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Effects of high-soy diet on S100 gene expression in liver and intestine of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The current study examines expression of S100 genes, a group of calcium-sensing proteins poorly characterized in fishes. In mammals, these proteins are known to play roles beyond calcium-signaling, including mediation of inflammatory processes. Some S100 proteins also serve as biomarkers for a variety of autoinflammatory conditions. It is well known that salmonids exhibit varying degrees of intestinal enteritis when exposed to alternative feed ingredients containing antinutritional factors, with soybean meal (SBM) being one of the best characterized. The etiology of soy-caused distal enteritis isn't entirely understood but displays similar histopathological alterations to the gut observed in human mucosal inflammatory bowel diseases. We sought to determine if teleost S100 genes show a concomitant response like that observed in mammals, utilizing rainbow trout fed high-soy diets as a model for intestinal inflammation. We examined expression of fourteen known salmonid S100 genes in the liver, first segment of the mid-intestine (proximal intestine), and second segment of the mid-intestine (distal intestine). After 12 weeks on a high-soy diet containing 40% SBM, we observed up-regulation of several S100 genes in the distal intestine (S100I2, A10a, V1, V2, and W), no changes in the proximal intestine, and downregulation of S100V2 in the liver. Overall, our results provide further knowledge of the expression of S100 genes and provide targets for future research regarding inflammatory processes in the rainbow trout gut.

1. Introduction

In comparison to humans and rodents, available information on S100 proteins in fishes is limited. Kraemer et al. [1] performed qPCR and *in situ* hybridization surveys on adult and larval zebrafish (*Danio rerio*), respectively. The intestine was not one of the ten tissues they examined in adult fish, but larval intestine showed strong expression of A10a. Bobe and Goetz [2] characterized A10b expression from tissues of post-ovulatory brook trout (*Salvelinus fontinalis*), with northern blot analysis also showing expression in the stomach and intestine, among other tissues. Cultured primary kidney cells of common carp (*Cyprinus carpio*) were induced to express S100A1, A10a, and A10b after exposure to recombinant IL-17A/F2a [3]. The crystal structure of zebrafish S100Z was determined, and found to possess a tetramerization interface similar to human S100A4 [4]. S100I has been found to be highly expressed in the skin of zebrafish, Atlantic salmon (*Salmo salar*), and channel catfish [5–7].

Additionally, S100 protein immunoreactivity has been utilized as a means of histologically identifying crypt neurons in zebrafish and mast cells in the chub (*Squalius cephalus*) [8,9]. Utilizing a general S100 antibody, Vigliano et al. [10] employed immunohistochemistry to observe developing rodlet cells in juvenile turbot (*Scophthalmus maximus*). The same results were observed in the intestines of rainbow trout, indicating that S100 immunoreactivity is a strong-indicator of rodlet cells, which are thought to play a role in immune function [11].

Our interest in S100 genes relate to the sensitivity of salmonids and other fishes to inclusion of soybean meal (SBM) in formulated diets, which is well described in causing enteritis in the distal intestine, anatomically characterized as the second segment of the mid-intestine [12–17]. The underlying mechanism for the negative immunological reactions observed after exposure to elevated levels of SBM have been postulated to include shifts in the composition of the intestinal microbiota, impairment of the barrier function of the intestine due to saponins and other antinutritional factors, an improper immune reaction to innocuous antigens, or through more complicated interactions between exogenous and endogenous factors [18–23].

Specific S100 proteins have been found to be associated with specific human diseases and have efficacy as diagnostic biomarkers [24].

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In regard to diagnosing and monitoring inflammatory bowel diseases in humans, the calgranulins (A8/A9 (calprotectin) and A12) have found the most clinical relevance, although there are isolated reports of the involvement of other S100 genes [24–27]. In humans, the calgranulins are specific to phagocytes, with calprotectin accounting for up to 40% of the cytosolic protein in circulating human neutrophils. At high extracellular concentrations, they induce apoptosis by sequestering zinc, while at low concentration they serve as receptor ligands and induce neutrophil chemotaxis and adhesion [25,28–30]. The sequestration of extracellular nutrient metals is a common component of the immune response as an attempt to inhibit extracellular pathogens, the dysfunction of which can result in the host-cell damage and inflammation present in many autoimmune inflammatory diseases [31,32]. As a ligand, calprotectin promotes inflammation by binding to Toll-like receptor 4 on phagocytes and cell proliferation by binding to RAGE [33]. As neither this S100 subfamily or RAGE are present in fishes, it is unknown if equivalent functionality exists in fishes [34].

Overall, there is little information on the expression of S100 genes in the gut of fishes. While Kraemer et al. [1] provided information of the abundance of S100 proteins in the developing intestinal tract of zebrafish, intestinal tissue was omitted from their survey of tissue RNA expression. Therefore, to provide further data on the expression of these genes, and the possible influence of inflammation in the liver, proximal (anatomically the first segment of the mid-intestine posterior to the pyloric caeca) and distal intestine, we surveyed these tissues for any observable changes in S100 RNA expression after exposure to a high (40%) SBM diet known to induce inflammation in the distal intestine of rainbow trout [12,17].

