Production of growth hormone transgenic Threespine Stickleback (*Gasterosteus aculeatus*) strains for biotechnology regulatory research

Kyle Wellband, Carling D. Gerlinsky, Taylor C. Gibbons, Dionne Sakhrani, Krista Woodward, Hamid Seshadri, Joelle Prevost, Shannan May-McNally, Monica Yau, Dolph Schluter, Robert H. Devlin

Fisheries and Oceans Canada
Science Branch, Pacific Region
Pacific Science Enterprise Centre
4160 Marine Drive
West Vancouver, BC V7V 1N6

2026

Canadian Technical Report of
Fisheries and Aquatic Sciences 3754

Fisheries and Oceans Canada | Pêches et Océans Canada

Canada

Canadian Technical Report of Fisheries and Aquatic Sciences

Technical reports contain scientific and technical information that contributes to existing knowledge but which is not normally appropriate for primary literature. Technical reports are directed primarily toward a worldwide audience and have an international distribution. No restriction is placed on subject matter and the series reflects the broad interests and policies of Fisheries and Oceans Canada, namely, fisheries and aquatic sciences.

Technical reports may be cited as full publications. The correct citation appears above the abstract of each report. Each report is abstracted in the data base *Aquatic Sciences and Fisheries Abstracts*.

Technical reports are produced regionally but are numbered nationally. Requests for individual reports will be filled by the issuing establishment listed on the front cover and title page.

Numbers 1-456 in this series were issued as Technical Reports of the Fisheries Research Board of Canada. Numbers 457-714 were issued as Department of the Environment, Fisheries and Marine Service, Research and Development Directorate Technical Reports. Numbers 715-924 were issued as Department of Fisheries and Environment, Fisheries and Marine Service Technical Reports. The current series name was changed with report number 925.

Rapport technique canadien des sciences halieutiques et aquatiques

Les rapports techniques contiennent des renseignements scientifiques et techniques qui constituent une contribution aux connaissances actuelles, mais qui ne sont pas normalement appropriés pour la publication dans un journal scientifique. Les rapports techniques sont destinés essentiellement à un public international et ils sont distribués à cet échelon. II n'y a aucune restriction quant au sujet; de fait, la série reflète la vaste gamme des intérêts et des politiques de Pêches et Océans Canada, c'est-à-dire les sciences halieutiques et aquatiques.

Les rapports techniques peuvent être cités comme des publications à part entière. Le titre exact figure au-dessus du résumé de chaque rapport. Les rapports techniques sont résumés dans la base de données *Résumés des sciences aquatiques et halieutiques.*

Les rapports techniques sont produits à l'échelon régional, mais numérotés à l'échelon national. Les demandes de rapports seront satisfaites par l'établissement auteur dont le nom figure sur la couverture et la page du titre.

Les numéros 1 à 456 de cette série ont été publiés à titre de Rapports techniques de l'Office des recherches sur les pêcheries du Canada. Les numéros 457 à 714 sont parus à titre de Rapports techniques de la Direction générale de la recherche et du développement, Service des pêches et de la mer, ministère de l'Environnement. Les numéros 715 à 924 ont été publiés à titre de Rapports techniques du Service des pêches et de la mer, ministère des Pêches et de l'Environnement. Le nom actuel de la série a été établi lors de la parution du numéro 925.

Canadian Technical Report of
Fisheries and Aquatic Sciences 3754

2026

Production of growth hormone transgenic Threespine Stickleback (*Gasterosteus aculeatus*) strains for biotechnology regulatory research

by

Kyle Wellband[a], Carling D. Gerlinsky[b], Taylor C. Gibbons[b], Dionne Sakhrani[a], Krista Woodward[a], Hamid Seshadri[a], Joelle Prevost[a], Shannan May-McNally[b], Monica Yau[b], Dolph Schluter[b], Robert H. Devlin[a]

[a.] Fisheries and Oceans Canada
Pacific Science Enterprise Centre
4160 Marine Drive
West Vancouver, BC V7V 1N6

[b.] Biodiversity Research Centre and Department of Zoology
University of British Columbia
6270 University Blvd
Vancouver, BC V6T 1Z4

Cat. No. Fs97-6/3754E-PDF ISBN 978-0-660-98133-8 ISSN 1488-5379
https://doi.org/10.60825/7q2x-vf65

Correct Citation for this publication:

Wellband, K., Gerlinsky, C.D., Gibbons, T.C., Sakhrani, D., Woodward, K., Seshadri, H., Prevost, J., May-McNally, S., Yau, M., Schluter, D., Devlin, R.H. 2026. Production of growth hormone transgenic Threespine Stickleback (*Gasterosteus aculeatus*) strains for biotechnology regulatory research. Can. Tech. Rep. Fish. Aquat. Sci. 3754: iv + 13 p. https://doi.org/10.60825/7q2x-vf65

ABSTRACT

Wellband, K., Gerlinsky, C.D., Gibbons, T.C., Sakhrani, D., Woodward, K., Seshadri, H., Prevost, J., May-McNally, S., Yau, M., Schluter, D., Devlin, R.H. 2026. Production of growth hormone transgenic Threespine Stickleback (*Gasterosteus aculeatus*) strains for biotechnology regulatory research. Can. Tech. Rep. Fish. Aquat. Sci. 3754: iv + 13 p. https://doi.org/10.60825/7q2x-vf65

Genetically modified organisms are widely used in agriculture in many parts of the world, yet longstanding questions remain regarding potential impacts from their purposeful or accidental entry into natural environments. Several species of growth hormone (GH) transgenic salmon have been produced and subjected to risk assessment research in contained simulated-natural environments to estimate their fitness and potential ecological consequences. Due to their long and complex life history, assessments of genetically modified salmon through a full life-history in multiple environments and across generations remains problematic. To explore whether some of these inherent limitations could be overcome using a smaller species, we developed a new GH-transgenic fish model for ecological risk assessment research using Threespine Stickleback (Gasterosteus aculeatus), a small temperate species that is extensively used in ecological and evolutionary research. GH-transgenic stickleback exhibit increased growth in the laboratory compared with non-transgenic fish consistent with increases observed for GH-transgenic salmon. This GH-transgenic fish model will assist in understanding the phenotypic effects and potential risks of GH-transgenic fish use, and, perhaps more importantly, further evaluate the validity of using surrogate species in risk assessment research.

