1999; Yada, 2007; O'Connor et al., 2008; Smith, 2010; Franz et al., 2016). We recently reported that IGF signalling is repressed in rainbow trout (*Oncorhynchus mykiss*) in response to bacterial and viral infections, with a striking upregulation of IGFBP subtypes that restrict IGFs from IGF-1R, and potentially modify immune function (Alzaid et al., 2016a,b). Interestingly, immune responsive genes from the IGF pathway showed a striking co-expression with pro-inflammatory cytokine and antiviral genes regulated by conserved immune signalling pathways (Alzaid et al., 2016b), supporting the hypothesis that mechanisms have evolved that limit growth investment as an intrinsic component of host defence.

What little is known about the regulation of GH and IGF pathway genes in teleost muscle following immune stimulation has come from *in vitro* work. Treatment of Atlantic salmon muscle cell cultures with the pro-inflammatory cytokine IL-1β resulted in upregulation of an

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IGFBP-6 subtype, suggested to repress IGF signalling (Pooley et al., 2013; Heidari et al., 2015). However, in a separate study, treatment of differentiated Atlantic salmon myotubes with the same cytokine resulted in limited expression changes in IGF system genes, despite the verified presence of muscle fibre atrophy (Garcia de la serrana et al., 2017). Given the dearth of knowledge in this area, an important goal of the current study was to perform a comprehensive *in vivo* analysis of expression responses for GH and IGF pathway genes following immune stimulation in teleost skeletal muscle.

GH transgenesis offers an ideal model to address mechanisms of cross-talk between growth and immunity. Salmonid species overexpressing GH in a wild-type (WT) genetic background show enhanced appetite, feed intake and food conversion (e.g. Devlin et al., 2009; White et al., 2016), as well as altered protein, lipid and carbohydrate metabolism (Raven et al., 2006; Leggatt et al., 2009; Higgs et al., 2009), with major effects on energy intake and processing that results in highly elevated growth rate and enhanced condition factor, including elevated levels of lipid stores (e.g. Devlin et al., 1994, 2004; Higgs et al., 2009). Rapid growth requires matched increases in energetic intake, and GH transgenic salmon will achieve suppressed growth potential (approaching WT) when provided a WT ration (Rise et al., 2006; Raven et al., 2008). GH transgenic salmon on a restricted ration have the same plasma IGF-I levels and liver *IGF-I* mRNA expression as WT, despite highly increased plasma GH (Raven et al., 2008), and possess reduced energy stores relative to WT and fully fed transgenic salmon (Higgs et al., 2009). This study system can be used to disentangle the impacts of GH from its influence on feed intake and growth rate. To date, however, there are no published reports addressing the impact of GH transgenesis on the response of skeletal muscle to immune challenge, although it is known that systematic immune function is reduced in GH transgenic salmon (Jhingan et al., 2003; Kim et al., 2013).

The aim of the current study was to characterise gene expression regulation linking growth to immune function within the skeletal muscle of coho salmon, focusing on the GH and IGF systems. We contrasted transcriptional responses of genes from both pathways, in addition to selected markers of immune and muscle growth status, to immune stimulation in three experimental groups, comparing WT animals with a GH transgenic strain achieving either maximal or supressed growth by ration manipulation. Our findings reveal a disruption to immune function and the regulation of growth–immune cross-talk in muscle of GH transgenic animals, with implications for the health of rapidly growing fish strains used in aquaculture, and for risk assessments concerning the impacts of transgenic salmon should they be released into natural environments.

MATERIALS AND METHODS

Experimental design

Experiments on coho salmon, *Oncorhynchus kisutch* (Walbaum 1792), were performed at Fisheries and Oceans Canada (DFO), West Vancouver, BC, Canada. This facility is designed to prevent the escape of transgenic fish to the natural environment. All work was done in accordance with guidelines of the Canadian Council on Animal Care, under a permit (#12-017) from the DFO's Pacific Regional Animal Care Committee. All studied fish were initially maintained under common garden conditions (4000 litre tanks supplied with 10.5±1°C aerated well water, natural photoperiod, at a density of <5 kg m^{-3}) and fed a commercial diet (Skretting Ltd, Vancouver, BC, Canada) twice daily at 09:00 h and 15:00 h (3% of body mass per day). Three experimental groups were generated after Oakes et al., 2007 and Raven et al., 2008: (i) 19-month-old WT animals fed to satiation throughout ontogeny ('WT'); (ii) 6-month-old GH transgenic animals fed to satiation throughout ontogeny (transgenic full ration: 'TF'); and (iii) 17-month-old GH transgenic animals fed to the WT satiety level throughout ontogeny (transgenic restricted ration: 'TR'). Using fish of different ages was necessary to standardise the confounding effects of body size, owing to different growth rates among the groups. The WT group was the offspring of parents collected at the Chehalis River in British Columbia, Canada (Devlin et al., 2004). The GH transgenic strain (M77) was originally produced by microinjecting the GH gene construct of sockeye salmon (*Oncorhynchus nerka*; OnMTGH1) into the eggs of WT parents from the same WT strain (Devlin et al., 1994, 2004), and subsequently maintained by crossing transgenic males with Chehalis River females at each generation to maintain a wild-type genetic background.

