Subfunctionalization of peroxisome proliferator response elements accounts for retention of duplicated fabp1 genes in zebrafish

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Abstract

Background: In the duplication-degeneration-complementation (DDC) model, a duplicated gene has three possible fates: it may lose functionality through the accumulation of mutations (nonfunctionalization), acquire a new function (neofunctionalization), or each duplicate gene may retain a subset of functions of the ancestral gene (subfunctionalization). The role that promoter evolution plays in retention of duplicated genes in eukaryotic genomes is not well understood. Fatty acid-binding proteins (Fabp) belong to a multigene family that are highly conserved in sequence and function, but differ in their gene regulation, suggesting selective pressure is exerted via regulatory elements in the promoter.

Results: In this study, we describe the PPAR regulation of zebrafish fabp1a, fabp1b.1, and fabp1b.2 promoters and compare them to the PPAR regulation of the spotted gar fabp1 promoter, representative of the ancestral fabp1 gene. Evolution of the fabp1 promoter was inferred by sequence analysis, and differential PPAR-agonist activation of fabp1 promoter activity in zebrafish liver and intestine explant cells, and in HEK293A cells transiently transfected with wild-type and mutated fabp1 promoter-reporter gene constructs. The promoter activity of spotted gar fabp1, representative of the ancestral fabp1, was induced by both PPARα- and PPARγ-specific agonists, but displayed a biphasic response to PPARα activation. Zebrafish fabp1a was PPARα-selective, fabp1b.1 was PPARγ-selective, and fabp1b.2 was not regulated by PPAR.

Conclusions: The zebrafish fabp1 promoters underwent two successive rounds of subfunctionalization with respect to PPAR regulation leading to retention of three zebrafish fabp1 genes with stimuli-specific regulation. Using a pharmacological approach, we demonstrated here the divergent regulation of the zebrafish fabp1a, fabp1b.1, and fabp1b.2 with regard to subfunctionalization of PPAR regulation following two rounds of gene duplication.

Keywords: Peroxisome proliferator activated receptor (PPAR), Dual luciferase assay, Fatty acid-binding protein, Subfunctionalization, Neofunctionalization, Nonfunctionalization, Teleost fishes, Gene promoter evolution, Zebrafish, Spotted gar
**Background**

Gene duplication is thought to facilitate increasing organismal complexity, but evolution does not accommodate redundancy. Duplication of genes can occur by unequal crossing-over during meiosis, replication slippage, retrotransposition, aneuploidy, or whole genome duplication [1]. The common fate of duplicated genes is loss of one copy owing to accumulated mutation and functional decay (non-functionalization) [2, 3]. Alternatively, both copies of a duplicated gene may be retained if one of the duplicates acquires a novel function (neofunctionalization), or the functions of the ancestral gene are subdivided between the duplicates (subfunctionalization) [2–4]. Non-, neo-, and sub-functionalization represent three possible fates of duplicated genes as described in the duplication degeneration complementation (DDC) model [2]. Both mutation of protein coding regions and the loss or gain of cis-acting regulatory elements in the promoters of duplicated genes may account for altered function of duplicated genes. Mutations in regulatory elements of promoters may affect tissue-, developmental stage- and stimulus-dependent transcript levels of duplicated genes [2–5].

Fatty acid binding proteins (Fabp), which belong to the multigene family of intracellular lipid-binding proteins, function as carriers of fatty acids, eicosanoids and other hydrophobic ligands to effectors in the cytosol and nucleus [6]. Previously, we observed that the promoters of the tandemly duplicated fapb genes of zebrafish, fapb1b.1 and fapb1b.2, differ in their regulation by peroxisome proliferator activated receptors (PPARs), where fapb1b.1 promoter activity was induced by PPAR, but fapb1b.2 promoter activity was not induced by PPAR [7]. The zebrafish fapb1a and fapb1b genes were generated by duplication of ancestral fapb1 gene owing to a whole genome duplication (WGD) event that occurred in the ray-finned teleost lineage approximately 325 mya [8–11]. Subsequently, the zebrafish fapb1b.1 and fapb1b.2 genes arose by tandem duplication of fapb1b, most likely by misaligned cross-over of homologous chromosomes during meiosis [12–14]. The zebrafish fapb1b.1 and fapb1b.2 genes are the only tandem duplicates of the multigene family of intracellular lipid-binding protein genes identified, thus far, in teleost fishes [12]. As a result, the zebrafish genome contains three extant fapb1 genes, fapb1a, fapb1b.1, and fapb1b.2. Spotted gar (Lepisosteus oculatus, order Lepisosteiformes) belongs to an order of teleost fishes that did not undergo a WGD, therefore, its genome contains a single copy of the fapb1 gene.

Zebrafish fapb1b.1 and fapb1b.2 differ in their responsiveness to dietary fatty acids: fapb1b.1 mRNA levels are increased in the intestine of linolenic acid-fed zebrafish, whereas fapb1b.2 mRNA levels are unaffected by linolenic acid [14]. Zebrafish fapb1a, fapb1b.1, and fapb1b.2 also differ in their responsiveness to the non-selective PPAR agonist, clofibrate [15]. fapb1a mRNA levels are increased in the liver of clofibrate-fed zebrafish, fapb1b.1 mRNA levels are increased in the heart of clofibrate-fed zebrafish, while fapb1b.2 mRNA levels are unaffected by clofibrate [15]. These findings implicate the PPARs in the differential regulation of the fapb1a, fapb1b.1 and fapb1b.2 genes in zebrafish [14, 15].

