Muscle fibre size optimisation provides flexibility to energy budgeting in calorie-restricted Coho salmon transgenic for growth hormone

Ian A. Johnston[1], Daniel Garcia de la serrana[1] and Robert H. Devlin[2]

[1] Scottish Oceans Institute, School of Biology, University of St Andrews, St Andrews, KY16 8LB, UK

[2] Fisheries and Oceans Canada, 4160 Marine Drive, West Vancouver, BC, V7V 1N6, Canada

Running title: Myogenesis in GH-transgenic salmon

Key words: Growth, myotube formation, transgenesis, optimal fibre size hypothesis

ABSTRACT

Coho salmon (*Oncorhynchus kisutch*) transgenic for growth hormone (GH) show substantially faster growth than wild-type (WT) fish. We fed GH-transgenic salmon either to satiation (1-year) (TF) or the same smaller ration of wild-type fish (2-years) (TR), resulting in groups matched for body size to WT salmon. The myotomes of TF and WT fish had the same number and size distribution of muscle fibres, indicating 2-fold higher rate of fibre recruitment in the *GH*-transgenics. Unexpectedly, calorie restriction was found to decrease the rate of fibre production in transgenics, resulting in a 20% increase in average fibre size and reduced costs of ionic homeostasis. Genes for myotube formation were down-regulated in TR relative to TF and WT fish. We suggest muscle fibre size optimisation allows the relocation of energy from maintenance to locomotion explaining the observation that calorie-restricted transgenics grow at the same rate as WT whilst exhibiting markedly higher foraging activity.

INTRODUCTION

Growth hormone (GH) has pervasive effects on growth rate, behaviour, feeding, metabolism, osmoregulation, smoltification and immunity in fish (Björnsson, 1997). Growth rate is substantially higher in *GH*-transgenic salmonid fish than in wild-type (WT), although the effects vary with promoter type and family origin (Leggatt et al., 2012). Fast growth in transgenics is associated with markedly higher appetite and feeding motivation than WT fish (Sundstrom et al., 2003).

GH binds to receptors in the liver and induces insulin-like growth factor-1 (IGF1) synthesis which is secreted into the general circulation, promoting growth in peripheral tissues such as skeletal muscle (Johnston et al., 2011). Binding of IGF1 to its receptor activates several downstream signaling cascades including the PI3K-Akt-TOR pathway to stimulate protein synthesis and inhibit protein degradation by the ubiquitin proteasome pathway (Johnston et al., 2011).

Primary effects of *GH*-transgenesis can be distinguished from secondary effects linked to appetite by comparing transgenics fed either to satiation (TF) or with the reduced ration (TR) of WT fish, producing groups of the same body size. *GH*-transgenic coho salmon (*Oncorhyncus kisutch*) fed to satiation had higher plasma GH and increased levels of tissue GH relative to WT (Raven et al., 2008). Plasma GH levels were higher still in TR fish whereas plasma and tissue IGF-1 concentrations were similar in TR and WT groups, reflecting similar feeding and growth rates (Raven et al., 2008). TR fish also maintain a much higher feeding motivation and are more active than WT fish (Sundstrom et al., 2003).

Teleosts recruit muscle fibres until they reach around 40-50% of the maximum adult size (Johnston et al., 2011). One report suggested that muscle in *GH*-transgenic coho salmon grew more by hyperplasia than hypertrophy than in WT fish (Hill et al., 2000), although in this study fibre number was not estimated. In order to test the hypothesis that *GH*-transgenesis enhances hyperplastic muscle growth we compared fibre number and diameter in WT, TF and TR groups of the same body length and measured expression of *gh*, *igf1* and genes required for myotube formation.

