Potential Roles of Arnt2 in Zebrafish Larval Development

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Abstract

The aryl hydrocarbon receptor nuclear translocator (ARNT) is a basic helix-loop-helix-PAS heterodimeric transcription factor that dimerizes with other basic helix-loop-helix-PAS proteins to mediate biological responses. The function of ARNT2 is poorly understood. Here we provide an initial characterization of the zebrafish *arnt2* null (*arnt2*^{-/-}) mutant to identify functions of Arnt2 during development. *Arnt2*^{-/-} mutant zebrafish develop normally until 120 hours postfertilization (hpf) when morphological changes and functional deficits occur. The C-start escape response initiated by either touch or startle stimuli is absent in the mutants. Brain ventricle size is markedly increased at 120 hpf. Heart ventricles are enlarged, with decreased ventricle wall thickness. A cardiac arrhythmia, characterized by missing beats, is also observed in the mutants. This is associated with bradycardia in *arnt2*^{-/-} larvae. Dilated liver sinusoids merge abnormally to form an extensive, labyrinth-like network of vascular channels. External appearance of *arnt2*^{-/-} larvae at 120 hpf is indistinguishable from wild type except that the swim bladder is not inflated. The *arnt2*^{-/-} mutants are not debilitated when phenotypic effects are first detected at 120 hpf that culminate in mortality, 4 days later around 216 hpf. Gross morphological assessment of the development of forebrain, midbrain, and hindbrain regions, neuromasts and Mauthner neurons, inner ear semicircular canals and otoliths, primary motor neurons, trigeminal ganglia, and trunk skeletal muscles, before or when the *arnt2*^{-/-} phenotype was observed, failed to demonstrate a difference from wild type. The only effect in *arnt2*^{-/-} larvae that occurred before 120 hpf was a decrease in expression of *sim1*, an Arnt2 dimerization partner, in the hypothalamus and ventral thalamus at 72 hpf. Further research is needed to determine if the primary functions of Arnt2 occur during the larval stage, when the phenotype is observed, or earlier in development.

Introduction

THE ARYL HYDROCARBON RECEPTOR nuclear translocators (ARNTs) are members of the basic helix-loop-helix-PAS family of transcription factors that also includes the aryl hydrocarbon receptor (AHR), hypoxia-inducible factor-1 alpha (HIF-1 α), and the *Drosophila* single-minded protein (SIM). Several isoforms of ARNT have been characterized and include ARNT (HIF-1 β), ARNT2, and ARNT3 (BMAL1, MOP3, JAP3, and ARNTL1). These proteins play signature roles in the detection of and adaptation to cellular changes, including responses to environmental contaminants, oxygen deprivation, biological rhythms, angiogenesis, and neuron development.¹⁻³

ARNTs differ from other PAS proteins in that they can dimerize with themselves as well as with other PAS proteins such as AHR, HIF-1 α , and endothelial PAS domain protein 1 (EPAS-1).⁴⁻⁶ For example, the AHR/ARNT heterodimer plays a pivotal role in mediating cellular responses to environ-

mental contaminants such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls and polyaromatic hydrocarbons through a well-characterized AHR/ARNT signaling pathway.⁷⁻¹⁰ The ligand-bound AHR/ARNT complex initiates metabolism of such toxicants by inducing the expression of phase I metabolizing enzymes, including cytochromes P450.¹¹⁻¹³

While it is thought that ARNT proteins may have redundant functions in certain tissue types later in development,¹⁴ they may have more distinct roles in the regulation of early development. For example, the ARNT2/SIM dimer is thought to be important for neuron survival and differentiation during development of the hypothalamus¹⁴⁻¹⁶ and AHR/ARNT is required for normal liver development.^{17,18} Additionally, ARNT2 forms a complex with HIF-1 α (or the closely related proteins HIF-2 α or HIF-3 α) in response to hypoxic conditions, initiating the expression of hypoxia-regulated genes such as vascular endothelial growth factor, glucose transporters, as well as several glycolytic enzymes.¹⁹⁻²²

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Multiple genes for ARNT, *arnt1* and *arnt2*, have been identified in mammals.^{5,23–25} Both genes are expressed in most organ tissues during early development; however, *arnt2* tissue distribution is more limited than *arnt1*.⁵ *Arnt1* is predominantly expressed in organs outside of the central nervous system (CNS), whereas *arnt2* is predominantly expressed in the developing CNS and kidney.²⁶ Initially, it was thought that fish had only one form of Arnt, *arnt2*, that has multiple splice variants.^{27–29} Zebrafish *arnt2* (*arnt2*) is primarily expressed in regions of the embryonic brain, liver, heart, and vascular endothelium.³⁰ Recently, a second *arnt* gene, *arnt1*, was identified in zebrafish.³¹ Unfortunately, temporal and spatial expression patterns of *arnt1* are not yet available.

In fish, the roles of Arnts are best characterized in relation to cellular responses after exposure to the prototype halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *In vitro*, molecular evidence demonstrated that one form of ARNT2, ARNT2b, formed a functional heterodimer with AHR2 that induced XRE-driven transcription in COS-7 cells treated with TCDD.^{28,32} In addition, *ahr2* and *arnt2a,b,c* mRNAs are co-localized in the same tissues as *cyp1a* after exposure of zebrafish to TCDD.³⁰ Although these results suggest that ARNT2 can form a functional dimer with AHR2 and may potentially contribute to TCDD-induced endpoints of developmental toxicity, ARNT1 is currently considered to be the main physiological dimerization partner for AHR2 *in vivo*^{31,33} leaving the physiological role for ARNT2 unclear.

Because ARNT2 proteins are dimeric partners for other PAS proteins such as HIF and SIM, it is likely that this transcription factor is important for numerous developmental pathways and functions in zebrafish tissues where *Arnt2* is expressed (regions of the embryonic brain, liver, heart, and vascular endothelium³⁰). This hypothesis is supported by research using a murine *arnt2*^{−/−} mutant model that identified ARNT2 as a necessary component for development of the hypothalamus^{15,16} and mice lacking ARNT2 die within 24 h of birth.¹⁶ *Arnt2* is the predominant form of Arnt in both *Fundulus* and zebrafish.^{28,34} In zebrafish, overexpression of dominant-negative *Arnt2* (a truncated splice variant of *Arnt2*, *Arnt2*×) is embryonic-lethal (embryos die around 96 hours postfertilization [hpf]),²⁷ suggesting that *Arnt2* also is important for the normal development of fish.

