Gene Expression and Development of the Air-breathing Organs (ABO) in Asian Catfish and Transfer of Putative ABO Transgenes into Channel Catfish (*Ictalurus punctatus*)

by

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Abstract

Tra catfish (*Pangasianodon hypophthalmus*) and bighead catfish (*Clarias macrocephalus*) are two freshwater air-breathing Asian species that belong to the order Siluriformes. Tra catfish have a swimbladder, whereas bighead catfish have both gills and modified gill structures that serve as an air-breathing organ (ABO) to allow aerial breathing. These two species are excellent models for investigating the development of the accessory air-breathing organ in teleosts. On the other hand, channel catfish (*Ictalurus punctatus*), one of the major aquaculture species in the United States, breathes only with gills and have no air-breathing ability. They easily die of low oxygen in water resulting into a massive economic loss. In this study, larvae of tra and bighead catfish were exposed to various hypoxic environment including atmospheric air for bighead catfish larvae. Early life stages of *P. hypophthalmus* from 5 to 10 days post fertilization (dpf) were identified as the developmental time points for the swimbladder and air-breathing ability. Likewise, for *C. macrocephalus*, 3, 5, 13, 14, 16, 17 and 26 dpf were selected as important time points for the formation of ABO. Tra catfish larvae of 11 dpf demonstrated 100 % survival at 0 mg/L of dissolved oxygen whereas bighead catfish larvae showed fully functional air-breathing at 26 dpf when exposed to the atmospheric air. The advancement of the ability to survive in hypoxia along with air-breathing function was consistent with the structural development of the ABO of the corresponding species. Previously conducted gene expression profiles of the two species were correlated with the gradual development of the ABOs during the early stages life stages to reveal the potential association of these genes with air-breathing function and formation of the ABO.

Hypoxia challenge coupled with histological and anatomical observation of tra catfish revealed the critical time points for the development of air-breathing function and the swimbladder. Comparative genomic analysis between channel catfish and tra catfish along with channel catfish and bighead catfish, identified species-specific genes for tra and bighead and RNA-seq analysis based on the transition towards survival during anoxia identified the potential candidate genes for air-breathing ability in tra and bighead catfish. From this list, *Grp, Cx3cl1* and *Hrg* were identified as the most likely contributors to the formation of swimbladder and air-breathing in tra catfish whereas in bighead catfish, *Fras1* along with Mb, Ngb and Hbae were found to play key roles in air-breathing. *Cx3cl1* was chosen to detect its tissue-specific expression with *in situ* hybridization in tra catfish larvae. The mRNA of *Cx3cl1* was detected in the esophageal

tissue of 1 dpf larvae and significantly increased at 2 dpf through the entire swimbladder region. During the later stages such as 5 to 6 dpf, the expression was found to decrease gradually and detected only in the mid portion and posterior end of the swimbladder. Previously conducted histological examination also detected the presence of swimbladder at 6 dpf in tra catfish larvae. These results provide further evidence that *Cx3cl1* has a role in the structural development of the swimbladder and air-breathing in tra catfish.

Since channel catfish (*Ictalurus punctatus*) breathes only with gills and cannot breathe in the air, to modify their response to hypoxia and to improve survival, *Grp*, *Hrg* and *Cx3cl1* transgenes isolated from tra catfish and *Fras1* transgene from bighead catfish, were knocked in into channel catfish genome through CRISPR/Cas9. The transgenes were driven by their native promoters. Different integration rates of these four transgenes were obtained at different life stages of transgenic channel catfish. However, significant differences in integration rate were observed only among the live fingerlings from the four types of transgenic groups ($P < 0.05$). The highest integration rate was observed in the *Grp* transgenic fingerlings (7.32 %). The integration rates were found to be negatively correlated with the size of the transgene constructs. Precise integration of ABO transgenes in channel catfish genome was confirmed by sequencing as well as the insertion of the complete sequence of *Grp* and *Cx3cl1* transgene in several individuals despite the large size of the transgene. Individuals transgenic for *Hrg* and *Cx3cl1* had significantly lower mean body weight (35.53 to 36.02 % for *Hrg* and 22.25 to 25.65 % for *Cx3cl1*) than the corresponding non-transgenics. However, *Grp* had no pleiotropic effect on growth. Although not statistically significant ($P>0.05$), *Fras1* transgenics had observed body weight 22.24 to 23.92 % larger than the non-transgenics. Transgenic individuals demonstrated more vulnerability to enteric septicemia of catfish (ESC) but more resistance to *Ichthyophthirius multifiliis* otherwise known as 'Ich'. Alterations in the morphology of the swimbladder of a sample of *Grp* transgenic individuals supports the hypothesis of the involvement of this gene in ABO development and structure. Optimization of sgRNA design to increase activity and lower off-target effects of CRISPR/Cas9 should be examined in future transgenic research. The study enhances the knowledge base concerning the adaptation of aquatic organisms to hypoxia, the formation and gradual development of air-breathing organs (ABO) as well as preliminary insights into the production of ABO transgenic channel catfish capable of surviving in low oxygen water.

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Chapter One: General Introduction

1.1 Problem of interest:

Catfish belongs to the order Siluriformes. Being one of the most taxonomically diverse orders it possesses over 12% of all fish species (about 33,000 total fish species) and 6.2% of all vertebrates (around 64,000 total species of vertebrates),[1] including 36 families, 478 genera and more than 3,000 species of fish.[2] Morphologically, catfish are cylindrical in shape, with a large mouth with at least a pair of barbel and dorsal and pectoral fins with spines.[3] Most of the families are scaleless with few exceptions such as Loricariidae and Callichthyidae which have bony dermal plates.[3]

Catfish farming is one of the largest aquaculture industries in the United States with Alabama, Arkansas and Mississippi as the top three states.[4] In the year 2003, the catfish industry achieved its highest production of 350 million kilograms followed by a drastic decline because of economic recession, increased costs and competition from imported products.[4] Despite the hindrances, approximately 214 million kg of catfish was processed in 2010, and it ranked sixth in the 'Top 10' fish and seafood consumption in the United States.[4] However, the production dropped to 138 million kg in 2011.[5] During 2015-2017 catfish production increased slightly and reached 150 million kg in 2017[6] and continues to grow slowly with 158 million kg of catfish produced in 2019.[5]

Channel catfish are native to east of the Rocky Mountains, south of Canada, north of Mexico, south of Delaware and from the Savannah River to Lake Okeechobee, Florida. The stocking of channel catfish (*Ictalurus punctatus*) initiated in the United States but were later extensively introduced to Europe, the Russian Federation, Cuba and portions of Latin America as well as Thailand and China leading to the global production to 432, 931 metric tons in 2016.[7]

Likewise, Asian catfish such as tra (*Pangasianodon hypophthalmus*), walking catfish (*Clarias batrachus*) and bighead catfish (*Clarias macrocephalus*) are being produced in substantial amounts. In Vietnam, 2/3 of its overall aquaculture production comes from tra catfish culture.[7] Abundance of freshwater in the Mekong delta combined with technological development has led

Vietnam to be one of the largest aquaculture producers in the world with 1.14 million metric tons of annual tra catfish production, resulting in 1.4 billion dollars of export revenue.[8-9] Walking catfish is widely distributed in Asia, the Indian subcontinent and Africa as well having a great economic value as a food fish.[10] The Asian catfish, *Clarias batrachus* is one of the most widely cultured food fish of India, Bangladesh, Thailand and Philippines.[11] *Clarias macrocephalus* is an important freshwater species distributed all through Southeast Asia.[12] Asthe main aquaculture species in Thailand, *Clarias* production is about 11,000 metric tons in recent years.[7] Moreover, the hybrid between *Clarias macrocephalus* female × *Clarias gariepinus* male has been cultured for over 20 years in Thailand.[13]

Morphologically, these species exhibit significant differences. Channel catfish have a large head and a fusiform body, whereas walking catfishes have a relatively smaller head and an elongated body shape.[14] Tra catfish have a very small head compared to their body size.[15]

These Asian catfish species also have some interesting evolutionary adaptations to a variety of environmental conditions. Bighead catfish (*C. macrocephalus*) are well adapted to hypoxic environments[16] while tra catfish (*P. hypophthalmus*) can also breath air when oxygen is low in water.[17] They have accessory breathing organs that help capture oxygen directly from the atmosphere. The air-breathing capability is a big advantage for these species to combat and survive in the hypoxic environment.The bighead catfish, *Clarias macrocephalus,* use both gills and modified gill structures for air breathing and can survive and move in a terrestrial environment for a significant time and distance as log as the skin remains moist.[18-19] The tra catfish can initiate facultative air breathing with its adapted swim bladder in oxygen-depleted waters.[17] In contrast, channel catfish does not possess an air breathing organ (ABO) and cannot directly breathe air.[20] North American cultured catfish do not tolerate low oxygen levels, sometimes resulting in catastrophic mortality and economic loss, and pond aeration is required at a significant cost to prevent their death. A long-term partial solution would be to create channel catfish with air-breathing capabilities like tra and bighead catfish. The transfer of putative structural and functional ABO genes from tra and bighead catfish into channel catfish could enhance air-breathing capability.

1.2 Anatomy and physiology of air-breathing in fish:

Most of the fish groups except sturgeons (Chondrostei) and herrings (Clupeomorpha) possess air-breathing fishes.[21] Several taxa developed a range of air-breathing organs such as modified gills, opercular or branchial cavities, swimbladder, skin, pharynx, pneumatic duct, stomach or intestine, lungs as a way of adaptation.

Some species use modified gills as accessory air-breathing organ (ABO). The efferent branchial arteries of anterior first and second gill arches act as an ABO provided with sites for gas exchange. The ventral aorta derives from the heart and then splits into a dorsal and a ventral section. The ventral section carries blood to the anterior gill arches flowing through the ABO and after that the blood goes back to the heart. The dorsal one delivers blood to the posterior gill arches (third and fourth) and continue toward the circulatory system that further transports oxygen-rich blood to other tissues.[22]

In *Clarias*, the ABO consists of four sections: (1) supra-branchial chamber, (2) gill fans, (3) branched-tree shaped labyrinth organ, and (4) respiration epithelium.[23] Gill fans arise from the second and third gill arches and extend to the suprabranchial chamber where they spilt into anterior and posterior recess. The labyrinth organs originate from the second and fourth gill arches reach correspondingly into the anterior and posterior recess.[22] Respiratory epithelial cells surround the suprabranchial chamber to retain its shape. Gills and accessory respiratory organs work as an integrated part of the circulatory system.[22]

In several groups of fish oxygen secretion or uptake is taken place in physostomus (open) swimbladder. For examples, Osteoglossomorpha has swimbladders that adhere to the body wall at the dorsal surface by a honeycomb-like parenchyma, but the ventral surface is made of a sturdy membrane that lacks respiratory structures. The tra catfish, *Pangasianodon hypophthalmus* has both well-developed gills and a modified swimbladder for air-breathing. Under normoxic conditions the standard metabolism of this fish is maintained by breathing in water and air-breathing becomes effective only during hypoxia.[24-25]

Other effective air-breathing fishes include Channiformes (snakeheads) and the perciform groups Blennioidei (blennies), Gobioidei (gobies), and Anabantoidei (climbing perches and other labyrinth fish). These groups possess a physoclistic (closed) swimbladder or no swimbladder at

all. For fishes in these groups as well as swamp eel, *Synbranchus*, the preferred location of air-breathing is in the pharynx and/or gill chambers. Anabantoidei and Channiformes have developed labyrinth organs, which are very specialized suprabranchial organs that are filled in by pushing air from the mouth and discharging water or by draining water into the mouth with the force from opercular contraction.[26] Climbing perch, *Anabus testudineus*, obtains around 54% of its oxygen requirement from the air when its body weight is between 29 and 51g.[27]

