Vergleichende Charakterisierung von Genen für eine differente Stresstoleranz bei der Regenbogenforelle (*Oncorhynchus mykiss*)

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Abkürzungsverzeichnis

ACTH	Adrenocorticotropin (engl. ***a**drenocortico**t**ropic **h**ormone*)
ALAS	**A**minol**ä**vulin**ats**ynthase
bio-FISCH M-V	**Bio**logisches **F**unktionales & **I**mmunologisches **S**creening zur **CH**arakterisierung regional selektierter Nutzfischarten in **M-V**
cDNA	komplementäre Desoxyribonukleinsäure (engl. ***c**omplementary **d**eoxyribo**n**ucleic **a**cid*)
DAMP	engl. ***d**amage **a**ssociated **m**olecular **p**attern*
DIREFO	**Di**fferent **re**sistente Regenbogen**fo**rellen
DNA	Desoxyribonukleinsäure (engl. ***d**eoxyribo**n**ucleic **a**cid*)
FLI	**F**riedrich-**L**öffler-**I**nstitut
gDNA	genomische DNA (engl. ***g**enomic **d**eoxyribo**n**ucleic **a**cid*)
GRASP	engl. ***G**enomics **R**esearch on Atlantic **S**almon **P**roject*
HPA-Achse	Hypothalamus-Hypophysen-Nebennierenrinden-Achse (engl. ***h**ypothalamic-**p**ituitary-adrenal **a**xis)*
HR	engl. ***h**igh **r**esponding*
HSP	**H**itze**s**chock**p**rotein
IPA	engl. ***I**ngenuity **P**athway **A**nalysis*
IPN	**I**nfektiöse **P**ankreasnekrose
KBE	**K**olonie-**b**ildende **E**inheiten
LR	engl. ***l**ow **r**esponding*
mRNA	Boten-Ribonukleinsäure engl. ***m**essenger **r**ibo**n**ucleic **a**cid*
NCBI	engl. ***N**ational **C**enter for **B**iotechnology **I**nformation*
PAMP	Pathogen-assoziierte molekulare Muster (engl. ***p**athogen-**a**ssociated **m**olecular **p**atterns)*
PSU	Bezugsgröße zur Angabe der Salinität engl. ***P**ractical **S**alinity **U**nits*
PRR	Rezeptoren zur Erkennung molekularer Muster

	(engl. ***p**athogen/**p**attern **r**ecognition **r**eceptors*)
qRT-PCR	quantitative Real-Time-Polymerase Kettenreaktion
	(engl. *quantitative **r**eal-**t**ime **p**olymerase **c**hain **r**eaction*)
RGT-Regel	**R**eaktions**g**eschwindigkeit-**T**emperatur-Regel (van-'t-Hoff'sche Regel)
subsp.	**Subsp**ezies/Unterart
SP	**S**tress**p**rotein
VHS	**v**irale **h**ämorrhagische **S**eptikämie
VHSV	**v**iraler **h**ämorrhagischer **S**eptikämie-**V**irus

1. Einleitung

1.1 Bedeutung der Fischzucht in der Aquakultur

Die Fischproduktion in der Aquakultur hat in den letzten zehn Jahren massiv an Bedeutung gewonnen. Die stark gestiegene Nachfrage nach qualitativ hochwertigem Fisch und Fischprodukten resultiert aus einer stetig wachsenden Weltbevölkerung und dem damit verbundenen gesteigerten Bedarf an Nahrungsmitteln sowie einer zunehmenden Popularität von qualitativ hochwertigem Fisch vor allem in Schwellenländern und den westlichen Industrieländern. Die Überfischung vieler natürlicher Bestände hat jedoch weltweit zu stagnierenden Mengen an Fangfisch verbunden mit einer langfristigen Verringerung der Biodiversität geführt. Um den zunehmenden Bedarf auch zukünftig decken zu können, ist eine nachhaltige Zucht von Speisefischen in regionalen Aquakulturen unumgänglich.

Derzeit stammen etwa 46 % der weltweit erzeugten Fischproduktion aus Aquakulturen (FAO 2010). Vor allem Süßwasserfische, Weichtiere, Krebstiere und diadrome[1] Wanderfische werden zur Zucht eingesetzt. In Deutschland dominieren vorzugsweise die zur Familie der *Cyprinidae* gehörenden Karpfen (*Cyprinus carpio*) und die der Familie der *Salmonidae* zuzuordnenden Regenbogenforellen (*Oncorhynchus mykiss*; Walbaum, 1792) den Besatz (BRAEMICK 2012). Im Jahr 2010 wurden in Deutschland in 96 % der mit Salmoniden besetzten Betriebe Forellen gezüchtet, was eine Ertragsmenge von ca. 22.000 t ausmacht (GALL *et al.* 2011). Forellen favorisieren vor allem unbelastetes, ganzjährig sauerstoffreiches und sommerkühles Wasser und werden daher hauptsächlich in Kaltwasseranlagen gehalten (BRAEMICK 2012). Obwohl die Verwendung standortangepasster Zuchtlinien eine etablierte Methode in der Aquakultur ist, wird in vielen Aquakulturbetrieben in Deutschland importierter Regenbogenforellenlaich verwendet. Er ist ganzjährig verfügbar und ein beständiger Forellenabsatz ist somit gewährleistet. Allerdings sind Importforellen nicht an regionale Umweltbedingungen angepasst. Insbesondere Umweltfaktoren wie schwankende Temperaturen und Wasserqualitäten sowie veränderte Keimspektren führen zu erhöhtem Stress und machen die Fische damit anfälliger für Infektionen, was zu hohen Verlustraten führen kann (MEYER 1991). Durch den Einsatz von Medikamenten und Vakzinen wird daher international versucht, Krankheiten entgegenzuwirken. In Deutschland ist aktuell nur ein Fischimpfstoff zugelassen (Bundesanzeiger-Veröffentlichung Nr. 369 vom 08.02.2012; Fundstelle BAnz AT 08.05.2012 B2). Er findet Anwendung gegen den Erreger der Rotmaulseuche *Yersinia ruckeri* bei Regenbogenforellen. Aufgrund der massiven

[1] Fische, die zum Laichen das Gewässer wechseln

Nebenwirkungen der meisten Fischarzneimittel für Fisch und Umwelt stellt der Medikamenteneinsatz in der Fischzucht keine Lösung dar. Der Vorteil regionaler, über Langzeitselektion erzeugter Zuchtlinien, liegt in ihrer genetisch determinierten und verbesserten Adaptation an die vorherrschenden Umweltbedingungen. Daraus resultieren stabile Wachstumsraten bei gleichzeitig geringen Verlusten, was sich in einer hohen Produktivität und Produktqualität widerspiegelt. Die Erzeugung und Verwendung robuster Zuchtlinien ist daher eine nachhaltige und zukunftsorientierte Lösung, um eine stabile Fischzucht in Aquakulturanlagen gewährleisten zu können.

1.2 Anforderungen an robuste Fischlinien in der Aquakultur

Die Zucht in der Aquakultur stellt besondere Herausforderungen an den verwendeten Fischbestand. In Becken- oder Teichanlagen wirkt eine Vielzahl biotischer und abiotischer Stressoren auf den Besatz. Neben Haltungsparametern wie Besatzdichte, Transport und dem allgemeinen Umgang mit den Fischen können vor allem die Parameter Wasserqualität, Temperatur und Infektionsdruck zu Stress führen und spielen daher für den Zuchterfolg eine entscheidende Rolle (OVERLI *et al.* 2005; PICKERING 1993).

Stress ist nach SELYE (1950) definiert als der veränderte Zustand eines Organismus, der durch die auf ihn einwirkenden Reize entsteht. Daraus resultieren Veränderungen der Körper-Homöostase. Um das physiologische Gleichgewicht wieder herzustellen, kommen verschiedene kompensatorische Mittel zum Einsatz (EVANS 2010; SELYE 1950). Stress ist demnach der Zustand einer bedrohten Homöostase, die als Reaktion auf den Stressor durch komplexe adaptive Prozesse aufrechterhalten wird (CHROUSOS 1998). Die Fähigkeit zur Ausbildung einer adäquaten Stressantwort ist somit essenziell für das Überleben von Organismen und zeigt dabei inter- und intraspezifische Variationen (HOCHACHKA *et al.* 2002). Es werden verschiedene Stufen der klassischen Stressantwort unterschieden, die in ihren grundlegenden Abläufen bei Fischen und höheren Vertebraten übereinstimmen (MAZEAUD *et al.* 1977; PICKERING 1981; PORTZ 2006; WEDEMEYER and MCLEAY 1981; WENDELAAR BONGA 1997). Im Allgemeinen kommt es zunächst zu einer kurzen Aktivphase. Auf diese folgen Veränderungen der körpereigenen Systeme, um die Stressantwort unter Kontrolle zu halten, und so die im belasteten Organismus beschleunigte Ausbreitung von Entzündungsherden zu verhindern.

Generell kann die Reaktion von Fischen auf Stressoren als das Zusammenspiel dreier Bereiche betrachtet werden. Die **(a)** primäre Stressantwort beinhaltet die schnelle

neuroendokrine Ausschüttung von Stresshormonen vor allem durch die Kopfniere der Fische. Dabei werden die cromaffinen Zellen des Nebennierenmarks über nervale Reizweiterleitung zur Sezernierung von Catecholaminen wie Adrenalin, Noradrenalin und Dopamin angeregt (RANDALL and PERRY 1992; REID *et al.* 1998). Zusätzlich vermittelt die HPA-Achse (im Mensch: Hypothalamus-Hypophysen-Nebennierenrinden-Achse) unter anderem über das Hypophysehormon Adrenocorticotropin (ACTH) die Abgabe des Cortikosteroids Cortisol in den Kreislauf (LEATHERLAND *et al.* 2010; OVERLI *et al.* 2007; WENDELAAR BONGA 1997). Cortisol seinerseits wirkt durch negative Rückkopplung auf die Sekretion des ACTHs. Die **(b)** sekundäre Stressantwort von Fischen beinhaltet eine Reihe biochemischer und physiologischer Veränderungen, die hauptsächlich als Folge der hormonellen primären Reaktion vermittelt werden (ALURU and VIJAYAN 2009; RANDALL and PERRY 1992; VIJAYAN *et al.* 1994a). Dazu gehören u. a. die Anpassung des Stoffwechsels, der Respiration, des Säure-Basen- und des Ionenhaushalts sowie der Immunantwort. Es kommt zu einer Veränderung der biochemischen und zellulären Blutzusammensetzung (u.a. Ionen-, Metabolitkonzentration) sowie daraus resultierenden Gewebsalterationen, wie z.B. dem Anschwellen und Verfärben der Milz. In Studien an dem Weißkehlmaulbrüter (*Oreochromis mossambicus*) sowie der Regenbogenforelle wurde nachgewiesen, dass die Stresshormone Adrenalin und Cortisol die Glukoseproduktion fördern, um den gesteigerten Energiebedarf zu decken (VIJAYAN *et al.* 1994b; VIJAYAN *et al.* 1997). In der Leber kommt es vermehrt zu Glykogenolyse und/oder Glukoneogenese (WENDELAAR BONGA 1997). Daher gilt die Erhöhung der Glukose-Konzentration im Plasma als metabolischer Indikator für Stress. Zusätzlich kommt es zur Ausschüttung von Stressproteinen (SPs), in Anlehnung an ihre ursprüngliche Entdeckung auch als Hitzeschock-Proteine (HSPs) bezeichnet. Sie wurden in allen bisher berücksichtigten Organismen nachgewiesen (FEDER and HOFMANN 1999; HOFMANN 2005). Sind Fische Stressoren langfristig ausgesetzt oder können sie die daraus resultierenden Anforderungen nicht (mehr) bewältigen, kann eine Verringerung des Wachstums, eine höhere Infektionsanfälligkeit oder auch eine reduzierte Reproduktionsfähigkeit die Folge sein, was im Allgemeinen als **(c)** tertiäre Stressantwort bezeichnet wird.

Auf zellulärer Ebene führt die Belastung durch einen Stressor üblicherweise zu Veränderungen oder Beschädigungen von Proteinen, DNA oder anderen wichtigen Makromolekülen. KÜLTZ *et al.*. (2005) definiert ein organismenübergreifendes relevantes Proteom, welches als Reaktion zum Einsatz kommt. Dieses umfasst unter anderem redoxsensible Proteine (z.B. die Aldosereduktase und die Glycerin-3-phosphat-

Dehydrogenase); Proteine, die Schäden wahrnehmen, reparieren und minimieren (molekulare Chaperone wie HSPs; DNA-Reparationsproteine wie MutS/L); sowie Proteine des allgemeinen Energiestoffwechsels (z.B. die Citrat-Synthase und die Enolase) (KÜLTZ 2005). Die zelluläre Stressantwort schätzt den Schaden ab, wirkt ihm entgegen und erhöht zumindest kurzfristig die Belastungstoleranz. Endgültig beschädigte Zellen werden durch aktiven Zelltod (Apoptose) beseitigt.

Insgesamt ist die Reaktion auf Stressoren allerdings differenzierter zu betrachten. Je nach Stressor erfolgt eine spezifische Reaktion, die ihrerseits hoch adaptiv ist. Eine Generalisierung ist daher nur für die Grundabläufe möglich. Neben den gut untersuchten Mechanismen der klassischen Stressantwort gibt es eine Vielzahl weiterer Parameter mit potenziellem Einfluss auf die adaptive Leistung des Organismus.

Voraussetzung für eine erfolgreiche Zucht in der Aquakultur ist dementsprechend die Minimierung der Umwelteinflüsse, die sich negativ auf die Produktivität auswirken. Die Anforderung an robuste Zuchtlinien besteht deshalb in einer erfolgreichen Adaption an Stressoren sowie eine schnelle, effektive Reaktion auf die auf den Fisch einwirkenden Stressoren. Dabei ist vor allem eine erfolgreiche Anpassung an Wasserqualität, Temperatur und Infektion entscheidend. Die Relevanz der einzelnen Parameter soll daher im Folgenden genauer erläutert werden.

1.3 Adaptation an Wasserqualität, Temperatur und Infektion

Für eine optimale Zucht in der Aquakultur ist die Qualität des Wassers von entscheidender Bedeutung. Eine schlechte oder suboptimale Wasserqualität ist ein starker Stressfaktor für die betroffenen Fische. Sowohl in extensiver[2] als auch in semiintensiver[3] und intensiver[4] Aquakultur wird zwischen offenen Kreislaufanlagen, die mit Durchflusswasser arbeiten und geschlossenen Anlagen mit in der Anlage zirkulierendem Wasser unterschieden. Viele Aquakulturanlagen stellen halboffene Systeme dar, in denen beide Wasserversorgungsvarianten eingesetzt werden. In offenen und halboffenen Anlagen kommt es zu direktem Nährstoff- und Sauerstoffeintrag durch die Umwelt sowie erheblichen saisonalen Schwankungen der Wassertemperatur. Zusätzlich wird die Wassersäule durch Wind in Bewegung gebracht. Anders als in geschlossenen Kreislaufanlagen verändern sich

[2] keine oder nur geringe Veränderung an gegebenen Umweltparametern; keine Futtermittel und Medikamente

[3] zusätzliche Gabe nährstoffreichen Futters

[4] umfassende Anpassung der gegebenen Umweltparameter; spezifisches Futter und Medikamentengabe

die Wasserparameter hier daher stetig, was eine Herausforderung an die Besatzfische darstellt. Fische stehen über Haut und Kiemen direkt mit dem Umgebungswasser in Verbindung. Ihr Gas- und Ionenaustausch findet vor allem über die Kiemenlamellen statt. Optimale Wasserbedingungen sind daher von essenzieller Bedeutung für ihre Homöostase.

Unter Wasserqualität wird im Allgemeinen die chemische, physikalische und biologische Beschaffenheit des Wassers verstanden. Sie wird bestimmt durch Verschmutzungsgrad, Temperatur, pH-Wert (H^+-Konzentration), Salzgehalt (Anzahl gelöster Ionen), Phosphat- ($PO4^{3-}$) und Stickstoffkonzentration (N), Wasserhärte (°dH) sowie Kohlendioxid- (CO_2) und Sauerstoffgehalt (O_2). Die verschiedenen Parameter sind dabei nicht losgelöst voneinander zu betrachten sondern stehen in direkter Wechselwirkung zueinander (PORTZ 2006). Die Ansprüche an die einzelnen Einflussgrößen variieren je nach Spezies, Entwicklungsphase und Erfahrungswert der Fische. Allgemeine artspezifische Richtwerte sind in diversen Studien angeführt (ADAMS 2002; PICKERING 1981; PORTZ 2006). Schlechte Wasserqualität führt zu einer Unterdrückung der primären Erregerabwehr und damit zu einer erhöhten Krankheitsanfälligkeit der Fische (MCLEAY 1975; WALTERS and PLUMB 1980).

Die Parameter der Wasserqualität dürfen nicht als statische, absolute Werte angesehen werden. Vielmehr sind z. B. die Gaslöslichkeit sowie die Aktivität der H^+-Ionen im Wasser und damit verbunden der pH-Wert temperaturabhängig. Auch die Korrelation von Temperatur und Sauerstoffgehalt ist ein kritischer Faktor. Die Sauerstoffsättigung im Wasser beträgt im Vergleich zu der in Luft nur ca. 0,04 % und sinkt zusätzlich mit steigenden Temperaturen und erhöhter Konzentration gelöster Stoffe im Wasser. Im Gegensatz dazu steigt der Sauerstoffbedarf der Fische bedingt durch die Korrelation der Temperatur mit der Stoffwechselrate (CLARKE and FRASER 2004; GILLOOLY *et al.* 2001). Der Stoffwechsel lebender Organismen folgt thermodynamischen Gesetzmäßigkeiten. Der RGT/Q_{10}-Regel folgend nimmt die Reaktionsgeschwindigkeit chemischer Reaktionen bei einem Temperaturanstieg bzw. -abfall von 283 K (entspricht 10 °C) um das Zwei- bis Vierfache zu bzw. ab. Die Temperatur beeinflusst die Struktur und Aktivität von Enzymen und anderen Proteinen nachhaltig (JAENICKE 1991; LONG *et al.* 2012; SOMERO 1995). Ihr Einfluss auf die Evolution von Proteinen wurde anhand einer Vielzahl vergleichender Studien der interspezifischen Enzymhomologien unterschiedlich temperaturadaptierter Arten nachgewiesen. Die ausreichende O_2-Aufnahme über Diffusion in den Kiemenlamellen von Fischen wird somit durch den steigenden Bedarf und den aber gleichzeitig nur geringen Vorrat an gelöstem Sauerstoff im Wasser zusätzlich erschwert.

Fische sind poikilotherme Organismen, ihre Körpertemperatur korelliert mit den Temperaturschwankungen des Umgebungswassers. Wie auch die Anforderungen an die Wasserqualität können die thermalen Ansprüche von Art zu Art teilweise erheblich variieren, wobei sich artspezifische Toleranzkurven mit letaler Ober- und Untergrenze erstellen lassen. Ihre Spannbreite ist dabei artspezifisch und steht in Korrelation mit der Akklimatisierungstemperatur. So toleriert zum Beispiel die ostafrikanische Barsch-Gattung *Tropheus* nur einen Schwankungsbereich von vier Grad (24 °C bis 27 °C), während viele *Teleostei* (echte Knochenfische) in Temperaturbereichen von 0 °C bis 30 °C leben können. Der optimale Temperaturbereich kann je nach individueller Anpassung, Entwicklungsstatus und Gesundheit der Fische variieren (FRY 1971; LONG *et al.* 2012; PICKERING 1981). Das Temperaturoptimum von Regenbogenforellen in der Aquakultur liegt nach Studien von SEDGWICK (1985) und YAMAZAKI (1991) zwischen 15 °C und 18 °C, wobei Wachstum zwischen 10 °C und 20 °C möglich ist und erst Temperaturen von unter 0 °C und über 25 °C letal wirken (SEDGWICK 1985; YAMAZAKI 1991). Die individuelle Geschwindigkeit, mit der eine Anpassung an die jeweilige Akklimationstemperatur erfolgen kann, ist ebenfalls artspezifisch und variiert zwischen 1 °C/Tag bei Goldfischen (*Carassius auratus*) sowie Plötzen (*Rutilus rutilus*) und nur 1 °C/Stunde bei der Barsch-Gattung *Girella* sowie diversen Salmoniden (Lachsfische) (FRY 1971).

Da es gerade in Teichanlagen und offenen Kreislaufanlagen zu erheblichen saisonalen Temperaturschwankungen kommen kann, sind die Anforderungen an den Toleranzbereich des Fischbesatzes hoch. Liegen die Temperaturen für einen Besatzfisch in der Nähe seines Toleranzbereiches, beziehungsweise weichen sie von seinem Optimum ab, so kann es zu Störungen des Ionenhaushalts und damit des osmotischen Gleichgewichts, des Stoffwechsels, der Nahrungsaufnahme, des Wachstums sowie der Fortpflanzungsfähigkeit und des Verhaltens kommen. Eine robuste Fischzuchtlinie zeichnet sich daher durch eine möglichst schnelle und effektive Anpassung der beeinträchtigten Funktionen aus, wobei die Wachstumsleistung möglichst konstant bleiben sollte.

Zusätzlich zu Wasserqualität und Temperatureinfluss stellen Infektionen eine große Herausforderung an den Fischbestand in der Aquakultur dar. Jährlich kommt es zu hohen Verlustraten durch bakterielle, virale oder parasitäre Infektionskrankheiten (FAO 2010; MEYER 1991). Salmoniden sind vor allem durch die bakteriell bedingte Rotmaulseuche (ausgelöst durch *Yersinia ruckeri*), die Furunkulose (ausgelöst durch *Aeromonas salmonicida*)

oder auch die virale hämorrhagische Septikämie (VHS, ausgelöst durch den Rhabdovirus VHSV) betroffen (O'BRIEN *et al.* 1994; ROSS *et al.* 1966; SKALL *et al.* 2005). Fischkrankheiten sind dabei nur selten monokausale Ereignisse, sondern entstehen meist aus dem Zusammenspiel von Pathogen, Fisch und Umwelt. Viele Pathogene sind opportunistisch, also erst bei physiologischer Beeinträchtigung des Fisches infektiös. Sie kommen im Wasser in hohen Konzentrationen vor, ohne Krankheiten auszulösen. Daher ist eine gute Balance der relevanten Einzelfaktoren der Wasserqualität wichtig für die Gesundheit und das Wachstum der Fische. Wird sie hingegen gestört, kommt es zu einem gehäuften Auftreten von stressassoziierten Infektionskrankheiten, verbunden mit vermindertem oder eingestelltem Wachstum (WEDEMEYER and MCLEAY 1981).

Das Immunsystem der Fische entspricht bis auf einige Abweichungen in allen grundlegenden Bestandteilen dem des Menschen (MAGNADOTTIR 2006; TORT *et al.* 2003). Auch bei Fischen wird zwischen dem schnellen, aber unspezifischen, angeborenen und dem zeitlich verzögerten, aber spezifischen, adaptiven Immunsystem mit humoraler und zellulärer Antwort unterschieden. Beide Immunreaktionen sind funktional miteinander verknüpft. Der sezernierte Schleim der Fischhaut mit antibakteriellen Molekülen wie Lysozymen und das Komplementsystem bilden die primäre Abwehr gegen Pathogene.

Erreger weisen auf ihrer Oberfläche spezifische Strukturen auf, sogenannte Pathogen-assoziierte molekulare Muster (PAMPs), die von speziellen Rezeptormolekülen des angeborenen Immunsystems, den PRRs (*pathogen/pattern recognition receptors*), erkannt werden (ZOU *et al.* 2010). Diese werden aufgrund ihrer Struktur in verschiedene Familien eingeteilt, dazu gehören u.a. TLRs (*Toll-like receptors*), C-Typ-Lektin-Rezeptoren oder auch NRLs (*NOD-like receptors*). Orthologe Mitglieder der PRR-Familien wurden auch in echten Knochenfischen nachgewiesen (AOKI *et al.* 2008; MAGNADOTTIR 2006; REBL *et al.* 2008). Erkennen PRRs ein Pathogen, so lösen sie über intrazelluläre Kaskaden die Initiierung früher Immunmechanismen aus (LEE and KIM 2007). Auch Makrophagen induzieren die Immunreaktion. Sie erkennen die Erreger durch ihre körperfremden Strukturen, nehmen sie auf und präsentieren sie anschließend an ihrer Oberfläche. B- und T-Lymphozyten des adaptiven Immunsystems erkennen die so präsentierten Antigene und leiten die aktive adaptive Immunantwort in Form der Antikörperproduktion ein (MEDZHITOV 2007).

Sowohl Stress als auch kritische Temperaturen haben einen Einfluss auf die Funktionalität des Immunsystems. Teile der humoralen als auch zellulären Immunantwort sind, im Gegensatz zur primären Antwort, temperaturabhängig (BLY and CLEM 1992; KUMARI *et al.* 2006; LANGSTON *et al.* 2002). Dazu gehört die T- und B-Zellen-Interaktion sowie die

Antikörperproduktion und -freisetzung. Die optimale Temperatur liegt dabei nahe der gewohnten Umgebungstemperatur. Bei Stress ausgeschüttete Hormone wirken im Fisch immunsupressiv. Das zentrale Nervensystem sowie Rückkopplungsschleifen des Stresshormons Cortisol führen zu einer Dämpfung der Immunreaktionen. Die Aktivität des Immunsystems wird durch das autonome Nervensystem kontrolliert und gebremst.

1.4 Untersuchungen zur intra- und interspezifischen Adaptation

In der Literatur finden sich diverse vergleichende Studien zur intra- und interspezifischen adaptiven Stressantwort bei Fischen (Tab. 1). So konnten u.a. genetisch determinierte Variationen in der Infektionsresistenz innerhalb und zwischen Fischbeständen nachgewiesen werden (CHEVASSUS and DORSON 1990; CIPRIANO *et al.* 2002; FJALESTAD *et al.* 1993).

Tab. 1: Ausgewählte vergleichende Untersuchungen zur intra- und interspezifischen adaptiven Stressantwort bei Fischen

Untersuchter Parameter	**Fischart bzw. -familie**	**Referenz**
intraspezifische Studien:		
Expression von HSP70, AGT/SERPINA8, ATP1A, ALAS in Populationen entlang eines Salinitätsgradienten	Flunder (*Platichthys flesus*)	LARSEN *et al.* (2008)
Resistenz nach Infektion mit IPN (Infektiöse Pankreasnekrose)	Regenbogenforelle (*O.mykiss*)	OKAMOTO *et al.* (1993)
Expression von HSP70 und ATP1A in zwei Populationen nach Salinitätsstress	Kabeljau (*Gadus morhua*)	LARSEN *et al.* (2011)
Microarray-Studie zur Expression von Stoffwechselgenen in Populationen entlang eines Temperaturgradienten	Killifisch (*Fundulus heteroclitus*)	WHITEHEAD and CRAWFORD (2006)
Vergleich des Cortisol- und Cortison-Levels nach Haltungsstress	Regenbogenforelle (*O.mykiss*)	POTTINGER and MORAN (1993)
interspezifische Studien:		
Resistenz nach Infektion mit Furunkulose	verschiedene *Salmonidae*	CIPRIANO *et al.* (1994); HOLTEN-ANDERSEN *et al.* (2012)
Vergleich des Cortisol-, Glukose-, Laktate- und Aminosäure-Niveaus im Plasma nach Haltungsstress	3 *Salmonidae*- und 3 *Cyprinidae*-Arten	POTTINGER (2010)

Eine der bestuntersuchten Beispiele für Adaptation an differente Umweltbedingungen ist der Killifisch (*Fundulus heteroclitus*) an der Ostküste Nordamerikas. Populationen, die Habitate mit einem Temperaturunterschied von 15 °C besiedeln, zeigen deutliche intraspezifische Unterschiede in der Expression des Gens *LDH* (Lactatdehydrogenase)

(SEGAL and CRAWFORD 1994) sowie weiterer Gene, die für essenzielle Proteine wie HSP70 und die Na/K-ATPase kodieren (FANGUE *et al.* 2006; SCOTT *et al.* 2004). Das Hauptaugenmerk bisheriger Studien zur adaptiven Stressantwort liegt in der Analyse der klassischen hormonellen Antwort durch Adrenalin und Cortisol, in Untersuchungen des Glukose- oder Laktat-Levels im Plasma sowie in Arbeiten an klassischen Stressproteinen, wie z.B. den HSPs.

POTTINGER (2010) vergleicht das Niveau von Cortisol, Glukose, Laktat und Aminosäuren im Plasma von je drei Arten der Cypriniden (Karpfenartige) und der Salmoniden (Lachsartige) unter Einfluss verschiedener Stressoren. Er konnte sowohl innerhalb der Familien als auch zwischen den Familien signifikante Unterschiede nachweisen, die auf artspezifische Unterschiede in den Schlüsselelementen der Stressantwort von Fischen hinweisen (POTTINGER 2010). Verschiedene vergleichende Studien zwischen Forellen, die je nach Höhe ihres Cortisol-Niveaus unter Stresseinwirkung in zwei Gruppen eingeteilt wurden (HR: *high responding*; LR: *low responding*), zeigten entscheidende Unterschiede in weiteren Bereichen der Stressantwort und Stressresistenz (OVERLI *et al.* 2005; OVERLI *et al.* 2007; PEMMASANI *et al.* 2011). Vergleichende Untersuchungen des Cortisol- und HSP70-Niveaus in Leber und Kiemen der Regenbogenforelle und der Tilapia Art *Oreochromis mossambicus* unter Temperaturstress lassen zusätzlich eine funktionelle Korrelation der hormonellen und zellulären Stressantwort vermuten (BASU *et al.* 2001). Viele Studien befassen sich näher mit der unterschiedlichen Stresstoleranz nah verwandter Arten. So haben z. B. NAKANO *et al.*. (2002) den Unterschied in der Toleranz zweier Groppen-Arten (*Cottidae*) auf ein unterschiedliches Niveau des HSP70 zurückgeführt (NAKANO and IWAMA 2002).

Der Schwerpunkt bisheriger Studien zur differenten Reaktion von Fischlinien auf Stressoren richtet sich auf die hormonellen und intrazellulären Schlüsselmoleküle der adaptiven Stressantwort. Die vorliegende Arbeit befasst sich hingegen mit der Identifikation und Untersuchung weiterer, an der adaptiven Stressantwort der Regenbogenforelle beteiligter Gene. Grundlage hierfür sind vergleichende initiale Studien an zwei gegenüber dem Erreger der Furunkulose, *Aeromonas salmonicidae*, different resistenten Zuchtlinien.

1.5 Die robuste Zuchtlinie BORN

Die Regenbogenforellenlinie BORN wurde im Verlauf einer 37-jährigen Selektion am Fischereiinstitut Born (Abb. 1) aus importierten *Steelhead*-Forellen (anadrome Wanderform) gezüchtet (ANDERS E. 1986) und ist an die lokalen Umweltbedingungen des Boddenwassers

der südwestlichen Ostsee angepasst. Diese umfassen saisonale Fluktuationen verschiedener biotischer und abiotischer Umwelteffekte wie des natürlichen Keimspektrums, des Eutrophierungsgrads, der Salinität [Brackwasser: 2.5 bis 6 *Practical Salinity Units* (PSU)] und stark schwankender Temperaturen von −0.3 °C bis 28 °C.

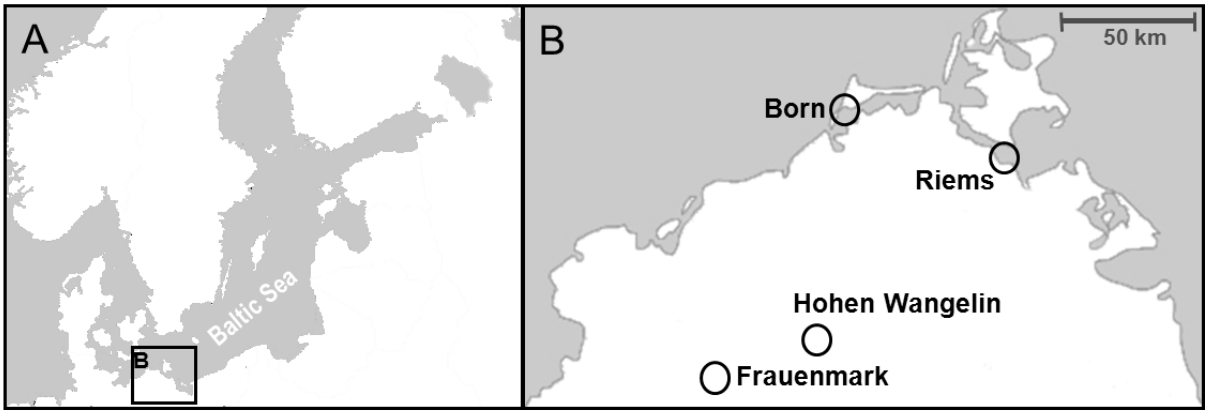

Abb. 1: Herkunft der BORN-Forelle

(A) Geographische Einordnung des Untersuchungsgebiets im Ostseeraum.

(B) Position der Aquakulturanlage, in der die regionale Linie BORN aus selektiver Züchtung entstanden ist (Anlage Born) sowie weitere Anlagen, in denen sowohl die Linie BORN als auch Importforellen gezüchtet (Anlage Frauenmark, Hohen Wangelin) bzw. zu Versuchszwecken gehalten werden (Insel Riems).

Die lokale Forellenlinie BORN erfüllt die in den Abschnitten 1.2 und 1.3 der Arbeit dargestellten Anforderungen an eine robuste Zuchtlinie. Zuchtbegleitende Studien zeigten unter anderem ihre erhöhte Resistenz gegenüber Pathogenen. So überleben im Vergleich zu handelsüblichen Importforellen mehr als dreieinhalb Mal so viele BORN-Forellen eine intraperitoneal injizierte Dosis mit 1 x 10^4 koloniebildenden Einheiten (KBE) des Furunkulose-Erregers *A. salmonicida* (Abb. 2A). Rund 20 % der regionalen Zuchtlinie überstehen weiterhin eine für importierte Forellen zu 100 % letale Dosis von 5 x 10^5 KBE (Köllner, FLI Riems, Deutschland). Eine mögliche Erklärung dieser Beobachtungen liefern zusätzliche vergleichende Untersuchungen, die ein schneller reagierendes adaptives Immunsystem der BORN-Forelle belegen (Köllner, FLI Riems, Deutschland).

Zusätzlich zu der erhöhten Erregerresistenz der BORN-Forelle konnte auch eine im Vergleich zu Importforellen stabile Wachstumsrate unter starken jährlichen Temperaturschwankungen nachgewiesen werden (Anders, LFA Born, Deutschland) (Abb. 2B). Obwohl diese ökonomisch bedeutsamen phänotypischen Ausprägungen der gesteigerten Robustheit der BORN-Forelle bekannt sind, gab es zu Beginn der vorliegenden Arbeit nur initiale vergleichende molekularbiologische Untersuchungen, die sich mit der

Aufklärung der dem Merkmal Robustheit zu Grunde liegenden genetischen Ursachen befassen (REBL *et al.* 2009a; REBL *et al.* 2009b; REBL *et al.* 2010).

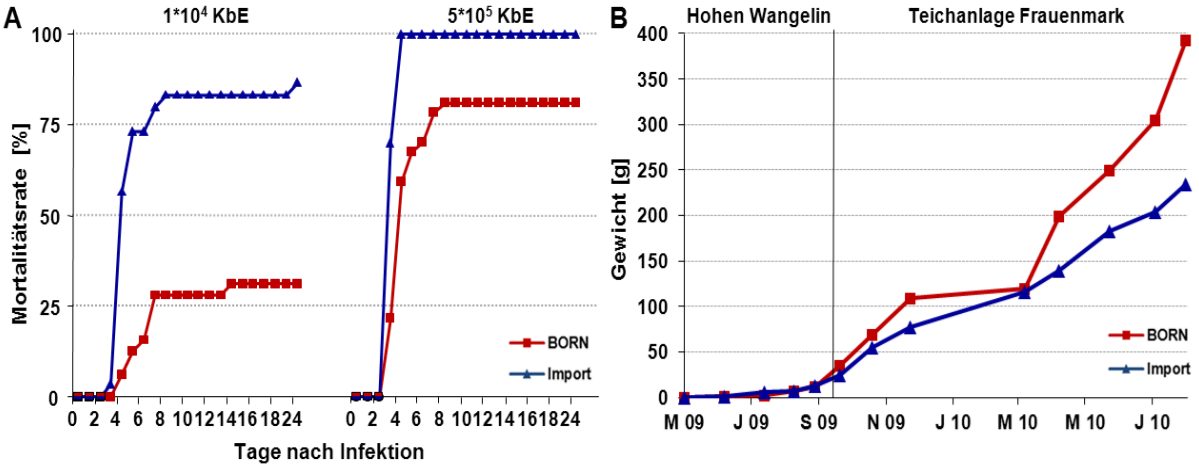

Abb. 2: Vergleichende Untersuchungen zur Sterberate nach Infektion und jahresspezifischen Gewichtszunahme von BORN-Forellen (rot) und handelsüblichen Importforellen (blau)

(A) Prozentuale Sterberate der BORN- und Import-Forellen nach *A. salmonicidae*-Infektion (je Gruppe n = 30). Dargestellt sind die Sterberaten nach einer Infektion mit $1x10^4$ und $5x10^5$ KbE (Kolonien bildende Einheit) des Erregers.

(B) Durchschnittliches Gewicht von BORN- und Import-Forellen im zeitlichen Verlauf (Mai 2009 bis Juli 2010) in den Aquakulturanlagen Hohen Wangelin und Frauenmark (je Messpunkt und Gruppe n = 50).

1.6 Hypothesen und Zielstellung

Die vorliegende Arbeit ist im Rahmen des vom Land Mecklenburg-Vorpommern geförderten DIREFO-Projektes (**Di**fferent **re**sistente Regenbogen**fo**rellen; 2009 - 2011, Projektnummer AU 08 026) entstanden und befasst sich mit der erhöhten Robustheit der lokalen Zuchtlinie BORN im Vergleich zu Importforellen[5]. Gegenstand der Untersuchung ist die Aufklärung der zu Grunde liegenden molekulargenetischen Mechanismen der differenten Stresstoleranz beider Linien. Dabei lautet die übergeordnete Hypothese:

Die erhöhte Robustheit der BORN-Forelle ist genetisch manifestiert.

Bisherige Studien zur Stressantwort im Fisch sind vor allem auf die bereits in Modellorganismen nachgewiesenen klassischen Parameter der humanen Reaktion beschränkt (vergleiche Tab. 1). Die Betrachtung weiterer Faktoren bietet daher die Möglichkeit, zusätzliche grundlegende Erkenntnisse zu erlangen. Da das Genom der Regenbogenforelle

[5] Importierte Forellenlinie Steelhead II-WA (Tacoma, USA)

noch nicht komplett entschlüsselt vorliegt, sind nur vergleichsweise wenige Sequenzen in den Datenbanken verfügbar. Funktionale Betrachtungen von Genen der Forelle erfordern deshalb zunächst deren strukturelle Analyse. Die Hauptziele der vorliegenden Arbeit lauten daher:

- **(i)** Gene und Stoffwechselwege zu identifizieren, die an einer alternativen Stressantwort der BORN-Forelle beteiligt sind;
- **(ii)** eine umfassende molekulargenetische Charakterisierung dieser Kandidatengene in der Forelle durchzuführen;
- **(iii)** den Einfluss der Kandidatengene auf die im Vergleich zu Importforellen erhöhte Robustheit der BORN-Forelle genauer zu bestimmen.

Neben molekulargenetischen Methoden zur Gencharakterisierung wurde anhand von Infektions- und Temperaturexperimenten der Einfluss von äußeren Stressoren auf die Expression der Kandidatengene in BORN- und Import-Forellen untersucht. Begleitende holistische Transkriptomanalysen auf der Basis von für Salmoniden spezifischen Microarrays wurden verwendet, um zusätzliche funktionale Daten zur differenten Expression beider Zuchtlinien zu erlangen (Abb. 3).

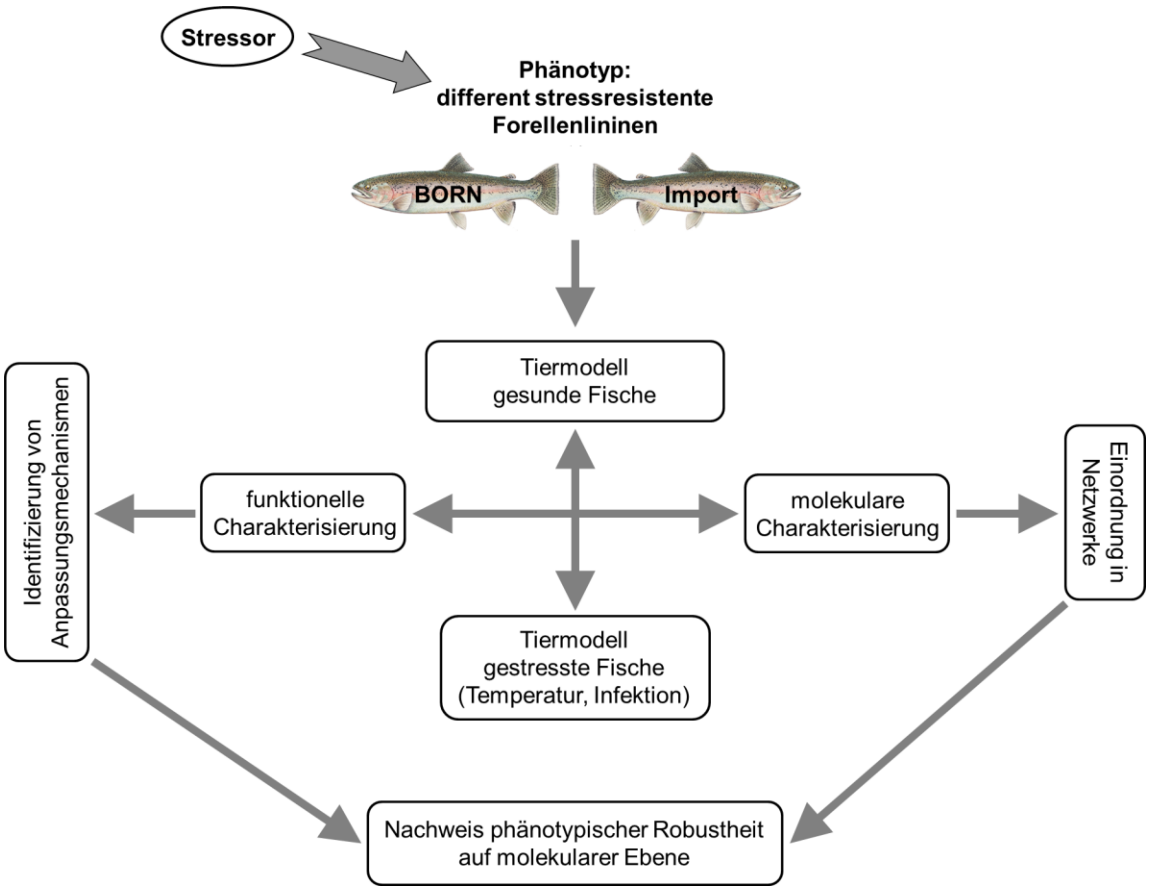

Abb. 3: Allgemeine schematische Darstellung der Arbeitszielstellungen

Vergleichende Microarray-Analysen bieten die Möglichkeit, eine differente Genexpression zwischen zwei Gruppen zu quantifizieren und so grundlegende Erkenntnisse über die unterschiedliche Regulation elementarer Abläufe in der Zelle zu erlangen. Weiterhin bietet sich die Möglichkeit, Gene zu identifizieren, die bisher nicht mit den betrachteten Zellprozessen in Verbindung gebracht wurden. Der Einsatz von Transkriptomanalysen bildet daher einen wichtigen Teil der Untersuchungen an der lokalen Zuchtlinie BORN im Vergleich zu Importforellen.

Ziel der vorliegenden Arbeit ist die Identifikation und Analyse von Genen, die zusätzlich zu klassischen Stressgenen an der adaptiven Stressantwort der BORN-Forelle beteiligt sind. Die Kandidatengene dieser Arbeit wurden aus einem grundlegenden Leber-Transkriptomvergleich zwischen gesunden BORN- und Importforellen ausgewählt. Aufgrund ihrer Funktion und differenten Expression zwischen beiden Forellenlinien ist ihr Einfluss auf die erhöhte Robustheit der BORN-Forellen naheliegend. Zusätzlich erfolgte die Vorauswahl von Genen für die Untersuchungen durch die Analyse themenrelevanter Literatur.
Die im Rahmen der Promotionsarbeit vorgelegten Studien befassen sich mit verschiedenen Teilbereichen der Untersuchungen zur adaptiven Stressantwort:

Eine schnelle und effektive Antwort des erworbenen Immunsystems der Fische ist von essenzieller Bedeutung für eine erfolgreiche Zucht in der Aquakultur und konnte für die BORN-Forelle bereits über die Aktivierung des MHC-II-Komplexes (*major histocompartibility complexes class II*) nachgewiesen werden (Köllner, FLI Riems, Deutschland). Die vorgeschaltete primäre Abwehr stellt jedoch die erste Barriere bei einer Infektion dar. Unterschiede in der Expression von Genen, die an der angeborenen und der erworbenen Immunantwort beteiligt sind, könnten daher auf eine differente Effektivität der Erregerabwehr der BORN- und Importforellen hindeuten (**Studie I, V**).

Die Aufrechterhaltung der Körperhomöostase ist ein entscheidender Faktor der adaptiven Stressabwehr. Eine Vielzahl an Mechanismen und Genen kommt dabei zum Tragen. Einige wichtige Bereiche wurden im Rahmen dieser Arbeit näher untersucht.

Ein Teilaspekt ist der effektive Schutz vor giftigen, die Zellteilung und das Zellwachstum beeinflussenden Substanzen. **Studie II** charakterisiert das Gen *PRR13* (*proline rich 13*), das einen erhöhten Schutz vor Zytostatika vermittelt und daher wichtig für die Resistenz der BORN-Forelle gegenüber natürlicherweise im eutrophen Wasser der Darß-Zingster Boddenkette vorkommenden Schadstoffe sein könnte.

Calcium spielt eine wichtige Rolle in der Signaltransduktion und der Aufrechterhaltung des Ionengleichgewichts der Zelle. Eine differente Aktivität Calcium-bindender Proteine unter Temperatur- oder auch Infektionsstress könnte daher durch die Regulation des Ca^{2+}-Haushalts der Zelle einen entscheidenden Faktor für die Adaptationsvorteile der BORN-Forelle darstellen (**Studien III+VI**).

Eisen-Schwefel-Cluster sind Bestandteile essenzieller Proteine des Metabolismus und des Ionentransports. Das [Fe-S]-Cluster-Gerüstprotein ISCU bildet und vermittelt die [Fe-S]-Cluster und stellt somit eine Nahtstelle für die Aufrechterhaltung entscheidender Stoffwechselwege dar. Sein möglicher Einfluss auf eine differente Regulation entscheidender Körperfunktionen der BORN-Forelle ist Bestandteil der **Studie IV.**

Der aktive Zelltod (Apoptose) wird durch Zellen des Immunsystems oder durch zelleigene Signale ausgelöst. Er bietet die Möglichkeit, beschädigte oder infizierte Zellen aktiv abzutöten und so ein Übergreifen auf umliegende Zellen zu verhindern. Ein Transkriptionsvergleich zwischen Schlüsselmolekülen des Apoptose-Signalwegs beider Forellenlinien sollte klären, ob die BORN-Forelle, anders als Importforellen, beschädigte oder infizierte Zellen durch Apoptose aktiv beseitigt und dadurch besser auf stressbedingte Schäden reagiert (**Studie VII).**

2. Einzelstudien

Studie I.

Identification of differentially expressed protective genes in liver of two rainbow trout strains.

Rebl, A., Verleih, M., Korytář, T., Kühn, C., Wimmers, K., Köllner, B., Goldammer, T.
Vet. Immunol. Immunopathol. (2012), **145**(1-2): 305-15.

kurze Zusammenfassung:
Diese Studie befasst mit sich mit der Analyse von Genen der primären Erregerabwehr, die in einem Leber-Transkriptomvergleich zwischen der lokalen Forellen-Zuchtlinie BORN und kommerziell in der Aquakultur eingesetzten Importforellen different reguliert waren. Unter anderem wurde die Expression der Gene bei 8 und 23°C und nach Pathogen-Infektion analysiert sowie das in der Forelle duplizierte Gen SERPINF2 erstmals in einem Salmoniden charakterisiert. Die Ergebnisse der Studie lassen den Schluss zu, dass die untersuchten Gene zu einer verbesserten ersten Barriere bei der Erregerabwehr von BORN-Forellen beitragen.

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Research paper

Identification of differentially expressed protective genes in liver of two rainbow trout strains

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ABSTRACT

Since 1975, the rainbow trout strain BORN (Germany) has been bred in brackish water from a coastal form imported from Denmark. Accompanying phenotypic monitoring of the adapted BORN trout until now revealed that this selection strain manifested a generally elevated resistance towards high stress and pathogenic challenge including lower susceptibility towards *Aeromonas salmonicida* infections in comparison to other trout strains in local aqua farms. We focus on the elucidation of both, genetic background and immunological basis for the increased survivorship to infections. A first comparison of gene expression profiles in liver tissue of healthy rainbow trout from the local selection strain BORN and imported trout using a GRASP 16K cDNA microarray revealed six differentially expressed genes evoking pathogen and wounding responses, LEAP2A (encoding for liver-expressed antimicrobial peptide), SERPINA1 (alpha-1 antitrypsin), FTH1 (middle subunit of ferritin), FGL2 (fibroleukin), CLEC4E (macrophage-inducible C-type lectin), and SERPINF2 (alpha-2 antiplasmin). Since the latter gene is not described in salmonid species so far, our first aim was to characterize the respective sequence in rainbow trout. Two trout SERPINF2 genes were identified, which share only 48% identical amino acid residues and a characteristic SERPIN domain. Second, we aimed to analyse the expression of those genes after temperature challenge (8 °C and 23 °C). Only FTH1 was upregulated in BORN and import trout after increase of temperature, while SERPINA1 and FGL2 were only elevated in import trout. Third, the expression of all named genes was analyzed after pathogen challenge with *A. salmonicida* subsp. *salmonicida*. As a main finding, we detected a comparably faster regeneration of LEAP2A mRNA abundance in BORN trout following bacterial infection. Ingenuity Pathways Analysis suggested a functional interplay among the mentioned factors and the pro-inflammatory cytokine TNF, whose stronger expression was validated in liver of BORN trout. This data indicate that the examined genes contribute to an improved first barrier against invading pathogens in BORN trout.

Abbreviations: aa, amino acid; ACTB, β-actin; CLEC4E, C-type lectin family 4, member E; EST, expressed sequence tag; FGL2, fibrinogen-like protein 2; FTH1, ferritin heavy polypeptide 1; IL6, interleukin 6; IPA, Ingenuity Pathways Analysis; LEAP2A, liver-expressed antimicrobial peptide 2A; ORF, open reading frame; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; SERPINA1, serpin peptidase inhibitor, clade A, member 1; SERPINF2, serpin peptidase inhibitor, clade F, member 2; TNF, tumor necrosis factor.