RÉSUMÉ

Wellband, K., Gerlinsky, C.D., Gibbons, T.C., Sakhrani, D., Woodward, K., Seshadri, H., Prevost, J., May-McNally, S., Yau, M., Schluter, D., Devlin, R.H. 2026. Production of growth hormone transgenic Threespine Stickleback (*Gasterosteus aculeatus*) strains for biotechnology regulatory research. Can. Tech. Rep. Fish. Aquat. Sci. 3754: iv + 13 p. https://doi.org/10.60825/7q2x-vf65

Les organismes génétiquement modifiés sont utilisés pour l'agriculture dans plusieurs régions du monde, mais des questions subsistent quant aux impacts potentiels de leur introduction intentionnelle ou accidentelle dans les milieux naturels. Plusieurs espèces de saumons transgéniques surexprimant l'hormone de croissance (HC) ont été produites. Afin d'estimer leurs conséquences écologiques potentielles, ces espèces ont été soumises à des études d'évaluation des risques dans des environnements confinés simulant les conditions naturelles. Mais, en raison de leur cycle de vie longue et complexe, l'évaluation des saumons génétiquement modifiés à travers plusieurs environnements et pendant plusieurs générations reste problématique. Afin de surmonter ces limites, nous avons développé un nouveau modèle de poisson transgénique surexprimant HC pour la recherche sur l'évaluation des risques écologiques en utilisant l'épinoche à trois épines (*Gasterosteus aculeatus*). L'epinoche à trois épines est une petite espèce largement utilisée dans la recherche écologique et évolutive. Nous avons démontré que les épinoches transgéniques surexprimant HC présentent une croissance accrue en laboratoire par rapport aux épinoches non transgéniques, une différence qui correspond aux augmentations de croissances observées chez les saumons transgéniques surexprimant HC. Ce modèle de poisson transgénique surexprimant HC permettra de mieux comprendre les effets phénotypiques et les risques potentiels liés à l'utilisation de poissons transgéniques surexprimant HC. Ce modèle également permettra d'évaluer la validité de l'utilisation d'espèces de substitution dans l'évaluation des risques.

Introduction

In Canada, the import and production of novel aquatic organisms that are products of biotechnology are regulated under the Canadian Environmental Protection Act (1999) and its associated New Substances Notification Regulations (Organisms) [NSNR(O)]. While authority for enforcing these regulations rests with the Ministers of Environment and Health (i.e., ECCC and HC), Fisheries and Oceans Canada has a Memorandum of Understanding with ECCC and HC to provide science advice to support regulatory decisions for novel aquatic organisms notified under the NSNR(O). To provide informed and timely decision-making within the legislated 120 days from notification, peer-reviewed science must exist prior to the notification of novel aquatic organisms.

Over the last 30 years, much of the interest in the production of fish that are products of biotechnology has focused on creating growth-enhanced stocks (e.g., Devlin et al., 1994, Du et al., 1992; Dunham et al. 1992; Nam et al. 2001). The Centre for Applied Biotechnology Regulatory Research at DFO's Pacific Science Enterprise Centre has long conducted research on growth-enhanced salmonids, specifically growth-hormone (GH) transgenic Coho Salmon and Rainbow Trout (Devlin et al. 2015). This research has quantified the impact of constitutively expressed growth-hormone on growth rates and a wide range of phenotypes related to fitness (see Devlin et al. (2015) for a summary). These data have revealed considerable uncertainty in using laboratory-derived data for risk assessments. For example, rearing GH-transgenic and non-transgenic fish in tanks results in large differences in body size, but when the same fish are reared in semi-natural stream environments, only a small difference in body size is detected (Sundström et al. 2007a). This genotype-by-environment interaction highlights the need to assess the effects of genetic modifications relative to wild-type, and to do so in multiple complex environments to more accurately estimate risks and associated uncertainties.

To date, transgenic salmon have been the model of choice to investigate basic questions about growth modification and its effects on fish phenotypes because of their relevance to industry for food production. However, conducting research with salmonids can be problematic due to their large size, long generation times, and complex life histories. In particular, conducting research to quantify the ecological impacts of GH-transgenic salmon if they escaped to the natural environment is challenged by the facts that 1) we cannot release transgenics into nature to study them, and 2) there are difficulties in simulating the ecology of salmon throughout their entire complex life-cycle (i.e., stream and oceanic conditions) in the lab. Thus, the development of additional, more experimentally tractable, transgenic species is crucial to assess potential ecosystem-level impacts of escaped GH-transgenics in the wild (Li et al. 2014) as well as to assess the generality of the effects of GH-transgenesis across species.

While the contemporary focus of genetic modification in fish has shifted to using modern gene-editing technologies (e.g., CRISPR), transgenic fish still represent important models to understand modifications that induce large or pleiotropic effects on phenotype. From a risk assessment perspective, research on additional GH-transgenic fish species, to determine the extent to which the pleiotropic effects of GH-transgenesis are conserved across species

and context, can provide valuable information about the degree to which we can rely on surrogate information. The use of surrogate information to inform risk is a common feature of risk assessments conducted for genetically modified fish in Canada (e.g., Devlin et al. 20215; DFO 2021). Confidence and certainty in risk assessment conclusions relies, in part, on the ability to trust surrogate information. Observing consistency, or a lack thereof, in the effects of a modification across species is informative for evaluating the generality of conclusions regardless of the technology used to produce the specific modification.