Some species have modified intestine as an ABO. In tropical silurids such as *Hypostomus, Lepidocephalichthys* and *Ancistrus*, the stomach works as a respiratory organ into which air is swallowed and regurgitated.[28-32] However, in *Misgurnus anguillicaudatus*, the air is breathed in a unidirectional way through the mouth and expelled through the anus[33] with gas exchange at the posterior part of the intestine.[34] This region is well vascularized with respiratory epithelium and capillaries to act as thin air-blood barrier (0.24-3.00 um).[35]

In obligatory air-breathing lungfishes such as South American lungfish (*Lepidosiren*) and African lungfish (*Protopterus*), paired lungs extend through the body cavity,[36] and they have much reduced gills.[37-38] These fishes are bimodal breathers as they use both gills and lungs for respiration, however, they are obligate breathers since they die without access to air [38-39]. The gills and skin of *Protopterus* are efficient at CO_2 removal but contribute only 10% of the total O_2 uptake.[40] They can aestivate and survive in extreme heat or for long dry periods by reducing the metabolic rate.[39, 41-42]

1.3 Histology:

Among 519 air-breathing fishes from different taxa, 38% belong to the order Siluriformes, the catfishes.[21] The structure of the gills of *Clarias mossambicus* and *Clarias gariepinus* have been found to be similar to other teleost fish.[43-45] Both the species have gills, labyrinth organ (LO) and suprabranchial chamber membrane (SBCM) as respiratory organs.[46] Gill arches have attached gill filaments , which consists of an anterior- and a posterior row of gill filaments where the anterior row is shorter than the subsequent posterior one. Gill rakers occur on the anterior portion of the gill arch and gill fans are made of cartilaginous gill rays. Secondary lamellae around the gill filaments are divided into numerous vascular channels of pillar cells and covered with epithelial

cells.[46] The labyrinth organ (LO) serves as the ABO, and it has tree-like branches that end in plump structures. The anterior LO arises from the second gill arch and is about one-third of the size of the posterior LO attached to the fourth gill arch. Both LOs are separated by prominent bony ridges into the respective epibranchial concavities. LOs are supported by a fibro-cartilaginous core and large mucus secretory cells scattered within the epithelial cells.[46] The surface area of labyrinth organs is highly vascularized with respiratory islets that are separated by pillar-like cells. Branching and surface vascularization of the LOs increases respiratory area and efficiency.

The suprabranchial chamber membrane is also vascularized with respiratory islets separated by non-vascular tracts. The inhalant aperture that opens into the suprabranchial chamber is formed by the gill fans. The remarkable similarities between the respiratory islets of LOs and SBCMs suggest that ABOs were derived from the gills.[46] The structure of vascular channels of the secondary lamellae of gills looks much like those in LOs and SBCMs. In all cases, they are separated by pillar-like cells.

Other fishes from Gonorynchiformes,[47] Characiformes and some species in Siluriformes, such as suborder Gymnotus and tra catfish (*Pangasianodon hypophthalmus*), use the swimbladder (SB) for aerial respiration.[48-49] Characiforms has a double-chambered SB, which is supported by collagenic fibrous walls.[30] Two types of epithelial cells cover the surface of fibrous wall inside the SB. The first one has thin respiration type epithelial cells, highly vascularized with numerous red blood cells, which cover most of the surface providing the swimbladder with major sites of gaseous exchange between air and blood. The second type are non- respiratory thicker cells with a brush border.[50]

The obligatory air-breathing lungfish such as the Australian lungfish (*Neoceratodus*) has a single elongated lung compartment with a thick cartilaginous structure.[51] The epithelial lining is comprised of capillaries scattered within cells which are alveolar type I and type II like-cells. These cells have large numbers of osmophilic bodies resembling mammalian lamellar bodies, which indicates the possibility of being the ancestral cell for alveolar type I and II found in the mammalian lung.[51] Moreover, they also possess a surfactant-like material that contains both SP-A and SP-B like proteins, which implies that the proteins involved in surfactant homeostasis in modern vertebrates were present even in the primitive lung.[51]

1.4 Evolution:

Fishes that are capable of air-breathing provide crucial evidence for the evolutionary transition of life from water to the terrestrial environment.[52] Around 350 million years ago, oxygen content of natural waterbodies started to decline with the increasing temperature and consumption of dissolved oxygen by decomposition of dead organic matter.[53] Fishes began to develop air-breathing organs at critically low levels of oxygen, which enabled them to uptake aerial oxygen rather than being limited to utilizing oxygen in water. More than 370 living air-breathing fish species are scattered among 49 families found around the world and involved in some sort of aerial respiration.[54] Air-breathers can surface to gulp air and can even slither on land for a considerable time.[55] Under hypoxic conditions, air-breathing fishes can boost the gill oxygen uptake ability by absorbing oxygen from the air through increasing vascularization in the buccal cavity. The obligatory air-breathers may die if they are denied access to air despite the water being saturated with oxygen.[52] The transition from aquatic to aerial breathing required a radical transformation of respiratory organs as a result of changes in gas transportation, physiology of ion transportation and excretion of nitrogenous waste products.[56] Typical fish gills that mediate gaseous exchange in water cannot uptake oxygen from air.[57] Several taxa have developed a range of air-breathing organs such as lungs, swimbladder, modified gills, opercular or branchial cavities, skin, pharynx, pneumatic duct, stomach or intestine as a way of adaptation. Swimbladders are used to balance hydrostatic pressure and sound navigation in non-air-breathing fishes. Some obligatory air-breathers have developed a fully functional lung, such as the Australian lungfish (*Neoceratodus*), which has features resembling the mammalian lung.[51] According to evolutionary biologists, the first air-breathing vertebrates were fishes and the air-breathing sarcopterygians (lobefin) form the Devonian ages are at the base of the ancestry extending from fishes of the Paleozoic era to the modern tetrapods.[58-59] For example, the fossil of the sarcopterygian fish *Styloichthys change* exhibits the character of the common ancestor of tetrapods and lungfish.[60] A recent discovery of the fossil of another sarcopterygian fish from the late Devonian in Arctic Canada also represents an intermediate organism between fin fish and tetrapods with limbs.[61]

1.5 Functional genomics:

Gene expression changes induced by hypoxia stress may stimulate various hypoxia survival mechanisms such as reducing metabolic rate and protein synthesis in different fish species.[62-64] *C. magur* is reported to show oxy-conforming response resulting in reducing oxygen consumption and activating anaerobic glycolysis.[65] Complex I (NADH-ubiquinone oxidoreductase), which is in the inner mitochondrial membrane, is considered as the key among the coenzymes of cellular respiration or oxidative phosphorylation.[66] The lack of ND4 protein, an important subunit of five complexes of the electron transport chain, has been found to cause the complete loss of activity of complex I l.[66] As a result, it lowers the rate of cellular respiration and may cause the shift from mitochondrial to anerobic glycolytic dependent energy production.

The transcription factors such as hypoxia inducible factors (HIF) are known to be the key regulators of hypoxic cell signaling.[67] Adaptation responses mediated by HIF primarily may result in upregulation of the transcript involved in cellular processes like angiogenesis,[68] erythropoiesis,[69] and/or glycolysis. The differential expression of genes metalloproteinase inhibitor 2 (TIMP2) (up-regulated) and protein phosphatase 3 catalytic subunit (PPP3C) (down-regulated) are reported to be involved in angiogenesis.[70] TIMP2 binds to matrix metalloproteinase (MMP-2) and regulates the proteolytic activity of it at the post-translational level.[71] Long term hypoxia can suppress the expression of TIMP2 that promotes the induced expression of MMPs and lowers the expression of TIMP2 helping angiogenesis.[71] It was also found that hypoxia regulates calcium signaling pathways, and calcium along with calmodulin increases the HIF-1 transcriptional activity by interacting with the hypoxia signal transduction pathway.[72]

Stress in fishes has been directly correlated with the physiological changes such as metabolism, respiratory and immune functions.[73] The up-regulation of genes involved in chemokines, components of the complement pathways and cytokines were found to be involved with innate immunity in *C. magur* under hypoxia stress.[70] Hypoxia has also been found to start the coagulation cascade through the activation of P selectin and tissue factors.[74] Trypsins are reported to be one of the evolutionary forerunners for coagulation proteases despite being produced in the pancreas and being involved in digestion.[75] Increased apoptosis in the spleen could result from an increase in trypsin expression under hypoxia.[76] Lowering oxygen can alter cytoskeletal and endoplasmatic stress gene expression in human.[77]

1.6 Air-breathing genes in fish:

Researchers have identified a number of putative ABO genes and their functions in air-breathing process. Some of these genes are found to be specific for air breathing while others are found to be related to this activity.

a. Myoglobin (mb):

Myoglobin plays an important role in oxygen transportation to muscles and heart and facilitates diffusion of oxygen in the respiratory chain as well as supports oxidative metabolism.[78-79] Mb is expressed in mammalian cardiac muscle during early embryonic stages and increases in later stages.[80] Adult Japanese medaka (*Oryzias latipes*), a species well adapted to tolerate hypoxia, shows strong upregulation of mb when exposed to low oxygen. In zebrafish (Danio rerio), maternal mb mRNA is present in 22 hpf (hour post-fertilization) and expression increased up to 50-fold between 18 and 31 hpf followed by a further increase of 10 to 20-fold at later stages, 4-5 dpf.[79, 81] The expression of mb steadily increases with the onset of blood circulation.[79, 82] In bighead catfish, *Clarias macrocephalus*, mb showed increased expression during 3-5 dpf.[83] Similar results were also found in zebrafish.[79, 82]

b. Hemoglobin alpha embryonic-3 (hbae3):

Hbae3 is one of the genes that play an active role in cellular oxygen supply helping aerobic metabolism.[79] It increases efficient oxygen transport from the respiratory surfaces to vital internal organs such as lungs, gills and skin.[84] In zebrafish, it is reported that biosynthesis of hemoglobin acts as a protective mechanism against hypoxia.[78] Since hbae3 is critical to oxygen transportation, it should be important to air-breathing fish.

c. Neuroglobin (ngb):

Neuroglobin is reported to be involved in oxygen dependent metabolism.[85-86] During early developmental stages in zebrafish, respiration is accomplished mainly by oxygen diffusion, and ngb expression remains low. After circulation starts at 4-5 dpf, ngb also increases together with mb.[84, 87] Similar ngb expression is found for bighead catfish at 5 dpf.[83]

d. Gastrin-releasing peptide (*Grp*):

Gastrin-releasing peptide (*Grp*), a bombesin-like peptide, plays a crucial role in pulmonary neuroendocrine cells (PNEC) and embryonic lung branching.[88-89] In the human fetal lung the expression of *Grp* was first detected between 9 to 10 weeks which increased up to 25 fold higher than in mature lung reaching a plateau from 16 to 30 weeks followed by lowering to an adult level at 34 weeks postpartum.[90] The momentary increase in *Grp* expression during the embryonic lung development at around 12 weeks perhaps indicates its involvement with lung development in humans. Moreover, *Grp* stimulates the formation of primitive air saccules along respiratory bronchioles and extension of airway epithelium.[90] This gene is also reported to regulate local blood flow, glandular secretion, and the activity of smooth muscle.[91] Addition of *Grp* in human cells increased endothelial cell migration and cord formation and induced angiogenesis *in vitro*.[92] In mice, similar evidence is found for expression of *Grp* in fetal lung branching during morphogenesis at embryonic day 12.[93] There is strong evidence that *Grp* is an ABO gene since the swimbladder of air-breathing fish species is thought to be the origin of the primitive lung.