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1. Introduction

Annual aquaculture production has more than tripled worldwide within the past 15 years (Sapkota et al., 2008) and its importance is increasing with the stagnating trend in capture fisheries and simultaneously rising fish consumption for an expanding human population. A key problem faced by fish farmers is the emergence and spread of serious aquatic pathogens, which rises, if water is reaching optimal pathogen growth temperatures. The Gram-negative bacterium *Aeromonas salmonicida* causes bacterial septicaemia in salmonids, called furunculosis (Reith et al., 2008). This severe systemic disease is responsible for considerable losses to the aquaculture industry yearly. A breeding program for rainbow trout (*Oncorhynchus mykiss*, Walbaum) is running since 36 years at the Fisheries Institute in Born, Germany (Anders, 1986), which is focused at provision of a robust line for local rainbow trout farming. Trout breeding occurs in brackish costal water of the southwestern Baltic Sea where fish are exposed to several seasonally changing biotic and abiotic stressors, such as natural pathogen challenge, heavy eutrophication, 2.5–6 practical salinity units (PSU), water temperatures from −0.3 °C to 28 °C, and varying oxygen content. By continuous selection for the trait survival rate for numerous generations, the breeding line BORN has been established, which is known to show a lower susceptibility towards pathogens among several other adaptation advantages (Anders, 1986). The genetic and immunological phenomena for these facts are underexplored. Aiming to evaluate and establish an animal model for studying mechanisms of stress regulation and immune defence in farmed fish, comparative analysis of BORN and import TCO steelhead II-WA (Tacoma, USA) rainbow trout are carried out to understand the genetic determined variation of host–pathogen interaction.

A salmonid cDNA microarray representing about 16,000 genes from trout and salmon (von Schalburg et al., 2008) was used to compare healthy BORN and import TCO trout in order to identify 'gatekeeper' genes that contribute to an efficiently functioning immune defence by blocking any pathogenic challenge before an infection is established. Several studies prove that the first barrier against infection in fish is represented by immune parameters like acute-phase proteins, lectins, lysozyme, and antimicrobial peptides (AMPs), reviewed by Magnadottir (2006). These immunorelevant molecules are synthesized in hepatocytes, some almost exclusively as reviewed by Parker and Picut (2005).

A first comparison of hepatic transciptome in healthy BORN and healthy import trout using microarray technology revealed 147 differentially expressed genes comprising 77 upregulated genes (Rebl et al., 2010) and 70 downregulated genes (Rebl et al., 2009) in BORN trout compared to import trout. Six of those are predicted to be responsible for an effective first barrier by responding to pathogen invasion and repairing lesions, i.e. LEAP2A – liver-expressed antimicrobial peptide 2A (a fish-specific variant of LEAP2/hepcidin-2; Krause et al., 2003; Zhang et al., 2004), SERPINA1 – serpin peptidase inhibitor clade A, member 1 (synonyms alpha-1 antiproteinase, antitrypsin; Hedin, 1906) and SERPINF2 – serpin peptidase inhibitor clade F, member 2 (synonyms alpha-2 antiplasmin, alpha-2-plasmin inhibitor, pigment epithelium derived factor; see review by Coughlin, 2005), FGL2 – fibrinogen-like protein 2 (synonym fibroleukin; Yuwaraj et al., 2001), ferritin heavy polypeptide 1 (FTH1, see review by Orino and Watanabe, 2008), and CLEC4E – C-type lectin domain family 4, family E (synonym macrophage-inducible C-type lectin, C-type lectin, superfamily member 9; Zhang et al., 2000). Since the complete cDNA sequence of SERPINF2 has not been isolated from salmonid species yet, we characterized the cDNA structure of two SERPINF2 genes from rainbow trout. Mammals possess only one copy of the SERPINF2 gene, whose gene product is the main physiologic plasmin inhibitor in plasma (Moroi and Aoki, 1976) playing a key role in the inhibition of fibrinolysis (Aoki et al., 1977).

Moreover, we proved the expression of the genes named above in healthy and infected BORN and import trout focusing on liver tissue as a central organ for acute-phase reactions that mediate an immediate response to injury and inflammation. These expression data will help in understanding the onset of immune responses in hepatic tissue.

2. Materials and methods

2.1. Preliminary sampling

For a preliminary holistic transcriptome analysis, clinically healthy two-year old rainbow trout from the local selection strain BORN (Born, Germany) and import TCO rainbow trout (Tacoma, USA; www.troutlodge.com) were acclimated separately for two weeks in freshwater aquaculture ponds at a regional aquaculture farm (Binnenfischerei Mecklenburg GmbH Schwerin; Frauenmark, Germany). Liver, spleen, and head kidney tissue samples were taken from six individuals of each strain and stored immediately in liquid nitrogen.

2.2. Experimental infection and temperature challenge experiment

Subsequent analyses regarding pathogen challenge and temperature stress were performed using BORN and import TCO rainbow trout, which were grown simultaneously to fingerlings in freshwater rearing tanks, followed by an adaptation to freshwater glass tanks.

For quantification of temporal candidate gene expression during immune response, 100 BORN and 100 import trout at age of 11–12-month were transferred and equally separated into four identical 300-L tanks with an average temperature at 15 °C. A total dosage of 1×10^4 cfu (colony forming unit) of *A. salmonicida* subsp. *salmonicida* was administered intraperitoneally (i.p.) to 72 fish of each strain. Prior to injection and sampling, fish were anesthetized in a benzocaine solution (50 ng/mL). Liver samples of four fish per strain and time point (0, 6, 12 h; 1, 2, 3, 7, 14 and 21 days p.i.) were taken after injection and stored in RNAlater solution (25 mM $Na_3C_6H_5O_7$; 9.9 mM EDTA; 5.3 M $(NH_4)_2SO_4$) until further use. The remaining 14 trout

per tank were considered as contact fish in order to monitor the contagion potential. Characteristic parameters of inflammation were recorded during the course of infection by flow cytometry as described elsewhere.

For quantification of the genes of interest in dependence on the temperature, 10-month old BORN (20 fish) and import TCO trout (20 fish) were separated in experimental 300-L tanks with 15 °C-tempered water two weeks before temperature change to minimize handling stress. In the experimental tanks with 10 fish of each strain, temperature was raised or lowered to 23 °C or to 8 °C by 1 °C per day and held at the respective temperature for one week allowing the fish to accommodate. Liver tissue samples were taken from 10 BORN and 10 import rainbow trout, respectively, and stored in RNAlater.

Total RNA from selected tissue of rainbow trout was individually extracted using TRIzol reagent (Invitrogen, Karlsruhe, Germany) and RNeasy Mini Kit (Qiagen, Hilden, Germany). Traces of genomic DNA were removed by DNase (RNase-free DNase Set; Qiagen) treatment for 15 min. Aarose gel electrophoresis determined the integrity of RNA.

2.3. Isolation and analyses of SERPINF2 genes from rainbow trout

Five microgram of total RNA from trout liver was reverse transcribed using Superscript II (Invitrogen) with Oligo-d(T)$_{24}$ to generate a cDNA template for the subsequent PCR amplification of SERPINF2A mRNA. The EST omykrbna507377 (GenBank accession: CB494163; Rise et al., 2004) was used to derive SERPINF2A-specific primers listed in Table 1. In order to obtain full-length trout SERPINF2A cDNA sequence, 5′- and 3′-RACE experiments were conducted using the Gene Racer Super Script™II RT Module (Invitrogen) according to a touchdown-PCR protocol. This included a 5-min pre-incubation step at 95 °C, a denaturing step at 94 °C for 30 s, an annealing step at temperatures decreasing from 68 to 59 °C during the first 10 cycles (temperature decrement of 1 °C per cycle) for 30 s, and an extension step at 72 °C for 2 min, followed by 30 cycles with 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C, and final extension at 72 °C for 7 min. A total of 41 cycles was performed.

The coding sequence of rainbow trout EST 143363 (EZ906697) corresponding to SERPINF2B gene was validated by PCR utilizing the primers listed in Table 1. Each nucleotide position was sequenced at least four times.

Phylogenetic analysis of conceptually translated (Expert Protein Analysis System proteomics server, Swiss Institute of Bioinformatics; http://www.expasy.ch/) amino acid sequences was performed using the Molecular Evolutionary Genetics Analysis package (MEGA v4) (Kumar et al., 2004).

2.4. Confirmation of differentially expressed genes by quantitative real-time RT-PCR

For quantification of candidate gene transcripts in selected tissues of healthy and challenged trout, quantitative real-Time RT-PCR (qRT-PCR) was carried out on the LightCycler® 480 System using the LightCycler® 480 SYBR Green I Master Kit (Roche, Mannheim, Germany). PCR was performed using the gene-specific oligonucleotides listed in Table 1 (tumor necrosis factor (TNF) primer sequences were adapted from those used by Teles et al., 2011) according to the following PCR program: initially 95 °C/10 min, then 40 cycles: 95 °C/15 s, 60 °C/10 s, 72 °C/20 s. A 183-bp β-actin (ACTB) or a 101 bp eukaryotic translation elongation factor 1 alpha 1 (EEF1A1; Bowers et al., 2008) gene fragment were amplified in parallel to serve as control for both RNA integrity and qRT-PCR success. Copy numbers were calculated relative to dilutions of PCR-generated fragments as external standards. Agarose gel electrophoresis on 3% gels allowed the separation of PCR products to assess product size and quality.

Parametric *T*-test or non-parametric Mann–Whitney *U*-test provided by IBM® SPSS® Statistics 19 evaluated statistical significance of the resulting data. In all of the tests, a two-tailed *P*-value of 0.05 or less evaluates significance. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, Ingenuity, CA, USA; www.ingenuity.com) served for generation of a network illustrating the functional relationship of the candidates.

3. Results

3.1. SERPINF2 is duplicated in rainbow trout

A preliminary microarray study comparing the hepatic transcriptome profiles of BORN trout in comparison to import TCO rainbow trout identified six genes associated with immunity and coagulation, i.e. LEAP2A (GenBank accession: NM_001124464; Zhang et al., 2004), SERPINA1 (NM_001124397; Mak et al., 2004), SERPINF2, FGL2 (NM_001140382; Leong et al., 2010), FTH1 (FTH-3, NM_001124549; Miguel et al., 1991) and the lectin CLEC4E (FJ607865; Zhang et al., 2000). All genes have been isolated from salmonid fish so far except SERPINF2 sequence. Thus, isolation and analysis of full-length SERPINF2 cDNA from trout was required for subsequent analysis.

We isolated a cDNA sequence of SERPINF2 from rainbow trout (FR677583) comprising 1921 bp. The initiation codon ATG at position 95 corresponds to the translational start in zebrafish (NP_001073479). The 513-bp 3′ UTR contains a less common, noncanonical "ATTAAA" poly(A) signal followed by the poly(A)tail 16 bp downstream. The putative 1314-bp open reading frame (ORF) encodes for a protein of 437 amino acids (aa).

Sequence comparison revealed that vertebrate SERPINF2 amino acid sequences share a comparably low degree of identity even among bony fish. 33.3% identical residues are to be found between SERPINF2 from human and trout. Trout SERPINF2 protein shares most identity with its counterpart in pufferfish *Takifugu rubripes* (55.6%). However, there is a second SERPINF2 gene in *T. rubripes*, which shows identities of only 39.4% with the ortholog in trout and 42.7% with its paralog in pufferfish. Duplicated SERPINF2 genes have also been found in zebrafish *Danio rerio*, but both share again only 40.2% sequence identities. The existence of duplicated SERPINF2 genes in teleostean species motivated our search for a second SERPINF2 gene in rainbow trout. BLAST analyses revealed the rainbow trout

Table 1
Gene-specific primers used in this study.

Primer name	Primer sequence (5′-3′)	Use
ACTB_LC_f	CCCTCCACCATGAAGATCAAGA	qRT-PCR, data normalization
ACTB_LC_r	GGGATGGGTACAGTCTGTTTAG	qRT-PCR, data normalization
CLEC4E_LC_f	ACAAGTAGATACCAACGGTCACT	qRT-PCR
CLEC4E_LC_r	TGCGATTTTCCCCATGTTTTAGG	qRT-PCR
EEF1A1_LC_f	TGATCTACAAGTGCGGAGGCA	qRT-PCR, data normalization
EEF1A1_LC_r	CAGCACCCAGGCATACTTGAA	qRT-PCR, data normalization
FGL2_LC_f	CAGCAACAAAATGGAGTCAGATAT	qRT-PCR
FGL2_LC_r	CTTGGGGCCTTTAGTTTCCATG	qRT-PCR
FTH1_LC_f	AACCGGATGATCAACTTGGAGAT	qRT-PCR
FTH1_LC_r	GAGTAAAATGCGTCCACCTCTC	qRT-PCR
IL6_LC_f	CAGCTTCTTCTTCAGCACGTTAA	qRT-PCR
IL6_LC_f	CGTAGACACCTCACCCAGAAC	qRT-PCR
LEAP2A_LC_f	GACATCTATCTATGCAAAACAACC	qRT-PCR
LEAP2A_LC_r	CATCTGCTTACTTTGGTAAAGTCA	qRT-PCR
SERPINA1_LC_f	TCACGCTTTAACAAGTGGCAAAAC	qRT-PCR
SERPINA1_LC_r	ATCAACAAACCAGCATGGACATC	qRT-PCR
SERPINF2A_f1	CCTAGACACCTGACTTCTCAGT	3′-RACE, qRT-PCR
SERPINF2A_f1nested	TTATTCCCACCTCTGGTATTGATT	3′-RACE
SERPINF2A_r1	AATCAATACCAGAGGTGGGAATAA	5′-RACE, qRT-PCR
SERPINF2A_r1nested	ACTGAGAAGTCAGGTGTCTAGG	5′-RACE
SERPINF2B_f1	TATACGAGCATCACTCATCTCCT	Sequence validation
SERPINF2B_r1	GACACGTTCACCTGGCCATTC	Sequence validation
SERPINF2B_f2	ATATCCCTTAAGTCTGCTCATTGA	Sequence validation
SERPINF2B_r2	ATCTGGATTTGCTTCCTCTCTGA	Sequence validation
SERPINF2B_LC_f	TGGGAGCCATGAATGAGACAGA	qRT-PCR
SERPINF2B_LC_r	TTGGGCTCAAACCCTGGTTGC	qRT-PCR
TNF_LC_f	GATACCCACCATACATTGAAGCA	qRT-PCR
TNF_LC_r	ATTTGGTTCCCCTGTAGCTCGA	qRT-PCR

EST 143363 (GenBank accession: EZ906697) with a complete 1218-bp ORF. We verified experimentally the coding sequence and corrected four positions (FR872890). The ORF can be translated into a 405-aa protein, in the following termed as SERPINF2B.

Both SERPINF2 aa sequences from rainbow trout share 48.3% identical and 31.1% similar residues. SERPINF2 sequences from bony fish and their homologs from human, chicken, and frog reveal on the one hand that no aa position is conserved within N- and C-terminus. On the other hand, a central domain shares still 14.9% identical aa and 20.3% strongly similar aa residues. This so called SERPIN domain is located at positions 54–393 and 27–366 within SERPINF2 protein A and B from rainbow trout, respectively. A sequence alignment of the respective domain from duplicated genes in trout, pufferfish, and zebrafish (Fig. 1A) highlights about one-third of conserved aa residues among the six piscine sequences. Only four residues are conserved among vertebrate species in SERPINF2A and B, respectively (not shown). Strikingly, all six piscine SERPIN domain sequences contain at least 12.2% leucine residues. An augmented number of ~12% leucine residues is generally present in full-length SERPINF2 proteins from vertebrates, some of them arranged in duplicate, triplicate or even in quintuplicate (full-length SERPINF2 from pufferfish *Tetraodon nigroviridis*; position 6–10). These leucine residues are obviously not arranged as leucine-rich repeats. However, they might provide a structural framework.

A phylogenetic tree (Fig. 1B) confirms that piscine SERPINF2 paralog proteins A and B are clearly separated with 99% bootstrap support. The separation of SERPINF2 A and B into different clades was repeated in a phylogenetic tree based on the SERPIN domain sequences (not shown).

The knowledge about both SERPINF2 sequences from rainbow trout allowed us to design specific oligonucleotides for qRT-PCR analyses, in order to confirm the results of the microarray pilot study.

3.2. *Elevated expression of four immunorelevant genes in liver of healthy BORN trout*

Quantitative real-time RT-PCR on liver tissue confirmed the microarray-predicted expression differences for the six genes related to immunity and hemostasis (Fig. 2A). Moreover, mRNA copy number of the respective genes was quantified in head kidney and spleen, which are mainly responsible for early immune responses in fish. The LEAP2 gene achieved the highest fold-change. The copy number of the respective gene was augmented in liver tissue of healthy BORN trout by 374% ($P = 0.02$) in comparison to import trout. Expression of SERPINA1 (+74%; $P = 0.008$), SERPINF2A (+63%; $P = 0.007$) and SERPINF2B (+25%; $P = 0.04$) was significantly higher in hepatic tissue,

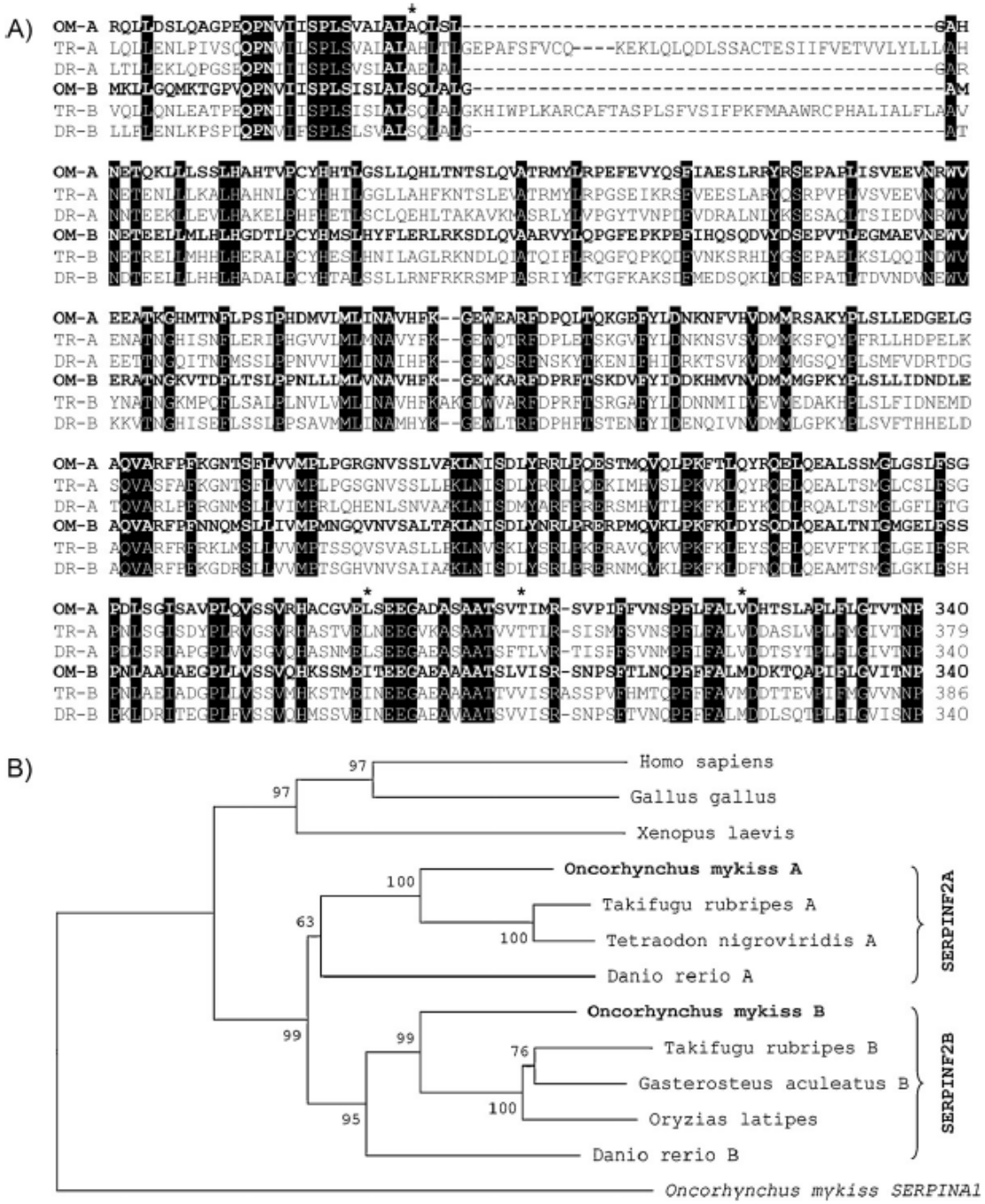

Fig. 1. (A) Amino acid sequence of the SERPIN domains from duplicated SERPINF2 genes (A and B) from rainbow trout (OM), Japanese pufferfish (TR), and zebrafish (DR). Identical residues are highlighted with black underlay. Asterisks above the alignment mark residues that are specific for either gene A or gene B. (B) Phylogenetic tree of SERPINF2 protein sequences using the Neighbour-Joining Method. The tree was rooted with rainbow trout SERPINA1 protein as outgroup. A bootstrap analysis based on 10,000 iterations was used to evaluate the robustness of the tree. The scale bar represents a genetic distance of 0.1 aa substitutions per site. The sequences and their corresponding GenBank (GB) and Ensembl (E) accession codes are *Homo sapiens* (human) SERPINF2: **AAH31592** (GB); *Gallus gallus* (chicken) SERPINF2: **XP_415807** (GB); *Xenopus laevis* (African clawed frog) SERPINF2: **NP_001087821** (GB); *Oncorhynchus mykiss* (rainbow trout; printed in bold face letters) SERPINF2A: **CBW45296** (GB), SERPINF2B EST: **EZ906697** (GB), SERPINA1: **CAD90255** (GB); *Takifugu rubripes* (Japanese pufferfish) SERPINF2A: **ENSTRUP00000012460** (E), SERPINF2B: **ENSTRUP00000007598** (E); *Tetraodon nigroviridis* (green spotted pufferfish) SERPINF2: **CAF97354** (GB); *Danio rerio* (zebrafish) SERPINF2A: **XP_688797** (GB), SERPINF2B: **NP_001073479** (GB); *Gasterosteus aculeatus* (three-spined stickleback) SERPINF2: **ENSGACP00000014384** (E); and *Oryzias latipes* (Japanese ricefish), **ENSORLP00000000609** (E).

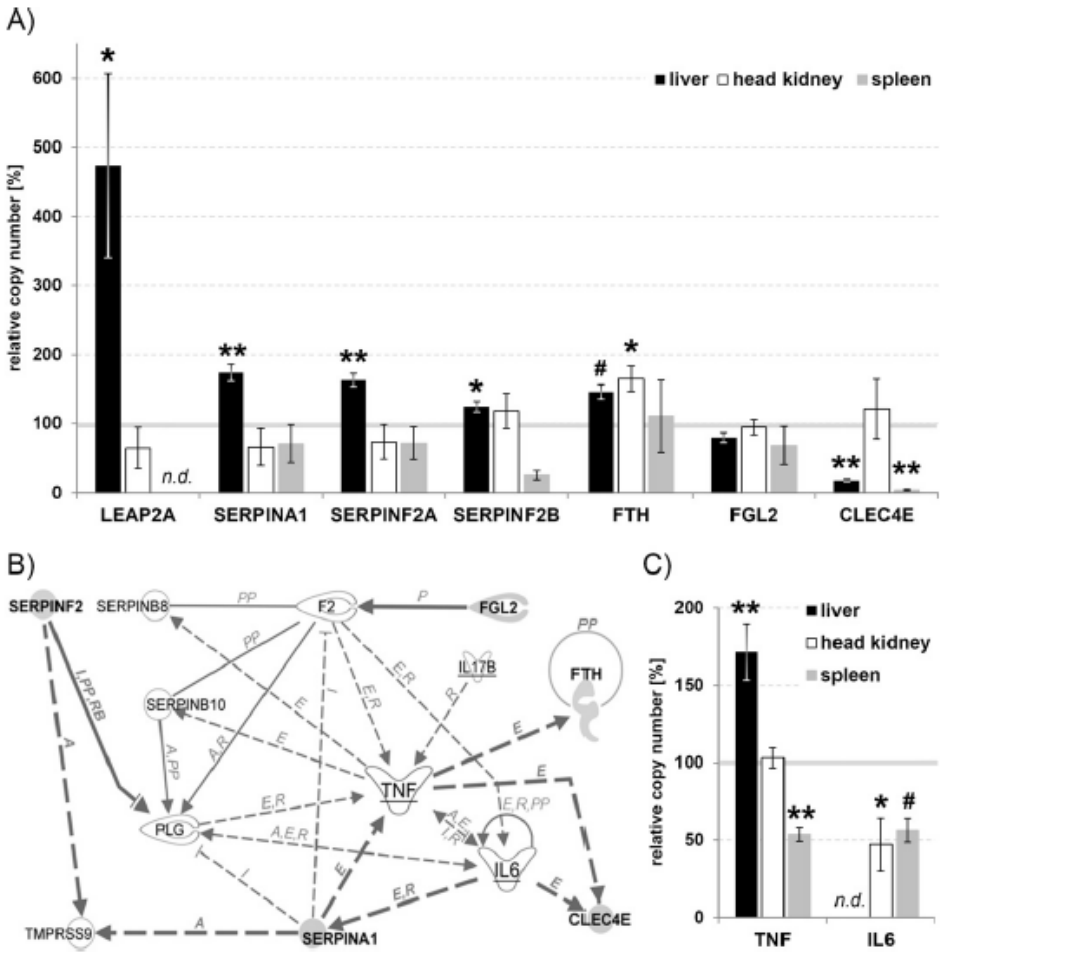

Fig. 2. (A) Gene expression differences between six healthy BORN and six import TCO trout held in open aquaculture. LEAP2A, SERPINA1, SERPINF2A, FTH1, FGL2, and CLEC4E transcripts were quantified by qRT-PCR in liver (black column), head kidney (white column), and spleen tissue (gray column). LEAP2A copy number measured in splenic tissue was not detectable (n.d.). ACTB transcript levels were non-significantly different. Mean values of the relative copy numbers from the respective import trout tissue were set as 100% (represented by a full gray line), and the means ± SEM from the corresponding BORN trout tissue are expressed as fraction hereof (represented by columns). Statistical significant expression differences are indicated by different symbols (**$P \leq 0.01$; *$P < 0.05$; #$P < 0.1$). (B) Graphical representation of an interaction network analysis generated by the Ingenuity Pathways Analysis Tool. Nodes represent the molecules, and edges represent the biological relationship between two nodes. At least one reference from the literature stored in the Ingenuity Pathways Knowledge Base supports each edge. Edges with various labels display the relationship of the involved peptides and proteins: protein–protein interaction (PP), activation (A), increment of expression (E), inhibition (I), proteolysis (P), regulation of binding (RB), and release/secretion (R). Candidate genes from the array analysis and associated relationships are printed in bold face letters and thick lines, respectively. Additional genes/gene products are IL6 (interleukin 6), IL17B (interleukin17B), F2 (prothrombin), PLG (plasminogen), SERPINB8 (serpin peptidase inhibitor clade B, member 8), SERPINB10 (serpin peptidase inhibitor clade B, member 10), TMPRSS9 (transmembrane serine protease 9), TNF (tumor necrosis factor). (C) TNF and IL6 transcript number in liver (black column), head kidney (white column), and spleen tissue (gray column) from each six healthy BORN and import trout. IL6 transcripts measured in liver were negligible low (n.d.). Mean values of the relative copy numbers from the respective import trout tissue were set as 100%. Error bars indicate standard error of the mean. Different levels of significance are indicated by different symbols (**$P \leq 0.01$; *$P < 0.05$; #$P < 0.1$).

whereas CLEC4E expression was significantly lower in liver (−82%; $P = 0.004$) and in spleen (−96%; $P = 0.01$) of BORN trout. A significant expression difference in liver has not been proven for FGL2 and only marginally for FTH1 (+46%; $P = 0.06$). However, FTH1 mRNA copy number was significantly elevated in head kidney of BORN trout (+65%; $P = 0.02$).

We used the Ingenuity Pathway Analysis software to visualize the suggested genes in a network structure (Fig. 2B) based on relationships and interactions that have been reported for mammalian species. The network comprises genes of the acute-phase and coagulation system including two additional members of the SERPIN family, SERPINB8 and SERPINB10, the transmembrane serine protease TMPRSS9 as well as the coagulation factors prothrombin/thrombin (F2) and plasminogen/plasmin (PLG). The extracellular antimicrobial peptide LEAP2A was not included in this network. The two cytokines TNF and

interleukin 6 (IL6) were identified as central key factors of the network. On the one hand, our data suggest the influence of SERPINA1 expression on TNF expression as well as a rather indirect influence on TNF expression by FGL2 *via* F2, and by SERPINF2 *via* PLG. On the other hand, TNF promotes the expression of FTH1 and CLEC4E. TNF and IL6 also mutually regulate their expression. IL6 stimulates the expression of SERPINA1 and CLEC4E, whereas FGL2 *via* F2 and by SERPINF2 *via* PLG indirectly regulate the IL6 expression.

Consequently, TNF and IL6 mRNA abundance was measured in BORN and import trout in order to detect strain-specific expression differences, which might influence or be influenced by the expression of the respective genes. Fig. 2C illustrates that hepatic expression of TNF gene was indeed elevated in BORN trout by 72% ($P = 0.01$) in comparison to import trout, though this basal TNF expression was relatively low. The respective CP (crossing point) values reflecting the cycle number, at which a significant increase of the fluorescence signal during qRT-PCR is first detected, ranged from 28.8 to 30.9. IL6 transcripts were not detectable in liver.

3.3. Similar expression of candidate genes in liver of both trout strains during infection, but clear depression of the lectin CLEC4E in BORN trout

BORN and import TCO trout were held under standardized conditions in freshwater tanks and infected with the economically important pathogen *A. salmonicida*. The temporal expression of the six microarray-suggested genes was measured in liver tissue.

In general, the expression values of hepatic gene expression in unchallenged BORN and import TCO trout maintained in water tanks corresponded to the results obtained preliminarily with healthy trout from both strains farmed in aquaculture ponds (Fig. 3A–F). Highest but marginally significant expression difference of 5.45-fold among BORN/import trout was measured for LEAP2A ($P = 0.08$). BORN trout showed furthermore an elevated expression for SERPINA1 (+394%; $P = 3.95 \times 10^{-5}$), SERPINF2A (+87%; $P = 0.24$), SERPINF2B (+68%; $P = 0.02$), FTH1 (+66%; $P = 0.02$), and a significantly depressed CLEC4E expression (−78.4%; $P = 9.16 \times 10^{-4}$) compared to import trout. A significant higher copy number was measured for FGL2 gene in BORN trout (+140%; $P = 0.03$) compared to import trout, inconsonant with the FGL2 expression data obtained in the initial analysis but in line with the microarray result.

The expression differences observed in healthy BORN and import trout were not detected throughout all time points after infection. It rather seems that copy numbers of the respective genes approached a similar level after pathogenic challenge in both strains. Four expression patterns shown in Fig. 3 are nevertheless remarkable.

First, LEAP2A copy number reached in both strains intermediately a minimal expression value (Fig. 3A). In contrast to BORN trout, this depression occurred fast in import trout within 6 h (−99.8%; $P = 0.004$). Furthermore, LEAP2A expression obviously recovered faster in BORN trout than in import trout ($P = 0.04$) returning to 59.3% of the original value (2.0% in import TCO trout) at day 21 p.i.

Second, both SERPINF2 genes were downregulated by at least 60% ($0.0002 \leq P \leq 0.07$) in both strains 3 days after infection (Fig. 3C). However, SERPINF2 transcript number regenerated in the later course of immune response. SERPINF2A expression was increased in challenged BORN trout after 21 days by even 151% ($P = 0.03$) in comparison to import trout, where expression values returned approximately to the initial value of healthy import trout (110.5%) at the same time point. On the other hand, SERPINF2B copy number was significantly upregulated by 166% ($P = 0.008$) in infected import trout at day 21 p.i. and concurrently induced by 158% ($P = 0.02$) in comparison to BORN trout that showed almost the same SERPINF2B mRNA level after 21 days as before *A. salmonicida* infection.

Third, in import trout, FTH1 expression decreased by −80% ($P = 0.005$) at day 3 p.i., but reached eventually a 60% increased mRNA level ($P = 0.03$) 21 days after infection (Fig. 3D). In BORN trout, FTH1 expression developed similarly, but 21 days after infection, only 67.4% ($P = 0.09$) of the original mRNA abundance in healthy individuals was present.

Fourth, CLEC4E expression showed different patterns in BORN and import trout after pathogenic challenge (Fig. 3F). CLEC4E expression was upregulated by 226% ($P = 0.002$) in BORN trout 1 day after infection and still elevated by 108% ($P = 0.03$) 21 days p.i. in comparison to control. In contrast, expression was upregulated by more than 150% ($P = 0.001$) in import trout at day 3 p.i., but only one-third of the original CLEC4E copy number was reached at day 21 p.i. with $P = 0.004$. Nevertheless, CLEC4E expression was generally lower in BORN trout.

Additionally, TNF and IL6 copy number was measured during infection (data not shown). As seen in the pilot study, TNF expression was increased in control BORN trout (+317%; $P = 0.1$), whereas IL6 copies were not quantifiable. However, the expression ratio changed at days 3 and 7, when TNF gene was depressed in BORN compared to import trout (−51% to −60%). At these time points, trout from both strains showed clinical signs of furunculosis, such as ascites, hemorrhages in liver, and inflamed intestine.

3.4. Temperature influences expression of SERPINA1, FTH1, and FGL2 genes

The above described experiments with rainbow trout held in aquaculture ponds for a pilot study as well as in water tanks under standardized conditions revealed that the basal fold-change difference in expression of the candidate genes between BORN and import trout varied partly to a large extent. Hypothesizing that temperature change could influence the expression level of those genes, we performed a subsequent experiment with healthy BORN and import trout maintained in water tanks with a temperature of either 8 °C or 23 °C (results not shown). We found a significant upregulation of SERPINA1 (+369%; $P = 0.05$) and FGL2 (+270%; $P = 0.02$) gene expression in import trout held at 23 °C compared to 8 °C trout. FTH1 was significantly upregulated in both, BORN (+136%; $P = 0.04$) and import trout (+111%; $P = 0.01$) after temperature increase from 8 °C to 23 °C. LEAP, SERPINF2A, and CLEC4E showed no

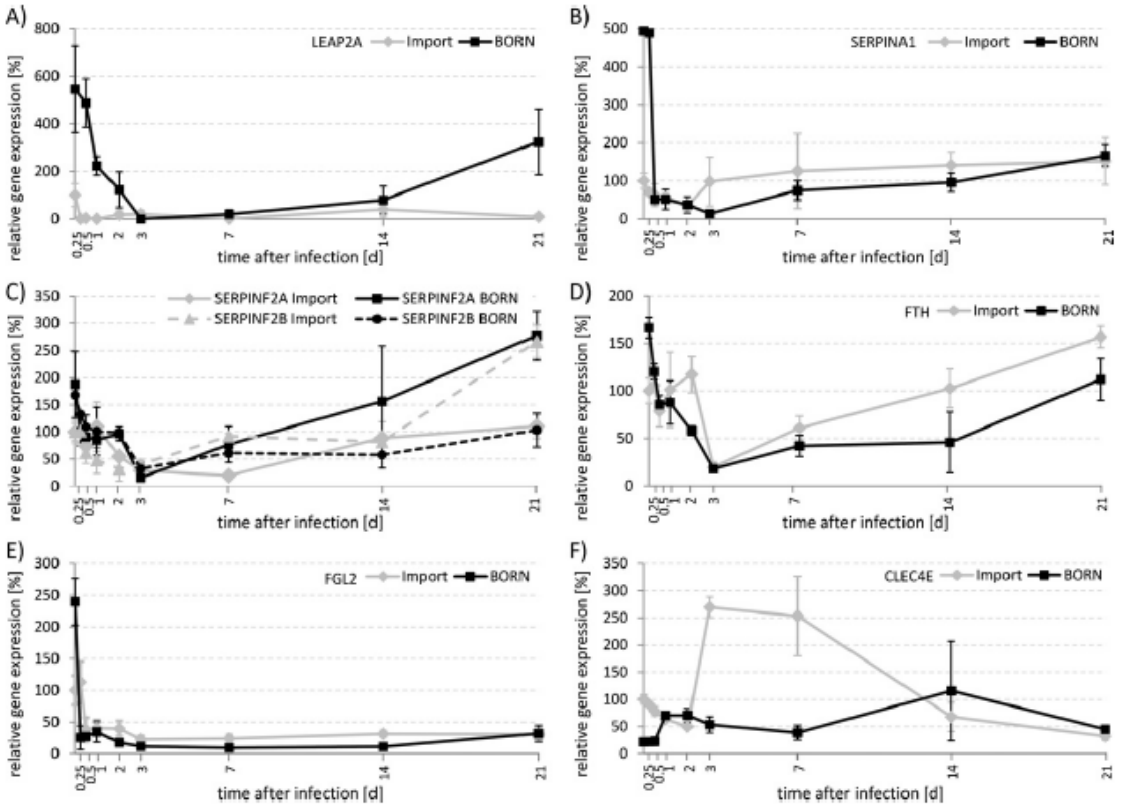

Fig. 3. Relative level of the immune-relevant genes (A) LEAP2A; (B) SERPINA1; (C) SERPINF2A (full lines) and B (broken lines); (D) FTH1; (E) FGL2; (F) CLEC4E in BORN (black lines) and import TCO trout (gray lines) after injection with *A. salmonicida*. Liver tissue was sampled from three trout of each strain at 0, 6, 12 h and 1, 2, 3, 7, 14, and 21 days after infection. Transcript numbers were normalized to EEF1A1 expression. Each data point represents the mean ± SEM; mean values from import trout (0 h) were set as 100%.

significantly different expression patterns in dependence on temperature.

4. Discussion

The top differential expressor among the immunorelevant genes in the liver of BORN and import trout was LEAP2A, a fish-specific gene variant of the antimicrobial peptide-encoding LEAP2 (Krause et al., 2003) with about a 5-fold expression difference in the performed experiments. Antimicrobial peptides have been isolated from a broad range of teleostean tissues (Smith et al., 2010) protecting the host by disrupting microbial cell membranes (Watts et al., 2001). Zhang et al. (2004) found the expression of LEAP gene variants 2A and 2B constitutively expressed in liver of rainbow trout. Our qRT-PCR data support the almost exclusive LEAP2A expression in trout liver, since we found a low LEAP2A copy number in spleen and almost no transcripts in head kidney. Martin et al. (2006) who have conducted a GRASP array experiment studying the response of Atlantic salmon (*Salmo salar*) against *A. salmonicida* (10^8 CFU i.p.) measured an 11-fold increase in expression within 48 h after infection. Since we could not detect any upregulation of the respective gene after injection with 10^4 CFU *A. salmonicida*, neither in BORN nor in import trout, induction of LEAP2A upregulation might be dose-dependent. This is in line with a report by Bao et al. (2006), who could not detect any LEAP2 gene upregulation in liver of catfish *Ictalurus punctatus* after infection with *Edwardsiella ictaluri*. Our investigations revealed a faster recovery of LEAP2A transcript level in BORN trout. This suggests that BORN trout regain earlier a part of an innate barrier against invasive pathogens, although LEAP2A seems to be predominantly essential in healthy individuals and hardly involved in inflammatory events. The importance of LEAP for piscine immune response to bacteria and viruses (Cuesta et al., 2008) may be based on its iron-regulatory role (Shi and Camus, 2006).

Iron is essential for the survival of bacterial pathogens (Jung and Kronstad, 2008). Possibly, the regulation of iron homeostasis by LEAP2 could be an alternative pathway alongside ferritin that functions as major iron storage protein (Miguel et al., 1991). Mammalian ferritin consists of 24 heavy and light chains (Orino and Watanabe, 2008). This acute-phase reactant is known to protect mammals against side effects of infectious and inflammatory conditions, e.g. reduction of reactive oxidant species. FTH1 functions as a rapid regulator of iron availability and other cellular processes, reviewed by Koorts and Viljoen (2007). Yamashita et al. (1996) reported enhanced transcription and

accumulation of FTH1 for trout. Though our qRT-PCR data revealed that FTH1 expression differed in healthy fish of BORN and import trout strains, only slight differences were detectable during infection on transcript level between both strains. On the other hand, we found FTH1 expression doubled after temperature increase, which might correlate *in vivo* with a strong proliferation of bacterial pathogens. To sum it up, competition for iron *via* ferritin and LEAP could prevent a systemic pathogen spread in BORN trout at a very early stage.

Aside from ferritin and LEAP, two further acute-phase genes were expressed to a higher extent in liver of BORN trout. These were the serine proteinase inhibitor (serpins) genes SERPINA1 and SERPINF2. SERPINA1 is the main acute-phase plasma proteinase inhibitor in blood, which neutralizes proteolytic virulence factors from bacteria and parasites (Hedin, 1906; Mak et al., 2004). SERPINF2 is the principle regulator of plasmin (see review by Coughlin, 2005) and therefore retards fibrinolysis, as plasmin remodels or even removes fibrin-rich clots by cleaving fibrin into degradation products. This is conceivably advantageous, since blood clots represent a physical barrier preventing bacterial invasion. Plasmin contributes furthermore to complement activation in mammals by cleaving C3 and C5 components (Amara et al., 2010). Eventually, SERPIN might also control complement reactivity through its plasmin-regulatory role.

Two different SERPINF2 genes are present in different teleostean species. In this study, duplicated SERPINF2 genes from rainbow trout have been identified and structurally characterized. Since salmonids underwent an additional round of genome duplication (Moghadam et al., 2005) even four SERPINF2 sequences might be present in trout genome. However, we only identified two different transcripts. Both conceptually translated proteins share a relatively low degree of conserved amino acid residues. It is imaginable that the different structures affect the formation and the stability of the resulting SERPINF2/plasmin complexes and hence the efficiency of plasmin inhibition.

Our SERPINF2A and -B transcript measurements indicate an expression of both genes with different kinetics in the two strains. In healthy BORN trout, mRNA abundance of both SERPINF2 genes increased compared to import trout. Three weeks after infection, SERPINF2A was upregulated in BORN trout, whereas SERPINF2B was upregulated in import trout each more than 2.6-fold in comparison to healthy fish. Salte et al. (1993) assessed SERPINF2 as a marker correlated with survival to furunculosis in salmon. They found that the lower SERPINF2 plasma level is in salmon the more fish is susceptible to furunculosis. However, it needs to be proven, whether SERPINF2A or -B or both genes are contributing to an improved resistance towards *A. salmonicida* in salmonid fish.

Similar to SERPINF2, the serine protease FGL2 functions as an immune coagulant (Yuwaraj et al., 2001). *A. salmonicida*-induced furunculosis signs include hemorrhage of the liver, provoking a protective coagulative response. However, we only found a strong FGL2 expression in healthy liver, being elevated in BORN trout compared to import trout. Nevertheless, mammalian FGL2 is a multifunctional protein that acts as a prothrombinase and possesses furthermore also immunosuppressive activity (Liu et al., 2008). In mouse liver, FGL2 expression is restricted to endothelial and Kupffer cells (Ding et al., 1998). This might explain the low FGL2 copy number during infection, which promotes the emigration of macrophages.

A network scheme generated by the IPA tool suggests a mutual activation of hepatic expression among the candidate genes SERPINA1, SERPINF2, FGL2, FTH1, and CLEC4E on the one hand as well as TNF and IL6 on the other hand. Though IPA networks are based on data for mammalian species, it has also been proven for teleosts that the pro-inflammatory signalling molecules TNF and IL6 are induced very fast after stimuli, which result from wounding and pathogen invasion (Bayne and Gerwick, 2001). Both genes have already been identified in rainbow trout (Glenney and Wiens, 2007; Iliev et al., 2007; Laing et al., 2001). Indeed, our qRT-PCR analyses verify that TNF expression was augmented in liver of BORN trout and possibly linked to the expression of the candidate genes. The detection of a comparably lower copy number of TNF and the respective genes in head kidney supports these findings.

Moreover, IPA predicted a relationship between coagulation cascade factors and CLEC4E. However, we could not verify the impact of TNF copy number on CLEC4E expression. CLEC4E mRNA copy number was significantly lower in BORN trout liver and spleen than in the respective import trout tissues. Mammalian CLEC4E is involved in sensing both fungal PAMPs (pathogen-associated molecular pattern) and DAMPs (damage-associated molecular pattern) released from damaged cells after traumata (Yamasaki et al., 2009). Unexpectedly, we found only in import trout a significant CLEC4E upregulation after 2 days of infection probably correlating with *A. salmonicida*-induced lesions. Also in BORN trout, CLEC4E expression was elevated in the course of infection, but less pronounced than in import trout, though two of three trout from each strain showed clear furunculosis symptoms at days 3 and 7 p.i.

5. Conclusion

In conclusion, expression of six genes responding to pathogen invasion and damaged epithelial surfaces was elevated in healthy rainbow trout of the local selection strain BORN in contrast to the import TCO strain. The expression of these genes, namely LEAP2A, SERPINA1, SERPINF2A and variant B, FTH1, and FGL2 may be concatenated with the expression of the potent cytokine TNF, which was also upregulated in liver of BORN trout. Altogether, these genes may contribute to maintain the health status of BORN trout that show enhanced resistance towards infections. BORN trout are therefore an excellent model to discover and investigate protective immune factors and mechanisms in comparison to susceptible rainbow trout.

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Studie II.

Molecular characterisation of PRR13 and its tissue specific expression in rainbow trout (*Oncorhynchus mykiss*).

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kurze Zusammenfassung:

Das Hauptaugenmerk dieser Studie lag auf der molekulargenetischen Charakterisierung des Gens *PRR13* in der Regenbogenforelle, dessen Produkt eine Resistenz gegen Zytostatika vermittelt. Neben der Analyse der Genstruktur wurde in vergleichenden gewebespezifischen Analysen eine fast durchgängig erhöhte Expression in BORN-Forellen festgestellt. Die Ergebnisse der Veröffentlichung lassen einen Einfluss des Gens auf die differente Robustheit von BORN- und Importforellen im Umgang mit durch lokale Brackwasserbedingungen hervorgerufenen Stress vermuten.

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Molecular characterization of PRR13 and its tissue-specific expression in rainbow trout (*Oncorhynchus mykiss*)

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Abstract The proline-rich protein 13 (PRR13) is reported to be a key regulator of the resistance to cytostatica by decreasing the copy number of the proapoptotic gene thrombospondin-1. We isolated and characterized the complete PRR13 gene sequence of rainbow trout (*Oncorhynchus mykiss*). The gene comprises four exons and three introns, the latter of comparatively short lengths (100–811 bp). The full-length PRR13 cDNA consists of 1,101 nucleotides, including an open reading frame of 563 bp, which is predicted to encode a 187 amino acid protein with a molecular mass of 18.8 kDa. A continuous stretch of ten serine residues at the C-terminus is highly conserved and characteristic for vertebrate PRR13, but not for other known proline-rich proteins. Phylogenetic analyses suggest a clear separation of teleostean PRR13 proteins and those from mammalian and reptilian species. Comparison of the tissue-specific PRR13 mRNA abundance in two strains of the rainbow trout coastal form (TCO Steelhead II-WA vs. BORN Steelhead II-Germany) revealed an increased expression in the BORN trout in nearly all examined tissues. The major expression differences were detected in gill (2.29-fold) and in liver tissue (2.16-fold). Hence, the increased PRR13 expression in BORN trout might cause improved protection from natural cytostatica and therefore support our assumption that PRR13 is a candidate gene possibly involved in the varying ability of the two rainbow trout strains to handle environmental stress under local conditions of the Southern Baltic.

Keywords Rainbow trout · Proline-rich protein · PRR13

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Introduction

Aquaculture contributes half of the fish consumed worldwide. In 2008, the share of salmon and trout in world fish trade stood at 11% (FAO Fisheries and Aquaculture Department 2009). Due to the growing demand of fish, it is particularly important to preserve the quality of the finished products and minimize the deterioration of product quality caused by stress. One option on this way is the generation of genetically and molecularly characterized breeds selected for survival under local environmental conditions. Following this goal, we have recently started to compare

the genetic potential of the commercially available rainbow trout strain TCO Steelhead II-WA adapted to fresh water conditions of its origin with the selection strain BORN Steelhead II-Germany, which serves as model for adaptation to local water conditions (Rebl et al. 2008). The BORN strain has been bred from a rainbow trout coastal form imported from Denmark in 1975. It shows an increased resistance towards different stress factors such as infection and pollution compared to other selection strains (Anders 1986). Initially, microarray hybridization experiments using the 16 K cDNA microarray chip (von Schalburg et al. 2008) with total mRNA as probe from liver tissue of healthy fish of both rainbow trout strains identified numerous different expressed genes potentially involved in stress regulation (Rebl et al. 2009). Whereas the genome-wide transcript analysis is under investigation, the different expression of the proline-rich protein 13 gene (PRR13) gave us occasion for its more precise molecular characterization and gene expression analysis.

PRR13 is characterized by a high amount of proline-rich residues. However, like PRR5, the PRR13 protein cannot be allocated to the known PROL family of small, highly basic and highly proline-rich proteins (Azen et al. 1984; Johnstone et al. 2005). A database search revealed that compared to other taxa, such as mammalian or reptilian only few proline-rich protein-encoding gene sequences are identified in bony fish. Independent from finding further proline-rich proteins in fish in future, at this time, full-length PRR13 gene sequence has been only sequenced in three-spined stickleback (Ensemble ENSGACT00000008858).

Whereas PRR13 gene sequences have been clarified for some species; however, the function of PRR13 is yet not well understood. PRR13 is reported to regulate the antiangiogenic and proapoptotic protein thrombospondin-1 (TSP-1) (Lih et al. 2006; van Amerongen and Berns 2006). By decreasing the expression of TSP-1, it conveys an enhanced resistance to apoptosis and taxanes (Papadaki et al. 2009). Taxanes are natural or synthetic cytostatica, which are used in combating of human cancer.

The present manuscript characterizes complete PRR13 gene and cDNA sequences from rainbow trout (*Oncorhynchus mykiss*) including tissue-specific mRNA expression as well as expression profiles in two rainbow trout strains.

Materials and methods

Tissue samples from two rainbow trout strains were isolated and flash frozen. Both strains represent the rainbow trout coastal form, but they are adapted to different environmental conditions. Whereas the strain TCO Steelhead II-WA (TCO; www.troutlodge.com) is an imported strain adapted to fresh water of high quality, the strain BORN Steelhead II-Germany (BORN) is adapted to local eutrophic water conditions of the Southern Baltic for numerous generations since 1975 (Anders, 1986). The BORN strain is based on a previously imported Danish rainbow trout coastal form.