An insertional mutant screen originally identified an *arnt2*^{−/−} mutant due to a poor touch response,³⁵ although an abnormal heart phenotype and motility problem were later observed (Hopkins laboratory, personal communication). The purpose of the present study was to further characterize the *arnt2*^{−/−} mutant phenotype so as to better define the role of *Arnt2* in zebrafish embryonic and larval development. Our findings suggest that *Arnt2* is a necessary transcription factor for normal development of the zebrafish heart, brain, liver, and possibly peripheral nervous system. Further, because *Arnt2* knockout mice are not viable, information gleaned from studies in zebrafish may provide insight into its roles in mammalian development as well.

Results

Arnt2^{−/−} larvae are true null mutants

Viable male and female *arnt2*^{+/−} zebrafish (*arnt2*^{hi1715}) generated in a large-scale retroviral mutagenesis screen³⁵ were used to produce wild-type (WT, *arnt2*^{+/+}) and *arnt2* null

mutant (*arnt2*^{−/−}) fish. Previously we showed by RT-PCR that the *arnt2*^{−/−} mutant does not express *arnt2a*, *arnt2b*, or *arnt2c* mRNA.^{33,36}

External morphology is unaffected in *arnt2*^{−/−} larvae

All *arnt2*^{−/−} larvae appeared normal upon gross observation at 120 hpf. The appearance of a representative WT and *arnt2*^{−/−} mutant is shown in Figure 1. The lower jaw appears larger in the mutant, but it is because its mouth is open; there is no difference in lower jaw size between the two genotypes. The only consistent difference was swim bladder inflation (Fig. 1). WT larvae inflated their swim bladder by 96–120 hpf, whereas *arnt2*^{−/−} mutants showed no swim bladder inflation at 120 or 148 hpf. Prasad *et al.*³⁶ also found that knockdown of *Arnt2* with a morpholino oligonucleotide caused no change in external morphology and failure of the swim bladder to inflate.

It is important to point out that none of the *arnt2*^{−/−} mutants assessed at 120 hpf were stunted in body growth, debilitated, or near death. Mortality occurred as a consequence of the mutation, but beginning later in time, around 216 hpf.

Startle and touch responses are impaired in *arnt2*^{−/−} larvae

Between 120 and 148 hpf, *arnt2*^{−/−} mutants failed to exhibit either a startle response to an acoustic/vibrational stimulus (tapping dish containing larva) or a touch response when touched on the head or tail. These results are consistent with those of a large-scale mutagenesis screen where *arnt2*^{−/−} mutants were described as being “touch insensitive.”³⁵

In Figure 2, the touch response of a representative WT larva (top panel) and an *arnt2*^{−/−} mutant (middle and bottom panels) is shown. After being touched on the head or tail, the WT larva exhibits a brisk C-start escape response. This is shown in the top panel for a WT larva touched on the head. The video recording was taken at 1 frame/0.07 s beginning when the head was touched and continuing for 0.14 s. In contrast, the *arnt2*^{−/−} mutant exhibits a very weak response when touched on the tail by moving only a short distance (middle panel) and fails to respond at all when touched on the head (bottom panel). *Arnt2*^{−/−} responses were recorded at the same speed as WT.

FIG. 1. *Arnt2*^{−/−} larvae have normal external morphology at 120 hpf. A representative WT larva and *arnt2*^{−/−} larva are shown. They have similar external morphology, except that swim bladder is not inflated in the mutant.

FIG. 2. The escape response to a touch stimulus is impaired in *arnt2*^{-/-} larvae at 120 hpf. When touched on the head a representative WT larva instantly reacts by swimming rapidly away from the mounted probe (top). The same brisk escape response is observed in the WT larva when touched on the tail (results not shown). In contrast, a representative *arnt2*^{-/-} larva at 120 hpf shows either a very weak response when touched on the tail (middle) or no response when touched on the head (bottom). The video images were recorded at 1 frame/0.07 s after the larva was touched. Results are representative of *n* = 6 WT or *arnt2*^{-/-} larvae.

The deficits in startle and touch responses by *arnt2*^{-/-} mutants raise questions about whether the central and peripheral motor and sensory nervous systems and skeletal muscles of the mutants develop normally. To find out, we assessed relevant endpoints of nervous system and skeletal muscle development at designated times from 24 to 120 hpf. Could we identify an early occurring deficit, morphological or functional, to explain the loss of the touch and startle responses?

Brain development appears unaffected in arnt2^{-/-} embryos

We began by grossly evaluating brain organogenesis in WT and *arnt2*^{-/-} mutants (Fig. 3). At 24 hpf, 96 h before loss of touch and startle responses are detected, dorsal (Fig. 3A, B) and lateral (Fig. 3C, D) views, respectively, of the head of a representative whole-mount WT and *arnt2*^{-/-} mutant show that forebrain, midbrain, and hindbrain regions of the two genotypes are indistinguishable. At 48 hpf, a dorsal view of the head of a WT and *arnt2*^{-/-} mutant show that the brain ventricles (Fig. 3E, F, white arrowheads) are similar in size and shape. Thus, at 24–48 hpf, no effect of the null mutation on zebrafish embryo brain development was detected that could explain loss of the touch and startle reflex at 120 hpf. This interpretation is further supported by similar expression of *krox20* and *pax6a* mRNA in the hindbrain rhombomeres of WT and *arnt2*^{-/-} mutants 24–48 hpf. The nearly identical pattern of expression at 24 hpf in the hindbrain of a representative WT and *arnt2*^{-/-} mutant, respectively, is shown for *krox20* (Fig. 3G, H, arrowheads) and *pax6a* (Fig. 3I, J, arrowheads).

FIG. 3. Early brain development appears normal in *arnt2*^{-/-} embryos at 24–48 hpf. Brain development in the *arnt2*^{-/-} mutant embryo was assessed by gross examination of the brain at the light microscopic level at 24 hpf. A dorsal view of the developing brain of a WT embryo and an *arnt2*^{-/-} embryo (A, B) and a lateral view of the brain of a WT embryo and an *arnt2*^{-/-} embryo (C, D) are shown. At 48 hpf a dorsal view of the brain of a representative WT embryo and *arnt2*^{-/-} embryo (E, F) is shown. White arrowheads point to ventricles in the brain of the WT and *arnt2*^{-/-} mutant, demonstrating that brain ventricles are similar in size and shape in the two genotypes (E, F). Overall, gross brain morphology of *arnt2*^{-/-} larvae at 24–48 hpf was indistinguishable from WT. Patterns of staining at 24 hpf are shown for whole-mount ISH of *krox20* in a representative WT embryo and *arnt2*^{-/-} embryo at 24 hpf (G, H, arrowheads) and of *pax6a* (I, J, arrowheads).