RESULTS AND DISCUSSION

TF coho salmon had a higher appetite and grew faster than WT recruiting fast muscle fibres at twice the rate, but showed a similar contribution of hyperplasia and hypertrophy to reach a given body length resulting in a similar average muscle fibre size (Supplementary Table 1; Fig. 1A,B) i.e. the hypothesis of an increased importance of hyperplasia in these transgenics was not supported (Hill et al., 2000). Unexpectedly, TR recruited 49% fewer fibres than WT on the same feeding regime and 59% fewer fibres than TF (Supplementary Table 1; Fig. 1B) ($P<0.05$). Since there were only

relatively small and not statistically significant differences in total muscle cross-sectional area between groups (Supplementary Table 1), this result reflects a greater contribution of fibre hypertrophy to growth in TR. The average fibre diameter (FD) was 20% greater in TR (49 μm) than TF and WT groups (41 μm) (Supplementary Table 1). We fitted probability density functions (PDF) to the distributions of FD and compared specific percentiles using Multi-Wilcoxon tests. The average PDF of the TR group was significantly different from the TF and WT groups (Kolmogorov-Smirnoff) ($P<0.05$). TR had larger diameter fibres across the whole range of fibre sizes i.e. increased hypertrophy was evident for cohorts of fibres with different birthdays (Fig. 1C and D). Growth hormone (*gh*) mRNA levels (Fig. 2A) were similar in TF and TR fish and massively higher than in WT fish as expected ($P<0.01$). In contrast *igf1* expression in muscle was significantly higher in TF than WT ($P<0.05$) reflecting higher food intake and growth (Sundstrom et al., 2003), whereas WT and TR were not significantly different from each other. The expression, of genes required for myoblast fusion during hyperplastic growth including *dock1*, *dock5*, *crkl*, *itgb1*, *cadh15* and *tmem8c* were 2 to 4-fold downregulated in TR relative to the other groups ($P<0.05$) (gene names are given in full in Supplementary Table 2).

Muscle fibre size is under strong evolutionary selection in fish and can be adjusted by altering the lifetime production of muscle fibres (Johnston et al., 2003). ATP-dependent ion pumping counteracts passive ion leak across the sarcolemmal membrane so that the energy cost of maintaining a negative resting membrane potential is proportional to fibre surface/volume (S/V) ratio. Thus large fibres are cheaper to maintain than smaller ones (Johnston et al., 2012; Jimenez et al. 2013). The optimal muscle fibre size (OFS) hypothesis envisages a trade-off between minimising S/V ratio on the one hand and avoiding diffusional constraints for diffusion of gas and metabolites on the other (Johnston et al., 2012, Johnston et al., 2003). Examples of fibre size optimisation over evolutionary time scales include the radiation of Antarctic notothenioid fishes following climatic cooling, resulting in a relaxation of diffusional constraints and “giant muscle fibres” through a dramatic reduction in body-size corrected fibre number (Johnston et al., 2003). Similarly, dwarfism in land-locked salmonid and stickleback populations was associated with a large reduction in fibre number relative to the large-bodied ancestral anadromous state, enabling a similar scaling of fibre diameter to body size (Johnston et al., 2012). The results of the present study indicate that, at least under certain circumstances, fibre size optimization may also operate within the lifetime of an individual.

The GH-axis is tightly coupled to the energy status of the fish and linked to feed intake via the expression of GH, IGF1 and their receptors (Raven et al., 2008). TR fish maintained a high GH output whilst experiencing a limitation in the supply of the amino acids and other nutrients required for growth. Our working hypothesis is that the uncoupling of the GH-axis from energy status directly affected some as yet unknown component of the signalling pathways regulating myotube formation

and hypertrophic growth, providing the stimulus for muscle fibre size optimization. GH-transgenics driven by the metallothionein-B promoter showed increased GH expression in all non-pituitary cell types including muscle and were not sensitive to the metabolic signals normally influencing endogenous GH regulation e.g. GH receptor expression was not increased with calorie restriction in TR as it was in WT (Raven et al., 2008). It is therefore possible that the effects of calorie restriction on fibre production differ between TR and WT fish, but this remains to be investigated. We found that the median fibre diameter was 20% higher in the TR than WT (Fig. 1B), expected to produce proportional energy savings in costs of ionic homeostasis. The energy saved could theoretically be reallocated to other aspects of the energy budget such as routine swimming activity, and may provide an explanation as to why TR fish were observed to exhibit much higher foraging activity whilst growing at a similar rate to WT fish fed the same diet.