Total RNA was isolated from adipose tissue, brain, fin, gill, head kidney, heart, intestine, liver, muscle, skin, spleen, stomach and trunk kidney. For RNA preparation, samples were homogenized individually in 1 ml TRIzol Reagent (Invitrogen, Karlsruhe, Germany), and RNA was extracted using the Nucleo Spin RNAII kit (Machery-Nagel, Düren, Germany) with in-column DNase treatment for 45 min. QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) was utilized to isolate DNA from flash-frozen liver tissue.

Five micrograms of total RNA from different tissues of rainbow trout was utilized for reverse transcription using Superscript II (Invitrogen). The generated cDNA was applied as a template for the subsequent PCR amplification of the PRR13 transcript. Atlantic salmon EST ssalrgb535055 (GenBank accession: CA063225; Rise et al. 2004) was used to derive PRR13-specific oligonucleotide sequences. Gene Racer Super Script™II RT Module (Invitrogen, Karlsruhe, Germany) was used to obtain the full-length PRR13 sequence *via* 3′- and 5′-RACE experiments, according to the manufacturer's protocol. RACE-PCR was performed by HotStarTaq® DNA polymerase (Qiagen, Hilden, Germany) using a touch-down cycling protocol. 5′-RACE products were subcloned into the pGEM®-T Easy Vector (Promega, LaJolla, CA, USA) and sequenced with the universal T7 and SP6 sequencing primers. Intron-flanking primers were designed on the basis of the orthologous PRR13 gene sequence in stickleback and then used to analyse the PRR13 gene structure. Each nucleotide position was sequenced at least four times.

To investigate PRR13 mRNA expression in various tissues, semi-quantitative RT–PCR was performed using the PRR13-specific primers

5′-GCAGGAGATCATCACTTTTTCAT-3′ (sense) and 5′-CTGCTTATCCCCCTGGTATG -3′ (antisense) to produce a 187-bp fragment. Moreover, a 101-bp EEF1A1 fragment was amplified as a reference control by utilizing the primers 5′-TGATCTA CAAGTGCGGAGGCA-3′ (sense) and 5′-CAGCAC CCAGGCATACT TGA-3′ (antisense).

Quantitative real-time RT–PCR (qRT–PCR) was performed to analyse PRR13 expression in two rainbow trout selection strains. A concentration of 1.5 µg of total organ RNA was converted to cDNA and used as template for real-time PCR using the Super Script™II kit (Invitrogen, Karlsruhe, Germany). The purified (High Pure PCR Product Purification Kit; Roche, Basel, Switzerland) products were analysed using the Light-Cycler 480 Instrument and Light-Cycler®480 SYBR Green I Master Kit (Roche, Mannheim, Germany). For amplification, rainbow trout PRR13-specific primers (primers see above) were used to create a 187-bp fragment. In addition, a 101-bp EEF1A1 fragment was amplified in order to normalize PRR13 gene expression (see above). The amplification profile initiated by a denaturation step of 10 min at 95°C, includes 40 cycles comprising: 15 s of denaturation at 95°C, 10 s of annealing at 60°C, extension at 72°C for 20 s and ultimately 5 s quantification at 83°C (PRR13) and 79°C (EEF1A1), respectively. qRT–PCR measurements were carried out in replica with mRNA samples from five individuals of each rainbow trout strain. Serial dilutions of PCR-generated PRR13 fragments (10^3–10^6) were applied, and calculated copy numbers served as external standards. Statistical significance ($P < 0.05$) was evaluated by using parametric *t*-test or non-parametric Mann–Whitney *U*-test.

Similarity searches of sequenced PRR13 DNA fragments and predicted amino acid sequence were performed using National Center for Biotechnology Information (NCBI) BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustAlW2 (Larkin et al. 2007) was applied for sequence alignments. The putative rainbow trout PRR13 protein was analysed by the ProtParam tool at the Expasy Proteomics Server (Wilkins et al. 1999) to reveal basic physical and chemical properties. A phylogenetic tree based on the deduced amino acid sequences was constructed by the Molecular Evolutionary Genetics Analysis (MEGA v3.1) package (Kumar et al. 2004).

Results and discussion

The PRR13 cDNA sequence of rainbow trout is 1,101 bp in length and comprises an open reading frame (ORF) of 563 bp, a 99-bp 5′ untranslated region (UTR) and a 417-bp 3′ UTR. The ORF encodes 187 amino acids. As a special feature, it includes a minisatellite, located 140 bp downstream the start codon. The minisatellite sequence TGGTATGGC CCC is 12 bp in length and repeated six times in a row. The first nucleotide of the minisatellite is substituted in the last three repetitions, which start with a cytosine instead of a thymin nucleotide. The alternative poly(A) signal AATAAT is located 53 bp upstream of the start of the poly(A) tail. The full-length sequence of the rainbow trout PRR13 cDNA has been deposited into NCBI GenBank (Accession number FN598572).

The rainbow trout PRR13 gene comprises four exons (GenBank: Accession number FN668729). This corresponds to the exon number in several other vertebrates, such as three-spined stickleback (*Gasterosteus aculeatus*), red throated anole (*Anolis carolinensis*), mouse (*Mus musculus*) and human (*Homo sapiens*) (Fig. 1a). Except of PRR13 from anole, all other sequences comprise an intron in front of the ORF, which differs in length from 304 bp (rainbow trout) to 904 bp (human). No PRR13 transcript variant has been identified in rainbow trout, which might be due to the relatively short intron length of the gene in this species. The splice site recognition across an intron ceases, when a threshold intron length of about 200 bp is reached (Fox-Walsh et al. 2005). A short distance between two splice sites seems to promote exon inclusion (Bell et al. 1998). For example, intron 2 from rainbow trout is very short compared to the respective intron of the human PRR13 gene (100 bp vs. 659 bp). Truly, human PRR13 features a transcript variant that results in a shorter exon 3 (GenBank Accession: NM_001005354).

The predicted amino acid composition of PRR13 in rainbow trout is unusually proline rich (Fig. 1b). The PRR13 protein contains 38 proline residues in the first 141 amino acids (20.3% of the entire protein) compared to 44 residues in human (30.3% of the entire protein). Additionally, seven histidines (eight in human) and 12 lysines (13 in human) are present in the C-terminal 46-amino acid segment. Noteworthy is

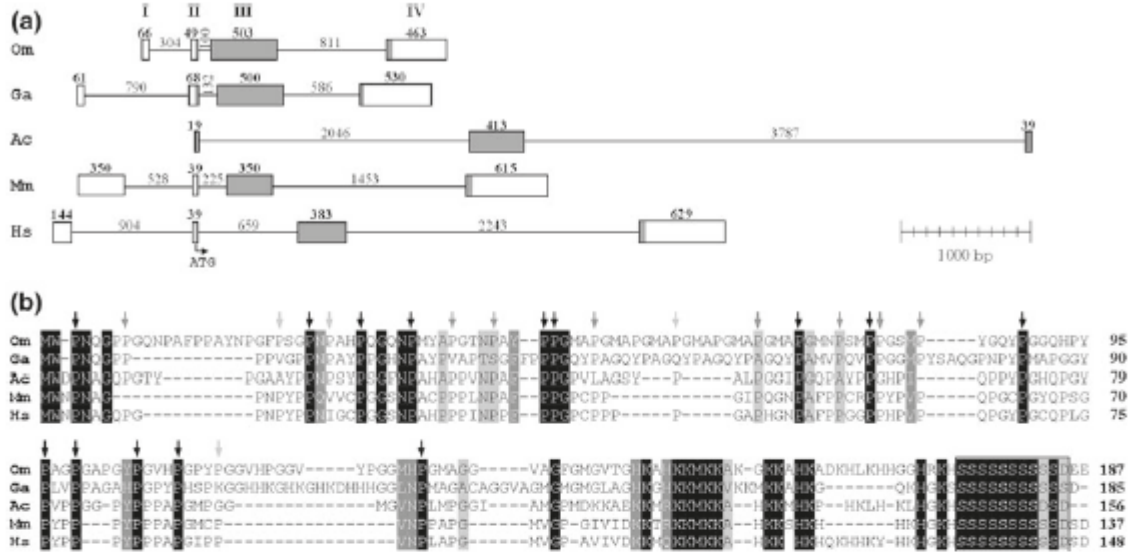

Fig. 1 Rainbow trout PRR13 gene (Om: *Oncorhynchus mykiss*, GenBank [GB]: FN668729) was compared regarding gene structure and predicted protein sequence with its homologues from fish (Ga: *Gasterosteus aculeatus*, Ensembl [E]: ENSGACP00000008839), reptile (Ac: *Anolis carolinensis*, E: ENSACAP00000000668) and mammals (Mm: *Mus musculus*, GB: NP_079661; Hs: *Homo sapiens*, GB: NP_060927). **a** Comparative illustration of exon/intron organization of fish PRR13 genes with its reptilian and mammalian homologues. The *boxes* represent exons; *white colour* depicts untranscripted regions and *grey colour* coding regions. Exons of rainbow trout PRR13 gene are numbered I-IV. Introns are represented as *scaled linking lines*. An *arrow marks* the position of the start codon ATG. **b** Alignment of predicted PRR13 protein sequences. Identical, strongly similar and weakly similar amino acid residues are labelled by *black*, *dark grey* and *light grey underlay*, respectively. Proline residues shared with PRR13 from rainbow trout are marked with *arrows*. *Black arrows* show matches in all sequences, dark *grey arrows* in four of five sequences and *light grey arrows* in three of five sequences

a continuous stretch of 10 serine residues at the C-terminus, which seems to be highly conserved, since it can also be found in all known PRR13 proteins of other species. Hence, it seems to be characteristic for the protein. Lih et al. (2006) revealed several putative α-helix motifs in the first 100 amino acids of human PRR13 and putative β-sheet structures near its C-terminal end. Due to the conserved amino acid residues, a homology in the allocation of putative α-helix motifs and β-sheet structures in the PRR13 protein from rainbow trout can be expected. The protein has a predicted molecular mass of 18.77 kDa and an isoelectric point of 9.79. The rainbow trout PRR13 protein shares highest identity with its homologue in stickleback (48.02%), followed by human (45.74%), red-throated anole (46.28%) and mouse (41.49%). However, PRR13 protein length differs among species. Trout PRR13 contains 50, 38 and 32 additional residues compared to PRR13 from mouse, human and anole, respectively. In contrast, PRR13-like proteins from trout and stickleback share a comparable number of residues (187 and 185 residues, respectively).

In order to investigate the evolutionary relationship between vertebrate PRR13 amino acid sequences, a neighbour-joining phylogenetic tree was constructed. Two mammalian and all yet known non-mammalian PRR13 sequences were compared (Fig. 2). Stickleback PRR12 was considered as an evolutionary outgroup. The phylogenetic analysis grouped PRR13 from rainbow trout and stickleback in a distinct clade with a bootstrap support of 92%. Teleost PRR13 proteins are distinctly separated from those of mammalian and reptile.

Semi-quantitative RT–PCR revealed high PRR13 transcript expression in fin, gill, head kidney, heart, stomach and trunk kidney. Moderate expressions were observed in intestine and spleen. Furthermore, low expression was detected in adipose tissue, brain, liver, muscle and skin tissue. The amount and quality of the cDNA was verified by amplification of EEF1A1 in each tissue. The confirmation that

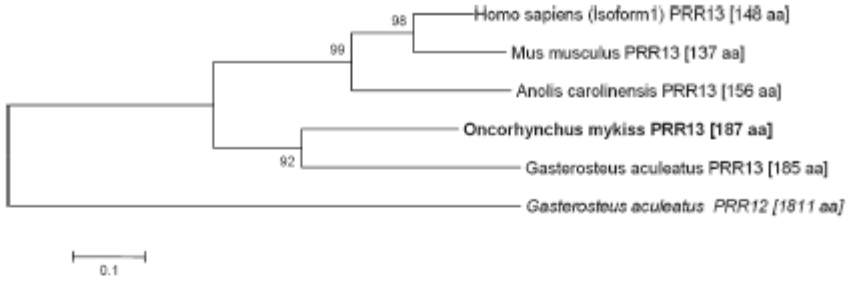

Fig. 2 A phylogenetic tree of selected mammalian, reptilian and piscine PRR13 sequences was constructed by MEGA v3.1 program using the neighbour-joining method. Bootstrap values based on 10,000 replicates are provided to support each partition. The tree was rooted with stickleback PRR12 as an outgroup (in *italic letters*). Rainbow trout PRR13 is labelled by *bold face letters*. Protein lengths (in amino acid residues) are given in *brackets*. The *scale bar* represents a genetic distance of 0.1 amino acid substitutions per site. The following protein sequences were used (GenBank [GB] and Ensembl [E] accession codes): rainbow trout *Oncorhynchus mykiss* (GB: FN598572), three-spined stickleback *Gasterosteus aculeatus* (E: ENSGACP00000008839 and ENSGACP00000006384), red-throated anole *Anolis carolinensis* (E: ENSACAP0000 0000668), mouse *Mus musculus* (GB: NP_079661) and human *Homo sapiens* (GB: NP_060927)

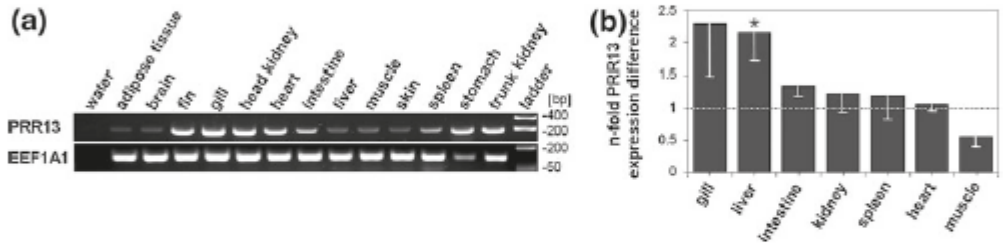

Fig. 3 a Semi-quantitative RT-PCR analysis of rainbow trout PRR13 mRNA expression in different tissues. A 187-bp PRR13 fragment was amplified in parallel with a 101-bp fragment of EEF1A1 to ensure cDNA quality. PCR products were separated in 3% agarose gel. b Real-time quantitative RT-PCR (qRT-PCR) analysis of total PRR13 mRNA expression in different tissues of five rainbow trout from selection strain BORN compared to the commercial strain TCO. Mean expression level in TCO rainbow trout tissues was set as 1.0 (*dotted line*). Means (-SEM) of mRNA expression from BORN rainbow trout tissue were expressed as fraction hereof (*black columns*). The significant expression difference ($P < 0.05$) in liver tissue is marked by an *asterisk*

PRR13 transcripts are broadly expressed in selected trout tissues is in line with findings from Lih et al. (2006), who revealed a general expression profile for human PRR13 transcripts with highest level in normal heart, kidney and leucocytes.

Proline-rich protein 13 (PRR13) mRNA expression was additionally analysed in seven tissues *via* qRT-PCR. Five fish from each rainbow trout selection strain were examined. PRR13 copy number was increased in all determined tissues of selection strain BORN compared to the commercial strain TCO except for muscle tissue where it is decreased (Fig. 3b). The highest fold-change difference was found in gills (2.29-fold), > liver (2.16-fold), > intestine (1.30-fold), > kidney (1.23-fold), > spleen (1.19-fold), > heart (1.05-fold), > muscle (0.56-fold). However, the PRR13 mRNA level was only significantly higher in BORN liver with $P < 0.02$. Even though the PRR13 mRNA abundance in gills of BORN trout is 2.29-fold higher than in gill tissue of the commercial strain TCO, this difference is not significant as a result of the high standard deviation. Human PRR13 protects cells from taxane cytotoxicity thus from natural cytostatica (van Amerongen and Berns 2006). Hence, the increased PRR13 expression in BORN trout might contribute to the protection of cells from damage caused by putatively toxic metabolites of cyanobacteria and algae present in the eutrophic habitat of this strain.

In conclusion, the report for the PRR13 gene in rainbow trout is the first description of a proline-rich protein in this species. The higher gene expression level in nearly all different tissues of the selection strain BORN in comparison with the strain TCO suggests its potential role in the advantage of this rainbow trout strain to cope with environmental stress.

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Studie III.

Comparative molecular characterization of the regucalcin (RGN) gene in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus marena*).

Verleih, M., Rebl, A., Köllner, B., Korytář, T., Anders, E., Wimmers, K., Goldammer, T. *Mol. Biol. Rep.* (2012), **39**(4): 4291-4300.

kurze Zusammenfassung:

Diese Veröffentlichung behandelt das Gen *RGN*, dessen Proteinprodukt durch seine Ca^{2+}-bindende Funktion an der Signaltransduktion sowie der Aufrechterhaltung des zellulären Ca^{2+}-Gleichgewichts beteiligt ist und somit vermutlich einen Beitrag zur Aufrechterhaltung der Körperhomöostase im Rahmen der adaptiven Stressabwehr liefert. Die Studie beinhaltet sowohl die Charakterisierung der *RGN*-Sequenz in der Forelle und dem Ostseeschnäpel als auch vergleichende Analysen der Genexpression in BORN- und Importforellen. Dabei zeigt sich in gesunden und gestressten Forellen beider Linien eine klar differente Regulation der *RGN*-Genexpression, was die Annahme unterstützt, dass RGN eine wichtige Rolle bei regenerativen Prozessen nach Umweltstress spielt.

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Comparative molecular characterization of the regucalcin (*RGN*) gene in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus marena*)

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Abstract The Ca^{2+}-binding protein regucalcin (RGN) is crucial for the regulation of Ca^{2+} ion homeostasis and signal transduction of cells. It is involved in the regulation of Ca^{2+}-dependent protein kinases and Ca^{2+} pump enzymes in cell membranes. Comparative transcriptome analysis in healthy fish of two aquacultured rainbow trout (*Oncorhynchus mykiss*) lines (BORN, TCO) varying in susceptibility to environmental stress identified significant differences in the expression of the *RGN* gene. Therefore, we firstly determined the full genomic DNA and cDNA sequence of *RGN* gene from rainbow trout and comparatively investigated the complete cDNA sequence in another salmonid fish dedicated for local aquaculture, the maraena whitefish (*Coregonus marena*). The sequence coding region translates for proteins of 298 and 299 amino acids (aa), respectively, indicating a high conservation of RGN proteins (95.7% aa identity) between the two related salmonids. In the second place, we generated *RGN* gene expression profiles after pathogen (*Aeromonas salmonicidae* subsp. *salmonicida*) and temperature (8 and 23°C) challenge in the two rainbow trout lines using salmon microarrays and quantitative RT-PCR. The profiles not only verified initially detected gene expression differences, they also display a tissue specific gene expression in dependence from the stressor and time. The differences in gene expression support our assumption that RGN might play a role in recovery of rainbow trout after environmental stress.

Keywords Regucalcin · SMP-30 · Ca^{2+}-binding · Salmon fish · Temperature stress · Infection

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Introduction

Calcium is crucial to a variety of cellular processes and functions. This metal ion is involved in signal transduction by acting as a second messenger, in muscle contraction or in the release of neurotransmitter [1, 2]. Terrestrial vertebrates have to cover their Ca^{2+} requirements through diet or the degradation of bone. In contrast, teleostean bone is extracellular and not suitable for Ca^{2+} extraction, but bony fishes are able to perform an active Ca^{2+} uptake mainly across the gill and intestinal epithelia [3]. Many Ca^{2+}-binding proteins like calmodulin or calpain share the EF-hand as a conserved motif to coordinate Ca^{2+} [4]. Nevertheless, RGN, also known as senescence marker protein-30 (SMP30) lacks this domain [5].

The first report on RGN protein function has been published for rat in 1978 [6]. Meanwhile, the regulatory role of RGN in calcium homeostasis of cells and its impact on the regulation of other physiological cell functions have been investigated. In higher vertebrates, *RGN* gene expression has been detected mainly in liver and kidney tissue [7]. The gene expression is enhanced after oral administration of

Ca^{2+} in rat liver and renal cortex cells [8]. Regucalcin is also reported to play an important role in intracellular Ca^{2+} homeostasis by controlling the amount of Ca^{2+} in cells through activating Ca^{2+} pump enzymes in the plasma membrane and regulating Ca^{2+}-dependent protein kinases like protein kinase C and protein phosphatases in the signal transduction [9]. Furthermore, it is involved in the regulation of nuclear DNA and RNA syntheses in liver cells [10, 11]. RGN has also been shown to have a suppressive effect on the proliferation of hepatoma cells in liver and renal cells [12, 13] as well as on cell death and apoptosis induced by various factors like lipopolysaccharide (LPS) and protein kinase inhibitors (e.g. dibucaine) [14]. Moreover, RGN has been suggested to be involved in the differentiation of hepatocytes and renal epithelia [7]. A recent study by Chakraborti and Bahnson [15] has shown that RGN binds not only to Ca^{2+} but also to Zn^{2+}, whereas only one metal ion is bound to the active site at one time.

We experienced *RGN* in a holistic transcriptome analysis after hybridization of mRNA from healthy liver tissue of two rainbow trout strains with a 16k salmon cDNA microarray chip [16]: TCO (Tacoma, USA) and BORN (Born, Germany). The expression analysis was performed after detection of a strain-specific susceptibility to local environmental stress such as pathogen and temperature challenge in aquaculture farms, reflected in different phenotypically traits such as growth and mortality. The different expression of *RGN* in both rainbow trout strains and the well investigated involvement of RGN in Ca^{2+} ion balance qualified *RGN* as a candidate gene for the description of the strain-specific adaptation potential and the recovery after environmental stress. In the present study, we isolated and characterized regucalcin gene sequences of rainbow trout and maraena whitefish, both used in regional aqua-farming. Gene expression was analyzed from selected tissues of healthy and challenged fish. Therefore, *RGN* mRNA expression was examined after temperature challenge at 8 and 23°C. Although rainbow trout is able to live at a temperature range from 0 to 25°C, its growth is limited to 10–20°C and the optimal temperature in aquaculture is of 15–18°C [17, 18]. We studied also *RGN* transcript level after infection of trout with the pathogen *Aeromonas salmonicida salmonicida*, which causes furunculosis in salmonid fishes [19] and is therefore responsible for considerable losses in aquaculture.

Materials and methods

Animal material and experimental design

For characterization of cDNA and genomic sequences as well as initial expression studies, one year old maraena whitefish as well as two year old rainbow trout of the local selection strain BORN (Born, Germany) and the imported strain TCO (Tacoma, USA) were purchased from a regional aquaculture farm (BIMES, Frauenmark, Germany). Both rainbow trout strains represent the anadromous form (steelhead trout) of the rainbow trout coastal form. Tissue samples have been taken from gill, head kidney, heart, intestine, liver, muscle, spleen as well as trunk kidney and were flash frozen in liquid nitrogen until further use.

TCO and BORN rainbow trout for challenge experiments have been grown simultaneously from eyed eggs in hatching jars to fingerlings in fresh water rearing tanks. This was followed by an adaptation to fresh water glass tanks and further growth from age of 7–8 months until start of the experiments at age of 11–12 month.

For the temperature experiment, 10–15°C acclimated fish of both strains were transferred into three 300 l tanks with a water temperature of 15°C, following an acclimation period of 2 weeks. Subsequently, water tanks were adjusted by 1°C per day from 15 to 8 and 23°C, respectively. After another week of acclimation at the appropriate temperatures organ samples (head kidney, liver, muscle and trunk kidney) of 8 fish per strain and temperature were taken and transferred into RNAlater (25 mM $Na_3C_6H_5O_7$; 9.9 mM EDTA; 5.3 M $(NH_4)_2SO_4$) at 4°C overnight and subsequent stored at −20°C until gene expression analysis.

For the infection experiment, 200 rainbow trout (100 of strain BORN and 100 of strain TCO) were divided among four 300 l tanks (50 animals per strain and tank). Doubling of tanks per strain allowed monitoring and exclusion of tank specific influences on fish. Thirty-seven fish per tank were infected intraperitoneal (i.p.) with a contagious dosage of 1×10^4 cfu (colony forming unit) of the pathogen *Aeromonas salmonicida salmonicida* including four control fish (0 h) of each strain. The remaining thirteen fish per tank have been used as i.p. untreated contact fish for monitoring the behavior of fish in general and for preservation of crowding behavior during the experimental period. All rainbow trout were anesthetized in a benzocaine/water (50 ng/ml) bath before injection and also before sampling. Samples of four fish per strain and time point were taken 0, 12, 24, 48, 72 h as well as 7, 14 and 21 days after infection. Between 72 h and day 7 first clinical furunculosis symptoms were visible. Prepared organs (liver, trunk kidney, muscle) were transferred into RNAlater until further use.

Isolation of nucleic acids

Tissue samples of organs mentioned above were homogenized individually in 1 ml Trizol (Invitrogen, Karlsruhe, Germany) followed by RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase

treatment for 30 min or DNA extraction by using QIAamp DNA Micro Kit (Qiagen). RNA and DNA quantity and quality was determined at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The integrity of RNA was verified by agarose gel electrophoresis using 1% agarose gels.

RGN gene structure from rainbow trout and maraena whitefish

Five µg of total RNA from each, rainbow trout and maraena whitefish, was utilized for reverse transcription using Superscript II (Invitrogen) following manufacturer's protocol. The cDNA was used for subsequent amplification of *RGN* transcript. Gene specific PCR primer pairs Om_RGN_f1, r1 and Om_RGN_f2, r2, listed in Table 1, were derived from a consensus sequence of overlapping rainbow trout ESTs (GenBank accession numbers: CB492687, CA349052, CA349053). The primer sets and further oligonucleotides designed from the transcript product (Tab. 1) were used for *RGN* gene sequence completion. Using a comparative PCR approach, rainbow trout specific primers listed above were used to amplify the *RGN* cDNA sequence of maraena whitefish. 5′- and 3′-RACE experiments were performed to obtain full-length *RGN* coding DNA sequences of both salmonids using the Gene Racer Super ScriptTMII RT Module (Invitrogen) in a touchdown-PCR (preincubation: 95°C; denaturation: 94°C for 30 s; first annealing step: decreasing temperature from 68 to 59°C, 10 cycles of 30 s; extension: 72°C for 2 min; second annealing step: 30 cycles with 94°C for 30 s, 60°C for 30 s, 72°C for 2 min; final extension: 72°C for 7 min). PCR products were analyzed by 1% agarose gel electrophoresis. The same touchdown-PCR program was used to create the genomic rainbow trout *RGN* sequence. Each nucleotide position of both cDNA and genomic DNA fragments was sequenced at least four times.

Table 1 Primer sequences used in this study

Primer name	Sequence (5′–3′)
Om_RGN_f1	CCTGATGGCATGTGTATTGACG
Om_RGN_r1	ACGTGACCTCAGCCTGTAAGG
Om_RGN_f2	TGTGGGAGGAGAAAGAAGGA
Om_RGN_r2	TAGACTCTGGGTCGCATGCT
Om_RGN_3′Race_f1	ACGGCATACGTTTTCAGTTG
Om_RGN_3′Race_f2	CCTGATGGCATGTGTATTGACG
Om_RGN_5′Race_r1	ACGTGACCTCAGCCTGTAAGG
Om_RGN_5′Race_r2	GCTATGTGATCGGAGAGGGA
Om_RGN_qRT_f	AGACAGGGGTGCGTTTGCAGA
Om_RGN_qRT_r	AAGCAACCGCAGGCAGGATGT
Om_EEF1A1_f	TGATCTACAAGTGCGGAGGCA
Om_EEF1A1_r	CAGCACCCAGGCATACTTGAA

RGN gene expression analysis

Quantitative real-time RT-PCR (qRT-PCR) was performed by utilizing the LightCycler Instrument 480 System and FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche, Mannheim, Germany) to determine *RGN* mRNA expression in healthy and challenged BORN and TCO rainbow trout. In detail, 1.5 µg of total organ RNA was reverse transcribed as mentioned above. A fragment of 152 bp was amplified by using gene specific primers Om_RGN_LC_f1, r1 listed in Table 1. In addition, a 101-bp *EEF1A1* fragment was amplified to normalize RGN gene expression utilizing gene specific primers OM_EF-1a_f1, r1 (Tab 1). After an initial denaturation at 95°C for 10 min, cycling conditions were as follows: 40 cycles consisting of denaturation at 95°C/15 s, annealing at 60°C/10 s, extension at 72°C/20 s and ultimately quantification at 79°C (*EEF1A1*) and 81°C (*RGN*), respectively. Serial dilutions of PCR-generated *RGN* fragments (10^3–10^6) were applied and calculated copy numbers served as external standards. The amplified PCR fragments were examined by melting curve analyses and agarose gel electrophoresis was performed to access product size and quality. Statistical significance ($P < 0.05$) was evaluated by using SPSS software (SPSS Inc., Version 15.0) performing parametric *t*-test, nonparametric Mann–Whitney *U*-test and one-way ANOVA applying the Bonferroni method. All experimental data are shown as mean ± SEM.

Statistical analyses

NCBI and Ensemble BLAST have been used to find sequence homology for *RGN* (http://blast.ncbi.nlm.nih.gov/Blast.cgi; http://www.ensembl.org/Multi/blastview). Sequence alignments were carried out using the ClustAlW program [20]. The RGN deduced amino acid sequence was analyzed for signal sequence and motif prediction using ExPASy Proteomics Server [21]. The Molecular Evolutionary Genetics Analysis (MEGA v3.1) package [22] was used to construct a phylogenetic tree based on the deduced RGN amino acid sequences. A bootstrap analysis based on 10,000 iterations was performed to evaluate the robustness of the tree.

Results and discussion

Regucalcin is evolutionary highly conserved

In this study, we isolated and characterized the cDNA sequences of *RGN* from the salmonid fishes rainbow trout

(GenBank accession: FR846198) and maraena whitefish (FR846199). The sequences are 1,323 and 1,158 bp in length (excluding polyadenylated tail). They comprise 5′ untranslated regions (UTR) of 198 and 60 and 3′ UTRs of 228 and 196 bp, respectively. We located two polyadenylation signals (AATAAA) in each of the two sequences: at nucleotide position 1,204–1,209 and 1,305–1,310 in rainbow trout as well as at position 1,070–1,075 and 1,140–1,145 in maraena whitefish. The poly(A)-tail is situated 114 or 13 and 82 or 12 bp downstream in trout and maraena, respectively. The protein encoding cDNA sequence of rainbow trout contains a deletion of three nucleotides in comparison to its orthologue in maraena whitefish situated three base pairs downstream the ATG start codon. This deletion leads to divergent open reading frame (ORF) lengths of 897 and 900 bp, respectively. Hence, rainbow trout *RGN* sequence encodes for a protein of 298 amino acids (aa) with a hypothetical molecular weight of 32.82 kDa and a calculated pI of 5.28. Maraena whitefish coding sequence results in a protein of 299 aa with a hypothetical molecular weight of 33.13 kDa and a calculated pI of 5.18.

The predicted amino acid sequences of rainbow trout and maraena whitefish regucalcin have no signal peptide. Sequence homology search using BLAST identified a SGL multi-domain (SMP-30/Gluconolactonase/LRE-like region). This domain family includes a SMP-30 region stretching from amino acid position 16–262 of rainbow trout regucalcin. RGN has also been shown to have a Zn^{2+} dependent gluconolactonase activity [15].

No significant homology with sequences of other known calcium-binding proteins of rainbow trout could be detected. Databank researches showed furthermore that the proteins lack the EF-hand motif [4], which functions as a Ca^{2+}-binding site in many other calcium-binding proteins like calmodulin or calpain. Shimokawa and Yamaguchi [5] showed that regucalcin from rats comprises a hydrophilic region between nucleotide position 100 and 200, which might function as a Ca^{2+}-binding site instead. A recent study on the crystal structure of regucalcin revealed that the protein comprises a 6-bladded β-propeller fold and that only one metal ion can be bound to the active site at one time. Furthermore, the Ca^{2+} ion was proven to coordinate with protein residues E17, N153 and D203 and three water molecules [15]. These amino acid positions are conserved in regucalcin of rainbow trout and maraena whitefish (Supplementary Figure S1).

We compared predicted RGN amino acid sequences from sixteen species including all known fish RGN sequences as well as the respective sequences of three mammalian, two avian as well as one reptilian and one amphibian species. In total, the proteins share 61.2% identical and similar amino acids. Former studies already indicated an evolutionary high conservation of regucalcin protein among tetrapods [23]. This suggests that the protein is of high biological importance depending on a well conserved structure, which seems to be essential to ensure its physiological function. In detail, due to their short evolutionary distance, the cDNA and derived amino acid sequences of rainbow trout and maraena whitefish are highly conserved. They share 95.3% of their nucleotides within the ORF leading to a remarkably high identity of 95.7% of the encoded proteins. Database researches revealed one additional entry for regucalcin of a salmonid species, namely the Atlantic salmon (*Salmo salar*). About 95.0% of the total aa residues from rainbow trout, maraena whitefish and atlantic salmon are identical, whereas only 1.0% is different at all. Due to the additional three nucleotides in the ORF of the *RGN* cDNA sequence from maraena whitefish, the predicted protein shares a serin (S) at the third amino acid position with its homologues from higher vertebrates, while regucalcin from rainbow trout and Atlantic salmon, lacks this amino acid. This deletion occurs furthermore only in regucalcin from pike (*Esox lucius*). In total, rainbow trout and Atlantic salmon differ at four positions of their predicted amino acid sequences, while trout or salmon and maraena differ in at least twelve positions.

As expected, the protein length of all species compared in Supplementary Figure S1 comprises 299 amino acid residues, except for those mentioned before as well as for stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*), whose proteins are one and four aa shorter, respectively. The deletion of stickleback RGN occurs after position 127. Zebrafish misses one aa after position 94 and three amino acids after position 127. Proteins of all known fish RGN share in total 69.9% identical, strongly and weekly similar amino acids.

We constructed a neighbor-joining phylogenetic tree based on all known regucalcin amino acid sequences from fish as well as the respective sequences of higher vertebrates with the program MEGA version 3.1 (Fig. 1). The tree elucidates the evolutionary relationship of regucalcin. RGN proteins of all salmonids form a cluster with their homolog from pike, all grouped together into the superorder Protacanthopterygii, whereas proteins of rainbow trout and Atlantic salmon show the closest relationship within this clade. They represent a sister group to regucalcin sequences of the Acanthopterygii, which are clearly separated on the one hand into the two pufferfishes (*Takifugu rubripes*, *Tetraodon nigroviridis*) and on the other hand into stickleback as well as medaka ricefish (*Oryzias latipes*). Interestingly, proteins from zebrafish and channel catfish (*Ictalurus punctatus*) form a separate group to all other teleostei, being closer related to regucalcin from tetrapods than to RGN from fish. Within the tetrapods,

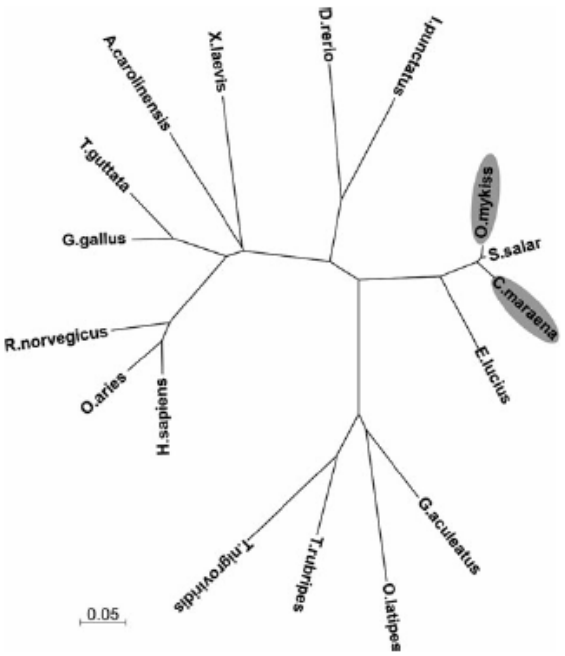

Fig. 1 Radial phylogenetic tree inferred from the 17 RGN amino acid sequences. Used sequences are listed in Table 2. The tree was constructed using MEGA 3.1 with neighbor-joining algorithms. Bootstrap analyses are based on 10,000 replicates. *Scale bar* indicates the number of changes per position for a unit branch length. Formation of RGN proteins from rainbow trout and maraena whitefish which are derived in this study are marked by *gray underlay*

there is a clear distinction of amphibian and reptilian regucalcin from those of birds and mammals.

The *RGN* gene from rainbow trout is structured into 8 exons and 7 introns (Fig. 2). The intron length varies from 73 bp (intron 5) up to 1,604 bp (intron 7). No *RGN* transcript variant has been identified in rainbow trout. Except of intron 1, which is located two base pairs upstream the ATG start codon all other introns are situated within the ORF. Only for zebrafish and stickleback a 5′-UTR-encoding exon is also known so far (compare Table 2). But since *RGN* sequences of pufferfishes submitted in databases are limited to coding regions, possible non-coding exons might not been proven yet. However, the *RGN* gene structure differs within the teleostei. While seven *RGN*-encoding exons are conserved in rainbow trout, stickleback, medaka ricefish and pufferfishes, only six coding exons are known for zebrafish. Coding exons two, five and six are highly conserved in teleost. In contrast, coding exons three and four of trout, stickleback, medaka ricefish and pufferfishes seems to be replaced by only one coding exon (exon three) in zebrafish, the intron between coding exons 3 and 4 seems to be completely removed. Zebrafish shares this feature with higher vertebrates, including clawed frog (*Xenopus laevis*), red throated anole (*Anolis carolinensis*), chicken (*Gallus gallus*) and human (*Homo sapiens*).

Furthermore, intron length differs between teleostean regucalcin genes. While green spotted puffer (*Tetraodon nigroviridis*) has relatively short introns reaching from 66 to 105 bp, introns of medaka ricefish reach lengths up to 5252 bp.

RGN mRNA expression reveals significant differences between two rainbow trout strains

The local selection strain BORN shows a higher adaptation potential towards the challenging conditions of brackish water and other stressors such as infection and pollution compared to the imported steelhead strain TCO [24, 25]. Initial hybridization experiments using a 16 k cDNA microarray chip [16] with total mRNA as sample from liver tissue of healthy fish of both rainbow trout strains identified numerous differentially expressed genes. *RGN* belonged to the top regulated genes [26, 27] with a fold change of 2.33 between both strains.

Regarding this, we compared the expression profile of regucalcin in eight different tissues of healthy BORN and TCO rainbow trout. The highest copy numbers (normalized to EEF1A1) could be detected in trunk kidney of both strains. In comparison, normalized *RGN* expression was lower in liver (0.4-fold), >intestine (0.8-fold), >gill (1.4-fold), >spleen (3.2-fold), >head kidney (4.4-fold), >heart

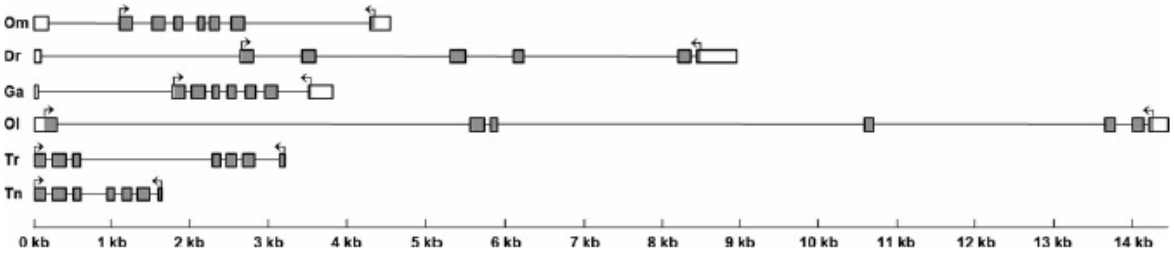

Fig. 2 Comparative genomic structure of rainbow trout *RGN* and its teleostean homologues (Table 2). Exons are represented by *boxes* and introns by *scaled linking lines*. Coding regions are highlighted in *grey*. *Black arrows* indicate the positions of the start and stop codons

Table 2 Sources of RGN nucleotide and protein sequences used in this study

Organism		Accession number	
Scientific name	Common name	Nucleotide ID	Protein ID
Oncorhynchus mykiss (Om)	Rainbow trout	FR846198	CCA61104
Coregonus marena (Cm)	Maraena whitefish	FR846199	CCA61093
Salmo salar (Ss)	Atlantic salmon	NM_001141394	NP_001134866
Esox Lucius (El)	Northern pike	BT079099	ACO13523
Danio rerio (Dr)	Zebrafish	NM_205746	NP_991309
Ictalurus punctatus (Ip)	Channel catfish	NM_001200368	NP_001187297
Gasterosteus aculeatus (Ga)	Three-spined stickleback	ENSGACT00000020320	ENSGACP00000020281
Oryzias latipes (Ol)	Medaka	ENSORLT00000023633	ENSORLP00000023632
Takifugu rubripes (Tr)	Japanese pufferfish	ENSTRUT00000028327	ENSTRUP00000028215
Tetraodon nigroviridis (Tn)	Green spotted puffer	ENSTNIT00000013714	ENSTNIP00000013520
Xenopus laevis (Xl)	African clawed frog	NM_001085655	NP_001079124
Anolis carolinensis (Ac)	Carolina anole	ENSACAT00000011967	ENSACAP00000011725
Taeniopygia guttata (Tg)	Zebra finch	XM_002197807	XP_002197843
Gallus gallus (Gg)	Chicken	NM_204729	NP_990060
Rattus norvegicus (Rn)	Rat	NM_031546	NP_113734
Ovis aries (Oa)	Sheep	NM_001130935	NP_001124407
Homo sapiens (Hs)	Human	Transcript1 NM_004683	NP_004674
		Transcript2 NM_152869	NP_690608

(5.1-fold) and muscle (13.0-fold) of BORN trout and intestine (0.3-fold), >gill (1.1-fold), >spleen (1.3-fold), >liver (1.5-fold), >muscle (5.0-fold), >head kidney (6.5-fold) and heart (8.0-fold) of TCO trout (Fig. 3). In human and rat, regucalcin is mainly expressed in liver and kidney cells while in mouse the expression is restricted to liver [28, 29]. Additionally, regucalcin was found to be moderately expressed in human heart and pancreas as well as in rat brain [30]. We also found *RGN* gene expression in liver and kidney of rainbow trout with the highest expression value in renal cells of both rainbow trout strains and a remarkable high mRNA level at least in hepatic cells of

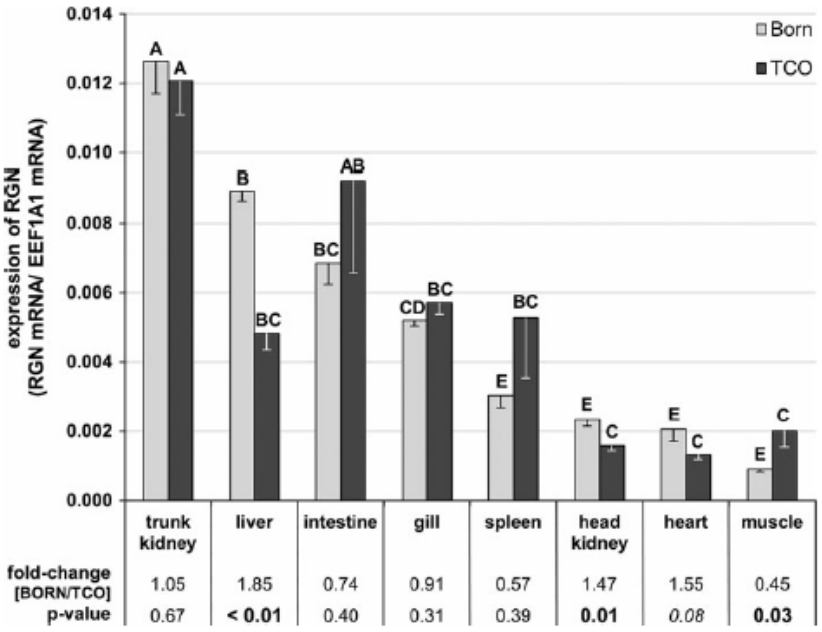

Fig. 3 qRT-PCR analysis of tissue specific *RGN* expression in six healthy rainbow trout of BORN (*grey column*) and TCO (*black column*) strain. Means (-SEM) are normalized to the housekeeping gene EEF1A1. *The table below the graph* shows the fold-change and *P*-value in tissue specific gene expression between both strains (ratio BORN/TCO), significantly and tendentially differences written in *bold* and *italic letters*, respectively. The mRNA gene expression of each strain individually was compared between examined tissues using one-way ANOVA applying the Bonferroni method. Means with different letters are significantly different ($P < 0.05$) from each other

BORN trout. But furthermore, we could demonstrate that regucalcin is ubiquitous expressed in rainbow trout. We found a remarkable high expression level in intestine, gill or spleen tissue and a comparably lower expression in head kidney, heart and muscle. Since the main organs for Ca^{2+} absorption in fish are gills and intestine [31], an elevated *RGN* expression in these organs is expected.

Moreover, the *RGN* expression profile shows relevant differences in the expression of both rainbow trout strains, confirming the results of the initial transcriptome analyses (see above). In detail, the transcript level of BORN trout was elevated significantly in liver by 0.85-fold ($P < 0.01$) and head kidney by 0.47-fold ($P = 0.01$) and it was tendentially increased in heart tissue by 0.55-fold ($P = 0.08$) compared to *RGN* expression of TCO trout. In contrast, we found a significantly enhanced mRNA expression in muscle tissue by 1.22-fold ($P = 0.03$) of TCO trout.

Liver and head kidney are important organs for metabolism and immune response, respectively. Regucalcin is shown to play an important role in liver and kidney of rats [32]. The protein has a suppressive effect on cell death and apoptosis [14] and regulates calcium-dependent metabolic enzymes like the cytosolic pyruvate kinase in liver cells [33]. Furthermore, it is involved in the regulation of calcium reabsorption in rat kidney. Therefore, the enhanced *RGN* expression in both organs of BORN trout might support a more purposeful maintenance of normal organ functions likely including Ca^{2+}-balance in this strain.

Regucalcin expression is altered in head kidney, trunk kidney and muscle but not in liver tissue in comparison of 8 and 23°C

On the basis of the differential adaptation potential of BORN and TCO rainbow trout and the fact that *RGN* belonged to the top regulated genes of the hybridization experiment mentioned above, we compared the expression of *RGN* in both strains after thermal and infection stress.

Samples were taken for each rainbow trout strain at 8 and 23°C, respectively. We evaluated the *RGN* mRNA expression in trunk kidney (highest transcript level in healthy fish of both strains, Fig. 3) as well as in liver, head kidney and muscle tissue (significantly different expression levels between healthy fish of both strains).

Significant expression differences could be detected in head and trunk kidney as well as in muscle tissue but not in liver (Fig. 4). *RGN* mRNA expression in liver was higher

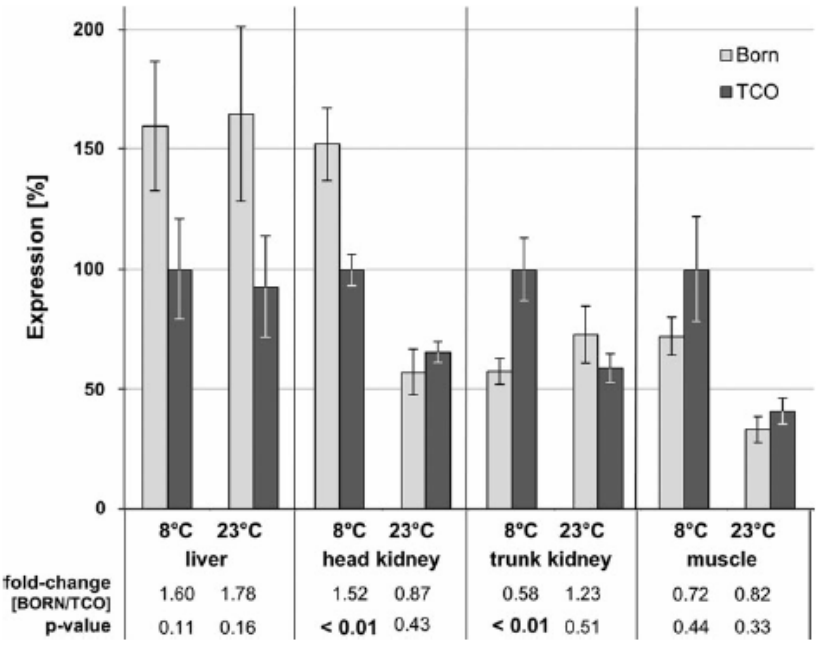

Fig. 4 Transcript level of rainbow trout RGN gene after temperature challenge at 8 and 23°C measured by qRT-PCR. *RGN* mRNA expression of eight BORN (*grey column*) and TCO (*black column*) rainbow trout was examined in liver, head kidney, trunk kidney and muscle tissue after maintenance at 8 and 23°C. Means (normalized to the housekeeping gene EEF1A1) of the tissue specific *RGN* gene expression in BORN rainbow trout at 8°C are set as 100%, the expression at 23°C as well as TCO gene expression at 8 and 23°C is shown as fraction hereof. *Error bars* indicate ±SEM. *Asterisks* indicate statistical different expressions with $P < 0.05$ (*) and $P < 0.01$ (**). *The table below the graph* shows the fold-change and *P*-value of the tissue and strain specific gene expression between 8 and 23°C, significantly differences in *bold letters*

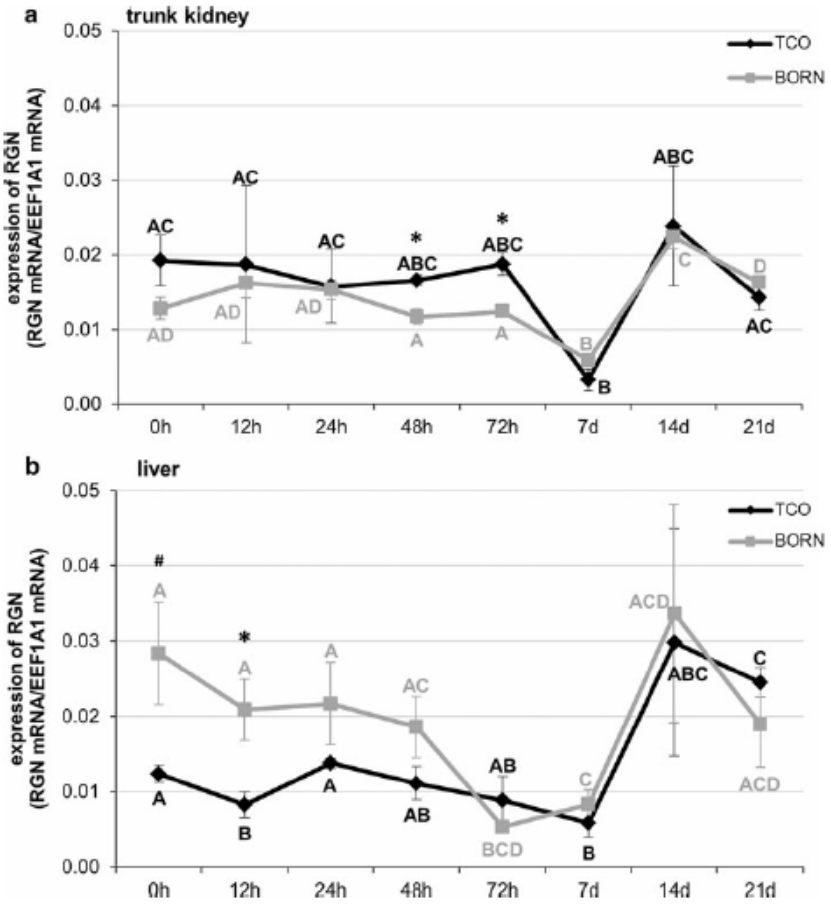

Fig. 5 qRT-PCR analysis of temporal *RGN* gene expression after infection with pathogen *Aeromonas salmonicida salmonicida* in **a** trunk kidney and **b** liver tissue. *Grey* (BORN rainbow trout) and *black* (TCO rainbow trout) symbols represent the mean values (±SEM) of *RGN* copy numbers normalized to housekeeping gene EEF1A1 at each time point. Marginally significant expression levels are indicated by *hash sign* (#). *Asterisks* indicate statistical different expressions between both strains with $P < 0.05$ (*). Means with different *capital letters* (A–D) indicate a significant ($P < 0.05$) different RGN gene expression within strain BORN and strain TCO between the different time points (0 h, 12 h, 24 h, 48 h, 72 h, 7 days, 14 days, 21 days)

in BORN rainbow trout than in TCO at both temperatures (8°C: +60%; 23°C: +78%) but the temperature change seemed to have no influence on the expression within each strain. In contrast, we observed a significant lower *RGN* transcript level in head kidney of both strains at 23°C in comparison to 8°C (BORN: −94%, $P < 0.01$; TCO: −34%, $P < 0.01$). In trunk kidney *RGN* transcript level in TCO trout was about 41% lower at 23°C compared to 8°C while relative *RGN* copy number of BORN trout remained nearly constant. Moreover, both strains showed a significant lower transcript level in muscle tissue at 23°C than at 8°C (BORN: $P < 0.01$; TCO: $P = 0.01$). Beside the strain-specific alterations in liver, expression differences between BORN trout and TCO trout could also be detected in head and trunk kidney. While the *RGN* expression in head kidney at 8°C was significant higher in BORN trout than in TCO (+52%, $P < 0.01$), it was significant lower in trunk kidney (−41%, $P < 0.01$). Expression differences could also be observed in muscle tissue at 8°C although they were not significant.