Sensory and motor nervous system development appears normal in arnt2^{-/-} embryos and larvae

Next, we sought to determine if loss of the startle and touch responses is preceded by failure of sensory or motor neurons to develop (Fig. 4). *Islet1* expression was assessed by

whole-mount *in situ* hybridization (ISH) in WT and *arnt2*^{-/-} mutants at 24 hpf (Fig. 4A, B). *Islet1* identifies CNS structures where primary neurons form, including the epiphysis, trigeminal ganglia, and prospective Rohon-Beard cells of the spinal cord. The staining pattern for *islet1* (black arrows) was similar in the two genotypes (Fig. 4A, B).

To determine whether Mauthner neurons that initiate the escape response to touch or vibration stimuli are affected, whole-mount immunohistochemistry (IHC) was performed on WT and *arnt2*^{-/-} mutants at 30 hpf (Fig. 4C, D). A primary antibody, 3A10, specific for Mauthner neurons was used. The pattern of staining was similar in the two genotypes, suggesting that development of the Mauthner neurons was not affected by the mutation (Fig. 4C, D).

The IHC staining patterns for ZNP1, which identifies primary motor neurons and trigeminal ganglia, are shown in the bottom two panels of Figure 4. ZNP1 immunostaining is similar in a representative WT and *arnt2*^{-/-} mutant at 30 hpf when assessed in a lateral view of the whole embryo (Fig. 4E, F) as well as in a dorsal view of the trunk of the embryo

(Fig. 4G, H). The similar staining pattern suggests that the null mutation for *arnt2* does not affect primary motor neuron development at 30 hpf.

Sensory systems located along the lateral line and in the inner ear are used by zebrafish to detect touch and/or vibration in the water. Impaired development of these systems could account for loss of the touch and startle responses. The startle response requires larvae to sense vibration in the water caused by a tap applied to the dish containing the larva. To determine if loss of the startle response in *arnt2*^{-/-} mutants is caused by defective inner ear development, larvae were assessed at 72 hpf (Fig. 5). Comparison of a representative WT and *arnt2*^{-/-} mutant (Fig. 5A, B) reveals that all inner ear structures, such as the semicircular canals and otolith including the maculae and cristae, are present. There are no differences in inner ear morphology between the two genotypes.

FIG. 4. Sensory neurons involved in the touch and startle responses appear normal in *arnt2*^{-/-} embryos at 24–30 hpf. Lateral views for whole-mount ISH of *islet1* in a representative WT larva and *arnt2*^{-/-} larva is shown at 24 hpf (A, B). The pattern of *islet1* staining in the brain (black arrow) is similar for the two genotypes, suggesting that regions where primary neurons form are developing normally. 3A10 IHC staining of Mauthner neurons is similar in a representative WT embryo and *arnt2*^{-/-} mutant embryo at 30 hpf (C, D). ZNP1 IHC to identify primary motor neurons and trigeminal ganglia is shown in a lateral view of a WT embryo and an *arnt2*^{-/-} embryo (E, F) and in a dorsal view (G, H) at 30 hpf. ZNP1 IHC is similar in the two genotypes. Results in all panels are representative of 5–9 embryos/genotype.

FIG. 5. Morphology of the inner ear, neuromasts, and primary motor neurons appears normal in *arnt2*^{-/-} larvae at 72–148 hpf. Structures of the inner ear, including the semicircular canals and otolith of a WT larva and an *arnt2*^{-/-} larva at 72 hpf, are similar (A, B). Neuromasts, the lateral line neurons that sense vibration in the water, are stained with DASPEI and appear as white dots on the lateral surface of WT and *arnt2*^{-/-} larvae (C, D). The DASPEI-stained neuromasts are similar in number and distribution in the two genotypes. ZNP1-stained primary motor neurons are shown in the trunk of a representative WT larva and *arnt2*^{-/-} larva at 148 hpf (E, F). Skeletal muscle of the trunk is also shown in these lateral views (E, F). Both ZNP1-stained primary motor neurons and trunk skeletal muscle of WT and *arnt2*^{-/-} larvae appear to be similar.

Neuromasts are mechanoreceptors of the lateral line that sense water vibration and touch. They were identified by DASPEI staining as white dots along the lateral line and head of a representative WT and *arnt2*^{-/-} larva at 120 hpf (Fig. 5C, D). This is the same time that *arnt2*^{-/-} mutants lose the touch and startle response. After evaluating several DASPEI-stained WT and *arnt2*^{-/-} mutants, it was concluded that the number and distribution of neuromasts on the lateral surface of the body and head of the two genotypes were not significantly different.

*Development of primary motor neurons and skeletal muscle appears to be unaffected in *arnt2*^{-/-} larvae*

Because impaired startle and touch responses were observed at both 120 and 148 hpf, we wanted to determine if primary motor neurons in the trunk are present at 148 hpf. A lateral view of the IHC staining pattern for ZNP1, which identifies primary motor neurons, was found to be similar in a representative WT and *arnt2*^{-/-} mutant larva at 148 hpf (Fig. 5E, F). This demonstrates that loss of the startle and touch responses in *arnt2*^{-/-} mutants is not caused by failure of the primary motor neurons that innervate skeletal muscle to develop. In addition, skeletal muscle histology in the trunk of a WT larva that exhibits normal startle and touch responses, and a *arnt2*^{-/-} mutant that is incapable of making either response are indistinguishable (Fig. 5E, F). Thus, not only are primary motor neurons that innervate skeletal muscle present in the *arnt2*^{-/-} mutant, but so is the skeletal muscle and it appears to have developed normally.