MATERIAL AND METHODS

Fish

Coho salmon (*Oncorhynchus kisutch*) were reared in a containment facility at Fisheries and Oceans Canada, West Vancouver. Wild-type (WT) fish were from the 2010 brood of Chehalis River strain (BC, Canada). The strain M77 transgenic coho salmon was derived from Chehalis River strain produced using the OnMTGH1 construct as previously described (Leggatt et al., 2012). Fish were reared in fresh water at 10 ± 1^{o}C with a natural photoperiod, and fed commercial diet (Skretting, Vancouver, Canada). The 2011 brood of GH transgenics were fed to satiation thrice daily (TF group). The 2010 GH transgenics (TR group) were fed the same ration as the WT group, to produce similar body weights (Supplementary Table 1). Experiments were conducted meeting Canadian Council for Animal Care guidelines, and were approved by the Department of Fisheries and Oceans Pacific Region Animal Care Committee under Animal Use Protocol (#10-016). TF, TR and WT groups showed no significant differences in fork length (FL), however, body mass was significantly higher in the TF than WT group (Supplementary Table 1) ($P<0.05$) and condition factor (Mass/FL3 x 100) was higher in the TF than either the TR or WT groups, reflecting differences in body shape (Fig. 1A). The total cross-sectional area of fast muscle at the position of the anal vent was not statistically different (Supplementary Table 1).

Muscle morphometry

A steak ~ 5 mm thick was made at the level of the anal vent. Three blocks were prepared to sample one entire half of the myotomal cross-section and frozen in isopentane (2-methyl butane) cooled to its freezing point in liquid nitrogen. Sections were stained with myosin ATPase to distinguish between

fibre types and hematoxylin-eosin for morphometric analysis (Johnston et al. 2012). Photographs of tissue sections were taken at a magnification of 200 times and 4 fields, selected at random, were photographed per block per fish. The cross-sectional areas of the entire fast muscle portion of the myotome and around 300 fast muscle fibres per block were digitised. In total, 800 fast muscle fibres were randomly selected per fish using a program written in R+ (http://www.r-project.org/) and smooth probability density functions (pdfs) fitted using a kernel function. The average smoothing parameter used (0.284) was similar between groups. Bootstrap techniques (n=1000) were used to distinguish underlying structure in the distributions from random variation. A non-parametric Kolmogorov-Smirnoff two-sample test was used to test the null hypothesis that the probability density functions of groups were equal over all diameters (see Johnston et al. 2012). Data on fish size and muscle cellularity parameters was tested for normality (Shapiro-Wilk test) and equal variance and analysed using a one-way ANOVA with pairwise multiple comparisons by the Holm-Sidak method (overall significance level equal to 0.05).

Gene expression analysis

Pure fast muscle was dissected from dorsal epaxial myotomes. Total RNA extraction, quality analysis and concentration protocols were as described previously (Macqueen et al., 2013). Tissues were sampled at a similar time of day for 6 fish per group and the RNA stored at -80^{o}C. A detailed description of the cDNA synthesis, primer design, qPCR reaction set-up, and data analysis is provided elsewhere (Macqueen et al., 2013) and Supplementary Table 2. Briefly, a total of 1ug of RNA from 6 individuals for each of the treatments (WT, TF, TR) was reverse transcribed to cDNA using Quantitec reverse transcription kit (QIAGEN, Manchester, UK) including a gDNA removal step. Minus reverse transcriptase (-RT) was performed using 1µg of RNA from a pool created with RNA from all samples. 6µl per sample were mixed with 7.5µl of SensiFAST SYBR Lo-ROX 2X master mix (Bioline, London, UK) containing 400nM of sense/antisense primers. Reactions were performed in duplicate in a Mx3005P Thermocycler (Agilent; Berkshire, UK), with 1 cycle of 2 min at 95^{o}C and x40 cycles of 5s 95^{o}C and 20s at 65^{o}C, followed by a dissociation curve analysis, which resulted in a single peak in all cases. The stability of the four housekeeping genes *rpl27*, *rpl13*, *ef1a* and ß-*actin* was analysed as previously described (Macqueen et al. 2013). *Rpl13* and *ef1a* were found to be the most stable reference genes (M=0.058). Normalization of gene expression was performed using the geometric average of rpl13 and ef1a. All expression values are expressed as arbitrary units. Expression between groups was compared using a one-way ANOVA with a Bonferroni post-hoc correction using SPSS21 statistical package (IBM).

COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS

IAJ and RHD conceived the study; RHD was responsible of fish husbandry and generation of the experimental groups. IAJ was responsible of the histological analysis. DGDLS was responsible of the RNA extraction and RT-qPCR analysis. IAJ and DGDLSC carried out the statistical analysis. IAJ wrote the manuscript with input from the other authors.

FUNDING

The study was supported by the Marine Alliance for Science and Technology for Scotland (Scottish Funding Council grant HR09011) and by the Canadian Biotechnology Strategy (RHD).

REFERENCES

Björnsson, B. T. (1997) The biology of salmon growth hormone: from daylight to dominance. *Fish Physiology* **17**, 9-24.

Hill, J. A., Kiessling, A., Devlin, R. H. (2000) Coho salmon (*Oncorhynchus kisutch*) transgenic for a growth hormone gene construct exhibit increased rates of hyperplasia and detectable levels of differential gene expression. *Can. J. Aquat. Sci*. **57**, 939-950.

Jimenez, A.G., Dillaman, R. M., Kinsey, S. T. (2013) Large fibre size in skeletal muscle is metabolically advantageous. *Nature Communications* 4, 2150, DOI: 10.1038.

Johnston, I. A., Fernandez, D., Calvo, J., Vieira, V. L. A., North, T. W., Abercromby, M., Garland, T. Jr. (2003) Reduction in muscle fibre number during the adaptive radiation of Notothenioid fishes: a phylogenetic perspective. *J. Exp. Biol*. **206**, 2595-2609.

Johnston, I. A., Bower, N. I., Macqueen, D. J. (2011) Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol*. **214**, 1617-1628.

Johnston, I. A., Kristjánsson, B. K., Paxton, C. G., Vieira, V. L. A., Macqueen, D. J., Bell, M. A. (2012) Universal scaling rules predict evolutionary patterns of myogenesis in species with indeterminate growth. *Proc. R. Soc. B.* **279**, 2255-61.

Leggatt, R., Biagi, C.A., Smith, J. L., Devlin, R. H. (2012) Growth of growth hormone transgenic coho salmon *Oncorhynchus kisutch* is influenced by construct promoter type and family line. *Aquaculture* **356–357**, 193–199.

Macqueen, D. J., Garcia de la serrana, D., Johnston, I. A. (2013) Evolution of ancient functions in the vertebrate insulin-like growth factor system uncovered by study of duplicated salmonid fish genomes. *Mol. Biol. Evol*. **30**, 1060-1076.

Raven, P. A., Uh, M., Sakhrani, D., Beckman, B. R., Cooper, K., Pinter, J., Leder, E. H., Silverstein, J., Devlin, R. H. (2008) Endocrine effects of growth hormone overexpression in transgenic coho salmon. *Gen. Comp. Endocrinol.* **159**, 26-37.

Sundstrom, L. F., Devlin, R. H., Johnsson, J. I., Biagi, C. A. (2003) Vertical position reflects increased feeding motivation in growth hormone transgenic coho salmon (*Oncorhynchus Kisutch*). *Ethology*, **109**, 701-712.

FIGURE LEGENDS

Figure 1. Analysis of muscle growth patterns in Coho salmon (*Oncorhynchus kisutch*).

(A) WT, wild type; TF, Growth-hormone (GH) transgenics fed to satiation; TR, calorie-restricted GH-transgenics growing at the WT rate. (B) Relationship between fibre number and fish length. WT (closed triangles), TF (closed circles) and TR (open circles). Lines were fitted by least squares regression. (C) Probability density functions (pdfs) of muscle fibre diameter. The dashed lines represent the average pdfs of groups and the solid line the pdf of the combined groups. The shaded area represents the 1000 bootstraps of the combined group. (D) Percentiles of fibre diameter for WT, TF and TR groups. Values represent Mean ± SE.

Figure 2. Gene expression analysis in coho salmon (*Oncorhynchus kisutch*) fast skeletal muscle.