In general, we observed significant changes in *RGN* mRNA expression for each strain in head kidney and muscle tissue as well as in trunk kidney of TCO at 23°C in comparison to 8°C, but not in liver tissue. It might be important for liver cell functions to keep a steady state level of *RGN* mRNA expression at stress conditions of high temperature, since the protein is involved the regulation of Ca^{2+} ion homeostasis and signal transduction of cells. The remarkable lower transcript level in the other three tissues might save energy for basic metabolic processes. Since almost no difference between both rainbow trout strains could be observed at higher temperatures, RGN seems to have no remarkable influence in a different temperature adaptation of both strains at least at examined temperature of 23°C.

RGN mRNA expression is elevated at day 14 after infection

Tissue samples were taken after 0, 12, 24, 48 and 72 h as well as at day 7, 14 and 21 to examine rainbow trout *RGN* gene expression after infection with the pathogen *A. salmonicida salmonicida*.

A 44 k salmonid oligo microarray chip was utilized in order to perform transcriptome analysis [34]. Transcriptome profiling studies using trunk kidney tissues of four time points (0 h, 72 h, 7 day, 21 day) from BORN and TCO trout (results not shown) revealed a decreased *RGN* transcript level at day 7 (BORN: 0.43-fold; TCO: 0.32-fold), which returns to the original transcript level (day 0) at day 21. The *RGN* gene expression in trunk kidney and liver tissue was furthermore determined for all time points via qRT-PCR (Fig. 5) to validate the chip results for trunk kidney in the first place and to gain a more profound insight in the expression profile after infection in the second place.

Only minor expression changes could be observed from hour 0 to 72 in trunk kidney of both strains. The transcript level was slightly different between TCO and BORN trout, being significantly higher in TCO trout after 48 and 72 h (for both $P = 0.02$). Astonishingly, *RGN* mRNA level decreases significantly at day 7 in both strains (BORN: $P = 0.02$; TCO: $P = 0.01$) and increases again at day 14, resulting in a peak expression level (BORN: $P < 0.01$). At day 21 the *RGN* copy number returns to the initial transcript level.

Changes of the *RGN* transcript level in liver tissue over the course of infection were comparable to that in trunk kidney. However, the decrease varied clearly between BORN and TCO rainbow trout. While the *RGN* mRNA amount in TCO trout decreased steady but continuously between 0 h up to 7 days ($P = 0.04$), it decreased faster in BORN trout, reaching its lowest point already 72 h after infection ($P = 0.03$). According to the expression profile in head kidney, the transcript level in liver increased highly, but due to the high standard derivation not significantly from day 7 to day 14.

Conclusions

In summary, the present data show a high conservation of rainbow trout RGN protein with its homologues from other species, including the SGL multi-domain, which is stretched nearly over the whole sequence length. This indicates the high biological importance of the protein. Investigations of the *RGN* expression profile revealed a gene regulation after temperature challenge and infection stress with considerable differences between the two rainbow trout strains BORN and TCO especially in liver tissue, suggesting the importance of regucalcin in the functions of this organ and confirming the significant expression differences between both strains we found in healthy rainbow trout. Taken together the results suggest a possible involvement of regucalcin in the different adaptation potential of both rainbow trout strains and the recovery after environmental stress.

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Studie IV.

Structural characterization and expression analyses of duplicated iron-sulfur cluster scaffold (ISCU) gene in salmonid fish.

Verleih, M., Rebl, A., Köllner, B., Korytář, T., Köbis, J. M., Kühn, C., Wimmers, K., Goldammer, T.

Gene (2013), **512**(2): 251-258.

kurze Zusammenfassung:

Die molekulargenetische Charakterisierung des Gens *ISCU* ist Inhalt der Studie IV. Das Protein ISCU reguliert wichtige Stoffwechsel- und Ionentransportprozesse der Zelle über die Bindung und Weitergabe von [Fe-S]-Clustern. In der Studie wurde die Duplikation des Gens in Regenbogenforelle und Ostseeschnäpel nachgewiesen und die entsprechenden Gensequenzen charakterisiert. Zusätzlich wurde ein spezifisches Expressionsprofil der Genvarianten in der Forelle erstellt. Insgesamt konnte trotz partiell differenter Genexpression in BORN- und Importforellen zwei Wochen nach einer Pathogen-Infektion der vermutete Einfluss des Gens auf die differente Robustheit der BORN- und Importforellen nicht bestätigt werden.

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Iron–sulfur cluster scaffold (ISCU) gene is duplicated in salmonid fish and tissue and temperature dependent expressed in rainbow trout

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ABSTRACT

The iron–sulfur cluster protein ISCU is a scaffold protein tasked with the building and mediation of iron–sulfur [Fe–S]-clusters. These are crucial for [Fe–S]-enzymes, which are involved in essential biological cell processes like metabolism or ion transport. Analysis of *ISCU* in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus maraena*) revealed the existence of two gene variants in each of the two salmonids. This study presents the characterization of the duplicated *ISCU* cDNA sequences in both species as well as the comparative functional analysis of the genes in healthy and affected fish of two rainbow trout strains differing in trait robustness under regional aquaculture conditions. Coding sequences of trout *ISCUA* and *ISCUB* genes are spanning over five exons. Open reading frames (ORF) of trout (*ISCUA*: 495 bp, *ISCUB*: 498 bp) and whitefish (*ISCUA* and *ISCUB*: 495 bp) genes encode for evolutionary highly conserved proteins and share 72% sequence similarity with human ISCU.
Transcriptome analyses comparing healthy fish of the local rainbow trout strain BORN and the import strain TCO revealed strain-specific expression patterns for *ISCU*. Expression analyses by quantitative RT-PCR indicated remarkable differences between the transcript level of the gene variants *ISCUA* and *ISCUB*. Moderate temperature challenge (8 °C and 23 °C) suggests a generally higher transcript level of the two gene variants at 8 °C in the liver, spleen, and gill of both strains. However, no remarkable differences between the strains occurred in the temperature-dependent *ISCU* gene expression profiles. The experimental infection with *Aeromonas salmonicida* resulted in a different *ISCU* gene expression in the gill and trunk kidney of both strains after two weeks, suggesting a specific role of the scaffold gene in rainbow trout strain BORN, regarding the recovery after infection. Although results partially reflect the expected strain- and tissue-specific *ISCUA* and *ISCUB* regulation in rainbow trout, the data do not support the assumed association of *ISCU* with the trait robustness.

1. Introduction

[Fe–S]-clusters represent inorganic cofactors in many proteins that have essential physiological functions. A complex system of different factors is involved in the biogenesis of eukaryotic [Fe–S]-clusters. The mitochondrial ISC (iron–sulfur cluster) system has its origin in similar eubacteria systems (Lill and Muhlenhoff, 2006). It is known that [Fe–S]-cluster maturation is performed by ISCU, which serves as a scaffold protein for the cluster assembly (Garland et al., 1999). Furthermore, the protein transfers the [Fe–S] cluster to the appropriate apo-[Fe–S] proteins, including many metabolic key enzymes, such as aconitase which is essential for the citric acid cycle (Beinert and Kennedy, 1993), NADH dehydrogenase that is part of the oxidative phosphorylation in mitochondria (Galante and Hatefi, 1979), or hydrogenases which are involved in anaerobic metabolism (Hampl et al., 2011). [Fe–S]-cluster enzymes often function as electron acceptors and donors and are therefore important for mediation of ion transport, gene regulation, and many other fundamental biological processes in the cell (Lill and Muhlenhoff, 2005).

In this study, we describe the [Fe–S] cluster mediating scaffold gene *ISCU* from rainbow trout, which showed distinct expression differences in an initial transcriptome study, comparing the two steelhead rainbow trout (*Oncorhynchus mykiss*) strains BORN (Born, Germany) and TCO (Tacoma, USA) (Rebl et al., 2012a). The local strain BORN has been bred in the Institute of Fisheries Born in brackish water for the last

Abbreviations: aa, amino acid(s); ARCN1, archain 1; cDNA, DNA complementary to RNA; DHDDS, dehydrodolichyl diphosphate synthase; EEF1A1, elongation factor 1-alpha; EST, expressed sequence tag; GRASP, Glaciogenic Reservoir Analogue Studies Project; IPA, Ingenuity Pathways Analysis; ISCU, iron–sulfur cluster scaffold protein; MARCH5, membrane-associated ring finger (C3HC4) 5; NADH, reduced form of nicotinamide adenine dinucleotide; NELL2, neural epidermal growth factor-like-like protein 2; ORF, open reading frame; PRR13, proline-rich protein 13; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; RGN, regucalcin; SNP, single nucleotide polymorphism.

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37 years. It shows a higher adaptation potential toward numerous environmental stressors present in semi-open aquaculture such as pollution, temperature fluctuations or pathogens, compared to the imported rainbow trout strain TCO, typically used in local fish farming (Anders, 1986; Rebl et al., 2011; Verleih et al., 2012). In this study, we aim to clarify the genetic background of this phenomenon. Holistic transcriptome studies of healthy and infected rainbow trout classified *ISCU* as one of the top-regulated genes. Comparative analyses using 16 k salmon cDNA microarrays (von Schalburg et al., 2008) hybridized with mRNA isolated from healthy liver and spleen tissue of BORN and TCO trout revealed expression differences of genes involved in various cell functions including ISCU (Rebl et al., 2012a). Information about a number of top regulated genes of this investigation was published previously (summarized in Fig. 1). For example, rainbow trout specific gene sequences were characterized for *PRR13* (proline-rich 13; mediates cytostatic resistance) (Verleih et al., 2010), *MARCH5* (membrane-associated ring finger [C3HC4] 5; involved in protein ubiquitination) (Rebl et al., 2011), *DHDDS* (dehydrodolichyl diphosphate synthase; participated in the formation of glycosyl carrier-lipid dolichol) (Rebl et al., 2009a), *NELL2* (neural epidermal growth factor-like-like protein 2; regulates cell growth and differentiation) (Rebl et al., 2012b), *ARCN1* (archain 1; involved in intracellular protein transport) (Rebl et al., 2009b) and *RGN* (regucalcin also named senescence marker protein-30; regulates Ca^{2+} ion homeostasis) (Verleih et al., 2012). The data provide a first insight into the likely involvement of these genes in the differing adaptation potential of the local selection strain BORN compared to imported rainbow trout.

The fact that ISCU is a key player of iron–sulfur cluster assembly together with results of the initial transciptome analysis indicated the gene as a probable candidate for the differing adaptation potential of BORN rainbow trout. Therefore, *ISCU* gene sequences were isolated and characterized from rainbow trout and comparatively from the close salmonid relative marena whitefish (*Coregonus maraena*), both used in regional aqua farming. Subsequently, we investigated tissue specific *ISCU* gene expression in BORN and TCO rainbow trout under common aquacultural conditions, following temperature challenge (8 °C and 23 °C) and after pathogen infection (*Aeromonas salmonicida*), respectively. *ISCU* mRNA expression might point to strain specific strategies in coping with challenging temperatures and infection since the protein is involved in the maturation of metabolic relevant

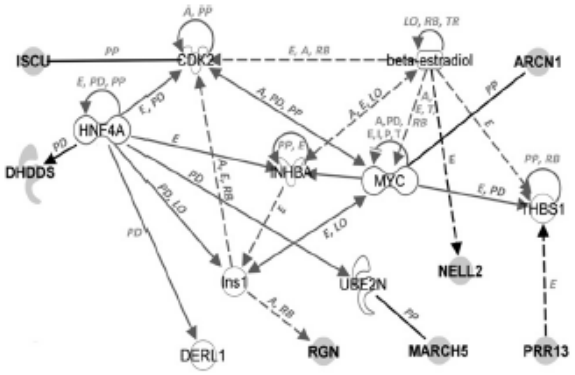

Fig. 1. Network of different expressed candidate genes (bold letters, shaded in gray) in liver of healthy BORN and TCO trout prepared by online tool IPA (Ingenuity® Systems, www.ingenuity.com). Abbreviations on the connections indicate the kind of relation: protein–protein interaction (PP), activation (A), support of expression (E), inhibition (I), proteolysis (P), secretion/release (S), protein–DNA binding (PD), proteolysis (P), localization (LO), binding regulation (RB), translocation (TR), transcription (T). Arrows specify direction and direct (continuous line) or indirect (dashed line) influence between genes. Bilateral arrows show reciprocal regulation. Blocked lines represent inhibitory regulation.

[Fe–S]-proteins. Therefore, iron as an essential nutrient for bacteria (Ratledge and Dover, 2000), is bound in the [Fe–S]-cluster matured by ISCU. In addition, the direct correlation between temperature and metabolism has been shown in various studies (Blank et al., 2007; Kieffer et al., 1994; White et al., 2012).

2. Material and methods

2.1. Experimental animals, temperature challenge and infection experiment

For initial transcriptome and sequence analyses, two-year old rainbow trout of the local selection strain BORN (Born, Germany) and the imported strain TCO (Tacoma, USA), held in freshwater aquaculture ponds, as well as one-year old maraena whitefish were obtained from fresh water aquaculture ponds at the Binnenfischerei Mecklenburg GmbH Schwerin (Frauenmark, Germany). Organ samples (liver, spleen, head kidney, trunk kidney, gill, musde, and brain) of six fish per strain were taken and frozen in liquid nitrogen immediately until further use.

For the temperature study, BORN and TCO trout were grown simultaneously from eyed eggs to fingerlings under similar conditions in fresh water, followed by an adaptation to fresh water glass tanks at age of 7–8 months and further growth. The experiment was carried out with 10-month old rainbow trout of both strains (20 fish each). Ten fish each were transferred into two separate 300 l freshwater tanks and adapted to 15 °C for two weeks. Following this initial acclimation phase, the water temperature was gradually adjusted by 1 °C per day until respective temperatures of 8 °C and 23 °C were reached. The final temperature was maintained for one week. Fish were sacrificed with an overdose of benzocaine and liver, spleen, gill and brain tissue were obtained from all fish and stored in RNAlater (25 mM $Na_3C_6H_5O_7$; 9.9 mM EDTA; 5.3 M $(NH_4)_2SO_4$) until further use.

For the infection experiment, 33 fish and four control fish (0 h) were treated and infected with the furunculosis-causing pathogen *A. salmonicida salmonicida* as described in Verleih et al. (2012). Gill samples were taken 0 h as well as 7 and 21 days after infection and transferred into RNAlater until further use.

2.2. Nucleic acid extraction

Flash frozen tissue samples were homogenized individually in 1 ml Trizol (Invitrogen, Karlsruhe, Germany) and total RNA or DNA was extracted by using RNeasy Mini Kit (Qiagen, Hilden, Germany) or QIAamp DNA Micro Kit (Qiagen). RNA and DNA quantity and quality were determined at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) while agarose gel electrophoresis was used to determine the integrity of RNA.

2.3. Isolation and structural analyses of ISCU gene from trout and whitefish

Trout *ISCUA* gene sequence was derived from three rainbow trout ESTs (GenBank accession numbers: BX076238, BX077022, and BX077023). Additional BLAST searches revealed two ESTs (DFCI database accession numbers: TC146269 and TC138863) according to a second *ISCU* gene variant. Based on the assembled rainbow trout sequence, gene specific primer pairs were derived. Assuming well conserved *ISCU* sequences among salmonid fish, trout specific primers were used to derive first sequence fragments of *ISCU* from maraena whitefish, which provided the basis to generate gene specific primers for both whitefish *ISCU* genes. All primers used in this study are listed in Table 1. Subsequent blast analysis (NCBI: http://www.ncbi.nlm.nih.gov/, GRASP: http://web.uvic.ca/grasp/ or DFCI: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon) identified 214 ETSs matching with ISCUA or ISCUB, but no further gene variant.

For sequence analyses, 5 µg of RNA from both, trout and whitefish, was reverse transcribed using Superscript II (Invitrogen) to derive a cDNA template following the manufacturer's protocol. In order to

obtain full-length trout *ISCU* cDNA sequence, 5′- and 3′-RACE experiments were conducted using the Gene Racer Super ScriptTMII RT Module (Invitrogen) and the following touchdown-PCR protocol: 10 cycles including 5 min pre-incubation (95 °C), 30 s denaturation (94 °C), annealing for 30 s (decreasing from 68 to 59 °C), and extension for 2 min (72 °C) followed by 30 cycles with 30 s denaturation (94 °C), annealing for 30 s (60 °C), extension for 2 min (72 °C) and final extension for 7 min (72 °C). The same touchdown-PCR program was used to amplify the genomic rainbow trout *ISCU* sequence. Each nucleotide position of cDNA fragments was sequenced at least four times.

Functional relationship of hepatic and splenic candidate genes was illustrated by using Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, Ingenuity, CA, USA; www.ingenuity.com). NCBI and Ensemble databases were searched with the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi; http://www.ensembl.org/Multi/blastview) to find sequence homologies. Basic physical and chemical properties of putative trout ISCU protein were analyzed by the ProtParam tool at the Expasy Proteomics Server (www.expasy.ch/cgi-bin/protparam) (Wilkins et al., 1999). For phylogenetic analyses, conceptually translated amino acid sequences (http://web.expasy.org/translate/) were compared by the Molecular Evolutionary Genetics Analysis (MEGA v3.1) package (Kumar et al., 2004) using the Neighbor-Joining method. Bootstrap values are based on 10,000 iterations.

2.4. Microarray analyses

An initial comparison of healthy BORN and TCO rainbow trout by microarray technology using a salmonid 16 k cDNA microarray (von Schalburg et al., 2008) identified 147 genes including *ISCU* as different expressed in liver and spleen tissue. Details on this holistic transcriptome analysis are described in Rebl et al. (2009a, 2012a). Further transcriptome analyses were achieved on a 44 k salmonid oligo microarray chip (Agilent, Waldbronn, Germany) to reveal expression differences in fish of both rainbow trout lines infected with a pathogenic dosage of *A. salmonicida* (Rebl et al., 2012b). For this, total RNA of trunk kidney and gill tissue from BORN and TCO rainbow trout were analyzed for three time points after infection (0 h, 7 and 21 days). For each time point, samples from two animals per strain were included in hybridization experiments. Expression profiling and statistical analyses were done according to MIAME Standards by ATLAS Biolabs GmbH (Berlin, Germany) (Wagner et al., 2007).

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
Amplification of ISCUA and ISCUB coding sequence from rainbow trout	
Om_ISCU_A_f1	CCGCCTACCACAAGAAGGTA
Om_ISCU_A_r1	ACCTGGCATCCACAATCTTC
Om_ISCU_B_f1	CGCTCGCATGGTTTCAGACTG
Om_ISCU_B_r1	CCTTGGTAGTAGGGTGCAGGA
Om_ISCU_A_f2	AGACAAACAGCGAACATGG
Om_ISCU_A_r2	ACTGGGAAACCACAGAATGC
Om_ISCU_B_f2	CATACAGAACACGGATATTGC
Om_ISCU_B_r2	CCCAATGGAACACACCCATA
Amplification of ISCUA and ISCUB coding sequence from maraena whitefish	
Cm_ISCU_A_f1	AGACAAACAGCGAACATGG
Cm_ISCU_A_r1	ACTGGGAAACCACAGAATGC
Cm_ISCU_B_f1	CATTTCAGACAAACCGTGAACA
Cm_ISCU_B_r1	AACATTGGCAAAGCCCTCAC
Amplification of ISCUA and ISCUB 3′- and 5′- ends from rainbow trout	
Om_ISCU_A_3′Race_f1	TGTGGCATTCTGTGATTTCC
Om_ISCU_A_3′Race_f2	TTCTAAAGGTGTCCCATCAACA
Om_ISCU_B_3′Race_f1	CATACAGAACACGGATATTGC
Om_ISCU_B_3′Race_f2	GCAACTGAGGTCTTTGCCAATG
Om_ISCU_A_5′Race_r1	ACCTGGCATCCACAATCTTC
Om_ISCU_B_5′Race_r1	GTAGGCAGCTTGGGCTTTATAT
Quantification of ISCUA, ISCUB, EEF1A1 from rainbow trout	
Om_ISCU_A_qRT_f	AAGCCAGCAACTAAGGGAGGAC
Om_ISCU_A _qRT_r	TACGTTGTATGGGTGGACATAAC
Om_ISCU_B_qRT_f	GCAACTGAGGTCTTTGCCAATG
Om_ISCU_B _qRT_r	GGGCTTGGTGTGTACACTGTG
Om_EEF1A1_f	TGATCTACAAGTGCGGAGGCA
Om_EEF1A1_r	CAGGACCCAGGCATACTTGAA

2.5. Expression analyses via quantitative real-time PCR

For comparison of *ISCU* gene expression in extracted tissues of BORN and TCO trout, 1.5 μg of total organ RNA was reverse transcribed (see above). In addition, quantitative RT-PCR (qRT-PCR) was carried out on the LightCycler Instrument 480 System by utilizing the FastStart DNA MasterPLUS SYBR Green I Kit (Roche, Mannheim, Germany). We designed oligonucleotides for amplification of 167 bp (*ISCUA*) or 183 bp (*ISCUB*) fragments using PSQ™ Assay Design software (Biotage, Uppsala, Sweden), listed in Table 1. Specific gene expression was normalized against a fragment of the housekeeping gene *EEF1A1*, whose expression served as a control for both RNA integrity and qRT-PCR success (Table 1). The PCR was performed as follows: 40 cycles including 10 min pre-incubation (95 °C), 15 s denaturation (95 °C), annealing for 10 s (decreasing from 60 °C), extension for 20 s (72 °C) and quantification for 5 s (79 °C for *EEF1A1*, 81 °C for *ISCUA*, and 79 °C for *ISCUB*). Copy numbers were calculated relative to dilutions of PCR-generated ISCU fragments as external standards. PCR products were separated by electrophoresis in 2% agarose gels to assess product size and quality. Statistical significance ($p<0.05$) was evaluated by performing parametric t-test and nonparametric Mann–Whitney U-test.

3. Results and discussion

3.1. Identification of duplicated ISCU genes

In this study, we isolated and characterized two *ISCU*-like cDNA sequences from rainbow trout and marena whitefish. Subsequently, they are termed as *ISCUA* [GenBank (GB) accession: rainbow trout FN598573; marena whitefish FN598574] and *ISCUB* (GB: rainbow trout HE648574; marena whitefish HE648575). Transcripts of both *ISCU* genes from trout and maraena share 82% and 93% identity, respectively. In detail, rainbow trout *ISCUA* and *ISCUB* cDNA sequences are 1130 and 1133 base pairs (bp) in length, excluding polyadenylated (polyA-) tail, respectively. The open reading frame (ORF) of gene variant B comprises 498 bp, three additional nucleotides compared to the coding sequence of variant A. Thus, the length of the predicted proteins differs in one amino acid between both variants. The polyA-tail is situated 614 bp (variant A) and 593 bp (variant B) downstream of the stop codon. Maraena whitefish *ISCUA* and *ISCUB* ORF-sequences are each 495 bp in length, leading to predicted proteins of 164 aa, respectively, with a hypothetical molecular weight of 1.77 kDa or 1.78 kDa and a calculated pI of 9.0 or 8.6. Furthermore, maraena *ISCUB* features a potential single nucleotide polymorphism (SNP) at position 94 of the ORF (G/A), which shifts the glutamic acid (E) at predicted protein position 32 to a lysine (K). The clear determination of this SNP needs further analyses. Trout and whitefish sequences feature the canonical polyA-signal AATAA.

An IscU-like domain is stretched over 115 amino acids including three strictly conserved cysteine residues at positions 64 or 65, 90 or 91 and 133 or 134 in rainbow trout ISCUA or ISCUB protein, respectively. These amino acids constitute the active binding side for the generated [2Fe–2S]/[4Fe–4S]-cluster. Thirteen ISCU specific trimerization sides have been identified using NCBI blast. They are essential for forming the protein specific asymmetric homotrimer. All ISCU specific features have also been detected in maraena whitefish ISCUA and ISCUB proteins.

The predicted protein sequences from both rainbow trout variants differ in fourteen amino acids. Ten of the different residues are strongly similar and three are weekly similar. Furthermore an additional alanine was found at position seven of protein variant ISCUB. In contrast, a higher sequence similarity was identified for the predicted ISCUA and ISCUB proteins in maraena whitefish. They differ only in ten residues, seven of these are strongly similar, two weakly similar and the predicted amino acid serine (S) at position 21 of ISCUA is substituted by tyrosine (Y) in ISCUB. Moreover, the proteins ISCUA and ISCUB in rainbow trout and in maraena whitefish share a sequence motive containing the amino acids Leu-Pro-Pro-Val-Lys (LPPVK), which is typical for ISCU family members from both, prokaryotes and eukaryotes (Hoff et al., 2002). It is located within the solvent-exposed loop close to the [Fe–S]-cluster assembly site (Dutkiewicz et al., 2003; Hoff et al., 2002). These five amino acid long peptide is necessary and sufficient for ISCU interactions with species specific chaperones (bacterial HscA, HscB; yeast Ssc1, Jak1; eukaryotes mtHSP70s) (Vickery and Cupp-Vickery, 2007). The chaperones may facilitate [Fe–S]-cluster construction and enhance rate and efficiency of their transfer to apo-[Fe–S] proteins. Especially the proline and the lysine residues of the sequence motif are important for high affinity binding of the co-chaperones (Hoff et al., 2003; Vickery and Cupp-Vickery, 2007). The interaction of chaperones and scaffold proteins seems to be compelling for [Fe–S]-protein maturation.

Salmonid ISCU protein sequences are evolutionary highly conserved. The alignment of duplicated rainbow trout and whitefish ISCU proteins shows 89.1% sequence identity with their homologues from Atlantic salmon (*Salmo salar*) and 54.2% sequence identity with all known ISCU proteins from teleost fish (data not shown). A comparison of ISCUA and ISCUB proteins to their mitochondrial co-ortholog from human (GB accession NP_998760) revealed a remarkable conservation of 72%. The phylogenetic relationship of the duplicated ISCU proteins from trout and whitefish in comparison with all known homologues from teleostean fish and selected sequences from higher vertebrates is displayed in Fig. 2A.

Trout ISCUB forms a clade with its homologue in salmon, being clearly separated (bootstrap of 81%) from ISCUB of whitefish and ISCUA from trout, salmon and whitefish which for their part are detached from whitefish ISCUB with a bootstrap value of 70%. Furthermore, ISCU variants of salmonid fish form a discrete cluster from pike (*Esox lucius*) with a bootstrap value of 92%. The amino acid alignment of all ISCU proteins from teleost fishes identified three identical sequence motifs between salmonid and pike ISCU proteins (data not shown): first, the N-terminal sequence motif AKKCITPLVV, second the C-terminal amino acid motif DxxIEAVKASN and third a central valine (V) instead of an alanine (A) residue in all other teleosts. According to these sequence motifs, it can be presumed that these mutations occurred in the ancestor of salmonid and pike and therefore a clear phylogenetic separation emerges. The separation of salmonid ISCU amino acid sequences from ISCU protein of pike is based on further sequence differences. Moreover, ISCU of sablefish (*Anoplopoma fimbria*), stickleback (*Gasterosteus aculeatus*), two pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*), medaka rice fish (*Oryzias latipes*), and zebrafish (*Danio rerio*) as well as rainbow smelt (*Asmerus mordax*) could not clearly been separated from their amphibian, reptile, bird and mammalian counterparts: the clawed frog (*Xenopus tropicalis*), the red throated anole (*Anolis carolinensis*), the zebra finch (*Taeniopygia guttata*), and human (*Homo sapiens*). This supports the presumption of a structural well conserved and therefore functional conservation of the scaffold protein ISCU during evolution (Johnson et al., 2005).

Duplicated rainbow trout genes *ISCUA* and *ISCUB* both consist of five exons and four introns. The corresponding introns differ in length and show no sequence similarity. The comparison of trout *ISCU* gene-structure with those of different species shows a high level of conservation. Fig. 2B exemplarily illustrates sequence conservation throughout the lengths of exons 2 (114 bp), 3 (111 bp) and 4 (79 bp) of duplicated *ISCU* genes from trout, zebrafish, stickleback and their co-ortholog from human. Notably, the coding region of duplicated *ISCU* genes from trout and zebrafish consists of the same exon-number, while *ISCUA* and *ISCUB* from stickleback contain an additional intron in either exon 1 or exon 5. The human *ISCU* gene codes for two isoforms, a cytosolic and nuclear isoform 1 that results from an additional exon in the 5′ coding region and a downstream start codon and a mitochondrial isoform 2. If the separated isoform locations in human correlate with similar locations in salmonids, it can be assumed that rainbow trout

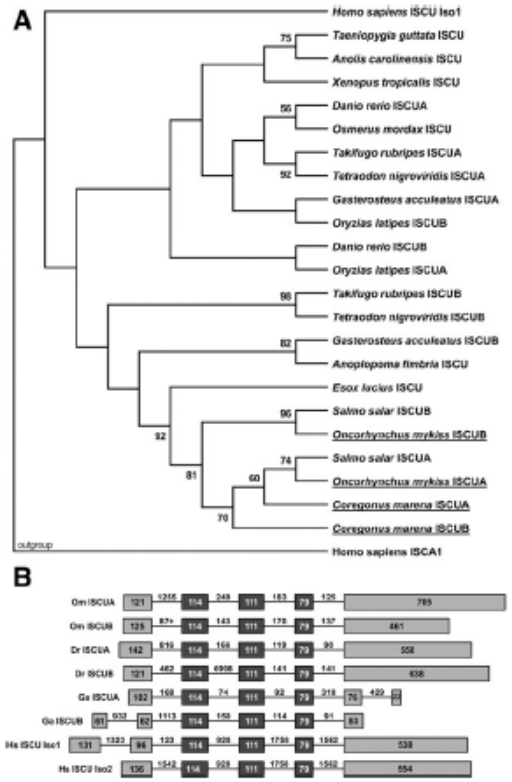

Fig. 2. (A) Phylogenetic tree of ISCU protein sequences. The dendrogram was constructed by using the neighbor-joining method of MEGA 3.1. Only Bootstrap values over 50% are shown, based on 10,000 replicates. Amino acid sequences used for this analysis (with corresponding GenBank [GB] and Ensembl [E] accession codes): *Coregonus maraena* (ISCUA, GB: CBI62790; ISCUB, GB: HE648575), *Oncorhynchus mykiss* (ISCUA, GB: CBI62789; ISCUB, GB: HE648574), *Salmo salar* (ISCUA, GB: AC169709; ISCUB, GB: AC169573), *Esox lucius* (GB: ACO13671), *Anoplopoma fimbria* (GB: ACQ58345), *Gasterosteus aculeatus* (ISCUA, E: ENSGACP00000011625; ISCUB, E: ENSGACP00000011627), *Tetraodon nigriviridis* (ISCUA, E: ENSTNIP00000018953; ISCUB, E: ENSTNIP00000011934), *Takifugo rubripes* (ISCUA, E: ENSTRUP00000036453; ISCUB, E: ENSTRUP00000028879), *Oryzias latipes* (ISCUA, E: ENSORLP00000010428; ISCUB, E: ENSORLP00000008679), *Danio rerio* (ISCUA, E: ENSDARP00000051606; ISCUB, E: ENSDARP00000033254), *Osmerus mordax* (GB: ACO09999), *Xenopus tropicalis* (GB: NP_989088), *Anolis carolinensis* (GB: XP_003229457), *Taeniopygia guttata* (GB: XP_002196892), *Homo sapiens* (Isoform1, GB: NP_055116). The tree is rooted by human ISCA1 amino acid sequence (GB: NP_112202), which functions as outgroup. Protein sequences resulting from nucleotide sequences derived in this study are underlined in black. (B) Exon–intron structure of duplicated *ISCU* genes from rainbow trout. Genomic *ISCU* structure of the salmonid (*Oncorhynchus mykiss* (Om) *ISCUA*, GenBank [GB]: HE648572 and *ISCUB*, GB: HE648573) compared with respective sequences from zebrafish (*Danio rerio* (Dr) *ISCUA*, Ensembl [E]: ENSDARG00000035596; *ISCUB*, E: ENSDARG00000026582), stickleback (*Gasterosteus aculeatus* (Ga) *ISCUA*, E: ENSGACG00000008801; *ISCUB*, E: ENSGACG00000016973) and human (*Homo sapiens* (Hs) E: ENSG00000136003). Lengths are given in bp. Exons are represented by scaled boxes, conserved exon lengths by dark gray, others by light gray underlay. Introns are symbolized by linking lines.

protein variants ISCUA and ISCUB might also be located in different cell parts. However, this aspect remains unsolved by our study.

3.2. Validation of microarray results

We analyzed the hepatic and splenic *ISCU* mRNA expression by qRT-PCR to validate results of previously performed 16 k salmonid cDNA microarray experiments, in which *ISCU* was one of the top regulated genes (liver: fold-change 5.00; spleen: fold-change 4.57) between imported and local rainbow trout strain. Since the gene is one of the key elements in the [Fe–S] cluster mediation and is therefore important for the functionality of [Fe–S] enzymes, the different expression pattern in liver and spleen of BORN and TCO trout is remarkable.

The qRT-PCR analysis validated the previous results: *ISCUA* was significantly higher expressed in liver of BORN trout (+30%, $p=0.03$) compared to TCO, while the mRNA level was significant lower in spleen (−60%, $p=0.01$). In accordance, a highly significant decrease in the transcript level of *ISCUB* could be revealed in spleen (−60%, $p=0.2*10^{-6}$) of BORN trout (Fig. 3A). Strikingly, the number of *ISCUA* and *ISCUB* gene copies differed by two orders or one order of magnitude in liver and spleen, respectively.

3.3. Tissue and strain specific ISCU gene expression

Subsequent to the above mentioned studies with BORN and TCO rainbow trout held in aquaculture ponds, we investigated the expression profiles of healthy and challenged trout kept in experimental tanks.

Thus, we examined the tissue- and strain-specific expression of duplicated *ISCU* genes to clarify the respective importance of *ISCUA* and *ISCUB* in metabolic- and immune-relevant tissues. Unexpectedly, *ISCUB* is highly expressed in brain with mRNA copy-numbers up to 620,000, which is in contrast to its expression value in all other examined tissues being throughout lower than 80,000 (Fig. 3B). In comparison to the cerebral *ISCUB* gene-expression, the mRNA level was on an average remarkable lower in the gill (x 74,000), >head–kidney (x 35,000), >spleen (x 27,000), trunk kidney (x 21,000), >muscle (x 11,000) and liver (x 1,600). In accordance to *ISCUB*, the *ISCUA* mRNA-level is also highest in brain being even enhanced with up to 1,100,000 copies. However, unlike *ISCUB* the *ISCUA* transcript level is also remarkable high in the trunk kidney (x̄ 540,000), spleen (x̄ 490,000), head kidney (x 410,000) and gill (x 400,000). The lowest ISCUA expression was detected in muscle (x̄ 100,000) and liver (x̄ 53,000), which remains still manifold compared to ISCUB expression in both tissues. The high energy-demand of brain is directly correlated with considerable needs of iron and [Fe–S]-clusters, since both are essential components of many enzymes of the ATP production (Pinero and Connor, 2000). Therefore it might be a logical consequence that the expression of *ISCUA* and *ISCUB* is highest in brain, compared to other tissues. The in particular high cerebral mRNA-level of *ISCUB* suggests a specific role of this gene variant in the respective tissue. Future analyses on protein level of the scaffold protein could provide more information on gene and tissue specific functions of the trout *ISCU* genes.

Significant different *ISCU* gene expression between the local selection strain BORN and imported TCO rainbow trout has only been detected in head kidney. *ISCUA* showed a significant higher transcript level in BORN than in TCO trout (BORN: 570,000, TCO: 240,000; $p=0.03$). The strain specific expression was furthermore different in muscle tissue, however, without significance. No significant strain specific expression differences have been determined for *ISCUB*, which is presumable due to the very low mRNA level measured in the examined tissues.

3.4. Influence of temperature challenge on ISCU gene expression

In addition to the general transcriptome and expression analysis mentioned above, we examined *ISCU* gene expression after exposing BORN and TCO rainbow trout to 8 °C and 23 °C for one week. Subsequently, *ISCUA* and *ISCUB* mRNA expression was measured in liver and spleen, according to significantly different expression levels of the gene variants comparing healthy fish of both rainbow trout strains (compare Figs. 1 and 3A). Additionally, *ISCU* gene expression was examined in gill and brain, which are the tissues with the highest *ISCUA* and *B* transcript levels in healthy trout (Fig. 3B).

The general expression differences of the duplicated *ISCU* gene have been confirmed by the temperature experiment. While the *ISCUA* copy number varied between 56,000 (liver, 8 °C) and 1,080,000 (brain, 8 °C) in BORN trout and 43,000 (liver, 8 °C) and 1,150,000 (brain, 8 °C) in TCO trout, the overall *ISCUB* transcript level was lower, varying between 1400 (liver, 8 °C) and 560,000 (brain, 23 °C) in the local selection strain and 1500 (liver, 8 °C) and 620,000 (brain, 15 °C) in the imported trout. Remarkably, we found a generally lower *ISCUA* and *ISCUB* transcript

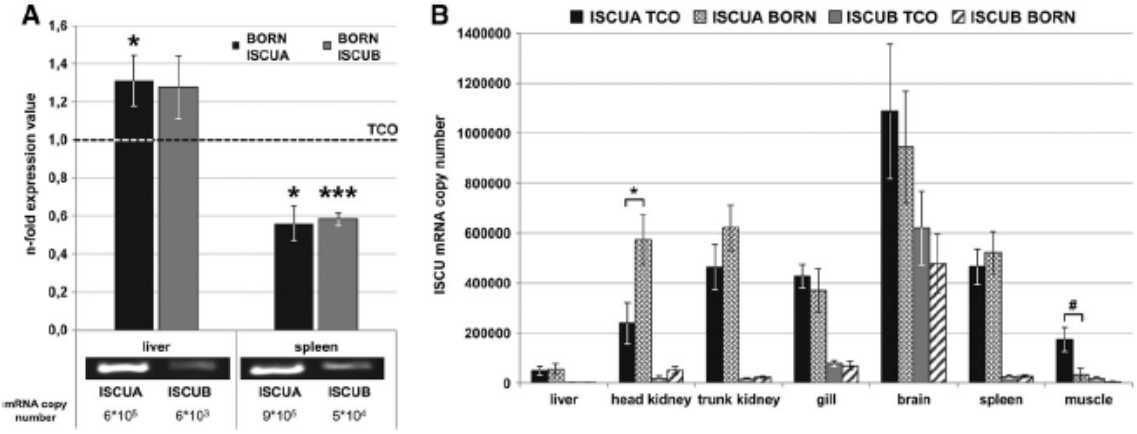

Fig. 3. (A) *ISCU* gene expression in hepatic and splenic tissue of rainbow trout strains BORN and TCO. Means (± SEM) are normalized to the housekeeping gene *EEF1A1*. *ISCU* gene expression in imported strain TCO was set as 1.0 (dotted line), the *ISCUA* (black column) and *ISCUB* (gray column) gene expression of the local strain BORN is shown as fraction hereof. Asterisks indicate statistical different expressions between both strains with $p<0.05$ (*) and $p<0.001$ (***). Absolute mRNA copy numbers of *ISCU* gene *A* and *B* in liver and spleen of BORN trout are shown in the table below the graph. A respective gel picture illustrates the differences in the expression value. (B) *ISCU* transcript level in seven rainbow trout tissues. The graph shows the average (± SEM) of *ISCUA* and *ISCUB* mRNA copy number from six clinical healthy steelhead trout from breeding strains TCO (black and gray columns) and BORN (black checkered and gray striped columns). Statistical significant expression differences are indicated by asterisk (*, $p<0.05$) and hash sign (#, $p<0.1$).

level at 23 °C than at 8 °C except for cerebral *ISCUB* (Fig. 4). In detail, *ISCUA* gene expression at 23 °C was significantly lower in spleen of both rainbow trout strains (BORN: −41%, p=0.0002; TCO: −37%, p=0.003) and in brain of BORN trout compared to 8 °C (−30%, p=0.02). Furthermore, it was lower at 23 °C in gill of BORN trout (−59%, p=0.06) and TCO (−40%, p=0.05) compared to 8 °C. *ISCUB* gene expression in TCO trout was depressed in liver, spleen and gill tissue at the higher temperature. However, in contrast to *ISCUA*, it was significantly elevated at 23 °C in brain (+53%, p=0.004).

Additionally, we found differences in *ISCU* gene expression of examined strains in brain and gill. The cerebral *ISCUA* transcript level at 23 °C varied by 25%, being significantly lower in BORN trout (p=0.04). The *ISCUB* mRNA level in brain was even highly significant between both lines, being lower in BORN trout as well (−33%, p=0.008). Strikingly, temperature challenge at 8 °C and 23 °C seemed to have only little influence on *ISCUB* gene expression of BORN trout in the liver, spleen and brain. The transcript level was only marginally different in gill, being lower at 23 °C than at 8 °C (−59%, p=0.06). This is in contrast to the *ISCUB* expression level in TCO trout, which differed between both temperatures.

Overall, the chosen temperatures seem to have influence on *ISCU* gene expression in general, but the expression is not strain specific.

3.5. ISCU gene expression after pathogen infection

Transcriptome analysis of gill and trunk kidney tissue at 0 h as well as 7 and 21 days after an infection with the pathogen *A. salmonicida* (Bernoth, 1997) revealed *ISCU* as one of the most upregulated gene in BORN rainbow trout three weeks after infection (gill: fold-change 107.22; p=0.02; trunk kidney: fold-change 87.47, p=0.08), indicating an infection-dependent role of *ISCU* gene expression in the local strain. To evaluate this, additional analyses of *ISCU* gene expression were performed by qRT-PCR. Fig. 5 elucidates the results of both experiments. Four 60-oligomeres located on the 44 k salmonid oligo microarray have clearly been allocated to *ISCU* gene sequences. Strikingly, the fold-change of *ISCU* gene expression in BORN trout relative to TCO trout differed between the oligomers, being throughout highest for 60-mer 1 (Fig. 5A). Here, the *ISCU* transcript level in the local strain was significantly elevated in gill compared to the imported strain (i) at starting point of the experiment (fold-change 55.93, p=0.04) and (ii) 21 days (see above) after infection. Findings for renal *ISCU* transcript level of 60-mer 1 were similar, being clearly elevated in BORN trout at each time point, although differences were not significant (Fig. 5B). Nevertheless, the additional expression analyses with specific primers for both trout gene variants could not confirm these findings (Figs. 5C, D). Branchial transcript level of *ISCUA* and *ISCUB* was found to be negligible lower in BORN trout compared to TCO trout at 0 h (*ISCUA*: 0.90-fold, p=0.54: *ISCUB*: 0.80-fold, p=0.45) and 21 d (*ISCUA*: 0.96-fold, p=0.49: *ISCUB*: 0.91-fold, p=0.19) after infection, and even significantly depressed at day 7 (*ISCUA*: 0.52-fold, p=0.04: *ISCUB*: 0.46-fold, p=0.03). Expression of renal *ISCUA* stayed approximately on an equal level during infection and although the *ISCUB* transcript level was slightly elevated in BORN trout at 0 h (0.50-fold, p=0.25), no remarkable differences in the strain specific expression of both gene variants were found during infection.

Transcriptome analyses using microarrays provide the possibility to study the molecular basis of phenotypic variation. But since the common ancestor of bony fishes underwent one genome duplication event 226 to 316 million years ago (Hurley et al., 2007) and another intraspecific genome duplication occurred most likely near the origin of salmonids between 25 and 100 million years ago, rainbow trout are now considered to be pseudo-tetraploid. Consequently, multiple gene copies might exist. Therefore, the different results of the expression analyses might be based on the fact that no specific determination was performed in regard to different *ISCU* gene variants, of which at least two could be described in this study. Although, the identified ISCU gene variants in rainbow trout and maraena whitefish correspond to findings in other species, the results cannot exclude the potential existence of further ISCU copies in both salmonids. The presented number of gene variants should therefore undergo a validation by DNA hybridization technologies, such as Southern blot, FISH, or species specific microarrays. In addition, the rainbow trout transcriptome is still not

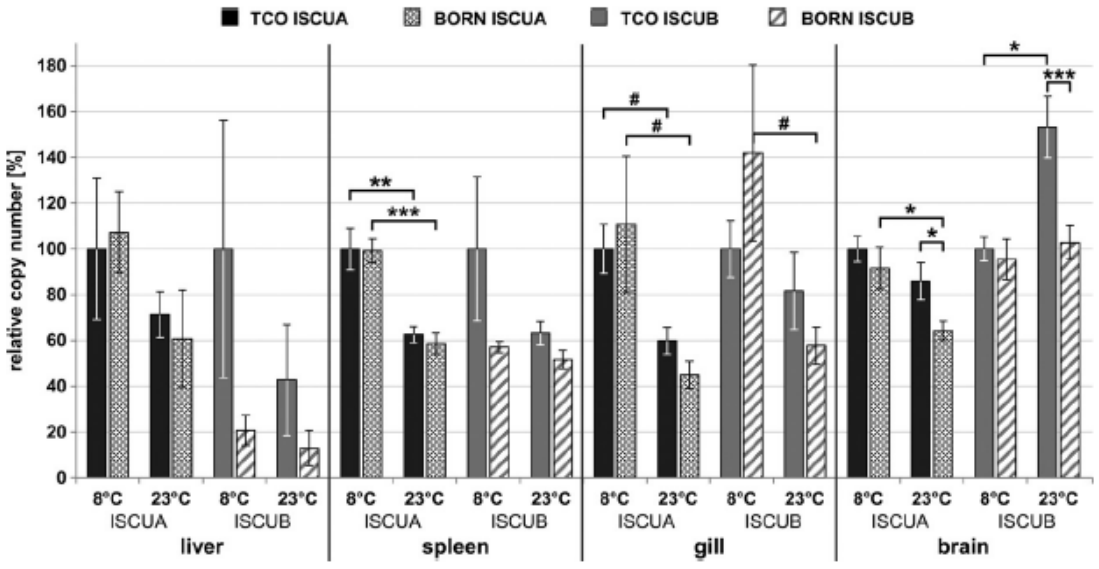

Fig. 4. Relative *ISCU* expression in liver, spleen, gill and brain after temperature challenge (8 °C and 23 °C). Averaged *ISCUA* and *B* copy numbers (±SEM) of TCO (black and gray column) and BORN (black checkered and gray striped) rainbow trout are normalized to the housekeeping gene *EEF1A1*. *ISCU* gene expression in TCO trout at 8 °C was set as 100% and the respective transcript level at 23 °C as well as mRNA level of BORN trout at 8 °C and 23 °C is shown as fraction hereof. Brackets with asterisk and hash sign indicate significant different gene expression (*, p<0.05; **, p≤0.01; ***, p<0.001; #, p<0.1).