For each experimental group, 60 animals [size-matched, immature and of unknown sex; mean mass±s.d. as follows: WT: 74.2±3.6 g, TF: 77.9±6.1 g, TR: 78.6±3.3 g; mean condition factor (K): calculated as $K=(M\times l^3)\times 100$, where M is mass (g) and l is length (mm), as follows: WT: 1.15, TF: 1.17; TR: 1.12] were marked by fin-clips and allocated into four separate 70 litre tanks prior to immune stimulation. The fish were then intraperitoneally injected with either: (i) polyinosinic–polycytidylic acid (Poly I:C) at 200 µg per 100 g fish mass (24 fish per tank per group); (ii) peptidoglycan (PGN) at 200 µg per 100 g fish mass (24 fish per tank per group); or (iii) phosphate-buffered saline (PBS) (i.e. control, 24 fish per tank per group). After treatment, fish were re-stocked into the 4000 litre tanks and maintained under the common garden design described above, with the exception that feed was not provided. The concentrations of Poly I:C and PGN used were based on past studies (Kono and Sakai, 2001; Jensen et al., 2002; Lockhart et al., 2004; Kono et al., 2004). At the point of sampling, fish were killed by a lethal dose of tricaine methanesulphonate (200 mg l^{-1}; Syndel Laboratories Ltd, Vancouver, BC, Canada; buffered in 400 mg l^{-1} sodium bicarbonate) after prior sedation using Aquacalm (1 mg l^{-1}; Syndel Laboratories). For each group, 10 fish were randomly sampled 30 h post-treatment. A panel of tissues, namely skeletal muscle, intestine, liver, head kidney and spleen, were rapidly team sampled. For all tissues except skeletal muscle, samples were fixed in RNAlater™ (ThermoFisher Scientific) overnight at 4°C and stored at −80°C. For skeletal muscle, the samples were split, with half fixed in RNAlater™ as described above and the other half flash frozen on dry ice. For the current study, the skeletal muscle samples were shipped on dry ice to the School of Biological Sciences, University of Aberdeen, UK, where samples were stored at −70°C until analysis. Samples fixed in RNAlater™ were used for all molecular analyses described below (N=5 fish per group per treatment; 45 samples).

Primer design

Details of primer pairs for 47 quantitative PCR (qPCR) assays performed in the study are provided in Table S1, including citations to previously published primers. Coho salmon genes of interest were initially acquired using Atlantic salmon and rainbow trout orthologues acquired from the NCBI database as queries in BLASTn searches against two published coho salmon transcriptomes (Kim et al., 2016; Garcia de la serrana et al., 2015) and a sequence capture data set that included target genes from the GH and IGF systems for coho salmon (Lappin et al., 2016; Robertson et al., 2017). When the current paper was in preparation, a high-quality genome was released for coho salmon (NCBI accession; GCA_002021735.1). Hence, a larger pool of gene models became available, which were used to check all coho

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salmon sequences targeted by qPCR; where possible, we report coho-specific accession numbers for all gene targets (Table S1). For most IGF system genes, we found that published primers from Atlantic salmon (Macqueen et al., 2013) and rainbow trout (Alzaid et al., 2016a,b) were conserved in coho salmon. New primer pairs were designed for *IGFBP-1A2* and *IGFBP-5B1* owing to significant mismatches between published primers and coho salmon. Salmonid-specific genes encoding GH are known for salmonids (previously named *GH1* and *GH2*) (e.g. McKay et al., 2004; Robertson et al., 2017) and both were identified in coho salmon (accession numbers in Table S1). We initially tested primers conserved across both GH duplicates and detected limited muscle transcript expression: because this primer pair binds both genes equally, we concluded that neither GH duplicate was sufficiently expressed to warrant design of additional primers. A past study identified salmonid-specific duplicates of GHR (*GHR1* and *GHR2*), including in coho salmon (Very et al., 2005), for which we designed new primer pairs that bind divergent regions among the duplicates (Table S1). Additional primers were used for marker genes known to be strongly upregulated by immune stimulation or to be directly involved in muscle growth and development (described in Castro et al., 2015; Alzaid et al., 2016a,b).

Quantitative gene expression analyses

Total RNA extraction and reverse transcription was performed as described previously (Alzaid et al., 2016b). RNA was extracted using TRIzol Reagent (Sigma-Aldrich), following the manufacturer's instructions. RNA quantity and purity were measured using A260/280 and A260/230 NanoDrop™ UV spectrophotometry (ND-1000, Thermo Scientific™) and RNA integrity confirmed by agarose gel electrophoresis. Reverse transcription was done using a QuantiTect Reverse Transcription Kit (Qiagen), including a step to remove genomic DNA. Transcript levels of the target genes were measured with qPCR using an Mx3005P qPCR System with Brilliant III Ultra-Fast SYBR Green (Agilent Technologies). The efficiency of qPCR assays was calculated using LinRegPCR (Ruijter et al., 2009). Data analyses were performed in GenEx (MultiD Analyses AB) using the variance-based algorithm NormFinder (Andersen et al., 2004) to test the suitability of five potential reference genes (*RpL4*, *RpS13*, *RpS29*, *ACTB* and *EF1A*) for data normalisation; this approach identified *RpL13* and *ACTB* as the most stable pair of reference genes across all samples (combined s.d.: 0.14). The same genes were the most stably expressed considering variation in expression across treatments (combined s.d.: 0.10) and fish groups (combined s.d.: 0.10), and were used to normalise the expression data for each experimental gene. Within GenEx, efficiency-corrected, normalised arbitrary transcript levels were placed on a relative scale that was quantitatively comparable across different genes.

Statistics

Statistical analyses were performed in Minitab v.18 (Minitab Inc.). Differences in baseline gene transcript levels among fish groups for the control animals (PBS injected) were identified using one-way ANOVA, with Tukey's *post hoc* test to reveal significant pair-wise differences among groups (i.e. WT–PBS versus TR–PBS versus TF–PBS). The effect of PGN and Poly I:C on gene expression was tested (separately for each immune mimic) using two-way ANOVA, including the effect of treatment, fish group and treatment × group interaction. When two-way ANOVA revealed a significant effect of treatment or a significant treatment × group interaction, we used Tukey's *post hoc* test to: (i) identify significant pair-wise differences within each fish group due to treatment (i.e. WT–control versus WT–PGN or WT–Poly I:C; TR–control versus TR–PGN or TR–Poly I:C; TF–control versus TF–PGN or TF–Poly I:C); and (ii) identify significant pair-wise differences in transcript levels among fish groups subjected to each immune treatment (i.e. WT–PGN versus TR–PGN versus TF–PGN; WT–Poly I:C versus TR–Poly I:C versus TF–Poly I:C). For all parametric analyses, we tested whether the fitted model residuals conformed to assumptions of normality (Anderson–Darling test) and homoscedasticity (Levene's test). Box–Cox transformations and, if necessary, a non-parametric test (Kruskal–Wallis) were employed when data failed to meet these assumptions.