Gene expression in fast skeletal muscle measured by qPCR for *gh*, *igf1*, *dock5*, *dock1*, *cadherin-15*, *tmem8c*, *itgb1* and *crkl* (see Supplementary Table 2 for abbreviations). Results represent Mean ± SE, 6 fish per group. Different letters indicate differences between means ($p<0.05$).

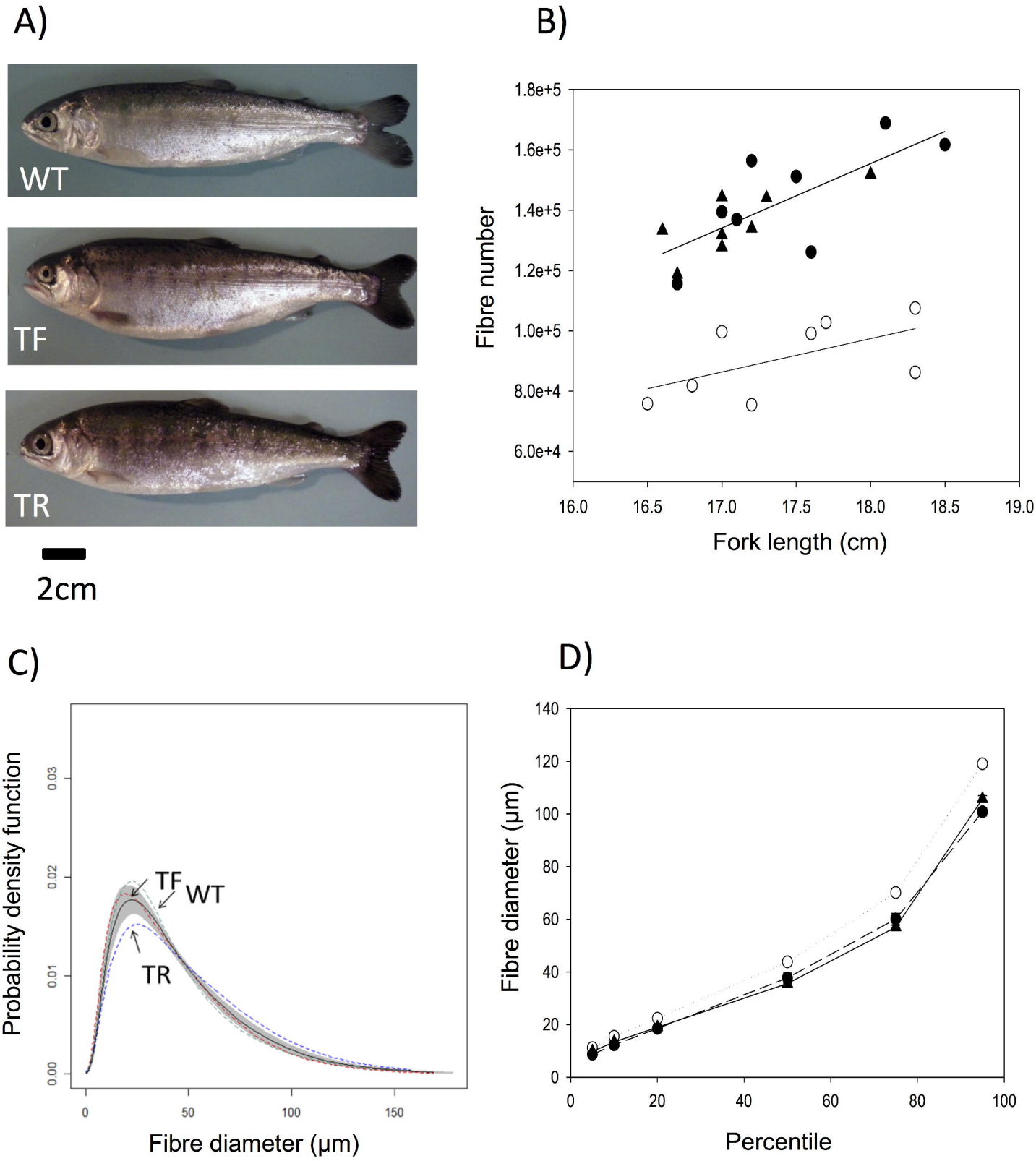
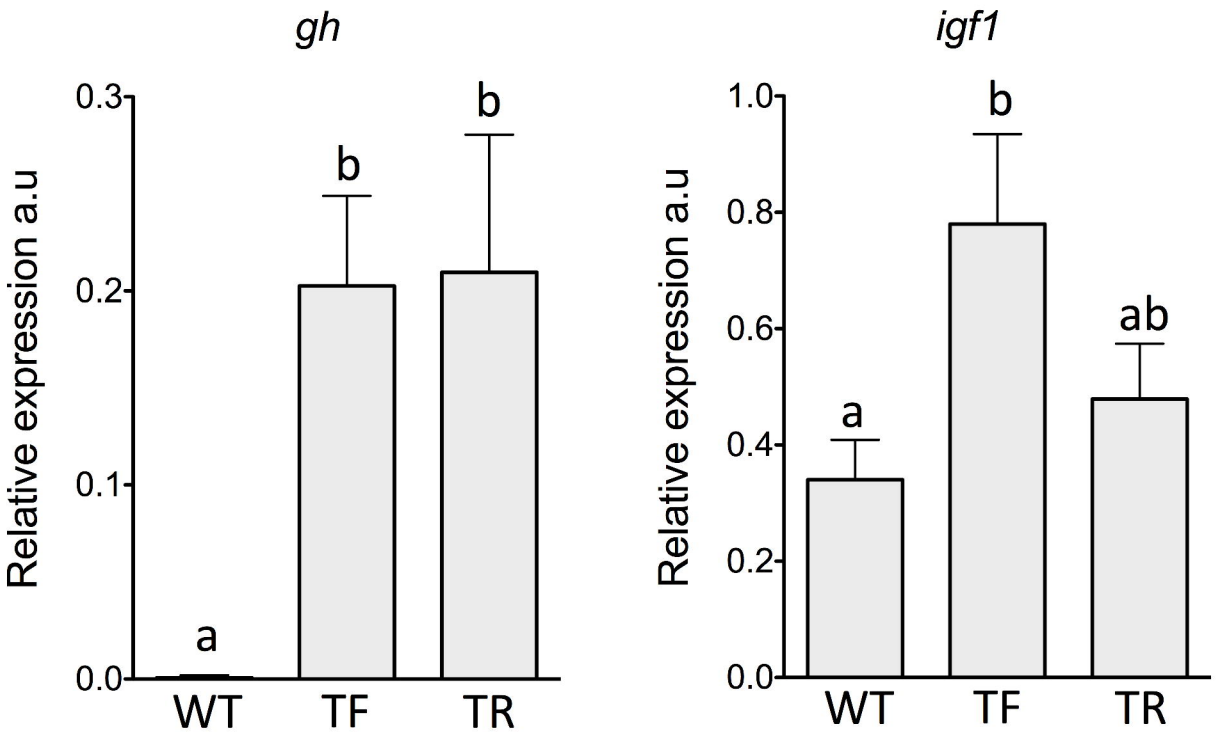