*Expression in brain of the Arnt2 dimerization partner, Sim1, is reduced in *arnt2*^{-/-} larvae*

A decrease in mRNA expression of the Arnt2 dimerization partner, Sim1, is detected in the *arnt2*^{-/-} larva brain at 72 hpf (Fig. 6). This is 48 h before disruption of the startle and touch responses. In a lateral view of a whole-mount WT and *arnt2*^{-/-} larva (Fig. 6, left top and left bottom), the intensity of *sim1* mRNA expression appears to be less in the *arnt2*^{-/-} mutant. To observe the *sim1* staining in this lateral view, it is necessary to remove the eyes of the euthanized larvae. Black arrows (Fig. 6, left) indicate the plane of a cross section of the larva head, containing eyes and brain, used to observe the brain pattern of *sim1* staining. Cross section of a WT brain (Fig. 6, right top) shows a distinct elliptical ring pattern of *sim1* staining in the ventral thalamus (vt) and two oval areas of bilaterally positioned *sim1* staining in the hypothalamus (h). In the *arnt2*^{-/-} mutant (Fig. 6, right bottom), *sim1* staining in both areas is reduced in intensity. This reduction in *sim1* expression at 72 hpf is significant because it occurred earlier than any other effect of the *arnt2* null mutation identified in the present study.

*Brain ventricle size is reduced in *arnt2*^{-/-} larvae*

It was previously shown, when assessed at 48 hpf, that the size of the brain ventricles in WT and *arnt2*^{-/-} embryos is similar (Fig. 3E, F). On the other hand, when assessed later at 120 hpf, the same time that startle and touch responses were lost, a striking increase in brain ventricle size of the *arnt2*^{-/-} mutant is evident (Fig. 7). A black arrow points to the ventricle of a WT larva brain at 120 hpf (Fig. 7, top) and to the same

FIG. 6. mRNA Expression of the Arnt2 dimerization partner, Sim1, is reduced in the hypothalamus and ventral thalamus of *arnt2*^{-/-} larvae at 72 hpf. ISH of *sim1* in a cross section of the brain taken through the ventral thalamus and hypothalamus of a representative WT larva and *arnt2*^{-/-} larva at 72 hpf shows reduced expression in the *arnt2*^{-/-} mutant (compare right top and right bottom). In the left panel, black arrows indicate the plane at which the cross section (shown in the right panel) was taken. Eyes of the representative WT larva and *arnt2*^{-/-} larva were removed after euthanasia (left top and left bottom) so that the pattern of *sim1* staining in the whole-mount brain could be seen in lateral view. vt, ventral thalamus; h, hypothalamus.

ventricle, but larger in size, in the *arnt2*^{-/-} mutant (Fig. 7, bottom). Both cross sections were taken at essentially the same location in the brain. This is indicated by the same brain structures being present in each section (tc, tectum; tg, tegmentum; h, hypothalamus; p, presumptive pituitary, to which a white arrow is pointing). So the difference in brain ventricle size is real, not an artifact of sectioning at different brain locations. The increase in brain ventricle size of the *arnt2*^{-/-} mutants is of particular interest because it is detected at the same time as loss of the startle and touch responses, 120 hpf.

*Heart ventricle size is increased and wall thickness decreased in *arnt2*^{-/-} larvae*

Morphology and function of the heart also was affected by the *arnt2* null mutation at 120 hpf (Fig. 8). In longitudinal section, the heart of a WT and *arnt2*^{-/-} mutant appears generally to be similar (Fig. 8A, left and right). The sinus venosus (SV), atrium (A), ventricle (V), and bulbous arteriosus (BA) are present in both. However, closer examination of the ventricle of the *arnt2*^{-/-} mutant (Fig. 8A, right) suggests that it is larger than WT (Fig. 8A, left). To determine if this is the case, a cross section of the ventricle was taken at three distinct locations along its anterior axis (Fig. 8A, left and right: vertical, red, dashed lines). The first cross section (Fig. 8B) was taken from the most anterior region of the heart where the ventricle joins the bulbous arteriosus, the second (Fig. 8C) was taken in the middle of the heart where cross-sectional diameter of the ventricle is greatest, and the third (Fig. 8D) was taken where the ventricle joins the atrium (D). At each location, ventricle

FIG. 7. Brain ventricle area is increased in *arnt2*^{-/-} larvae at 120 hpf. Cross section of the brain of a representative WT larva shows a small ventricle to which a black arrow is pointing (top panel). On the other hand, a cross section taken through the same region of the brain of a *arnt2*^{-/-} mutant reveals a large brain ventricle (black arrow, bottom panel). The two cross sections were taken at similar locations along the anterior–posterior axis of the brain using the tectum, tc, and presumptive pituitary, p (white arrow), for orientation of the sections. h, Hypothalamus; tg, tegmentum; pc, parachordal cartilage.

cross-sectional area is larger and ventricle wall thickness seems smaller in the *arnt2*^{-/-} mutant.

Mean ventricle cross-sectional area and mean ventricle wall thickness, as a function of the individual cross section number, for 8–10 cross sections taken in an anterior to posterior direction through the ventricle, are shown for WT and *arnt2*^{-/-} mutant (Fig. 8E, line graphs: top, area; bottom, thickness). The results show that in each individual cross section, taken at similar locations along the ventricle, cross-sectional area is greater and ventricle wall thickness is less in the *arnt2*^{-/-} mutant (Fig. 8E, top and bottom line graphs).

Mean ventricle area and mean ventricle wall thickness, based on all cross sections, also were significantly different between the two genotypes (Fig. 8F, bar graphs, left and middle). Overall, these findings suggest that the *arnt2*^{-/-} heart ventricle increased its size by being stretched, because ventricle wall thickness is less, rather than by increasing cardiomyocyte proliferation in the ventricle.