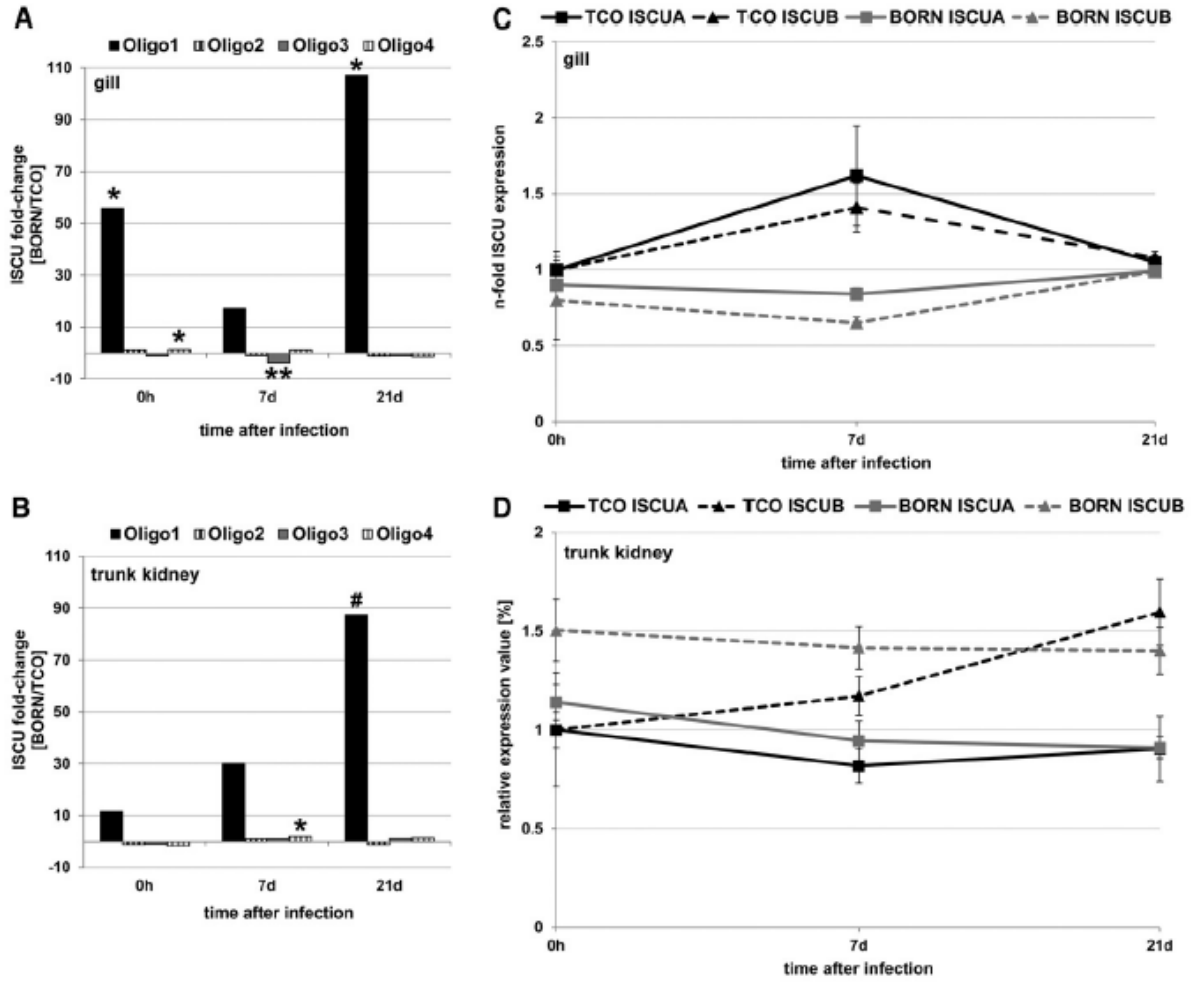

Fig. 5. Comparative analyses of branchial and renal *ISCU* gene expression after pathogen infection. Fold change (local trout strain BORN/imported strain TCO) of microarray data (A+B) is shown for 60-oligomeres 1–4 (black column, black striped column, gray column and gray striped column) at 0 h, 7 d and 21 d after infection. Statistical different expressions are marked with asterisks (*, $p<0.05$; **, $p\leq 0.01$). *ISCUA* (solid lines) and *ISCUB* (dashed lines) gene expression of BORN (gray symbols) and TCO (black symbols) rainbow trout analyzed by qRT-PCR (C+D) is represented for three time points (0 h, 7 d, and 21 d). *ISCUA* and *ISCUB* gene expression in TCO trout after 0 h was set as 1.0 and the respective transcript level at days 7 and 21 as well as mRNA level of BORN trout after 0 h, 7 d and 21 d is shown as fraction hereof. Means (±SEM) are normalized to the housekeeping gene *EEF1A1*.

sufficiently characterized. Most 60mers spotted on the 44 k salmonid oligo microarray are based on EST or cDNA sequences from Atlantic salmon (roughly 80% salmon and 20% trout oligos) (Jantzen et al., 2011). Despite the fact that trout and salmon are very closely related species, the cross-species hybridization with trout targets might affect the quantitative measurement.

4. Conclusion

We identified and characterized the sequence structure and evolutional conservation of the gene variants *ISCUA* and *ISCUB* in rainbow trout and maraena whitefish, respectively, and analyzed the functional activity of *ISCU* comprehensive by gene expression analysis. We detected tissue-specific and temperature-dependent expression patterns of duplicated *ISCU* gene from rainbow trout. Our data show that both gene variants are differently expressed in metabolic- and immune-relevant tissues and that the expression of gene variant B is almost restricted to brain. Investigations on *ISCU* gene expression of the two rainbow trout strains BORN and TCO underline previous revealed differences, which distinguish both strains in dealing with unstable conditions in semi-open aquaculture that is regionally favored. However, the performed analyses support only partially an active role of *ISCU* genes in metabolic maintenance of the previously determined higher robustness of the rainbow trout strain BORN compared to the import strain TCO. Pinning of *ISCU* as trait associated candidate gene needs therefore further investigation.

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kurze Zusammenfassung:
Ubiquitin-Ligasen regulieren nachweislich membranständige Immunrezeptoren. Diese Studie umfasst molekulargenetische Untersuchungen des für eine Ubiquitin-Ligase kodierenden Gens *MARCH5*. Zum einen wurde die Gensequenz in der Regenbogenforelle und dem Ostseeschnäpel charakterisiert. Dabei zeigte sich, dass MARCH5 in beiden Salmoniden dupliziert vorliegt. Vergleichende Expressionsanalysen in der Forelle legten zum anderen gewebe- und zellspezifische sowie eine in BORN- und Import-Forellen teilweise differente Expression der *MARCH5*-Genvarianten offen. Untersuchungen der Genexpression im Rahmen einer viralen Infektion weisen außerdem auf eine Beteiligung der Genvariante *MARCH5A* an der spezifischen Erregerabwehr der Forelle hin.

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MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection

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ABSTRACT

Ubiquitination regulates the activity, stability, and localization of a wide variety of proteins. Several mammalian MARCH ubiquitin E3 ligase proteins have been suggested to control cell surface immunoreceptors. The mitochondrial protein MARCH5 is a positive regulator of Toll-like receptor 7-mediated NF-κB activation in mammals. In the present study, duplicated MARCH5-like cDNA sequences were isolated from rainbow trout (*Oncorhynchus mykiss*) comprising open reading frames of 882 bp (MARCH5A) and 885 bp (MARCH5B), respectively. Trout MARCH5A and MARCH5B-encoding sequences share only 65% sequence identity. Phylogenetic analyses including an additionally isolated MARCH5-like sequence from whitefish (*Coregonus maraena*) suggest that teleosts possess an additional MARCH5 gene copy resulting from a fish-specific whole genome duplication. Coding sequences of MARCH5A and MARCH5B genes from trout are distributed over six exons. Hypothetical MARCH5 proteins from trout comprise four transmembrane helices and a single motif similar to a RING variant domain (RINGv) including eight highly conserved cysteine and histidine residues. A 'reverse-northern blot' analysis revealed furthermore a MARCH5B Δexon5 transcript variant. Both MARCH5 genes from trout show a strain-, tissue- and cell-specific expression profile indicating different functional roles. Fish-specific MARCH5A gene for instance might be involved in defense mechanisms, since *in vivo*-challenge with the viral pathogen VHSV caused a significant 1.7-fold elevated copy number of the respective gene in gills four days after infection, whereas MARCH5B transcript level did not increase.

1. Introduction

Ubiquitination is the posttranslational attachment of the polypeptide ubiquitin to target proteins, reviewed in [1]. This multi-enzyme process is carried out by a cascade of concerted reactions involving ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-protein ligase E3, reviewed in [2]. Unlike to E1 and E2, E3 ubiquitin ligases display substrate specificity. Eleven members of the MARCH (membrane-associated RING-CH) ubiquitin E3 ligase family have been identified in human so far [3]. The majority shares an N-terminal C_4HC_3-type RING (really interesting new gene) finger [4]. Furthermore, two transmembrane spans are characteristic for MARCH proteins, although MARCH5 bears 4 and MARCH6 even 13 transmembrane domains, whereas MARCH7 and MARCH10 lack the respective domain.

The precise physiological function of the MARCH family remains as yet unknown, but there is evidence for an association with immune defense [5]. Therefore, MARCH family members have also been termed as MIR (modulators of immune recognition). It has been hypothesized that MARCH-dependent ubiquitination allows internalization, recycling or lysosomal degradation of cell surface immunoreceptors, reviewed in [6]. Human MARCH4 and MARCH9 were suggested to influence surface expression of MHC-I (major histocompatibility complex, class I) molecules [4]. Similarly, human MARCH1 and MARCH8 proteins have been identified as potent regulators of MHC-II surface expression [7]. Furthermore, human MARCH8 is reported to influence the expression of the co-stimulatory molecule CD86 (B7-2) [8]. Both MHC and CD86 molecules are essential for antigen presentation and/or the subsequent

Abbreviations: aa, amino acid; EST, expressed sequence tag; mAb, monoclonal antibody; MARCH, membrane-associated RING-CH; MIR, modulator of immune recognition; MHC, major histocompatibility complex; ORF, open reading frame; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; RING, really interesting new gene; RINGv, RING-variant; TLR, Toll-like receptor; VHSV, viral hemorrhagic septicaemia virus.

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activation of effector immune cells. MARCH9 has been suggested to control expression of the intercellular adhesion molecule ICAM1, which is important for the onset and manifestation of inflammatory responses [9]. However, TLR (Toll-like receptor)-signaling and other dendritic cell maturation signals are known to counteract ubiquitination of immunoreceptors [5]. It might be noteworthy that the MARCH-homolog MIR proteins have been initially identified in double-stranded DNA virus KSHV (Kaposi's sarcoma-associated herpes virus) [10] as down-modulators of MHC-I.

Human MARCH5 is reported to participate in the regulation of mitochondrial networks [11]. It has been shown that MARCH5 acts as a critical regulator of mitochondrial division and interconnection in mammals [12]. Most likely the MARCH5-dependent ubiquitination and subsequent degradation of the dynamin 1-like protein and further members of the mitochondrial scission complex plays a central role. Furthermore, it has been hypothesized that MARCH5 also interacts with the membrane protein mitofusin 2 promoting mitochondrial fusion. The balanced regulation of mitochondrial fission and fusion rates contributes to the cellular fitness concerning for example essential mechanisms like Ca^{2+} buffering [13]. Recently, it has been shown that human MARCH5 protein positively regulates TLR7 signaling by ubiquitination of TANK (TRAF family member-associated NF-κB activator) [14]. TANK is known as an inhibitor of TLR-dependent NF-κB activation by suppressing the autoubiquitination of the downstream factor TRAF6 (TNF receptor-associated factor 6).

In this manuscript, MARCH5-like genes from the salmonid fishes rainbow trout and maraena whitefish are characterized including bioinformatic analyses and expression profiles in healthy trout and after infection with the single-stranded RNA rhabdovirus VHSV (viral hemorrhagic septicaemia virus), causing severe hemorrhages in different organs and tissues. VHSV is a serious threat for salmonid aquaculture industry with high mortality rates predominantly among rainbow trout and Atlantic salmon, reviewed in [15].

2. Materials and methods

2.1. Sampling and nucleic acid extraction

Two-year old farmed rainbow trout (*Oncorhynchus mykiss*) of the imported strain TCO steelhead II-WA or of the local selection steelhead strain Born (BORN), and one-year old maraena whitefish (*Coregonus maraena*) were purchased from Binnenfischerei Mecklenburg GmbH Schwerin (Frauenmark, Germany).

In order to isolate MARCH5 cDNA and genomic DNA sequences as well as to investigate its tissue-specific expression, tissues (adipose tissue, gills, head kidney, heart, intestine, liver, muscle, and spleen) from six healthy imported trout and six healthy BORN trout as well as liver tissue from three healthy maraena whitefish were collected and immediately frozen in liquid nitrogen. For RNA isolation, flash-frozen tissue samples were homogenized individually in 1 ml TRIzol Reagent (Invitrogen, Karlsruhe, Germany). RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase treatment for 30 min. DNA was isolated from flash-frozen liver tissue using QIAamp DNA Micro Kit (Qiagen). DNA and RNA quantity was assessed at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The integrity of RNA was determined by agarose gel electrophoresis.

2.2. Isolation of MARCH5 sequences from rainbow trout and maraena whitefish

Subsequent to BLAST searches, we designed oligonucleotides (Sigma–Aldrich, Taufkirchen, Germany) specific for MARCH5A utilizing two ESTs with the GenBank accession numbers CB490059 and CX722962 as well as for MARCH5B utilizing three overlapping ESTs with the accession codes CA343783, CR376203, and BX085190 (Table 1).

RNA was extracted from livers of steelhead rainbow trout and maraena whitefish. 5 μg of total RNA were reverse-transcribed using Superscript II™ (Invitrogen) to generate a cDNA template for PCR amplification of MARCH5A and 5B fragments. In order to obtain full-length trout MARCH5A cDNA sequence, 5′- and 3′-RACE experiments were conducted using the Gene Racer Super Script™II RT Module (Invitrogen) according to a touchdown-PCR protocol. This included a 5-min pre-incubation at 95 °C, a denaturing step at 94 °C for 30 s, an annealing step at temperatures decreasing from 68 to 59 °C during the first 10 cycles (temperature decrement of 1 °C per cycle) for 30 s, and an elongation step at 72 °C for 2 min, followed by 30 cycles with 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C, and final elongation at 72 °C for 7 min. A total of 41 cycles was performed.

Coding MARCH5 mRNA sequence of maraena whitefish was generated using primer pairs CM_MARCH_f1, -r1; and CM_MARCH_f2, -r2 (Table 1) according to a 30-cycle PCR protocol.

Introns of both MARCH5 genes were generated in touchdown-PCRs using genomic trout DNA as template and the oligonucleotides listed in Table 1. In order to amplify intron 1, a genomic walking library from rainbow trout DNA was established using the BD GenomeWalker™ Universal Kit (BD Biosciences, Erembodegem, Belgium).

Each nucleotide position of both cDNA and genomic DNA fragments was sequenced at least four times.

2.3. Southern blot analysis on RACE PCR fragments

3′-RACE PCR products from spleen and gill tissue amplified with OM_MARCH5B_f2 gene-specific oligonucleotide (Table 1) were run together with a PCR-generated MARCH5B fragment as a positive control on 2% agarose gel and washed twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and twice in neutralization solution (0.5 M Tris–HCl [pH 7.5], 1.5 M NaCl). DNA was transferred on a positively charged nylon membrane (Roche, Mannheim, Germany) by overnight capillary blotting in 20× SSC and eventually UV-cross-linked. In parallel, a 169-bp digoxigenin-labelled probe for hybridization reaction, which corresponds to exon 4 of trout MARCH5 was synthesized using the oligonucleotides OM_MARCH5-SB_f and -r (Table 1) and the PCR DIG Probe Synthesis Kit (Roche). Prehybridization and overnight hybridization were carried out at 43 °C in DIG Easy Hyb solution (Roche). Membranes were washed twice in low stringency buffer (2× SSC, 0.1% SDS) at room temperature for 5 min each and then twice in high stringency buffer (0.1× SSC, 0.1% SDS) at 43 °C for 20 min each. The blots were visualized according to the DIG Nucleic Acid Detection Kit (Roche).

2.4. Flow-sorting of trout lymphocytes

Leukocytes from head kidney, spleen, gills, and intestine were prepared and incubated with anti-CD8α monoclonal antibodies (mAb) in mixed medium (MM), i.e. Iscove's DMEM/Ham's F-12 (Sigma–Aldrich, Steinheim, Germany) at a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS) and 0.1% sodium azide for 30 min. The cells were washed twice with MM and incubated with FITC-conjugated goat anti-rat IgG (H + L) (Jackson ImmunoResearch, Newmarket, England) for 20 min. Flow-sorting was performed with a MoFlo™ high speed cell sorter (Dako, Eching, Germany). The lymphocytes were sorted into two populations, anti-CD8α mAb-positive and anti-CD8α mAb-negative cells. After

Table 1
Primers used in this study.

Primer Name	Sequence (5′–3′)	Positions within cDNA/gene/EST (GenBank accession code)
amplification of MARCH5A 3′-end from rainbow trout:		
OM_MARCH5A-3R_f	CGACACAGAGGACAGCGGAAA	177–197 (CB490059)
OM_MARCH5A-3Rn_f	TTTAGGAGGTGCATTACATTTCCA	295–318 (CB490059)
amplification of MARCH5A 5′-end from rainbow trout:		
OM_MARCH5A-5R_r	ACCACACCGGCAGCAGTGAAT	64–44 (CX722962)
OM_MARCH5A-5Rn_r	CACTGTCCATCTGCGGAGGAA	24–4 (CX722962)
amplification of MARCH5A coding sequence from maraena whitefish:		
CM_MARCH_f1	TCCATTCACTGCTGCCGGTGT	303–323 (FN550946)
CM_MARCH_f2	ACCGCCACATCCTCAACTACC	800–820 (FN550946)
CM_MARCH_r1	TGGGCTCTACAGTGTACACACA	1252–1231 (FN550946)
CM_MARCH_r2	TCTATCTCCCCTCTACTTCTCC	958–937 (FN550946)
amplification of MARCH5B coding sequence from rainbow trout:		
OM_MARCH5B_f1	TTCAACAAGGCCAAGCTACC	225–244 (CA343783)
OM_MARCH5B_f2	ATCATGGTGGGCTCCATTTA	270–289 (CR376203)
OM_MARCH5B_r1	GTAGGTGACAGCGGTCCAGT	308–289 (CR376203)
OM_MARCH5B_r2	GCTGCTCAGAGGGGTACAAG	506–487 (BX085190)
amplification of MARCH5A intron sequences from rainbow trout (together with Genome Walker primer AP1 and AP2)		
OM_MARCH5AIntr1_r1	ACCACACCGGCAGCAGTGAAT	428–408 (FN400889)/574–554 (FN400890)
OM_MARCH5AIntr2_f1	GGCTCGATGAGAAGCAGAAA	263–282 (FN400889)/254–273 (FN400890)
OM_MARCH5AIntr2_r1	ATAGACTGTCCCCACCACCA	444–425 (FN400889)/590–571 (FN400890)
OM_MARCH5AIntr3_f1	TGGTGGTGGGGACAGTCTAT	425–444 (FN400889)/571–590 (FN400890)
OM_MARCH5AIntr3_r1	TCTGCCACAGCCTCAGTATG	625–606 (FN400889)/965–946 (FN400890)
OM_MARCH5AIntr4_f1	CATACTGAGGCTGTGGCAGA	606–625 (FN400889)/946–965 (FN400890)
OM_MARCH5AIntr4_r1	CCACCAATTGTGCAAGTTCT	1247–1228 (FN400890)
OM_MARCH5AIntr5_f1	TCTCCTCATTTTGTCTGTCATAGT	1152–1175 (FN400890)
OM_MARCH5AIntr5_r1	TACACCTTCAACACTCCCTTCAT	1764–1742 (FN400890)
probe synthesis for Southern Blotting:		
OM_MARCH5-SB_f	GTGGGCCATAAGAAGGGCTTG	487–507 (FN400889)
OM_MARCH5-SB_r	CAATAGCAGCTGTAGTTTAGAGG	654–632 (FN400889)
quantification of MARCH5A/MARCH5B/ACTB/EEF1A1 from rainbow trout:		
OM_MARCH5A_LC_f	ATTTCCAAACAGAGAACTTGTCCA	1110–1133 (FN400889)
OM_MARCH5A_LC_r	TGGGCTCTACAGTGTACAGACA	1264–1243 (FN400889)
OM_MARCH5B_LC_f	CCATCCTGGGTGGGATTGCG	828–847 (FN677805)
OM_MARCH5B_LC_r	GCTCCACCCACTCCCCCTTT	996–977 (FN677805)
OM_ACTB_LC_1	CCCTCCACCATGAAGATCAAGA	1015–1036 (AF157514)
OM_ACTB_LC_r1	GGGATGGGTACAGTCTGTTTAG	1197–1176 (AF157514)
OM_EEF1A1_LC_f1	TGATCTACAAGTGCGGAGGCA	136–156 (NM_001124339)
OM_EEF1A1_LC_r1	CAGCACCCAGGCATACTTGAA	236–216 (NM_001124339)

sorting, total RNA was extracted from 30,000 cells of each cell population using RNeasy Micro Kit (Qiagen). The production and characterization of mAbs against rainbow trout CD8α has been described in [16].

2.5. VHSV infection

VHSV strain 861 was grown on EP/F cells (FLI cell culture collection) and titrated according to the method of Reed and Muench. One year old steelhead rainbow trout were kept in a semicircular water system in 300 L tanks at 15 °C and fed commercial dry pellets. Two groups of 25 fish each were intraperitoneally infected with the VHSV strain 861 at a dosage of 100 $TCID_{50}$/100 µl/fish, while a control group of the same size was injected with the same amount of cell culture medium. Fish were anesthetized with benzocaine (Sigma–Aldrich) and organ samples (spleen, head kidney and gills) were collected at day 0, 2, 4, and 7 after injection from both infected and control fish. Organ pieces of about 100 mg were subsequently transferred into RNAlater (Quiagen), stored overnight at 4 °C and transferred to −20 °C until RNA extraction. RNA was robot-extracted (Tecan, Männedorf, Switzerland) from organ pieces of 10–15 mg using the NucleoSpin II Kit (Macherey-Nagel, Düren, Germany).

2.6. Quantitative real-time RT-PCR

Quantitative Real-Time RT-PCR (qRT-PCR) was carried out using the LightCycler® 480 Instrument and the LightCycler® 480 SYBR Green I Master Kit (Roche). First strand cDNA was synthesized from 1.5 or 0.9 µg of total organ RNA from healthy or VHSV-infected trout by utilizing the Super Script™II Kit (Invitrogen). Products were purified using High Pure PCR Product Purification Kit (Roche). The trout MARCH5A- or MARCH5B-specific oligonucleotides OM_MARCH5A_LC_f and -r or OM_MARCH5B_LC_f and -r (Table 1) were used to quantify a 157-bp or a 169-bp fragment, respectively. For MARCH5 mRNA quantification in healthy or VHSV-infected trout, a 183-bp β-actin (ACTB) or a 101-bp eukaryotic elongation factor-1 (EEF1A1) fragment was amplified in parallel to serve as a control for both RNA integrity and qRT-PCR success utilizing the primers OM_ACTB_LC_f1 and -r1 or OM_EEF1A1_LC_f1 and -r1 [17], respectively (Table 1).

Quantification was performed in repeated runs with an initial denaturation step of 10 min at 95 °C, and then 40 cycles as follows: 15 s of denaturation at 95 °C, 10 s of annealing at 60 °C, 20 s of elongation at 72 °C, and ultimately 5 s quantification at 78 °C (MARCH5A), 79 °C (EEF1A1), 83 °C (MARCH5B), and 84 °C (ACTB), respectively. Copy numbers were calculated relative to dilutions of PCR-generated MARCH5 fragments as external standards (10^3–10^6 copies). PCR products were separated in 3% agarose gels to assess product size and quality. The housekeeping gene copy number was in all cases non-significantly different, except for quantification in flow-sorted lymphocytes. For this reason, we decided to normalize MARCH5 transcript number to EEF1A1.

Statistical significance of expression data was assessed with SPSS software (SPSS Inc., Version 15.0) using parametric *t*-test, non-parametric Mann and Whitney *U*-test or one-way ANOVA applying

the Bonferroni method. In all tests, a two-tailed *P*-value of 0.05 or less was considered significant.

2.7. Computational analyses

NCBI and Ensembl BLAST searches were conducted for sequence comparisons. Sequence alignments were carried out by ClustAlW multiple alignment [18]. Basic physical and chemical properties were analyzed by the ProtParam tool at the Expasy Proteomics Server [19]. Transmembrane helices of the MARCH5 protein were predicted with TMHmm v2.0 included in the HUSAR v3.0 package (DKFZ-Heidelberg, Germany).

Phylogenetic analysis of amino acid sequences was conducted using the Molecular Evolutionary Genetics Analysis package (MEGA4) [20]. The dendrogram was reconstructed with the Neighbor-Joining method based on Poisson-corrected distances and optimized manually. Node robustness was evaluated on a bootstrap analysis based on 10,000 iterations.

3. Results

3.1. Rainbow trout encodes a second MARCH5 gene producing transcript variants

In the present study, we isolated a full-length MARCH5-like cDNA sequence from rainbow trout (*O. mykiss*) with RACE technique based on two ESTs (GenBank accessions: CB490059, CX722962). The complete cDNA sequence of trout MARCH5 gene comprises 1318 bp (FN400889). The ORF of 882 bp is flanked by 120 and 316 bp of 5′- and 3′-UTR, respectively. In addition, a homologous sequence (FN550946) was isolated from maraena whitefish (*C. maraena*), a close relative to rainbow trout. Both fishes are classified as Salmoniformes. The ORF of maraena whitefish MARCH5-encoding cDNA is shorter by 3 bp due to an anticipated TAG stop codon. However, 3′-untranslated regions of maraena whitefish and rainbow trout MARCH5-encoding sequences share 91% identity and a canonical poly(A) signal AATAAA separated by 14 nucleotides from the poly(A) tail.

MARCH5 nucleotide sequences from rainbow trout and maraena whitefish share highest identity with each other (97%) and with a partial MARCH5 sequence of another salmonid fish, Atlantic salmon (*Salmo salar*, BT072360) representing about $^{3}/_{4}$ of the putative ORF. Database searches revealed that salmon possesses a second MARCH5 gene with lower sequence homology (NM_001140034). Moreover, two MARCH5 variants are also present in zebrafish (*Danio rerio*). This finding motivated us to search in the GRASP database for ESTs encoding a second rainbow trout MARCH5 variant. Three overlapping ESTs (CA343783, CR376203, BX085190) were identified, the assembled sequence was subsequently experimentally proven and unknown positions were corrected (FN677805, in the following termed as MARCH5B). The 885-bp ORF of MARCH5B is 3 bp or 6 bp longer than the firstly identified "MARCH5A" sequences from rainbow trout or maraena whitefish, respectively, and shares only 65% or 66% sequence identity with both MARCH5A sequences. In contrast, MARCH5B sequences from rainbow trout and its full-length counterpart in salmon show 94% identity.

The genomic structures of both trout MARCH5 genes (MARCH5A: FN400890; MARCH5B: FR749991) were determined by comparing cDNA and genomic sequences. The coding sequences of both, trout MARCH5A and MARCH5B gene includes six exons, although we detected additionally a MARCH5B Δexon5 splice variant *via* a 'reverse-northern blot' utilizing trout cDNA from spleen and gill tissue as template (Fig. 1A).

Fig. 1B depicts the exon/intron composition of both trout MARCH5 genes corresponding generally with its homologs from zebrafish as well as human. The lengths of the exon 2, 3, and 5 are throughout conserved. It might be noteworthy that the introns of MARCH5A gene from trout are not longer than 464 bp, whereas three introns within the paralogues MARCH5B gene comprise more than 1000 bp. Corresponding introns share accordingly no sequence homology. However, MARCH5A does not feature generally short introns, since zebrafish includes four-digit introns in both MARCH5 genes. The mammalian counterparts also contain extremely long introns (up to 29,335 bp in human). Remarkably, MARCH5 gene from three-spined stickleback *Gasterosteus aculeatus* contains an additional intron within the first exon.

As special features, trout MARCH5A gene contains a $(GT)_{39}$ dinucleotide microsatellite within intron 1, 35 bp downstream of the start codon and 9 bp downstream of exon 1. MARCH5B includes a $(TG)_{9}$ dinucleotide microsatellite located in intron 4, 67 bp downstream of exon 4.

3.2. MARCH5 proteins from trout and maraena contain characteristic domains

The ORF of MARCH5A gene from rainbow trout encodes a putative protein of 293 aa (CAZ64332), whereas its counterpart from maraena whitefish (CBE70290) is one amino acid residue shorter. Both proteins have a hypothetical molecular weight of 32 kDa and a theoretical pI of 8.5 as predicted by the ProtParam tool at the Expasy Proteomics Server. The conceptually translated trout MARCH5B protein includes an additional amino acid residue compared to its paralog. The theoretical pI of MARCH5B is 9.0.

Four transmembrane helices have been predicted for MARCH5A proteins from rainbow trout and maraena whitefish as well as for

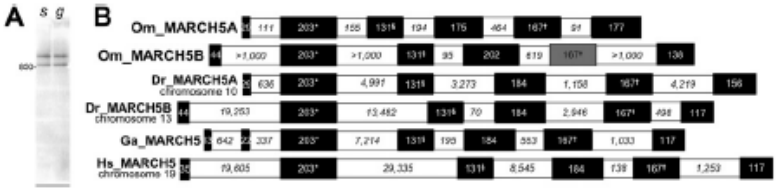

Fig. 1. A) Detection of a MARCH5B transcript variant by southern blotting on RACE PCR fragments. 3′-RACE fragments were generated using total oligo-(dT) cDNA from spleen (s) and gills (g), immobilized and hybridized with a MARCH5 probe specific for exon 4. The lower band represents the MARCH5B Δexon5 splice variant, which is shorther in length by ~170 bp corresponding to exon 5; a characteristic marker band is indicated in bp on the left. To validate the Δexon5 splice variant, 3′-RACE fragments from splenic and branchial cDNA were cloned and sequenced. Procedure was repeated three times with different cDNA samples, a representative blot is shown here. B) Comparative illustration of MARCH5 gene structures from different vertebrates. The figure compares the lengths of exons (scaled black boxes) and introns (graduated white boxes) from duplicated MARCH genes in trout with those in zebrafish (Dr_MARCH5A, Ensembl accession: ENSDART00000039187; Dr_MARCH5B, ENSDART00000043069), as well as single copy MARCH genes in stickleback (Ga_MARCH5, ENSGACT00000003872), and human (Hs_MARCH5, ENST00000358935). Lengths are given in bp. The gray box represents a spliceable exon. Conserved exon lengths in ortholog and paralog MARCH5 genes are marked with a symbol (asterisk: trout MARCH5 exon 2; paragraph: exon 3; cross: exon 5).

MARCH5B variant from trout spanning from position (i) 100 to 122 or 105 to 127, respectively; (ii) 137 to 159 or 142 to 164; (iii) 205 to 224 or 219 to 238; and (iv) 234 to 251 or 248 to 265 (Fig. 2) corresponding to the respective positions in the human MARCH5 protein [12]. Hence, MARCH5B Δexon5 splice variant lacks one transmembrane helix. In contrast to full-length MARCH5B protein, both ends of the truncated MARCH5B variant are most likely situated at different sides of the membrane comparable to (human) MARCH6 ligase containing also an odd number of transmembrane domains.

A single motif similar to a RING variant domain (RINGv) was found in both hypothetical MARCH5A protein sequences from residue 12 to 66 and in MARCH5B protein from residue 17 to 71 corresponding to the consensus pattern C-X_2-C-X_{10-45} (15 in trout and whitefish)-C-X-C-X_7-H-X_2-C-X_{11-25} (18 in trout and whitefish)-C-X_2-C (in short C_4HC_3). RING domain sequences characteristically comprise a cluster of cysteine and histidine residues that coordinate zinc ions [21]. MARCH5 amino acid sequences from rainbow trout and maraena whitefish contain one histidine residue at position 41 (variant A) or 46 (variant B; Fig. 2), as well as seven cysteine residues from position 12 to 66 (variant A) or 17 to 71 (variant B) that might constitute cysteine bridges, which are typical for E3 ubiquitin ligases. As expected, the respective amino acid residues are well conserved among MARCH5 proteins from vertebrates as illustrated by the amino acid sequence alignment in Fig. 2. Moreover, the comparison visualizes the presence of fish-specific MARCH5 proteins, namely MARCH5A, whereas piscine MARCH5B proteins resemble tetrapod MARCH5 proteins represented by MARCH5 from chicken *Gallus gallus* and human. Fig. 2 accents a total of 56 amino acid residues specific for either MARCH5A or MARCH5B as indicated by arrows. It clearly reveals first that the N-termini of MARCH5A variants from trout, whitefish, zebrafish, and pike are five amino acid residues shorter than MARCH5B variants from trout, salmon, and zebrafish as well as from chicken. Second, the C-termini of the selected piscine MARCH5A sequences are significantly (six to 20 residues) longer than piscine MARCHB variants as well as tetrapod MARCH5. Furthermore, species-specific deletions or insertions are present in MARCH5 proteins, e.g. an additional stretch of six amino acid residues after position 188 within trout and salmon MARCH5B protein.

3.3. *MARCH5 genes are duplicated in several teleostean species*

The mammalian MARCH family consists of eleven members (MARCH1 to -11) showing a distinct tissue distribution [6]. Our searches at the Ensemble genome browser and in GenBank revealed that the teleostean fishes zebrafish *D. rerio*, three-spined stickleback *G. aculeatus*, Japanese rice fish *Oryzias latipes*, Japanese pufferfish *Takifugu rubripes*, and spotted green pufferfish *Tetraodon nigroviridis* encode presumably only eight MARCH genes, MARCH2, -4, -5, -6, -7, -8, -9, and -11. Zebrafish possesses a MARCH1 gene (NCBI protein accession code: CAX14354), at present uniquely among teleosts. Similarly, MARCH3 has been experimentally validated only in *Salmonidae* so far. Recently, we isolated a MARCH3 cDNA sequence from rainbow trout (NCBI: FR851411). In parallel, the Atlantic salmon's ortholog was published (NCBI: NP_001135117). MARCH10 is obviously absent in all piscine species. Although clawed frog's genome lacks also MARCH10, it does encode MARCH3. In brief words, the MARCH family in non-vertebrates seems to differ from the mammalian one. Moreover,

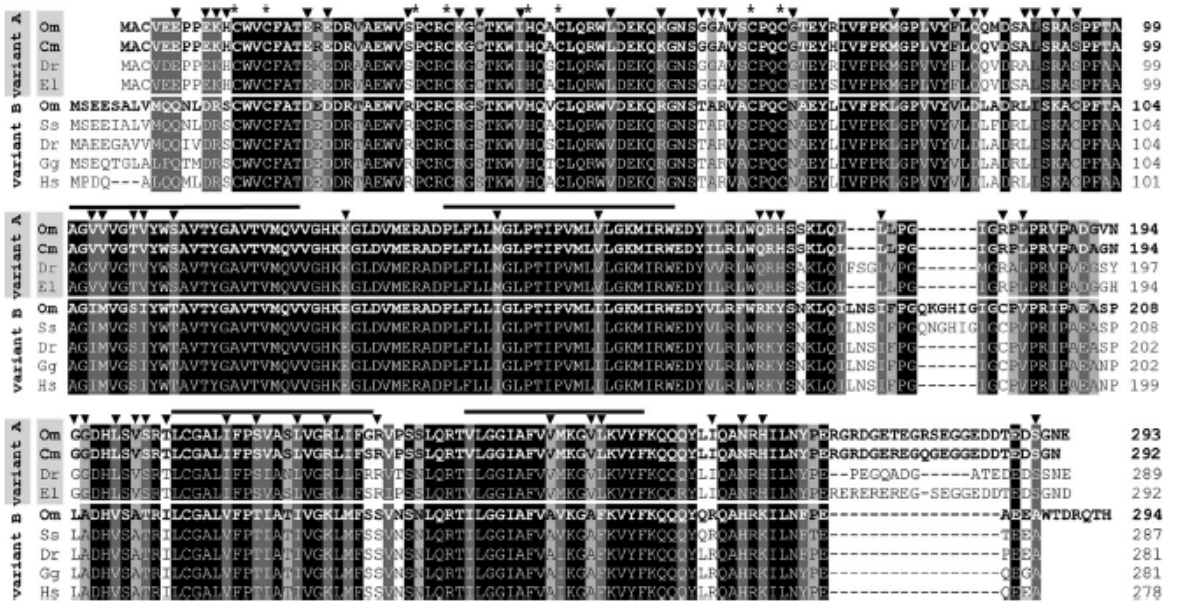

Fig. 2. Multiple alignment of MARCH5 amino acid sequences. The comparison includes MARCH5 variant A (highlighted in gray along the left margin) and variant B sequences from the teleostean species rainbow trout (Om, variant A, GenBank accession: CAZ64333; variant B, CBK39084), maraena whitefish (Cm, CBE70290), zebrafish (Dr, variant A, NP_956033; variant B, NP_001076296), northern pike (El, ACD13898), and Atlantic salmon (Ss, ACD33369), as well as chicken (Gg, NP_001012924), and human (Hs, NP_060294). Sequences that have been generated for the present study are printed in bold face letters. Amino acids are numbered along the right margin. Identical amino acids are highlighted in black; strongly similar amino acids are printed in white letters with dark gray underlay; weakly similar amino acids are printed in black letters with light gray underlay. Conserved residues of the RINGv domain, which is critical for ubiquitin transfer activity of several E3 ubiquitin ligases, are indicated with an asterisk above the respective alignment section. Arrows mark amino acid residues, which are specific for either MARCH5A or MARCH5B variants. Four predicted transmembrane domains of MARCH5 proteins from rainbow trout and maraena whitefish are indicated with a solid line.

it appears that some MARCH family members are duplicated in bony fish, i.e. MARCH6, -9, and MARCH5, described here.

MARCH5-like amino acid sequences from rainbow trout, maraena whitefish, and Atlantic salmon (ACN58677) share 95–97% sequence identity. In comparison with human MARCH family members, MARCH5A proteins from trout and whitefish show higher homology to MARCH5 (NP_060294; 61%–62%) than to human MARCH1 to -4 and -6 to -11 (≥14%). MARCH5B from trout shares even 94% sequence identity with human MARCH5 protein, but a similarly low identity with human MARCH5 paralogs (5%–19%). To support the assumption that both trout sequences described here are co-orthologs of the mammalian MARCH5 protein, a comprehensive phylogenetic dendrogram including representatives of all members of the MARCH E3 ubiquitin ligase family was reconstructed (Fig. 3). The dendrogram contains all human MARCH amino acid sequences and their respective piscine counterparts as well as MARCH5 proteins from insect *Culex quinquefasciatus*, frog *Xenopus tropicalis*, reptile *Anolis carolinensis* and bird *G. gallus*.

The dendrogram clearly separates the MARCH proteins into seven major families, (i) MARCH1/-8, (ii) MARCH2/-3, (iii) MARCH4/-9/-11 including two piscine MARCH9 gene variants, (iv) MARCH5 including piscine A and B gene variants, (v) MARCH6 including two piscine gene variants, (vi) MARCH7, and (vii) MARCH10 without piscine members. Among the MARCH5 proteins, the insect amino acid sequence is clearly separated (bootstrap confidence level: 100%) from vertebrate counterparts. MARCH5 protein from mosquito contains indeed a few additional sequence features that are absent in vertebrate MARCH5 proteins.

This phylogenetic analysis proves that MARCH5 sequences from rainbow trout and maraena whitefish are related to tetrapod MARCH5. Moreover, the dendrogram reveals that piscine MARCH5 proteins are separated into distinct clades with 77% bootstrap support. On the one hand, MARCH5A amino acid sequences from zebrafish and pike *Esox lucius* form a sister group to MARCH5A proteins from the salmonids trout, salmon and whitefish with 88% bootstrap confidence. On the other hand, MARCH5B clade comprises sequences from salmon, zebrafish, and trout which are grouped with MARCH5 from stickleback, rice fish, pufferfishes and even chicken and human.

3.4. MARCH5A gene is highly expressed in the immunorelevant tissues spleen, gills, and head kidney

We analyzed the expression of MARCH5A and MARCH5B gene in eight different tissues of six clinically healthy steelhead rainbow trout *via* qRT-PCR using oligonucleotides specific for either trout MARCH5A or -5B (Table 1). The highest concentration of MARCH5A copies has been found in immune-related tissues, i.e. spleen, gills, head kidney (Fig. 4A). MARCH5A mRNA abundance was significantly lower ($P = 0.0045$) in heart, intestine and liver. Hence, 18.8 ± 1.7-fold more MARCH5A copies were detected in splenic compared to hepatic tissue. These data are contrasted by MARCH5B gene, which is more equally expressed among the selected tissues (Fig. 4B). The highest MARCH5B mRNA concentration is present in heart, followed by head kidney, gills, and spleen. Liver shows again lowest mRNA copy number, but cardiac MARCH5B expression is only 1.6 ± 0.4-fold higher than hepatic expression. These tissue-specific expression profiles are furthermore reflected in a different MARCH5A/B transcript number. In head kidney, gills, spleen as well as muscle and adipose tissue, 0.4- to 1.2-fold more MARCH5A transcripts are present than MARCH5B copies. *Vice versa*, liver, gut, and heart contain 3.4- to 4.6-fold more MARCH5B than MARCH5A copies.

Assuming an importance of a pronounced MARCH5A expression in the immunocompetent tissues spleen, gills, and head kidney, we wanted to examine, to which extent MARCH5A and MARCH5B are expressed in leukocytes of the respective organs. However, Fig. 4C and D illustrates that more MARCH5B copies were detected in both, anti-CD8α mAb-positive and -negative leucocytes, namely branchial lymphocytes, >pronephrocytes, >splenocytes.

Besides the tissue- and cell-specific expression differences, we also found strain-specific differences. We are investigating the genomic potential of a local rainbow trout selection strain BORN, which has been bred since 1975 at the fishery institute in Born in brackish costal water of the southwestern Baltic Sea with varying temperature, between 2.5 and 6 practical salinity units (PSU), differently strong pollutant and pathogen pressure [22,23]. MARCH5A mRNA level is significantly lower in muscle tissue of the selection strain BORN in comparison to the imported steelhead strain (fold change of 0.47 ± 0.07, $P = 0.005$; Table 2). In the other seven tissues, the expression of MARCH5A in both trout strains was non-significantly different. In contrast, MARCH5B expression was significantly lower in all tissues examined (head kidney, spleen, gills, heart, intestine, adipose tissue, muscle), except for liver, with fold changes ranging from 0.40 ± 0.07 (muscle, $P = 0.002$) to 0.72 ± 0.05 (head kidney, $P = 4 \times 10^{-4}$) compared to imported steelheads (Table 2).

3.5. MARCH5A is up-regulated in gills after VHSV

We evaluated both, MARCH5A and MARCH5B mRNA expression levels in spleen, gills and head kidney after *in vivo* challenge with VHSV. The respective organs were chosen, since they showed highest MARCH5A mRNA abundance. Samples were taken at day 0, 2, 4, and 7 and quantified *via* qRT-PCR analysis. Fig. 5A illustrates that on the one hand, expression of MARCH5A gene remained on a similar level in spleen and head kidney during evaluation period. On the other hand, MARCH5A expression slightly increased in gill tissue (1.61-fold change, $P = 0.34$) two days after VHSV infection, peaked significantly at day 4 post-challenge (2.68 ± 0.83-fold change, $P = 0.03$) and almost returned to control levels (1.13 ± 0.31-fold change, $P = 0.67$) one week after infection. In contrast, expression of MARCH5B in gills is not elevated, but eventually slightly depressed at day 7 post-challenge (0.61 ± 0.14-fold change) with a marginal statistical significance ($P = 0.06$). Similarly, MARCH5B copy number is significantly down-regulated in spleen four days (0.65 ± 0.06-fold change, $P = 0.01$) and seven days (0.53 ± 0.15-fold change, $P = 0.04$) after challenge. In head kidney, significant changes of the MARCH5B mRNA level were not apparent.

4. Discussion

4.1. Teleosts possess a tetrapod-ortholog and a fish-specific MARCH5 variant

We isolated two MARCH5 DNA sequences from rainbow trout (*O. mykiss*) termed as variant A and B. Both MARCH5 variants are transcribed and encode MARCH5-characteristic amino acid residues and motifs. However, both genes contain specific intron sequences and the putative proteins comprise distinct amino acid positions that allow separation of variant A from variant B. Phylogenetic analyses suggest MARCH5 variant A as a fish-specific gene, whereas variant B seems truly ortholog to the respective gene in tetrapods. Variant A was not identified in tetrapods, but has been found in maraena whitefish (*C. maraena*) in the present study as well as in zebrafish [24], salmon, and northern pike [25] in the frame of cDNA sequencing projects. Zebrafish, as well as salmonids and the closely related pike evolved early among bony fishes. Large scale sequencing and analyses of gene loci in bony fish have

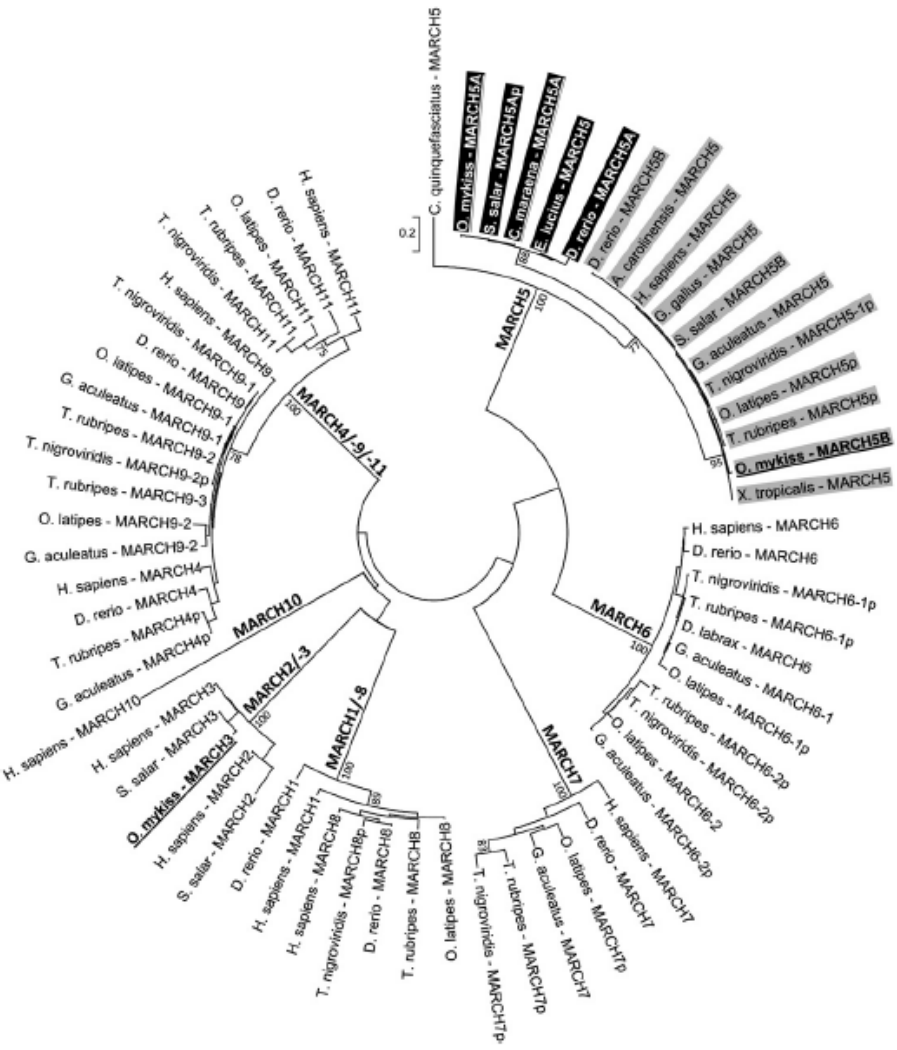

Fig. 3. Circular phylogenetic tree of MARCH amino acid sequences. The MEGA4 dendrogram was reconstructed with the Neighbor-Joining Method using the Poisson correction distance model. Numbers at the nodes indicate the percentage of bootstrapping after 10,000 replications. Only bootstrap values above 75% are indicated. The scale bar represents a genetic distance of 0.2 amino acid substitutions per site. The tree includes MARCH protein sequences from the following species (with corresponding GenBank [GB] and Ensembl [E] accession codes): *Anolis carolinensis* (MARCH5, GB: XP_003224783), *Coregonus maraena* (MARCH5A, GB: CBE70290), *Culex quinquefasciatus* (MARCH15, GB: XP_001862231), *Danio rerio* (MARCH1, GB: CAX14354; MARCH4, GB: NP_001038876; MARCH5A, GB: NP_956033; MARCH5B, GB: CAX13539; MARCH6, GB: XP_002660875; MARCH7, GB: NP_001108052; MARCH8, GB: NP_001154907; MARCH9, GB: XP_001339845; MARCH11, GB: XP_001338632), *Dicentrarchus labrax* (MARCH6, GB: CBN81886); *Esox lucius* (GB: ACO13898), *Gallus gallus* (MARCH5, GB: NP_001012924), *Gasterosteus aculeatus* (MARCH4, E: ENSGACP00000008045; MARCH5, E: ENSGACP00000003859; MARCH6-1, E: ENSGACP00000003448; MARCH6-2, E: ENSGACP00000019168; MARCH7, E: ENSGACP00000020033; MARCH9-1, E: ENSGACP00000015512; MARCH9-2, E: ENSGACP00000000754), *Oncorhynchus mykiss* (MARCH3, GB: CCA64454; MARCH5A, GB: CAZ64333; MARCH5B, GB: CBK39084), *Oryzias latipes* (MARCH5, E: ENSORLP00000000908; MARCH6-1, E: ENSORLP00000009594; MARCH6-2, E: ENSORLP00000016019; MARCH7, E: ENSORLP00000005266; MARCH8, E: ENSORLP00000009376; MARCH9-1, E: ENSORLP00000020760; MARCH9-2, E: ENSORLP00000019029; MARCH11, E: ENSORLP00000007969), *Salmo salar* (MARCH2, GB: NP_001135370; MARCH3, GB: NP_001135117; MARCH5A, GB: ACN58677; MARCH5B, GB: ACI33369), *Takifugu rubripes* (MARCH4, E: ENSTRUP00000027579; MARCH5, E: ENSTRUP00000010692; MARCH6-1, E: ENSTRUP00000022643; MARCH6-2, E: ENSTRUP00000013780; MARCH7, E: ENSTRUP00000043983; MARCH8, E: ENSTRUP00000008831; MARCH9-1, E: ENSTRUP00000006082; MARCH9-3, E: ENSTRUP00000047405; MARCH11, E: ENSTRUP00000003675), *Tetraodon nigroviridis* (MARCH5, E: ENSTNIP00000006615; MARCH6-1, E: ENSTNIP00000010989; MARCH6-2, E: ENSTNIP00000009099; MARCH7, E: ENSTNIP00000012345; MARCH8, E: ENSTNIP00000011522; MARCH9-1, E: ENSTNIP00000021407; MARCH9-2, E: ENSTNIP00000005476; MARCH11, E: ENSTNIP00000010823), and *Xenopus tropicalis* (MARCH5, GB: NP_001116950). Additionally the tree includes human amino acid sequences of MARCH1 (GB: NP_060393), -2 (GB: NP_001005415), -3 (GB: NP_848545), -4 (GB: NP_065865), -5 (GB: NP_060294), -6 (GB: NP_005876), -7 (GB: AAH65014), -8 (GB: AAH66988), -9 (GB: NP_612405), -10 (GB: AAH35021), and MARCH11 (GB: NP_001096032). MARCH5A and -5B sequences are highlighted with black and gray underlay, respectively. Sequences isolated in our laboratory are underlined. The letter 'p' indicates partial sequences.