A)
WT
TF
TR
2cm
B)
Fibre number
1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
16.0
16.5
17.0
17.5
18.0
18.5
19.0
Fork length (cm)
C)
Probability density function
0.00
0.01
0.02
0.03
TF
WT
TR
0
50
100
150
Fibre diameter (µm)
D)
Fibre diameter (µm)
140
120
100
80
60
40
20
0
0
20
40
60
80
100
Percentile

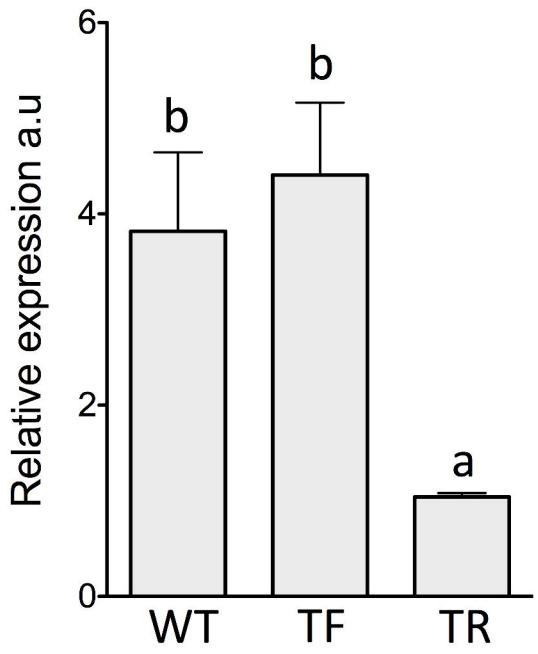
Fig.1

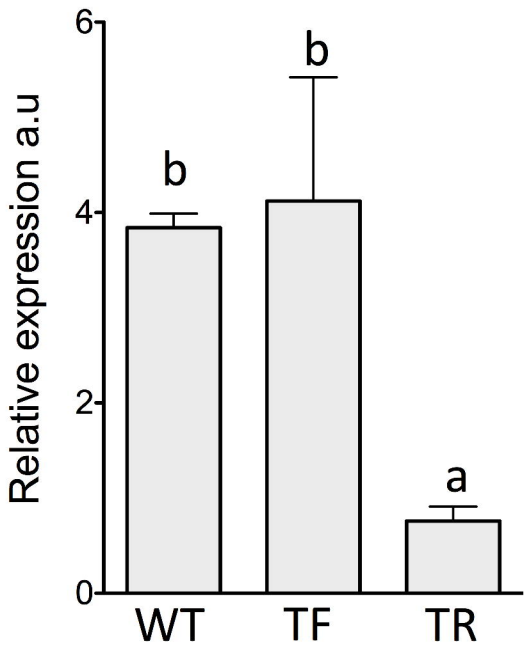
A)

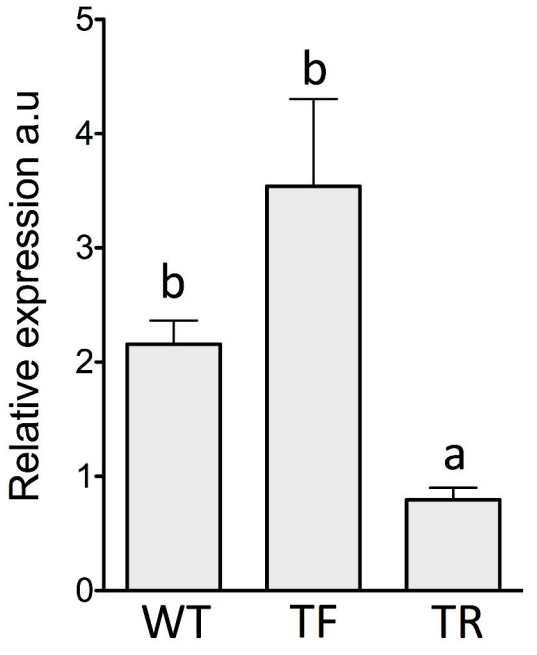

gh
Relative expression a.u
0.3
0.2
0.1
0.0
a
b
b
WT
TF
TR

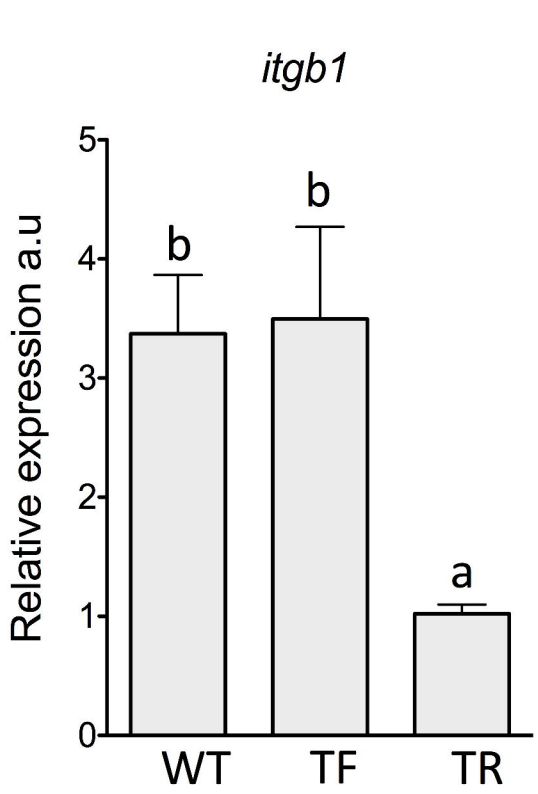
igf1
Relative expression a.u
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
WT
TF
TR