PPARs are nuclear receptor transcription factors that bind, and are activated by, free fatty acids and eicosanoids [16–18]. Upon activation, PPARs heterodimerize with the retinoid X receptor (RXR) and bind to a PPAR response element (PPRE) located in the promoters of many vertebrate genes, including fapb genes [16–18]. The consensus sequence for the vertebrate PPRE is defined as 5’-CAAAAAACAGGTCAANAGGTTCA-3’ [16–18]. Binding of the PPAR to a PPRE may cause increased or decreased gene expression, depending on the gene [16–18]. Three PPAR isoforms have been identified across vertebrate species: PPARα, PPARγ, and PPARβ/δ [16–18]. While PPARα and PPARγ are expressed in many vertebrate tissues, PPARβ/δ expression is limited to the skin, adipose, and brain [16–18]. A PPRE may be PPAR isoform-selective (i.e., a PPRE that preferentially binds PPARα relative to PPARγ) [16, 17]. A PPRE with high sequence identity in the 5’ flanking region (5’-FR) (underlined: 5’-CAAAAAACAGGTCAANAGGTTCA-3’) to the consensus PPRE exhibits greater activation of transcription at promoters by the isoform PPARα compared to the isoform PPARγ, whereas PPARγ binding is less-dependent on the 5’ FR than PPARα [16–18]. Both PPARα and PPARγ bind to the direct repeat element (DRI) (underlined 5’-CAAAAAACAGGTCAANAGGTTCA-3’) of the PPRE to activate transcription [16–18]. A PPRE with low sequence identity in the 5’ FR and high sequence identity in the DRI, therefore, may be PPARγ-selective [16–18], as is apparent for fapb1b.1 promoter activity, which displays PPARγ-selectivity in liver and intestine explant tissue and fapb promoter-reporter gene constructs in the human embryonic kidney cells, HEK293A [7].

The objective of this study was to investigate divergent, PPAR-dependent transcriptional regulation at the promoters of the zebrafish (Danio rerio) fapb1a, fapb1b.1 and fapb1b.2 genes, and the spotted gar fapb1 gene (representative of the ancestral fapb1 gene) in order to determine the molecular mechanisms that led to the retention of the three fapb genes in zebrafish following the teleost-specific WGD event and subsequent local (tandem) duplication event. To define teleost fapb1 promoter evolution, the regulation of zebrafish fapb1a, fapb1b.1, and fapb1b.2 gene promoters was investigated by three approaches: (1) assay of gene transcripts in liver and intestine explant cultures treated with PPAR-agonists; (2) identification of
putative PPREs in the zebrafish fabp1a, fabp1b.1, and fabp1b, and the spotted gar fabp1 promoters by in silico analysis; and (3) in HEK293A cells using wild-type and mutagenized zebrafish fabp1a, fabp1b.1, and fabp1b.2, and spotted gar fabp1 promoters fused to the luciferase reporter gene, to determine the promoter-specific regulation of fabp1 genes by PPARα and PPARγ. We applied a comparative pharmacological approach to spotted gar fabp1 and zebrafish fabp1a, fabp1b.1, and fabp1b.2 promoter activity across a wide range of PPAR agonist concentrations in the absence or presence of PPAR antagonists. In this way, it was possible to model evolutionary processes for PPAR isoform-selectivity through readily quantifiable measurements of agonist potency, efficacy, and specificity.

Results
Differential induction of zebrafish fabp1a, fabp1b.1 and fabp1b.2 transcription by PPAR agonists in zebrafish liver and intestine explant culture

The genomic organization of the fabp1 gene of spotted gar and, the fabp1a, fabp1b.1 and fabp1b.2 genes of zebrafish is highly conserved; each gene consists of four exons, they share between 57 – 75 % mRNA sequence identity (Fig. 1a) and 40 – 71 % amino acid sequence identity (Fig. 1b), but differ markedly in their promoter sequences (Fig. 1c). The duplicated zebrafish fabp1 genes also exhibit differential regulation by linolenic acid and by the PPAR-agonist, clofibrate [12, 14, 16].

To determine if fabp1a, fabp1b.1 and fabp1b.2 mRNA levels are induced by PPAR activation, the steady-state levels of duplicated fabp transcript were quantified in explant tissue derived from zebrafish liver and intestine treated with the PPARα agonist, WY14,643, or the PPARγ agonist, rosiglitazone. fabp1a mRNA transcripts were detected in both liver and intestine. The PPARα agonist, WY14,643, increased fabp1a transcript levels 109-fold, while the PPARγ agonist, rosiglitazone, increased fabp1a transcript levels 60-fold. Induction of fabp1a transcriptional initiation by PPAR agonists was only in intestine explant tissue and not in liver explant tissue (Fig. 2). Transcriptional induction of the zebrafish fabp1a, therefore, appeared to be PPARα-selective as WY14643 resulted in higher fabp1a mRNA levels than rosiglitazone treatment (Fig. 2). fabp1b.1 mRNA levels were increased 13-fold following WY14643 treatment, and 26-fold following rosiglitazone treatment in liver, but not intestine (Fig. 2). Based on the differential regulation of these duplicated genes by PPAR agonists, fabp1b.1 induction appears PPARγ-selective as rosiglitazone treatment resulted in higher fabp1b.1 mRNA levels than WY14643 treatment (Fig. 2), fabp1b.2 mRNA was detected in both liver and intestine, but the steady-state level of fabp1b.2 transcripts was not changed in liver or intestine explant tissue by either rosiglitazone or WY14643.
treatment (Fig. 2). These data demonstrate that transcription of fabp1a and fabp1b.1, but not fabp1b.2, is induced by PPAR in zebrafish liver and intestine explant tissue.

**In silico analyses**

Promoter sequences of the zebrafish fabp1a, fabp1b.1, and fabp1b.2, and spotted gar fabp1 genes, 5’ of their transcription start sites (TSS), were analyzed in silico for the presence of putative PPREs. Sequences of 3,308 bp for zebrafish fabp1a, 3,059 bp for zebrafish fabp1b.1, 3,218 bp for zebrafish fabp1b.2, and 3,283 bp spotted gar fabp1 were retrieved from databases using the conserved non-coding sequence (CNS) discovery pipeline (v. 3.0) [19] (Additional file 1) and putative PPREs identified by the algorithm, MatInspector (v. 8.1). The length (in bp) of the promoter fragments retrieved was chosen by the CNS discovery pipeline as the region within 4,000 bp 5’ upstream of the TSS containing > 60 % of transcription factor binding motifs with > 60 % sequence identity to the vertebrate transcription factor binding site [19].