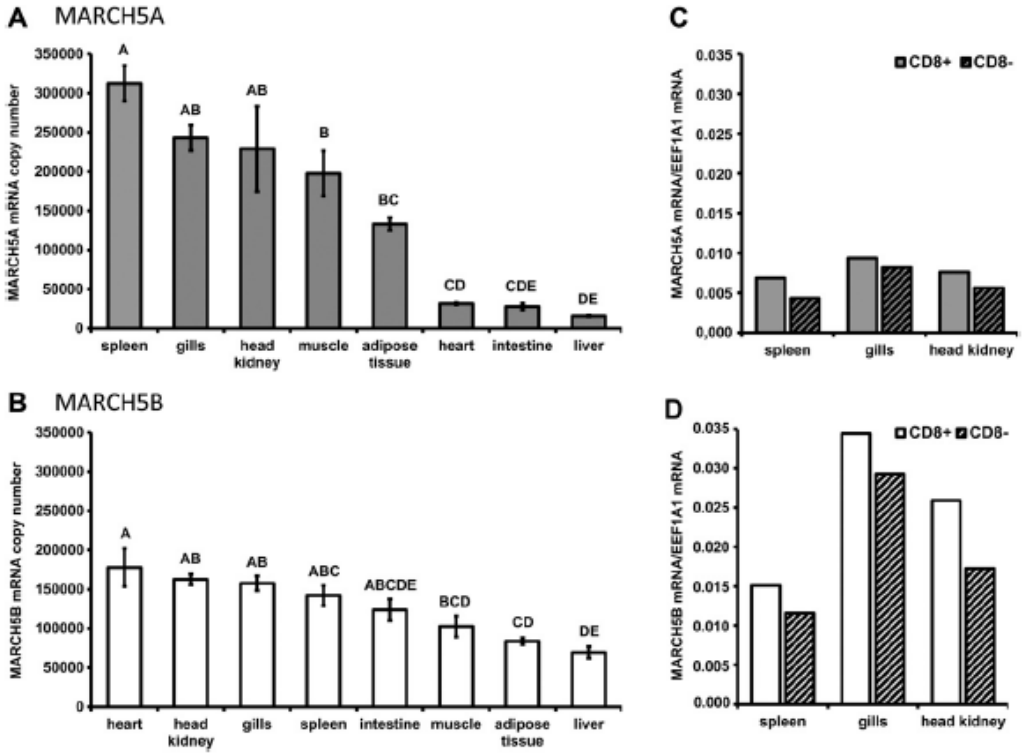

Fig. 4. (A) MARCH5A and (B) MARCH5B transcript number in 75 ng reverse-transcribed RNA isolated from selected tissues of six clinically healthy steelhead rainbow trout. Each bar represents the mean ± SEM. ACTB and EEF1A1 copy numbers were measured in parallel and showed no significant alteration. Different capital letters (A–E) above the bars indicate statistically significant ($p < 0.01$) differences in MARCH5A and -B expression between the different tissues detected by the post-hoc Bonferroni test for each gene. (C) MARCH5A and (D) MARCH5B copy number in anti-CD8α mAb-positive and -negative (open and hatched columns, respectively) leukocytes. Relative expression of MARCH5A and MARCH5B gene against EEF1A1 level in splenocytes, pronephrocytes and branchial lymphocytes is shown. The experiment was repeated with corresponding results.

indicated that teleosts contain duplicate copies of many single copy human genes [26]. Hence, MARCH5 duplicates might be the result of the third round of whole genome duplication (3R) in vertebrates [27]. This 3R duplication event occurred 226–316 million years ago near the origin of bony fishes [28]. Since teleosts are thought to be monophyletic [29], MARCH5A is expected to be present in all teleostean species. The question remains, why teleosts retained both MARCH5 gene copies. Neither variant A nor variant B has been silenced, since expression was confirmed for both. In the case of two parallely expressed duplicate genes, it is possible that both gene products fulfill different tasks (neofunctionalization) or partition the ancestral function (subfunctionalization) [30]. But indeed there are only sparse examples of neofunctionalization or subfunctionalization in fish like the antifreeze glycoprotein gene in Antarctic fishes that derived from a trypsinogen-like protease gene [31] or two closely related protocadherin 15 genes, pcdh15a and pcdh15b, that are required exclusively for function and morphology in the ear and eye, respectively [32]. Nevertheless, we cannot exclude a subfunctionalization phenomenon regarding the MARCH5 genes from trout since both proteins share a lower degree of conservation. Both genes show additionally different expression patterns.

Table 2
MARCH5A and MARCH5B expression ratio between imported and BORN trout.

Tissue	MARCH5A		MARCH5B	
	Expression difference BORN/Import	P-value	Expression difference BORN/Import	P-value
spleen	0.88	0.36	**0.71**	**0.01**
gills	0.97	0.74	**0.68**	**0.01**
head kidney	1.16	0.54	**0.72**	**0.0004**
white muscle	**0.47**	**0.005**	**0.40**	**0.002**
adipose tissue	0.89	0.37	**0.48**	**0.0006**
heart	1.04	0.73	**0.64**	**0.03**
intestine	1.36	0.12	**0.60**	**0.04**
liver	1.15	0.06	0.79	0.22

Values in bold are significant ($P < 0.05$).

4.2. *Duplicated MARCH5 genes from rainbow trout show different expression patterns and might perform different physiological demands*

Pronounced MARCH5A mRNA copy numbers have been detected in the immunocompetent organs spleen, head kidney, and gills from rainbow trout. Spleen and head kidney are the major lymphoid and hematopoietic organs of fish, reviewed in [33]. Recently, a gill lymphoid structure termed as "interbranchial lymphoid tissue" has been reported as a quantitatively very important site of T cell aggregation [34] representing an immune-competent barrier against the external. Although MARCH5B gene shows also highest expression values in those three organs together with heart, the MARCH5B copy number in the other organs is not clearly lower as seen in the MARCH5A expression profile. In liver for example, MARCH5A accounts for ~5% of the respective expression

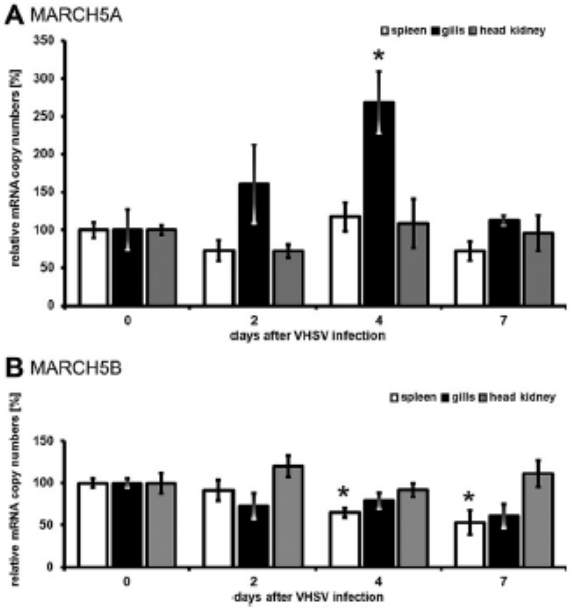

Fig. 5. Relative MARCH5A (A) and MARCH5B (B) copy number after infection of steelhead rainbow trout with VHSV. Spleen (white), gills (black), and head kidney (gray) from three trout at each time point were examined. Each bar represents the mean ± SEM; mean values of MARCH5A and MARCH5B mRNA number from not infected trout were set as 100%. Asterisks indicate significantly different MARCH5 expression levels with $P < 0.05$ compared to the respective tissue of healthy trout.

in spleen, while MARCH5B amounts to ~49%. Expression of both MARCH5 genes in leukocytes from spleen, head kidney, and gills of healthy fish has been proven, although a prevailing MARCH5A copy number compared to the MARCH5B transcript level could not be found. This indicates that MARCH5A expression is elevated in another cell type. It might be conceivable that MARCH5A expression is down-regulated in leukocytes to limit the production of proinflammatory mediators in healthy fish. In this case, TANK might be hardly ubiquitinylated by MARCH5A and is supposed to repress TLR-mediated NF-κB activation. Indeed, a significant increase of MARCH5A copy number by 1.7-fold was observed in gills four days after viral infection. Matsuki et al. suggested an alternative scenario hypothesizing that surface molecules are MARCH-dependently degraded and substituted with newly synthesized proteins to guarantee efficient defense mechanisms [35]. This immunomolecule metabolism might also explain MARCH5A up-regulation in gills of infected fish. Salmonid gills are equipped with extremely thin mucous membranes and may therefore depend on excellent defense mechanisms.

However, a more or less steady MARCH5A mRNA copy number in spleen and head kidney might doubt the involvement of MARCH5 in piscine immune mechanisms. Whether the elevated MARCH5A expression in gills supports the TLR-dependent NF-kB activation needs to be proven. However, our current transcriptome profiling study exploiting gill tissues from rainbow trout infected with *Aeromonas salmonicida* subsp. *salmonicida* shows a comparable up-regulation of MARCH5A gene (results not shown). In detail, a 60-mer sequence (derived from a *D. rerio* sequence with the TIGR accession TC101431) corresponding to trout MARCH5A indicated a significant up-regulation at day 7 after infection compared to healthy animals. This microarray result was verified in a qRT-PCR (2.40 ± 0.24-fold change, $P = 0.03$, $n = 4$). On the other hand, a sequence corresponding to MARCH5B (derived from an annotated *S. salar* EST sequence with the GenBank accession NM_001140034) indicated almost no alteration of expression level (−1.01 to 1.13 within an evaluation period of 21 days, verified by qRT-PCR).

As discussed before, several MARCH proteins including MARCH1, -3, and -10 appear to be absent in some teleostean species, whereas others including MARCH5, -6, and -9 are probably duplicated. The varying number of MARCH proteins among mammalian and non-mammalian vertebrates might indicate alternate functional spectra of the MARCH family members in different species. Regarding human MARCH5, two functions have been described. Firstly, MARCH5 regulates mitochondrial fission and fusion [11]. Secondly, MARCH5 affects TLR signalling [14]. It might be assumed that piscine MARCH5 proteins share the original functions of ancestral MARCH5 factor, i.e. MARCH5A controls immune mechanisms and MARCH5B modifies mitochondrial morphology according to the subfunctionalization theory [30].

Moreover, we found MARCH5A and -5B differentially expressed in several tissues of two trout strains, the economically important steelhead trout TCO and the local steelhead selection strain BORN. A decreased MARCH5B expression appears characteristic for BORN trout probably correlating with decreased ubiquitination in BORN trout tissues, which influences protein degradation/recycling processes. Unfortunately, the physiological role of MARCH5 and its paralogs in teleosts as well as in mammals is not completely understood. The structural characterization and expression analysis of two trout MARCH5 genes and the MARCH5B splice variant provides the basis for further research work.

5. Conclusions

In summary, the present manuscript discusses the differently composed MARCH gene family among vertebrates. It is likely, that some MARCH family members are absent in most fishes, whereas others are duplicated compared to mammals. We provide evidence for the existence of a duplicated MARCH5 gene in at least some teleostean species. In rainbow trout, both MARCH5 genes show characteristic expression profiles in two strains, different tissues and cell types suggesting a similar, but not overlapping activity. MARCH5A might be involved in immune regulation, but further research is required to clarify its definite physiological role in fish.

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VI. Duplicated NELL2 genes show different expression patterns in two rainbow trout strains after temperature and pathogen challenge

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kurze Zusammenfassung:

Studie VI widmet sich der Analyse des Gens *NELL2* in der Regenbogenforelle, welches für ein extrazelluläres Glykoprotein kodiert. NELL2 ist an der Differenzierung und dem Wachstum von Zellen beteiligt. Zusätzlich ist das Ca^{2+}-bindende Protein in die Signaltransduktion und die Regulation des Ionengleichgewichts der Zelle involviert. Inhalt der Studie ist die molekulare Charakterisierung des Gens in der Regenbogenforelle sowie Analysen der spezifischen Genexpression im Laufe der Embryonalentwicklung. Außerdem wurde die *NELL2*-Expression nach einer Pathogen-Infektion und leichtem Temperaturstress vergleichend zwischen der lokalen Zuchtlinie BORN- und Importforellen untersucht. Es zeigte sich, dass *NELL2* in der Forelle dupliziert ist, wobei eine Genvariante ubiquitär, die andere gehirnspezifisch exprimiert wird.

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Duplicated *NELL2* genes show different expression patterns in two rainbow trout strains after temperature and pathogen challenge

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ABSTRACT

Mammalian neural epidermal growth factor-like-like 1 and 2 genes (*NELL1* and *NELL2*) encode multifunctional glycoproteins involved in cell growth regulation and differentiation. We isolated two closely related *NELL2* transcript sequences from rainbow trout (*Oncorhynchus mykiss*). These conceptually translated *NELL2a* and *-b* sequences share 83% identical residues and the NELL-typical structure. Phylogenetic analyses suggest that bony fish possess two *NELL* genes, though these are either present as *NELL1/2* pair corresponding to their mammalian orthologs or as a *NELL2a/2b* combination that might have arisen by a fish-specific duplication event. Both trout *NELL2* genes are highly expressed at early developmental stages. In adult rainbow trout, *NELL2a* copies were detected in each tissue analyzed, whereas *NELL2b* is abundantly expressed only in brain. Cerebral *NELL2a/b* gene expression seems to be temperature-independent, whereas *NELL2a* gene expression is clearly down-regulated in gill and up-regulated in muscle tissue after temperature elevation. Infection with *Aeromonas salmonicida* leads to a considerable increase in *NELL2a/b* copy number in trunk kidney at day 7 p.i. Moreover, comparative qRT-PCR revealed different *NELL2a/b* expression pattern in two rainbow trout strains, imported rainbow trout TCO and a local trout selection strain BORN that is known to be resistant to several biotic and abiotic stressors.

1. Introduction

The *NEL* gene has originally been isolated from a chick embryonic cDNA library as an epidermal growth factor (EGF)-like protein strongly expressed in neural tissue (Matsuhashi et al., 1995). Avian *NEL* mRNA is ubiquitously present in all embryonic tissues, but restricted to neural tissues after hatching and has been discussed as a receptor or a ligand in neuron-specific signal transduction. The mammalian *NEL*-like genes *NELL1* and *NELL2* have been discovered and characterized in human (Watanabe et al., 1996), rat (Kuroda et al., 1999), and mouse (Zhang et al., 2002). Fluorescence in situ hybridization revealed that both human *NELL* genes are located on different chromosomes, namely 11p15.1-p15.2 (*NELL1*) and 12q13.11-q13.12 (*NELL2*) (Watanabe et al., 1996) indicating their origin from large-scale duplications (Lynch and Conery, 2000). NELL proteins act as homotrimers. Characteristically, NELL proteins possess calcium-binding sites in their EGF-like repeat domains suggesting their contribution to calcium-dependent cellular events (Kuroda et al., 1999). Both, NELL1 and NELL2 factors are protein kinase C-activated by these EGF-like domains supporting the assumption that NELL proteins act as cell signaling molecules critical for growth and development (Kuroda and Tanizawa, 1999). NELL1 has also been identified as a potent regulator of intramembranous and endochondral ossification (Desai et al., 2006) in close interaction with bone morphogenetic protein 2 (BMP2) (Cowan et al., 2007; see also review by Zhang et al., 2010).

NELL proteins activate the mitogen-activated protein (MAP) kinase signaling cascade (Cowan et al., 2007) regulating a variety of intracellular processes including proliferation, differentiation, death, and survival. On the one hand, NELL1 is suggested to induce apoptosis in association with increased Fas and Fas-L synthesis (Zhang et al., 2006) and to suppress tumor growth (Mori et al., 2006). On the other hand, expression of recombinant NELL2 in a mammalian cell model showed increased cell survival under cell death-inducing conditions through extracellular signal-regulated kinase 1 and 2 (ERK1/2; Choi et al., 2010). Moreover, NELL1 has been detected in early and late pre-B cell lines, but not in FACS-purified B cells. NELL2 has been found in B and T cells, monocytes and natural killer cells as well as in leukemic cell lines (Luce and Burrows, 1999). Recently, an alternative *NELL2* splice variant termed as NELL2-Tsp, has been isolated in rat and suggested as a negative regulator of NELL2 (Kim et al., 2010).

Abbreviations: aa, Amino acid; CR, Cysteine-rich domain; Dpf, days post fertilization; EEF1A1, Eukaryotic translation elongation factor 1; EGF, Epidermal growth factor; EST, Expressed sequence tag; GSP, Gene-specific primer; NELL, Neural epidermal growth factor-like-like; ORF, Open reading frame; PRKCB1, Protein kinase C, β-1; qRT-PCR, Real-time fluorescence-based quantitative reverse transcription polymerase chain reaction; RACE, Rapid amplification of cDNA ends; TSPN, N-terminal thrombospondin-like domain; vWF, von Willebrand factor domain.

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The present manuscript characterizes two *NELL2* cDNA sequences from teleost rainbow trout (*Oncorhynchus mykiss*) and compares their expression profiles at different developmental stages and in different tissues as well as after temperature and pathogen challenge in two rainbow trout strains, the imported rainbow trout strain TCO Steelhead II-WA and the local selection strain BORN Steelhead II-Germany. A rainbow trout selective breeding program with imported Danish steelhead trout nurtured in semi-closed aquaculture began in 1975 at the fishery institute Born using the brackish water from shallow parts of the Southern Baltic Sea. Rainbow trout as considered cold-water fish are very sensitive to changes in water temperature, which may vary considerably in aquaculture (Fowler et al., 2009). Especially, increasing water temperatures induce stress responses based on numerous physiological modifications to regain and maintain homeostasis. Not less important are relevant pathogens, i.e. *Aeromonas salmonicida* subsp. *salmonicida*. This Gram-negative bacterium causes the severe systemic disease furunculosis (Reith et al., 2008), which is responsible for considerable losses to the salmonid aquaculture industry.

Long-time selected BORN trout show an increased resistance toward different abiotic and biotic stressors in comparison to the common steelhead trout in local aqua farms (Anders, 1986). Transcriptome-wide microarray analyses were performed to investigate the genetic potential of the robust selection strain BORN as a model for adaptation to local water conditions (Rebl et al., 2009; Verleih et al., 2011). In two approaches, we compared clinically healthy rainbow trout from the strains BORN and TCO (i) unchallenged (15 °C; Rebl et al., 2012) and (ii) acclimated to 8 °C or 23 °C (A. Rebl, unpublished data) and found *NELL2* gene differently expressed in both cases, though the array did not discriminate between the two similar gene copies *NELL2a* and *NELL2b*. However, these findings motivated us to characterize the *NELL2* genes in rainbow trout.

2. Materials and methods

2.1. Preliminary sampling

Preliminary NELL2 expression analyses were performed using two-year old rainbow trout (*Oncorhynchus mykiss*) of the local strain Born Steelhead II-Germany (BORN) or of the imported trout TCO Steelhead II-WA (import; www.troutlodge.com). Both trout strains represent the coastal form. Trout were acclimated for 2 weeks in separate freshwater aquaculture ponds at the fish farm BIMES (Binnenfischerei Mecklenburg GmbH Schwerin, Frauenmark, Germany). Adipose tissue, brain, fin, gill, heart, intestine, liver, muscle, skin, spleen, head kidney, and trunk kidney from five individual fish from each strain were collected and flash-frozen in liquid nitrogen until further use.

To detect the expression patterns of NELL2 genes during development, we sampled egg and larval material as well as fry from import trout at the Institute for Fisheries LFA-MV (Born and Hohen Wangelin, Germany). These samples comprised 30- and 38-dpf 'eyed eggs' (10 and 2 days, respectively; before start of hatching) and 45-dpf alevins (2 days after final hatching) maintained at 8 °C in upwelling incubators; and 78-dpf fry fish without yolk sac (18 days after first feeding) maintained at 9 to 10 °C in holding tanks. Fry fish were killed by an overdose of benzocaine. Samples were immediately transferred into RNAlater (Qiagen, Hilden, Germany) and stored at −80 °C until RNA extraction was performed.

2.2. Pathogen and temperature challenge experiment

Subsequent challenge experiments were conducted using BORN and TCO rainbow trout reared simultaneously to fingerling stage in freshwater tanks under constant conditions including a water temperature of 15 °C. Trout were further maintained in 800-liter freshwater tanks. Fourteen days prior to challenge, fish were transferred in experimental 300-liter tanks, in order to minimize handling stress influencing the qRT-PCR data.

For the temperature experiments, the temperature of both 15 °C experimental tanks was raised or lowered by 1 °C/day to moderate stress temperatures of 23 °C or to 8 °C, eventually holding the respective 'stress' temperature for 1 week. Brain, gill, trunk kidney, and muscle tissue samples were taken from seven 10-month old BORN and seven 10-month old import rainbow trout and stored in RNAlater solution.

For infection study, 72 BORN and 72 import trout aged 11–12 months were intraperitoneally (i.p.) infected with a total dosage of 1×10^4 cfu (colony forming unit) *A. salmonicida* subsp. *salmonicida*. Before injection and sampling, fish were anesthetized in a benzocaine solution (50 ng/ml). Gill, trunk kidney, and muscle tissue samples of four fish per strain were taken 0, 12, 24, 48, 72 h as well as 7, 14 and 21 days after injection. Severe signs including pale, swollen, hemorrhagic liver; intestinal hemorrhages; lytic white muscle; necrotic kidney occurred in BORN and import trout 72 h and 7 d after infection.

2.3. RNA preparation

Flash frozen tissue samples were at first powdered under nitrogen. Both, tissue powder and tissue samples stored in RNAlater solution were homogenized individually in 1 ml TRIzol Reagent (Invitrogen, Karlsruhe, Germany) using Precellys 24 homogenizator (PEQLAB Biotechnologie, Erlangen, Germany). RNA was extracted (RNeasy Mini Kit, Qiagen) with in-column DNase treatment for 30 min. RNA quantity was assessed by absorbance at 260/280 nm at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was determined by electrophoresis in a 1% agarose gel.

2.4. Isolation and analyses of two rainbow trout NELL2 cDNA sequences

The human cDNA sequence encoding *NELL2* (NM_001145107) was used to identify the rainbow trout ESTs 654245 (CA376007), 37452654 (CA341843), as well as 1315861 (CX262257) employing the NCBI BLAST tool. Alignment of the ESTs 37452654 and 1315861 revealed 15.5% altered sequence positions. They were thus utilized to derive trout-specific *NELL2* oligonucleotide primer sequences displayed in Table 1. All primers used in this study were designed with PSQ™ Assay Design software (Biotage, Uppsala, Sweden). 5 µg of total RNA extracted from fins of rainbow trout was reverse transcribed using SuperScript II (Invitrogen, Karlsruhe, Germany) to generate a cDNA template. In order to obtain full-length cDNA sequence,

Table 1
Primers used in this study.

NELL2a primers (5′–3′)	*NELL2b* primers (5′–3′)	Technique
CATCCTCCTGCACATCACCTG*	GACAGCTTGCTGGAGGTCTTG*	5′RACE PCR
CGTTGTCCAGCACCCAGATC*	GATCTTCTGCACCAGCCCATG*	5′RACE nested PCR
CACGGATGAGCACGGCATCC	CTGGACCAAACACAGAACAGAAT	3′RACE PCR
CAGTGCAAGAATGGACATATCTG	CCCCCTGTGCCTTTAATCTGG	3′RACE nested PCR
CAGATCTGGGTGCTGGACAAC		3′RACE for Southern Blot
GACCTGTTCTGCTGTCCAGAG		Southern Blot: probe generation
GGCACTGCTGGCAGTTCTCC*		Southern Blot: probe generation
GTTACATCAGGATCGATGACTAC	GATACATCCGGATCGATGACTAT	qRT-PCR
CACAGGCCATCACACATCGCT*	CACAAACCATCACACAAAGCTC*	qRT-PCR

Asterisks "*" indicate antisense oligonucleotides.

5′- and 3′-RACE experiments were conducted with the Gene Racer SuperScript II RT Module (Invitrogen, Karlsruhe, Germany), following the manufacturers protocol. Amplificates from at least three different PCR reactions were sequenced.

Sequence alignments were created using the ClustAlW program (Combet et al., 2000). The domain structure was predicted from the SignalP 3.0 server (Bendtsen et al., 2004), the TMHMM 2.0 server (Krogh et al., 2001), and the SMART server (Letunic et al., 2009).

The Molecular Evolutionary Genetics Analysis package MEGA v3.1A reconstructed a phylogenetic dendrogram of amino acid sequences using the Neighbour–Joining Method and the Poisson correction parameter (Kumar et al., 2004).

2.5. Southern blot analysis on RACE PCR fragments

3′-RACE PCR products were obtained with oligonucleotides that share 100% identity with both *NELL2* variants (Table 1). Fragments were run on 2.5% agarose gel that was subsequently washed twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and twice in neutralization solution (0.5 M Tris–HCl [pH 7.5], 1.5 M NaCl) followed by DNA transfer on a positively charged nylon membrane (Roche, Mannheim, Germany) by overnight capillary blotting in 20× SSC at 4 °C. In parallel, a 115-bp digoxigenin-labeled *NELL2* probe covering a region with 88% identity between both *NELL2* sequences from trout was synthesized using the oligonucleotides listed in Table 1 and the PCR DIG Probe Synthesis Kit (Roche). After UV-cross-linking, prehybridization and overnight hybridization were carried out at 43 °C in DIG Easy Hyb solution (Roche). Membranes were washed twice in low stringency buffer (2× SSC, 0.1% SDS) at room temperature for 5 min each and then twice in high stringency buffer (0.1 ×SSC, 0.1% SDS) at 43 °C for 20 min each. The blots were visualized according to the DIG Nucleic Acid Detection Kit (Roche).

2.6. Quantitative RT-PCR

Semi-quantitative reverse transcriptase PCR (RT-PCR) and Real-time fluorescence-based quantitative RT-PCR (qRT-PCR) were set up with primers that are specific for *NELL2a* or *NELL2b* (Table 1) generating 175-bp fragments. In order to avoid target amplification from genomic templates, we designed an antisense RT-PCR oligonucleotide, which is homolog to the sequence bridging exon 15 and 16 in the orthologous gene from pufferfish *Tetraodon nigroviridis* (Ensemble accession: ENSTNIT00000019296), assuming conserved exon/intron boundaries. Primers were validated and PCR conditions were optimized by use of agarose gel electrophoresis to ascertain that amplification yielded a single product of the predicted size. A 101-bp fragment of the housekeeping gene *EEF1A1* (eukaryotic translation elongation factor 1 alpha 1) was amplified in parallel as an internal control using oligonucleotides designed by Bowers et al. (2008). cDNA was prepared using an oligo-d(T) primer [25 pmol/μL] and Super Script™ II kit (Invitrogen, Karlsruhe, Germany).

For semi-quantitative RT-PCR, Hot Star Taq Plus (Qiagen) amplified *NELL2a* and *NELL2b* fragments from a cDNA input equivalent of 24 ng of total RNA. Amplified DNA fragments were electrophoresed in 3% agarose gels. PCR was repeated twice with samples from three different trout.

For fluorescence qRT-PCR carried out on the LightCycler 480 Instrument, the LightCycler 480 SYBR Green I Master Kit (Roche) was utilized to analyze the cDNA equivalent of 150 ng total RNA. Standard curves for both *NELL* genes and the reference gene *EEF1A1* were generated based on the crossing point (CP) values of 10-fold dilutions of gene-specific, PCR-generated fragments (10^3 to 10^6 copies). The slope and y-intercept were obtained via linear regression. The absolute copy numbers of the respective genes were calculated on the basis of linear regression of the standard curve. Eventually, each target gene expression value was divided by the mean expression value of the housekeeping gene.

Statistical significance of *NELL2a* and *NELL2b* expression data was assessed with SPSS software (SPSS Inc., Version 15.0) using parametric *t*-test in case of normal distribution as evaluated with Kolmogorov–Smirnov test or non-parametric Mann–Whitney *U*-test. All differences were regarded significant at the $p<0.05$ level, while differences with $0.05<p<0.1$ will be referred to as a tendency in the data.

3. Results and discussion

3.1. Both NELL sequences from trout resemble mammalian NELL2

We isolated two full-length NEL-like cDNAs from rainbow trout by RACE PCR, which were termed as *NELL2a* (GenBank accession: NM_001199152) and *NELL2b* (NM_001164067). Coding sequences of both *NELL* genes share 83% identity with each other and >64% identity with human *NELL2*. Both cDNA sequences comprise 3247 or 3186 bp excluding polyadenylated tail, and encode an ORF of 2439 bp and 2445 bp, respectively. Conceptual translation reveals that NELL2a and NELL2b encode for 812- or 814-aa precursor proteins with a hypothetical molecular weight of 89.9 and 90.2 kDa, respectively.

Both NELL proteins share the same motifs and domains (Fig. 1). A secretion signal peptide was predicted for the first 19 aa, however, no transmembrane domains within both NELL2 proteins could be identified. The signal peptide is followed by a 186-aa thrombospondin-type laminin G domain (N-terminal thrombospondin-like domain, TSPN). This TSPN module includes a central 69 aa long region with 8 histidine residues (11.6%) within both NELL2 aa sequences from rainbow trout. In comparison, chicken NEL and NEL-like protein as well as human NELL2 show even eleven histidine residues (15.9%), whereas human NELL1 has only 6 histidine residues in the respective region (8.8%).

Within trout NELL2 proteins, the TSPN is followed by a 19-aa coiled coil region corresponding to its counterpart in the mammalian NELL homologue (Kuroda et al., 1999). This motif is strongly conserved among NELL proteins from trout, chicken, and NELL2 proteins from mammals (89.5% identical and 10.5% strongly similar aa). Nevertheless, the coiled coil region of mammalian NELL1 proteins shares significantly lower sequence identity with the respective region in mammalian NELL2 molecules (52.6% identical and 26.3% strongly similar aa).

The C-terminal two thirds of the NELL proteins from rainbow trout include five 53-aa to 62-aa cysteine-rich von Willebrand factor (vWF) domains (vWF1, 57 aa; vWF2, 62 aa and 61 aa, respectively; vWF3, 53 aa; vWF4, 56 aa and 57 aa, respectively; vWF5, 53 aa) flanking six EGF-like domains (EGF1 comprising 38 and 40 aa, respectively; EGF2: 41 aa; EGF3: 40 aa; EGF4: 28 aa; EGF5: 46 aa; EGF6: 35 aa). The vWF domain is found in various plasma proteins, which are typically involved in multiprotein complexes. It has been assumed that mammalian NELL monomers associate into homotrimers through vWF domains or the coiled coil region (Kuroda et al., 1999).

The six EGF-like domains vary in length from 28 aa to 46 aa. Each domain includes at least six conserved cysteine residues (Fig. 1). EGF-like domains are essential for functionality in more than 100 eukaryotic membrane and secreted proteins (Van Zoelen et al., 2000). They mediate calcium-dependent protein to protein interactions related to growth regulation and differentiation. Indeed, three of six EGF-like domains within both trout NELL2 proteins correspond to calcium-binding domains (EGF-like domain 2, 5, and 6), which is in line with findings for mammalian NELL proteins (Kuroda et al., 1999). NELL factors are reported to be phosphorylated by protein kinase C, β−1 (PRKCB1) via the EGF-like domains in order to transmit developmental signals (Kuroda and Tanizawa, 1999).

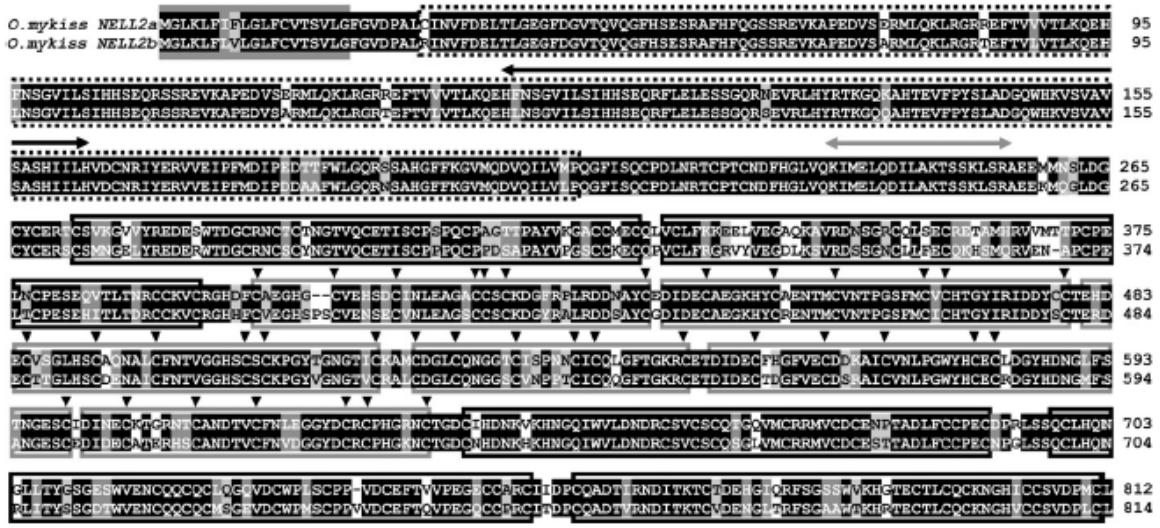

Fig. 1. Alignment of NELL2 amino acid sequences from rainbow trout.NELL2a and NELL2b sequences are presented in single letter code. Gaps are shown as dashes. Identical amino acids are highlighted in black; strongly similar amino acids are printed in white letters with dark gray underlay; weakly similar amino acids are printed in black letters with light gray underlay. The predicted N-terminal signal peptide is indicated by gray underlay. An adjacent thrombospondin-like module is framed by a dotted black line. Five von Willebrand factor domains are framed by a solid black line. Six EGF-like domains are framed by a solid gray line; conserved cysteine residues are marked with black triangles above the gray frame. A histidine-rich region is indicated by a black arrow above the respective scheme section. A coiled coil region is indicated by a gray arrow.

Amino acid sequence alignments show that trout NELL2a shows most identity with the respective full-length sequences from pufferfishes *Takifugu rubripes* (Ensembl Peptide ID: ENSTRUT00000042861) (85.2%) and *Tetraodon nigroviridis* (ENSTNIP00000019068) (84.2%) as well as Japanese ricefish (medaka; *Oryzias latipes*; ENSORLP00000003776) (84.8%). Trout NELL2b shares highest degree of identities with NELL sequence from Atlantic salmon (*Salmo salar*; GenBank accession: NP_001133618) (90.0%). However, in comparison with NEL or NEL-like protein from chicken (*Gallus gallus*), only 63–64% or ~70% aa residues are identical. It might be noteworthy that both NELL2 variants share more identical aa residues in trout (82.8%) than in zebrafish (*D. rerio*; 53.3%). NELL2 proteins in zebrafish show a very low conservation within the segment between the coiled coil region and the C-terminal EGF-like domain with 37.9% identical aa residues, whereas trout NELL2 genes share 74.8% identities in the respective region.

Furthermore, both NELL proteins from chicken share a higher degree of identity (89.5%) than NELL1 and NELL2 in mammals, e.g. mouse (54.3%), rat (54.1%), and human (54.8%) due to a comparably lower sequence conservation (~30% identities) of particularly vWF1 and EGF1 domains.

3.2. Fishes may possess different NELL pair combinations

All NELL protein sequences from teleost species available to date in NCBI and Ensembl databases have been arranged in a phylogenetic Neighbour-Joining tree together with NELL proteins from human, chicken, and clawed frog *Xenopus tropicalis* as well as from insect *Apis mellifera* (Fig. 2A). Human epidermal growth factor sequence was used as outgroup. The dendrogram illustrates the clear separation of NELL1 and NELL2 protein sequences. Both human NELL1 and NELL2 sequences are grouped with its non-teleost orthologues. Correspondingly, NELL1 proteins from both pufferfishes and stickleback *Gasterosteus aculeatus* form an independent clade with a bootstrap confidence value of 100%. The phylogenetic tree depicts furthermore that teleost NELL2 sequences may be subdivided into a NELL2a and a NELL2b cluster with 91% bootstrap support. It seems that fishes possess either a *NELL1*/*NELL2* (pufferfishes, stickleback) or a *NELL2a*/*NELL2b* gene couple (zebrafish, salmonids). This assumption is supported by the synteny between *NELL1* and *NELL2* loci in human and pufferfishes (Fig. 2B). On the other hand, *NELL2a* and *NELL2b* genes from zebrafish are both located adjacent to genes, which are also adjacent to human *NELL2* genes, whereas orthologue genes neighboring human *NELL1* are located downstream of zebrafish *NELL2a*.

We were unable to find an EST encoding a trout *NELL1* variant or a zebrafish *NELL1* gene in Ensembl and NCBI databases that are expected to contain several thousands of development- and growth-related ESTs from trout oocytes and embryos. It is still speculation whether or not *NELL1* gene copy has been lost in fishes that possess a duplicated *NELL2* gene. Due to a closely structural relatedness, *NELL2* copy could have adopted the function of *NELL1*, which has evolved as a pseudogene through degenerated mutations according to a theory by Lynch and Conery (2000). Chicken also seems to compensate the obvious lack of a *NELL1* ortholog with two *NEL(L)* genes corresponding to mammalian *NELL2*.

3.3. Both NELL2 genes from trout show a distinct developmental and tissue-specific expression profile

NEL and *NEL*-like genes have been reported to be linked with growth and differentiation. Quantitative RT-PCR recording the expression of both *NELL2* genes at early stages of rainbow trout development revealed considerable *NELL2a* and *NELL2b* transcript numbers. The respective crossing point values (that are inversely proportional to the copy number) for *NELL2a* (ranging from 18.0 to 21.5) and *NELL2b* (20.8 to 26.0) indicate that *NELL2a* expression is comparably stronger in eyed eggs (30 and 38 dpf, respectively), larval fish (45 dpf) and fry (78 dpf). *NELL2a* transcript number peaks 2 days before hatching and decreases by 73.7% in free-swimming fry fish (Fig. 3A), whereas *NELL2b* mRNA level tends to be higher after hatching by about 40% ($p=0.095$) (Fig. 3B).

These results contrast the findings in birds, where *NEL* gene has been detected ubiquitously in embryonic tissues of 10- and 17-day old embryos, but was hardly detectable in non-neural tissues after hatching (Matsuhashi et al., 1995).

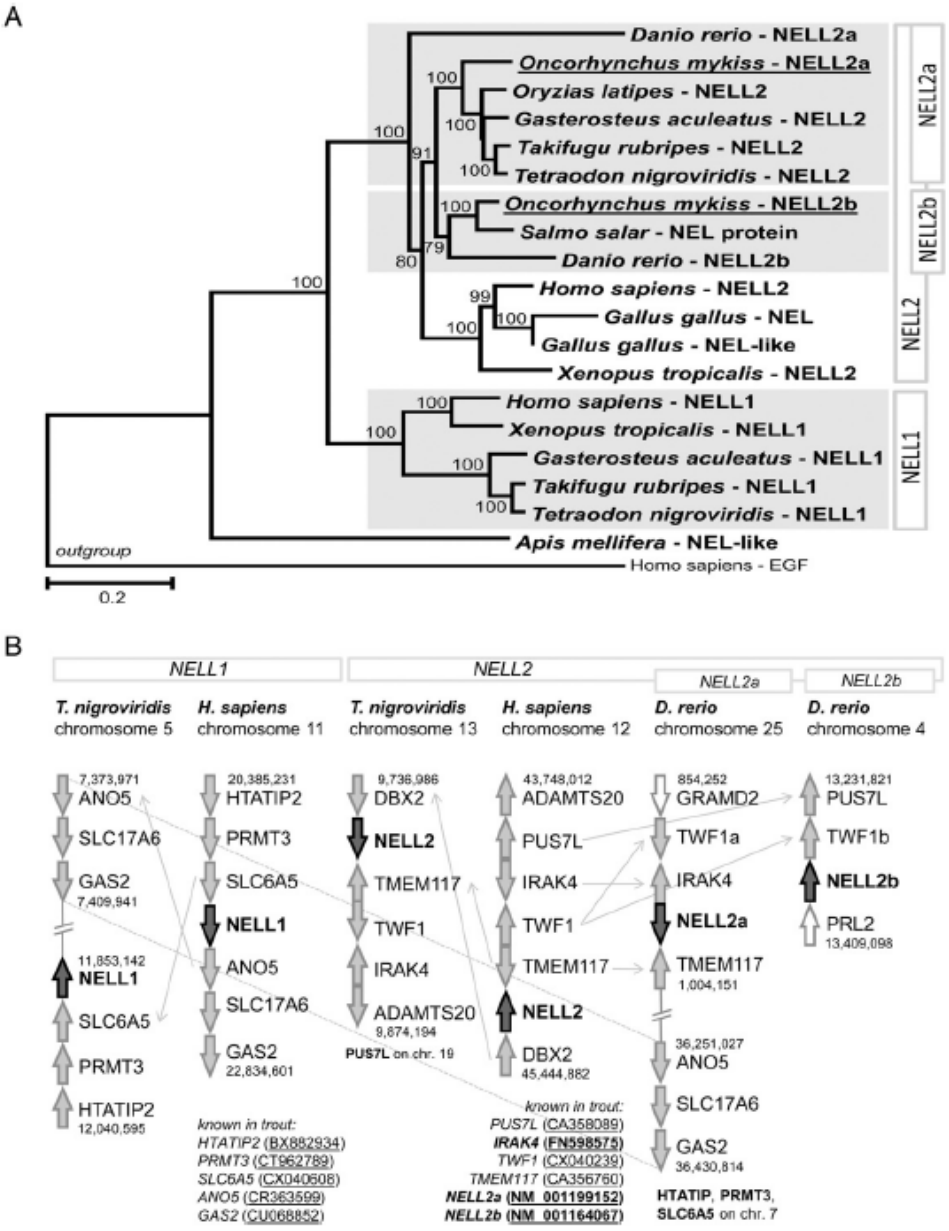

Fig. 2. (A) Neighbour-Joining phylogenetic tree of full-length NEL and NEL-like protein sequences. Bootstrap values (10,000 replicates) are shown as percentages in the branches. The tree includes protein sequences from the following species (with corresponding GenBank [GB] and Ensembl [E] accession codes): *Apis mellifera* (GB: XP_395215), *Gallus gallus* (NEL, GB: JP0076; NEL, NP_001025911), *Danio rerio* (NELL2, GB: XP_002661425, E: ENSDARP00000111598; NELL2b, GB: XP_002661425), *Gasterosteus aculeatus* (partial NELL1, E: ENSGACP00000022585; partial NELL2, E: ENSGACP00000005265), *Homo sapiens* (NELL1, GB: NP_006148; NELL2, NP_006150), *Oncorhynchus mykiss* (NELL2a, GB: NP_001186081; NELL2b, GB: NP_001157539; both underlined), *Oryzias latipes* (NELL2, E: ENSORLP00000013776), *Salmo salar* (NEL, GB: NP_001133618), *Takifugu rubripes* (NELL1, E: ENSTRUP00000040947; NELL2, E: ENSTRUT00000042861), *Tetraodon nigroviridis* (NELL1, E: ENSTNIP00000012484; NELL2, E: ENSTNIP00000019068), *Xenopus tropicalis* (partial NELL1, GB: AAI21468; NELL2, GB: NP_001090754). Human EGF precursor (GB: NP_001954) serves as an outgroup. The scale bar represents a genetic distance of 0.2 amino acid substitutions per site. (B) Synteny between human and teleost *NEL* loci. The comparison includes *NELL1* loci from human (Ensemble gene ID: ENSG00000165973) and pufferfish (ENSTNIG00000009609) as well as *NELL2* loci from human (ENSG00000184613), pufferfish (ENSTNIG00000015980), and zebrafish (*NELL2a*, ENSDARG00000090690; *NELL2b*, ENSDARG00000062797). Thick gray arrows represent genes found in synteny and indicate their orientation; official gene symbols were used. *NEL* genes were highlighted with black arrows. Thin gray arrows indicate the positions of orthologue genes up- and downstream of *NELL* genes. The location of the depicted gene groups within the respective chromosome are given in bp. The figure is not scaled. Ortholog sequences from rainbow trout available in GenBank are listed below the scheme; full-length sequences are printed in bold face letters.

The expression of trout *NELL2a* and *NELL2b* gene was furthermore examined in twelve different tissues from adult rainbow trout maintained in aquaculture ponds (Fig. 3C). *NELL2a* mRNA copies were detected in all tissues analyzed including brain, fin, gill, muscle, skin, and spleen, which showed higher mRNA amounts in comparison to adipose tissue, head and trunk kidney, intestine, and liver. In

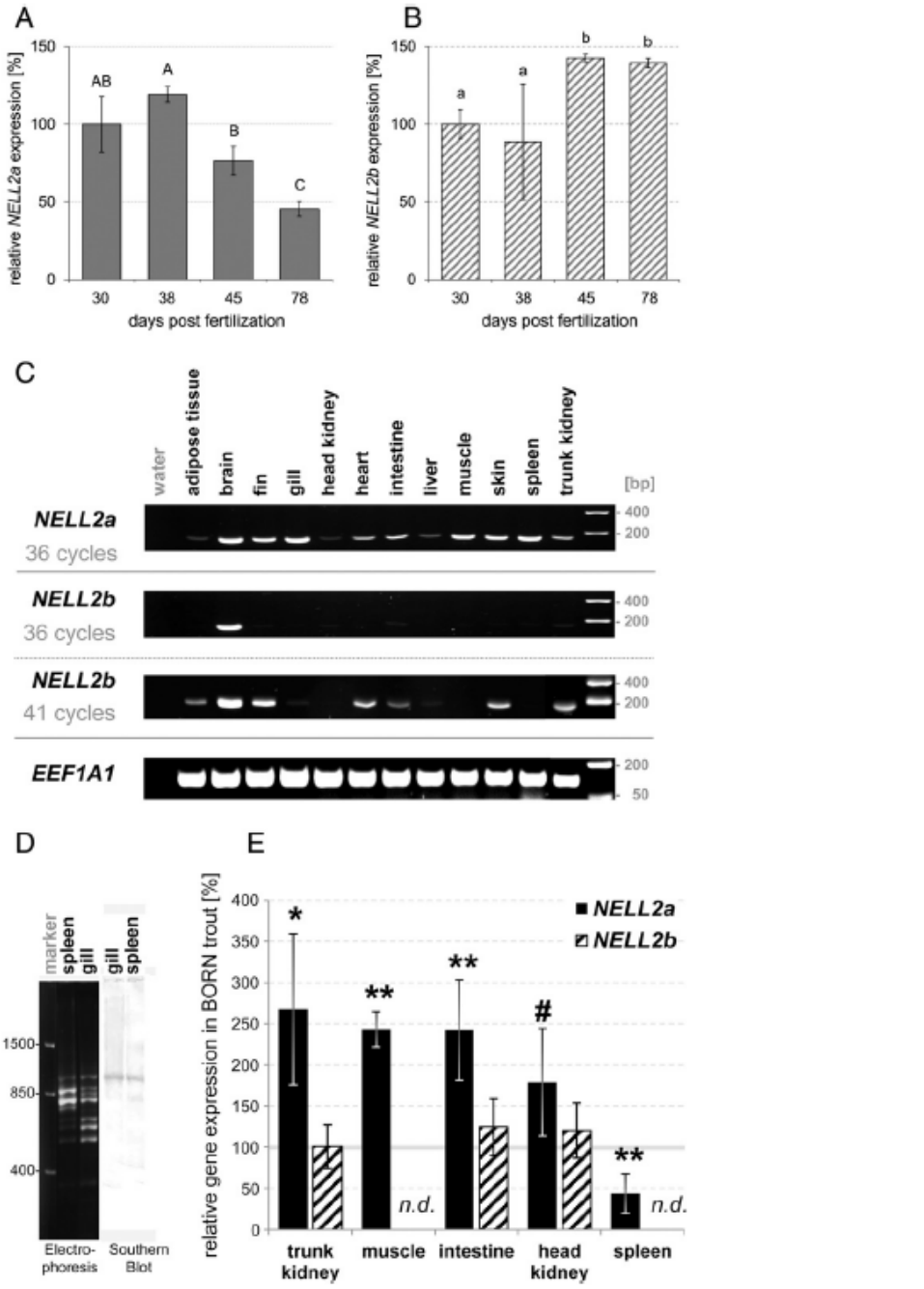

Fig. 3. Quantitative RT-PCR analysis of (A) *NELL2a* and (B) *NELL2b* transcript level during development. Bars represent the relative normalized means of five individual samples (±SEM). Expression in 30-dpf eggs was set as 100%. Differences in expression with $p<0.01$ or $p=0.095$ between the developmental stages are indicated by different capital letters (A–C) or small letters (a, b), respectively. (C) Tissue-specific expression profile of *NELL2a* and *NELL2b* gene from rainbow trout determined by semiquantitative RT-PCR. *NELL2a* and *NELL2b* fragments were generated in a standard PCR comprising 36 or 41 cycles. *EEF1A1* was employed as an internal control. The twelve examined tissues are indicated above each lane. A representative mRNA distribution profile obtained by repeated PCR analysis is shown here since similar results were obtained with three healthy individuals. (D) 'Reverse northern blot' analysis on 3'-RACE products from spleen and gill tissues. 3'-RACE PCR products were separated by electrophoresis on a 2.5% agarose gel (shown on the left). The blot shown on the right proves only one *NELL2* band with the expected length of ~980 bp characteristic for *NELL2a* (compare with the marker bands in bp on the left margin). (E) qRT-PCR analysis of *NELL2a* (filled columns) and *NELL2* (hatched columns) transcript level in trunk kidney, muscle, intestine, and spleen from rainbow trout. Normalized mean values of the relative copy numbers from the respective import trout tissue ($n=5$) were set as 100% (represented by a solid gray line), only the normalized means from the corresponding BORN trout tissue ($n=5$) are shown in the diagram. Error bars indicate SEM. # indicates an expression difference with $p=0.08$; * represents a significant difference ($p=0.03$), ** indicates highly significant differences ($p\leq0.01$) between *NELL2a* expression in BORN and import trout. Columns for *NELL2b* expression in muscle and spleen are missing due to negligible low copy numbers (n. d.).

contrast, *NELL2b* was strongly expressed only in brain. In mammals, a predominant *NELL1* and *NELL2* expression has been proven in adult and fetal brain along with a very weak expression in kidney (Watanabe et al., 1996).

Regarding the discordant expression profiles of *NELL1/2* and *NELL2a/b* genes in mammals, birds and trout, respectively, it seems that both *NELL* gene pairs might fulfill different physiological demands. Strikingly, trout *NELL2a* gene appears to be ubiquitously expressed, whereas the restricted *NELL2b* expression profile resembles mammalian one. Interestingly, 3′-RACE performed with low stringency on cDNA isolated from spleen and gills generated multiple bands suggesting the assumed existence of several *NELL* gene or transcript variants. However, a 'reverse-northern blot' validated our previous observations that only one *NELL2* transcript is present in the respective tissues (Fig. 3D). Sequencing of the further fragments confirmed the amplification of other sequences, predominantly troponin transcripts.

Though *NELL2b* expression in gill, head kidney, and spleen could be proven via LightCycler qRT-PCR with a ~6-fold higher cDNA input, the measured copy numbers were very low or even negligible. Regarding expression differences between BORN and import rainbow trout, in five tissues a different *NELL2a* mRNA abundance was detected validating our previous microarray results (Fig. 3E). Whereas *NELL2a* copy number was significantly lower in splenic tissue of BORN trout by 56.5% ($p=0.002$), *NELL2a* mRNA abundance was significantly elevated in trunk kidney by 167.6% ($p=0.03$), in muscle by 143.0% ($p=0.006$), and in intestine by 142.2% ($p=0.01$) of BORN trout in comparison to import rainbow trout. In addition, *NELL2a* copy number was elevated in head kidney of BORN trout in comparison to import trout, although this difference was not significant ($p=0.08$). *NELL2b* showed similar expression values in both strains.

These expression data reveal that *NELL2a* gene from trout shows not only a tissue-specific expression pattern, but also a strain-specific one, whereas *NELL2b* does not.

