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Fish Physiology and Biochemistry

Stress and expression of cyclooxygenases (cox1, cox2a, cox2b) and intestinal eicosanoids, in Atlantic salmon, Salmo salar.

--Manuscript Draft--

Manuscript Number:	FISH1165R3
Full Title:	Stress and expression of cyclooxygenases (cox1, cox2a, cox2b) and intestinal eicosanoids, in Atlantic salmon, Salmo salar.
Article Type:	Original research
Keywords:	eicosanoid cascade; midgut; hindgut; Liver; muscle; gill; spleen; kidney; prostaglandins; gene expression; prostacyclins, isoprostanes, lipoxins
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Manuscript Region of Origin:	
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Response to Reviewers:	We have checked the manuscript for spelling erros. For the notations of genes and proteins as suggested by the referee, we have doen the following, which is the current convention on labelling. We hope to have eliminated the errors by now. Changes are marked in red in teh document.

	COX1 large capital letters, denotes the protein in animals Cox1 first letter large capital followed by small letters, denotes protein in fish Cox1 (in italics) first letter capital, followed by small letters, denotes genes in animals cox (in italics) small letters, denotes genes in fish

Stress and expression of cyclooxygenases (*cox1*, *cox2a*, *cox2b*) and intestinal eicosanoids, in Atlantic salmon, *Salmo salar*.

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Keywords: eicosanoid cascade, midgut, hindgut, liver, muscle, gill, spleen, kidney, prostaglandin, gene expression, prostaglandins, prostacyclins, isoprostanes, lipoxins

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Abstract

Prostaglandin H synthetases (cyclooxygensases) catalyze the initial reactions leading to prostanoids in animals. They form interesting links between diet and fish physiology as the type and nature of eicosanoids are affected by dietary lipid sources. Their expression is likely to be affected by tissues and also altered environmental conditions leading to altered amount and ratio of eicosanoids. These mechanisms are however poorly understood in fish. In the present study, Atlantic salmon *Salmo salar* L. (1000g, 10°C, seawater) were subjected to acute chasing stress. Liver, kidney, spleen, gill, muscle, midgut and hindgut were extracted before and 1h post stress and analyzed for mRNA expression of *cox1*, *cox2a* and *cox2b*. Intestinal samples were furthermore sampled over 24h for both *cox* expression and eicosanoid content. Results show a highly variable but consecutively expression of *cox1*, *cox2a*, *cox2b* in most tissues analyzed. Low levels were only found for *cox2a* in liver and *cox2b* in liver and kidney. The study reveals the general trend that *cox1* is about 10 times the level of *cox2b* which again is about 10 times the level of *cox2a*. *Cox2b* shows the highest level of expression in the gills indicating a possible higher requirement for this protein in gills. Imposing stress to the fish induce a temporal increase in the expression of *cox2a* in the midgut while the gene expression of the other genes is not affected in any of the tissues analyzed. There is however a general tendency to increased expression of both *cox2* genes that merits further studies. Stress had a profound effect on the intestinal eicosanoid content which showed a general decrease in midgut sections after stress that persisted for at least 24h.

Introduction

In modern salmonid aquaculture, shortage in marine type oils force the feed industry to include increasing amounts of vegetable oils into salmonid diets. These are characterized by having a lower content of n-3 polyunsaturated fatty acids (PUFA), and a higher content of n-6 PUFA. The fatty acids are incorporated into cellular membranes in a ratio corresponding to their dietary content (Henderson and Tocher 1987), where they serve many central homeostatic and immunologic functions (de Pablo and de Cienfuegos 2000; Vance 2008; Lebman and Spiegel 2008). A significant part of these processes are mediated through the action of eicosanoids including prostaglandins (PG) and prostacyclins. They affect most aspects of life including vascular tone and permeability allowing blood plasma exudation and tissue oedema (Homaidan et al. 2002; Smith 2008). The main fatty acid substrates are arachidonic acid (20:4n-6, AA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (DHA, 22:6n-3). The synthesis of PG is initiated by the release of fatty acids from cellular membranes by phospholipase A_2 (PLA_2) followed by processing through several pathways. One main pathway is the lipoxygenase pathway (Rowley et al. 1995). Another main pathway is through the prostaglandin H synthase (or cyclooxygenases, Cox) cascade eventually leading to the release of PG's, prostacyclins and thromboxanes (Smith 2008).

In mammals there are two paralogous proteins of cyclooxygenase, COX1 and COX2. Although the products are similar, COX1 is generally assumed to be constitutively expressed performing homeostatic and maintenance functions, while COX2 is induced following pathophysiological triggers such as inflammation, neuronal degeneration, cancer or endogenous triggers such as growth factors, cytokines, endotoxin and neuronal depolarization. However, studies have indicated that this image is more complex, and both constitutively expressed *Cox2* and inducible *Cox1* genes have been described somewhat depending on tissue (Breder et al. 1995; Harris et al. 2001). They also seem to be differentially regulated, and have shown to have different function in knock-out mice, whereas COX1 seems to have housekeeping function while COX-2, accounts for the elevated production of prostaglandins (Dinchuk et al. 1995; Langenbach et al. 1995; Morham et al. 1995). In teleosts there are additional copies of *cox2*, probably due to genome duplication. In rainbow trout and zebrafish there are two *cox2* genes, which both display functional characteristics of *cox-2* (*cox2a/b*), and one *cox1* gene (Ishikawa et al. 2007).

The current change in salmonid farmed diets towards higher levels of n-6 PUFA has led to some concern that the eicosanoid cascades can change in fish affecting aspects like ion regulation and immunology. The reason is that the eicosanoids from n-6 PUFA are generally more potent than n-3 PUFA (Calder 2006). This can for example enhance severity of inflammatory responses to external stimuli like vaccination (Gil-Martens 2010) which has become a necessity in intensive aquaculture. There is also some information that stress may affect the eicosanoid cascade, with many similarities to inflammatory processes (Oxley et al. 2010). Increased knowledge of these mechanisms is essential for a future healthy aquaculture industry.