RESULTS

GH transgenesis alters baseline expression of GH and IGF system genes in skeletal muscle

We first assayed the baseline mRNA levels of all tested GH and IGF pathway genes in the muscle of unstimulated control fish for the three experimental groups (Fig. 1; Table 1). For the mRNAs encoding hormones, *GH* was expressed at low levels in all groups, *IGF-I* expression was not different across the groups, whereas *IGF-II* expression was significantly elevated (by ∼2.3-fold) in TR versus WT (Fig. 1A; Table 1). *IGF-II* levels were substantially (∼20-fold) higher than *IGF-I* in all groups (Table 1). Among the assayed receptors, *GHR1* transcript levels were significantly lower in TF versus WT and TF versus TR comparisons (by ∼4.5- and 2.5-fold, respectively), whereas *GHR2* expression was not significantly different across groups (Fig. 1B; Table 1). Expression of *IGF1R-a2* was higher than other IGF1R family genes (i.e. *IGF1R-a1* and *IGF1R-b*) in all groups, and significantly higher in TF versus WT, by ∼2.4-fold (Fig. 1C; Table 1). The expression of four out of 11 IGFBP family member genes differed significantly between the three groups (Table 1). No muscle expression was detected for *IGFBP-1B1*, *-1B2*, *-2B1*, *-2B2*, *-3B1*, *-3B2*, *-6A1* and *-6A2*. *IGFBP-1A2* transcript levels were significantly higher (by ∼3.8-fold) in TF versus WT but were not significantly different comparing TF and TR (Fig. 1D). Conversely, *IGFBP-3A1*, *IGFBP-5B1* and *IGFBP-6B2* were each most highly expressed in the TR group and always significantly higher than WT (and significantly higher than TF for *IGFBP-5B1* only) (Fig. 1E–G; Table 1). For the tested markers of muscle growth status, most were not differentially expressed across groups, including *FBXO32* (Tacchi et al., 2010), which encodes an E3-ubiquitin ligase involved in structural protein turnover, *TNNI2* and *MYL1*, which encode sarcomere proteins, and *myoG*, which is a transcription factor for myogenic differentiation (Table 1). However, transcript levels of *MyoD1a* (Macqueen and Johnston, 2006), a transcription factor for myogenic determination and differentiation, were significantly elevated in TF versus WT (Fig. 1H; Table 1).

GH transgenesis alters skeletal muscle immune gene expression

To assess skeletal muscle responses to PGN, we measured transcript levels for markers of pro-inflammatory cytokines (*TNF-α*, *IL-1β* and *IL-8*) and acute-phase proteins (*SAA* and *HAMP*) (Fig. 2A; Table S2) after Castro et al., 2015. A response to PGN was detected in each group, evidenced by a significant induction of all tested marker genes barring *TNF-α* in TF (Fig. 2A; Table S2). The lack of *TNF-α* response in TF was coupled with a respective 5.4- and 4.1-fold higher baseline expression versus WT and TR (Fig. 2A; Table S2). In addition, the magnitude of observed responses of *TNF-α*, *IL-1β* and *IL-8* was distinct among the fish groups; being

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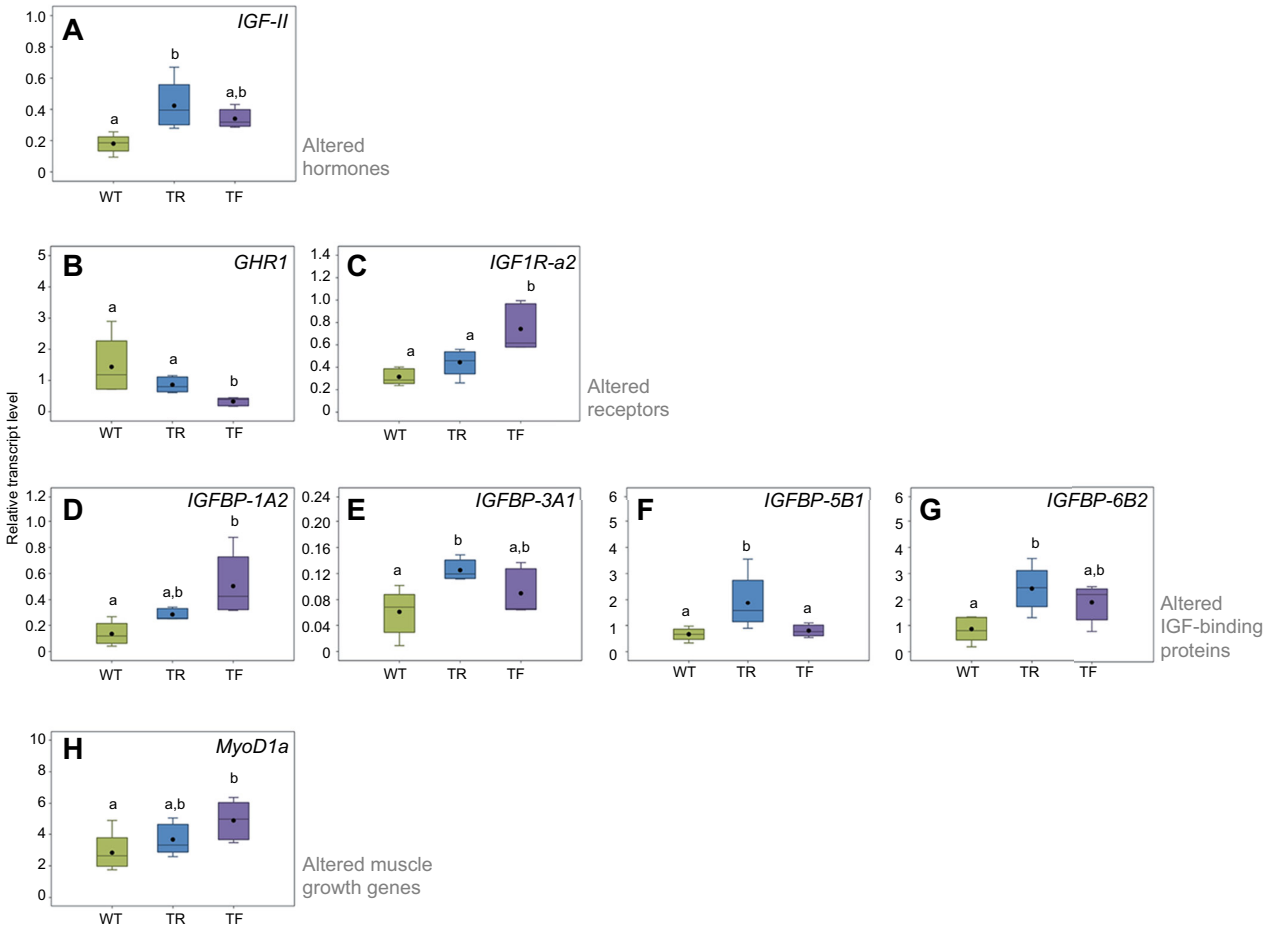