Threespine Stickleback (*Gasterosteus aculeatus*; hereafter: stickleback) is a species of fish that has been widely studied as a model for investigating adaptation and speciation processes. Populations of stickleback have diverged countless times as a result of freshwater colonization from marine environments and along benthic-limnetic axes in freshwater (Bell & Foster, 1994; Colosimo et al., 2005; Schluter, 2009). Body length and size at reproduction are linked with fitness (Schluter, 1995; Wootton, 1984), and populations exhibit growth differences in response to abiotic environmental factors (Gibbons et al., 2016, 2017; Marchinko & Schluter, 2007). Furthermore, the ecological consequences of phenotypic and genetic variation in stickleback have been well-studied (Harmon et al., 2009; Rudman et al., 2019; Rudman & Schluter, 2016). It has been demonstrated throughout this body of research that the ecology of stickleback ponds can be recreated in artificial ponds. This means that experimental manipulation and biocontainment can be achieved while still adequately simulating the natural ecology of this species. Stickleback represent an ideal model for investigation of: 1) the effects of GH-transgenesis on phenotypic and life history traits, 2) comparisons of these effects with those observed in existing transgenic fish, 3) assessments of the extent of genotype-by-environment effects, 4) estimates of the overall influence of GH-transgenesis on fitness, and 5) assessment of potential ecosystem-level impacts that could arise from the release of GH-transgenic fish. Here we describe the production of GH-transgenic stickleback lines to support future research on the generality of the effects of GH-transgenesis on fish species as well as provide a model for testing ecosystem-level impacts of GH-transgenic fish under a simulated escape event.

Materials and Methods

Fish husbandry and breeding

Adult Threespine Stickleback (*Gasterosteus aculeatus*; hereafter: stickleback) were collected from an anadromous population at the mouth of the Little Campbell River in British Columbia, Canada (49°00’52”N, 122°45’33”W) during their normal breeding season (late May to early August 2014-2019). Gravid females and males in breeding condition (the latter indicated by blue eyes and a red throat) were transported to the University of British Columbia (UBC). Collections from Little Campbell River were conducted under the following collection and transport permits: British Columbia Fish Collection Permit: 2014) NA-SU14-93473, 2015) MRSU15-163200, 2016) MRSU16-229407, 2017) SU17-258923, 2018) MRSU18-288855, 2019) MRSU19-454239; Fisheries and Oceans Canada permit to

transfer live fish: 2014) 13348, 2015) 13581, 2016) 13863, 2017) 14054, 2018) 14282, 2019) 119081.

All fish were held in 100 L aquaria housed within an environmental chamber at UBC. Fish were reared on dechlorinated tap water with the salinity adjusted to 5 ppt using Instant Ocean® sea salt. Temperatures were maintained at 17°C (summer) or 7-8°C (winter) and rearing occurred under lighting that mimicked a natural spectrum and photoperiod. Gravid females were observed in the lab until their eggs were ovulated as indicated by a swollen abdomen with a box-like corner at the cloaca, and were then used in artificial crosses to generate offspring (performed as in Marchinko and Schluter (2007)). For eggs undergoing microinjection with the transgene (see below), the egg clutch was covered and allowed to incubate at room temperature for approximately one hour or until the blastodisc was visible. Fertilized eggs were put into hatching cups (plastic cups with the bottom removed and replaced with fine mesh screen) in aquaria supplied with gentle aeration. Eggs were observed daily, and any with arrested development or visible fungus were removed. Methylene blue was added to tank water (until water was pale blue) to inhibit fungal growth. At the end of experiments, fish were euthanized in 0.5 g/L tricaine methanesulfonate (Syndel Canada, Nanaimo, BC) buffered to pH 7.5 with sodium bicarbonate. To prevent release of live stickleback fish or gametes to nature, all wastewater from aquaria was treated for 24 hours with commercial bleach. The procedures of these experiments were conducted under the approval of a University of British Columbia animal care certificate A18-0001.

Gene construct and microinjection of embryos

The gene construct (CMVGaGHmCh; synthesized by Genscript Biotech Inc., Piscataway, NJ) contained the stickleback growth hormone coding regions from the ATG start codon to the TAG stop codon (GaGH) linked by a 74 bp TATAA box and 546 bp CMV promoter regions, and terminated with a 274 bp SV40 sequence containing a AATAAA polyadenylation site (Figure 1). The construct also contained a reporter gene for a red fluorescent protein variant (mCherry) driven by an EF1-alpha promoter. The entire construct was flanked by Tol 2 ends that have been shown to facilitate construct insertion frequency in some species (Kawakami, 2004). PCR primer sequences used to amplify the CMVGaGHmCh construct and amplicon sizes are shown in Table 1.

Tol2 L200 Arm

GTTTAAACTCTAGAACTAGTGATCTGCTGGGCTTGCTGAAGGTAGGGGGTCAAGAACCAGAGGTGTAAAG
TACTTGAGTAATTTTACTTGATTACTGTACTTAAGTATTATTTTTGGGGATTTTTACTTTACTTGAGTAC
AATTAAAAATCAATACTTTTACTTTTACTTAATTACATTTTTTTAGAAAAAAAAGTACTTTTTACTCCTT
ACAATTTTATTTACAGTCAAAAAGTACTTATTTTTTGGAGATCACTTATTTAAATGGGCCCGGGCCTGCA
GGCTCGAG

Inverse complement of Thymidine Kinase terminator

AGGGGATGCTATGGCAGGGCCTGCCGCCCCGACGTTGGCTGCGAGCCCTGGGCCTTCACCCGAACTTGGG
GGGTGGGGTGGGGAAAAGGAAGAAACGCGGGCGTATTGGCCCCAATGGGGTCACGGTGGGGTATCGACAG
AGTGCCAGCCCTGGGACCGAACCCCGCGTTTATGAACAAACGACCCAACACCCGTGCGTTTTATTCTGTC
TTTTTATTGCCGTCATAGCGCGGGTTCCTTCCGGTATTGTCTCCTTCCGTGTTTCAGTTAGCCTCCCCC