In a previous work, we observed that intestinal *cox2a* expression was increased 1h following stress, but not the eicosanoid content (Oxley et al. 2010). It was hypothesized that this could be due to a delayed increase in eicosanoid production, which could help understand the long term effects of increased permeability seen in salmon intestines up to 48h post an acute stressor (Olsen et al. 2005). It is also possible that other isoforms of the cox family could be involved in the stress response such as *cox1* and *cox2b* previously identified in other fish species (Ishikawa et al. 2007). The current study first aimed at identifying and monitoring the general gene expression of *cox1*, *cox2a* and *cox2b* in various tissues before and 1h post stress as this has not been done previously in Atlantic salmon. Next, we monitored the timeline response over 24h of midgut and hindgut gene expression of these isoforms post acute stress. To relate to eicosanoid production, we also assessed the main prostanoids and selected non-prostanoids using LC-MS, and the relationship compared between production and gene expression.

Materials and methods

Experimental animals, diets and stress

Twenty five immature Atlantic salmon postsmolts (1,000 ± 150 g) were hatched and bred using eggs obtained from AkvaGen A/S (Tingvoll, Norway) and distributed equally between 5 × 1.5m^2 indoor fibreglass tanks at Matre Research Station (Institute of Marine Research, Matredal, Norway). Tanks were supplied continuously with seawater and maintained at a constant temperature of 10.1°C (± 0.1°C) and O_2 saturation of >80%. Fish were kept under a natural lighting regime and fed to satiation twice a day using a standard commercial diet (EWOS Ltd, Norway) suitable for the size of fish. To ensure intestines were clear of digesta before commencing the stress experiment, fish were unfed for 48 hours.

Five control fish (t=0) were collected, anaesthetised in 0.4% (w/v) benzocaine and sacrificed by a sharp blow to the head. Immediately after sampling, the water level in the remaining tanks was lowered to 10 cm and the fish chased with a net for 15 min to represent acute stress. One hour post-stress (t = 1), 5 fish from the second tank were anaesthetised sacrificed for analysis. Sampling continued in subsequent tanks at 3, 8 and 24 hours. Immediately after anaesthetisation, blood was withdrawn from the caudal vein using heparinised syringes, centrifuged at 11,000 rpm for 1 min, and plasma stored at -80°C for analysis of cortisol. The intestine was then removed from each fish and the intestinal lumen washed with saline. Midgut and hindgut segments, liver, head kidney, spleen, gill, and white muscle were sampled for gene expressional studies, where a small piece of tissue (~5 mm^3) were flash frozen in liquid nitrogen. In order to assess the *cox* activity in intestinal cell layers, control fish intestinal enterocytes were collected from the intestinal sections with the aid of a glass slide, and, with the resulting muscular layer, frozen in liquid nitrogen. Larger intestinal samples (ca 1g) were also flash frozen in liquid nitrogen for subsequent analysis of intestinal eicosanoid production.

Cortisol analysis

Plasma cortisol was analysed by ELISA (RE52061 IBL-International, Hamburg, Germany).

Eicosanoid analysis

Frozen intestinal mucosa was homogenised and extracted for LCMS analysis as

previously described (Oxley et al. 2010) using PGB2-d4 as an internal standard. Extracts were subsequently purified by SPE (Masoodi and Nicolaou 2006) and evaporated to dryness under a gentle stream of nitrogen. Samples were resuspended in 25 µl of ethanol and analysed by tandem mass spectrometry coupled to liquid chromatography (LC/ESI-MS/MS). The LC- system was an Agilent 1200 Series (Agilent Technologies Inc., CA,USA) with binary pump, variable volume injector, and a thermostated auto sampler. HPLC separation was conducted at 20^{o}C using a gradient solvent mixture of two mobile phases. Mobile phase A was 10 med mer ammonium acetate (aq) with pH adjusted to 8.5 with ammonia solution. Mobile phase B was methanol. Ten µL of the sample was injected onto a Luna Phenyl-Hexyl column (3µm, 150 x 2 mm; Phenomenex Inc., USA) at a gradient of: B 0.1 min 0%; B 18 min 40-60%; B 1 min 60-100%; B 6 min 100%, 5 min B 0% at flow 0.25 mL/min. The mass spectrometer used was an Agilent 6410 Triple Quad LC/MS (Agilent Technologies Inc., CA, USA) equipped with an electrospray source. Source parameters included: gas temp 350^{o}C, gas flow 12 l/min, nebulizer 40 psi, capillary 4000 V. Multiple Reaction Monitoring (MRM) for data acquisition and negative ion detection was used (Table 1). MassHunter software (Agilent Technologies Inc., CA, USA) was used for HPLC system control, data aquistion and data processing.

Cyclooxygenase-1 and -2 gene expression

Total RNA was extracted from tissue samples using the FastRNA Pro Green Kit (Qbiogene) and DNase-treated according to manufacturer's instructions (Invitrogen). Quantity and quality of isolated RNA was assessed by NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only samples with a 260/280 nm absorbance ratio >1.8 were approved. First-strand cDNA was synthesised from 250 ng of total RNA using a Reverse Transcription Core Kit using random hexamers according to manufacturer's instructions (Eurogenetec, Seraing, Belgium). All quantitative real-time PCR (qPCR) reactions were run on a 7900 HT Fast Real-Time PCR system using taqman mastermix (Applied Biosystems). Each reaction (25 µl) contained 2 µl of cDNA diluted 1:5 in double-distilled H_20, 12.5 µl of taqman PCR master mix and 0.9µM F/R primers. qPCR reactions conditions were: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer and probe sequences for *cox-1*, *cox-2a*, *cox2b* designed using Primer express (Applied Biosystems) and are shown in Table 2. *ef1α* was used as a reference gene (Olsvik et al. 2005). Reaction efficiencies of target and reference

genes were validated by a log standard curve dilution of input RNA used for cDNA synthesis (500, 250, 125, 62.5ng). In the validation experiment 500, 250, 125, 62.5ng of RNA was used for cDNA synthesis, and the slope of log input amount of RNA versus delta Ct was for *cox1*/ *ef1α* = 0.064 , *cox2a*/ *ef1α*= 0.054 and *cox2b*/*ef1α* = -0.0011 which is < 0.1, which show that there was comparable efficiencies between target and normalization gene. All samples and standard curves were run in triplicate reactions along with non-template (ntc) and reverse-transcription (-RT) controls. Relative expression levels were calculated using the Comparative Ct method (ABI User Bulletin #2 for ABI 7700 sequence detection system).

Statistical analysis

All gene expression data was subjected to Kolmogorov-Smirnov test for Gaussian distribution. If there was variability in Gaussian distribution, the data were subjected to an unpaired *t*-test with Welch's correction when comparing expression levels. Otherwise data was subjected to one-way ANOVA with Bonferroni's *post-hoc* test. Data analyses were performed using GraphPad Prism 5.0 (La Jolla, CA 92037, USA). A $P<0.05$ value was taken to indicate statistical significance. Eicosanoid content was assessed using SPSS ver 11 software using standard GLM procedures followed by Tukey's post hoc test.