e. Fractalkine-like isoform X1 (*CX3CL1*):

Fractalkine-like isoform X1 (*CX3CL1*), otherwise known as chemokine ligand 1, has been reported to have increased expression by lung microvascular endothelium in response to hypoxia which induces phenotypic switching, proliferation, and muscle expansion in smooth muscle cells. [94] The human lung increases the air exchange area and capillary length to counteract pulmonary alveolar.[94] Ma et al. (2021) found that this gene to be a potentially important one in the development of swimbladder and air-breathing function in tra catfish.[83]

f. Histidine rich glycoprotein (*HRG*):

HRG is mostly found in plasma fluid, and is considered to play different roles in the human blood, for instance, angiogenesis, vascularization, coagulation, and immunity.[95] When angiogenesis occur, *HRG* binds to thrombospondin (TSP) and TSP-1 to prevent antiangiogenic effect of TSP-1 since it is a powerful inhibitor of angiogenesis.[96-97] Li et al. (2018) considered the genes related to angiogenesis as a way of adaptation for the air breathing organ to maintain the gas exchange efficiently for the adaptation of air-breathing fish in low-oxygen terrestrial condition.[98]

g. Cytochrome c oxidase (COX):

Cytochrome-c, a small heme protein, associated with the inner membrane of the mitochondria, plays a key role in electron transport. Cytochrome oxidase is a well-known respiratory enzyme that conserves energy released by the reduction of oxygen to water. Bremer et al. (2016) found that COX activity was increased by 4.1-fold when stimulated by cold acclimation comparative to warm-acclimated goldfish.[99] In many fish species, mitochondrial biogenesis is induced by low temperature.[100] However, Duggan et al. (2011) reported that in dace (*Chrosomus eos*), goldfish (*Carassius auratus*), and zebrafish (*Danio rerio*) not all the subunits of COX such as COX4- 1, COX5A1, COX6B1, COX6C, and COX7C are cold-responsive genes.[101]

h. PET117 Cytochrome C Oxidase Chaperone (PET117):

PET117 is a protein coding gene. In humans, it functions similarly as cytochrome c oxidase (cox) biogenesis in yeast (*Saccharomyces cerevisiae*).[102] PET117 is reported to interact with myofibrillogenesis regulator 1 (MR-1S) and with some COX subunits in presence of PET100 and assists COX biogenesis in higher eukaryotes.[103]

i. Fibronectin 1b (fn1b):

Sun et al. (2021) indicated a vital role of fibronectin 1b (fn1b) in loach (*Misgurnus anguillicaudatus*) barbel air-breathing. They found a distinctive central vascular structure in loach barbel with a short blood–gas diffusion distance similar to the posterior intestine which serves as ABO in this fish. In addition, the distance of loach barbels became significantly shorter under acute hypoxia.[104] Previously it was reported that the expression levels of fn1b in air-breathing organs of rice-field eel and snakehead fish were significantly higher than in gills.[105] Therefore, this gene may play important role in air-breathing.

j. Hypoxia inducible factors (HIF):

Hypoxia inducible factors (HIF) are known as the key regulators of hypoxic cell signaling.[67] Adaptation responses mediated by HIF primarily may result in upregulation of transcript involved in cellular process like angiogenesis,[68] erythropoiesis,[69] and/or glycolysis. Mohindra et al. (2013) has observed the up-regulated response of hypoxia inducible factors (HIF-1a and -2a) in *C. batrachus*.[106] Hypoxia also increases calcium signaling pathways and calcium

along with calmodulin increases HIF-1 transcriptional activity by interacting with the hypoxia signal transduction pathway.[72]

k. Olfactory receptor class A related 1 (ora1):

Fish can recognize odorants and other chemical compounds in water. Fish species usually possess olfactory receptor gene family of six genes- ora1-ora2, ora3-ora4 and ora5-ora6 related to olfaction.[107] Li et al. (2018) identified all the six ora genes in *Clarias batrachus* genome including 15 copies of ora1.[98] Moreover, the coelacanth (*Latimeria chalumnae*), an ancient lobe-finned fish, which is ideally close to tetrapods form evolutionary oint of view, possesses all the ora genes along with an expansion in the ora1-ora2 gene-pair.[108-109] Despite having no true air-breathing function, expansion of ora1 genes might be a way of adaptation for the transition from water to land to help recognize airborne chemicals.[98]

l. Sulfotransferase 6b1 (Sult6b1):

Sulfotransferase enzymes function increases the hydrophilicity of the target xenobiotics and other harmful contaminants for excretion by coupling with a sulfate group.[110] Being an air-breathing fish, *C. batrachus* often live in drying water, which have a higher concentration of toxic chemicals coming from land. Li et al. (2018) identified 12 copies of sult6b1 in the *C. batrachus* genome suggesting that it has tolerance to toxic chemicals.[98] *C. batrachus* also showed an overall better tolerance to three widely distributed xenobiotics than two other air-breathing fish species such as the Asian stinging catfish (*Heteropneustes fossilis*) and spotted snakehead (*Channa punctatus*).[111] This gene is not directly related to air-breathing but can improve the chance of better living both in water and land.

m. Complex I (NADH-ubiquinone oxidoreductase):

Complex I is located on the inner membrane of mitochondria and plays a vital role as coenzyme of cellular respiration or oxidative phosphorylation.[66] Lack of ND4 protein may completely diminish the activity of complex I.[66] Down regulation of this component lowered the respiration rate in *C. magur* under hypoxia suggesting the shift from mitochondrial to anaerobic glycolytic energy production.[70]

n. Metalloproteinase inhibitor 2 (TIMP2):

TIMP2 is involved in angiogenesis. It binds to matrix metalloproteinase (MMP-2) to regulate its proteolytic activity at post-translational level.[71] The expression of TIMP2 was suppressed during long term hypoxia to induce the expression of MMP-2 and favor angiogenesis in human endothelial cells.[71]

o. Protein phosphatase 3 catalytic subunit (PPP3C):

PPP3CA plays a vital role in cell proliferation and signaling cascades, facilitated out of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) in human.[112] Knocking down PPP3CA increased the vascular endothelial growth factor (VEGF) induced angiogenesis processes.[112] Nevertheless, the influence of hypoxia on the expression of PPP3C has not been thoroughly studied.

p. Complement component 3 (C3):

In *C. magur*, 13 immune responsive genes were found to be up-regulated in complement and coagulation system.[70] These pathways take action as the first line of defense against injurious stimuli and invading pathogens.[113] Among them, the complement component 3 (C3) is found to play an important role in the development of pulmonary arterial hypertension in hypoxia exposed mice.[114]

q. Cytoskeletal and endoplasmatic stress genes:

Lowering oxygen can alter cytoskeletal and endoplasmatic stress genes in humans.[77] PVALB, a calcium-binding protein, interacted with the sarcoplasmic reticulum for the muscle contraction-relaxation cycle in carp.[115] It was up regulated in *C. magur* under hypoxia.[70, 116]

r. Heat shock proteins genes:

Heat shock proteins are known to control essential cellular processes such as folding, sorting, degradation, resolubilization of proteins, and assembly of proteins into larger aggregates.[117] In C. magur, under hypoxic conditions, the transcript level of three heat shock proteins genes (HSC71, 90 alpha and 10) were highly regulated by hypoxia stress.[116]

s. Superoxide dismutase (SOD):

SOD is considered as a sentinel gene for detoxification of the oxidative stress induced harmful products.[118] Mohindra et al. (2016) found that SOD was upregulated due to hypoxia induced oxidative stress in *C. magur*.[116]

t. Trypsins:

Hypoxic stress induced inflammation or injury can cause up-regulation of trypsin in spleen of *C. magur*.[116] Inflammatory reactions have also been reported to activate the release of proteases from lysosomes.[119] Therefore, increased level of trypsin expression under hypoxia may perhaps be in response to increased apoptosis in spleen.[76]

1.7 CRISPR/Cas9 in transgenic fish:

Aquaculture industries are facing challenges developing aquatic organisms with enhanced phenotypic attributes such as rapid growth, high fertility, disease resistance, hypoxia tolerance, salinity tolerance among other traits. Investigation on molecular mechanisms of genes associated with desired traits along with sequencing and characterization of genomes has led to understanding genomic function more precisely. Various artificial nuclease systems have been created for genome editing. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the two initial techniques that were widely used as engineered nucleases.[120-121] In recent years, genome editing using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) became the most popular genome editing technology as an alternative to ZFN and TALEN. CRISPR/Cas9 is a prokaryotic immune system that can be applied to edit genes within organisms even in eukaryotes.[122] CRISPR/Cas9 contains a Cas9 nuclease and a sequence-specific CRISPR RNA (crRNA), which guides Cas9 to DNA cleavage and produces double-strand breaks at target sites, followed by cellular DNA repair resulting in desired insertions, deletions or substitutions at specific sites.[123] The rapid improvement of CRISPR/Cas9 technology has resulted in a tremendous proliferation of cell and molecular biology studies and medical research on human diseases.[122] Gene editing with targeting accuracy of CRISPR/Cas9 offered a more comprehensive model to rationalize the balance between target recognition and nuclease activation.[124] Moreover, the development of multiple engineered

sgRNAs with various target sequences can guide Cas9 to the corresponding sites within the same cells.[125] Mutation of genes which control complex traits as well as multiple members of gene families is a potential application of this technique.[126-127]

CRISPR/Cas9 knock-out system:

Gene editing in fish by disrupting gene sequence with knock-out models can be easily accomplished but the efficiency of insertion of foreign genes, known as knock-in, into endogenous genomic loci is limited.[128-129] The cell can repair an induced double-stranded break (DSB) in two ways, non-homologous end-joining (NHEJ) where there is no donor DNA and homology directed repair (HDR) which requires a donor DNA.[130-131] The NHEJ is error-prone and can cause insertion, deletion or substitution of some nucleotides which is preferred for gene knock-out. Since it occurs throughout the cell cycle and is a dominant repair system in vertebrates, the efficiency of NHEJ mediated mutation is high.[132]

Use of CRISPR/Cas9 caused successful somatic mutation at a protein deglycase (DJ-I) locus with 87 % efficiency in medaka.[131] CRISPR/Cas9-mediated knock-out zebrafish models are also being used to model several diseases.[133-134] Several desired phenotypes have been produced with loss of gene function through mutation in farmed fish.[135-138] High rates of mutagenesis have been achieved in the target protein-encoding sites of myostatin, a negative regulator of skeletal muscle mass, in channel catfish (*Ictalurus punctatus*) and red sea bream (*Pagrus major*), respectively. A 30 % enhancement of mean body weight of gene-edited channel catfish fry and a 16 % increase in skeletal muscle or the edible muscles of a market size red sea bream were generated though knocking out myostatin gene .[135, 138] The disruption of muscle suppressor gene *mstn* in common carp (*Cyprinus carpio*) has significantly whereas, other mutated genes such as sp7, runx2 and spp1 showed severe bone defects.[137] In channel catfish, up to 93 % mutation rate was obtained when the toll-interleukin 1 receptor domain-containing adapter molecule (TICAM 1) and the rhamnose binding lectin (RBL), two immune-related genes correlated with disease progression, were knocked out using CRISPR/Cas9.[138] However, further investigation is required on how TICAM 1 and RBL gene knockout effect the immune response and disease resistance of channel catfish.