Two putative PPREs were identified in the fabp1a promoter fragment that had 66.7 % and 87.9 % (indicated by the purple rectangle in Fig. 3) sequence identity to the consensus sequence for the vertebrate PPRE (5’ – 3’, respectively) (Additional file 1) [16, 17]. Five putative PPREs were identified in the fabp1b.1 promoter fragment that exhibited 65 %, 68.1 %, 82.3 % (indicated by the red rectangle in Fig. 3), 65.1 % and 76.2 % sequence identity to the consensus sequence for the vertebrate PPRE (5’ – 3’, respectively) (Additional file 1) [16, 17]. No PPREs were identified within the fabp1b.2 promoter fragment (Additional file 1). The PPRE at −2,710 bp relative to the TSS in fabp1a displayed high sequence identity to the PPRE consensus in the 5’ FR (5’-CAAAAC-3’), but not the DRI (5’-AGGTCANAGGTCA-3’) region of the
PPRE (Additional file 1). In contrast, the PPRE sequence at −1,232 bp relative to the TSS in fabp1b.1 displayed high sequence identity to the vertebrate PPRE consensus in the DR1, but not 5′FR (Additional file 1). The 5′FR of the PPRE enhances the binding of PPARα to the PPRE and, thereby, imparts PPARα-selectivity to a PPRE. The 5′ FR is not required, however, for PPARγ binding to the PPRE and, thus, is not crucial for PPARγ-dependent gene regulation [16, 17]. Based on this observation, the putative PPRE identified at −2,710 bp in the fabp1a promoter appears to be PPARα-selective, whereas the PPRE at −1,232 bp in fabp1b.1 was PPARγ-selective, which is consistent with the induction of the steady-state levels of fabp1a and fabp1b transcripts in zebrafish explant intestine cultures treated with PPARα- and PPARγ-specific agonists (Fig. 2).

Four putative PPREs were identified in the spotted gar fabp1 promoter that had 75.6 % (indicated by the purple rectangle in Fig. 3), 64.7 %, 74.3 % (indicated by the red rectangle in Fig. 3), and 64.8 % sequence identity to the consensus sequence of the vertebrate PPRE (5′−3′, respectively). Similar to the PPRE in the zebrafish fabp1a gene, the putative PPRE present at −1,953 bp relative to the TSS of spotted gar fabp1 displayed high sequence identity to the PPRE consensus sequence in the 5′FR (henceforth referred to as PPRE-1). Like the PPRE in zebrafish fabp1b.1, the putative PPRE at −539 bp relative to the TSS of spotted gar fabp1 displayed high sequence identity to the PPRE consensus in the DRI region (henceforth referred to as PPRE-2) [16, 17, 20, 21]. Taken together, these data suggest that the spotted gar fabp1 promoter might be regulated by both PPARα- and PPARγ-selective PPREs [16, 17, 20, 21]. Furthermore, these data suggested that the PPREs with preferential binding affinity for PPARα and PPARγ, respectively, in the ancestral (spotted gar) fabp1 gene were subdivided between fabp1a and fabp1b.1 subsequent to the WGD event that occurred in ray-finned fish.

Analyses of zebrafish fabp1a, fabp1b.1 and spotted gar fabp1 promoter activity in HEK293A cells
To determine functionality of putative PPREs in the fabp1a and fabp1b.1 promoters, 3,300 bp of fabp1a and 2,847 bp of fabp1b.1 5′ upstream of their respective TSS were PCR-amplified from zebrafish genomic DNA and cloned into the pGL3-Basic promoter-reporter plasmid. In the pGL3-Basic plasmid, the 3,300 bp of fabp1a and 2,847 bp of fabp1b.1 were fused to the firefly luciferase gene for functional promoter assays. All promoter fragments displayed similar basal promoter activity to the TK promoter in HEK293A cells (data not shown). Treatment of HEK293A cells transfected by the zebrafish fabp1a promoter construct with 1 nM − 1 mM WY14643 (PPARα agonist) for 24 h induced in fabp1a promoter activity (Fig. 4a). WY14643-dependent fabp1a promoter activity was inhibited by the PPARα-selective antagonist GW6471 [rightward shift in the concentration-response curve (CRC) and greater EC50 (Table 1, Fig. 4a). WY14643-dependent fabp1a promoter activity was not inhibited by the PPARγ-selective antagonist T0070907 (Table 1, Fig. 4a). Treatment of HEK293A cells transfected with the fabp1a promoter construct with 1 nM − 1 mM rosiglitazone (PPARγ agonist) for 24 h also increased fabp1a promoter activity (Fig. 4b). Rosiglitazone-dependent fabp1a promoter activity was inhibited by T0070907, but not by GW6471 (Table 1, Fig. 4b). WY14643 was a more potent PPAR agonist for induction of fabp1a promoter activity than rosiglitazone, indicating the zebrafish fabp1a gene contained at least one functional, PPARα-selective, PPRE (Table 1).

Treatment of HEK293A cells transfected by the fabp1b.1 promoter construct with WY14643 induced fabp1b.1 promoter activity (Fig. 4c). WY14643-dependent fabp1b.1 promoter activity was inhibited by GW6471, but not by T0070907 (Table 1, Fig. 4c). Treatment of HEK293A cells transfected by the fabp1b.1 promoter construct with rosiglitazone also increased fabp1b.1 promoter activity (Fig. 4d). Rosiglitazone-induced fabp1b.1 promoter activity was inhibited by T0070907, but not by GW6471 (Table 1, Fig. 4d). Rosiglitazone was a more potent agonist of fabp1b.1 promoter activity than WY14643, indicating that the zebrafish fabp1b.1 promoter contained a functional, PPARγ-selective PPRE (Table 1).

Site-directed mutagenesis was used to confirm the functionality of putative PPREs identified in the zebrafish fabp1a and fabp1b.1 promoters. Mutagenesis of the 5′ FR was expected to reduce the affinity of PPARγ to the PPRE (i.e. a rightward shift in the CRC), whereas mutagenesis of the DRI expected to generally reduce PPAR affinity to the promoter [16–18]. The 5′ FR and DRI elements of the fabp1a PPRE (−2,710 bp) and the fabp1b.1 PPRE (−1,232) were individually mutagenized (Fig. 5a, b).