Results

Plasma cortisol

Plasma cortisol increased from 2 ng l^{-1} before stress to an average of 118 ng l^{-1} one hour post stress (Fig 1). However, the effect was transient, and by 3h, the level had reached 6 ng l^{-1} that was indistinguishable from pre-stress levels. All other measurements up to 24 h were on the baseline.

Gene expression in different tissues

Gene expression of *cox1* was at a similar level in muscle, midgut and hindgut, while the expression was significantly lower in liver compared to all other tissues measured (Fig. 2). Kidney and spleen displayed the highest expression levels of *cox1* while gills displayed significantly lower expression while significantly higher than midgut, hindgut and muscle. Expression of *cox2a* showed a 1-100 times lower expression (depending on tissue) in comparison to *cox1*. There was also a major diversity in expressional level between tissues (Fig. 2). Gills and muscle expressed the highest level of *cox2a*. For the other tissues, expression was lower, and not detected in kidney and liver (Fig. 2B). *Cox2b* was significantly higher expressed in most tissues compared to *cox2a*. Whereas the expression was detected to be highest in the gill and at a similar lower level in muscle, midgut and hindgut, while the expression was subsequently lower in spleen and kidney and not detected in the liver. Previous studies have reported expressional differences between midgut and hindgut for *cox2a* (Oxley et al. 2010), we could not detect any differences in expression of the *cox* genes between hindgut and midgut. However in our preparation we had used the whole intestine including muscle and enterocytes. To clarify if the differet observations could be due to the presence of muscle tissue in the preparation we micro dissected enterocytes and muscle from midgut and hindgut. Gene expression analysis of intestinal mucosa (enterocytes) and intestinal muscle tissue showed that *cox-1* was evenly distributed between the two compartments (Fig 3). But for both *cox2a* and *cox2b*, midgut enterocytes mRNA level was significantly lower compared to midgut intestinal muscle while no differences were detected in the hindgut (Fig 3)

Gene expression in response to acute stress

For *cox1*, there was not detected any response to acute stress in any of the tissues analyzed (0h-1h, Fig. 4A). Gene expression was analyzed in all tissues at 2 time points (0h-1h) and

at 5 time points for the gut (0h, 1h, 3h, 8h and 24h) after stress. For *cox2a* the midgut responded to acute stress by upregulation of expression, one hour after stress exposure and returned to baseline expression 3 hours after stress exposure ($p<0.05$, Fig. 4B). *Cox2b* did not significantly respond to stress in any of the tissues analyzed (Fig. 4C).

Eicosanoid content in intestine

Resolvins E_1, D_1, Leucotriene (LT) C_4 and LTB_4 were below the detection limit of the current setup, and could not be quantified. Of the remaining eicosanoids, it was a clear trend that those originating from AA dominated over those originating from EPA (Fig. 5). For example PGE_2 was by far the most predominant prostaglandin constituting 816 and 1053 ng g^{-1} in midgut and hindgut segments of unstressed fish respectively (Fig 3A), while PGE_3 was found at 235 and 186 ng g^{-1} respectively (Fig. 5B). However, it was still the second most predominant eicosanoid. Likewise, $PGF_{2\alpha}$ was found at 30.7 and 27.6 ng g^{-1} (Fig. 5C), compared to 14.9 and 9.8 ng g^{-1} for $PGF_{3\alpha}$ in midgut and hindgut segments respectively (Fig 5D). However, in the latter case midgut contained almost twice the level as hindgut before stress. PGD_2 was highest in the midgut reaching 108.2 ng g^{-1} compared to 61.1 ng g^{-1} in hindgut (Fig 5E). For the stable products of PGI, 6-keto-$PGF_{1\alpha}$ (Fig. 5F; stable product of PGI_2), hindgut contained 77.3 ng g^{-1} compared to only 33.1 ng g^{-1} in midgut. For the EPA derived counterpart d17-6-keto-$PGF_{1\alpha}$ (Fig. 5G; stable product of PGI_3), the tissue distribution was fairly similar, and the tissue content ranging between 10.6 and 13.9 ng g^{-1}. Lipoxin A_4 was analyzed as a representative of the lipoxygenase pathway, with an intermediate concentration, 43.3 and 30.8 ng g^{-1} in midgut and hindgut segments respectively (Fig. 5H). Finally, the isoprostane representatives' 8-iso-15-keto-$PGF_{2\alpha}$ (Fig 5I) and 8-iso-15- keto-$PGF_{2\alpha}$ (Fig 5J) were only found in minute amounts in both segments. The former appeared to be more prevalent in midgut than hindgut while no difference was observed for the latter. Subjecting the fish to acute stress had a striking effect on the eicosanoid content in midgut, but only marginally in hindgut. For the major eicosanoids PFE_2, PGE_3, PGD_2, $PGF_{2\alpha}$, $PGF_{3\alpha}$, stress caused a significant reduction in the midgut, either immediately following stress, or sometime after 1h (Figure 5). The reductions were generally in the range of 50%. These reductions were also rather persistent, and in most cases, there was no recovery even at the last sampling 24h post stress. The only deviation was found in 6-keto-$PGF_{1\alpha}$ and d17-6-keto- $PGF_{1\alpha}$ respectively where the concentration increased markedly 1h post stress (Figure 6). However, the increase was transient and the level then either returned to baseline level after 3h (6-keto-

$PGF_{1\alpha}$), or was reduced (d17-6-keto-$PGF_{1\alpha}$) like most of the other eicosanoids. Both isoprostanes (8-iso-$PGF_{2\alpha}$ and 8-iso-15-keto-$PGF_{2\alpha}$) examined were also reduced in the midgut following stress while Lipoxin A_4 was unaffected until 24 h post stress where the level was significantly increased.

Hindgut was on the other hand relatively unaffected by stress, and no significant tendency was found for PGE_2, PGE_3, PGD_2 or $PGF_{2\alpha}$. $PGF_{3\alpha}$ did however show a transient increase at 1 and 3h post stress before returning to baseline levels. The only obvious trend was that for PGI derivates that were reduced over time. The isoprostane 8-iso-15-keto-$PGF_{2\alpha}$ also showed a transient increase at 1 and 3h post stress, and was then reduced to below detection level at 24h. This did not occur for 8-iso-$PGF_{2\alpha}$ where the level was unaffected by stress.