CRISPR/Cas9 knock-in system:

Knock-in of exogenous DNA fragments is performed through homology directed repair (HDR). In this process a homologous DNA template is used to promote DNA repair through homologous recombination (HR) to integrate a desirable DNA sequence at the target site.[139] HDR has been reported to have lower rate of DSB repair by HR compared [140-141] to NHEJ as it occurs only during the late S and G2 phase and not throughout the cell cycle with an efficiency of homology directed repair ranges between 1-10%.[142-143] Control of the cell cycle events and regulation of expression of key repair pathway proteins can enhance the HDR,[144] but there is chance of altering the cellular response to DNA damage at other non-target sites in the genome.[145] Therefore, designing optimal DNA donor templates, such as a plasmid donor with at least 1–2 kb of total homology can provide successful integration of exogenous genes by creating large sequence changes in the presence of target cleavage.[146-147] For small sequence changes, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) sequences are used.[145] Targeting key factors such as manipulating the cell cycle phase, stimulating HDR-promoting factors, such as the RAD51 recombinase, or inhibiting NHEJ factors by small molecules or shRNA-mediated silencing have also been attempted by researchers to enhance the more precise HDR pathway and/or suppress the error prone NHEJ pathway.[148-149]

Designing an optimal donor DNA template can provide increased HDR frequencies, which is advantageous for genetic knock-in studies.[145] Hisano et al. found that modification of the donor plasmid by using short homologous sequences (20–40 bp) flanked by two sgRNA target sequences (also known as double cut donors) resulted in precise integration rate ranging between 60-77 % of an exogenous mCherry or eGFP gene into the targeted genes (tyrosinase and krtt1c19e) in zebrafish.[150] However, some researchers reported that the efficiency of recombinatorial repair increases proportionately with the length of homology arms.[136, 145, 151] The efficiency of HDR as well as precise integration rate was increased form 63 % to 77 % in 293 T cells when the length of homology arms was increased from 50 bp to 300 bp.[151] Although some studies achieved 12-58 % HDR rate by using longer homology arms that were 1 kb or more,[145, 152] shorter homology arms provide more accessibility for insertion in the target site through the simultaneous cleavage of genomic and plasmid DNA.[151]

Targeting the non-coding sequences using the CRISPR/Cas9 system:

The bulk of noncoding DNA in eukaryotic genome was thought to be junk with no known purpose.[153] Recent studies have discovered that these regions are integral to the function of cells, particularly the control of gene activity.[154-155] Noncoding sequences act as regulatory elements and as structural elements such as repetitive DNA sequences.[156] They also provide instructions for certain kinds of RNA molecules such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs) and long noncoding RNAs (lncRNAs).[155, 157] Protein coding sequences are targeted mostly to generate small insertion and deletion (indel) mutations to disrupt the open reading frames of protein coding genes.[125, 136] On the other hand, mutation of noncoding sequences using CRISPR/Cas9 system is difficult since small indels caused by a single mutation sometimes do not produce loss of function.[158] Since non-coding sequences are intended to affect the expression of neighboring or distant genes through signaling, guiding, sequestering or scaffolding molecules, targeting these regions either through loss-of-function or gain-of-function approaches is sometimes helpful .[159-160] However, this approach might affect multiple genes resulting in off-target effects. Some studies have reported successful large genomic deletion in mammalian cells and animal models such as mouse and zebrafish by using dual guide RNAs (gRNAs).[161-162] Deletion mutations of up to 900 bp were reported recently in channel catfish targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) using a single guide RNA.[138] Moreover, desirable non-coding sequences mutants were generated in Nile tilapia (*Oreochromis niloticus*) through CRISPR/Cas9 system .[158] Some researchers used single or dual gRNA guided Cas9 nuclease and short ssDNA as a donor to create specific deletion of noncoding sequences including microRNA and 3' untranslated region (UTR) in tilapia.

1.8 Off-target effects in CRISPR/Cas9 system:

Despite being an efficient and versatile tool for a wide variety of genome editing applications, off-target effects are a major concern for the CRISPR/Cas9 system. These occur mainly due to the unintended cleavage of DNA at sites whose sequences show mismatches with the guide RNA (gRNA).[163] It can often lead to inadvertent effects on the organism.[164] In many instances, non-recognition of Cas9/gRNA of more than three mismatched DNA sites; Cas9/gRNA

does not recognize and edit DNA sites with any number of mismatches (within 10–12 bp) near the protospacer adjacent motif (PAM) and the higher the Cas9/gRNA concentration, the greater the possibility of off-target effects.[165-166]

The off-target effects can be minimized by modifying the gRNA to create a truncated version of less than 20 nucleotides which impairs the gRNA-DNA duplex stabilities at the off-target sites and improves target specificity and decreases off-target cleavage by 5000-fold.[166-167] Another method is to control the concentration of Cas9-gRNA complex by titrating the amount of Cas9 and gRNA delivered into the cells.[168] It may improve the specificity of Cas9 to cleave DNA at the target cut site.[165] Direct delivery of purified Cas9 protein and gRNA is important and can reduce off-target effects because Cas9-gRNA complex cleaves chromosomal DNA almost immediately after introduction to cells and degrades rapidly.[168-169] Moreover, use of Cas9 nickase mutant or dimeric Cas9 protein complexed with pairs of gRNAs, may strengthen the on-target stability of the CRISPR/Cas9 system.[170] In this way double checkpoints are initiated for target recognition by increasing the number of mutated base pairs at the target site, thus reducing off-target frequency.[170-171] Yet, these strategies could present challenges in gene delivery since they need complex components of the CRISPR/Cas9 system.[172] Off-target effects might also be cell-type-specific and dependent on the integrity of double-stranded breaks (DSBs) repair pathways of a particular cell type.[173] This would not be a problem with early embryo cells since they are not differentiated.

1.9 *In situ* hybridization (ISH):

The *in situ* hybridization **(**ISH) is used to detect specific nucleic acid sequences in morphologically preserved cells or embryos. It connects microscopic topological information to gene activity at the DNA or mRNA level. ISH to whole-mount embryos is a method commonly used to illustrate the expression pattern of developmentally regulated genes. Initially, around the 1980s, radio-actively labeled DNA probes were used in ISH to detect expression of genes on histological sections, and were extensively used in many model organisms including zebrafish.[174] During the 1990s, chemically labeled antisense RNA probes made the analysis of gene expression patterns in whole-mount tissues or embryos quicker and easier.[175-177] Use of *in vitro* synthesized

RNA tagged with digoxygenin uridine 5′-triphosphate to determine gene expression patterns gradually made the ISH protocol even strong and consistent.[178] It allows visualization of gene expression at single-cell resolution, including genes expressed at low levels. After hybridization, the transcript is visualized immunohistochemically using an anti-digoxygenin antibody conjugated to alkaline phosphatase, the substrate of which is chromogenic. Expression patterns of more than 10,000 zebrafish genes have been established by using this protocol.[179-180] The highest mRNA expression of C9 was detected in the liver of southern catfish (*Silurus meridionalis*) with RT-PCR and *in situ* hybridization using digoxigenin (DIG)-labeled RNA probe indicating that liver was indeed the main supplier of C9.[181]

1.10 Objectives:

The objectives of this study were to describe the anatomical development of the air breathing organ in tra and bighead catfish, to correlate anatomical development to air breathing gene expression and to utilize *in situ* hybridization (ISH) to verify the tissue specific expression of a putative ABO gene of tra catfish. The next goal was to transfer putative ABO genes from tra and bighead catfish and determine any phenotypic effects from the ABO transgenes in channel catfish.

Chapter Two: Anatomical Development of Air-breathing Organs (ABO) in Asian Catfish

2.1 Abstract:

Tra catfish (*Pangasianodon hypophthalmus*) and bighead catfish (*Clarias macrocephalus*) are two freshwater fish species of the order Siluriformes with air breathing capabilities. *P. hypophthalmus* has a swimbladder while *C. macrocephalus* has both gills and modified gill structures serving as an air breathing organ (ABO) to allow aerial breathing. These two species provide excellent models for studying the development of the accessory air-breathing organ in teleost fish. In this study, larvae of these species were exposed to various hypoxic environment including atmospheric air for bighead catfish larvae. Six early life stages of *P. hypophthalmus* from 5 to 10 days post fertilization (dpf) were identified as the time of development of swimbladder and air-breathing ability and seven development stages such as 3, 5, 13, 14, 16, 17, 26 dpf were selected as important time points for formation of accessory air-breathing organ (ABO) in *C. macrocephalus*. 11 dpf tra catfish larvae obtained 100 % survival at 0 mg/L of dissolved oxygen whereas bighead catfish larvae of 26 dpf demonstrated fully functional air-breathing when they were exposed directly into air. The development of the ability to survive in hypoxic condition as well as air-breathing function was consistent with the structural development of the ABO of the corresponding species. The gradual development of these organs in early stages of life was correlated to previously conducted gene expression profile of the two species to indicate the prospective association of these genes with air-breathing function and formation of ABO. This study provides insights to the adaptation of aquatic organisms to hypoxia, and formation and gradual development of air breathing organs (ABO).

2.2 Background:

Teleost possess a range of air-breathing fishes except for sturgeons (Chondrostei) and herrings (Clupeomorpha).[21] Several taxa developed a range of air-breathing organs such as lungs,

swimbladder, modified gills, opercular or branchial cavities, skin, pharynx, pneumatic duct, stomach or intestine as a way of adaptation.

Among 519 air-breathing fishes from different taxa, 38% belong to Siluriformes, the catfish order.[21] The catfishes are bottom dwelling organisms that often live in a poorly oxygenated environment. Some use their swimbladder as an air-breathing organ. The swimbladder is divided into two parts- the anterior and the posterior. The anterior part is attached to the Weberian apparatus that aids in hearing. The tra catfish (*Pangasianodon hypophthalmus*), a facultative air-breather, has modified swimbladder for air-breathing. The walking catfish, *Clarias*, has branched organs that extend from the branchial chambers, in contrast to the swimbladder strategy. However, *Heteropneustes*, the stinging catfish, has unbranched organs that are well penetrated into the muscles forming a sac like structure.[23, 182-184]

In several groups of fish, oxygen secretion or uptake takes place in the physostomus (open) swimbladder. For example, Osteoglossamorpha has a swimbladder that adheres to the bodywall at the dorsal surface with a honeycomb like parenchyma, but the ventral surface is made of sturdy membrane that lacks respiratory structures.

Effective air-breathing fishes include Channiformes (snakeheads) and the perciform groups Blennioidei (blennies), Gobioidei (gobies), and Anabantoidei (climbing perches and other labyrinth fish). These groups possess a physoclistic (closed) swimbladder or no swimbladder at all. In these groups as well as swamp eel, *Synbranchus*, the preferred location of air-breathing is the pharynx and/or gill chambers. Anabantoidei and Channiformes have developed very specialized suprabranchial organs known as labyrinth organs, which are that are filled in by pushing air from the mouth and discharging water or by draining water into mouth with the force from opercular contraction.[185] The respiratory surface in the climbing perch (*Anabas*) is the modified gill chamber epithelium. However, in walking catfish (*Clarias*) the respiratory surface is developed from the secondary lamellae of modified gill arches, which extend into branched organs.[182] There are similarities among the fine gas-exchange surfaces in the genera *Channa, Clarias* and *Anabas* despite being taxonomically different. Each of them has complicated superficial blood channels that strongly force blood cells towards the exchange surface resulting in a thin diffusion distance of less than 0.5 μm.[186] In this experiment, embryos and larvae of tra and bighead catfish were dissected to observe the morphological changes of ABOs and to correlate

the subsequent development of ABOs with their behavior and survival during hypoxia challenge in different environment.