Bradycardia and a cardiac arrhythmia occur in arnt2^{-/-} larvae

The heart rate of *arnt2*^{-/-} larvae gradually slowed beginning around 120 hpf in some, but not all of the mutants. By 144 hpf, bradycardia was more apparent, and this is evi-

FIG. 8. Heart ventricle enlargement, bradycardia, and cardiac arrhythmia in *arnt2*^{-/-} larvae at 120 and 144–148 hpf. In a representative longitudinal section of the heart (A) of a WT larva (left) and an *arnt2*^{-/-} larva (right) at 120 hpf, the ventricle of the *arnt2*^{-/-} mutant appears slightly larger than WT. Transverse sections of the ventricle at 120 hpf were taken along its anterior–posterior axis: anterior (B), middle (C), and posterior (D) for WT (left) and *arnt2*^{-/-} mutant (right), respectively. A, Atrium; BA, bulbous arteriosus; SV, sinus venosus; V, ventricle. Line graphs (E) show ventricle cross-sectional area as a function of section number (top line graph) and ventricle wall thickness as a function of section number (bottom line graph). WT (line, open circle) and *arnt2*^{-/-} (line, solid triangle) results shown in the line graphs are for *n* = 7 larvae/genotype at 120 hpf. Mean ventricle area and ventricle wall thickness based on all cross section measurements of the ventricle also are shown for WT and *arnt2*^{-/-} mutant's heart (F, bar graphs, right and middle). Heart rate assessed in WT and *arnt2*^{-/-} larvae at 144 hpf (G) revealed the presence of bradycardia in *arnt2*^{-/-} larvae (WT, *n* = 4; *arnt2*^{-/-}, *n* = 11). Asterisk indicates a significant difference (*p* < 0.05).

denced by a significant decrease in mean heart rate of 191 beats/min, WT versus 166 beats/min *arnt2*^{-/-} mutant (Fig. 8G, bar graph, right; *p* < 0.05). Bradycardia also was observed when larvae were assessed at 148 hpf; mean heart rate in WT larvae at this time was 180 beats/min compared to 135 beats/min in *arnt2*^{-/-} (results not shown).

Beginning at 120 hpf, a cardiac arrhythmia was detected in the *arnt2*^{-/-} mutants that became progressively worse over time. There was no temporal pattern to the occurrence or duration of the arrhythmia. It was characterized by missing heartbeats resulting in extended filling and over inflation of the atrium (see Supplemental Movie, available online at www.liebertonline.com). At 120 hpf, 36% of *arnt2*^{-/-} larvae had a cardiac arrhythmia; by 148 hpf, 64% of the mutants exhibited 1–6 arrhythmic beats/min.

Liver sinusoid morphology is altered in arnt2^{-/-} larvae

Figure 9 shows two sections of the liver of a WT larva at 120 hpf (low and high magnification, A and C) and a representative *arnt2*^{-/-} larva (low and high magnification, B and D). The liver (l) of both genotypes (A and B) is positioned below the esophagus (e) and above and to the left of the yolk (y) when viewed at low magnification. A red arrowhead in each panel points to a liver sinusoid that is a vascular channel in the zebrafish larva liver. The parenchyma of the zebrafish liver is different from that of mammals; it lacks portal and central veins, and hepatocytes are not arranged in cords or lobules with sinusoids connecting portal and central veins. Rather, the normal zebrafish liver is a nonlobular, tubular organ. It has fewer sinusoids than the mammalian liver, and they are irregularly distributed between the hepatocytes as shown for a representative WT liver (Fig. 9A, C).

The major difference in liver morphology between WT and *arnt2*^{-/-} mutant zebrafish larvae at 120 hpf is that both the size and structure of the sinusoids are quite different. In WT liver these vascular channels are scattered throughout the parenchyma and appear small and round, and the total parenchymal area occupied by the sinusoids is small relative to that occupied by hepatocytes (Fig. 9A, C). In a representative

FIG. 9. Altered liver sinusoid morphology in *arnt2*^{-/-} larvae at 120 hpf. Photomicrographs of sections of the liver of a representative WT larva (**A**, **C**) and *arnt2*^{-/-} mutant (**B**, **D**) are shown. Compared to WT, the *arnt2*^{-/-} liver is characterized by enlarged sinusoids engorged with blood that have merged to form large labyrinth-like vascular channels. A red arrowhead points to a blood-filled sinusoid in each of the sections. e, Esophagus; l, liver; y, yolk; g, glomerulus; pd, pronephric duct; bd, bile duct.

arnt2^{-/-} mutant liver, on the other hand, the sinusoids are dilated, are engorged with blood, and merge to form an extensive, labyrinth-like network of vascular channels (Fig. 9B, D).

Discussion

Conservation of the AHR/ARNT system across piscine, avian, and mammalian species suggests that it has physiological functions in addition to its role as a defense mechanism against environmental contaminants.⁶ Evidence from murine studies suggests that the closely related ARNT and ARNT2 homologs, in addition to mediating cellular responses to toxicants, have both unique and overlapping functions *in vivo* that are essential for normal development.¹⁴ The recent identification and characterization of *arnt1*³¹ suggests the same holds true for fish; however, little is known about the roles of either ARNT protein in the regulation of early development.

Primary function of Arnt2 may occur at the larval stage of development

Effects on startle and touch responses, brain ventricle size, heart ventricle size, cardiac rhythm, heart rate, and liver sinusoid morphology were first identified in *arnt2*^{-/-} mutants during the larval stage of development. The effects were not secondary to stunted growth nor were the mutants in a debilitated state and near death when the effects were detected. Rather, all *arnt2*^{-/-} larvae appeared to be normal upon gross examination at 120 hpf, except that the swim bladder was not inflated. It was only after careful histological and/or functional assessment of the brain, heart, and/or liver that adverse effects on these organs were discovered. Death of the *arnt2*^{-/-} larvae did not occur until 216 hpf, 4 days after the phenotypic effects were first detected. Overexpression of Arnt2x, a dominant-negative form of Arnt2, caused most microinjected zebrafish larvae to die about 96 hpf.²⁷ In the mouse, *arnt2*^{-/-} fetuses seem to develop normally upon gross observation but die perinatally.¹⁴

The late occurring onset of effects in *arnt2*^{-/-} zebrafish suggests that Arnt2 may have its primary function during larval development. This is the stage when all morphological and functional effects of the null mutation were found. The only effect detected earlier was a decrease in expression of the Arnt2 dimerization partner, Sim1, in the ventral thalamus and hypothalamus at 72 hpf. However, this is still during the larval stage of development, which supports the hypothesis that the primary function of Arnt2 occurs at this time. The alternative hypothesis is that Arnt2 is necessary and essential earlier, during the embryonic stage of development. If this is the case, the cluster of organ-specific morphological and functional responses observed at 120 hpf in *arnt2*^{-/-} larvae would be secondary or tertiary responses caused by loss of the primary Arnt2 response during embryonic development. Of course, a primary response of Arnt2 was not identified during embryonic development in the present study. Further research is needed to identify the primary function of Arnt2 and stage of development at which it occurs.