3.4. NELL2a gene expression is elevated in trout white muscle after temperature increase

Subsequent to our preliminary *NELL2* expression studies with BORN and import trout held in aquaculture ponds, we conducted challenge experiments with trout maintained in experimental water tanks under standardized conditions. In order to determine the expression of *NELL2a* and *NELL2b* genes at different temperatures suggesting a potential involvement in thermoregulation, BORN and import trout were exposed to 8 °C and 23 °C for 7 days. A previous transcriptome analysis indicated a clear temperature-dependent *NELL2* expression difference in gill and trunk kidney tissue of import and BORN trout, respectively (A. Rebl, unpublished data), though data were not specifically determined for *NELL2a* and *NELL2b*, since the 60-mer sequence placed on the microarray slide allows the hybridization with both transcripts. Hence, copy numbers of both *NELL2* genes were quantified with gene-specific oligonucleotides in brain (where pronounced *NELL* mRNA abundances in trout and almost exclusive *NELL* expression in mammals is present), gill (to validate the initial microarray results), trunk kidney, and muscle (which showed both the largest expression differences among the two trout strains).

As seen in the preliminary expression study, *NELL2b* gene could again not be measured in gill and muscle tissue. Fig. 4 illustrates that *NELL2a* copy number was significantly ($p<0.006$) depressed by >50% in gill tissue of both trout strains at 23 °C compared to 8 °C. In trunk kidney of import trout, *NELL2a* expression was decreased by tendency (−60.1%; $p=0.07$) and *NELL2b* expression was highly (−71.4%; $p=0.003$) down-regulated at 23 °C. These results contrast *NELL2a* expression in muscle, which is up-regulated (+57.4%, $p=0.007$) in import trout at 23 °C compared to 8 °C. In brain of import trout acclimated to 8 °C, only the *NELL2b* transcript number tended to be lower (−22.2%, $p=0.08$) compared to trout at 23 °C.

Besides, significant strain-specific expression differences are present for *NELL2a* at 8 °C in gill (−24.2% in BORN with $p=0.05$) and muscle (+100.4% BORN with $p=7.5\times10^{-5}$) as well as for *NELL2b* at 23 °C in trunk kidney (+157% in BORN with $p=0.01$).

Altogether, *NELL2* transcript level remained almost constant in brain even under mild laboratory-induced temperature stress. Strikingly, only gill tissue showed a clear temperature-induced *NELL2a/2b* expression difference in both rainbow trout strains. Furthermore, both *NELL2* genes were (if detectable) at least slightly down-regulated at 23 °C in gill and trunk kidney compared to 8 °C, whereas *NELL2a* was up-regulated in muscle.

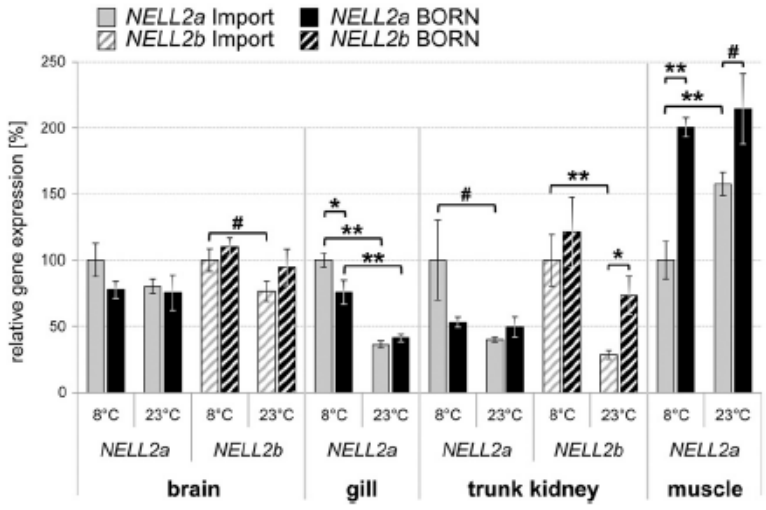

Fig. 4. qRT-PCR analysis of *NELL2a* (filled columns) and *NELL2b* (hatched) transcripts in brain, gill, trunk kidney, and muscle of import (gray) and BORN trout (black) at 8 °C and 23 °C. Relative quantities normalized to *EEF1A1* mRNA expression are presented as mean transcript levels ± SEM ($n=7$). *NELL2a* and *NELL2b* copy numbers from import trout acclimated to 8 °C were set as 100%, whereas the relative mRNA abundance from import trout (held at 23 °C) and BORN trout (8 °C and 23 °C) are expressed as fraction hereof. # indicates expression differences with $p\leq0.08$, * represents significant differences with $p\leq0.05$, ** represents highly significant differences with $p<0.01$.

The question remains, whether this expression feature in muscle emphasizes NELL2a protein's importance for physiology or not. The steroid hormone cortisol is released in response to stress and has been shown to induce hypercalcemia in trout (Björnsson et al., 1987). Calcium ions are crucially involved as intracellular messengers in the regulation of many forms of cellular activities (reviewed by Berridge, 1997; Cheng and Lederer, 2008). Based on data from a mammalian cDNA array, it has been discussed that NELL2 may stimulate many cellular events related to the signaling of intracellular calcium (Kim et al., 2002), since it possesses calcium-binding EGF-like domains. Though the physiological role of NELL proteins in mammals is not well understood so far, not to speak of fish, it seems that NELL proteins are multifunctional factors influencing the expression of numerous genes and participating in several pathways, which regulate development, growth, survival and apoptosis. Accordingly, BORN trout may profit from an elevated basal *NELL2* expression in muscle tissue compared to import trout (see Figs. 3E,4), as NELL2 has been described as a marker for the early phase of hypaxial muscle differentiation in birds. *NELL2* transcripts have been detected in dermomyotome (Nelson et al., 2002), which comprise muscle precursor cells that give rise to all skeletal muscle derivatives. Moreover, Nelson et al. (2004) postulated that members of the *NELL* gene family promote the differentiation of those cells, in which they are expressed. In trout, NELL2a may be concerned in maintenance of muscle cells. Mammalian NELL1 has been proven to support osteoblast cell differentiation and mineralization (Zhang et al., 2010). Therefore it might be possible that NELL stimulates the development of fish bones, though further research is needed to prove this hypothesis.

3.5. Infection with *A. salmonicida* provokes up-regulation of renal *NELL2* gene expression in selected BORN trout

A second experiment was conducted to evaluate the role of trout *NELL2* during bacterial infection with *A. salmonicida* in gill, trunk kidney and muscle tissue, which showed already significant expression differences after temperature challenge. *A. salmonicida* is the causal agent of furunculosis that may be characterized by wounds with small necrosis or even deep lesions surrounded by necrotic muscle and pronounced dermatitis (Paterson et al., 1980; Wichardt et al., 1989).

Gill is known as an immunocompetent organ in trout harboring abundant T cells (Koppang et al., 2010). However, in gill tissue of BORN trout, *NELL2a* transcript level remained almost constant (Fig. 5A). This suggests that *NELL2a*, which has been reported to be expressed in a variety of mammalian immune cells (Luce and Burrows, 1999), is not involved in branchial immune response after experimental i.p. infection. It may be doubted if gills did get in contact with the pathogen, as we did not observe signs of infection in this tissue.

Regarding trunk kidney, BORN and import trout showed a different NELL2 expression pattern (Fig. 5B). Whereas in import trout *NELL2a* transcript level doubled (+120.2% compared to healthy individuals) and *NELL2b* copy number decreased (−69.0%) until day 21 of infection, in BORN trout a sudden significant increase in *NELL2a* expression (+116.2%) occurred at day 7 p.i. accompanied by an increased *NELL2b* gene expression (+57.4%). At this time point, BORN and import trout showed severe signs including pale, swollen, hemorrhagic liver; intestinal hemorrhages; lytic white muscle; and necrotic kidney.

Also in white muscle, *NELL2a* was up-regulated (+144.3% and +85.5%) at day 14 after challenge in BORN and import trout, respectively (Fig. 5C). NELL2 might mediate for instance enhanced cell survival, originally suggested by Choi et al. (2010). Mammalian NELL1 has furthermore been proven to affect directly or indirectly the expression of a number of genes, e.g. two collagen types,

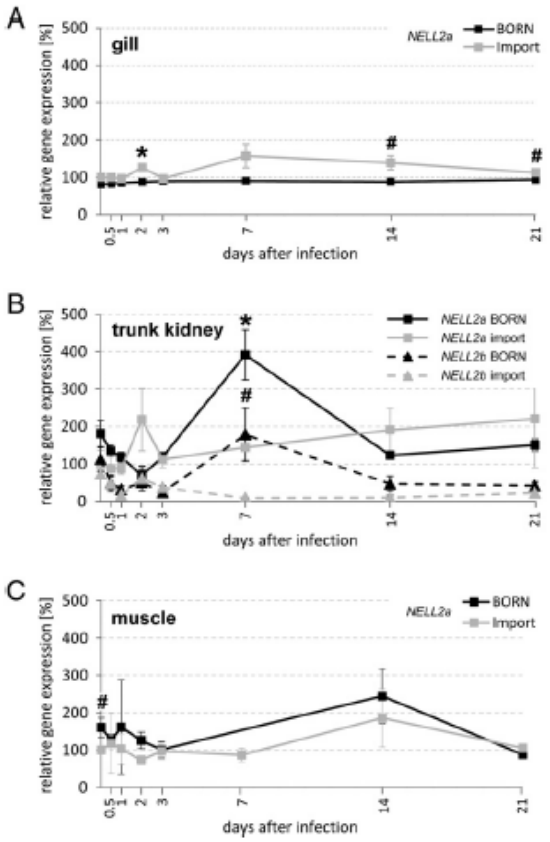

Fig. 5. Relative level of *NELL2a* (full line, square symbols) and/or *NELL2b* genes (broken line, triangular symbols) in (A) gill; (B) trunk kidney; and (C) muscle of BORN (black lines) and import trout (gray lines) after intraperitoneal injection with *A. salmonicida*. Tissues were sampled from three trout of each strain at 0, 12 h and 1, 2, 3, 7, 14, and 21 days after infection. Each data point represents the normalized mean values ± SEM; mean values from import trout were set as 100%. # indicates expression differences with $p<0.1$, * represents a significant difference with $p<0.05$.

COL12A1, *COL6A1*; tenascin XB; thrombospondin-3 (Desai et al., 2006), which are all implicated in wound healing. Therefore NELL could be involved in repair mechanisms after *A. salmonicida* infection. The increased *NELL2a* expression in trout kidney, a tissue tasked with hematopoiesis comparable to mammalian bone marrow, might also indicate that NELL2a is involved in signaling processes leading to the development of myeloid and lymphoid cells during later infection. Luce and Burrows (1999) have already reported that NELL expression is detectable in a restricted developmental stage of myeloid cells.

4. Conclusions

Taken together, the protein sequence deduced from the two thrombospondin-1/EGF-like NELL cDNA sequences from rainbow trout contain several domains which have been reported for their mammalian and avian counterparts before. *NELL2* gene pair in trout may have arisen from a recent duplication event. RT-PCR reveals that *NELL2a* and *NELL2b* show different tissue distribution profiles. *NELL2b* gene is mainly expressed in brain, whereas *NELL2a* shows a more widespread expression. A differential *NELL2a* gene expression is striking in several tissues of an import rainbow trout strain and the robust BORN trout strain.

These differential expression features are also remarkable after temperature change and *A. salmonicida* stimulation. An up-regulation of *NELL2a* expression in muscle under both challenge conditions might reflect its importance for survival. The elevated robustness of BORN trout might be supported by the comparably higher synthesis of the multifunctional NELL proteins, which are involved in numerous physiological processes.

Acknowledgments

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VII. Comparative analyses of apoptosis-related candidate genes in rainbow trout: molecular characterization and transcriptome analyses.

Verleih, M., Rebl, A., Köllner, B., Korytář, T., Kühn, C., Wimmers, K., Goldammer, T. *Schriftenreihe des FBN* (2011), **18**: 39-42.

kurze Zusammenfassung:
Im Rahmen dieser Studie wurde die spezifische Regulation von Schlüsselgenen der Apoptose im Infektionsverlauf untersucht. Dazu wurde ein Vergleich des Kopfniere-Transkriptoms infizierter Fische der lokalen Zuchtlinie BORN und Importforellen durchgeführt. Dabei war in beiden Zuchtlinien vor allem sieben Tage nach Infektion eine Aktivierung von Genen des aktiven Zelltods erkennbar. Allerdings zeigten sich Differenzen im zeitlichen Verlauf sowie in spezifischen Regulationsmechanismen der Apoptose. Die Ergebnisse der Studie geben einen ersten Hinweis für eine differente Regulation der Apoptose in BORN- und Importforellen nach Infektion.

Day of the doctoral student, 19 Mai 2011, FBN Dummerstorf

Comparative analyses of apoptosis-related candidate genes in rainbow trout: molecular characterization and transcriptome analyses

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1 Introduction

Programmed cell death, known as apoptosis [1], facilitates the active elimination of damaged, mutated or infected tissue [2] and therefore promotes the maintenance of normal tissue functions. The molecular mechanisms of the mammalian apoptosis pathway are comprehensively examined and evolutionary well conserved [3;4]. Cysteine-aspartic proteases, termed as caspases represent the central protein family involved in programmed cell death. Apoptosis is initiated by extracellular death ligands, e.g. the cytokines FASLG (Fas ligand) and TNFα (tumor necrosis factor-alpha) that bind to their specific membrane-bound death receptors. The following intracellular signalling cascade activates the caspases, which in turn regulate the final apoptotic steps. Beside this extrinsic pathway, an intrinsic cascade is known, which activates the caspases by the release of CYCS (cytochrome c, somatic) and DIABLO (direct IAP-binding protein with low pI).

The use of fish as a model organism for apoptosis studies has been recently recommended since teleosts can be studied under extreme environmental conditions that cannot be easily investigated in mammals, e.g. fluctuations of temperature, salinity or oxygen content [5]. Several crucial molecules of the programmed cell death in commercially important rainbow trout have been characterized except for initial CASP3 (caspase-3) and effector CASP8 (caspase-8) gene. In parallel to the structural characterization of those factors, we compared the strain-specific expression profiles of apoptosis-related genes *via* transcriptome analyses in two aquacultured rainbow trout (*Oncorhynchus mykiss*) lines after a pathogen infection: the local selection strain BORN (Germany) and the imported strain TCO from Tacoma (USA). Compared to TCO strain, the BORN strain shows an increased resistance towards different stress factors such as infection and pollution [6]. A different regulation of several molecular

factors, putatively involved in the defense of stress and infection has been described recently [7;8]. Therefore, both lines serve as a suitable model organism to study varying adaptation to environmental stress.

2 Materials and methods

Eleven to twelve month old BORN and TCO rainbow trout, grown under identical conditions in a regional aquaculture farm (BIMES, Frauenmark, Germany) for nine month and then transferred and adapted to glass tanks for further three months, were infected intraperitoneally (i.p.) with a dosage of $1x10^4$ cfu (colony forming unit) of the pathogen *Aeromonas salmonicida* subsp. *salmonicida*. Trunk kidney tissue samples of four fish per strain were taken 0 h (control) and 72 h as well as 7 d and 21 d after infection, transferred into RNAlater, and subsequently homogenized in 1 ml Trizol (Invitrogen, Karlsruhe, Germany). RNA extraction was conducted using RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase treatment for 30 min or DNA extraction by using QIAamp DNA Micro Kit (Qiagen). The integrity of RNA was verified by agarose gel electrophoresis.

Subsequently, total RNA of trunk kidney tissue was hybridized to a 4x44K Agilent salmonid oligo microarray according to a standard protocol by ATLAS Biolabs GmbH (Berlin) [9]. For each time-point, samples from two animals per strain were included in hybridization experiments. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

3 Results and discussion

We compared the temporal expression of 47 apoptosis-related genes present on the salmonid microarray chip in both rainbow trout lines BORN and TCO after infection with the intracellular gram-negative bacterium *A. salmonicida* subsp. *salmonicida*. The expression profile in trunk kidney revealed significant differences within both strains, in particular 7 days after infection compared to uninfected fish (Fig.1).

Both strains show no significant regulation of apoptosis-related genes 72 hours after infection in comparison to uninfected control fish except for TANK (TRAF family member-associated NF-κB activator) which is up-regulated in BORN trout (1.71-fold, p= 0.006) and responsible for blocking the NF-κB (nuclear factor NF-kappa-B) activation. In contrast, significant regulation of in total ten apoptosis-related genes could be detected 7 d after infection. In BORN rainbow trout, (i) the anti-apoptotic gene BCL-2 (B-cell CLL/lymphoma 2) is -4.03-

fold (p= 0.003) down-regulated and (ii) the pro-apoptotic effector CASP 9 (caspase-9) gene is 1.90-fold (p= 0.04) enhanced.

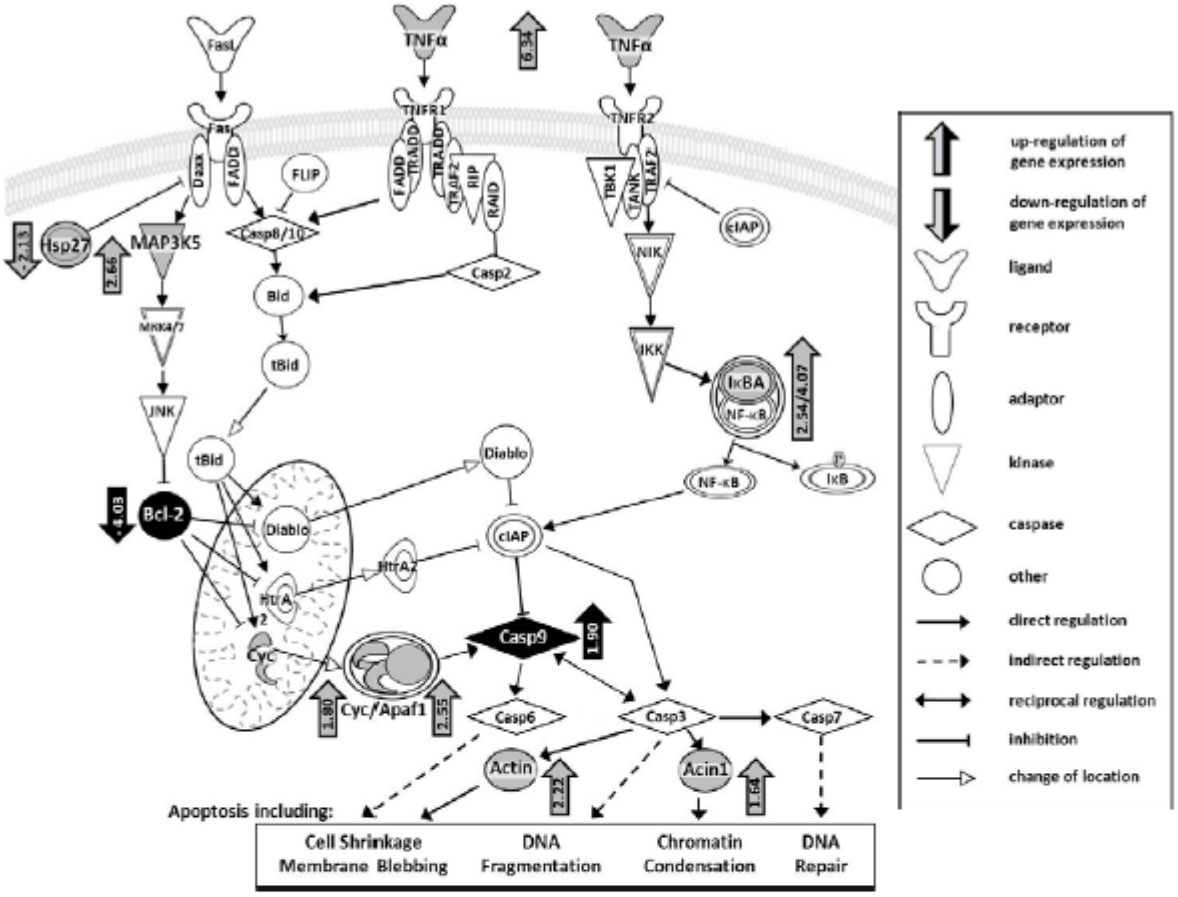

Fig.1: Regulation of apoptosis-related genes in trunk kidney after pathogen infection. Transcript level 7 d after infection was compared to uninfected rainbow trout (0 h). Significantly ($p < 0.05$) regulated genes of BORN and TCO trout are represented by black and grey symbols, respectively; gene-specific fold-change is indicated in black and grey arrows, respectively.

Eight apoptosis-related genes are significantly regulated in TCO trout compared to healthy trout. The cell death-inducing death-receptor ligand TNFα is elevated 6.34 fold (p= 0.01) as well as the pro-apoptotic genes MAP3K5 (mitogen-activated protein kinase kinase kinase 5; 2.66-fold, p= 0.025), CYCS (1.80-fold, p= 0.037) and APAF1 (apoptotic peptidase activating factor 1; 2.6-fold, p= 0.045). Additional, gene expression of HSP27 (heat shock protein 27) inhibiting Fas-mediated apoptosis, is decreased -2.13-fold (p= 0.009), whereas the transcript levels of two putative NF-kB inhibitor gene variants for NFKBIA (nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha) are elevated 2.54/4.07-fold (p= 0.03/0.009). Furthermore, we detected a significant up-regulation of ACTA2 (smooth muscle aortic alpha-actin; 2.22-fold, p= 0.011) and ACIN1 (apoptotic chromatin condensation inducer in the nucleus; 1.64-fold, p= 0.009), which are both involved in the final steps of apoptosis, leading to cell shrinkage, membrane blebbing and chromatin condensation. Three

weeks after infection, no significant gene regulation could be observed in BORN trout. However, effector caspase 3 transcript level was elevated 1.69-fold (p= 0.006) in TCO trout at this time point.

In summary, we detected a pro-apoptotic gene regulation in both rainbow trout lines after pathogen infection. It seems, however, that BORN and TCO trout use different distinctive regulation mechanisms. Interestingly, in TCO trout, eight genes of the programmed cell death were regulated, located at different steps of three apoptotic pathways. In BORN trout, the regulation was restricted to two genes of the Fas-mediated apoptosis. Verification of the results *via* qRT-PCR is underway. The data support and consolidate our hypothesis of a genetically determined optimized adaptation of BORN trout to local habitats and environmental conditions. This strain is therefore suggested as a valuable alternative in local aquaculture farms.

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3. Diskussion

Fische in der Aquakultur sind aufgrund artifizieller Haltungssysteme vermehrt Umweltfaktoren ausgesetzt, die Stress auslösen und sie damit vor besondere Herausforderungen stellen. Eine schnelle und effektive physiologische Anpassung der Fische an kritische Umweltbedingungen ist daher essenziell, um die Homöostase des Organismus aufrecht zu erhalten und langfristige Beeinträchtigungen zu vermeiden. Das Zusammenspiel einer Vielzahl molekulargenetischer und metabolischer Regulatoren sowie Faktoren ist dafür notwendig. Für Regenbogenforellen der Zuchtlinie BORN wurde gezeigt, dass sie unter lokalen Bedingungen typische Stressoren in der Aquakultur besser tolerieren (z.B. Temperaturschwankungen) oder bekämpfen (z.B. bakterielle Infektion) können als importierte Forellen, deren genetische Selektion in anderen Habitaten erfolgt (siehe Abschnitt 1.5). Im Ergebnis dieser ersten Untersuchungen wurde der Merkmalsbegriff der Robustheit geprägt. Er definiert und fasst die verschiedenen Anpassungsstrategien der unterschiedlichen Forellenlinien nach einer Stresssituation zusammen. Dabei wird angenommen, dass der phänotypischen Ausprägung eine genetische Determinierung zu Grunde liegt. Die vorliegende Arbeit befasst sich mit der Untersuchung von Genen, die Einfluss auf die differente Robustheit lokaler und importierter Regenbogenforellen haben könnten. Die Analysen umfassen die molekulargenetische Charakterisierung der Kandidatengene und vergleichende Transkriptomanalysen zwischen der lokalen Zuchtlinie BORN und Importforellen nach Stress durch Infektion und Temperatur. Dadurch soll der Zusammenhang zwischen der differenten Robustheit beider Zuchtlinien und einer zu Grunde liegenden genetischen Manifestierung näher spezifiziert werden.

In der folgenden Diskussion wird zunächst auf die Auswahl der Kandidatengene eingegangen. Anschließend folgen die Diskussion und Einordnung der in den Einzelstudien dargestellten Ergebnisse sowie ein Fazit und ein Ausblick.

3.1 Kandidatengenauswahl

Eine differente Expression von Genen spiegelt sich in einer unterschiedlichen Ausprägung betroffener Zellprozesse wider. Daher können ungleich regulierte Gene der BORN-und Importforelle relevant für die Ausprägung der erhöhten Robustheit der lokalen Zuchtlinie sein. Um entsprechende Gene identifizieren zu können, wurde ein holistischer Transkriptomvergleich zwischen beiden Forellenlinien durchgeführt. Die klassische Reaktion auf äußere Stressoren ist in vielen Einzelstudien im Fisch bereits gut untersucht. Bei der

Kandidatengenauswahl dieser Arbeit wurden vor allem Gene berücksichtigt, die im Gegensatz zu klassischen Stressgenen, wie z.B. HSP-kodierende Gene, an der adaptiven Stressantwort der BORN-Forelle beteiligt sind. Zusätzlich wurden weitere relevante Gene für die differente Robustheit anhand vergleichender Studien in der Literatur ausgewählt und in die Untersuchungen miteinbezogen. Mithilfe des Analyse-Programms IPA (*Ingenuity Pathway Analysis*) wurden funktionale Netzwerke basierend auf Erkenntnissen aus humangenetischen Studien erstellt, um die Verbindung der Gene untereinander zu verdeutlichen (Abb. 4 A, B). Die Kandidatengene *ISCU*, *PRR13*, *MARCH5* und *NELL2* sowie die Gene *FGL2*, *CLEC4E*, *FTH1*, *CASP3*, *CASP7*, *CASP8*, *SERPINF2*, *TPT-1* und *SERPINA1* werden dabei je einem Netzwerk zugeordnet, das Kandidatengen *RGN* beiden Netzwerken. Es stellt somit ein Bindeglied zwischen den Netzwerken dar. Netzwerk A fasst vor allem die Kandidatengene zusammen, die an Entwicklung, Wachstum, Ionentransport sowie der Aufrechterhaltung der Homöostase der Zelle beteiligt sind. Regulatorische Moleküle wie der DNA-Bindefaktor *MYC* (*V-MYC avian myelocytomatosis viral oncogene homolog*) sowie der Transkriptionsfaktor *HNF4A* (*Hepatocyte nuclear factor 4 alpha*) stellen Schnittstellen dar. Die in Netzwerk B eingeordneten Gene sind der Immunantwort sowie der Apoptose zuzuordnen. Einen zentralen Punkt bildet dabei der Transkriptionsfaktor NF-kB (*nuclear factor NF-kappa-B*), der unter anderem Teile der Immunantwort sowie des aktiven Zelltods reguliert (MERCURIO and MANNING 1999).

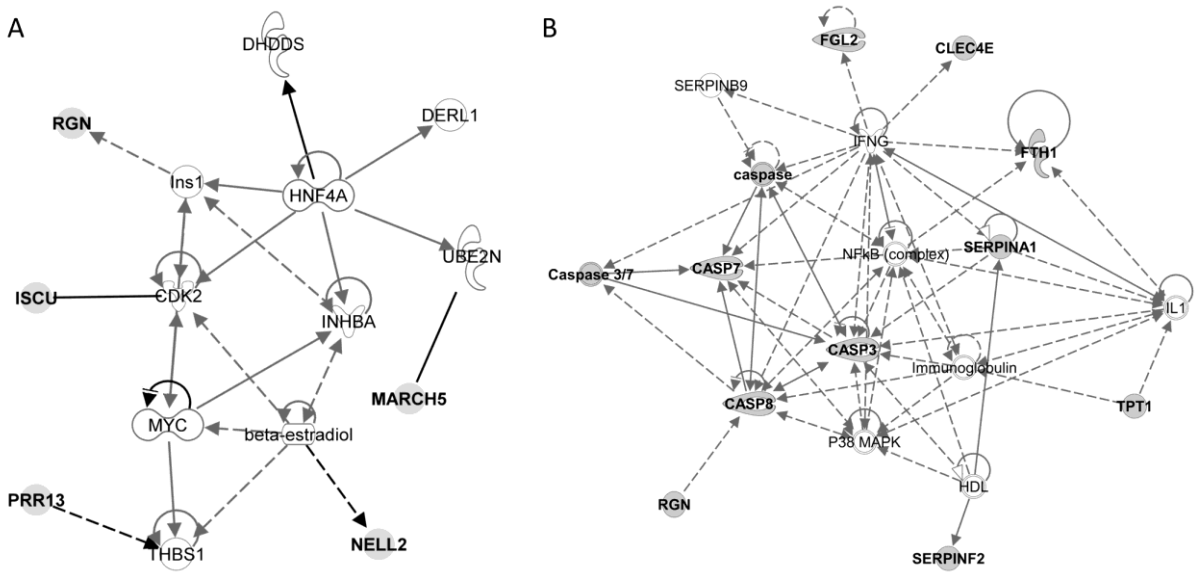

Abb. 4: (A+B) Interaktionsnetzwerke der Kandidatengene dieser Arbeit (Fett, grau unterlegt) erstellt mit dem Programm IPA (*Ingenuity Pathways Analysis*, Ingenuity®). Durchgezogene Pfeile in der Graphik stehen für eine direkte, unterbrochene Pfeile für eine indirekte Interaktion zwischen den Genen. Jede Verbindung repräsentiert mindestens eine registrierte Referenz in der *Ingenuity® Pathway Knowlegdge Base* Datenbank.

3.1.1 Holistischer Transkriptomvergleich des Lebergewebes

Mithilfe eines Transkriptomvergleichs wurden zunächst grundlegende Unterschiede zwischen der lokalen Zuchtlinie BORN und Importforellen aufgedeckt. Dazu wurde gesundes Lebergewebe beider Zuchtlinien auf einen 16k Salmoniden-cDNA-Microarray (GRASP: *Genomics Research on Atlantic Salmon Project*; VON SCHALBURG *et al.* 2008) hybridisiert und different exprimierte Gene identifiziert. Die Leber eignet sich als zentrales Stoffwechselorgan der Fische besonders gut für vergleichende Untersuchungen adaptiver Prozesse und ist als ein wesentliches Organ der Stressachse an der direkten und indirekten Stressantwort beteiligt. Sie wurde bereits im Rahmen anderer Studien für Transkriptomvergleiche zwischen Forellenlinien verwendet (PEMMASANI *et al.* 2011). So verglichen PEMMASANI *et al.*. (2011) das hepatische Transkriptom zweier sich in der Intensität ihrer Stressreaktion deutlich unterscheidender Forellenlinien und identifizierten so potentielle Genmarker.

Von den 68 different regulierten Genen (19 nicht annotiert; *fold-change* ≥ 2.0) wurden elf für weitere Untersuchungen ausgewählt: *RGN* (*Regucalcin*; auch *SMP30 - senescence marker protein-30*), *NELL2* (*neural epidermal growth factor-like-like 2*), *PRR13* (*proline-rich 13*), *MARCH5* (*membrane-associated ring finger [C3HC4] 5*), *ISCU* (*iron-sulfur cluster scaffold*), *FTH1* (*middle subunit of ferritin*), *SERPINA1* (*alpha-1 antitrypsin*), *SERPINF2* (*alpha-2 antiplasmin*), *LEAP2* (*liver-expressed antimicrobial peptide*), *FGL2* (*fibroleukin*), *CLEC4E* (*macrophage-inducible C-type lectin*). Für die Mehrzahl dieser Gene lagen bis *dato* keine forellenspezifischen Sequenzen vor. Als Auswahlkriterium dieser Gene diente, neben der signifikant unterschiedlichen Regulation zwischen den beiden Forellenlinien, ihr Einfluss auf Zellprozesse, die eine Rolle in der allgemeinen Stressreaktion spielen. Die Produkte der Kandidatengene *RGN* und *NELL2* sind vor allem am Calcium-Transport beteiligt und somit wichtig für die Regulation der Ca^{2+}-Homöostase der Zelle (KURODA and TANIZAWA 1999; TAKAHASHI and YAMAGUCHI 1995). Calcium ist ein zentrales Element zellulärer Signalweiterleitung (BERRIDGE 1997; MACHACA 2010). Die Aufrechterhaltung und Anpassung des Ionenhaushalts ist ein entscheidender Faktor der zellulären Stressreaktion. Zusätzlich legen Untersuchungen von KURODA and TANIZAWA (1999) eine Beteiligung von NELL2 als Signalmolekül an Wachstum und Differenzierung nahe (KURODA and TANIZAWA 1999). Das Protein könnte daher eine wichtige Rolle bei der Regenerationsphase nach akuter Belastung spielen. Das Gen *ISCU* kodiert für ein wichtiges Aufbau- und Transportprotein von Eisen-Schwefel-Clustern [Fe-S-Cluster] (GARLAND *et al.* 1999). Diese Cluster sind unter anderem Kofaktoren elementarer Enzyme des Stoffwechsels, z.B. der NADH-Dehydrogenase

(BEINERT and KENNEDY 1993; GALANTE and HATEFI 1979; HAMPL *et al.* 2011). Wie schon in Abschnitt 1.2 beschrieben, wird der Stoffwechsel unter Stress vor besondere Herausforderungen gestellt. Die weiterhin im Leber-Transkriptomvergleich signifikant different regulierten Gene *MARCH5*, *LEAP2*, *SERPINA1*, *FTH1*, *FGL2*, *CLEC4E* und *SERPINF2* kodieren für Proteine, die an der primären Immunantwort beteiligt sind und daher eine wichtige Rolle in der Pathogenabwehr der BORN-Forelle spielen könnten. Ein weiteres Kandidatengen ist *PRR13*, dessen Produkt im Menschen eine Resistenz gegen Zytostatika vermittelt (PAPADAKI *et al.* 2009). Die erhöhte Expression des Gens in der BORN-Forelle könnte auf einen Einfluss von PRR13 auf den Umgang der robusten Linie mit von Verschmutzung beeinträchtigter Wasserqualität hinweisen. In derselben Studie von PAPADAKI *et al.*. (2009) konnte die Regulation des proapoptotischen Proteins Trombospondin (TSP-1) durch PRR13 nachgewiesen werden und damit der Einfluss des Proteins auf die Aktivierung des aktiven Zelltods, welcher ein wichtiger Teil der zellulären Stressreaktion eines Organismus darstellt.

3.1.2 Kandidatengene aus der Literatur

In verschiedenen Studien wird auf die Bedeutung des aktiven Zelltods im Rahmen der Stressantwort eines Organismus hingewiesen (KÜLTZ 2005; SANCHEZ *et al.* 2011; WENDELAAR BONGA 1997). Bei einer stressinduzierten anhaltenden Überschreitung der individuellen Toleranzgrenze kommt es neben passivem (Nekrose) vor allem zu aktivem Zelltod (Apoptose). Der durch Stress ausgelöste Cortisolanstieg im Plasma sorgt nachweislich für eine Aktivierung apoptotischer Prozesse in der Zelle (MACKENZIE *et al.* 2006; WEYTS *et al.* 1998). Anders als durch Nekrose kann der Körper durch Apoptose zielgerichtet beschädigte Zellen abtöten, um eine Ausbreitung der Beschädigung oder Infektion auf umliegende Zellen zu verhindern und ihre Erneuerung zu ermöglichen. So zeigen z.B. Untersuchungen an Kiemengewebe und Epithelzellen der Buntbarsch-Art *Oreochromis mossambicus* sowie des Karpfens *Cyprinus carpio* nach Einfluss unterschiedlicher Stressoren eine hohe Rate an Apoptose und Nekrose verbunden mit vermehrter Mitose (WENDELAAR BONGA 1997). Dies spricht für eine gerichtete Erneuerung der Kiemenzellen, um einer Beschädigung entgegenzuwirken.

Der zielgerichtete und effektive Einsatz von Apoptose als letztes Mittel, um eine langfristige Beschädigung der Körperzellen durch Stressoren abzuwehren, stellt somit einen Vorteil in der Bewältigung von Stress dar. Die grundlegenden Abläufe und Moleküle der

Apoptose stimmen in Mensch und Fisch überein (DOS SANTOS *et al.* 2008; KRUMSCHNABEL and PODRABSKY 2009; YAMASHITA 2003). Eine adaptive Anpassung der zielgerichteten Aktivierung des aktiven Zelltods könnte neben anderen Faktoren zu der erhöhten Robustheit der BORN-Forelle beitragen. Um dies zu untersuchen, wurden Gene, die für Schlüsselmoleküle der Apoptose kodieren der Liste der Kandidatengene dieser Studie hinzugefügt: *CASP3* (Caspase 3, *apoptosis-related cysteine peptidase*), *CASP7* (Caspase 7) und *CASP8* (Caspase 8) sowie das regulatorische Gen *TPT1* (*tumor protein, translationally-controlled 1*). Ihre Gensequenzen waren bisher in der Regenbogenforelle noch nicht charakterisiert. Während es sich bei Caspase 8 um eine Initiator-Caspase handelt, die bei Aktivierung die nachgeschaltete Kaskade auslöst, sind die Caspasen 3 und 7 Effektor-Caspasen, die durch die Aktivierung nachgeschalteter Proteine zur endgültigen Zerstörung der Zelle führen (DOS SANTOS *et al.* 2008; SALVESEN and DIXIT 1997). Für TCTP (t*ranslationally-controlled tumor protein*), Transkript des Gens *TPT1*, wurde hingegen eine antiapoptotische Wirkung nachgewiesen (SUSINI *et al.* 2008). Das Protein wurde ursprünglich im Mausmodel als Tumorprotein identifiziert. Neuere Studien ordnen es der Klasse der Hitzeschockproteine mit Funktionen eines Chaperons zu (GNANASEKAR *et al.* 2009).

3.2 Neue Gensequenzen in der Forelle

Für die Mehrzahl der Kandidatengene lagen zu Beginn der Arbeit noch keine Sequenzen für die Regenbogenforelle vor. Ihre molekulare Charakterisierung bildete daher die Grundlage für alle weiteren funktionalen Analysen und zukünftige Untersuchungen.

Im Rahmen der vorliegenden Arbeit konnten die Gen-Sequenzen von zwölf Kandidatengenen in der Forelle aufgeklärt und in der Online-Datenbank NCBI (*National Center for Biotechnology Information*, http://www.ncbi.nlm.nih.gov/) abgelegt werden (Tab. 2). Anhand der isolierten Sequenzen konnten im weiteren Verlauf genspezifische Primer für vergleichende Expressionsstudien konzipiert werden. Eine Herausforderung bei der Sequenzanalyse und Untersuchungen des Transkriptoms stellt die Problematik der Genduplikation dar. Im Gegensatz zu allen anderen Vertebraten unterliefen die echten Knochenfische vor etwa 226 bis 316 Millionen Jahren einer weiteren Verdopplung ihres Genoms. Während angenommen wird, dass diese Tetraploidisierung durch Rediploidisierung wieder aufgehoben wurde (AMOUTZIAS *et al.* 2010), ist eine erneute Genomduplikation innerhalb der Salmoniden, die vor 25 bis 100 Millionen Jahren stattfand, heute teilweise noch nachweisbar (HURLEY *et al.* 2007). Salmoniden gelten daher als pseudo-tetraploid.

Demzufolge kann ein Gen bei Forellen in mehrfacher Kopie vorhanden sein. Im Rahmen der Arbeit wurde für fünf der untersuchten Kandidatengene mehr als ein Gen nachgewiesen. Für *ISCU*, *NELL2*, *MARCH5* und *SERPINF2* wurden je zwei Gene gefunden. Für *CASP3* konnten drei Gene in der Forelle identifiziert werden, von denen die cDNA-Sequenz einer Genvariante bereits in der NCBI Datenbank abgelegt wurde. Ein Problem, dass aus der Duplikation von Genen entsteht, ist die teilweise sehr hohe Sequenzhomologie zwischen den Varianten, vor allem im kodierenden Bereich (z.B. bis zu 91% bei *ISCUA* und *ISCUB*). Insbesondere das Ableiten genspezifischer Primer für Expressionsanalysen wird dadurch erheblich erschwert. Auch Microarray-Analysen an Salmoniden werden durch dieses Phänomen beeinträchtigt. Oft sind die auf Microarrays aufgebrachten Oligomere nicht ausreichend spezifisch für eine der jeweiligen Genvarianten. Die Ergebnisse holistischer Transkriptomanalysen bei Salmoniden können daher durch fehlerhafte Hybridisierung verfälscht werden.

Tab. 2: GenBank Einträge (NCBI) der im Rahmen der Arbeit ermittelten Gen-Sequenzen in der Regenbogenforelle

Gen		**Datenbank ID**		
Gensymbol	**Genprodukt**	**mRNA**	**Protein**	**gDNA**
PRR13	Proline-rich protein 13	NM_001195175	NP_001182104	FN668729
ISCUA	Iron-sulfur cluster scaffold protein a	NM_001171861	NP_001165332	HE648572
ISCUB	Iron-sulfur cluster scaffold protein b	NM_001256846	NP_001243775	HE648573
RGN	Regucalcin	NM_001246349	NP_001233278	FR846200
NELL2A	Neural epidermal growth factor-like-like protein 2 a	NM_001199152	NP_001186081	---
NELL2B	Neural epidermal growth factor-like-like protein 2 b	NM_001164067	NP_001157539	---
MARCH5A	Membrane-associated ring finger [C3HC4] 5 a	FN400889	CAZ64332	FN400890
MARCH5B	Membrane-associated ring finger [C3HC4] 5 b	FN677805	CBK39084	FR749991
CASP8	Caspase 8	HE608242	CCE39579	---
CASP3	Caspase 3	NM_001246335	NP_001233264	---
SERPINF2A	Alpha-2-antiplasmin a	FR677583	CBW45296	---
SERPINF2B	Alpha-2-antiplasmin b	FR872890	CCB84818	---

Insgesamt weisen die abgeleiteten Proteinsequenzen in der Forelle überwiegend eine sehr hohe Konservierung mit den entsprechenden Sequenzen anderer Fische und höherer Vertebraten auf. Dies spricht für eine hohe biologische Bedeutung und lässt eine funktionelle Konservierung vermuten. Vergleichsweise geringere Sequenzhomologien fanden sich für das noch kaum untersuchte Protein PRR13 und das durch *SERPINF2* kodierte Alpha-2-antiplasmin. Allerdings liegen für PRR13 nur zwei weitere Einträge in den Datenbanken NCBI und ENSEMBL (www.ensembl.org) vor, die nicht einem Säugetier zuzuordnen sind. Daher war ein Sequenzvergleich fast nur mit Säugersequenzen möglich.

Insgesamt konnten die Sequenzen der noch nicht in der Forelle charakterisierten Kandidatengene im Rahmen der Arbeit beschrieben werden. Sie bildeten im weiteren Verlauf das Grundgerüst der vergleichenden Expressionsanalysen und ermöglichen in Zukunft weiterführende Untersuchungen, wie z.B. merkmalsassoziierte Studien oder auch Genkartierungen.

3.3 Analyse des Expressionsprofils der Kandidatengene

Anhand von semi-quantitativen und quantitativen Untersuchungen wurde die gewebespezifische Expression der Kandidatengene in der Forelle näher analysiert. Die quantitative Analyse bietet dabei den Vorteil einer ersten Einordnung des Transkriptlevels und die Möglichkeit des direkten quantitativen Vergleichs zwischen verschiedenen Genen. Allerdings ist dabei zu beachten, dass die gemessene Transkriptmenge der mRNA nicht zwangsläufig die Anzahl des translatierten Proteinmoleküls widerspiegelt, da weitere Regulationsmechanismen der Proteinbiosynthese die Proteinexpression von der Genexpression entkoppeln. Dennoch liefert die Erfassung der spezifischen Genexpression zu einem definierten Zeitpunkt unter definierten Bedingungen erste grundlegende Informationen über funktionelle Zusammenhänge zwischen Stressfaktor und stressassoziierten Genen sowie zu möglichen molekularen Antworten des Organismus auf die jeweilige Stresssituation.

Zusätzlich zu gewebespezifischen Untersuchungen wurde der Einfluss von typischen Stressoren in der Aquakultur (Infektion und Temperatur) auf die Genexpression von Forellen der lokalen Zuchtlinie BORN sowie Importfischen vergleichend betrachtet. Damit wurde ein möglicher Zusammenhang zwischen der spezifischen Expression der Kandidatengene und der differenten Robustheit zwischen den Forellenlinien untersucht.

3.3.1 Duplizierte Gene mit spezifischer Expression

Im Laufe der Genomevolution verlieren duplizierte Gene oft ihre bis dahin vorhandene Funktion im Organismus und werden zu funktionslosen Pseudogenen. Behalten hingegen beide Gene ihre Funktionalität, können sie vollkommen verschiedene Aufgaben haben (Neofunktionalität) oder auch gemeinsam die ursprüngliche Funktion des Vorfahrengens erfüllen, die dieses vor der Duplikation inne hatte (Subfunktionalität) (FORCE *et al.* 1999). Meist sind Genduplikate kurz nach dem Duplikationsereignis noch redundant. Werden sie nicht im Evolutionsverlauf ausgeschaltet, entwickeln sie sich bei hohem selektivem Druck

sowohl in ihrer Expression als auch in der Übereinstimmung der Sequenzen ihrer kodierenden Genbereiche auseinander, neue Genfamilien können entstehen (LI, *et al.*., 2005).

Bei den im Rahmen der Arbeit nachgewiesenen duplizierten Forellengenen handelt es sich nicht um im Laufe der Evolution stillgelegte Gene. Für alle konnte eine Expression in der Regenbogenforelle nachgewiesen werden (vergleiche Studien IV, VI, VII). Auffallend dabei ist, dass die jeweiligen Genvarianten ein divergentes Expressionsmuster aufweisen. Die Expression von *MARCH5A* ist vor allem auf die Immunorgane des Fisches, Kopfniere und Milz sowie die Kiemen beschränkt, während *MARCH5B*-Transkripte ubiquitär nachweisbar sind (vergleiche Studie VI). Ähnliches zeigt sich auch für die duplizierten Gene *ISCUA* und *ISCUB* sowie *NELL2A* und *NELL2B*. Sowohl *ISCUB* als auch *NELL2B* werden im Gegensatz zu *ISCUA* und *NELL2A* fast ausschließlich im Gehirn transkribiert (vergleiche Studien IV, VII). Die differenten Expressionsmuster der duplizierten Gene lassen zunächst eine jeweils eigenständige Funktion vermuten. Allerdings weisen neofunktionelle Gene nur noch marginale Sequenzhomologien auf. Die duplizierten Gene *MARCH5A* und *B*, *ISCUA* und *B* sowie *NELL2A* und *B* zeigen hingegen einen vergleichsweise hohen Grad an Übereinstimmung ihrer kodierenden Sequenzen. Daher erscheint eine subfunktionelle Aufgabenteilung wahrscheinlicher. Um dies zu klären, sind zukünftige Analysen der Proteinfunktion nötig.

Interessanterweise zeigen die duplizierten Gene *MARCH5* und *NELL2* auch im Vergleich der beiden Forellenlinien ein unterschiedliches Expressionsmuster. Dies könnte auf eine abweichende Bedeutung der Genvarianten in BORN- und Importforellen hindeuten. Um diesen Sachverhalt ausreichend zu klären, sind ebenfalls weitere funktionelle Untersuchungen auf Proteinebene nötig, die nicht Teil dieser Arbeit waren.

3.3.2 Grundlegend differente Genexpression von BORN-und Importforellen in stoffwechsel- und immunrelevanten Organen

Mittels quantitativer Expressionsanalyse *via* qRT-PCR wurde das Transkriptlevel der Kandidatengene in verschiedenen stoffwechsel- und immunrelevanten Organen gesunder BORN- und Importforellen untersucht und die Ergebnisse des 16k-cDNA Leber-Chips validiert. Dabei wurden grundlegende Unterschiede zwischen den Forellenlinien vor allem im zentralen Stoffwechselorgan Leber, in den Immunorganen Niere und Milz sowie in den direkt mit der Umwelt in Kontakt stehenden Kiemen offen gelegt.

Die Ergebnisse des Microarray-basierten Vergleichs des Leber-Transkriptoms konnten für die Kandidatengene verifiziert werden (vergleiche Studien I-VI). Die zentrale Rolle der Leber sowohl als Stoffwechselorgan als auch als wichtiger Ort der Stressantwort lassen der differenten Expression der Kandidatengene in BORN- und Import-Forellen eine besondere Bedeutung zukommen. Die selektive Zucht der BORN-Forelle unter Brackwasserbedingungen könnte zu einer Anreicherung von Genotypen geführt haben, die in der Folge auch eine Anpassung der Expression von Genen wie *RGN* bewirken. Diese kontrollieren den Ionen-Haushalt der Forelle und stellen dadurch einen Anpassungsvorteil dar. In verschiedenen Studien wurde RGN bereits eine zentrale Rolle in der Wahrung der Ca^{2+}-Homöostase in Leber und Niere von Säugern nachgewiesen (FUJITA *et al.* 1999; TAKAHASHI and YAMAGUCHI 1995). Für die oben genannte Annahme der angepassten Genotypen spricht auch die signifikant höhere Expression des Gens *PRR13*, dessen Proteinprodukt eine erhöhte Resistenz gegen natürliche Zytostatika vermittelt (PAPADAKI *et al.* 2009).

Die vor allem in der Leber der BORN-Forellen im Vergleich zu Importforellen erhöhte Expression von Genen der Akuten-Phase Reaktion des Immunsystems wie *LEAP2A*, *SERPINA*, *SERPINF2* und *FTH1* könnte für die lokale Zuchtlinie einen generellen Vorteil im primären Schutz des Organs vor pathogenen Erregern bedeuten. Proteine der Akute-Phase-Reaktion des Immunsystems werden primär in der Leber synthetisiert. Sie stellen auch im Fisch die erste unspezifische Verteidigung gegen Invasoren dar und schützen vor Gewebeschädigungen sowie akuten oder chronischen Infektionen (BAYNE and GERWICK 2001; KUSHNER 1982).

Nur teilweise bestätigt werden konnte die signifikante höhere hepatische Expression von *FGL2* in der lokalen Zuchtlinie. Die direkte Verifizierung mittels qRT-PCR bestätigte die erhöhte Expression des immunregulatorischen Gens nicht. Dennoch konnte im Rahmen des Temperatur- und Infektionsexperiments durchaus ein signifikanter Expressionsunterschied des Gens in der Leber nachgewiesen werden (vergleiche Studie I).

3.3.3 Expression nach Temperaturstress und Infektion

Als poikilotherme Organismen hat die Wassertemperatur direkten Einfluss auf den Metabolismus und die meisten physiologischen Reaktionen von Fischen (FRY 1971). Gerade in nicht geschlossenen Aquakulturanlagen ist die Belastung durch starke saisonale Temperaturschwankungen für die Besatzfische hoch. Zusätzlich stellen auch bakterielle und

virale Infektionen eine Belastung dar und führen nicht selten zum Tod. Eine möglichst effektive Reaktion des Immunsystems ist daher überlebenswichtig. In ersten Vergleichen von BORN- mit Importforellen konnte ein anhaltendes Wachstum der lokalen Zuchtlinie unter stark erhöhten Sommertemperaturen sowie eine deutlich höhere Überlebensrate nach Infektion beobachtet werden (vergleiche Abb. 2A, B). Daraus resultiert die Annahme, dass die BORN-Forelle besser an lokale äußere Stressoren angepasst ist. Der Vergleich des Lebertranskriptoms (vergleiche Punkt 3.1.1) beider Forellenlinien sowie eine umfangreiche Literaturrecherche lieferten erste Anhaltspunkte dafür, dass diese Adaptationsleistung auch auf eine differente Regulation der betrachteten Kandidatengene zurückzuführen ist. Da bis zu dieser Arbeit für die Mehrzahl der Kandidatengene weder Gensequenzen und damit auch keine Expressionsstudien vorlagen, handelt es sich hierbei um erste grundlegende Untersuchungen.