Treatment of HEK293A cells transfected by the fabp1a Δ5 FR promoter construct with WY14643 shifted the CRC to the right compared to non-mutated fabp1a (Table 1, Fig. 5c). Treatment of HEK293A cells transfected with the fabp1a ΔDR1 promoter construct with WY14643 shifted the CRC to the right compared to non-mutated fabp1a and fabp1a Δ5 FR (Table 1, Fig. 5c). Treatment of HEK293A cells transfected by the fabp1a Δ5 FR promoter construct with rosiglitazone shifted the CRC compared to non-mutated fabp1a (Table 1, Fig. 5d).
The CRC was shifted to the right in HEK293A cells transfected with the fabp1a ΔDR1 promoter construct and treated with rosiglitazone compared to non-mutated fabp1a and and fabp1a Δ5′FR (Table 1, Fig. 5d). These data demonstrate that the PPRE at −2,710 was functional and regulated, in part, by PPARα as mutagenesis of the 5′FR consistently affected PPAR-dependent induction of promoter activity [17]. Moreover, the DR1 element of the PPRE is required for the binding of all PPARs, a finding further supported by the functional-ity of the PPRE at −2,710 bp of the fabp1a promoter fragment [16–18].

WY14643 did not change the CRC for promoter activity in HEK293A cells transfected by the fabp1b.1 Δ5′FR promoter construct compared to non-mutated fabp1b.1 promoter construct (Table 1, Fig. 5e). WY14643 treatment shifted the CRC to the right in HEK293A cells transfected by the fabp1b.1 ΔDR1 promoter construct compared to non-mutated fabp1b.1 promoter (Table 1, Fig. 5f). Although the EC50 was shifted slightly to the right, the rosiglitazone produced a potent and fully efficacious response in HEK293A cells transfected by the fabp1b.1 Δ5′FR promoter compared to non-mutated fabp1b.1 promoter (Table 1, Fig. 5e). These observations provide compelling evidence that the zebrafish fabp1b.1 promoter region contains a functional, PPARγ-selective PPRE at −1,232 bp as rosiglitazone was a more potent agonist of PPAR induction of fabp1b.1 promoter activity than WY14643, and the DR1 element, not the 5′FR, was the major regulator of PPAR potency in these assays (Table 1). Since neither mutagenesis of the 5′FR or DR1 in fabp1a or fabp1b.1 abolished transcriptional induction of these fab genes by PPAR agonism, and no change in Emax was observed, additional, functional PPREs are likely present in both the fabp1a and fabp1b.1 promoters.

Given that both fabp1a and fabp1b.1 promoters contained functional, PPAR subtype-selective PPREs, we assayed the responsiveness of the orthologous spotted gar fabp1 gene promoter activity to PPAR isoform-specific agonism. A 3,283 bp fragment of the spotted gar fabp1 promoter, 5′ upstream of its TSS, was PCR-amplified from spotted gar genomic DNA and cloned into the pGL3-Basic promoter-reporter plasmid 5′ of the firefly luciferase gene. Treatment of HEK293A cells transfected by the fabp1 promoter construct with WY14643 resulted in a bell-shaped CRC for fabp1 promoter activity (Table 2, Fig. 6a). Co-treatment with WY14643 and T0070907 did not change CRC of spotted gar fabp1 promoter activity (Table 2, Fig. 6a). Co-treatment with WY14643 and GW6471 abolished the
Table 1. Pharmacological characterization of PPAR induction of zebrafish *fabp1a* and *fabp1b.1* promoter activity

|                          | WY14643 | Rosiglitazone |
|--------------------------|---------|---------------|
|                          |         |               |
| *E*<sub>max</sub> (RLU)  | 152.30 ± 5.57 | 152.80 ± 7.24 |
| EC<sub>50</sub> (μM)     | 0.29 (0.17–0.49) | 0.26 (0.28–0.23) |

Data derived from Figs. 4 and 5 presented as the mean ± SEM or 95% confidence intervals (brackets)

* <i>P</i> < 0.05 compared to WY14643 treatment alone within promoter
** <i>P</i> < 0.05 compared to rosiglitazone treatment alone within promoter
*** <i>P</i> < 0.05 rosiglitazone treatment compared to matched WY14643 treatment within promoter, as determined via two-way ANOVA followed by Bonferroni’s post-hoc analysis for *E*<sub>max</sub> or by non-overlapping confidence intervals (EC<sub>50</sub>). n = 3
Table 2 Pharmacological characterization of PPAR agonist and antagonist regulation of spotted gar fabp1 promoter activity

|                    | WY14643 Upward Slope | Downward Slope |
|--------------------|-----------------------|----------------|
|                    | GW6471                | T0070907       |
| E_{max} (RLU)      | 80.6 ± 7.40           | 97.5 ± 3.78    |
|                    | 10.9 ± 4.64           | 8.86 ± 3.50    |
| EC_{50} (μM)       | 0.12 (0.07–0.28)      | 0.54 (0.43–0.65)* |
| Rosiglitazone      |                       |                |
| Upward Slope       | GW6471                | T0070907       |
| E_{max} (RLU)      | 56.3 ± 2.40           | 57.4 ± 2.44    |
|                    | 5.28 ± 4.80           | −0.42 ± 2.73   |
| EC_{50} (μM)       | 0.009 (0.006–0.017)   | 0.016 (0.012–0.021) |

Data derived from Fig. 6 presented as the mean ± SEM or 95% confidence intervals (brackets)

*P < 0.05 compared to agonist treatment alone within the upward slope

**P < 0.05 compared to matched treatment between upward and downward slopes, as determined via two-way ANOVA followed by Bonferroni’s post-hoc analysis for E_{max}, E_{min}, or by non-overlapping confidence intervals (EC_{50}). n = 3–4
bell-shape of the fabp1 CRC and shifted the slope of the CRC to the right compared to the upward slope of the WY14643 CRC (Table 2, Fig. 6a). In contrast to WY14643, treatment of HEK293A cells (transfected with the fabp1 promoter) with rosiglitazone resulted in a concentration-dependent increase in fabp1 promoter activity (Table 2, Fig. 6b). The CRC for fabp1 promoter activity in cells treated with rosiglitazone was not changed by co-treatment with GW6471, but was shifted to the right by co-treatment with T0070907 (Table 2, Fig. 6b). Based on the results of these functional assays, we conclude that spotted gar fabp1 promoter activity was inducible by both PPARα and PPARγ. However, higher concentrations of WY14643 (PPARα agonist), but not rosiglitazone (PPARγ agonist), reduced promoter activity of the spotted gar fabp1 genes suggesting an inhibitory effect of PPARα. This inhibitory effect of the PPARα agonist may be mediated by recruitment of a transcriptional repressor to the PPRE-bound PPARα. Alternatively, this inhibitory effect may be mediated by steric hindrance of PPARα (i.e. a non-specific cross-activation of PPARγ at high concentrations of WY1463).