Inverse complement of mCherry mRNA

TTACTTGTACAGCTCGTCCATGCCGCCGGTGGAGTGGCGGCCCTCGGCGCGTTCGTACTGTTCCACGATG
GTGTAGTCCTCGTTGTGGGAGGTGATGTCCAACTTGATGTTGACGTTGTAGGCGCCGGGCAGCTGCACGG
GCTTCTTGGCCTTGTAGGTGGTCTTGACCTCAGCGTCGTAGTGGCCGCCGTCCTTCAGCTTCAGCCTCTG
CTTGATCTCGCCCTTCAGGGCGCCGTCCTCGGGGTACATCCGCTCGGAGGAGGCCTCCCAGCCCATGGTC
TTCTTCTGCATTACGGGGCCGTCGGAGGGGAAGTTGGTGCCGCGCAGCTTCACCTTGTAGATGAACTCGC
CGTCCTGCAGGGAGGAGTCCTGGGTCACGGTCACCACGCCGCCGTCCTCGAAGTTCATCACGCGCTCCCA
CTTGAAGCCCTCGGGGAAGGACAGCTTCAAGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTG
GAGCCGTACATGAACTGAGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCACCCTTGGTCACCTTCA
GCTTGGCGGTCTGGGTGCCCTCGTAGGGGCGGCCCTCGCCCTCGCCCTCGATCTCGAACTCGTGGCCGTT
CACGGAGCCCTCCATGTGCACCTTGAAGCGCATGAACTCCTTGATGATGGCCATGTTATCCTCCTCGCCC
TTGCTCACCAT

Inverse complement of EF1a promoter

GGTGGCGACCGGTGGATCCGTCGAGGAATTCTTTGCCAAAATGATGAGACAGCACAACAACCAGCACGTT
GCCCAGGAGCTGTAGGAAAGAGAAGAAGGCATGAACATGGTTAGCAGAGGGGCCCGGTTTGGACTCAGAG
TATTTTATCCTCATCTCAAACAGTGTATATCATTGTAACCATAAAGAGAAAGGCAGGATGATGACCAGGG
TGTAGTTGTTTCTACCAATAAGAATATTTCCACGCCAGCCAGAATTTATATGCAGAAATATTCTACCTTA
TCATTTAATTATAACAATTGTTCTCTAAAACTGTGCTGAAGTACAATATAATATACCCTGATTGCCTTGA
AAAAAAAGTGATTAGAGAAAGTACTTACAATCTGACAAATAAACAAAAGTGAATTTAAAAATTCGTTACA
AATGCAAGCTAAAGTTTAACGAAAAAGTTACAGAAAATGAAAAGAAAATAAGAGGAGACAATGGTTGTCA
ACAGAGTAGAAAGTGAAAGAAACAAAATTATCATGAGGGTCCATGGTGATACAAGGGACATCTTCCCATT
CTAAACAACACCCTGAAAACTTTGCCCCCTCCATATAACATGAATTTTACAATAGCGAAAAAGAAAGAAC
AATCAAGGGTCCCCAAACTCACCCTGAAGTTCTCAGGATCGGTCGACCTGCAGGAAGCTTCAGCTAGAAC
TCGCCGCAGACCCGGTGAGGAAGAGAGCGAACCGGGCCTTAACCACCCTTTATATAGCCGCCTCTACTGG
GCGGGGATTAACCATGACATCATCAAGTCCAGAATTCCCAAAGTGCTAGAGGCGGGGTCTTGACAGAACA
TTCAGCCTGTAAGCGTCTTGTATACTACAACTCCCAGTAGCACTAGGGCGGATGCAGCTGTTGAGTGAAA
CGCGCGAGTTATCACGAAGTTAGGGCGAAAGGAAGGGTGGCACTCCCTAGTGCGTCATAAGCTAGCTTGC
ATGCCTGAGAATTTCAGAATGTAATGATACCTTTGTTAGATAAGGCTGTTTACATCTGATAGTGGACCTT
AAGCCGACACTTAAATGATAAAAACGGCAAAGAATTGCAAGTTTGATTTGCATTGGAAAGGGTCGCTGGC
TTTTGTGTTACACGCCCCTTATTTGTGCTTGATTAGATGATCCCCCTG

CMV Promoter region

CTCGAGCCTGCAGGCGCGCCATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAAT
AATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGG
TAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG
GTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTA
CGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT
GACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAAC
GGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGA
GGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTGGGATCCCGTCGCTTACCGATTCAGAATGGTTGATA
TCGCGGCCGC

***Gasterosteus aculeatus* GH mRNA**

CGCCACCATGAACAGAGTCATCCTCCTGCTGTCGGTGGTGTTTCTGGGCGTGTCTACTCAGCCAATCACA
GACGGCCGTCTGTTTTCCATCGCTGTGGGTAGAGTCCAACACCTCCACCTGCTCGCTCAGAGACTCTTCT
CGGACTTTGAGAGTTCTCTGCAATCAGAAGAGCAACGTCAACTCAACAAAATCTTTCTCCAAGATTTCTG
CAACTCTGATTACATCATCACCCCCGTCGACAAGCACGAGACCCAACGTAGCTCTGTGTTGAAGCTATTA
TCCATCTCCTATCGATTGGTCGAGTCCTGGGAGTTTCCCAGTCGTTCCCTGTCTGGTGGTTCTGCTCCCA
GAATCCAGATTTCCCCCAAACTGTCGGAGCTAAAGACGGGAATCCTCCTTTTGATCAAGGCCAATCAGGA
TGGAGCAGATCTTTTCCCTGACAGCTCCGCCCTACAGCTCGCTCCTTATGGAAACTACTATCAAAGTCTG
GGGGCTGACGAGTCGCTGAGAAGAACATATGAACTACTTGCCTGTTTTAAGAAAGACATGCACAAGGTGG
AGACCTACCTGACTGTGGCTAAATGTCGACTCTCTCCAGAAGCCAACTGCACTTTGTAG

SV40 Terminator (polyadenylation site)

GGCCGGCCCACCGCGGTGGAGCTCGAATTAATTCATCGATGATGATCCAGACATGATAAGATACATTGAT
GAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTG
CTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCA
GGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTAT
GATCCTC

Tol2 Arm

GGGCCCGGGCATTTAAATGATCTAGCAGATCTAATACTCAAGTACAATTTTAATGGAGTACTTTTTTACT
TTTACTCAAGTAAGATTCTAGCCAGATACTTTTACTTTTAATTGAGTAAAATTTTCCCTAAGTACTTGTA
CTTTCACTTGAGTAAAATTTTTGAGTACTTTTTACACCTCTGTCAAGAACCATATGCCGGTACCACGTTG
TATCTAGAACTAGTGTTTAAAC

Figure 1: DNA sequence structure of gene construct CMVGaGHmCh. Major functional sequence blocks are shown for Tol 2 transposition, thymidine kinase terminator, mCherry, EF1alpha promoter, CMV promoter, *Gasterosteus aculeatus* GH mRNA, SV40 terminator.