2.3 Materials and methods:

2.3.1 Ethics statement:

All experimental procedures involving animal care and tissue collection were approved by the Auburn University and Can Tho University Institutional Animal Care and Use Committees. All animal related procedures were performed following the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the United States and in Vietnam.

2.3.2 Experimental animals and tissue collection:

Tra catfish and bighead catfish embryos were produced at Can Tho University, Vietnam. Tra catfish embryos were hatched within 1-day post fertilization while hatching of bighead catfish embryos were completed in about 24-30 hours. Samples were collected every 24 hours over a 30-day period post-hatch based on a hypoxia challenge experiment where dissolved oxygen (DO) concentration was gradually reduced from 4 to 0 mg/L. A total of 20-50 eggs/embryo/fry from both species were collected at each sampling. Three sets of samples were taken at each time point and later used for anatomical and gene expression analysis. At each sampling, eggs/embryos/fry were euthanized with 200 ppm buffered MS-222, and then stored as three separate sets. The first set was placed in 4% paraformaldehyde and sealed with screw top lids[187] and the second and third set were preserved in 70% methanol and RNA-later solution (Thermo Fisher Scientific). Samples were shipped to the US on dry ice and immediately preserved at appropriate temperature upon arrival. The 4% paraformaldehyde samples were kept at room temperature, whereas methanol and RNA-later samples were kept at -20°C and -80°C respectively.

2.3.3 Hypoxia challenge:

Hypoxia challenge for both species was done previously as a part of this study. Experimental animals were exposed to a hypoxic environment to monitor their behavior and survival.

Anoxia challenge for tra catfish (*Pangasianodon hypophthalmus*)

Anoxia challenge was performed at Can Tho University, Vietnam, to assess the tolerance of tra catfish and their behavioral changes to anoxic conditions at 27°C. Tra catfish larvae were challenged each day from 2-12 dpf (days post fertilization). A group of 20 larvae was stocked in a two-liter container with aeration at the beginning and then the dissolved oxygen level was gradually lowered by bubbling nitrogen gas into the water until a 0 mg/L dissolved oxygen concentration was reached. Dissolved oxygen concentration was measured by DO meter. Control treatment was set by placing another group of 20 larvae into a second container of the same volume provided with oxygen. Dissolved oxygen levels, larval behavior and survival rate were measured and recorded at 15 minutes interval. The experiment was continued daily up to 12 days post-fertilization when all 20 fish survived without oxygen supply. The fish demonstrated facultative air breathing by this time point.[188]

Anoxia challenge for bighead catfish (*Clarias macrocephalus*)

Two different oxygen challenge experiments were done at Can Tho University, Vietnam, to determine the air breathing ability of *C. macrocephalus*. After completion of yolk sac absorption, larvae of age 3 dpf were challenged with low dissolved oxygen each day from 3 to 20 dpf. Fifteen to twenty larvae were kept into a two-liter container provided with oxygen as a control treatment. Another group was kept in a similar set up without oxygen supply and dissolved oxygen level was reduced by bubbling nitrogen gas into the water until the DO concentration reached to 0 mg/L. Dissolved oxygen concentration was measured by DO meter. Dissolved oxygen levels, larval behavior and survival rate were monitored and recorded every 10 minutes interval. The results of response to hypoxia were represented graphically for different time points. Since adult *Clarias* can leave the water and move on land and live for a substantial amount of time if the skin remains moist, another oxygen challenge experiment was conducted in a petri dish to mimic that situation. Larvae were exposed directly to the air and challenged each day from 3 dpf to 27 dpf. A group of 15-20 larvae were placed in a petri dish without water and their skin was kept wet by adding drops of water while forcing the fish to obtain O_2 from the air. A control treatment was set up in a in an aerated two-liter water-filled tank as before. Likewise, larval behavior and survival rate were also monitored and documented every 10 minutes.[83]

2.3.4 Staining of larval fish:

Bighead catfish larvae were chemically stained to have better visibility of the gill tissue during dissection while no stain was used for tra catfish larvae. Staining of larval fish samples was performed according to a standard protocol for whole-mount bone staining of small fish with some modifications.[189] Alcian Blue and Alizarin Red S were used as the staining solution. Alcian Blue stains the cartilage while Alizarin Red S is used to stain the bones.

Fish larvae were fixed in 4% paraformaldehyde and shaken for 72h on a horizontal shaker at room temperature for 72 hrs. Fixation solution was discarded after that. Samples were immersed in 30% ethanol for 10 minutes and then in 50% ethanol for another 10 minutes.

Samples were stained in Alcian Blue stain solution at room temperature overnight with gentle agitation on a shaker. Alcian Blue powder was dissolved in 50% ethanol and incubated at 37°C and shaken until it totally dissolved. Then 95% ethanol was added until the final concentration of ethanol was 70%, and the final concentration of Alcian Blue was 0.4%. A 100 ml Alcian blue stain solution consisted of 5ml 0.4% Alcian Blue, 70ml 95% ethanol, 12ml $MgCl_2$ and 13ml deionized water.

Samples were rehydrated in 50% ethanol for 10min, then 30% for 10min, and finally in deionized water for 2 hours. After rehydration, samples were digested in 50mg/ml trypsin for 12-16h. After digestion, 1% KOH was added for the preliminary transparent treatment for 1-2 hours. Then samples were kept in 60% ether for an hour for degreasing.

After degreasing, 3ml Alizarin Red staining solution was added to each sample and stained overnight. Then 0.5g of Alizarin Red S powder was dissolved in 100ml deionized water to make 0.5% Alizarin Red S. Alizarin Red staining solution was made up of 0.4ml 0.5% Alizarin Red S and 10ml 0.5% KOH.

After removing the staining solution, 3ml of deionized water was added and inverted few times to mix well. Next, 3ml of 1.5% bleaching solution was added. After that, the samples were kept at room temperature for 30 minutes keeping the lid of the centrifuge tube open.

Bleaching solution was discarded, and the samples were kept in 3ml of transparent agent 1 for 12-16 hours and then in transparent agent 2 for another 12-16 hours (transparent agent 1: 20%

glycerol and 1% KOH, transparent agent 2: 50% glycerol and 0.25% KOH). Skeletal structure and development status was observed under the dissecting microscope.

2.3.5 Dissection of swimbladder and ABO of larval fish

Based on previously performed hypoxia challenges and the response of the fish to hypoxic environment, certain developmental time points were selected for dissection of swimbladder and ABO of larval fish. For tra catfish larvae 5 to 10 dpf were selected, whereas for bighead catfish 3, 5, 13, 14, 16, 17, 24, 26, 28 and 30 dpf were taken.

Dissection of swimbladder of tra catfish:

Tra catfish larvae were transferred from 4 % paraformaldehyde and transferred to a 2 % agar plate with tweezers. The entire fish was submerged in 3-4 mL PBS and gently laid on its side. To manipulate larvae easily under the microscope and to minimize light refraction, the volume of PBS on the working surface (petri dish with agar) was adjusted. The petri dish was placed under a dissecting microscope with 25X magnification. The fish trunk was held with tweezers for stabilization. The abdomen was ripped longitudinally from the anus to the operculum region with another pair of sharp tweezers. The skin and muscles on one side of the body were removed slowly and gently to expose the internal organs. This step needs time and caution to not damage the organs. Visceral organs ventral to the swimbladder were removed very carefully without damaging the delicate swimbladder. Some gonadal tissue attached to it was also removed. The swimbladder begins at the posterior region of the head and extends beyond the anus. In tra catfish, the swimbladder has a cone shaped structure (Fig. 5).

Dissection of modified gills of bighead catfish:

The preparation for dissection of the bighead catfish larvae to be dissected was the same as described above for tra. After securing with tweezers, the fish was cut halfway, and the trunk was separated from the anterior portion of the body. Then, the operculum was slowly removed on one side to expose the gill structures. The same procedure was repeated to open the gills on the other side. The tissues around the anterior part of the intestine that are connected around branchial chamber were removed. The modified gills/labyrinth organ were located inside the supra-branchial chambers attached to the 4th gill arch. Surrounding tissues were removed very carefully without

tearing the supra-branchial chambers. To remove the tissues between the skull and gills, the anterior part of mouth was held with tweezers in one hand and slowly the gills were pulled upward with another pair of sharp tweezers. Gentle pressure had torn the tissue underneath the gills. After extracting the gills, the supra-branchial covers were removed cautiously. This step was critical as the modified gills inside would otherwise be broken. The supra-branchial chambers are made of a very thin layer of epithelial tissue and the modified gills are connected closely to them. Being thin and delicate these structures reduce the diffusion distance for gaseous exchange.

2.4 Results:

2.4.1 Anoxia challenge for tra catfish (*Pangasianodon hypophthalmus*):

A low oxygen challenge was performed to assess the survival of tra catfish in anoxic conditions as a function of age.[187] Details are attached in Appendix A. At 2 dpf, when the oxygen was declined to 0 ppm, the survival of tra larvae was 0% (Figure 1-A). Larvae swam normally after removal of aeration from the container at the beginning. After 15 minutes, when the dissolved oxygen level dropped below 2.1 mg/L, the fish showed signs of oxygen stress by swimming rapidly at the surface. The movement of fish was reduced dramatically at 1.3 mg/L DO after 30 minutes. Fifteen minutes later, all fish stopped swimming and sank to the bottom and died at 0.7 mg/L DO.

At 4 dpf, within 15 minutes of removal of the aeration, the oxygen concentration dropped quickly from 5 mg/L to 1.5 mg/L and fish started to swim sluggishly. After 30 minutes from the beginning, the fish were observed to swim rapidly near the surface when the dissolved oxygen level dropped to 1.1 mg/L. After 45 minutes, half of the fish dropped to the bottom of the tank at 0 mg/L DO but no fish died at this point. The dissolved oxygen concentration was continued at 0 mg/L up to 55 minutes and all the tra catfish larvae were confirmed to be dead.

The larvae exhibited similar behavior in hypoxic conditions at 6 dpf. The challenge began with a dissolved oxygen level of 4.7 mg/L. After 15 minutes, oxygen concentration was reduced to 1.2 mg/L and the fish were actively swimming at the surface water. DO was dropped to 0.7 mg/L within next fifteen minutes. At 45 minutes, the DO concentration reached at 0 mg/L and 50% of the population fell to the bottom. None of them could survive after 60 minutes.

Throughout the time, the fish showed a gradual increase in the ability to survive in hypoxia. At 8 dpf, the oxygen challenge started at 4.6 mg/L when the fish were actively swimming within the container. Dissolved oxygen level dropped from 0.9 mg/L to 0.6 mg/L within 15 to 30 minutes from the beginning. At 45 minutes, when the DO level decreased to 0 mg/L, thirty percent of the fish dropped to the bottom. At 60 minutes, 70% of the fish were dead followed by 100% death within the next 10 minutes.