The two putative PPREs were identified in the spotted gar fabp1 promoter region, one located at −1,953 bp upstream of the TSS (PPRE-1), a location similar to the functional PPRE identified in the zebrafish fabp1a, and a second PPRE located at −539 bp (PPRE-2). Site-directed mutagenesis was used to confirm the functionality of these putative PPREs in the spotted gar fabp1 gene. The DR1 regions of the PPRE-1 and PPRE-2 promoters normalized to Renilla luciferase activity driven by the TK promoter in HEK293A cells treated with 1 nM – 10 μM WY14643 (PPARα agonist) or 10 nM T0070907 (PPARγ agonist) for 24 h. C Site-directed mutagenesis was used to alter the DR1 PPAR binding sites of two fabp1 PPREs at −1,953 bp (ΔPPRE-1) or −539 bp (ΔPPRE-2). D, E Firefly luciferase activity driven by the fabp1 promoter activity by WY14643 (PPARα agonist) or ΔPPRE-2 promoters normalized to Renilla luciferase activity driven by the TK promoter in HEK293A cells treated with 1 nM – 10 μM WY14643 (d) or rosiglitazone (e). Data are mean ± SD. *P < 0.001 compared to agonist alone or ‘No mutation’, as determined via two-way ANOVA followed by Bonferroni’s post-hoc test. n = 3 – 4

Fig. 6 PPAR induction of the spotted gar fabp1 promoter was PPARα- and PPARγ-selective. a, b) Firefly luciferase activity driven by the fabp1 promoter normalized to Renilla luciferase activity driven by the TK promoter in HEK293A cells treated with 1 nM – 10 μM WY14643 (PPARα agonist) (a) or rosiglitazone (PPARγ agonist) (b) ± 240 nM GW6471 (PPARα antagonist) or 10 nM T0070907 (PPARγ antagonist) for 24 h. C Site-directed mutagenesis was used to alter the DR1 PPAR binding sites of two fabp1 PPREs at −1,953 bp (ΔPPRE-1) or −539 bp (ΔPPRE-2). D, E Firefly luciferase activity driven by the fabp1 promoter activity by WY14643 (PPARα agonist) or ΔPPRE-2 promoters normalized to Renilla luciferase activity driven by the TK promoter in HEK293A cells treated with 1 nM – 10 μM WY14643 (d) or rosiglitazone (e). Data are mean ± SD. *P < 0.001 compared to agonist alone or ‘No mutation’, as determined via two-way ANOVA followed by Bonferroni’s post-hoc test. n = 3 – 4
shaped CRC of promoter activity observed following WY14643 treatment of the transfected HEK293A cells (Table 3, Fig. 6d). ∆PPRE-1 or ∆PPRE-2 of fabp1 also shifted either curve to the right compared to the upward slope of the non-mutated fabp1 promoter (Table 3, Fig. 6d). WY14643 induced spotted gar ∆PPRE-1 fabp1 promoter activity much more than the promoter activity of the spotted gar ∆PPRE-2 fabp1 promoter activity (Table 3, Fig. 6d). The ∆PPRE-2 fabp1 promoter rosiglitazone CRC was shifted to the right compared to the non-mutated fabp1 promoter (Table 3, Fig. 6e). The ∆PPRE-1 fabp1 promoter and non-mutated fabp1 promoter displayed similar responses to rosiglitazone CRC. We conclude from these functional promoter assays using the PPARα- and the PPARγ-specific agonists, that PPRE-1 on the spotted gar fabp1 promoter was PPARα-selective and mediated both increases (concentrations of PPAR agonist < 100 nM) and decreases (concentrations of PPAR agonist > 100 nM) in promoter activity. PPRE-2 was PPARγ-selective.

**Discussion**

In this study, we employed a pharmacological approach to define the PPAR selectivity, potency, and efficacy of PPAR-dependent regulation in the promoters of the zebrafish fabp1a, fabp1b.1 and fabp1b.2 genes. We observed that the zebrafish fabp1a promoter contained a functional, PPARα-selective PPRE, while the zebrafish fabp1b.1 promoter contained a functional, PPARγ-selective PPRE. The spotted gar fabp1 promoter contained two functional PPREs: a PPARα-selective PPRE (PPRE-1) and a PPARγ-selective PPRE (PPRE-2). These results are consistent with previous publications that: (1) the steady-state level of fabp1a and fabp1b.1 mRNA and hnRNA levels are induced in adult zebrafish fed a linolenic acid- or clofibrate-rich diets, and this transcriptional activation is mediated by PPAR [14, 15], and (2) that the fabp1a and fabp1b.1 promoters are functionally-selective for PPARα and PPARγ, as described here and in a previous report [7].

The spotted gar fabp1 promoter served as a representative of the ancestral fabp1 gene that gave rise to fabp1a and fabp1b following the teleost WGD [9]. The two functional PPREs identified in the spotted gar fabp1 promoter were oriented such that PPRE-1 was PPARα-selective and PPRE-2 was PPARγ-selective. Pharmacological analyses and site-directed mutagenesis demonstrated that both the spotted gar PPRE-1 of fabp1 and the similarly-aligned zebrafish PPRE of fabp1a (Fig. 1) were more responsive to PPARα-agonists and antagonists than to PPARγ-agonists and antagonists, based on promoter activity assays, suggesting that the fabp1a PPRE at −1,953 bp was derived from the PPARα-selective PPRE-1 in the ancestral fabp1 prior to the teleost WGD. Furthermore, pharmacological analyses and site-directed mutagenesis demonstrated that both the PPRE-2 of spotted gar fabp1 and the similarly-aligned zebrafish PPRE of fabp1b.1 (Fig. 1) were both more responsive to PPARγ agonists and antagonists than to PPARα agonists and antagonists, as assayed by the induction of promoter activity, suggesting that the fabp1b.1 PPRE at −539 bp was derived from an ancestral PPARγ-selective PPRE-2 present in the spotted gar (ancestral) fabp1 gene prior to its duplication following the teleost WGD.