Role of Arnt2 in development of the startle and touch responses

The C-start escape response consists of orchestrated, sequential movements to effect an escape from a directed

stimulus. This reflex involves the integration of sensory input from Rohon-Beard neurons in the spinal cord and motor output from the hindbrain and a large number of interneurons.³⁷⁻⁴⁵ *Arnt2*^{-/-} larvae were predominantly unreactive to vibrational or tactile stimuli, suggesting that Arnt2 is associated with a part of the neural network that coordinates this response. Neuromasts are present in the *arnt2*^{-/-} mutants, which together with the overall intact structure of the inner ear suggests that auditory sensation, as well as vibrational sensation (lateral line), is not affected by loss of Arnt2. The mutants also appear to develop the necessary mechanosensory apparatus during embryogenesis, including the Rohon-Beard neurons, Mauthner neurons, trigeminal ganglia, and primary motor neurons, to respond to external stimuli. However, we cannot rule out the possibility that higher brain centers (e.g., brain stem neurons as described by Liu and Fetcho⁴⁰) are not affected. Foreman and Eaton⁴⁶ suggest that mechanical properties of the skeletal muscles regulate the size and timing of the ipsilateral and contralateral trunk muscle contractions that define the escape trajectory. Therefore, structural and functional aberrations in developing neuromuscular synapses could also contribute to loss of the startle and touch responses. It remains to be determined if immobility of *arnt2*^{-/-} larvae is caused by a deficit at the level of the sensory nervous system, motor nervous system, and/or skeletal muscle. Functional assessment of the sensory and motor systems of *arnt2*^{-/-} mutants will be required to determine the Arnt2 site of action. Startle and touch responses have not been assessed in *arnt2*^{-/-} mouse fetuses¹⁴ or zebrafish larvae overexpressing a dominant-negative Arnt2x.²⁷ Failure to build dorsal and ventral skeletal muscle in the pectoral fins has been reported in zebrafish larva with overexpressed dominant-negative Arnt2x.²⁷

Role of Arnt2 in brain development

Zebrafish brain development has been well characterized.⁴⁷ First the neural keel, a transient condensed primordium with no lumen, forms at the midline by epithelial infolding. Rounding of the neural keels results in a neural rod that later hollows to form the neural tube⁴⁸; however, before cavitation, regional morphogenesis occurs at the anterior neural keel that leads to brain development. During segmentation, a uniform brain rudiment gradually becomes more sculptured through the formation of neuromeres. Those that correspond to the diencephalon and telencephalon (forebrain) and mesencephalon (midbrain) appear first; the primordium of the hypothalamus and epiphysis appear from the expanding diencephalon during late somitogenesis. The tectum and tegmentum form from the division of the midbrain primordium. Seven neuromeres (rhombomeres) subdivide the hindbrain. The cerebellum is among the last regions to form, but can be seen caudally at the midbrain-hindbrain boundary before the start of the pharyngula period. During this period (36–48 hpf), *arnt2* mRNAs have been located in the zebrafish brain, specifically in the neuroepithelium and ependyma lining of the ventricles.³⁰ Similarly, Arnt2 is expressed in the murine brain during development of the paraventricular and supraoptic nuclei, important integration centers for output of the hypothalamus to the pituitary.¹⁵

Early formation of the *arnt2*^{-/-} mutant brain occurs as normal. However, by 120 hpf, enlarged brain ventricles

become a major part of the mutant phenotype in zebrafish. In *arnt2*^{-/-} mouse fetuses, the third ventricle of the brain is also enlarged. The cause of the greater brain ventricle size is undetermined, but previous studies suggest that it may be due to altered brain circulation⁴⁹ that may affect cerebrospinal fluid (CSF) formation by the choroid plexus. Little is known about CSF formation and brain ventricle development in fish. Normal enlargement of the brain ventricles during chick embryogenesis is thought to be dependent on positive CSF pressure.⁵⁰ In dogs, a reduction in circulation to the brain resulted in a decreased rate of CSF production.⁵¹ Likewise, a reduction or absence of CSF in zebrafish mutants exhibiting cardiovascular dysfunction was suggested as a possible cause of poor brain ventricle inflation.⁴⁹ Schier *et al.* proposed an association between increased blood flow or blood pressure and enlargement of brain ventricles by showing that zebrafish mutants with reduced body length such as *no tail* and *floating head* (suspected to have a shorter vascular system and increased blood flow or blood pressure) had enlarged brain ventricles.⁴⁹ Whether there is a causal association between the altered cardiovascular development and function and altered brain ventricular development in the *arnt2*^{-/-} mutants remains to be seen.

In mice the ARNT2/SIM1 heterodimer plays an essential role in the development of secretory neurons in the supra-optic, paraventricular, and anterior paraventricular nuclei of the hypothalamus.^{15,16} Secretory neurons lost from these hypothalamic nuclei regulate pituitary function by secreting oxytocin, arginine-vasopressin, thyrotropin-releasing hormone, corticotropin-releasing hormone, and somatostatin.^{15,16} Their absence in *arnt2* null mice disrupts hormonal homeostasis, and because the neurons no longer extend neurites to the median eminence and posterior pituitary, these areas are hypocellular in *arnt2*^{-/-} mice.^{15,16} It is not known if neuroendocrine cell lineages are lost from the hypothalamic nuclei of *arnt2*^{-/-} zebrafish. However, zebrafish larvae overexpressing dominant-negative Arnt2x exhibit necrosis and degeneration of the midbrain at 96 hpf.²⁷ Whether the subtle reduction in *sim1* expression in the hypothalamus and ventral thalamus of *arnt2*^{-/-} mutant zebrafish at 72 hpf in the present study is secondary to a possible loss of neurons from the hypothalamus and ventral thalamus and/or an increase in brain ventricle size is unknown. It may also be that Arnt2 is required to interact with other PAS proteins besides Sim1 in the hypothalamus and ventral thalamus to maintain normal development.

Role of Arnt2 in heart development

In zebrafish, the primitive heart tube is formed between 24 and 26 hpf. It loops at approximately 30–36 hpf, eventually placing the atrium dorsal to the ventricle with the sinus venosus developing as the inflow tract to the atrium and the bulbous arteriosus forming as the outflow tract from the ventricle.⁵² During the next few days (48–120 hpf), the heart valves form and extensive trabeculation occurs within the ventricle, which is followed by pectinate muscle development in the atrium at 120 hpf. Expression of *arnt2* begins at 36 hpf in the pericardium and vascular endothelium, and at 48 hpf it is expressed in the endocardium.³⁰ Expression of *arnt2* in the heart at these times is consistent with its potential role in early development of the heart.