At 9 dpf, the challenge began at 5.4 mg/L dissolved oxygen. The oxygen concentration was gradually lowered to 1.6 mg/L, 0.8 mg/L and then within 45 minutes from the beginning and fish were actively swimming near the surface. The DO concentration was decreased to 0 mg/L after 50 minutes. At 75 minutes, 27% of the fish were found dead. The mortality remained the same over the next 50 minutes followed by an additional 13% death. The challenge was ended with a 60% survival at 120 minutes. From this point onwards, a large portion of the larval fish remained alive for an extended period.

When the fish were 10 dpf, the challenge set up at an oxygen concentration of 4.6 mg/L. Fish were observed to swim actively throughout the container at 0.9 mg/L of DO for the first 15 minutes. Half of them were found to swim at the surface showing signs of stress when DO concentration dropped to 0.5 mg/L at 30 minutes of the experiment. Fifteen minutes later, all the fish started gulping air at the surface at 0 mg/L of dissolved oxygen. At 55 minutes, 13% of the fish died followed by additional 7% death within the next 10 minutes. The challenge was ended after 120 minutes with 80% survival of tra catfish larvae at 0 ppm DO.

At 11dpf the challenge began at 4.6 mg/L. Fish were actively swimming at the bottom of the container as before for the first 40 minutes when the dissolved oxygen concentration decreased from 1 to 0.5 mg/L. After 50 minutes, the dissolved oxygen level fell to 0 mg/L and fish came up to the midwater column and were swimming actively, sometimes gulping air near the surface. The challenge was stopped after 120 minutes with 100% survival of the fish.

During this experiment, the temperature was maintained at 27 ℃. Fish showed behavior indicative of stress at DO concentration less than 2 mg/L for 15 min at early stages of life around 6 dpf. No survival was found when the anoxic (0 mg/L) condition was continued for 25 min. Nevertheless, some larvae of this age had reached to a point of gas exchange activity since they displayed air gulping as a mechanism to survive hypoxia. Larvae of 8 dpf obtained 100 % survival

at 0.6 mg/L DO and sustained for a while at 0 mg/L. Form 9 dpf, a significant increase in survival rate (60 %) at 0 mg/L DO was observed and continued to increase during the following days. At 10 dpf and 11 dpf, survival rate of the larvae was 80 % and 100 %, respectively, when the challenge was continued up to 120 min. Behavioral changes in response to hypoxia as well as increased survival rate over the developmental stages indicate that during 9-10 dpf, air-breathing ability was well developed and by 11 dpf, tra catfish larvae obtained complete air-breathing ability along with fully developed swimbladder.

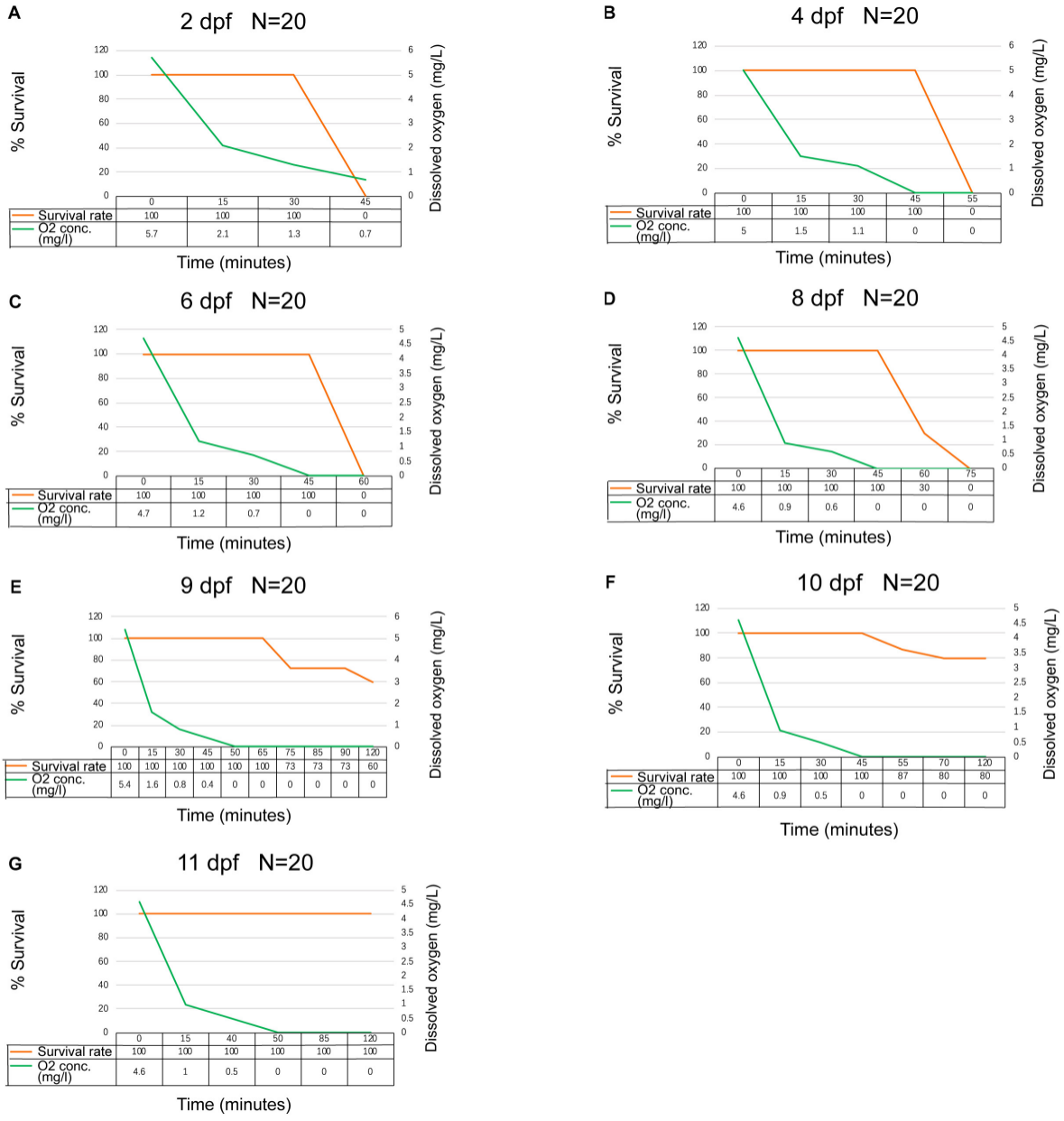

Figure 1. Anoxia challenge for tra catfish (*Pangasianodon hypophthalmus*) at seven early developmental stages (adapted form Ma et al (2021)). Dissolved oxygen concentrations (mg/L) and survival curve for tra catfish at 2, 4, 6, 8, 9, 10, and 11 days post fertilization (dpf) (A–G), during a low dissolved oxygen challenge. Dissolved oxygen was reduced by bubbling nitrogen into the water to strip oxygen. Catfish were considered as moribund when the opercular movement stopped.[188]

2.4.2 Anoxia challenge for bighead catfish (*Clarias macrocephalus*):

Low oxygen challenge experiments were performed to assess the survival of *C. macrocephalus* in 0 ppm dissolved oxygen (DO) (Appendix B) and in an aerial environment (Appendix C). The changes in survival rate over time is illustrated in Figure 2.

At 3dpf, the challenge was started at 4.15 mg/L dissolve oxygen (Fig. 2-a). At the beginning the fish were actively swimming at the bottom of the container. After 50 minutes, when the DO was dropped to 0.26 mg/L, the survival rate was 93.3 % and most of the fish came to the surface and few stayed at the bottom. DO was dropped to 0.20 mg/L within the next 20 minutes when most of the fish became weak and fell to the bottom. The movement of the fish was stopped after 70 minutes from the beginning of the experiment at 0.19 mg/L of DO followed by 100 % death at 0.17 mg/L of DO within the next 8 minutes.

For 5 dpf larvae, the oxygen concentration dropped from 4.3 to 2.02 mg/L within 15 minutes after removal of aeration and fish were swimming at the bottom. They continued to swim slowly, and some came up to surface when dissolved oxygen level dropped from 2.02 to 0.43 mg/L within the next 60 minutes (Fig. 2-b). After 25 minutes, all the fish started to swim slowly near the surface when the concentration of dissolved oxygen was 0.35 mg/L. Within the next 5 minutes, DO level dropped to 0.33 mg/L when 3 fish died while others were erratically swimming and gradually becoming weak. All fish fell to the bottom and stopped moving within the next 15 minutes when DO was reduced to 0.31 mg/L. The challenge was terminated after 125 minutes form the initiation with 100 % mortality at 0.30 mg/L dissolved oxygen.

The dissolved oxygen challenge was started at 3.85 mg/L for 13 dpf fish when they were found actively swimming within the container (Fig. 2-c). 45 minutes later, the dissolved oxygen level dropped to 0.67 mg/L, and some of them started to swim up to the surface and moved slowly. At 80 minutes the dissolved oxygen level decreased to 0.36 mg/L having all fish moving slowly at the surface and 80% survival was reported at this stage. Within the next 45 minutes, DO was lowered to 0.2 mg/L causing some fish to die while others were very weak. All fish stopped moving and fell to the bottom and died within the next 30 minutes at 0.1 mg/L of DO.

At 14 dpf, the challenge was started at 4.55 mg/L dissolved oxygen. Within 15 to 45 minutes oxygen level was reduced from 2.33 to 0.68 mg/L. Most of the fish were slowly swimming at the bottom while few others came up to the surface. At 80 minutes, the DO concentration decreased to 0.38 mg/L and all fish came up to the surface moving slowly. After 10 minutes from then, the DO level was decreased to 0.32 mg/L with three fish died. Within next 10 to 20 minutes, fish started to fall to the bottom and the survival rate was decreased from 80 to 53.3 % (Fig. 2-d). DO was decreased further to 0.13 mg/L within the next 10 minutes with 20 % survival of the fish. All fish died within following 10 minutes. The challenge was concluded at 0.09 mg/L dissolved oxygen.

When the fish were 16 dpf, the challenge was initiated with a DO level of 4.41 mg/L, and they were swimming normally. 30 minutes later, oxygen concentration dropped to 0.98 mg/L and some fish swam to the surface and moved slowly. At 80 minutes, the DO level was decreased further to 0.37 mg/L when the first fish died (Fig.2-e). Within the following 50 minutes, DO was decreased from 0.37 to 0.17 mg/L and survival rate was also decreased from 93.3 to 26.7 %. During this time, most of the fish fell on the bottom and eventually died. Mortality remained the same for the next 10 minutes, but fishes were becoming very weak. Within the next 25 minutes, DO was dropped to 0.08 mg/L and all fish were dead.

The oxygen challenge began at 4.38 mg/L with 17 dpf fish. The dissolved oxygen concentration dropped to 0.47 mg/L within 60 minutes from the beginning. DO dropped to 0.28 mg/L at 110 minutes with 93.3 % survival (Fig. 2-f). Over the next 35 minutes, DO concentration was dropped to 0.13 mg/L and most of the fish became very weak to move and survival was 20 %. The challenge was continued up to 162 minutes at 0.08 mg/L with no survival.

At 21 dpf, the DO concentration was 5.03 mg/L at the beginning of the challenge and the fish were actively swimming through the water column. After an hour, fish started to move slowly at the surface when DO was dropped to 0.44 mg/L. All fish were observed to come to the surface within the next 45 minutes at 0.17 mg/L of DO. Some fish continued to fall and turn back to the surface without dying up to 135 minutes of the experiment at 0.12 mg/L DO. After 5 minutes, when DO concentration dropped to 0.11 mg/L, they started to die (Fig. 2-g). Some fish were also observed to jump out of the surface at 0.05 to 0.07 mg/L DO. All fish were dead at 0.03 mg/L after195 minutes. Fishes of 24 dpf were observed to have similar behavior and mortality pattern like 21 dpf (Fig. 2-h).