Aufgrund des Einflusses der Kandidatengene auf die Aufrechterhaltung der Zell-Homöostase, der Kontrolle wichtiger Stoffwechselenzyme, der primären Immunantwort und Wundheilung sowie der Apoptose bestand die Erwartung, dass sie an der adaptiven Stressreaktion der BORN-Forelle beteiligt sind und sich dies auch in ihrem jeweiligen Expressionsprofil nach thermaler Belastung und Infektion widerspiegelt. Um die Regulation der Kandidatengene zu untersuchen, wurde ihre Expression in BORN- und Importforellen in einem Temperaturversuch (Temperaturanpassung an 8 °C und 23 °C) sowie parallel in Infektionsexperimenten mit dem Erreger *A. salmonicida* sowie dem Rhabdovirus VHSV miteinander verglichen. Zusätzlich lieferten entsprechende Vergleiche des Transkriptoms mittels Agilent 44k Oligo-Microarrays weitere Anhaltspunkte für darüber hinaus an der differenten adaptiven Stressreaktion beteiligte Gene.

Ein temperaturabhängiges Expressionsniveau konnte für *ISCUA* und *B*, *RGN*, *NELL2A* und *B*, *SERPINA1*, *FGL2*, und *FTH1* in beide Forellenlinien beobachtet werden, nicht aber für *LEAP*, *SERPINF2A* und *CLEC4E*. Das Expressionslevel von *RGN*, *ISCUA* und *B* sowie *NELL2* ist in den meisten untersuchten Geweben bei 23 °C deutlich niedriger als bei 8 °C. Ein Grund hierfür ist in der starken energetischen Belastung der Forellen unter dieser kritischen Temperatur zu suchen. Bei Stress kommt es, wie bereits beschrieben, zu einer Umschichtung des Energieverbrauchs (BARTON 1987). Die Expression der Gene scheint in den entsprechenden Organen entsprechend abgesenkt zu werden. Diese Beobachtung machten auch PEMMASANI *et al*. (2011). Sie analysierten das hepatische Expressionsprofil in Regenbogenforellen, die stark bzw. vergleichsweise schwach auf Stressoren reagieren. Auch

sie gehen davon aus, dass die herunterregulierten Gene eine Reduzierung des Energieverbrauchs der Forellen widerspiegeln (PEMMASANI *et al.* 2011). In einer früheren Studie konnten CAIRNS *et al*. (2008) zeigen, dass die Herunterregulierung von Genen im Zuge einer Belastung von Forellen durch Stress bereits zwei Stunden nach Beginn des Stresses einsetzt und bis zu sieben Tage danach anhält (CAIRNS *et al.* 2008).

Im Gegensatz zu der allgemeinen Absenkung des Expressionsniveaus der Kandidatengene bleibt die Expression von *RGN*in beiden Forellenlinien in der Leber bei 8 °C und 23 °C nahezu gleich. Für die Leber als wichtigstes Stoffwechselorgan ist die Aufrechterhaltung der Homöostasie in den Zellen gerade unter für den Organismus stressigen Umständen besonders wichtig. RGN ist in der Leber vor allem maßgeblich involviert an der Aufrechterhaltung der Ca^{2+}-Homöostase (TAKAHASHI and YAMAGUCHI 1995) sowie der Differenzierung von Hepatozyten (FUJITA *et al.* 1999). Die Vermutung liegt nahe, dass durch die Wahrung eines konstanten Transkriptlevels in der Leber, der Ablauf der durch das Gen regulierten Prozesse gewährleistet ist.

Basierend auf seiner Ca^{2+}-Transportfähigkeit wird für NELL2 ebenfalls ein Einfluss auf zelluläre Signalprozesse vermutet (KIM *et al.* 2002). Zusätzlich haben NELSON *et al*. (2002) in ihren Untersuchungen eine Beteiligung von NELL2 an der Differenzierung von Muskelzellen nachgewiesen und diese Aussage in einer späteren Studie um die Annahme erweitert, dass *NELL2* Einfluss auf die Differenzierung der Zellen hat, in denen es exprimiert wird (NELSON *et al.* 2004). Die Expression von *NELL2A* ist im Muskel der BORN-Forelle bei 23 °C im Vergleich zu 8 °C signifikant erhöht, was vermutlich auf die zusätzliche Wichtigkeit der oben genannten Funktionen des kodierten Proteins unter Stresseinfluss zurückzuführen ist und für die BORN-Forelle einen Vorteil bei der Regeneration des Gewebes darstellen könnte.

Ähnlich wie bei der Temperaturänderung auf 8 °C bzw. 23 °C, konnte im Verlauf des Infektionsversuchs eine Verringerung der Expression einiger Kandidatengene beobachtet werden. Das Transkriptlevel von *LEAP2A*, *SERPINA2A* und *B*, *FGL2*, *SERPINA1* und *FTH* fällt in den ersten drei Tagen nach Infektion sehr deutlich ab, erhöht sich aber nur teilweise im weiteren Verlauf wieder. Im Gegensatz dazu ist eine Erhöhung der Expression von *NELL2A* und *B*, *MARCH5A* und *B* sowie *CLEC4E* zu definierten Zeitpunkten im Infektionsverlauf zu beobachten. Wie schon im Vergleich der Expression in gesunden Forellen zeigen *NELL2A* und *B* sowie *MARCH5A* und *B* ein spezifisches Expressionsmuster der Genvarianten im Infektionsverlauf bzw. eine spezifische Expression der Varianten in den beiden Forellenlinien. Diese Beobachtung unterstützt die bereits angeführte Vermutung einer teilweise spezialisierten Funktion der Genvarianten.

Des Weiteren konnten Differenzen in der temperatur- und infektionsabhängigen Genexpression der Kandidatengene in BORN- und Importforellen offen gelegt werden. So wird *NELL2* temperaturabhängig vor allem in Muskel und Körperniere der Fische different exprimiert. An Tag 7 nach der Infektion mit *A. salmonicida* zeigt vor allem *NELL2A* (signifikant), aber auch *NELL2B* (marginal signifikant) eine deutliche Hochregulierung seines renalen Transkriptlevels in der BORN-Forelle. Sowohl Importforellen als auch die lokale Zuchtlinie wiesen zu diesem Zeitpunkt deutliche Symptome der Furunkulose auf. Übereinstimmend mit der muskulären Expression des duplizierten Gens nach leichtem Temperaturstress, liegt auch hier die Vermutung nahe, dass der Einfluss von NELL2 auf die Ca^{2+}-abhängige Signalweiterleitung sowie die Zelldifferenzierung in der BORN-Forelle zu diesem fortgeschrittenen Zeitpunkt der Infektion besonders wichtig ist. Nicht eindeutig geklärt werden kann, ob die Importforelle die mit dem Gen verknüpften Aufgaben different bewältigt oder eventuell aufgrund der fortschreitenden Infektion nicht mehr zu einer entsprechenden Regulation fähig ist.

Das Akute-Phase-Gen *CLEC4A* zeigt im Infektionsverlauf in Importforellen eine deutlich markantere Regulation als in BORN-Forellen. Die Expression des Gens in BORN-Forellen ist an Tag 1 deutlich erhöht und bleibt bis Tag 21 im Verhältnis zum Ausgangswert erhöht. In Importforellen hingegen steigt das Transkriptlevel erst an Tag 3, dafür aber deutlich stärker an und sinkt bis Tag 14 noch unter das Ausgangsniveau zurück. *CLEC4A* ist durch die Erkennung von PAMPs und DAMPs (*damage associated molecular pattern*) an der Immunantwort beteiligt (YAMASAKI *et al*. 2009). Die etwas verzögerte aber damit heftigere Reaktion der Import-Forellen könnte einerseits auf eine effektivere Funktion von CLEC4A in Import-Forellen hindeuten. Andererseits könnte die schnellere und konstantere, dafür aber schwächere Erhöhung der Expression von *CLEC4A* in der BORN-Forelle auch auf eine bereits besser adaptierte Reaktion der lokalen Zuchtlinie auf Infektionen hinweisen.

Ein deutlicher Anstieg der Transkriptmenge bei Wassertemperaturen von 23 °C ist für die Akute-Phase-Gene *SERPINA1* und *FGL2* in Importforellen und für *FTH1* in BORN-Forellen nachweisbar. Die Expression von *FTH1* in der lokalen Zuchtlinie hat sich im Verhältnis zu ihrem Niveau bei 8 °C sogar verdoppelt. *FTH1* kodiert für eine Untereinheit des Protein-Komplexes Ferritin, dem zentralen zellulären Eisenspeicher-Protein. Da bakterielle Erreger Eisen benötigen (JUNG and KRONSTAD 2008), konkurriert der Erreger bei einer Infektion des Fisches mit dem essenziellen eisenbindenden Protein Ferritin aktiv um das Metall. Die erhöhte Expression von *FTH1* in der BORN-Forelle könnte folglich zu einem Vorteil für die lokale Zuchtlinie führen, da Ferritin die Verbreitung von Pathogenen im Organismus durch

vermehrte Eisenbindung einschränken könnte. Allerdings konnten nach *A. salmonicida*-Infektion nur marginale Unterschiede in der Expression des Gens in BORN- und Importforellen nachgewiesen werden. Hier wäre ebenfalls eine deutlich erhöhte *FTH1* Expression in der lokalen Zuchtlinie zu erwarten gewesen, um von einer spezifischen adaptiven Anpassung der Expression des Gens ausgehen zu können.

Das ebenfalls für ein eisenbindendes Protein kodierende Gen *ISCU* ist zwar auf dem Agilent 44k Oligo-Microarray an Tag 7 und 21 nach Infektion in Kieme und Kopfniere hochsignifikant verschieden zwischen den beiden Forellenlinien reguliert, aber diese Ergebnisse konnten unter Verwendung der qRT-PCR nicht validiert werden. Allerdings ist hierbei zu beachten, dass das Gen *ISCU* in der Forelle dupliziert vorliegt. Die auf den Microarray aufgebrachten Oligomere wurden allerdings, aufgrund bisher fehlender Forellensequenzen des duplizierten Gens in den Datenbanken, nicht eindeutig den Varianten *ISCUA* oder *ISCUB* zugeordnet, wodurch es zu Fehlhybridisierungen und damit falschen Ergebnissen kommen kann.

Insgesamt konnte für die Mehrzahl der Kandidatengene eine temperatur- und/oder infektionsabhängige Regulation der Expression in beiden Forellenlinien belegt werden. Aus diesen Beobachtungen lässt sich schließen, dass die entsprechenden Gene einen Einfluss auf die Regulation von Zellprozessen haben, die an der Reaktion auf Temperatur- und/oder Infektionsstress in der Forelle beteiligt sind. Zusätzlich spricht die differente Expression einiger Gene in der lokalen Zuchtlinie BORN - im Vergleich zu Importforellen - für eine durch natürliche Selektion bedingte spezifische Anpassung der BORN-Forelle an die lokalen Haltungs- und Zuchtbedingungen im Boddenwasser der Ostsee.

3.3.4 Differente Aktivierung der Apoptose in BORN- und Importforellen nach Infektion

Im Rahmen der Analyse der Genregulation nach Infektion wurde auch die Aktivierung der Apoptose vergleichend zwischen den beiden Forellenlinien untersucht. Neben anderen adaptiven Anpassungen könnte auch eine differente Regulation dieses aktiven Prozesses eine unterschiedliche Robustheit der beiden Zuchtlinien bei einer Infektion beeinflussen. Da die deutliche Vergrößerung und dunkle Verfärbung der Niere auf eine starke Belastung durch den Erreger hinwies, wurde die renale Genregulation zu vier Zeitpunkten der Infektion analysiert. Insgesamt wurde die Regulation von 47 an Abläufen der Apoptose beteiligten Genen des verwendeten Agilent 44k Oligo-Microarrays untersucht. Unter ihnen die Kandidatengene *CASP3*, *CASP7* und *CASP8*.

Insgesamt zeigt sich, dass in beiden Forellenlinien die Apoptose nach Infektion aktiviert wird. Da sowohl BORN als auch Importforellen im Infektionsverlauf deutliche Merkmale der Erkrankung Furunkulose aufwiesen, war eine Aktivierung des aktiven Zelltods in beiden Zuchtlinien zu vermuten. Allerdings sind regulatorische Unterschiede vor allem 7 Tage nach der Infektion mit *A. salmonicida* zu erkennen. Zu diesem Zeitpunkt sind in Importforellen acht Gene des Apoptose-Signalwegs signifikant reguliert, davon sieben hoch- und eines runterreguliert. Signifikant erhöht ist unter anderem die Expression des Liganten *TNFα* (*tumor necrosis factor-alpha*), durch dessen Bindung an entsprechende Rezeptoren der Zelle eine Aktivierung oder auch Hemmung der Apoptose über die Caspase-Kaskade bzw. den Transkriptionsfaktor NF-kB ausgelöst wird (RATH and AGGARWAL 1999). Allerdings war die renale Expression eines NF-kB-Inhibitors an Tag 7 ebenfalls signifikant erhöht, so dass eine pro-apoptotische Reaktion vermutet werden kann. Unterstützt wird diese Annahme durch die ebenfalls verstärkte Expression der für finale Proteine der Apoptose kodierenden Gene *ACTA2* (*smooth muscle aortic alpha-actin*) und *ACIN1* (*apoptotic chromatin condensation inducer in the nucleus*). Ihre Transkripte lösen zusammen mit anderen Proteinen die endgültige Zersetzung der Zelle aus.

In der BORN-Forelle ist 7 Tage nach Infektion die Expression des Apoptose-hemmenden Gens *BCL-2* (*B-cell CLL/lymphoma 2*) gesenkt und die des Gens der *CASP9* erhöht. Dies spricht für die Aktivierung der Apoptose in der lokalen Zuchtlinie über den intrinsischen Weg der Kaskade. Dieser wird durch die Freisetzung von Cytochrom C aus den Mitochondrien und anschließender CASP9-Aktivierung ausgelöst (DOS SANTOS *et al.* 2008). Auffallend ist, dass in den BORN-Forellen deutlich weniger Gene zu den untersuchten Zeitpunkten reguliert sind als in Importforellen. Vermutlich spiegelt dies eine zeitliche Verschiebung der entsprechenden Abläufe der Apoptose in der lokalen Zuchtlinie wider. Allerdings kann dieser Zusammenhang nur durch die vergleichende Analyse weiterer Zeitpunkte geklärt werden.

4. Fazit und Ausblick

Ziel der vorliegenden Arbeit war es, Gene zu identifizieren und molekulargenetisch zu charakterisieren, die an der erhöhten Robustheit und Stresstoleranz der lokalen Zuchtlinie BORN im Vergleich zu importierten Forellen beteiligt sind. Auf Basis eines holistischen Leber-Transkriptomvergleichs gesunder Forellen beider Linien sowie Literaturrecherche wurden fünfzehn Kandidatengene identifiziert. Aufgrund ihrer Funktion sowie ihrer differenten Regulation in beiden Forellenlinien wurde angenommen, dass sie, ergänzend zu klassischen Stressgenen, eine wichtige Rolle bei der adaptiven Stressreaktion spielen.

Die Charakterisierung der Sequenzen der Kandidatengene war ein Hauptziel der vorliegenden Arbeit. Es konnte gezeigt werden, dass viele der Gene angesichts einer additiven Genomduplikation innerhalb der Salmoniden in der Regenbogenforelle mehrfach vorliegen. Infolge der hohen Sequenzhomologien der duplizierten Gene wurden die anschließenden Analysen der spezifischen Genexpression erschwert. Durch die Charakterisierung der jeweiligen Gensequenzen liefert die Arbeit einen wesentlichen Beitrag für zukünftige funktionale Untersuchungen der Gene. Interessant wären dabei die Analyse ihrer spezifischen Funktion in der Regenbogenforelle im Vergleich zum Menschen sowie eine Verknüpfung dieser zellulären Funktionen mit einer phänotypischen Merkmalsausprägung.

Was den Einfluss der Kandidatengene auf die differente Robustheit der beiden Forellenlinien betrifft, müssen die Ergebnisse der vergleichenden Expressionsanalysen der Gene in gesunden Forellen sowie nach Belastung durch kritische Temperaturen und Infektion differenziert betrachtet werden. Zwar konnte für die Kandidatengene eine differente Expression zwischen gesunden Forellen der lokalen Linie BORN und Importforellen nachgewiesen werden, allerdings zeigten sich nur bei einigen der Gene grundlegende Unterschiede zwischen gestressten Fischen beider Zuchtlinien. Daher kann zwar auf eine unterschiedliche Bedeutung der Gene in beiden Forellenlinien geschlossen werden, ein Einfluss auf die erhöhte Stresstoleranz der BORN-Forelle ist allerdings nicht eindeutig nachzuweisen. Dieser Aspekt bedarf weiterer Analysen. Dabei sei außerdem darauf verwiesen, dass im Rahmen der vergleichenden holistischen Expressionsanalysen zwischen temperaturgestressten sowie durch Infektion belasteten Forellen beider Linien weitere signifikant different regulierte Gene identifiziert werden konnten (siehe Anhang). Diese stellen mögliche zukünftige Kandidatengene für Untersuchungen zur erhöhten Robustheit regionaler Zuchtlinien dar.

Abschließend kann festgestellt werden, dass die vorliegende Arbeit einen wichtigen Beitrag zur Aufklärung der Haupthypothese des DIREFO-Projektes liefert, die erhöhte Robustheit der BORN-Forellen sei genetisch manifestiert. Gestützt wird die Hypothese durch im Rahmen der Arbeit nachgewiesene eindeutige Varianzen in der Genregulation sowohl zwischen gesunden als auch gestressten Fischen der Zuchtlinie BORN und Importforellen. Allerdings waren Untersuchungen zur Korrelation dieser Unterschiede mit der funktionellen und phänotypischen Ausprägung in den Regenbogenforellen nicht Bestandteil der vorliegenden Arbeit. Dieser Zusammenhang sollte in zukünftigen Projekten, wie dem von 2012 – 2014 geplanten „Campus bio-FISCH M-V (**Bio**logisches **F**unktionales & **I**mmunologisches **S**creening zur **CH**arakterisierung regional selektierter Nutzfischarten in **M-V**, Projektnummer VI-560/7308-4)“, genauer untersucht werden. Die Ergebnisse der vorliegenden Arbeit bilden daher eine Grundlage für diese weiterführenden Untersuchungen.

5. Zusammenfassung

Die zunehmende Überfischung der Weltmeere bei gleichzeitig weltweit steigendem Bedarf an hochwertigem Fisch und Fischerzeugnissen hat die wirtschaftliche Bedeutung der nachhaltigen Fischzucht in Aquakulturanlagen in den letzten Jahren markant ansteigen lassen. Der Einsatz möglichst robuster Zuchtlinien als Besatz ist dabei besonders wichtig, um stressbedingte Verluste durch typischerweise in der Aquakultur auftretende äußere Stressoren wie z. B. saisonale Temperaturschwankungen, Infektionen oder auch Verschmutzung möglichst gering zu halten. Die an Brackwasser adaptierte lokale Regenbogenforellenlinie BORN hat sich im direkten Vergleich mit kommerziell in der Aquakultur eingesetzten Importforellen als besonders robust in ihrer Reaktion auf Stressoren gezeigt. Über die dem Merkmal Robustheit zugrundeliegenden genetischen Ursachen war zu Beginn der vorliegenden Arbeit nur wenig bekannt. Ziel dieser Arbeit war es daher, einen Beitrag zur Aufklärung der molekulargenetischen Mechanismen der differenten Stresstoleranz von BORN- und Importforellen zu leisten. Dafür sollten Gene und Stoffwechselwege identifiziert und molekulargenetisch charakterisiert werden, die zusätzlich zu klassischen Stressgenen an der differenten Stressantwort der BORN-Forelle beteiligt sind.

Auf der Basis eines holistischen Leber-Transkriptomvergleichs gesunder Regenbogenforellen beider Zuchtlinien konnten elf Kandidatengene (*PRR13, ISCU, NELL2, MARCH5, RGN, FGL2, LEAP2, SERPINA1, SERPINF2, CLEC4E, FTH1*) identifiziert werden, vier weitere (*CASP3, CASP7, CASP8, TPT-1*) wurden aus der Literatur gewählt. Im Rahmen der Arbeit wurde die Mehrzahl der Gene in der Regenbogenforelle erstmalig strukturell charakterisiert und beschrieben. Die Sequenzen bildeten die Grundlage der vergleichenden Expressionsanalysen der Arbeit und stellen einen grundlegenden Beitrag für zukünftige funktionelle Analysen der Gene in der Regenbogenforelle dar. Des Weiteren zeigte sich, dass fünf der Kandidatengene (*ISCU, NELL2, SERPINF2, CASP3*) in der Forelle mehrfach vorliegen, was auf eine zusätzliche Duplikation des Genoms der Salmoniden in der Evolution zurückzuführen ist. Analysen der Expression ergaben, dass es sich dabei nicht um stillgelegte, sondern um transkribierte Gene handelt. Auch konnte gezeigt werden, dass die Genvarianten ein divergentes Expressionsmuster in der Forelle sowie teilweise auch zwischen der lokalen Zuchtlinie BORN und Importforellen aufweisen.

Im Weiteren ergaben vergleichende Untersuchungen zwischen gesunden Forellen der lokalen Zuchtlinie BORN und Importforellen markante Unterschiede in der Expression der Kandidatengene, was auf eine differente Bedeutung der Gene in beiden Linien schließen lässt.

Die Expressionsanalyse der Kandidatengene nach Stress durch kritische Temperaturen sowie Infektion zeigte ebenfalls Unterschiede in der Genregulation beider Zuchtlinien, allerdings nur partiell, so dass eine klare Korrelation zwischen Expression der Kandidatengene und erhöhter Stresstoleranz der BORN-Forelle nicht eindeutig nachzuweisen ist. Weiterführende funktionale Untersuchungen sind erforderlich, um diesen Zusammenhang eindeutig zu klären.

Insgesamt liefert die vorliegende Arbeit einen wesentlichen Beitrag zur Aufklärung der Annahme, dass der erhöhten Robustheit und Stressresistenz der lokalen Forellenlinie BORN eine genetische Ausprägung zugrunde liegt.

6. Summary

Successful and purposeful fish farming has been gaining in importance increasingly during the years, due to the worldwide rising demand for fish and fish products and the in parallel continuously overfishing of the oceans. Therefore the forming of robust breeding-lines is of high relevance to minimize losses caused by stressful conditions like annual temperature fluctuations, infection or pollution. The local rainbow trout selection strain BORN is raised in brackish water of the Baltic Sea. It has been shown to be less susceptible towards different stressors than imported breeding-lines commercially used in aquaculture farms. Little is known about the genetic background of this phenomenon so far. Hence, the objective of this thesis was to contribute to the understanding of the distinct stress tolerance of BORN and imported rainbow trout. Therefore, the aim was to identify and characterize candidate genes that are, additionally to already known genes responding to stress, involved in the different stress response of BORN trout.

The eleven candidate genes *PRR13, ISCU, NELL2, MARCH5, RGN, FGL2, LEAP2, SERPINA1, SERPINF2, CLEC4E* and *FTH1* were identified by comparative transcriptome analysis of liver tissue; the four candidate genes *CASP3*, *CASP7*, *CASP8* and *TPT-1* were selected by literature research. In the present study, the majority of the candidate genes are molecularly characterized in rainbow trout, being first described in this salmonid at all. The respective sequences were essential for the comparative expression analysis of this study and will contribute to functional investigations in the future. Moreover, the survey revealed that due to an additional round of genome duplication in salmonid fish, more than one gene copy of the candidate genes *ISCU*, *NELL2*, *MARCH5*, *SERPINF2* and *CASP3* has been identified in rainbow trout. Expression analyses gave evidence of the functionality of these genes on mRNA level. Additionally, the gene variants showed a deviating expression pattern in trout in general, as well as a partially different mRNA expression in BORN and imported rainbow trout.

Comparative analyses between healthy rainbow trout of the local selection strain BORN and an imported breeding-line revealed remarkable differences in the expression of the candidate genes. This led to the conclusion that they might be of different functional importance in both lines. Expression analyses of the candidate genes after stress caused by infection and challenging temperatures also reflect these strain specific expression profiles. Since these findings were only partially, a clear correlation between gene expression and elevated stress tolerance of BORN trout cannot be proven. Further functional analyses are necessary to clarify this relationship in detail.

All in all, this thesis substantially contributes to the hypothesis of a genetically manifestation of the higher robustness and stress tolerance of the local breeding-line BORN.

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8. Anhang

Anhang I:

Initialer Leber-Transkriptomvergleich der vorliegenden Arbeit:

16k Salmoniden-cDNA-Microarray (GRASP); Leber BORN-Forellen gesund vs. Importforellen gesund

(68 regulierte Gene: 33 Gene hochreguliert, 35 Gene runterreguliert)

Gen-Bank ID (NCBI)	Genname	fold-change	p-value
CB497981	Ubiquitin carboxyl-terminal hydrolase isozyme L3	**5.32**	7.46558E-06
CB514334	UNKNOWN	**4.08**	2.24613E-08
CB496481	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial precursor	**3.70**	1.77784E-08
CB494163	Alpha-2-antiplasmin precursor	**3.22**	9.30821E-14
CB499782	ADP-ribosylation factor 4	**3.20**	5.78596E-15
CB505295	40S ribosomal protein S3a	**3.10**	2.16251E-08
CA039942	UNKNOWN	**3.03**	5.77746E-05
CA055538	UNKNOWN	**2.96**	3.92137E-08
CA038885	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	**2.76**	9.92738E-10
CA050472	UNKNOWN	**2.75**	2.95702E-10
CB493710	PREDICTED: Danio rerio hypothetical LOC556945 (LOC556945), mRNA	**2.63**	1.01552E-06
CB501721	UNKNOWN	**2.61**	1.03344E-10
CB512162	UNKNOWN	**2.56**	5.93814E-07
CA056704	Radixin	**2.49**	0.000114138
CK990379	Tenascin precursor [Tenascin-C in serum: a questionable tumor marker.]	**2.33**	4.81487E-11
CB516516	Proline-rich protein 12	**2.30**	6.41231E-15
CB512918	ADAM 23 precursor	**2.27**	0.000110674
CB517229	PREDICTED: Gallus gallus similar to KIAA0938 protein (LOC417869), misc RNA	**2.25**	0.001267904
CB514370	Tripartite motif-containing protein 16	**2.23**	5.71679E-05
CA038296	UNKNOWN	**2.18**	0.000140062
CB504316	UNKNOWN	**2.14**	6.9711E-06
CA057957	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	**2.13**	6.35075E-05
CA043877	DNA-directed RNA polymerases I, II, and III subunit RPABC2	**2.12**	0.000364018
CA059573	UNKNOWN	**2.12**	0.001003371
CB490914	Fumarylacetoacetase	**2.12**	4.25311E-05
CB492283	interferon inducible protein 1 [Oncorhynchus mykiss]	**2.08**	5.87289E-11

Gen-Bank ID (NCBI)	Genname	fold-change	p-value
CB511924	UNKNOWN	**2.08**	3.63103E-05
CB494112	Ribosome-binding protein 1	**2.08**	3.22026E-05
CA055486	Fibroleukin precursor	**2.05**	1.36977E-14
CA063996	THO complex subunit 4	**2.05**	0.001094653
CB498311	Retinol-binding protein II, cellular	**2.05**	3.05739E-05
CA063234	Cornichon homolog 4	**2.05**	0.000984481
CB509952	UNKNOWN	**2.02**	2.40736E-09
CA051867	steroidogenic acute regulatory protein	**0.48**	0.000515913
CB494195	Reticulon-3	**0.45**	4.23381E-07
CB502459	Transcription factor MafB	**0.44**	1.66454E-06
CA057292	UNKNOWN	**0.44**	2.84157E-14
CB488781	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes	**0.44**	3.37502E-11
CB492687	Regucalcin	**0.43**	1.55921E-05
CA042290	antimicrobial peptide 2A (LEAP-2A) Oncorhynchus mykiss liver-expressed	**0.42**	2.4917E-08
CA057553	UNKNOWN	**0.41**	0.000230822
CA062966	UNKNOWN	**0.40**	2.17788E-06
CB493963	LIM domain-binding protein 3	**0.39**	1.69673E-20
CB496739	Fructose-bisphosphate aldolase A	**0.39**	6.57777E-07
CB511033	Diamine acetyltransferase 1	**0.37**	5.65105E-07
CA057213	Aldehyde dehydrogenase, mitochondrial precursor	**0.36**	1.03332E-06
CA060384	Amyloid-like protein 2 precursor	**0.35**	8.08129E-07
CB514640	Ras GTPase-activating-like protein IQGAP1	**0.34**	5.23892E-05
CA054580	Transforming protein RhoA precursor	**0.34**	1.40876E-08
CA052085	Coatomer subunit delta	**0.34**	2.1563E-12
CB510137	UNKNOWN	**0.31**	1.43211E-11
CA054581	Ephexin-1	**0.29**	2.99764E-05
CB512660	UNKNOWN	**0.29**	1.37331E-05
CA057293	UNKNOWN	**0.26**	4.41414E-15
CA063225	Proline-rich protein 13	**0.25**	0.001262863
CB512504	Four and a half LIM domains protein 1	**0.25**	1.12135E-08
CB493689	Protein kinase C-binding protein NELL2 precursor	**0.25**	3.82532E-09
CA051589	UNKNOWN	**0.23**	5.00594E-05
CB514106	Vesicle-associated membrane protein-associated protein A	**0.23**	3.30074E-12

Gen-Bank ID (NCBI)	Genname	fold-change	p-value
CA037556	Alpha-1-antitrypsin homolog precursor	**0.21**	4.01414E-18
CB494189	Ubiquitin	**0.21**	3.53974E-20
CA061276	Iron-sulfur cluster assembly enzyme ISCU, mitochondrial precursor	**0.20**	9.97567E-21
CA055404	Dehydrodolichyl diphosphate synthase	**0.18**	6.85677E-10
CB498239	Protein-arginine deiminase type-2	**0.18**	1.29986E-26
CA057512	E3 ubiquitin-protein ligase MARCH5	**0.13**	5.27486E-07
CA061481	Sel-1 homolog precursor	**0.08**	8.58639E-05
CA059431	UNKNOWN	**0.08**	4.34679E-07
CB494589	Glycogen phosphorylase, muscle form	**0.07**	0.000468018

Anhang II:

Exemplarisch für die vergleichenden Transkriptomanalysen dieser Arbeit:

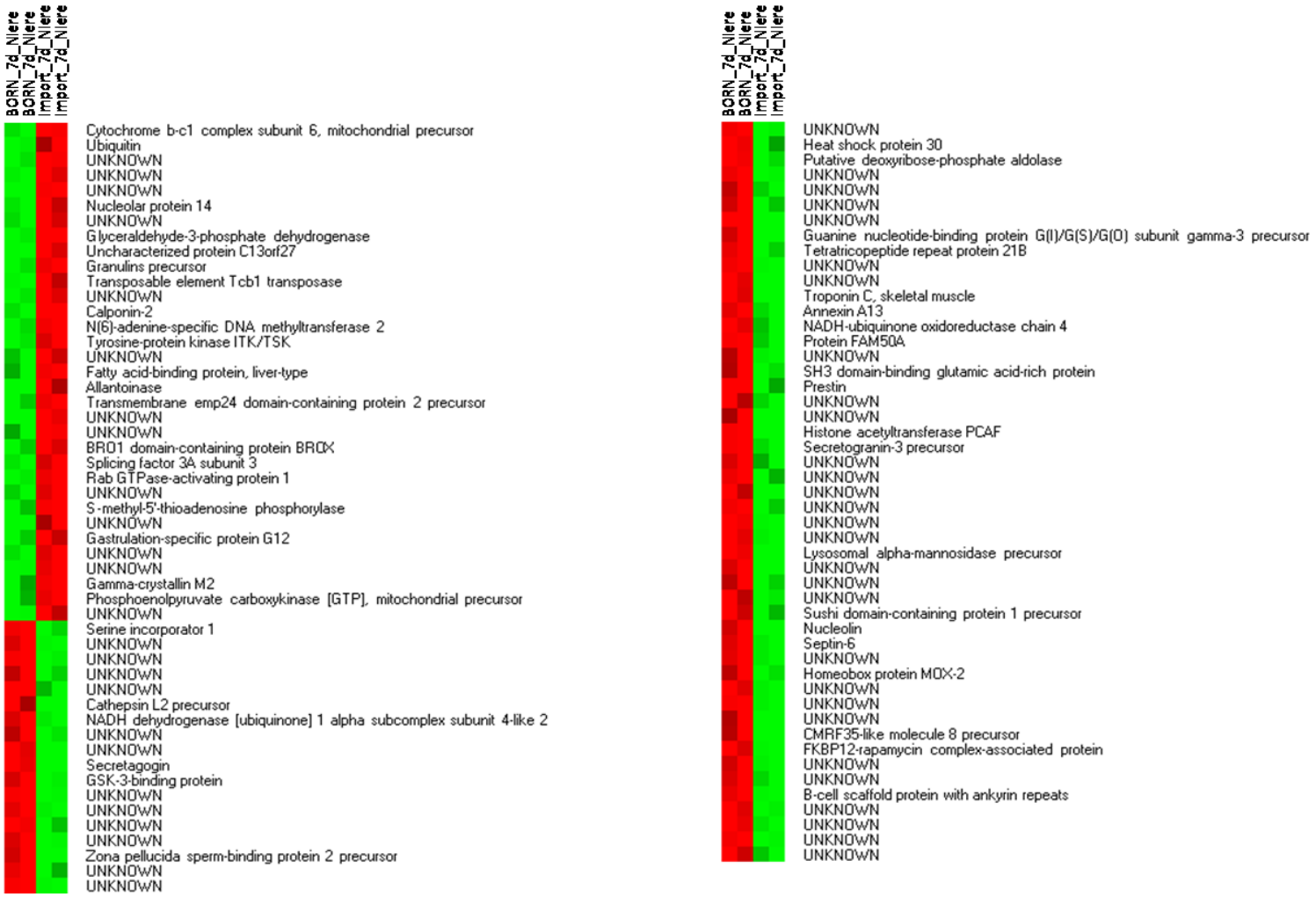
44k Oligo-Microarray (Agilent): Infektion mit *A. salmonicida* Niere BORN 7d vs. Import 7d

(Top 100 der regulierten Gene: 33 hochreguliert, 77 runterreguliert)

Gen-Bank ID (NCBI)	Genname	fold-change	p-value
A_05_P484307	Cytochrome b-c1 complex subunit 6, mitochondrial precursor	**689.88**	0.004
A_05_P431587	Ubiquitin	**191.39**	0.046
A_05_P443252	UNKNOWN	**164.96**	0.004
A_05_P485457	UNKNOWN	**101.32**	0.006
A_05_P276169	UNKNOWN	**85.33**	0.000
A_05_P452722	Nucleolar protein 14	**85.25**	0.020
A_05_P470742	UNKNOWN	**83.97**	0.016
A_05_P251714	Glyceraldehyde-3-phosphate dehydrogenase	**72.15**	0.002
A_05_P466192	Uncharacterized protein C13orf27	**64.27**	0.006
A_05_P478967	Granulins precursor	**48.26**	0.005
A_05_P376427	Transposable element Tcb1 transposase	**32.48**	0.020
A_05_P422042	UNKNOWN	**32.12**	0.008
A_05_P411697	Calponin-2	**31.81**	0.004
A_05_P417552	N(6)-adenine-specific DNA methyltransferase 2	**25.75**	0.006
A_05_P279712	Tyrosine-protein kinase ITK/TSK	**24.34**	0.010
A_05_P327037	UNKNOWN	**23.58**	0.035
A_05_P251329	Fatty acid-binding protein, liver-type	**21.53**	0.033
A_05_P355682	Allantoinase	**21.48**	0.039
A_05_P446062	Transmembrane emp24 domain-containing protein 2 precursor	**21.44**	0.014
A_05_P363462	UNKNOWN	**17.74**	0.002
A_05_P272874	UNKNOWN	**16.90**	0.038
A_05_P260854	BRO1 domain-containing protein BROX	**16.56**	0.021
A_05_P435887	Splicing factor 3A subunit 3	**14.82**	0.012
A_05_P309057	Rab GTPase-activating protein 1	**14.24**	0.003
A_05_P372147	UNKNOWN	**13.94**	0.016
A_05_P377677	S-methyl-5'-thioadenosine phosphorylase	**12.56**	0.016
A_05_P326407	UNKNOWN	**11.41**	0.042
A_05_P443722	Gastrulation-specific protein G12	**10.58**	0.036
A_05_P431932	UNKNOWN	**10.34**	0.008
A_05_P350122	UNKNOWN	**9.89**	0.003
A_05_P439872	Gamma-crystallin M2	**9.76**	0.041
A_05_P455477	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial precursor	**9.70**	0.033

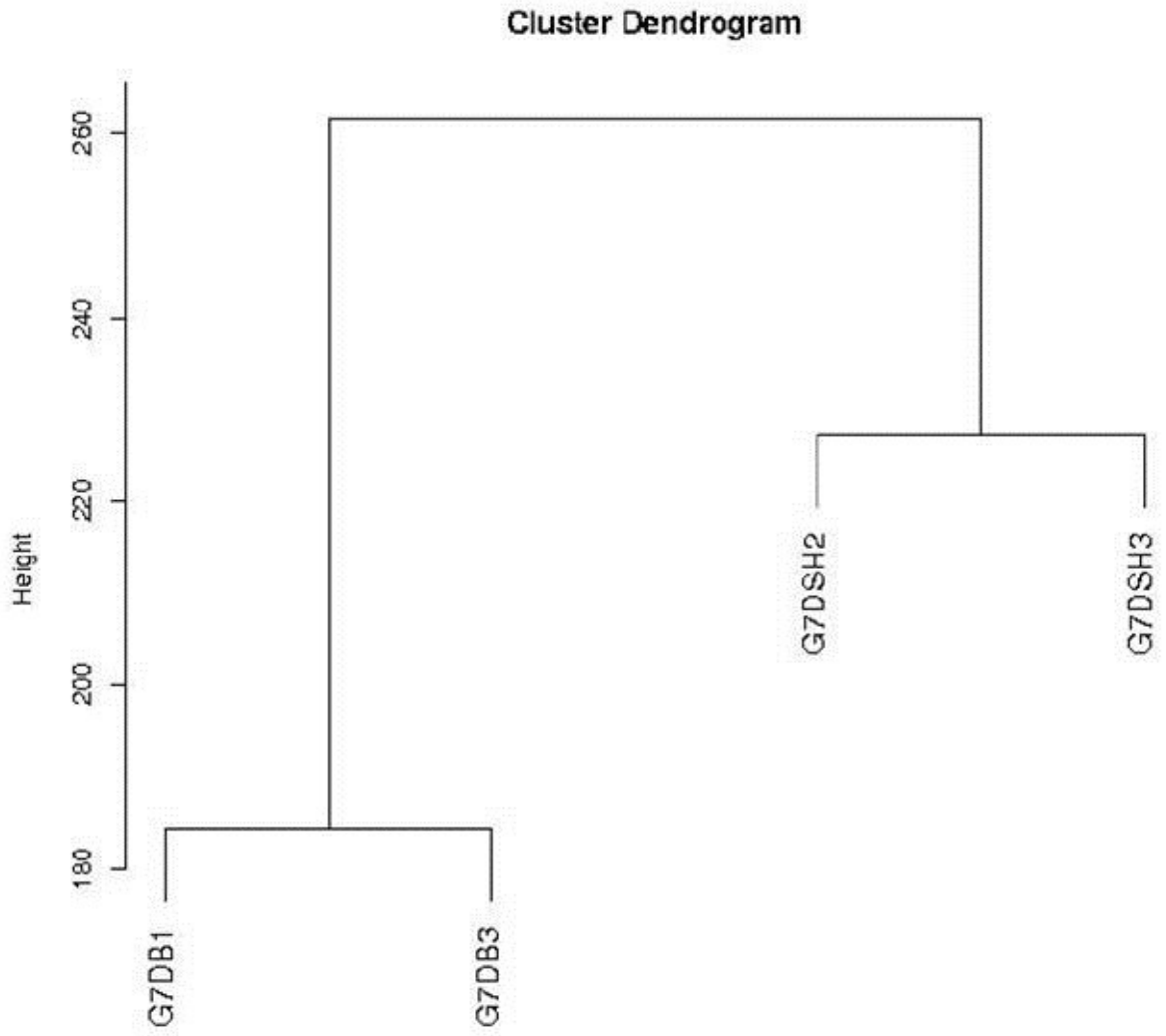
Gen-Bank ID (NCBI)	Genname	fold-change	p-value
A_05_P454262	UNKNOWN	**9.67**	0.022
A_05_P452267	Serine incorporator 1	**-729.28**	0.007
A_05_P276745	UNKNOWN	**-69.70**	0.006
A_05_P312482	UNKNOWN	**-47.56**	0.001
A_05_P348272	UNKNOWN	**-39.12**	0.036
A_05_P265774	UNKNOWN	**-38.50**	0.022
A_05_P386177	Cathepsin L2 precursor	**-25.45**	0.043
A_05_P301312	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4-like 2	**-21.91**	0.009
A_05_P358437	UNKNOWN	**-20.54**	0.028
A_05_P315207	UNKNOWN	**-20.15**	0.003
A_05_P279287	Secretagogin	**-20.11**	0.002
A_05_P266624	GSK-3-binding protein	**-18.24**	0.009
A_05_P450767	UNKNOWN	**-17.17**	0.000
A_05_P325987	UNKNOWN	**-17.14**	0.004
A_05_P448057	UNKNOWN	**-16.24**	0.017
A_05_P325932	UNKNOWN	**-15.76**	0.007
A_05_P441777	Zona pellucida sperm-binding protein 2 precursor	**-15.66**	0.011
A_05_P257294	UNKNOWN	**-15.66**	0.032
A_05_P255729	UNKNOWN	**-15.54**	0.001
A_05_P319242	UNKNOWN	**-15.47**	0.001
A_05_P351892	Heat shock protein 30	**-14.98**	0.045
A_05_P271244	Putative deoxyribose-phosphate aldolase	**-14.66**	0.006
A_05_P354532	UNKNOWN	**-13.83**	0.002
A_05_P457237	UNKNOWN	**-13.57**	0.034
A_05_P401642	UNKNOWN	**-13.54**	0.032
A_05_P346622	UNKNOWN	**-13.44**	0.000
A_05_P487887	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-3 precursor	**-13.15**	0.011
A_05_P328152	Tetratricopeptide repeat protein 21B	**-13.13**	0.013
A_05_P287777	UNKNOWN	**-12.95**	0.001
A_05_P351512	UNKNOWN	**-12.87**	0.003
A_05_P388112	Troponin C, skeletal muscle	**-12.77**	0.006
A_05_P449722	Annexin A13	**-12.65**	0.009
A_05_P471962	NADH-ubiquinone oxidoreductase chain 4	**-12.62**	0.022
A_05_P372592	Protein FAM50A	**-12.08**	0.011
A_05_P297002	UNKNOWN	**-11.19**	0.031
A_05_P277487	SH3 domain-binding glutamic acid-rich protein	**-11.19**	0.040
A_05_P410072	Prestin	**-11.03**	0.039
A_05_P358842	UNKNOWN	**-11.02**	0.043

Gen-Bank ID (NCBI)	Genname	fold-change	p-value
A_05_P327637	UNKNOWN	**-10.95**	0.042
A_05_P391592	Histone acetyltransferase PCAF	**-10.72**	0.000
A_05_P389087	Secretogranin-3 precursor	**-10.49**	0.004
A_05_P490392	UNKNOWN	**-10.38**	0.048
A_05_P388417	UNKNOWN	**-10.34**	0.034
A_05_P279477	UNKNOWN	**-10.24**	0.020
A_05_P426662	UNKNOWN	**-10.19**	0.013
A_05_P303212	UNKNOWN	**-9.82**	0.001
A_05_P393327	UNKNOWN	**-9.76**	0.005
A_05_P466277	Lysosomal alpha-mannosidase precursor	**-9.56**	0.001
A_05_P445092	UNKNOWN	**-9.52**	0.006
A_05_P360637	UNKNOWN	**-9.41**	0.036
A_05_P356952	UNKNOWN	**-9.21**	0.029
A_05_P298782	Sushi domain-containing protein 1 precursor	**-9.16**	0.047
A_05_P392762	Nucleolin	**-9.16**	0.011
A_05_P486562	Septin-6	**-9.14**	0.006
A_05_P336262	UNKNOWN	**-9.12**	0.004
A_05_P293827	Homeobox protein MOX-2	**-9.07**	0.032
A_05_P414302	UNKNOWN	**-9.05**	0.006
A_05_P331567	UNKNOWN	**-8.98**	0.001
A_05_P336592	UNKNOWN	**-8.96**	0.030
A_05_P357997	CMRF35-like molecule 8 precursor	**-8.92**	0.027
A_05_P339117	FKBP12-rapamycin complex-associated protein	**-8.92**	0.006
A_05_P491747	UNKNOWN	**-8.91**	0.012
A_05_P484767	UNKNOWN	**-8.82**	0.015
A_05_P330767	B-cell scaffold protein with ankyrin repeats	**-8.75**	0.002
A_05_P290972	UNKNOWN	**-8.74**	0.005
A_05_P487427	UNKNOWN	**-8.72**	0.005
A_05_P297397	UNKNOWN	**-8.55**	0.001
A_05_P473552	UNKNOWN	**-8.54**	0.033



Anhang III:

Exemplarisch für die vergleichenden Transkriptomanalysen dieser Arbeit: Heatmap der Top 100 der regulierten Gene des 44k Oligo-Microarrays (Agilent) nach Infektion mit *A. salmonicida* (Niere BORN 7d vs. Import 7d)



Anhang IV:

Exemplarisch für die vergleichenden Transkriptomanalysen dieser Arbeit: Cluster Dendrogram des 44k Oligo-Microarrays (Agilent) nach Infektion mit *A. salmonicida* (Niere BORN 7d vs. Import 7d)

Erklärung zum eigenen Anteil an den Veröffentlichungen

Hiermit erkläre ich, dass mein Anteil an den in der vorliegenden Arbeit zusammengefassten Veröffentlichungen wie folgt ist:

Publikation I

Identification of differentially expressed protective genes in liver of two rainbow trout strains.
Rebl, A., **Verleih, M.**, Korytar, T., Kuhn, C., Wimmers, K., Köllner, B., Goldammer, T.
Vet Immunol Immunopathol (2012), **145**(1-2): 305-15.

- Mitarbeit an der Probenahme sowie der Durchführung des Infektionsversuchs
- Mitarbeit an der Erhebung der Expressiondaten
- Diskussion und Interpretation der Daten
- Korrektur des Manuskripts

Publikation II

Molecular characterisation of PRR13 and its tissue specific expression in rainbow trout (*Oncorhynchus mykiss*).
Verleih, M., Rebl, A., Köllner, B., Korytar, T., Kotterba, G., Anders, E., Wimmers, K., Goldammer, T.
Fish Physiol Biochem (2010), **36**(4): 1271-6.

- Mitarbeit an der Probenahme
- Durchführung der molekulargenetischen Analysen
- Statistische Auswertung der Daten
- Diskussion und Interpretation der Daten
- Verfassen des Manuskripts inklusive Literaturrecherche sowie Erstellung der Abbildungen

Publikation III

Comparative molecular characterization of the regucalcin (RGN) gene in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus marena*).
Verleih M, Rebl, A., Köllner, B., Korytar, T., Anders, E., Wimmers, K., Goldammer, T.
Mol Biol Rep (2012), **39**(4): 4291-4300.

- Mitarbeit an der Probenahme sowie der Durchführung des Temperatur- und Infektionsversuchs
- Durchführung der molekulargenetischen Analysen
- Statistische Auswertung der Daten
- Diskussion und Interpretation der Daten
- Verfassen des Manuskripts inklusive Literaturrecherche sowie Erstellung der Tabellen und Abbildungen

Publikation IV

Structural characterization and expression analyses of duplicated iron-sulfur cluster scaffold (ISCU) gene in salmonid fish.
Verleih, M., Rebl, A., Köllner, B., Korytář, T., Köbis, J.M., Kühn, C., Wimmers, K., Goldammer, T.
Gene (2013), **512**(2): 251-258.

- Mitarbeit an der Probenahme sowie der Durchführung des Temperatur- und Infektionsversuchs
- Durchführung der molekulargenetischen Analysen
- Statistische Auswertung der Daten
- Diskussion und Interpretation der Daten
- Verfassen des Manuskripts inklusive Literaturrecherche sowie Erstellung der Tabellen und Abbildungen

Publikation V

MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection.
Rebl, A., Köbis, J.M., Fischer, U., Takizawa, F., **Verleih, M.**, Wimmers, K., Goldammer, T.
Fish Shellfish Immunol (2011), **31**(6): 1041-50.

- Mitarbeit an der molekulargenetischen Charakterisierung der Gensequenzen
- Mitarbeit an der Erhebung der Expressiondaten
- Diskussion und Interpretation der Daten

Publikation VI

Duplicated NELL2 genes show different expression patterns in two rainbow trout strains after temperature and pathogen challenge
Rebl, A., **Verleih, M.**, Köllner, B., Korytář, T., Goldammer, T.
Comp Biochem Physiol B Biochem Mol Biol (2012), **163**(1): 65-73.

- Mitarbeit an der Probenahme der Entwicklungsstadien sowie Mitarbeit an der Probenahme und der Durchführung des Temperatur- und Infektionsversuchs
- Mitarbeit an der Erhebung der Expressiondaten
- Diskussion und Interpretation der Daten
- Korrektur des Manuskripts

Publikation VII

Comparative analyses of apoptosis-related candidate genes in rainbow trout: molecular characterization and transcriptome analyses.
Verleih, M., Rebl, A., Köllner, B., Korytář, T., Kühn, C., Wimmers, K., Goldammer, T.
Schriftenreihe des FBN (2011), **18**: 39-42.

- Mitarbeit an der Probenahme sowie der Durchführung des Infektionsversuchs
- Durchführung der molekulargenetischen Analysen
- Diskussion und Interpretation der Daten
- Verfassen des Manuskripts inklusive Literaturrecherche sowie Erstellung der Abbildung

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Publikationen und Tagungsbeiträge

Publikationen

Verleih, M., Rebl, A., Köllner, B., Korytar, T., Kotterba, G., Anders, E., Wimmers, K., Goldammer, T. (2010). Molecular characterization of PRR13 and its tissue-specific expression in rainbow trout (*Oncorhynchus mykiss*). Fish Physiol Biochem 36, 1271-1276.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich oder wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 05.02.2013

Marieke Verleih

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