2. Materials and methods

2.1. Diets

Rainbow trout diets containing > 30% SBM have been demonstrated in the past to elicit inflammatory responses in the intestine [12,14,35]. Accordingly, two diets were formulated on a digestible nutrient basis to provide 40% digestible protein and 20% crude fat, while containing 0% or 40% SBM (Table 1). Both diets were balanced for digestible protein and supplemented with methionine, lysine, and threonine to meet or exceed all known nutrient requirements [36]. Yttrium oxide was also included at 0.01% as an indigestible inert marker to quantify nutrient digestibility.

Table 1
Diet Formulation (%DM) of experimental diets.

Ingredient	Soy 0	Soy 40
Menhaden Fish Meal	30.00	0.00
Soybean Meal 48%CP	0.00	40.00
Chicken 42 - ADF	11.50	11.50
Corn Protein Concentrate	11.50	11.50
Menhaden fish oil	14.72	16.35
Wheat gluten meal	0.16	1.30
Wheat flour	23.29	5.54
Lecithin	1.00	1.00
Stay-C 35	0.15	0.15
Vitamin premix ARS 702	1.00	1.00
TM ARS 640	0.10	0.10
NaCl	0.28	0.28
Magnesium Oxide	0.06	0.06
Potassium chloride	0.56	0.56
Monocalcium Phosphate	0.60	4.80
Choline Cl 50%	1.00	1.00
DL-Methionine	0.33	0.63
Lysine HCl	2.17	2.56
Threonine	0.40	0.49
Tryptophan	0.00	0.00
Taurine	1.00	1.00
Biofix Plus	0.00	0.00
Yttrium oxide	0.10	0.10
Astaxanthin	0.08	0.08
Total	*100%*	*100%*

Diets were mixed and co-ground without fish oil and lecithin using an air-swept pulverizer (model 18-H, Jacobson, Minneapolis, USA) to a particle size of less than 250 μm. Diets were manufactured using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with six barrel sections and 18-s transit time through the barrel. The material was not steam conditioned prior to extrusion and water was added to barrel section 2. The 3.5- mm floating pellets were dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) at 102 °C until moisture levels were below 7%, and cooled prior to vacuum oil infusion (A.J. Mixing, Ontario, Canada). Diets were stored in a cool dry environment until utilized.

2.2. Fish culture and feeding

Fifteen rainbow trout (88 ± 1.6 g; Troutlodge Inc., Sumner, WA) per tank were stocked into a recirculating system consisting of six 320-L poly tanks connected to a common bio-filter with spring water inflow of approximately 20% makeup. Water temperatures were maintained at 15 °C with a 14:10 light:dark cycle for the duration of the experiment. A sample of 10 fish taken from the initial population were euthanized with an overdose of MS-222 at a concentration of 250 ppm (Syndel USA, Ferndale, USA) and stored frozen at −20 °C for chemical analysis to determine protein and energy retention efficiencies at the experiment's completion. Fish were fed twice daily, 6 days per week, for 12 weeks to apparent satiation. Consumed feed was recorded weekly. All fish in each tank were counted and weighed every 3 weeks. All fish were handled and treated in accordance with the USFWS procedures according to the Guidelines for Use of Fishes in Research [37].

2.3. Sample collection

At the termination of the trial, three fish per tank (n = 3/tank; n = 9/treatment) were euthanized for whole-body proximate composition, and three additional fish per tank were dissected to quantify relative liver size (hepatosomatic index) and viscera (viscerosomatic index).

Samples of proximal and distal intestine from five fish per tank (n = 5/tank; n = 15/treatment) were excised; proximal being defined as the point from the last pyloric cecum to the point where intestinal diameter and smooth muscle visually increase in size (approximately half the distance from the last pyloric cecum to the vent) with the proximal sample being taken approximately 1 cm anterior and the distal sample approximately 1 cm posterior to this location (i.e. samples were taken of the first segment of the mid-intestine and second segment) of the mid-intestine (distal intestine). Feces were collected from the remaining fish 16-h post feeding to measure nutrient digestibility by sedating the fish in 50-ppm MS-222 until equilibrium was lost, removing the fish from the water and drying the fish to prevent water contamination of the feces. Gentle pressure was then applied to the abdomen approximately one third of the distance from the anus to the gill plate. Fecal matter was excreted into a weigh boat and transferred into micro-centrifuge tubes, snap frozen in liquid nitrogen, and stored at −80 C for analysis. Remaining feces were lyophilized, and nutrient and yttrium content were measured to calculate apparent nutrient absorption and digestibility [38]. Protein and energy retention efficiencies were determined according to the methods of Gaylord and Barrows [39].