Initially, gross heart morphology of *arnt2*^{-/-} mutant larvae appears normal compared with their WT siblings. The sinus venosus migrates dorsally from the base of the yolk, and the heart loops so that the ventricle and atrium are positioned along side each other in the pericardium. However, the ventricle is significantly enlarged and its wall is thinner than in WT larvae, suggesting that Arnt2 is required for normal heart development in zebrafish. This is further supported by the bradycardia and cardiac arrhythmia observed in *arnt2*^{-/-} larvae. The arrhythmia, characterized by missing beats, results in prolonged overfilling of the atrium; it shows no distinct pattern of occurrence (see Supplemental Movie) and is atypical in comparison to other mutants with heart phenotypes^{53,54} or larvae exposed to polyaromatic hydrocarbons.⁵⁵ Whether the bradycardia observed in *arnt2*^{-/-} mutants is a cardiac-specific effect of the mutation or a secondary effect caused by loss of a primary Arnt2 function has yet to be determined. In zebrafish embryos overexpressing dominant-negative Arnt2x, severe pericardial edema, defective atrium and ventricle, and a slow heart beat were observed.²⁷ To our knowledge, the *arnt2*^{-/-} fetal mouse heart has yet to be examined for phenotypic changes.

Mechanism(s) underlying the *arnt2*^{-/-} heart phenotype is unknown, but may involve alterations in gene expression or signaling in the heart that manifest later as altered ventricle morphology and altered heart physiology. For example, Arnt2 may be required to interact with PAS domains of genes important for cardiac development and/or the regulation of heart rhythm and rate. Of these genes, *hif* is a possible candidate. In mice, HIF-1 α -deficient embryos exhibit cardio bifida and abnormal neural crest migration, suggesting that HIF-1 α is necessary for cardiac development by modulating neural crest migration and ventricle formation.⁵⁶ HIF-2 α (EPAS1)-deficient murine embryos display pronounced bradycardia thought to be associated with altered catecholamine homeostasis, and die during mid-gestational embryonic development from circulatory failure.⁵⁷ Because cross talk between hypoxia signaling and the AhR pathway has been demonstrated in fish,³⁶ it is possible for dimerization to occur between Hif1 α or Hif2 α and Arnt2. Therefore, loss of a Hif/Arnt2 dimer, if it exists, could potentially lead to cardiac abnormalities as suggested by the murine studies.

Role of Arnt2 in liver development

In comparison to mammalian liver, teleost hepatocytes are rarely seen in cords or lobules. In zebrafish larva, the liver is a nonlobular, tubular organ. Sinusoids are fewer in number than in mammals and are irregularly distributed between the hepatocytes. In *arnt2*^{-/-} larvae at 120 hpf, we discovered that the liver sinusoids are dilated and appear to merge with one another to form a labyrinth-like network of vascular channels. Aberrant vascularization of the liver is not uncommon in other *arnt* null mutant vertebrates.

Several isoforms of ARNT have been identified in mammals and are termed ARNT, ARNT2, and ARNT3,^{58,59} and ARNT is the well-established dimerization partner of HIF-1 α .⁵⁹ Both *arnt*^{-/-} and *hif-1 α* ^{-/-} mice have vascular phenotypes, although the ARNT mutant appears less sensitive.^{60,61} There has been no report of a vascular phenotype in the liver of *arnt2*^{-/-} mouse fetuses. Thus far, disrupted vascular development has only been observed in *arnt*^{-/-} mice^{18,20,62} and

mice generated with a hypomorphic *arnt* allele.¹⁷ In these hypomorphs, a rare vascular phenotype was exhibited in which the ductus venosus failed to close. Injection of trypan blue showed that perfusion of the murine liver failed to occur as the dye flowed straight from the portal vein, through the ductus venosus, and out through the inferior vena cava. This was in contrast to WT mice that showed a perfusion of primary and secondary portal branches. Further, poor vasculogenesis and angiogenesis have been reported in mice deficient for ARNT.^{18,20,62}

As the zebrafish liver lacks central and portal veins, a direct comparison with the murine liver is not possible. Altered hepatic vascularization in *arnt2*^{-/-} zebrafish larvae could be the result of occluded vascular channels or sinusoid enlargement resulting from increased volume of blood passing through fewer sinusoids, or could be associated with cardiovascular dysfunction. Further, such impacts on vascular development may be associated with an absence in the expression of Hif/Arnt-regulated genes such as vascular endothelial growth factor, which is essential for normal vascular development. These hypotheses remain to be tested.

In summary, the presence of several forms of Arnt in zebrafish, along with different expression patterns, reflects their complicated regulatory paradigm. The function of ARNT2 in vertebrate development remains obscure as much of the work investigating the roles of these proteins has focused on their role in AHR-mediated responses to environmental contaminants^{58,59} and because of the fact that ARNT2 knockout mice are not viable. Characterization of the *arnt2* null phenotype in zebrafish has shown that Arnt2-mediated signal transduction pathways play key roles in heart, brain, liver, and possibly peripheral nervous system development. Further research is needed to identify the role of individual Arnt2 isoforms in producing these organ system-specific responses and to delineate the full spectrum of Arnt2 isoform-mediated regulatory events during development.

Materials and Methods

Zebrafish lines and embryos

Viable *arnt2*^{+/-} zebrafish (*arnt2*^{hi1715}) were of the TAB-14 background strain (Tubingen/AB cross no.14). This line of fish was generated in a large-scale retroviral mutagenesis screen^{35,63} and was a generous gift of Nancy Hopkins from the Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. Previously, it was shown by RT-PCR that *arnt2*^{-/-} mutants do not express *arnt2a*, *arnt2b*, or *arnt2c* mRNA.³³ Embryos were raised and maintained at 28°C as previously described⁶⁴ with daily 50% water changes.

Arnt2 genotyping

After all analyses, embryos were genotyped. Two primer sets were used for genotyping larvae as described by Prash et al.³³ Primers 1715-C5 (CGGAAATGTCGCTGTTGTTAGT-TGTG) and 1715C-3 (GAACTGAGTTTGCGCGTTTGAGAC) flank the knockout viral insertion and were used to distinguish *arnt2*^{-/-} mutants from WT and HET larvae. The 1715C-5 and SFGR (TGCGATGCCGTCTACTTTGA) primers were used to distinguish WT from HET larvae (SFGF lies within the

insert). DNA was extracted from part of the tail of each euthanized larva. Tail clips were incubated in DNA extraction buffer (0.01 M Tris, 2 mM EDTA, 0.2% Triton-X, and Proteinase-K 0.2 mg/mL) at 55°C for 2.5 h, and then heated at 100°C for 10 min before centrifugation. Sequences were amplified by PCR using GoTaq polymerase (Promega, Madison, WI), and PCR products were examined by gel electrophoresis.