Fig. 1. Growth hormone (GH) and insulin-like growth factor (IGF) pathway genes with altered baseline expression comparing wild-type and GH transgenic fish. Box plots are shown for genes where a significant effect (P<0.05) was observed by one-way ANOVA comparing the three tested fish groups (N=5 fish per group). Black circles within box plots show mean transcript levels. Different letters indicate significant differences among fish groups (Tukey's *post hoc* analysis). Full data (including means±s.d.) are given in Table 1.

highest in WT, intermediate in TR and lowest or non-existent in TF, leading to a significant treatment × group interaction (Fig. 2A; Table S2). In contrast, *SAA* and *HAMP* showed a highly significant induction in all groups, without any treatment × group interaction (Fig. 2A; Table S2).

To assess skeletal muscle responses to Poly I:C, we measured transcript levels for markers of cytokines and proteins involved in the antiviral response (*IFN-a*, *γ-IP*, *Mx*, *LMP2* and *RSAD2*) (Fig. 2B; Table S2) after Castro et al., 2015. Each tested marker was significantly induced in WT and TR but not TF (Fig. 2B; Table S2). The responses of these genes showed a striking and consistent difference among groups, with between ∼17- and 470-fold induction across the five genes in WT, compared with between ∼1.6- and 34-fold in TR and no upregulation in TF, leading to a highly significant treatment × group interaction (Fig. 2B; Table S2).

GH transgenesis alters GH and IGF system expression responses to immune stimulation

PGN altered the expression of several GH and IGF pathway genes, evidenced by significant overall treatment effects, and/or significant treatment × group interaction effects, reflecting different responses among fish groups (Table 2). PGN had no effect on the expression of mRNAs encoding GH or IGF-I but *IGF-II* was significantly upregulated in WT and downregulated in TR (Table 2). PGN altered the expression of mRNAs encoding all tested receptors, with some genes showing a significant treatment effect (*GHR1*, *GHR2* and *IGF1R-b*) and these and others showing a significant treatment × group interaction (*GHR1*, *IGF1R-a1* and *-a2*) (Table 2). *GHR1* was significantly downregulated in WT, unchanged in TR and upregulated in TF by >5-fold, whereas *IGF1R-a2* showed a reciprocal pattern of induction in WT and downregulation in TF (Table 2). Seven genes encoding IGFBPs showed a significant treatment × group interaction and were typically upregulated in WT (significant effect for *IGFBP-1A1* and *-6B1*) and downregulated in GH transgenic fish (significant effect for *IGFBP-3A2* and *-6B2* in TR, and for *IGFBP-1A2*, *-2A* and *-3A2* in TF) (Table 2). For several IGFBP genes, the response to PGN in WT led to transcript levels that were significantly higher than observed in GH transgenic fish, including *IGFBP-1A1* (WT>TR and TF), *IGFBP-2A* (WT>TF) and *IGFBP-6B1* (WT>TR) (Table 2). Considering the marker genes for muscle growth status, we observed no significant responses to PGN for genes encoding sarcomeric proteins (Table 2). Whereas *myoG* showed a significant treatment × group interaction, there were no significant differences in expression among the three groups (Table 2). *MyoD1a* showed a significant downregulation in TF compared with WT (Table 2).

Poly I:C altered the expression of several GH and IGF pathway genes, which typically showed distinct responses between WT and GH transgenic animals (Table 3). Among the tested hormones,

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Table 1. Differences in baseline expression of growth hormone (GH) and insulin-like growth factor (IGF) system genes comparing wild-type with transgenic coho salmon

Gene	*P*-value	WT transcript level	TR transcript level	TF transcript level
Hormones				
GH	0.77	0.01±0.01	0.01±0.00	0.01±0.01
IGF-I	0.33	0.01±0.01	0.02±0.01	0.02±0.01
IGF-II	0.01	0.18±0.06^{a}	0.42±0.15^{b}	0.34±0.06^{a,b}
Receptors				
*GHR1**	0.001	1.43±0.90^{a}	0.86±0.24^{a}	0.32±0.13^{b}
GHR2	0.24	0.73±0.67	0.56±0.13	0.43±0.09
IGF1R-a1	0.40	0.07±0.03	0.08±0.02	0.09±0.02
IGF1R-a2	0.001	0.31±0.07^{a}	0.44±0.12^{a}	0.74±0.21^{b}
IGF1R-b	0.36	0.07±0.02	0.10±0.03	0.09±0.04
IGF-binding proteins				
IGFBP-1A1	0.49	0.13±0.08	0.13±0.05	0.18±0.08
IGFBP-1A2	0.01	0.13±0.09^{a}	0.28±0.04^{a,b}	0.50±0.23^{b}
*IGFBP-2A**	0.09	0.22±0.09	0.41±0.10	0.83±1.08
IGFBP-3A1	0.02	0.06±0.03^{a}	0.13±0.02^{b}	0.09±0.04^{a,b}
IGFBP-3A2	0.06	0.20±0.07	0.43±0.20	0.42±0.16
*IGFBP-4**	0.69	1.03±1.30	0.91±0.42	0.65±0.14
IGFBP-5A	0.10	0.02±0.02	0.03±0.03	0.01±0.01
*IGFBP-5B1**	0.01	0.66±0.23^{a}	1.87±1.01^{b}	0.80±0.22^{a}
IGFBP-5B2	0.18	0.75±0.57	1.23±0.19	0.97±0.27
IGFBP-6B1	0.07	0.17±0.06	0.42±0.22	0.27±0.14
IGFBP-6B2	0.01	0.86±0.47^{a}	2.42±0.83^{b}	1.93±0.69^{a,b}
Muscle growth status markers				
FBXO32	0.47	13.70±15.80	5.10±0.94	9.90±9.99
TNNI2	0.52	487.72±369.77	337.85±181.83	324.16±90.35
MYL1	0.89	230.07±136.61	198.63±75.88	221.97±92.12
myoG	0.16	0.58±0.39	0.95±0.16	0.69±0.28
MyoD1a	0.04	2.83±1.21^{a}	3.66±0.97^{a,b}	4.88±1.22^{b}