2.4. Histological analysis of intestine

Sections of dissected distal and proximal intestine were dehydrated in ethanol, flushed with xylene and paraffin embedded. Tissue from each section was thin sliced (3 μm) crosswise on the longitudinal axis

and stained with hematoxylin and eosin (H&E). A semi-continuous scoring system (ranging from 0 to 5) was used to evaluate mucosal fold height, goblet cell number, lamina propria thickness and cellularity, supranuclear vacuolization, eosinophilic granulocyte infiltration and sub-epithelial mucosa appearance in the intestine adapted from Urán et al. [40] and Venold et al. [41]. A score of 0–1 represented normal variation in morphology and scores > 2 indicating morphological changes consistent with increasing severity of intestinal inflammation. Slides were prepared and scored randomly at the Bozeman Fish Health Center and subsequently at the Hagerman Fish Culture Experiment Station using light microscopy (Zeiss Axioscope A1, Carl Zeiss Ltd, Cambridge, UK) with the evaluators blinded to treatment. The non-parametric, Mann-Whitney Rank Sum Test, with a Yates continuity correction (Sigmaplot 14.0, Systat Software, Inc. San Jose, CA) was used to evaluate differences in histological scores with significance assigned at $\alpha < 0.05$.

2.5. RNA extraction and reverse transcription

Total RNA was extracted from each tissue with TRIzol® reagent (ThermoFisher Scientific, Waltham, USA). Briefly, small pieces of tissue were placed in 2-mL round-bottom centrifuge tubes containing 1 mL of TRIzol® and homogenized in a bead mill (MixerMill 200, Retsch GmbH, Haan, Germany). The homogenate was centrifuged at 12,000 g at 4 °C for ten minutes, with the supernatant being transferred to fresh 1.5 mL tubes. Two hundred uL of chloroform was added to each tube, which were then vigorously shaken for 15 seconds before being incubated at room temperature for ten minutes. Tubes were then centrifuged at 12,000 g at 4 °C for fifteen minutes, with the supernatant being transferred to fresh 1.5 mL tubes. Five hundred uL of isopropanol was added to each tube, which were then mixed gently before incubating overnight at −20 °C. The precipitated RNA was pelleted by centrifuging at 12,000 g at 4 °C for fifteen minutes, washed with cold 75% ethanol, and resuspended in nuclease-free water. RNA quantity and purity were determined spectrophotometrically (Nanodrop 1000, ThermoFisher Scientific, Waltham, USA). After individual sample concentrations were determined, resuspended RNA was pooled with equal contributions of RNA from each sample for each tank (n = 5/tank n = 15/treatment). Pools were treated with DNase (DNase I, Invitrogen, Carlsbad, USA) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, ThermoFisher Scientific, Waltham, USA) according to manufacturer's instructions. The resulting cDNA was aliquoted and stored at −80 °C for later analysis.

2.6. Primer design and amino acid alignment

Utilizing sequence data provided by Kraemer et al. [1], we performed BLAST (Version 2.7.1, [42]) searches and obtained sequences of all S100 genes in rainbow trout (Table 2). Primers were designed to be intron spanning using Primer3 (Version 2.4, [43]) and were checked for specificity by performing BLAST searches against the most recently published rainbow trout genome assembly (GCA_002163495.1) [44].

In addition, we produced an alignment and percent identity matrix (Figs. 1–2) of rainbow trout S100 amino acid sequences with Clustal Omega 1.2.4 (default parameters with the BLOSUM62 substitution matrix) [45]. The alignment was visualized with Pfaat 2.0.117, highlighting residues with ≥50% conservation [46]. The EF-hand motifs are denoted by solid (α-helices) and dotted (N-terminal S100- and C-terminal canonical-loop) bars. Matches to known EF-hand motifs were checked by scanning sequences against the PROSITE collection of motifs [47].

2.7. QPCR and data analysis

Pooled samples in plates were amplified in duplicate (ViiA 7 Real-Time PCR System, Life Technologies, Carlsbad, USA) according to manufactures instructions (Fast SYBR™ Green Master Mix, Life Technologies, Carlsbad, USA) with a melt curve to check for product specificity. Of the three reference genes evaluated (ribosomal protein S15 (RPS15), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and acidic ribosomal protein (ARP)), RPS15 was utilized for the intestinal tissues and GAPDH for the liver. Expression efficiencies were 97–101% in the intestinal tissues and 93–97% in the liver. The Cq value of the reference gene was subtracted from each gene of interest to obtain ΔCq values. After testing for homogeneity and homoscedasticity, one-way ANOVA with Tukey-Kramer *post-hoc* tests ($\alpha < 0.05$) were performed with untransformed ΔCq values using SAS® version 9.4 (PROC-GLM, Cary, SC).

Table 2
Primer sequences of rainbow trout S100 genes and reference genes used in qPCR.