Previous studies have focused on non-quantitative or semi-quantitative data derived from electrophoretic mobility shift assays to determine the specificity of PPARs interaction with PPREs [16–18, 22]. The unique pharmacological approach used in this study to define the

### Table 3 Pharmacological characterization of spotted gar fabp1 promoter PPRE mutants

|          | WY14643 |               |               |               |               |               |               |               |               |               |               |               |
|----------|---------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|          | Upward Slope |                |               | Downward Slope |                |               |               |               |               |               |               |               |
|          | No mutation | ∆PPRE-1      | ∆PPRE-2      | No mutation | ∆PPRE-1      | ∆PPRE-2      |               |               |               |               |               |               |
|          |          |              |              |              |              |              |               |               |               |               |               |               |
| E<sub>max</sub> (RLU) |         |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
| E<sub>min</sub> (RLU) |         |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
| EC<sub>50</sub> (μM) |         |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
|          | Rosiglitazone |                |               |               |               |               |               |               |               |               |               |               |
|          | Upward Slope |                |               | Downward Slope |                |               |               |               |               |               |               |               |
|          | No mutation | ∆PPRE-1      | ∆PPRE-2      | No mutation | ∆PPRE-1      | ∆PPRE-2      |               |               |               |               |               |               |
|          |          |              |              |              |              |              |               |               |               |               |               |               |
| E<sub>max</sub> (RLU) |         |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
| E<sub>min</sub> (RLU) |         |              |               |         |              |               |               |               |               |               |               |               |
|          |          |              |               |         |              |               |               |               |               |               |               |               |
| EC<sub>50</sub> (μM) |         |              |               |         |              |               |               |               |               |               |               |               |

Data derived from Fig. 6 presented as the mean ± SEM or 95% confidence intervals (brackets)

*P < 0.05 compared to agonist treatment alone within the upward slope

**P < 0.05 compared to matched treatment between upward and downward slopes, as determined via two-way ANOVA followed by Bonferroni’s post-hoc analysis for E<sub>max</sub>, E<sub>min</sub>, or by non-overlapping confidence intervals (EC<sub>50</sub>, n = 3
regulation and its evolution of promoter activity provided quantitative data, which supports the contention that the 5′ FR is directly involved in PPARα-, but not PPARγ-, dependent promoter activation [17]. Furthermore, this work supports earlier findings that the DR1 regulates general PPAR-dependent promoter activation [16–18, 22].

The data reported here raise two questions. First, how did the divergent transcriptional regulation of the zebrafish fabp1a, fabp1b.1, and fabp1b.2 genes by PPARs arise? Second, why was this divergent regulation of fabp1a, fabp1b.1, and fabp1b.2 by PPARs not selected against? To answer these questions, we must consider the data from this study and how it might be integrated into the existing model of gene duplication, particularly in teleost fishes. The spotted gar fabp1 gene, used here as a surrogate for the ancestral fabp1 gene promoter, contained at least two functional PPREs that resembled the zebrafish fabp1a and fabp1b.1 promoter PPREs, respectively, in both their location relative to the TSS, and their PPAR-isoform selectivity. The existing model of gene duplication in teleosts suggests that zebrafish fabp1a and ancestral fabp1b genes arose by WGD [9, 12, 14], whereas the zebrafish fabp1b.1 and fabp1b.2 arose by tandem duplication of the fabp1b gene during misaligned unequal crossing over during meiosis [12]. From these data, we can construct a model for divergent regulation of the fabp1 genes within the context of the DDC model (Fig. 7) [2–4]. The most straightforward explanation for the retention of fabp1a, fabp1b.1 and fabp1b.2 in the zebrafish genome is the ancestral teleost fabp1 gene was duplicated during a WGD event, which was later followed by a tandem duplication specific to zebrafish. We showed here that increasing concentrations of the PPARα-selective agonist, WY14643, enhanced and then repressed spotted gar fabp1 promoter activity (Fig. 6). While one might invoke recruitment of a transcriptional repressor of the spotted gar fabp1 promoter activity, this is not the most parsimonious explanation. Elimination of either PPRE-1 or PPRE-2 abolished the biphasic response of spotted gar fabp1 promoter activity suggesting PPARα-dependent functional antagonism of the spotted gar fabp1 promoter activity, which occurred via an interaction or competition between the two identified functional PPREs (Fig. 6). No functional antagonism was observed in the zebrafish fabp1a and fabp1b.1 promoters (Fig. 5). We suggest that following the teleost WGD, mutations may have accumulated independently in the PPARα-selective PPRE of the zebrafish fabp1a promoter, and in the PPARγ-selective PPRE of the zebrafish fabp1b promoter, leading to elimination of these elements in their respective promoters and loss of the functional antagonism observed in the spotted gar fabp1 promoter (Fig. 7). Zebrafish fabp1a and fabp1b promoters, therefore, underwent subfunctionalization relative to fabp1 with regard to PPAR isoform specificity. Subsequent tandem duplication of the zebrafish fabp1b gene resulted in a PPARγ-selective PPRE in fabp1b.1 and the loss of a functional PPRE in the zebrafish fabp1b.2 promoter with retention of basal promoter activity (Fig. 7) [7]. Zebrafish is unique among teleosts for having three fabp1 genes [12]. These data demonstrate that fabp1a and fabp1b.1 genes have retained their functional regulation by PPAR, and therefore their association with PPAR-dependent metabolic and hormonal signaling pathways [16]. In contrast, the local (tandem) duplicate fabp1b.2 is retained in the genome, but its transcription is not modulated by either dietary fatty acids or PPAR agonists. As such, the zebrafish fabp1b.2 gene does not appear to be

![Fig. 7 PPAR subfunctionalization of the fabp1 promoters following the teleost whole genome duplication. An ancestral fabp1 promoter contained two PPREs that had high sequence identity to the consensus sequence for PPREs. These PPREs were retained in the spotted gar fabp1 gene promoter. In zebrafish, fabp1a and the ancestral fabp1b underwent subfunctionalization. fabp1a retained the PPARα-selective PPRE and fabp1b retained the PPARγ-selective PPRE, following whole genome duplication. Further subfunctionalization of promoter function occurred following the tandem gene duplication of zebrafish fabp1b resulting in fabp1b.1 and fabp1b.2. Passage of time and accumulation of mutations is represented from left to right. PPREs are represented by purple (1, α) or red (2, γ) rectangles. PPRE loss is represented by smaller, more transparent rectangles. zf, zebrafish; sg, spotted gar; WGD, whole genome duplication; TGD, tandem gene duplication.](image)
associated with PPAR-dependent physiological processes [7, 12, 14]. This conclusion is consistent with cross-species analyses that have shown genes retained from WGD events often belong to signalling networks, whereas local gene duplication events are more likely to acquire network-independent functions [23, 24]. Additional research is required to understand what other regulatory elements have undergone neo-, non-, or subfunctionalization in the zebrafish fabp1 promoters compared to the spotted gar fabp1 promoter [3, 4, 25–28]. Our observations provide an example of increasing intra-organismal complexity through the subfunctionalization of response to PPAR stimuli among gene duplicates.