Data shown are for control (phosphate-buffered saline-injected) fish and represent relative transcript levels (means±s.d., *N*=5) shown as arbitrary units normalised to two empirically validated reference genes (*RpL13* and *ACTB*). The data are quantitatively comparable across genes and among fish groups. Probability values are from one-way ANOVA testing for an effect of fish group. Different superscript letters highlight significant differences in transcript levels among groups according to Tukey's *post hoc* test. The symbol '*' shows genes where the data required Box–Cox transformation.

IGF-II showed a significant downregulation in both transgenic groups (Table 3). For the tested receptors, *GHR1* was significantly induced in TF, whereas *IGF1R-a2* was significantly induced in WT but significantly downregulated in TF, and *IGF1R-b* showed significant downregulation in both GH transgenic groups (Table 3). The expression of seven genes encoding IGFBPs was affected by Poly I:C, with *IGFBP-1A2* showing significant upregulation in WT and significant downregulation in both GH transgenic groups, leading to WT having significantly greater transcript levels (Table 3). *IGFBP-1A1* mRNA was significantly downregulated in TF specifically and was significantly more abundant in WT than TF following Poly I:C treatment (Table 3). *IGFBP-2A*, *IGFBP-5B2*, *IGFBP-6B1* and *IGFBP-6B2* were significantly downregulated in both GH transgenic groups (Table 3). Considering the markers of muscle growth status, a significant treatment × group interaction was observed for *TNNI2*, with the TF transcript level post-treatment significantly higher than WT (Table 3). *MyoD1a* was significantly downregulated by Poly I:C in TF (Table 3).

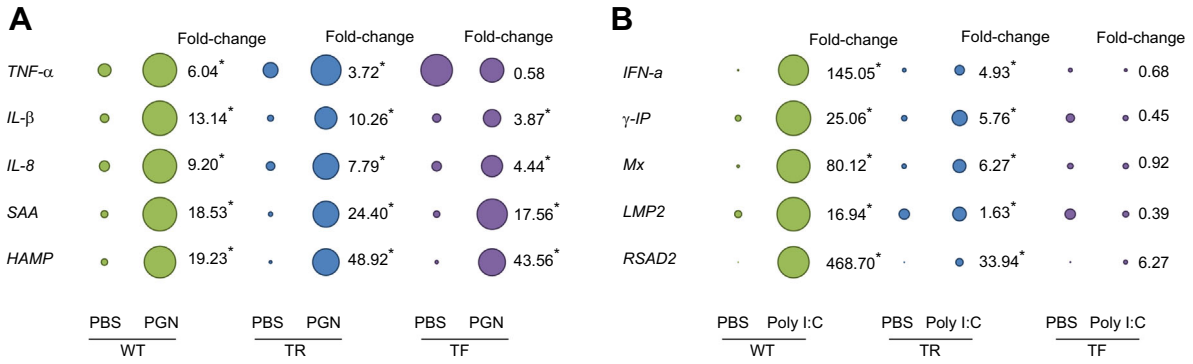

Fig. 2. Impact of immune stimulation on the expression of host defence genes comparing wild-type and growth (GH) transgenic fish. (A) Peptidoglycan (PGN) treatment. (B) Polyinosinic–polycytidylic acid (Poly I:C) treatment. Transcript expression is presented as bubble plots of sizes comparable among fish groups for each shown gene. The bubble area is proportional to mean transcript levels (*N*=5 fish per group). Two-way ANOVA revealed that all the shown genes were significantly induced (*P*<0.05) by the immune stimulations (Table S2) and Tukey's *post hoc* test identified significant changes in gene expression between control and treated fish, shown by asterisks next to accompanying fold-change values (calculated by dividing mean treatment by mean control, *N*=5 per mean). Full data (means±s.d.) are given in Table S2. PBS, phosphate-buffered saline.

Table 2. Effects of peptidoglycan (PGN) on expression of growth hormone (GH) and insulin-like growth factor (IGF) system genes comparing wild-type with transgenic coho salmon groups