Gene	Accession	Primers
S100A1	XM_021559274.1	*f. TTC CTG GCG GCA AGT AAG* *r. CAG CAA CCA AGA TGA CAA ACT C*
S100A10a	XM_021571618.1	*f. GGC TTC CTC AAG TCT CAG AAG* *r. GTT CAC CTC TCC ATC ACC ATT AG*
S100A10b	XM_021621938.1	*f. GCA ACA CAC TGA GCA AGA AG* *r. TCC TTC TGG GAC TTC AGG A*
S100A11a	XM_021589013.1	*f. AAG GCG TTT GCT GGA AGT* *r. TCA TCA GCG TTC TTA ACG AAG T*
S100A11b	XM_021560736.1	*f. TGT CAA GAA CGC CAG CGA* *r. CCT TGC TTG TTG GCA AGT TT*
S100B	XM_021571442.1	*f. GCC CTT ACA GGG CAA GT* *r. GTC CAA TTC CGA GTC TCC ATC*
S100P	XM_021578278.1	*f. GAC CTT TGA CAA GTA CTC TGG A* *r. CTT AGA AGC CTT CAG GAG AGA AG*
S100I1	XM_021595301.1	*f. CAT CGC CTC CTT CCA CAA GT* *r. ACT TGT CAC TGG CTT TCC CC*
S100I2	XM_021598338.1	*f. GCT TGG AGA GAT CAT GGG GAA AA* *r. GCC ATC TGA GTT AGC GTC CA*
S100W	XM_021598332.1	*f. CCA GAG TTC CAC GGA AAG G* *r. GAG TAG CCA AGA ACA TGG AGA A*
S100U	XM_021588983.1	*f. GTA TCT GAA GTC TGC CAA AGG G* *r. AGG AAC TCT CTG TTC CAG TCA*
S100V1	XM_021593175.1	*f. TGG TAA GTC AGT TCC ATG GTG* *r. CTG CTG TCT TGT CAA GGG T*
S100V2	XM_021572132.1	*f. TTA CGA CTG GAG CGT CAG A* *r. CCT CCA GAA GTG ATT GAA GGT G*
S100Z	XM_021601314.1	*f. GGA ACG ACG GAG ACA AAT ACA* *r. TTT CTG AGA CAT GAG AAA GTC AGT*
RPS15	NM_001165174.2	*f. ACA GAG GTG TGG ACC TGG AC* *r. AGG CCA CGG TTA AGT CTC CT*
GAPDH	XM_021571945.1	*f. AAG TGA AGC AGG AAG GTG GAA* *r. CAG CCT CGC CCC ACT TGA TG*
ARP	XM_021610240.1	*f. GCT GTA AAA GCG ATC CTT CG* *r. ATT GTC TGC ACC CAC AAT GA*

3. Results

There were no differences between final body composition, feed intake, and growth performance between the experimental groups (Table 3). Survival was 100% in both groups. Histological scores differed between control ($x = 0.9 \pm 0.1$) and experimental groups ($x = 2.8 \pm 0.1$) indicating mild but significant inflammation consistent with distal enteritis was achieved with 12 weeks of feeding the 40% SBM diet ($p < 0.001$, $U = 925.5$). Scores between proximal intestinal samples in either group were not significant. Additionally, no significant morphological changes were observed between proximal and distal intestinal samples (first segment of the mid-intestine and second segment of the mid-intestine).

We found marked differences in the conservation of the EF-hand motifs within the rainbow trout S100 amino acid sequences (Fig. 1). *In silico* analysis of the PROSITE database with these sequences show the canonical-hand motif being more conserved (not found in S100B, P, V1, and V2) than the S100-hand (not found in S100A11a, A11b, W, U, V1,