Conclusions

The present subfunctionalized state of PPAR responsiveness in the zebrafish fabp1a, fabp1b.1, and fabp1b.2 promoters may represent a form of segregation avoidance such that three gene products sharing similar function are expressed in different tissues, under different developmental or environment conditions [27, 28]. These data demonstrate the divergent, PPAR isoform-specific regulation of zebrafish fabp1a, fabp1b.1, and fabp1b.2 in relation to their subfunctionalization across evolutionary history using a unique pharmacological approach.

Methods

Zebrafish and spotted gar fabp1 promoter sequences

Promoter sequences for zebrafish fabp1a, fabp1b.1, and fabp1b.2 and spotted gar fabp1 genes were obtained using the CNS Discovery Pipeline (v. 3.0) created and described by Turco et al. [19]. The source code for the CNS Discovery Pipeline 3.0 is available for download at https://github.com/gturco/find_cns with instructions for installation at (https://github.com/gturco/find_cns/blob/master/INSTALL.rst) [19]. CNS Discovery Pipeline was run using default settings except that the filter for promoter regions containing gene-coding regions was removed. The input was the zebrafish Zv9 whole genome assembly (GenBank Assembly ID GCA_000002035.2) and spotted gar Linkage group LG2 LepOcu1 representative genome assembly (GenBank Assembly ID GCA_000242695.1, Gene symbol LOC102694982) [29]. The length of promoter fragments retrieved by the CNS discovery pipeline was determined as the region within 4,000 bp 5' of the TSS containing > 60 % sequence identity to the consensus of vertebrate transcription factor binding motifs [19]. The resulting “.fasta” output files for the fabp promoters and their corresponding genes were used to design PCR primers to clone fabp promoter fragments (Additional file 1).

Identification of putative PPREs in teleost fabp1 promoters by in silico analysis

Promoter sequences were analyzed for putative PPREs using MatInspector (v. 8.1) with the Genomatix ElDorado genomes database and the vertebrate matrix group. The PPRE was defined as 5'-CAAAACCTAGGTGTCANAGG TCA-3' [16–18]. The mismatch threshold was set to 35 % (i.e. transcription factor sites were identified if they were 65 % similar to the corresponding IUPAC string).

Cell culture

Primary zebrafish cell culture methods were adapted from Kan et al. [30]. Primary explant cell cultures of zebrafish liver and intestine were obtained from adult male fish. Fish were euthanized with tricaine (10 % v/v) and rinsed with 70 % ethanol in sterile phosphate-buffered saline (PBS). The liver and intestine were dissected, rinsed once with PBS, and incubated in 0.25 % trypsin-EDTA (Gibco, Oakville, ON) for 5 min at room temperature. Tissue was suspended in trypsin-EDTA by pipette and centrifuged at 500 x g for 5 min at room temperature. Cells were resuspended in media containing 50 % Leibovitz’s L-15, 35 % high glucose DMEM, 15 % Ham’s F-12, 5 % FBS, 0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL bovine insulin, and 50 ng/mL human EGF (Gibco) and maintained 28 °C, 100 % atmospheric air on poly-D-lysine-coated cell culture plates. Primary zebrafish cells were maintained for 48 h prior to drug treatment. Media was changed daily. All protocols were in accordance with the guidelines outlined by the Canadian Council on Animal Care. All animal protocols were approved by the Carleton Animal Care Committee at Dalhousie University prior to start of this study.

Human embryonic kidney 293A (HEK293A) cells were obtained from Cedarlane (Burlington, ON). HEK293A cells were maintained at 37 °C, 5 % CO2 in DMEM containing 10 % FBS and 104 U/mL Pen/Strep. HEK293A cells express PPARα and γ [31], which was confirmed by sequencing the RT-PCR products (data not shown).

Cloning of zebrafish fabp1a, fabp1b.1, and fabp1b.2, and spotted gar fabp1 promoter fragments into the pGL3-basic plasmid

DNA fragments containing the zebrafish fabp1a, fabp1b.1 and fabp1b.2, and spotted gar fabp1 promoter region were amplified from genomic DNA by PCR. Genomic DNA was isolated from frozen liver using the GenElute Genomic DNA Miniprep kit according to the manufacturer’s instructions (Sigma-Aldrich, Oakville, ON). The PCR contained: 2 mM MgCl2, 0.5 μM forward and reverse primers (Additional file 2), 0.3 mM dNTPs, 1 U Taq DNA polymerase, and 40 ng genomic DNA. PCR conditions were: 95 °C for 10 min; 35 cycles of 95 °C for
30 s, 57 °C for 30 s, 72 °C for 6 min; and 72 °C for 10 min. PCR products were resolved by gel electrophoresis and purified using the GenElute Gel Extraction kit (Sigma- Aldrich). Purified fabp1a, fabp1b.1, and fabp1b.2 PCR products were digested with MluI and HindIII according to the manufacturer's instructions (Fermentas, Burlington, ON). The purified spotted gar fabp1 PCR product was ligated into pGEM-T easy vector (Fermentas) at 16 °C overnight using T4 DNA ligase according to the manufacturer's instructions (Invitrogen, Burlington, ON). The spotted gar fabp1 promoter fragment was excised from pGEM-T by digestion with Ncol and SacI according to the manufacturer's instructions (Fermentas). fabp1a, fabp1b.1, and fabp1b.2 PCR products were ligated into pGL3-Basic (Promega, Madison, WI) at 16 °C overnight using T4 DNA ligase according to the manufacturer's instructions (Invitrogen). The resulting plasmids (pfabp1a, pfabp1b.1, pfabp1b.2, and pfabp1) were propagated in ampicillin-resistant DH5α competent E. coli (New England Biolabs, Whitby, ON) and purified using the GenElute Plasmid Midiprep kit (Sigma-Aldrich). The pHRL-TK plasmid was obtained from Promega.