Table 1: PCR primer sequences for amplification of DNA from putative transgenic fish. CMVGaGHmch represents the transgene construct and was used to identify transgene-positive stickleback. The Ectodysplasin gene (Eda) was used as a positive control because both transgenic and non-transgenic stickleback possess this gene. T_m indicates the predicted melting temperature of the primer.

Gene target	Primer name	Sequence (5'-3')	T_m
CMVGaGHmch	mCh-270F	AGCCCATGGTCTTCTTCTGC	60
CMVGaGHmch	mCh-624R	CACGAGTTCGAGATCGAGGG	59
CMVGaGHmch	CMV-225F	ATATGCCAAGTACGCCCCCT	61
CMVGaGHmch	CMV-406R	GGAGACTTGGAAATCCCCGT	59
CMVGaGHmch	GaGH-88F	CATCGCTGTGGGTAGAGTCC	59
CMVGaGHmch	GaGH-426R	GCTCCATCCTGATTGGCCTT	60
CMVGaGHmch	GaGH-611R	TGCAGTTGGCTTCTGGAGAG	59
Eda	GaEdaF4	CTATAGTCAGGTAGAAGTGA	50
Eda	GaEdaR4	AAGTTGAGGTAGTACACCT	52

Microinjections were performed in late summer 2014. After artificial fertilization, eggs were confirmed to be at the one or two-cell stage under a dissecting microscope, and then moved to a petri dish of agar with small depressions such that blastodiscs were in the same orientation, tilted approximately 45 degrees from vertical. The agar depressions, in combination with a glass suction tube fitted with a rubber foot switch, secured eggs for injection. The plasmid containing this construct was injected into eggs along with Tol 2 transposase (TPase). The injection solution contained Tris (1 mM, pH 7.0), phenol red for visibility (0.5%), and construct and Tol2 plasmid DNAs (100 µg/µL each). This solution was injected into the blastodisc until the disc was half-covered with phenol red. Eggs were injected with approximately 1 nL (100 ng) of each gene construct using a 1-2 µm diameter glass needle and a Narishige microinjector (Model IM300; Narishige Corporation, Japan).

Genetic screening for transgenesis

Testing for the presence/absence of the transgene in individual fish was conducted using the polymerase chain reaction (PCR) with primers specific to the transgene (Table 2). Positive amplification of a band of DNA using transgene specific primers indicated the transgene material was present in a given individual. Fish were allowed to reach at least 25 mm standard length before fin clipping to ensure sufficient tissue could be removed with a minimal impact on survival. Fin clipping was performed under anesthesia (50-75 mg/L tricaine methanesulfonate, buffered to pH 7.5 with sodium bicarbonate). For large fish, a 2 x 2 mm portion of the top lobe of the caudal fin was removed; for smaller fish, approximately 1 mm of the entire distal edge was removed. Fin clips were divided in two. One was placed in 95% ethanol for preservation and the other was used immediately for DNA isolation. DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN, Inc., Toronto, ON, Canada).

Table 2: PCR cycling conditions for initial screening of transgene-positive F0 fish.

Fish ID#	Forward primer	Reverse primer	PCR cycling conditions
1-40	CMV-225F	CMV-406R	94°C x 5 min, 35 x (94°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
1-40	CMV-225F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
41-60	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
41-60	GaGH-88F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
61-82	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 1 min), 72°C x 6 min, 4°C hold.
61-82	GaGH-88F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 1 min), 72°C x 6 min, 4°C hold.
174-202	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
174-202	GaGH-88F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
203-252	GaGH-88F	GaGH-426R	94°C x 5 min, 36 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 45 sec), 72°C x 5 min, 4°C hold.

203-252	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
203-252	CMV-225F	CMV-406R	94°C x 5 min, 36 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
203-252	GaGH-88F	GaGH-426R	94°C x 5 min, 36 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.

PCR reactions were performed with 1 µL of extracted DNA in 20 µL reactions containing 1X PCR buffer, 200 µM dNTPs, 1.5 mM MgCl2, 750 nM forward and reverse primers (Table 2), and 0.5 U AmpliTaq Gold® DNA polymerase (ThermoFisher Scientific Inc., Mississauga, ON) . Various primer combinations were used to detect the presence of the transgene as well as confirm the ability to positively amplify stickleback DNA (Table 2). Cycling conditions to screen F0 fish for the presence of the transgene were specific to primer pairs and to different batches of fish (Tables 2 and 3). Certain F0 fish with inconclusive initial results were subjected to follow-up PCR screening to definitively determine their transgenic status (Table 3). All PCR products were visualized on a 2% agarose gels. F1 generation fish were screened for transgenic status using quantitative real-time PCR. The sequence of the qPCR primers was, CMVGaGHmch-F: TGTACGGTGGGAGGTCTATATAAGC, and CMVGaGHmch-R: CCACCGACAGCAGGAGGAT. Reactions contained 1X Fast SYBR® Green Master Mix (Thermo Fisher Scientific Inc., Mississauga, ON), 1 µM forward and reverse primers and 0.75 µL of sample DNA in a final volume of 10 µL. Cycling conditions for qPCR were 95°C for 20 sec followed by 40 cycles of 95°C for 3 sec, 55°C for 30 sec.