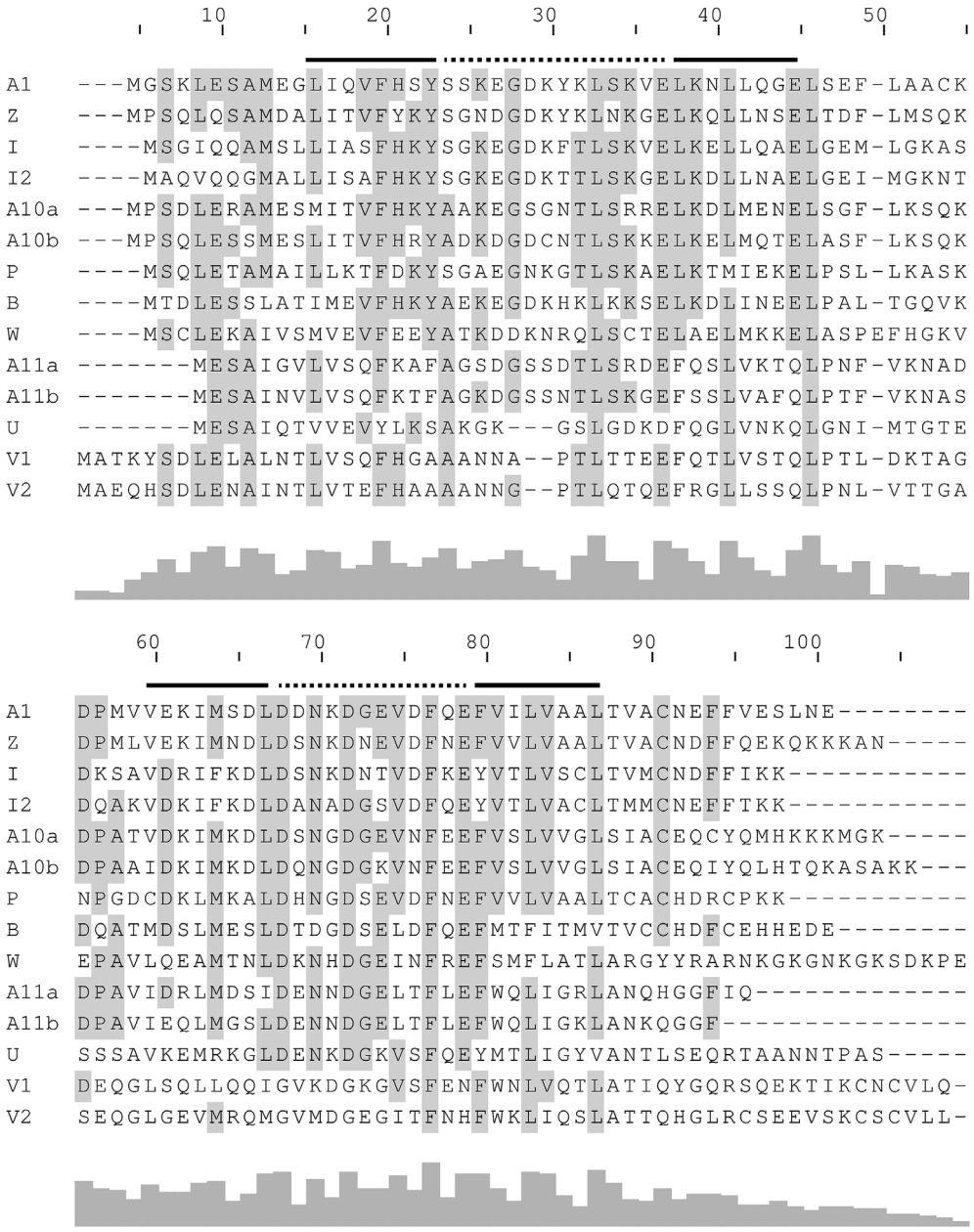

Fig. 1. Amino acid alignment of rainbow trout S100 proteins. EF-hand motifs are marked by solid (α-helices) and dotted (N-terminal S100-hand and C-terminal canonical-hand) bars. Residues with ≥50% conservation across all S100 proteins are shaded.

and V2) [47]. S100V is most highly divergent of the S100 genes found in fishes, having lost key residues in both EF-hands [1,26]. In addition, they show the lowest amino acid sequence identity (Fig. 2) among rainbow trout S100 paralogs (56%) when compared to I (70%), A10 (73%), and A11 (79%).

In the distal intestine, a significant increase in expression was observed in S100A10a, I2, V1, V2, and W in fish fed 40% SBM (Fig. 3). In addition, there was an observed nonsignificant decrease in S100A1. No treatment effects were observed in the proximal intestine. In the liver, expression of S100V2 was significantly lower in fish fed 40% SBM. No significant differences were observed for S100A1, A10b, I1, and U in the three tissues examined. Expression of S100B was not detected, while expression of S100A11a, A11b, P, and Z were too low for confident evaluation in all three tissues, along with S100I1 in the liver.

The overall pattern of expression for these genes was similar in all three tissues, with S100A1 and I1 showing the lowest expression, V2 and W showing low-intermediate expression, A10b and U showing intermediate expression, and A10a, I2, and V1 showing the highest levels of expression (Table 4). Of the three pairs of paralogs examined, differential expression was observed in each. Depending on the tissue, the expression of S100I2 was higher than I1, V1 was higher than V2, and A10a higher than A10b.

4. Discussion

The S100 family of proteins is a member of a superfamily of calcium-binding proteins that possess a canonical twelve amino acid EF-hand motif and a fourteen-amino acid S100-specific calcium binding motif in the N-terminal domain (Fig. 1) [48,49]. Each motif is composed of a helix-loop-helix, with calcium binding within the loop of the EF-hand producing a conformational change between its two helices and exposing a hydrophobic pocket. In addition to calcium, S100 proteins are capable of binding other divalent cations: magnesium, manganese, zinc, and copper [50,51]. They typically function as dimers,

	A1	Z	I	I2	A10a	A10b	P	B	W	A11a	A11b	U	V1	V2
A1		66	55	53	52	52	49	40	31	31	36	24	19	19
Z	66		55	52	52	52	52	37	27	30	34	21	20	22
I	55	55		70	43	45	46	36	24	26	29	24	19	17
I2	53	52	70		43	43	46	40	24	26	31	28	17	14
A10a	52	52	43	43		73	47	38	37	36	36	21	25	22
A10b	52	52	45	43	73		46	36	34	37	39	22	22	19
P	49	52	46	46	47	46		42	30	30	32	19	23	22
B	40	37	36	40	38	36	42		26	29	32	24	19	22
W	31	27	24	24	37	34	30	26		31	33	24	21	22
A11a	31	30	26	26	36	37	30	29	31		79	32	36	34
A11b	36	34	29	31	36	39	32	32	33	79		32	39	35
U	24	21	24	28	21	22	19	24	24	32	32		21	25
V1	19	20	19	17	25	22	23	19	21	36	39	21		56
V2	19	22	17	14	22	19	22	22	22	34	35	25	56	

Fig. 2. Percent identity matrix of rainbow trout S100 amino acid sequences computed from amino acid alignment.

although higher order oligomers are also known. They are involved in numerous cellular processes, with both intracellular and extracellular roles. Intracellularly, they are involved in calcium homeostasis and regulation of cell differentiation/proliferation [26,52]. Extracellularly, they are known to act as cytokine-like ligands and several are involved in sequestering zinc and copper [53].