Discussion

In this study we identified and monitored expression of two genes previously not studied in Atlantic salmon, *cox1* and *cox2b*, before and after acute stress. We have documented a significant expressional disparity between the genes *cox1*, *cox2a* and *cox2b* in tissues monitored. C*ox2a* clearly responds to stress in the intestine, and although no significant reponse to stress was found in any other tissues for *cox1* and *cox2b*. The main eicosanoids in the intestines originated from arachidonic acid followed those from eicosapenatenoic acid. The main eicosanoids were PGE, PGF, PGD and PGI. Other pathway products were lipoxin, and isoprostanes. Resolvins E and D, and leucotriens C and B were below detection limits. The general response to stress was a general decrease in midgut sections after stress that persisted for at least 24h. This decrease in eicosanoid content could be an attempt by the animal to prevent increased intestinal permeability.

Mammalian species contain two cox genes, *Cox-1* and *Cox-2*, and these genes have also been found in many fish species including zebrafish (Ishikawa et al. 2007) and rainbow trout (Ishikawa and Herschman 2007). In addition, both fish species have also been shown to possess two isoforms of *cox-2*, termed *cox-2a* and *cox-2b*. The current work verifies the existence of *cox-1*, *cox-2a* and *cox-2b* in Atlantic salmon. Sequence analysis reveals that the salmon *cox2a* sequence shows 96% and 76% similarity to the rainbow trout *cox2a* and *cox2b*. In addition searching through the salmon genome we identified a *cox2b* homologue in salmon (97% similarity to rainbow trout *cox2b*) clearly indicating that there are at least two variants of the *cox2* genes in Atlantic salmon. It is however possible due to the tetraploidy of salmon that more paralogs are present. In zebrafish (Ishikawa et al. 2007) the cox genes; *cox1*, *cox2a* and *cox2b* displays a similar pattern for liver, kidney and gut as we detected in the salmon. These results might indicate that the cox genes have some functional conservation for these tissues. However what is novel in this study, is the lower expression level of *cox2a* isoform compared to *cox2b* and the differential tissue expression of the two genes.

Interestingly, both *cox-1* and *cox2b* were found at a high level in the gills. The relatively higher level of *cox2a* (previous COX2) in gills than other tissues also agrees with previous data in Atlantic salmon (Ingerslev et al. 2006). However, in the present study, the level of *cox2b* was tenfold higher than *cox2a*. In view of the notably higher expression than other tissues, these data suggests a special role of *cox2b* in salmonids gills. Interestingly, this differential expression of isoforms was not found in zebrafish,

although *cox2a* appeared to have a higher expression level in gills than *cox2b* (Ishikawa et al. 2007).

As for gills, intestinal homeostasis is highly regulated by eicosanoids and their receptors. They are involved in cell growth, barrier functions, ion balance and general immunology (Wang et al. 2005, Ferrer and Moreno 2010). The constitutively and even expression of *cox-1* in both anterior and posterior part of the intestine, and also mucosa and deeper cellular tissue supports the function as a homeostatic gene. For *cox2a* and in particular *cox2b* however, we observed a very low expression in anterior enterocytes compared to posterior enterocytes. This agrees previous notions in Atlantic salmon *cox2a* (Oxley et al. 2010), and may be linked to, in part, ion regulation and water absorption. In rats, PGE_2 will reduce water and chloride absorption (Hodeify and Kreydiyyeh 2007). How there mechanisms are regulated in fish remains to be elucidated.

Stress is a complex and conserved and in part catecholamine driven mechanism (Wendelaar Bonga 1997) that in many ways resemble inflammatory processes. This initial fast response is followed by a slower and more persistent increase in circulating glucocorticoids. In salmon, cortisol peaks after around 1h before returning to baseline levels some hours later (Olsen et al. 2002). One main function of glucocorticoids is their ability to contain the inflammatory process, and they are still one of the most powerful anti-inflammatory agents used. In mammalian immune cells, they inhibit the production of pro-inflammatory mediators like IL-1, IL-2 and TNF, mostly through glucocorticoid receptors (Russo-Marie 2004). They also inhibit eicosanoid synthesis by inhibiting COX2 and $cPLA_2$ and to a lesser extent COX1 (Masferrer and Seibert 1994; Russo-Marie 2004). This down-regulation of eicosanoid synthesis is however not global however, and several studies have shown that glucocorticoids will increase eicosanoid synthesis in many cells including amnion cells (Zakar et al. 1995). In the present study, *cox1* appeared unaffected by stress which was expected due to its presumed constitutive function performing homeostatic and maintenance processes. Furthermore, stress significantly increased gill *cox2a* in midintestine. This agrees with previous notions on stress induced *cox2a* upregulation in rainbow trout liver (Wiseman et al. 2007). However the *cox2b* did not respond to stress in any tissue measured and *cox2a* only responded in the midgut. These results implies that at least not *cox2a* is involved in any acute stress response in Atlantic salmon, while *cox2a* is only in the midgut. However, the time window for a stress reponse could affect the result, and a gene expressional reponse to stress in either/or

cox2a or *b* have thereby not been measured. Further studies at other time points may clarify this issue. In addition, the relative differential expressional response between *cox2a/b* could also be a subfunctionalization of the gene product. Alternatively, it is possible that the tetraploid salmon genome might contain additional *cox2* genes which may respond differentially both in gill intestine and other organs.

Regardless of type of eicosanoid analyzed, it was evident that PGE's were the main eicosanoids in salmon intestines. The clear preference of production of PGE_2 over PGE_3, and a general preference of n-6 eicosanoids over their n-3 counterparts (eg $PGF_{2\alpha}$ and $PGF_{3\alpha}$, and also the stable nonenzymatic hydrolytic products of PGI_2 and PGI_3, 6-keto-$PGF_{1\alpha}$ and d17-6- keto-$PGF_{1\alpha}$ respectively) clearly indicates the importance of these metabolites despite AA by far has the lowest concentration in cellular membranes compared to EPA and DHA. This further suggests the possible role of phosphatidylinositol phospholipids, being enriched in AA as an important contributor of AA for eicosanoid production, although definitive data are still not available (Tocher 2003). It is also likely that other mechanisms in the eicosanoid signaling pathway have evolved discriminating in favor of AA. In brook trout for example recombinant *cox1* and *cox2* clearly had a clear discrimination towards AA and against EPA and DHA as substrate (Liu et al. 2006). Similar discriminations in other enzymes as phospholipase A_2 mobilizing precursors could be expected as well.