Table 3: PCR cycling conditions for re-screening of selected F0 fish to confirm transgenesis.

Fish ID#	Forward primer	Reverse primer	PCR cycling conditions
185, 186, 177	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
185, 186, 177	GaGH-88F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
185, 186, 177	mCh-270F	mCh-624R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 56°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
185, 186, 177	CMV-225F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 1 min), 72°C x 7 min, 4°C hold.
11,78	mCh-270F	mCh-624R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.

11,78	CMV-225F	CMV-406R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
11,78	CMV-225F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 1 min), 72°C x 7 min, 4°C hold.
11,78	GaGH-88F	GaGH-426R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.
11,78	CMV-225F	GaGH-611R	94°C x 5 min, 35 cycles of (94°C x 30 sec, 55°C x 30 sec, 72°C x 1 min), 72°C x 7 min, 4°C hold.
103, 119, 122, 124, 134, 90	GaEdaF4	GaEdaR4	94°C x 5 min, 35 cycles of (94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec), 72°C x 5 min, 4°C hold.

Growth of F1 fish in the laboratory

F1 generation offspring were created by artificial reproduction of transgenic individuals and wild caught fish as described above. All fish were held in separate 100 L aquaria by family group. Growth and survival in the laboratory was measured for F1 fish from five families (two A-line families, three B-line families described below) at three sampling points during development starting as soon as fish were large enough to survive handling (fall 2015 to summer 2016). Because different families were initiated on different dates, the timing of sampling points for each family were not synchronized in terms of date, but rather age. Only length was measured at the first sampling point (range of 135 to 165 days old, depending on family). At sampling points two (260 to 261 days old) and three (366 days old), fish were measured for both length and wet weight. Fish were genetically sampled and genotyped as described above to determine transgenic status. GH-transgene positive fish were marked with injectable elastomer tags to identify them at subsequent sampling time points. We statistically analyzed the growth data using linear mixed-effects models implemented in the 'lme4' package in R v4.2.0 (R Core Team 2022). In these models, genotype and line were designated as fixed effects and stickleback family was a random effect. In total, we constructed five linear mixed-effects models to analyze mass and length data at three sampling points during development: 1-3) three linear mixed-effects models for length: at sampling point one, two, and three; 4-5) two linear mixed-effects models for mass: at sampling point two and three (mass was not measured at sampling point one). We note that growth was not analyzed in a controlled, randomized, growth trial, but merely collected as observational data to evaluate if the transgene positive fish are larger than their non-transgenic siblings.

Results

Microinjection success and generation of F1 and F2 transgenic generations

In the founder (F0) generation, 1,048 embryos were microinjected with the CMVGaGHmCh gene construct + TPase, with an average egg survival rate of 15.4%. Of the 161 fish that survived the microinjection procedure and lived until they were large enough to be screened for transgenesis, four fish, two males and two females, tested positive for the CMVGaGHmCh transgene construct (a transgenesis success rate of 0.38%). All four of these transgenic F0 fish were reared to adulthood and subsequently became sexually mature.

The four transgenic F0 fish were bred with wild-type Little Campbell River fish to produce F1 offspring. Two of the transgenic F0 fish, one female and one male, produced transgenic F1 offspring and they were designated as the "A-line" and the "B-line," respectively. Overall, 27.3% of F1 B-line fish were screened for transgenesis (359 of 1,315 fish), of which 17.8% tested positive for the transgene (64 of 359 fish). All fish from the A-line were screened for transgenesis because they did not exhibit as much variability in size as the B-line. Of the total A-line progeny, 7.6% tested positive for the transgene (26 of 340 fish).

Growth of fish in the laboratory

The transgenic genotype significantly increased all metrics of fish size measured at each sampling point of the F1 fish (Figure 2). Transgenic individuals were 26% longer than non-transgenic fish at sampling point one (fish age: 132-162 days, $F_{1,258} = 134.3$, $p < 0.001$; Figure 2A), 36% longer (fish age: 260-262 days, $F_{1,125} = 235.3$, $p < 0.001$) and 161% heavier ($F_{1,125} = 212.3$, $p < 0.001$) at sampling point two (Figure 2B), and 35% longer (fish age: 366 days, $F_{1,105} = 173.4$, $p < 0.001$; Figure 2A) and 141% heavier ($F_{1,105} = 124.5$, $p < 0.001$) at sampling point three (Figure 2B). The differences between transgenic lines in size were similarly consistent. The increase in size caused by the transgene was greater in the B-line than in the A-line (interaction between genotype and line: length: sampling point one: $F_{1,258} = 43.4$, $p < 0.001$, sampling point two: $F_{1,125} = 39.8$, $p < 0.001$, sampling point three: $F_{1,105} = 16.0$, $p < 0.001$; mass: sampling point two: $F_{1,125} = 30.8$, $p < 0.001$, sampling point three: $F_{1,105} = 14.3$, $p < 0.001$). On average the difference between wild-type and transgenic B-line individuals was 48% longer and 241% heavier for transgenic individuals, and on average the difference between wild-type and transgenic A-line individuals was 17% longer and 68% heavier for transgenic individuals.

Discussion

We have produced GH-transgenic lines of stickleback and in doing so we have also created a diagnostic molecular genetic assay for characterization of GH-transgenic stickleback carrying the CMVGaGHmCh construct. The two GH-transgenic stickleback lines generated in this work were both consistently larger-bodied than their non-transgenic siblings of the same age. This growth effect manifested early in life and persisted into the adult stage consistent with salmonid GH-transgenic growth patterns including those in: Atlantic salmon (Du et al. 1992), Coho Salmon (Devlin et al 1994), Rainbow Trout (Devlin et al. 2001), and other GH transgenic lines (see Devlin et al. 2015). The observed variability in GH-transgene transmission efficiency to F1 and the variability in growth performance between lines is similar to that previously described for Coho Salmon (Devlin et al. 2004). In salmonids, GH-transgenesis has pleiotropic effects that extend beyond simple growth

metrics (Devlin et al. 2015). While we have thus far only presented evidence of accelerated growth, these GH-transgenic stickleback will be a useful model for testing cross-species generality of the pleiotropic effects of GH-transgenesis.