There have been 21 S100 genes identified in the human genome, four of which are singletons while the remainder are clustered on chromosome 1. The large number of S100 genes in this cluster are members of the S100A subfamily, with the four singleton genes located elsewhere: S100B on chromosome 21, S100G on the X chromosome, S100P on chromosome 4, and S100Z on chromosome 5. Phylogenetic analysis has grouped these genes into four subgroups: A1/A10/A11/B/P/Z, A2/A3/A4/A5/A6, A13/A14/A16, and A7/A8/A9/A12/G [54,55]. Of these subgroups, the only one present in fishes is A1/A10/A11/B/P/Z, the ancestral subgroup that arose approximately 500 million years ago [1,54,55]. The most ancient of these are S100A1, B, and Z, as these are also present in cartilaginous fishes. In addition to the six genes shared between teleost fishes and higher vertebrates, an additional eight are presently found only in teleosts [1]. Kraemer et al. [1] adapted to the nomenclature of mammalian S100 genes, and named them S100I (previously characterized as ictacalcin), Q, R, S, T, U, V, and W. Because of strong negative selection following gene duplication events, only W was found to be in all the teleost genomes analyzed [1]. For instance, rainbow trout lack the S100Q, R, S, and T subfamilies, possess singles copies of A1, B, P, U, W, and Z, and possess paralogs of A10, A11, I, and V. In addition, rainbow trout S100V1, V2, and U are among the S100 genes with deletions in the S100-hand, others of which include human A10 (Fig. 1) [56]. Like human S100A10, these deletions may result in the protein always being in the active conformation. Depending on the species of fish, similar deletions have been found in S100A10 and B [1].

In this study, inclusion of 40% SBM in the diet of rainbow trout resulted in measurable increases in several S100 proteins in the distal intestine (S100I2, A10a, V1, V2, and W), no changes in the proximal intestine, and downregulation of S100V2 in the liver. The genes of highest expression in the three tissues examined were S100I2, A10a, and V1. Of these, S100I is the most well characterized to date. Porta et al. [5] confirmed the calcium-binding ability of S100I and detected it the epithelial tissue of the olfactory rosette, barbel, skin, and gill. Additional evidence for its function as a calcium-sensing protein was observed in green spotted puffer (*Dichotomyctere nigroviridis*), where its expression increased in response to reduced environmental calcium [57].

S100I is an abundant protein in the skin and mucus of Atlantic salmon and channel catfish [7,58]. The data reported by Easy and Ross [7] was the first evidence of presence of an extracellular S100 protein in a teleost. The protein they identified as ictacalcin is S100I2, and it was found to be present in mucus of both infected and noninfected fish. In channel catfish, S100I was the most abundant mRNA transcript detected by Karsi et al. [58], accounting for almost 5% of all transcripts. In addition to the skin, S100I expression is increased in the gill of Atlantic salmon infected with sea lice [7,59]. Conversely, Its expression was downregulated in the anterior kidney of channel catfish following vaccination with attenuated *Edwardsiella ictaluri* [60].

In addition to S100I, A10a and A10b were also significantly expressed and showed changes between treatments in the distal intestine. S100A10a is a highly specific marker of the developing intestinal tract in larval zebrafish while A10b mRNA was found to be expressed in every tissue they examined [1]. Bobe and Goetz [2] observed high expression of S100A10b in the skin, gills, heart, and ovary of brook trout, with lower expression observed in the stomach, intestine, and spleen.

The increase in S100A1, A10a, and A10b in carp kidney cells treated

Table 3
Comparative growth, feeding performance, and proximate composition (mean ± SD) of rainbow trout after twelve weeks on diets containing either 0 or 40% soybean meal.

	Initial Mass (g)	Final Mass (g)	Feed Intake (g/d)	FCR	PRE	ERE
Soy 0	88.2 ± 1.09	377.8 ± 4.9	1.72 ± 0.00	1.03 ± 0.00	37.7 ± 0.84	54.5 ± 0.90
Soy 40	89.2 ± 0.91	382.8 ± 14.2	1.71 ± 0.02	1.02 ± 0.02	38.0 ± 1.57	57.8 ± 2.82
	Moisture (%)	Fat (%)	Protein (%)	Energy (cal/g)	HSI	VSI
Soy 0	65.7 ± 0.66	15.0 ± 0.86	16.5 ± 0.32	2269.5 ± 45	1.20 ± 0.10	9.16 ± 0.75
Soy 40	66.5 ± 0.82	14.2 ± 1.05	17.0 ± 0.38	2339.5 ± 66	1.02 ± 0.02	8.18 ± 0.33

Feed conversion ratio (FCR); Protein retention efficiency (PRE); Energy retention efficiency (ERE); Hepatosomatic index (HSI); Viscerosomatic index (VSI).