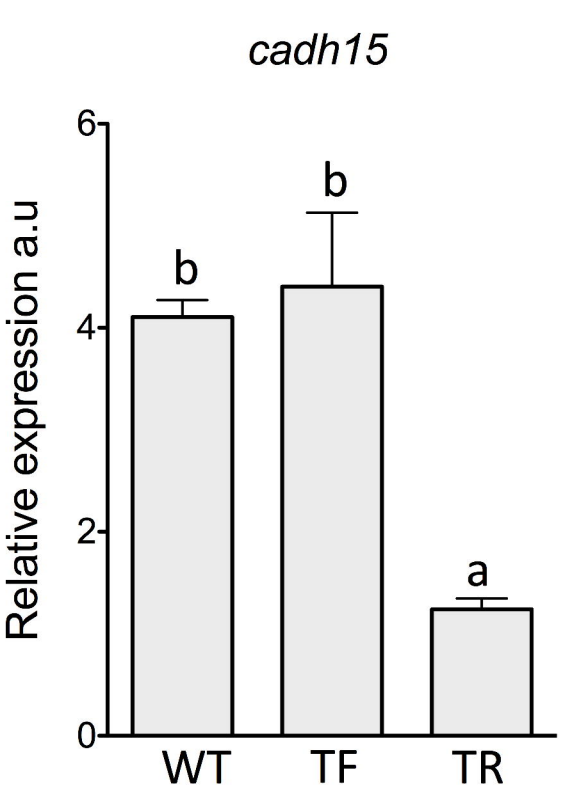
B)


dock1
Relative expression a.u
6
4
2
0
b
b
a
WT
TF
TR


dock5
Relative expression a.u
6
4
2
0
b
b
a
WT
TF
TR


crkl
Relative expression a.u
5
4
3
2
1
0
b
b
a
WT
TF
TR


itgb1
Relative expression a.u
5
4
3
2
1
0
b
b
a
WT
TF
TR


cadh15
Relative expression a.u
6
4
2
0
b
b
a
WT
TF
TR

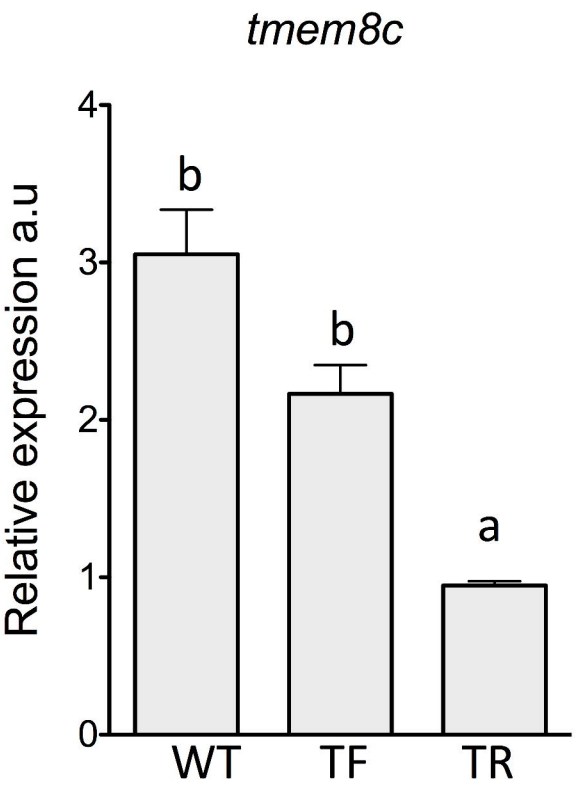

tmem8c
Relative expression a.u
4
3
2
1
0
b
b
a
WT
TF
TR

Fig.2