In a previous study and in this study, we observed that subjecting Atlantic salmon to acute stress did cause an up regulation of *cox2a* mRNA level in midgut and hindgut 1h post stress without having any impact on the actual eicosanoid production (Oxley et al. 2010). However, up-regulation has also been noted after 24 in rainbow trout liver (Wisman et al. 2007), and could lead to long lasting post-stress effects in the intestines. For example, impairment of intestinal barrier functions is a known consequence of PGE_2 up-regulation in mammalian tissue (Ferrer and Moreno 2010). Interestingly, long term impairment (up to 48h) of intestinal barrier functions has been observed in salmonids following stress (Olsen et al. 2002; Olsen et al. 2005). Whether these mechanisms are linked to altered eicosanoid production, remains to be elicudated. Although our data do agree with previous reports on an immediate up regulation of *cox2a* mRNA in salmon intestine (Oxley et al. 2010) the level returned to baseline levels within 3h suggesting that this is not a percistent response. We also observed a general reduction in intestinal prostaniod content implies a close-down of synthesis. It is possible that this is an attempt to reduce the increased intestinal permeability (Olsen et al. 2005). However reduced

prostanoid content could also indicate that the intestinal mucosa diplays lower proliferation/differentiation which might lead to less efficent nutrient uptake as a resonse to stress. Although the experiment ended after 24 h eicosanoid levels had not been re-established by that time indicating a long-term effect of the stressor. In addition to eicosanoids, we also included some other components in our analysis. Lipoxin A_4 was included as a representative on the lipoxin pathway. It appeared to be less regulated, except for a massive and yet unexplained increase in midgut after 24h. Furthermore, as stress may increase the peroxidative load of intestines, we also included two isoprostanes in the analysis. However, their level was very low, and fairly close to detection limits. If oxidative stress is viewed through these compounds, stress does not seem to cause any major oxidative stress.

To conclude this study reports the expression of *cox1*, *cox2a*, *cox2b* in a number of adult tissues in Atlantic salmon. The study reveals that *cox1* is about 10 times the magnitude of *cox2b* which again is about 10 times the magnitude of *cox2a*. *Cox2b* also shows the highest level of expression in the gills indicating that it might be involved in some functional aspect of the gill. There is a possibility that this high expression could be related to gill function since *cox2* expression has been shown to be positively correlated to salinity in tilapia (Tine et al 2011), however this assumption remains to be elucidated. Imposing stress to the fish induced a temporal increase in the expression of *cox2a* in the midgut while the gene expression of the other genes was not affected in any of the tissues analyzed. The differential reponse in between tissues could either be explained by a subfunctionalization of this gene in the intestine, another time-window for reponse or a specific stress response mechanism in the intestine. Also, stress had a profound effect on the intestinal eicosanoid content which showed a general decrease in midgut sections after stress and percisted for at least 24h.

Acknowledgements

The project was supported by the WEALTH project (Welfare and health in sustainable aquaculture, EU FP6 project no: No 501984). The authors are also indebted to Karen Anita Kvestad, IMR, Matre, and Laila Unneland, IMR, Bergen, for excellent technical assistance and Dr. Anthony Oxley for assisting in the setup and sampling process.

Table 1. Multiple reactions monitoring (MRM) transitions of LC/ESI-MS/MS analysis of selected eicosanoids.

Compound	Ret time, minutes	MRM (m/z) quantifier	MRM (m/z) qualifier	Fragmentor (V)	Collision energy (V)
6-keto $PGF_{1\alpha}$	10.5	369 → 163	369 →245	80	25
Resolvin E_1	11.2	349 → 195	349 → 161	140	15
PGE_3	12.0	349 → 269	349 → 313	110	10
$PGF_{3\alpha}$	12.8	351 → 307	351 → 193	140	19
8-*iso*-$PGF_{2\alpha}$	13.2	353 → 193	353 → 247	150	25
8-*iso*-15-keto $PGF_{2\alpha}$	13.4	351 → 315	351 → 289	120	10
PGE_2	14.4	351 → 271	351 → 315	110	12
$PGF_{2\alpha}$	14.7	353 → 193	353 → 309	160	25
Δ^{17}-6-keto-$PGF_{1\alpha}$	15.1	395 → 185	395 → 83	130	29
PGD_2	15.2	351 → 271	351 → 315	110	12
LipoxinA_4	17.8	351 → 115	351 → 217	120	13
PGB2*d*4	19.2	337 → 179		110	18
Resolvin D_1	18.1	375 → 215	375 → 141	120	13
LTC_4	18.2	624 → 272	624 → 606	135	20
LTB_4	22.3	335 → 195	335 → 317	135	10

Table 2. Primer and probe sequences used in the present study.

Gene	Acc.No	Primer F	Primer R	Probe
cox-1	BT045745	**5'CTGGTGAGGAGGAGATAGCC3'**	**5'GTAGAACTCCAGCGCATCAA3'**	**6-FAM-CACCATAGAGCTCCTCCAGCTCCC-TAMRA**
cox-2a	AY848944 **(Ingerslev et al. 2006)**	**5'ATTGCCAAAGGACTGCCCTA3**	**5'TGAGGATCCGGAATAAACCG3'**	**6-FAM-GAAGAGCAGTGCTCCCAGAT-TAMRA**
cox-2b	A combination of two exons in: gnl|ti|2271399401 gnl|ti|2271399401	**5'GGTGCTGGATGGAGAGGTGTAC 3'**	**5'GGCCGAACGCCTCATG3'**	**6-FAM-CACTACCCTCCCCATGTCCCTGAGTCTC-TAMRA**
ef1-α	AF321836 (Olsvik et al. 2005)	**5'CCCCTCCAGGACGTTTACAAA 3'**	**5' CACACGGCCCACAGGTACA 3'**	**6-FAM-ATCGGTGGTATTGGA-MGB**

Figure legends

Fig. 1. Plasma cortisol levels in Atlantic salmon subjected to acute stress.

Fig. 2. The graph is showing relative gene expression of *cox1*, *cox2a* and *cox2b* in selected tissues of Atlantic salmon. On the Y-axis the used tissues are presented. On the X-axis the relative abundance of the transcripts are shown in relation to the normalization factor *elongation factor 1α*. The letters a, b, c, d, e and f demarcates significant differences in gene expression between tissues and genes ($p<0.05$). Data is presented +/- S.E.M.