Gene	*P*-value treatment	*P*-value treatment × group interaction	WT fold-change; transcript level	TR fold-change; transcript level	TR fold-change; transcript level
Hormones					
GH	0.66	0.71			
IGF-I	0.67	0.29			
IGF-II	0.18	<0.0001	2.11*; 0.38±0.04^{a}	0.54*; 0.23±0.03^{a,b}	0.62; 0.21±0.08^{b}
Receptors					
GHR1‡	0.02	<0.0001	0.33*; 0.47±0.21^{a}	1.80; 1.55±0.46^{b}	5.05*; 1.64±0.58^{b}
GHR2	0.02	–			
IGF1R-a1	0.19	0.002	1.48; 0.09±0.02^{a}	0.66; 0.05±0.02^{b}	0.58; 0.05±0.02^{b}
IGF1R-a2	0.88	<0.0001	2.70*; 0.84±0.16^{a}	1.13; 0.35±0.11^{b}	0.37*; 0.27±0.05^{b}
IGF1R-b	0.03	0.09			
IGF-binding proteins					
IGFBP-1A1	0.97	<0.0001	2.25*; 0.30±0.09^{a}	0.50; 0.07±0.02^{b}	0.47; 0.09±0.05^{b}
IGFBP-1A2	0.01	<0.0001	2.15; 0.29±0.11^{a}	0.46; 0.13±0.05^{a}	0.32*; 0.16±0.05^{a}
IGFBP-2A‡	0.22	0.001	1.86; 0.40±0.13^{a}	0.68; 0.28±0.07^{a,b}	0.23*; 0.19±0.03^{b}
IGFBP-3A1	0.27	0.78			
IGFBP-3A2	0.01	0.001	1.77; 0.35±0.13^{a}	0.33*; 0.14±0.02^{a}	0.39*; 0.17±0.09^{a}
IGFBP-4	0.05	–			
IGFBP-5A	0.57	0.02	0.89; 0.02±0.01^{a}	0.19; 0.01±0.01^{a}	3.96; 0.03±0.02^{a}
IGFBP-5B1‡	0.90	0.09			
IGFBP-5B2	0.58	0.47			
IGFBP-6B1	0.87	0.001	2.81*; 0.48±0.24^{a}	0.32; 0.13±0.07^{b}	0.80; 0.22±0.11^{a,b}
IGFBP-6B2	0.05	0.002	1.89; 1.62±0.35^{a}	0.51*; 1.24±0.52^{a}	0.57; 1.10±0.38^{a}
Muscle growth status markers					
FBXO32	0.02	0.23			
TNNI2	0.17	0.06			
MYL1	0.11	–			
myoG	0.98	0.04	1.55; 0.90±0.14^{a}	0.75; 0.71±0.13^{a}	0.88; 0.61±0.17^{a}
MyoD1a	0.002	<0.0001	1.19; 3.38±0.87^{a}	0.82; 3.00±0.65^{a,b}	0.30*; 1.46±0.33^{b}

Fold-change values (underlined: calculated by dividing the mean PGN treatment by the mean control transcript levels, *N*=5 fish per mean) are given for genes where a significant treatment × group interaction was recorded by two-way ANOVA (the symbol '*' indicates a significant expression change according to Tukey's *post hoc* test). For the same genes, relative transcript levels are shown post-PGN treatment (means±s.d., *N*=5) and represent arbitrary units normalised to two empirically validated reference genes (*RpL13* and *ACTB*) that are quantitatively comparable across genes and among fish groups (different superscript letters highlight significant differences among groups according to Tukey's *post hoc* test). The symbol '‡' shows genes where the data required Box–Cox transformation. The symbol '–' shows genes where a Kruskal–Wallis test was done testing for a treatment effect only.

DISCUSSION

Animals have evolved defence mechanisms to eradicate pathogens via pathways that are energetically costly (e.g. Bonneaud et al., 2003; Lochmiller and Deerenberg, 2000). Recent studies in fish have revealed that the expression of genes from the IGF system is altered during immune responses to bacterial and viral infections, consistent with growth downregulation, theoretically allowing energetic resources to be invested in immune function (Pooley et al., 2013; Alzaid et al., 2016a,b). Until the current study, little attention had been given to the regulation of growth following immune stimulation in fish skeletal muscle, which is the most important tissue for growth investment. In addition to demonstrating baseline differences in GH and IGF system gene expression between wild-type and GH transgenic coho salmon, we demonstrate that muscle immune status is disrupted by GH transgenesis as well as the impacts of GH on growth and physiological status. This observation was coupled to striking differences in GH and IGF system regulation post-immune stimulation, suggesting 'normal' cross-talk between muscle growth and immune function is disrupted by GH transgenesis. As differences in GH and IGF system expression caused by GH transgenesis in coho salmon have been reported elsewhere (e.g. Raven et al., 2008; Devlin et al., 2009), we focus discussion around novel links between growth and immune function.

GH transgenesis disrupts immune status of skeletal muscle

In response to PGN, the cytokines *IL-1β*, *IL-8* and *TNF-α* were strongly induced in WT, confirming an inflammatory response. Whereas TR and TF groups showed a comparable response, the magnitude of cytokine induction was lower, and particularly attenuated in TF, which failed to upregulate *TNF-α*. These findings suggest that the GH transgenic fish have a reduced inflammatory response, particularly when maximal growth rate is being achieved under satiation feeding. Another potential explanation is that the inflammatory response has distinct temporal dynamics in GH transgenic and WT fish, which were not captured in our study (30 h post-treatment). However, genes encoding the acute-phase proteins Serum amyloid A and Hepcidin were induced at comparable levels in all fish groups, indicating that GH transgenesis does not compromise all effectors of innate immunity in muscle, quantitatively or in terms of temporal dynamics. Altered expression of pro-inflammatory cytokines in GH transgenics indicates disruption in cross-talk between skeletal muscle and immune function (reviewed in Pillon et al., 2013). This could relate to differences in the number of immune cells residing within muscle, e.g. macrophages and granulocytes, given past reports that GH transgenic salmon develop fewer white cells than wild-type fish (Kim et al., 2013). Constitutive upregulation of *TNF-α* in the TF group suggests that growth rate and/or nutritional status modulate the impact of GH on *TNF-α* regulation and could imply a permanent inflammatory state that classically would be deemed pathological (Pillon et al., 2013). TNF-α, along with other pro-inflammatory cytokines including IL-1 family members, also positively regulate muscle development (e.g. Chen et al., 2007; Costamagna et al., 2015). Hence, disrupted cytokine expression has implications for myogenesis in addition to health

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Table 3. Effect of polyinosinic–polycytidylic acid (Poly I:C) on expression of growth hormone (GH) and insulin-like growth factor (IGF) system genes comparing wild-type with transgenic coho salmon groups