pfabp1a, pfabp1b.1, and pfabp1 mutant plasmids were generated by PCR-based site-directed mutagenesis. The pfabp1a PPRE 5′ FR at −2,710 bp was mutated from 5′-CCAAAG-3′ to 5′-TCGGGT-3′ and the PPRE DR1 at −2,710 bp was mutated from 5′-ACAAAG-3′ to 5′-CGTGGA-3′. The pfabp1b.1 PPRE 5′ FR at −1,232 bp was mutated from 5′-CTAAAC-3′ to 5′-TCGGGT-3′ and the PPRE DR1 at −1,232 bp was mutated from 5′-TAAGGT-3′ to 5′-CGGAAC-3′. The pfabp1 PPRE-1 (−1,953 bp) was mutated from 5′-CCCTAA-3′ to 5′-TTTCTG-3′ and PPRE-2 (−539 bp) was mutated from 5′-AGGACA-3′ to 5′-TAAAGT-3′. Reactions were composed of 2 mM MgCl2, 0.5 mM forward and reverse mutagenic primers (Additional file 2), 0.3 mM dNTPs, 1 U Taq DNA polymerase, and 40 ng plasmid DNA. PCR conditions were: 95 °C 1 min, 18 cycles of 95 °C 50 s, 60 °C 1 min, and 68 °C 8 min, followed by a final extension at 68 °C for 10 min. Input plasmid was removed by digestion with the methylation-insensitive DpnI (5 U) in 1X FastDigest Green Buffer in a final volume of 20 μL (Fermentas, Burlington, ON) for 1 h at 37 °C. The constructs of wild-type and mutated zebrfish and spotted gar promoters was confirmed by DNA sequencing of the promoter-reporter gene constructs prior to transfection of HEK293A cells (data not shown).

Transfection, PPAR agonist and antagonist treatment, and the dual luciferase assay
Transfections of HEK293A cells was performed using lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen) with 400 ng of pfabp1a, pfabp1b.1, pfabp1b.2, pfabp1, or pGL3-Basic (background control), and 200 ng pHRL-TK. The luciferase activity of the pHRL-TK plasmid containing the Renilla luciferase gene under the regulation of the cytomegalovirus thymidine kinase (TK) promoter was used to normalize firefly luciferase activity under the regulation of zebrfish promoters. Luciferase activity was quantified according to the manufacturer's instructions (Promega).

HEK293A and primary zebrafish cells were treated with rosiglitazone (PPARγ agonist), WY14643 (PPARα agonist), T0070907 (PPARγ antagonist), GW 6471 (PPARα antagonist), or vehicle (0.5 % DMSO) at the concentrations and times indicated [32, 33]. All PPAR agonists and antagonists were purchased from Sigma-Aldrich.

Quantitative reverse transcriptase PCR
RNA was extracted from HEK239 cells using Trizol® (Invitrogen). Reverse transcription reactions were carried out with SuperScript III® reverse transcriptase (+RT; Invitrogen), or without (−RT) as a negative control for use in subsequent PCR experiments according to the manufacturer's instructions. Two micrograms of RNA were used per RT reaction. qRT-PCR was conducted using the LightCycler® system and software (version 3.0; Roche, Laval, QC). Reactions were composed of a primer-specific concentration of MgCl2 (Additional file 2), 0.5 μM each of forward and reverse primers (Additional file 2), 2 μL of LightCycler® FastStart Reaction Mix SYBR Green I, and 2 μL cDNA to a final volume of 20 μL with dH2O (Roche). The PCR program was: 95 °C for 10 min, 50 cycles of 95 °C 10 s, a primer-specific annealing temperature (Additional file 2) for 5 s, and 72 °C for 10 s. Experiments always included sample-matched −RT controls, a no-sample dH2O control, and a standard control containing product-specific cDNA of a known concentration. cDNA abundance was calculated using the △△CT method and normalized to GAPDH levels [34].

Statistical analyses
CRCs were fit using non-linear regression analyses [variable slope (four parameters) and Bell-shaped] in GraphPad Prism (v. 5.0). Statistical analyses were conducted by one-way ANOVA followed by Tukey’s post-hoc test or two-way ANOVA follow by Bonferroni’s post-hoc test, as indicated. Homogeneity of variance was confirmed using Bartlett’s test. All results are reported as the mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated, from at least three independent experiments.

Additional files
Additional file 1: Conserved non-coding sequence files (fasta) analyzed in this study. Putative PPREs are indicated as green 65–74.9 %, yellow 75–84.9 %, orange > 85 % sequence similarly to the defined PPRE consensus.
sequence. Red indicates primer binding sites for PCR. Light blue indicates regions of repetitive TAT sequences. DNA sequences determined by sequencing PCR-cloned promoter fragments of spotted gar or zebrafish genomic DNA. (PDF 38 kb)

**Additional file 2: Table S1.** Synthetic oligonucleotides used for cloning fabp1 (spotted gar), fabp1a (zebrafish), fabp1b.1 (zebrafish), and fabp1b.2 (zebrafish) promoter fragments, mutagenesis of the fabp1, fabp1a, and fabp1b.1 PPREs, and quantification of fabp1a, fabp1b.1, and fabp1b.2 transcripts via qRT-PCR in this study. (PDF 82 kb)

**Abbreviations**

SFR, 5' flanking region; CNS, conserved non-coding sequence; CRC, concentration-response curve; DDC, duplication-degeneration-complementation; DR1, direct repeat region; fabp, fatty acid-binding protein; HEK, human embryonic kidney cell line; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; RT, reverse transcriptase; SEM, standard error of the mean; SD, standard deviation; sg, spotted gar; TGD, tandem gene duplication; TK, thymidine kinase; TSS, transcription start site; WGD, whole genome duplication; zf, zebrafish

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**Availability of data and material**

The data sets supporting the results of this article are available in the Dryad repository, [http://dx.doi.org/10.5061/dryad.q03n7] (35).

**Authors’ contributions**

RBL, EMD-W, and JMW conceived the study. RBL conducted the experiments and analyzed the data. RBL, EMD-W, and JMW contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

All protocols requiring euthanized zebrafish were in accordance with the guidelines detailed by the Canadian Council on Animal Care. Zebrafish animal protocols were approved by the Carleton Animal Care Committee at Dalhousie University prior to beginning the study.

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