Fig. 3. The graph is showing relative gene expression of *cox1*, *cox2a* and *cox2b* in midgut and hindgut muscle and enterocytes respectively. On the Y-axis the used tissues are presented. On the X-axis the relative abundance of the transcripts are shown in relation to the normalization factor *elongation factor 1α*. The letters a, b, c and d demarcates significant differences in gene expression between tissues and genes ($p<0.05$). Data is presented +/- S.E.M.

Fig. 4. The graphs are showing relative gene expression of *cox1* (A), *cox2a* (B) and cox2b (C) in response to acute stress in selected tissues of an Atlantic salmon. Above the X-axis the tissues assayed are presented. On the X-axis time after acute stress are presented (hours). On the Y-axis the relative abundance of the transcripts are shown in relation to the normalization factor *elongation factor 1α*. The letters a and b demarcates significant differences in gene expression ($p<0.05$). Data is presented +/- S.E.M.

Fig 5. Content of eicosanoids in midgut and hindgut sections (ng g-1 wet weight) in Atlantic salmon before (t=0), and 1, 3, 8 and 24h post stress. A: PGE_2, B: PGE_3, C: $PGF_{2\alpha}$, D: $PGF_{3\alpha}$, E: PGD_2, F: 6-keto-$PGF_{1\alpha}$, G: d17-6-keto-$PGF_{1\alpha}$, H: lipoxin A4 (lipoxidase pathway), I: 8-iso- $PGF_{2\alpha}$ (isoprostane), J: 8-iso-15-keto-$PGF_{2\alpha}$ (isoprostane).

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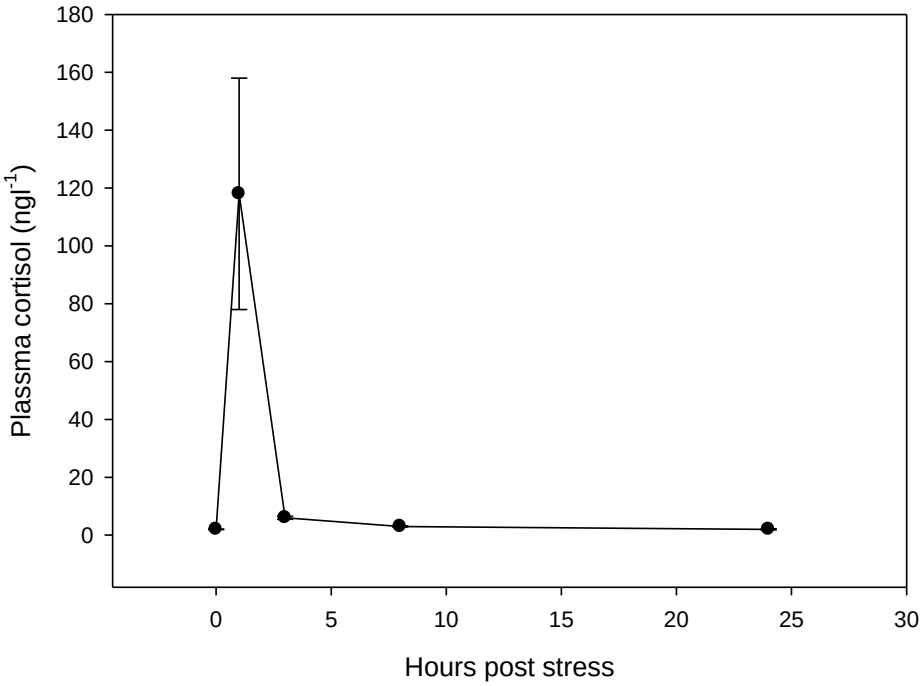

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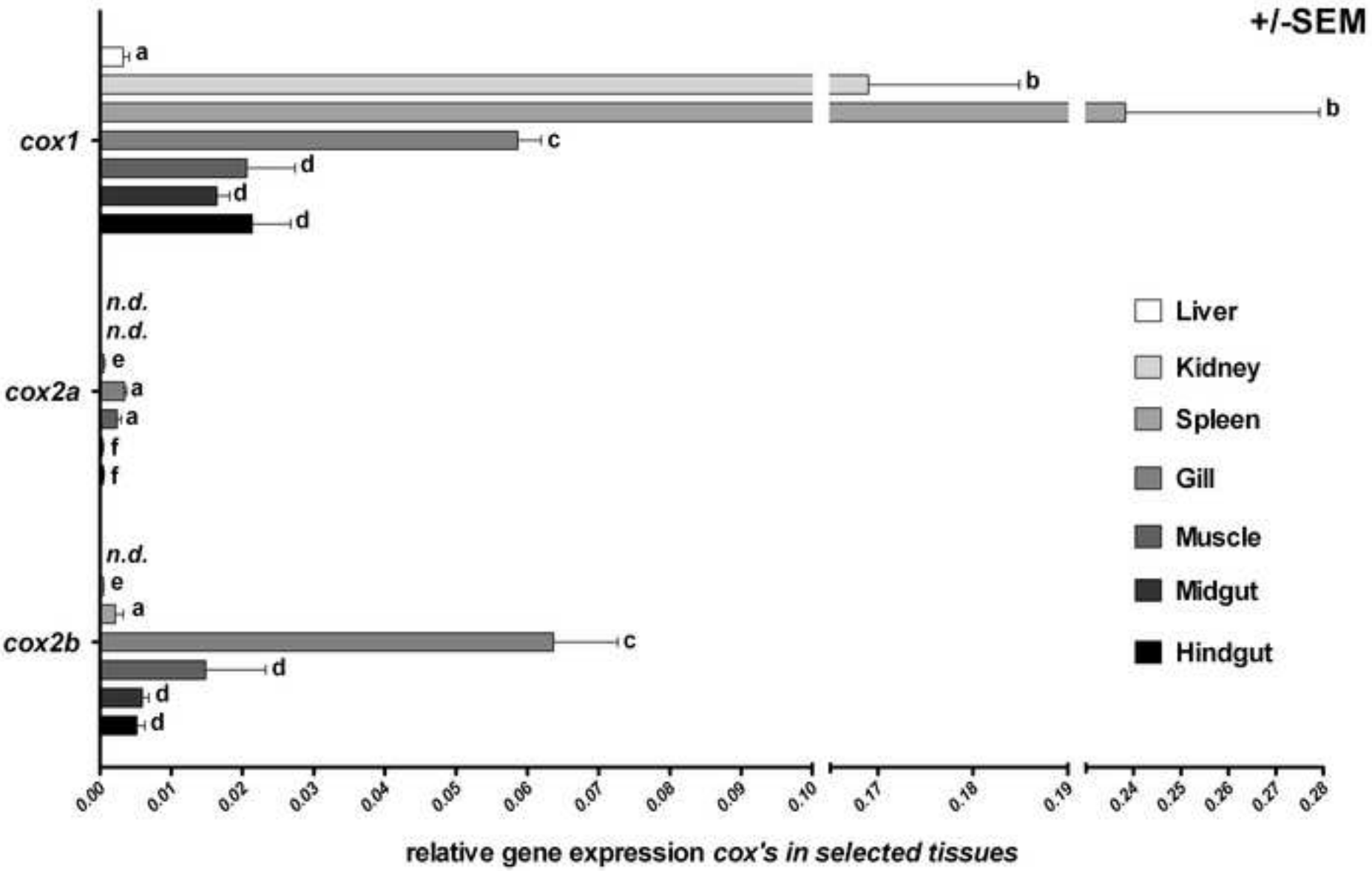