At 25 dpf, the challenge was started with 4.31 mg/L of DO. Within the next 170 minutes, DO was gradually dropped to 0.04 mg/L with no mortality. During this time, fish exhibited similar behavior like 21 and 24 dpf in response to hypoxia. However, the ability to withstand the hypoxic condition was significantly increased since the first mortality was observed at 0.04 mg/L of DO after 175 minutes from the initiation. The challenge was terminated at 420 minutes with 53.3 % survival at 0.03 mg/L of dissolved oxygen (Fig. 2-i).

At 26 dpf, the challenge began with 4.57 mg/L of DO. In the next 170 minutes, DO was slowly dropped to 0.04 mg/L without any death. Fish were observed to respond in the same way as 25 dpf. The first mortality was observed at 0.02 mg/L of DO after 305 minutes from the initiation. However, the challenge was terminated at 420 minutes with 46.7 % survival at 0.03 mg/L of dissolved oxygen (Fig. 2-j)

A similar trend was observed in terms of the behavior and mortality during the hypoxia challenge for 28 and 30 dpf larvae with significant increases in survival rate. In both cases, the challenge was concluded at DO concentration of 0.03 mg/L with 60 % and 86.7 % survival of the fish respectively (Fig. 2-k and l).

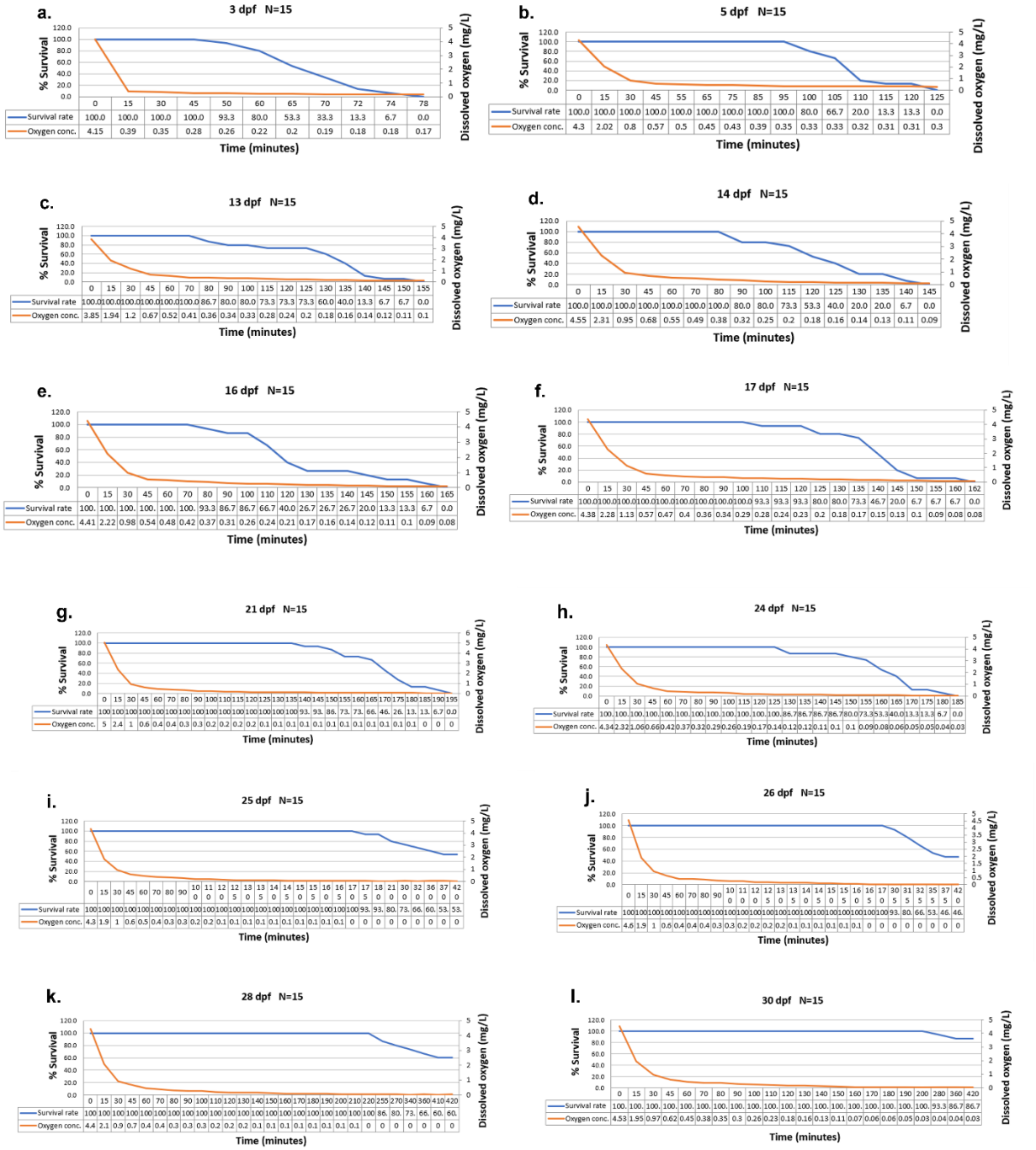

Figure 2. Anoxia challenge for *Clarias macrocephalus* at 12 early developmental stages. Dissolved oxygen concentrations (in blue, right y axis) and survival curve (in orange, left y-axis) for *Clarias macrocephalus* at 3, 5, 13, 14, 16, 17, 21, 24, 25, 26, 28 and 30 days post-fertilization (dpf) was plotted at the low dissolved oxygen challenge (a-i). Dissolved oxygen level was reduced

by replacing with the nitrogen stripping. Fish were considered as moribund when the opercular movement stopped.

Along with the tank experiment, another challenge experiment was done where *Clarias macrocephalus* larvae were exposed directly to the air in a petri dish, starting from 3 to 30 dpf. At 3 dpf, challenge was started with 30 fish (two petri dish with 15 fish each) and two fish died within 20 min followed by four more after 30 min (Fig. 3-a). By 80 minutes, the rest of the fish died. Similar trend was observed for 5 dpf larvae (Fig. 3-b) but the survival rate within the first hour was more than double compared to 3 dpf (86.7 % vs 40 %). At 13 dpf, all the fish survived during the first 40 minutes which is 20 minutes longer than at 5 dpf (Fig. 3-c). The first mortality was found at 40 minutes and none of them survived after 135 minutes. Similar results were found at 14 dpf (Fig. 3-d) and 16 dpf (Fig. 3-e) when the ability to survive in the air was increasing gradually since the first dead fish was found at 60 min and 100 min, respectively. For 14 dpf, no fish survived after 150 min. On the other hand, 36.7 % fish survived the entire 7 h experiment at 16 dpf. In 17 dpf and 24 dpf, a large proportion of fish remained alive throughout the 7 h challenge, with a 43.3 % and 56.7 % survival rate, respectively (Fig. 3-f and g). The survival rate was increased in the later stages of life; for example, 24 dpf (83.3 %) and 25 dpf (96.7 %) (Fig. 3-h and i). From 26 dpf onwards, 100 % survival of the fish was obtained (Fig. 3-j).

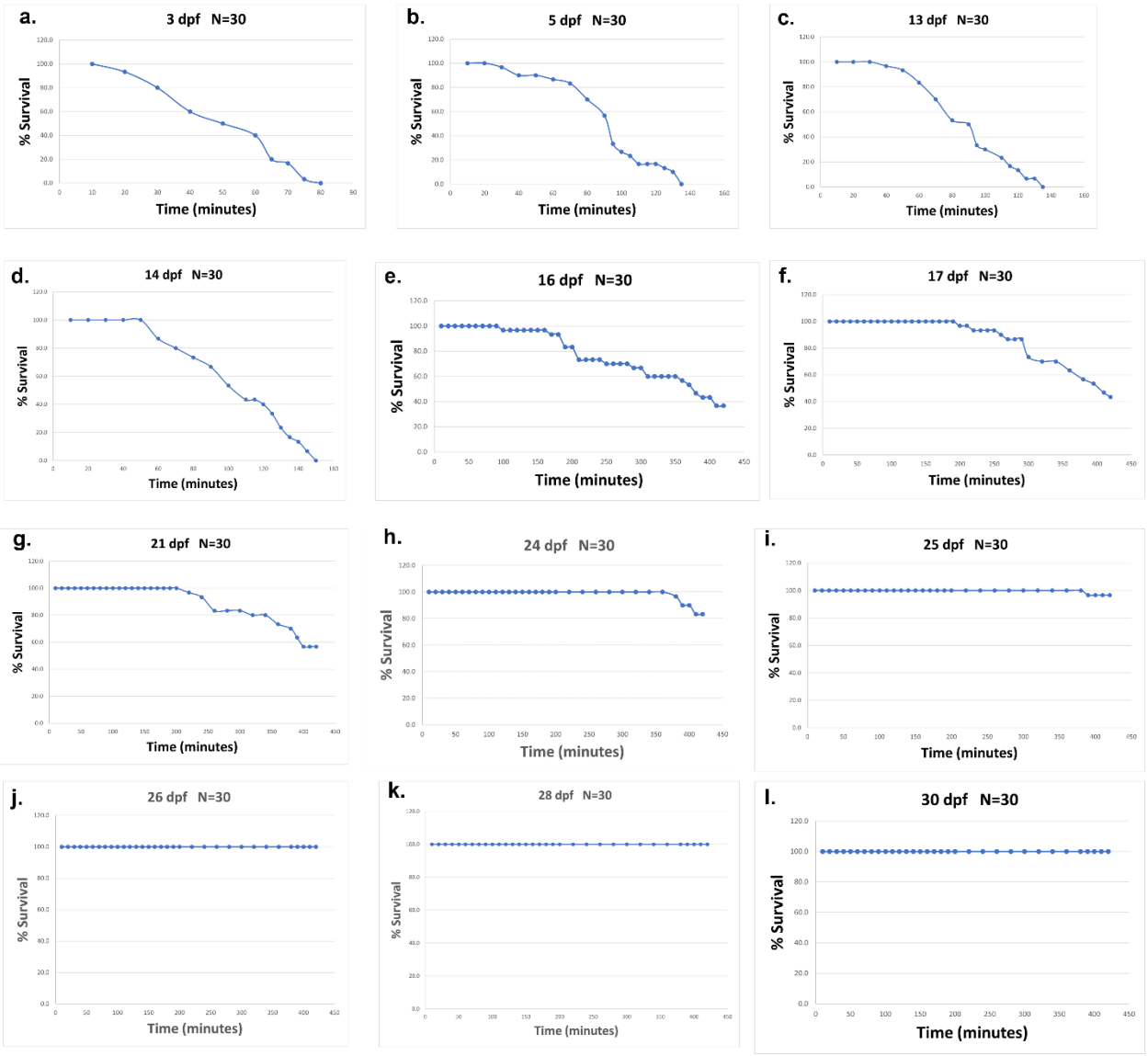

Figure 3. Survival curve was plotted for *Clarias macrocephalus* exposed to the aerial environment at 3, 5, 13, 14, 16, 17, 21, 24, 26, 28 and 30 days post-fertilization (dpf). Fish were determined moribund when the opercular movement ceased.