Gross morphology and histology

Arnt2^{-/-} embryos and larvae were observed daily using a dissecting microscope to assess gross morphology compared to WT fish. WT and *arnt2*^{-/-} mutant larvae were fixed at 120 hpf in BT fixative (4% paraformaldehyde/4% sucrose/0.1 M sodium phosphate/0.15 mM calcium chloride⁶⁴). Fixed larvae were oriented in 1% agarose for cross or longitudinal sectioning and dehydrated in a graded series of alcohol before infiltration and embedding with JB-4 resin (Polysciences, Warrington, PA). Embedded larvae were sectioned at 4 µm and stained with Giemsa (Sigma, Saint Louis, MO). Longitudinal sections were assessed for general histopathology. Images of the heart were taken at 40× magnification with a Nikon eclipse TE300 microscope. Cross sections were assessed for heart ventricle area and heart ventricle wall thickness using Scion Imaging software. An average of eight measurements of ventricle wall thickness from each section throughout the ventricle was used to compare WT and *arnt2*^{-/-} individuals (*n* = 7 larvae/genotype).

Startle and touch response assays

Larvae (120 hpf) were placed into a 3-cm-diameter well (1 fish/well) and allowed to acclimate for 5 min before being tested. Larvae were stimulated with a series of taps (1 tap/s) on the edge of a six-well plastic culture plate containing the larvae (*n* = 6/group) as previously described.⁶⁵ Video recordings of the C-start startle reflex in WT and *arnt2*^{-/-} mutant larvae were taken using a Leica MZ16 stereomicroscope with an Optronics Microfire digital camera. Larvae were visually assessed for behavioral responses such as speed of movement, distance traveled, and changes in direction after each stimulus. The response was characterized as no response (no movement), poor response (less than one body length in distance traveled with limited motion), or good response (rapid movement throughout the chamber). A duplicate group of larvae were also subjected to a touch stimulus. Using a pipette tip, a gentle touch stimulus was applied at the head and tail of WT and *arnt2*^{-/-} mutant larvae. Behavioral responses were assessed as described above.

Whole-mount ISH

Whole-mount ISH was used to determine if the pattern or intensity of staining for *sim1* (zebrafish *single-minded* homolog⁶⁶), as well as for *krox20*, *pax6a*, and *islet1*,⁶⁷ were altered in the brain of *arnt2*^{-/-} mutant zebrafish. ISH was performed as described by Oxtoby and Jowett⁶⁸ using digoxigenin (DIG)-labeled probes (labeled in accordance with the Roche DIG RNA labeling protocol) and detected using anti-DIG alkaline phosphatase (AP; 1:4000, v:v) and BCIP/NBT substrate. Control embryos and larvae not incubated in riboprobe were also exposed to the substrate to assess possible background

staining. Stained embryos or larvae were embedded in JB-4 resin and sectioned transversely at 6 μ m.

Lateral line and inner ear sensory systems

To determine whether the number or distribution of mechanoreceptive neuromasts (receptors of the lateral line) along the body of the *arnt2*^{-/-} mutants were affected, larvae at 120 hpf were immersed in fresh water containing 250 μ M of the vital dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI; Sigma) for 5 h to stain neuromasts.^{69,70} Larvae were rinsed in DASPEI-free water and orientated in 3% methylcellulose before fluorescence imaging of the cells at 10 $\times$ magnification with a Nikon eclipse TE300 microscope. Morphological structures of the inner ear, including the semicircular canals and otoliths, were also observed.

Neuron IHC

To determine whether *arnt2* mutants have an altered neuronal phenotype, whole-mount IHC was performed on 30, 50, and 148 hpf embryos ($n=5-9$) using primary antibody supernatants for ZNP1 and 3A10 (Developmental Studies Hybridoma Bank, Iowa City, IA). ZNP1 identifies primary motor neurons and trigeminal ganglia, while 3A10 identifies Mauthner neurons. All embryos were fixed in BT-fix and dehydrated in an ethanol/PBS series and stored at -20°C. After rehydration, embryos were permeabilized with 0.1% type 1A collagenase for 15–75 min and washed with phosphate buffered saline with 0.1% Tween-20. Embryos were rinsed and then blocked for 1 h in incubation buffer (5% sheep serum, 0.2% BSA, 1% DMSO, and 0.1% Tween-20 in PBS) before incubation with 1:1 antibody supernatant/incubation buffer overnight at 4°C. After washing several times with incubation buffer, embryos were incubated in a 1:500 dilution of the appropriate secondary antibody: goat anti-mouse IgG-AP or IgG1-AP (Santa Cruz Biotechnology, Santa Cruz, CA). AP-conjugated antibodies were stained with NBT/BCIP substrate following the manufacturer's instructions. Imaging was performed using a Leica MZ16 stereomicroscope with an Optronics Microfire digital camera.

Heart rate and cardiac arrhythmia

Heart rate was determined for WT and *arnt2*^{-/-} null zebrafish at 72, 120, 125, 144, and 148 hpf ($n=4-15$ larvae/group). For each larva, heart rate was visually determined in real time during three 20-s intervals and converted to beats/min.

The number of arrhythmic events that occurred in a 30-s period for each larva at 120–125 hpf or in a 60-s period at 144 and 148 hpf was also recorded. An arrhythmic event was defined as a transient disruption in normal cardiac rhythm (see Supplemental Movie) and categorized as extra beats, missed beats, or discordant beats.

Statistical analysis

Statistical analysis of the data was performed using Statistica 6.0 software and is presented as mean $\pm$ standard error of the mean. One-way analysis of variance (ANOVA) was used to detect differences between *arnt2*^{-/-} mutant and WT zebrafish. Data were evaluated for homogeneity of variance and for normality before ANOVA. Multiple comparisons

were conducted using Dunnett's test with significant differences identified at $p < 0.05$.

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Disclosure Statement

No competing financial interests exist.

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