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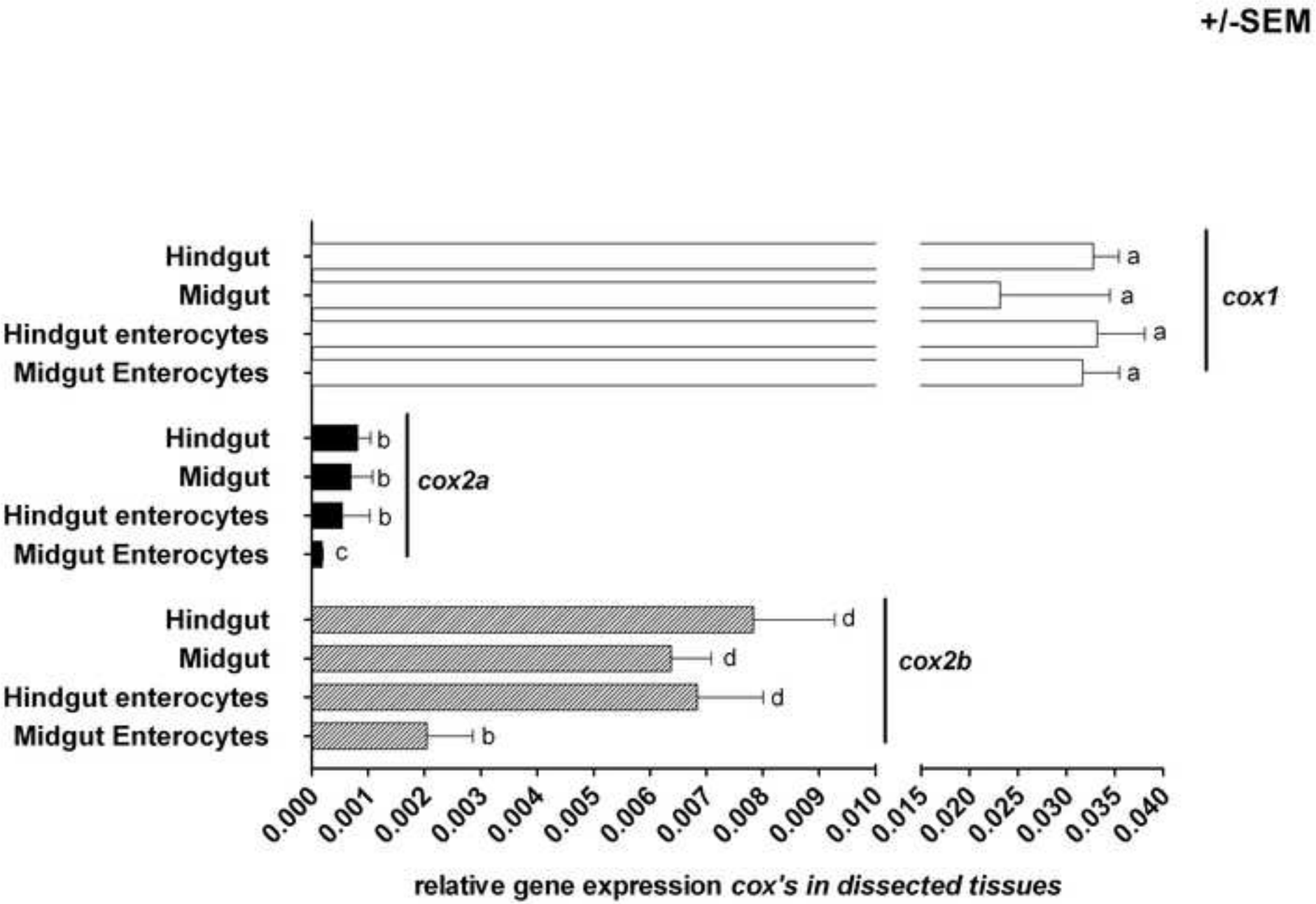