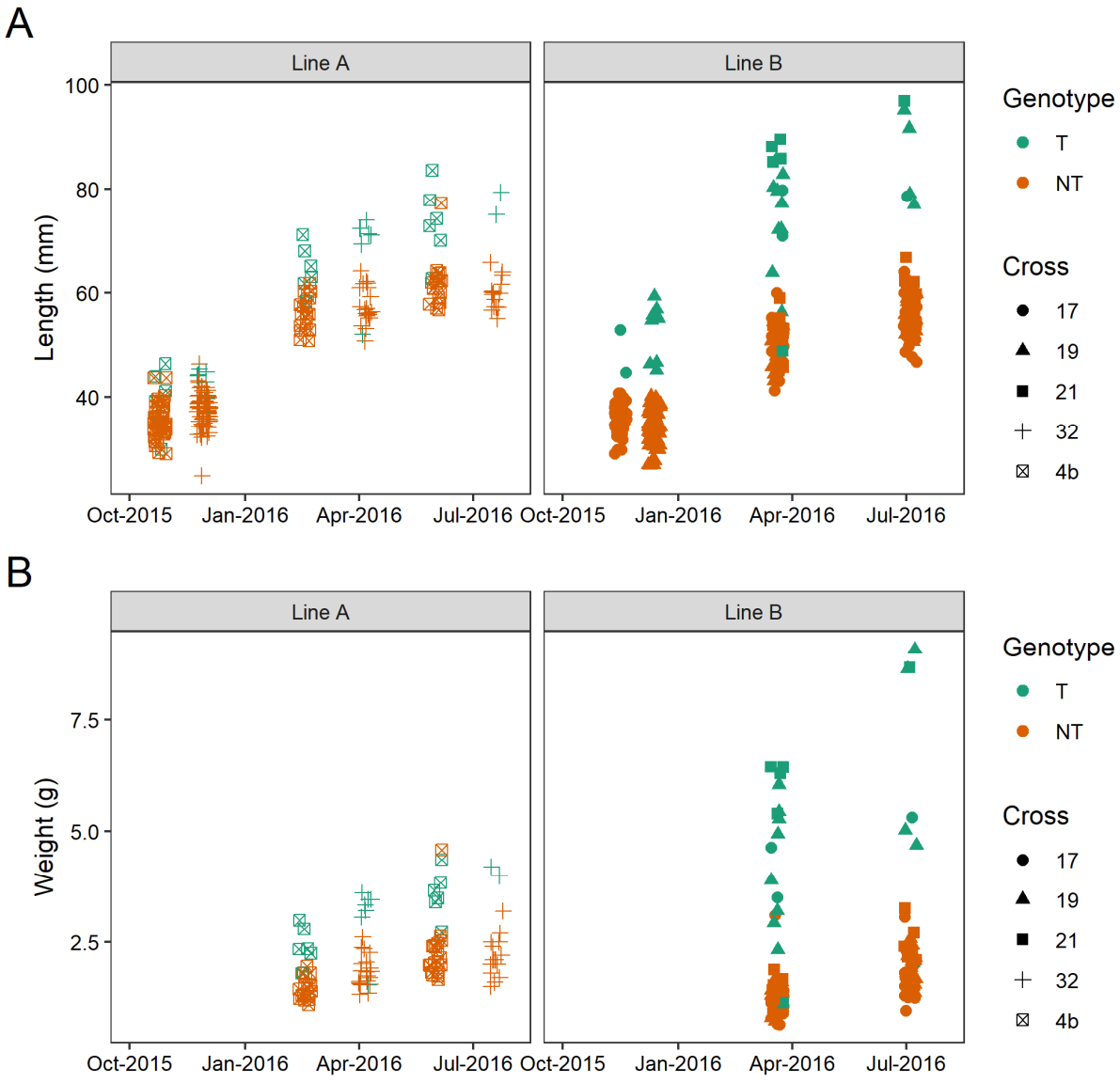

Figure 2: Growth of non-transgenic (NT) and transgenic (T) F1 stickleback of the A-line and the B-line at three sampling points during development in the laboratory. (A) = Length (mm); (B) = Weight (g).

A number of studies have previously demonstrated that the ecology of sticklebacks can be recreated in artificial ponds (Harmon et al., 2009; Rudman et al., 2019; Rudman & Schluter, 2016). This fact means that both experimental manipulations and the biocontainment necessary for experimenting on genetically-modified organisms could be achieved, while still adequately simulating the natural ecology of this species. The GH-transgenic

stickleback we have created thus represents a potentially useful model for testing hypotheses about the possible ecological consequences of GH-transgenic fish if they entered natural environments. While tropical fish species (e.g., *Danio rerio*, *Oryzias latipes*) are commonly used for transgenic models in biomedical research and are easily lab raised, their habitats and ecology are not reflective of the Canadian environment (Leggatt et al. 2018) and thus are less-suitable for future ecological risk research than a temperate species like Threespine Stickleback.

Conclusions

The present research has developed a new GH transgenic fish model to better understand transgenic effects across species and aid in the generation of risk assessment data to support regulatory processes. The use of stickleback, a small fish species with a one-year life cycle that responds strongly to GH transgenesis, can allow improved acquisition of specific data such as the nature of genotype-by-environment interactions and background genetic influences on transgenic phenotype. Perhaps more importantly (due to costs and time required to conduct risk assessment research) is determination of whether data from one transgenic species can be reliably used to evaluate a different species. Differences among species likely vary due to physiological and behavioral phenotypic differences, as well due to differences in experimental design, which ultimately generates uncertainty in risk assessment conclusions. Thus, this GH-transgenic stickleback model may best be used to enhance our identification and understanding of major factors and associated uncertainties affecting fitness and ecological consequences among GH transgenic species.