Gene	*P*-value treatment	*P*-value treatment × group interaction	WT fold-change; transcript level	TR fold-change; transcript level	TR fold-change; transcript level
Hormones					
GH	0.48	0.63			
IGF-I	0.74	0.15			
IGF-II‡	<0.0001	0.001	1.20; 0.22±0.03[a]	0.43*; 0.18±0.05[a]	0.46*; 0.15±0.08[a]
Receptors					
GHR1‡	0.05	<0.0001	0.44; 0.64±0.38[a]	1.34; 1.15±0.14[a]	3.80*; 1.24±0.30[a]
GHR2‡	0.33	0.38			
IGF1R-a1	0.56	0.06			
IGF1R-a2	0.17	<0.0001	2.16*; 0.68±0.13[a]	0.73; 0.32±0.08[a,b]	0.42*; 0.31±0.07[b]
IGF1R-b	0.03	<0.0001	1.70; 0.12±0.04[a]	0.34*; 0.03±0.01[b]	0.32*; 0.03±0.01[b]
IGF-binding proteins					
IGFBP-1A1	0.07	0.02	1.40; 0.18±0.11[a]	0.61; 0.08±0.03[b]	0.21*; 0.04±0.02[b]
IGFBP-1A2‡	0.05	<0.0001	2.52*; 0.34±0.10[a]	0.43*; 0.12±0.04[b]	0.30*; 0.15±0.04[b]
IGFBP-2A‡	0.05	0.001	2.91; 0.64±0.73[a]	0.46*; 0.19±0.06[a]	0.24*; 0.20±0.13[a]
IGFBP-3A1	0.02	0.14			
IGFBP-3A2	0.25	0.001	2.38; 0.47±0.21[a]	0.49; 0.21±0.07[a,b]	0.42; 0.18±0.12[b]
IGFBP-4‡	0.70	0.61			
IGFBP-5A‡	0.14	0.29			
IGFBP-5B1‡	0.70	0.11			
IGFBP-5B2	0.01	0.03	1.16; 0.87±0.22[a]	0.50*; 0.61±0.08[a]	0.54*; 0.52±0.26[a]
IGFBP-6B1‡	<0.0001	0.01	0.90; 0.15±0.05[a]	0.20*; 0.09±0.04[a]	0.33*; 0.09±0.06[a]
IGFBP-6B2	0.01	0.01	1.44; 1.24±0.33[a]	0.54*; 1.30±0.54[a]	0.42*; 0.81±0.32[a]
Muscle growth status markers					
FBXO32‡	0.20	0.13			
TNNI2	0.20	0.03	0.60; 295.6±59.1[a]	1.42; 481.5±195.7[a,b]	2.08; 676.0±218.4[b]
MYL1	0.33	0.72			
myoG	0.71	0.04	1.67; 0.97±0.35[a]	0.73; 0.70±0.06[a]	0.98; 0.67±0.23[a]
MyoD1a	<0.0001	0.01	0.83; 2.34±0.57[a]	0.60; 2.19±0.19[a]	0.37*; 1.79±0.43[a]

Fold-change values (underlined: calculated by dividing the mean Poly I:C treatment by the mean control treatment transcript levels, *N*=5 fish per mean) are given for genes where a significant treatment × group interaction was recorded by two-way ANOVA (the symbol '*' indicates a significant expression change according to Tukey's *post hoc* test). For the same genes, relative transcript levels are shown post-Poly I:C treatment (means±s.d., *N*=5) and represent arbitrary units normalised to two empirically validated reference genes (*RpL13* and *ACTB*) that are quantitatively comparable across genes and among fish groups (different superscript letters highlight significant differences among groups according to Tukey's *post hoc* test). The symbol '‡' shows genes where the data required Box–Cox transformation.

status. GH transgenic coho salmon were previously shown to have higher susceptibility to the bacterium *Aeromonas salmonicida* (Kim et al., 2013). These data imply systematic attenuation of innate immunity, but more data will be needed to confirm this finding in primary immune tissues, as the immune gene expression data presented here was restricted to skeletal muscle.

In response to Poly I:C, antiviral gene markers showed robust expression induction in skeletal muscle of WT fish. However, the same genes showed a lower magnitude of induction in TR and were not induced in the TF group. These antiviral genes are effectors of Type-I interferon signalling (e.g. Martin et al., 2007), which controls viral replication by inhibiting host translation and protein synthesis (Haller et al., 2007; Sadler and Williams, 2008). Our results may be explained by the presence of ubiquitous signalling towards new protein synthesis in the TF group through the IGF–PI3K–Akt–mTOR pathway downstream of GH (Egerman and Glass, 2014). Past studies have provided evidence for cross-talk between interferon signalling and this pathway (e.g. Kaur et al., 2008), providing possible mechanisms by which an antiviral response is attenuated in favour of protein synthesis. However, as markers for Type-I interferon signalling showed attenuated expression in the TR group, which have wild-type growth rate and IGF-I levels (Raven et al., 2008), our findings imply a direct role for GH in limiting expression of these antiviral genes. Future work should establish whether the absence of antiviral gene induction in GH transgenic salmon muscle is a consequence of systematic attenuation in antiviral immunity or linked to this tissue's key role as a target for growth. While our findings are also compatible with an altered timing of the antiviral response, the striking observed difference between WT and GH transgenic fish, and indeed between TR and TF, is difficult to rationalise solely due to distinct temporal dynamics.

GH transgenesis alters GH and IGF system expression responses to immune stimulation

The attenuation in immune gene expression in GH transgenics was coupled to complex changes in expression of GH and IGF system genes upon immune stimulation. As the same wild-type genetic background was maintained in all fish studied (i.e. differing genetically only by the presence of a GH transgene), the WT group provides a control for the gene expression responses of GH transgenics to immune stimulation. The hypothesis that effective immune function is aided by re-allocation of resources during infection ('growth-immune trade-off hypothesis') predicts that the WT group, which has presumably evolved under selection to balance growth and immune investment, should restrict growth by either inhibiting pro-growth gene expression or upregulating growth-inhibiting genes. Reciprocally, GH transgenic fish, particularly the TF group, where rapid growth rate is being realised, should have reduced scope for the same molecular responses if ubiquitous growth signalling overrides normal growth-immune trade-offs. Considering our data, we find some support for such notions, although in other cases the findings are counter-intuitive to the same ideas.