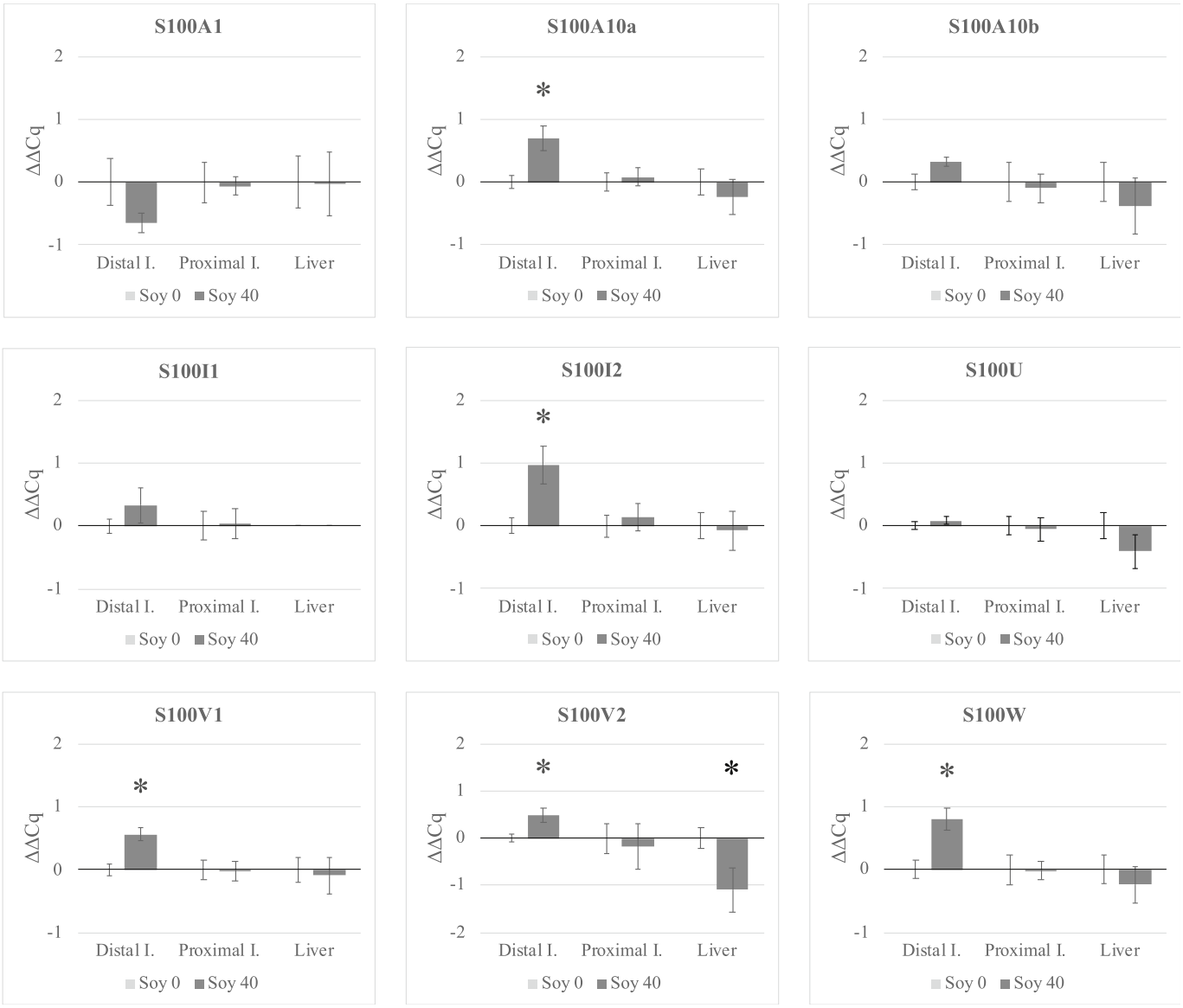

Fig. 3. ΔΔCq of S100 genes in distal intestine, proximal intestine, and liver. Asterisks mark significant differences ($p < 0.05$) between treatment groups. Error bars represent sample SEM.

Table 4
ΔCq of S100 genes for each tissue referenced to RPS15 (intestine) or GAPDH (liver). SEM represents pooled standard error.