Acknowledgments

This research was funded by Fisheries and Oceans Canada and the Canadian Regulatory System for Biotechnology to RHD and KW, and by the Canada Foundation for Innovation, the University of British Columbia, and Discovery grants from the Natural Sciences and Engineering Research Council (NSERC) to DS. Assistance with fish husbandry by Keith Sorenson is much appreciated. We thank two anonymous reviewers for helpful comments that improved the quality of the manuscript.

References

Bell, M.A., Foster, S.A. 1994. Introduction to the evolutionary biology of the threespine stickleback. In M.A. Bell & S.A. Foster (Eds.), The evolutionary biology of the threespine stickleback. Oxford University Press. Oxford, UK. pp. 1-27.

Colosimo, P.F., Hosemann, K.E., Balabhadra, S., Villarreal Jr, G., Dickson, M., Grimwood, J., Shmutz, J., Myers, R.M, Schluter, D., Kingsley, D.M. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. Science, **307**(5717): 1928-1933. https://doi.org/10.1126/science.1107239

Devlin, R.H., Yesaki, T.Y., Biagi, C.A., Donaldson, E.M., Swanson, P., Chan, W.-K. 1994. Extraordinary salmon growth. Nature, **371**(6494): 209-210. https://doi.org/10.1038/371209a0

Devlin, R.H., Biagi, C.A., Yesaki, T.Y., Smailus, D.E., Byatt, J.C. 2001. Growth of domesticated transgenic fish. Nature, **409**(6822): 781-782. https://doi.org/10.1038/35057314

Devlin, R.H., Biagi, C.A., Yesaki, T.Y. 2004. Growth, viability and genetic characteristics of GH transgenic coho salmon strains. Aquaculture, **236**(1–4): 607-632. https://doi.org/10.1016/j.aquaculture.2004.02.026

Devlin, R.H., Sundström, L.F., Leggatt, R.A. 2015. Assessing ecological and evolutionary consequences of growth-accelerated genetically engineered fishes. Bioscience, **65**(7): 685-700. https://doi.org/10.1093/biosci/biv068

Du, S.J., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R., Hew, C.L. 1992. Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Nat. Biotechnol. **10**(2): 176-181. https://doi.org/10.1038/nbt0292-176

Dunham, R.A., Ramboux, A.C., Duncan, P.L., Hayat, M., Chen, T.T., Lin, C.M., Kight, K., Gonzalez-Villasenor, I., Powers, D.A. 1992. Transfer, expression, and inheritance of salmonid growth hormone genes in channel catfish, Ictalurus punctatus, and effects on performance traits. Mol. Mar. Biol. Biotechnol. **1**: 380-389.

Gibbons, T.C., Rudman, S.M., Schulte, P. M. 2016. Responses to simulated winter conditions differ between threespine stickleback ecotypes. Mol. Ecol. **25**(3): 764-775. https://doi.org/10.1111/mec.13507

Gibbons, T.C., Rudman, S.M., & Schulte, P.M. 2017. Low temperature and low salinity drive putatively adaptive growth differences in populations of threespine stickleback. Sci. Rep. **7**(1): 16766. https://doi.org/10.1038/s41598-017-16919-9

Harmon, L.J., Matthews, B., Des Roches, S., Chase, J.M., Shurin, J.B., Schluter, D. 2009. Evolutionary diversification in stickleback affects ecosystem functioning. Nature, **458**(7242), 1167-1170. https://doi.org/10.1038/nature07974

Kawakami, K. 2004. Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. Methods Cell Biol. **77**: 201-222. https://doi.org/10.1016/s0091-679x(04)77011-9

Leggatt R.A., Dhillon R.S., Mimeault C., Johnson N., Richards J.G., Devlin R.H. 2018. Low-temperature tolerances of tropical fish with potential transgenic applications in relation to winter water temperatures in Canada. Can. J. Zool. **96**(3): 253-260. https://doi.org/10.1139/cjz-2017-0043

Li L., Pitcher T.J., Devlin R.H. 2014. Potential risks of trophic impacts by escaped transgenic salmon in marine environments. Environ. Conserv. **42**(2): 152-161. https://doi.org/10.1017/S0376892914000319

Marchinko, K.B., Schluter, D. 2007. Parallel evolution by correlated response: Lateral plate reduction in threespine stickleback. Evolution, **61**(5): 1084-1090. https://doi.org/10.1111/j.1558-5646.2007.00103.x

Nam, Y.K., Noh, J.K., Cho, Y.S., Cho, H.J., Cho, K.-N., Kim, C.G., Kim, D.S. 2001. Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach Misgurnus mizolepis. Transgenic Res. **10**(4): 353-362. https://doi.org/10.1111/j.1749-7345.2001.tb00461.x

Rudman, S.M., Schluter, D. 2016. Ecological impacts of reverse speciation in threespine stickleback. Curr. Biol. **26**(4): 490-495. https://doi.org/10.1016/j.cub.2016.01.004

Rudman, S.M., Goos, J.M., Burant, J.B., Brix, K.V., Gibbons, T.C., Brauner, C.J., Jeyasingh, P.D. 2019. Ionome and elemental transport kinetics shaped by parallel evolution in threespine stickleback. Ecol. Lett. **22**(4): 645-653. https://doi.org/10.1111/ele.13225

Schluter, D. 1995. Adaptive radiation in sticklebacks: Trade-offs in feeding performance and growth. Ecology, **76**(1): 82-90. https://doi.org/10.2307/1940633

Schluter, D. 2009. Evidence for ecological speciation and its alternative. Science, **323**(5915): 737-741. https://doi.org/10.1126/science.1160006

Sundström, L.F., Lõhmus, M., Tymchuk, W.E., Devlin, R.H. 2007. Gene-environment interactions influence ecological consequences of transgenic animals. Proc. Natl. Acad. Sci. USA. **104**(10): 3889-3894. https://doi.org/10.1073/pnas.0608767104

Wootton, R.J. 1984. A functional biology of sticklebacks. University of California Press. Berkeley, CA.