In response to both PGN and Poly I:C, the GH receptor *GHR1* was upregulated in TF, with post-treatment transcript levels higher than WT, with WT downregulating *GHR1* in response to PGN. As GH receptors are required for GH signalling, their upregulation in the TF group suggests augmentation of GH signal and therefore allocation into growth during immune stimulation, whereas downregulation observed in WT to PGN should inhibit growth. These data are consistent with the presence of a growth-immune trade-off in the WT group following treatment with a bacterial immune mimic, a response that is either absent (TR) or even highly misregulated (TF) in GH transgenics.

Many IGFBP-encoding genes were differentially regulated in WT versus GH transgenic muscle in response to immune stimulation, indicative of distinct local regulation of IGF signalling. IGFBP genes were systematically downregulated in GH transgenics, usually to a greater extent in TF than TR, but were never significantly downregulated in WT and in several cases upregulated (e.g. *IGFBP-1A* genes for PGN and Poly I:C; *IGFBP-6B1* for PGN). IGFBPs play complex roles in regulating the availability of free IGFs to IGF1R, and have diverse IGF-independent functions (reviewed in Garcia de la serrana and Macqueen, 2018). IGFBP-1 sub-types inhibit IGF signalling by sequestering IGFs in the extracellular environment and are major carriers of circulating IGF (Shimizu and Dickhoff, 2017). While their autocrine roles in teleost muscle are poorly understood, IGFBP-1A subtypes are thought to have evolved local functions, and are upregulated by catabolic signals in muscle (Garcia de la serrana and Macqueen, 2018). The upregulation of *IGFBP-1A* genes in WT fish to a level higher than TR and TF should therefore restrict IGF signalling and hence growth upon immune stimulation, whereas downregulation in GH transgenic fish should increase IGF availability to IGF1R. The observed downregulation of *IGFBP-2A* to immune stimulation in GH transgenics may serve a similar purpose, as IGFBP-2 is credited with IGF-inhibiting functions in muscle (Swiderski et al., 2016). IGFBP-6-encoding genes were either upregulated in WT fish by immune stimulation (i.e. *IGFBP-6B1* for PGN) or downregulated in GH transgenics (e.g. *IGFBP-6B1* and *-6B2* for Poly I:C in TR and TR). IGFBP-6 subtypes are considered negative regulators of growth (Garcia de la serrana and Macqueen, 2018) and are known to be induced by pro-inflammatory signals in Atlantic salmon muscle (Pooley et al., 2013). The reciprocal regulation of many IGFBPs during immune stimulation in TR and TF versus WT fish implies disruption in normal molecular cross-talk between growth and immunity owing to GH and its ability to impact growth rate and nutritional status. Past work in other salmonid species suggests that upregulation of growth-inhibiting IGFBP genes upon immune activation is driven by Type-I interferon signalling for antiviral responses or TNF-α/IL-1β cytokine signalling for inflammatory responses to bacteria (Pooley et al., 2013; Alzaid et al., 2016b). Considering the apparent attenuation of the same immune pathways observed in our study, we hypothesise that similar molecular mechanisms are thought to be involved in coho salmon, and contribute to the altered IGFBP regulation observed in GH transgenesis.

While the above data are consistent with the growth-immune trade-off hypothesis, the regulation of other IGF system genes was consistent with the activation of IGF signalling in wild-type fish in response to immune stimulation. For example, transcript levels for IGF1R subtypes increased in WT fish in response to PGN (*IGF1R-a1*) and Poly I:C (*IGF1R-a2*). The same genes were significantly downregulated in the TF group, to a level below WT. As IGF1R is required to activate the PI3K–Akt–mTOR pathway, this implies an activation of muscle growth in WT but downregulation in GH transgenics achieving maximal growth. In a similar fashion, *IGF-II* was upregulated in WT in response to PGN, and downregulated in GH transgenics in response to PGN (TR only) and Poly I:C (TR and TF). Such findings are incompatible with the growth-immune trade-off hypothesis but nonetheless again demonstrate that the normal WT response of growth genes to immune stimulation is disrupted by GH transgenesis. Such data highlight the extensive complexity of the interaction between growth and immune function in muscle that represents a promising direction for ongoing research effort, which should take into account additional tissues, and perhaps more deeply dissect molecular signatures through transcriptomic or proteomic approaches.

Broader perspectives

This study demonstrates that GH transgenesis, and associated changes in growth rate under different nutritional regimes (feed intake and energy status), disrupts immune function in coho salmon muscle, impacting normal cross-talk with growth systems. It will be important to determine whether similar immune-related effects occur in the muscle of domesticated fish strains, where artificial selection for fast growth has occurred across multiple generations with opportunity for co-adaptation and compensatory selective responses. Together with past reports of altered immune function in GH transgenics (Jhingan et al., 2003; Kim et al., 2013), our finding that the muscle of GH transgenic salmon achieving maximal growth lacked any detectable antiviral response, and showed a baseline upregulation of *TNF-α* expression, has implications for the health/welfare of GH transgenic strains destined for aquaculture, where rapid growth is the central goal of production. Finally, our data have ramifications for environmental risk assessments (Devlin et al., 2015) tasked with determining the fitness and potential impacts of genetically engineered fishes should they enter natural environments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.-H.K., R.H.D., S.A.M.M., D.J.M.; Methodology: J.-H.K., R.H.D., D.J.M.; Validation: A.A.; Formal analysis: A.A., D.J.M.; Investigation: A.A., J.-H.K., R.H.D., D.J.M.; Resources: R.H.D., S.A.M.M., D.J.M.; Data curation: A.A., D.J.M.; Writing - original draft: A.A., D.J.M.; Writing - review & editing: J.-H.K., R.H.D., S.A.M.M., D.J.M.; Visualization: A.A., D.J.M.; Supervision: R.H.D., S.A.M.M., D.J.M.; Project administration: J.-H.K., R.H.D., S.A.M.M., D.J.M.; Funding acquisition: R.H.D., S.A.M.M., D.J.M.

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Supplementary information

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