Gene	Distal Intestine			Proximal Intestine			Liver		
	Soy 0	Soy 40	*SEM*	Soy 0	Soy 40	*SEM*	Soy 0	Soy 40	*SEM*
S100A1	5.70	6.35	*0.377*	7.49	7.55	*0.479*	6.80	6.83	*0.377*
S100A10a	−0.03	−0.73	*0.148*	−0.40	−0.47	*0.053*	−2.04	−1.80	*0.129*
S100A10b	2.44	2.12	*0.107*	3.13	3.23	*0.233*	0.75	1.13	*0.319*
S100I1	5.95	5.62	*0.182*	7.64	7.61	*0.189*			
S100I2	−0.11	−1.08	*0.204*	−0.90	−1.05	*0.143*	−1.98	−1.90	*0.156*
S100U	2.91	2.83	*0.028*	3.06	3.11	*0.098*	2.65	3.06	*0.120*
S100V1	1.25	0.68	*0.086*	1.28	1.30	*0.085*	0.65	0.74	*0.121*
S100V2	5.62	5.14	*0.096*	5.97	6.15	*0.380*	3.31	4.42	*0.288*
S100W	5.61	4.81	*0.162*	5.73	5.74	*0.146*	3.56	3.81	*0.142*

with IL-17AF2a may provide for a potential mechanism for the increase observed in this study [3]. Previous work in Atlantic salmon, as well as data from our own lab with rainbow trout, show that IL-17 expression is a component of the acute mucosal inflammatory response in the distal intestine of salmonids, as well as in other fishes [15,20,61]. Marjara et al. [15] observed a 200-fold increase in the two paralogs for interleukin-17A/F2 (IL-17A/F2) expression in Atlantic salmon fed a diet containing 20% SBM. Additional evidence for the importance of IL-17A/F2 was reported by Krogdahl et al. [20], reporting a > 30-fold increase in IL-17A/F2 expression in Atlantic salmon fed a FM diet containing 10 g/kg purified soy saponin. Rainbow trout IL-17A/F2 was recently characterized by Monte et al. [62] where highest constitutive

expression was observed in the intestine. IL-17A is recognized in higher vertebrates for its importance in inflammatory responses in mucosal tissue [63]. As the key cytokine expressed by type 17 helper T cells (Th17), the Th17 immune response is characterized by high numbers of neutrophils. The intrusion of eosinophilic/neutrophilic granulocytes is also a significant characteristic of soybean meal-induced enteritis [64]. Considering that rainbow trout are more tolerant to inclusion of SBM, Sealey et al. [65] reported no significant change in the expression of tumor necrosis factor α in rainbow trout fed diets containing 0, 10, or 20% SBM. However, inclusion of over 35% SBM is observed to produce detrimental histological changes in the distal intestine [12,66].

S100V1 showed higher levels of expression in each of the three tissues examined (Table 4) and was significantly upregulated in the distal intestine of fish fed the 40% soy diet. While S100V2 showed similar upregulation in the distal intestine, it also displayed a significant decrease in the liver. S100V1 and V2 are of additional interest for no longer possessing functional EF-hands, due to deletion of position 6 in the S100-hand and losses of conserved residues at positions 1 (Asp → Gly), 3 (Asn → Lys/Met), 5 (Asp → Gly), and 12 (Glu → Asn/His) in the canonical-hand [48,67]. Moreover, the degree of sequence divergence between the two paralogs (56% sequence identity) is greater than that of any of the other paralogs (Fig. 2). Further work on the expression and protein abundance of S100V will be necessary to better understand the significance of these changes.

S100 antibodies have been utilized in previous research in the intestines of fish for the investigation of mast cells and rodlet cells [9–11,68,69]. Rodlet cells were first recognized in the late 19th century, the cells are characterized by a fibrous coat and numerous dense inclusions, the appearance for which the cells are named. Their function is not completely understood, but current evidence suggests a role in host-defense, possessing anti-microbial and anti-helminthic functions with rodlets being released into the lumen of the intestine in response to infection [11,68,69]. They have been exclusively found in fish and have been described in numerous species, although the distribution varies from species to species and from individual to individual. Regarding salmonids, Reite [68] observed very few rodlet cells in the intestinal epithelia of farmed salmon and trout, while greater numbers were observed in wild fish, often concomitant with helminth infections. Vigliano et al. [10] hypothesized that S100 proteins play a role in the contractile release of rodlets.

In summary, we have examined the expression of S100 genes in the liver and intestine of rainbow trout and noted differences in expression between two dietary treatments containing either 0% or 40% SBM. Fish fed the 40% soy diet showed higher expression of S100A10a, I2, V1, V2, and W in the distal intestine and reduced expression of V2 in the liver. To our knowledge, there is no previously published information concerning S100V and W. The phylogenetic analysis by Kraemer et al. [1] groups S100V with A11 and U, while W is groups closest to the mammalian group of A2-6. We observed no changes in the expression of these genes in the proximal intestine, indicative of how it differs from the distal intestine in both its function and its higher tolerance to high SBM diets. S100I2 and A10a were the most highly expressed, and our data indicate that S100I remains an interesting target for further study. It should also be noted that post-translational modification and longevity of mature proteins within cells may often have the most consequential effects and gene expression *per se* may not altogether explain or correlate well with inflammation level [70]. That said, these gene expression data do provide essential information and a starting point for future studies where S100 proteins can be compared and evaluated.

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