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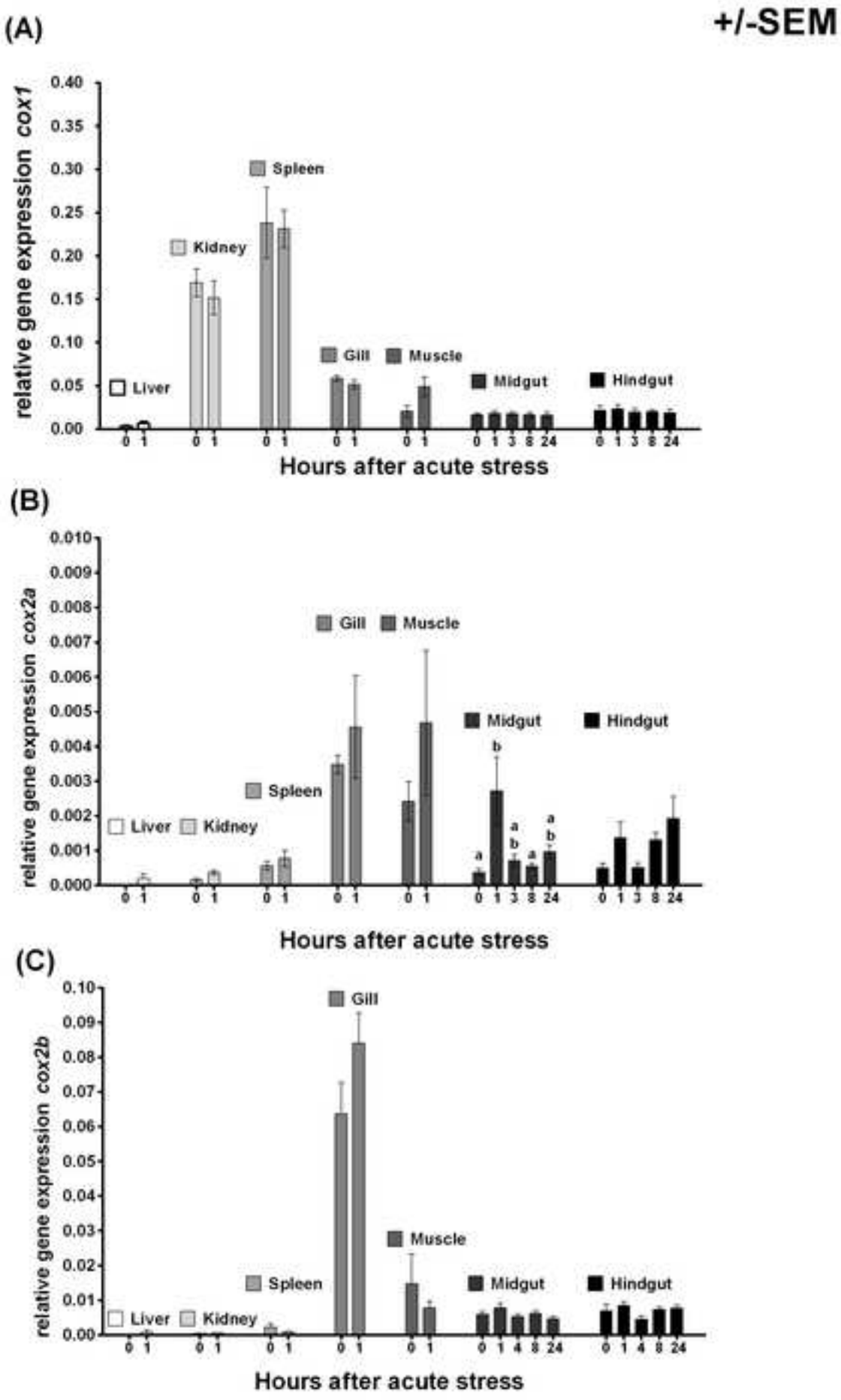

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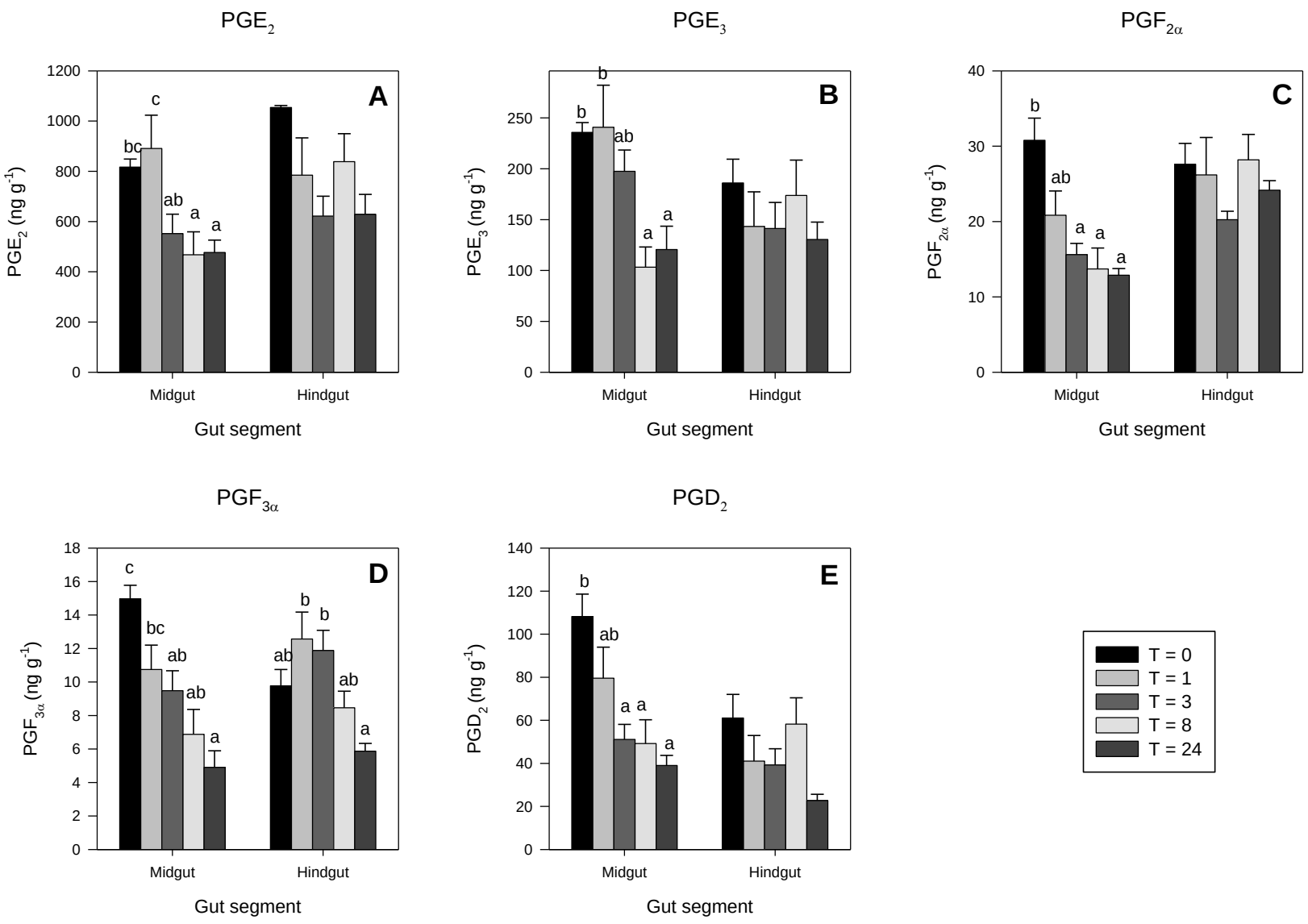

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