Clarias macrocephalus larvae were tolerant of low oxygen even at 3 dpf. Although there is low survival during the first 16 dpf, survival rate was observed to increase gradually from 17 dpf. However, when they were exposed into the air and forced to breathe air, from 3 dpf to 14 dpf all fish died. But at 16 dpf, it appeared that 36.7 % of the population had developed air-breathing capability. By 24 dpf, 83.3 % fish had developed air-breathing ability. Though 100 % survival was

not obtained until 26 pdf, but a significant amount of air-breathing was observed by 17 dpf and gradually increased from that point.

2.4.3 Anatomical analysis of the swimbladder of tra catfish (*Pangasianodon hypophthalmus*):

In juvenile and adult tra catfish, swimbladder extends from the posterior of the head to the tail beyond the anus (Fig. 4). During the early life stages, tra larvae have very delicate tissue which were very difficult to dissect to reveal the internal organs. Especially prior to absorption of yolk sac, organs in the abdominal region are not clearly visible, and the gastrointestinal structures are not fully developed.

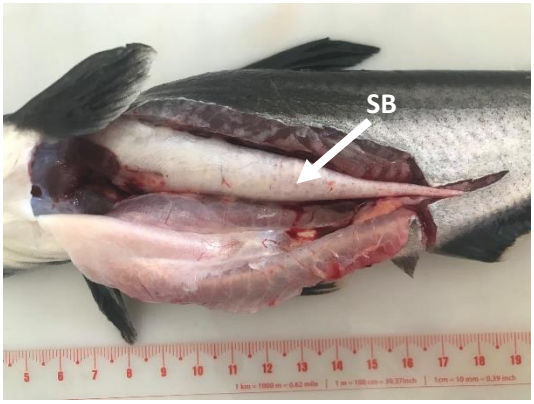

Figure 4. The swimbladder (SB) of a juvenile tra catfish (*Pangasianodon hypophthalmus*) produced in Can Tho University, Vietnam.

The swimbladder of tra catfish was not detected until 4 dpf. At 5 dpf, a rudimentary structure was seen that emerged from an outgrowth of the esophagus. This structure was an elongated tube located on ventral side of the vertebral column and extended up to anus (Fig. 6-a). This organ became the swimbladder in later stages. Unfortunately, the tissues were very soft, possibly due to long-term preservation, and so most of the abdominal region was ruptured.

Previously conducted comparative transcriptome analysis between tra and channel catfish identified 76 tra catfish specific genes responsible for air-breathing in tra catfish.[188] In a time series expression analysis, these genes were categorized into six different clusters. Cluster 1, 3 and 5 showed 48 genes with peak expression at 2 and 4 dpf which might be the key genes for air-

breathing function. This might be since the swimbladder increases in size at 6 dpf concomitant with the onset of air-breathing as revealed during hypoxia challenge (Fig.5-cluster 1, 3, 5). That means the genes which contribute for the structural development of swimbladder and air-breathing function would be anticipated to be turned on before 6 dpf, or earlier such as 4 dpf or 2 dpf. In other words, these genes may have increased expression with the formation of air-breathing as well as development of the swimbladder over time.

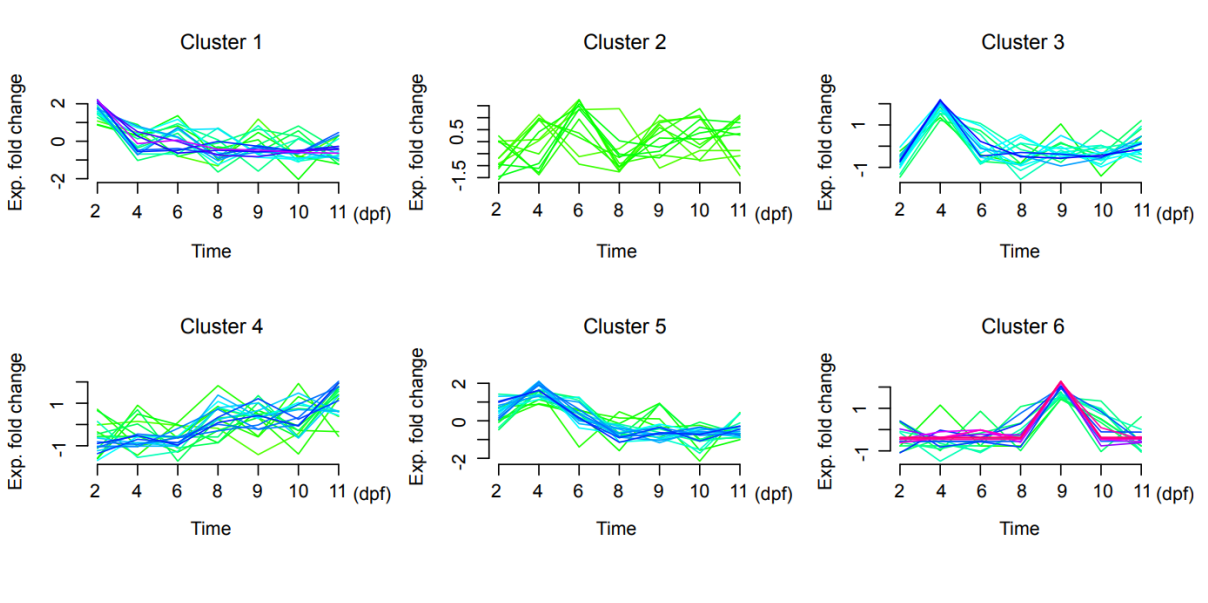

Figure 5. Time series expression profiles for tra catfish (*Pangasianodon hypophthalmus*) specific genes grouped into six different clusters (adapted from Ma et al (2021)). dpf: days post fertilization.[188]

At 6 dpf, a moderate increase in the dimension of the organ was found. It formed an elongated cone shape (Fig. 6-b). At this stage the yolk sac was fully absorbed. The newly observed swimbladder was located ventral to the notochord and outside of the peritoneal cavity and it covered a major portion of the body cavity. During oxygen challenge larvae were found to develop free swimming and regulation of orientation under water at 6dpf. At 0.7 mg/L, larvae of this age had improved survival ability. The gene expression data confirmed the onset of air breathing by this age.

At 8 dpf, the size of the swimbladder had increased slightly and the other internal organs had developed more as well (Fig. 6-c). The ventral side of the swimbladder was attached to the

peritoneal lining. At this stage survival in hypoxic condition was increased slightly. They could survive at 0.6 mg/L oxygen for a short period of time.

By 9 dpf, the outer layer swimbladder had become sturdier and increased in size. The larvae showed a longer survival rate of 60% at 0 mg/L of dissolved oxygen. At 10 dpf a distinct change in morphology were observed and the muscular structure became slightly thicker, while it had expanded significantly. Gene expression profile also showed 9 genes with peak expression values at 9 dpf perhaps contributing to a massive expansion in the size of the swimbladder (Fig. 5-cluster 6). Although it was not possible to dissect the whole organ due to damaged tissue from abdomen towards the anus, it was evident that the swimbladder was functional at hypoxia since survival rate was increased to 80% at 0 mg/L. Larvae of age 11 dpf displayed a very similar structure to swimbladder with a significant expansion of size. They had 100% survival at this stage during anoxia challenge. Since different fish grow and develop at different rates, 100 % survival during anoxia means all have reached air-breathing by that age. Both the hypoxia challenge and histology observation demonstrated that before 11 dpf, the fish did not possess full air-breathing ability.[188]

Form the observation of hypoxia challenge and RNA-seq analysis, it is evident that from 6 dpf, the tra catfish swimbladder served as an ABO and the air-breathing ability was fully functional at 11 dpf. Later, from the list of key genes for development of swimbladder and air-breathing function, *Grp* (gastrin-releasing peptide-like isoform X2), *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) and *Hrg* (histidine-rich glycoprotein) were selected for CRISPR/Cas9 mediated knock-in into channel catfish genome. *Grp* and *Cx3cl1* were found to show peak expression in cluster 3 and *Hrg* in cluster 5 at 4 dpf.[188]

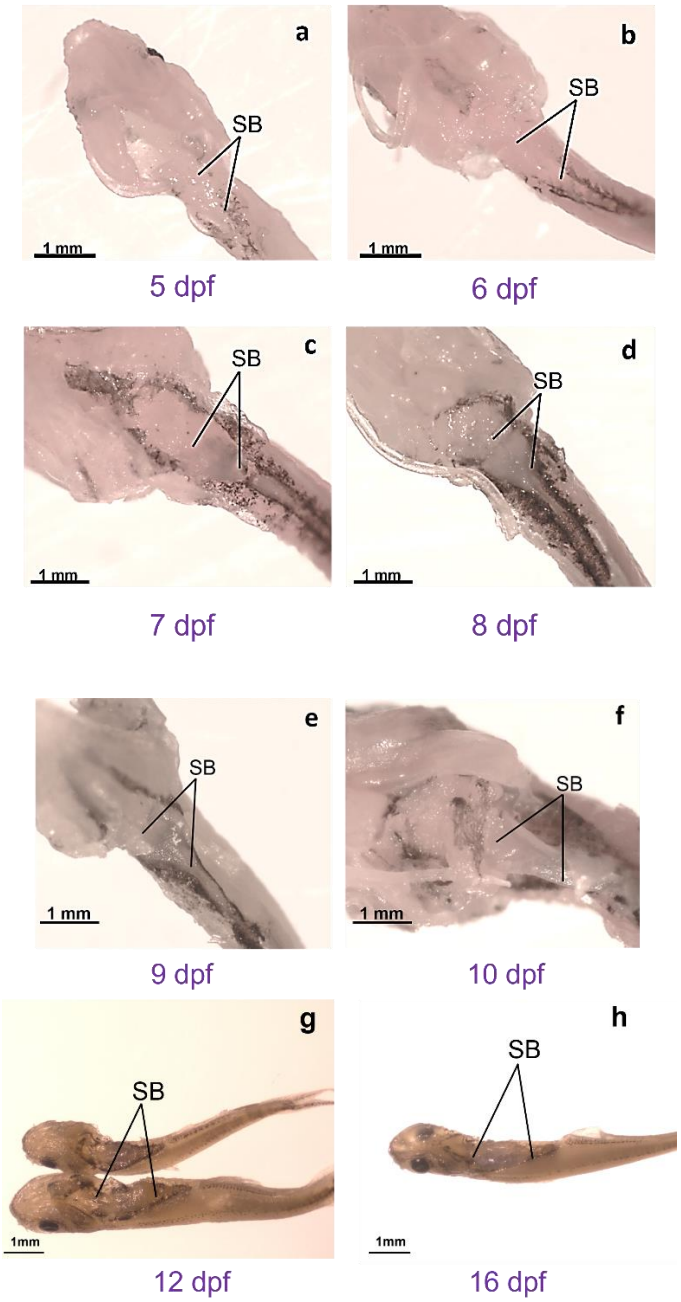

Figure 6. The ventral view of swimbladder (SB) of tra catfish (*Pangasianodon hypophthalmus*) during eight early developmental stages (a-h)

2.4.4 Anatomical analysis of gills of bighead catfish (*Clarias macrocephalus*):

In juvenile and adult bighead catfish, the accessory respiratory organ or labyrinth organ originates form the second and third gill arches and forms a branched tree like structure (Fig. 7)

During early life stages, the gills were very soft, and ABO was not detected. The size of the organ was increased in later stages.

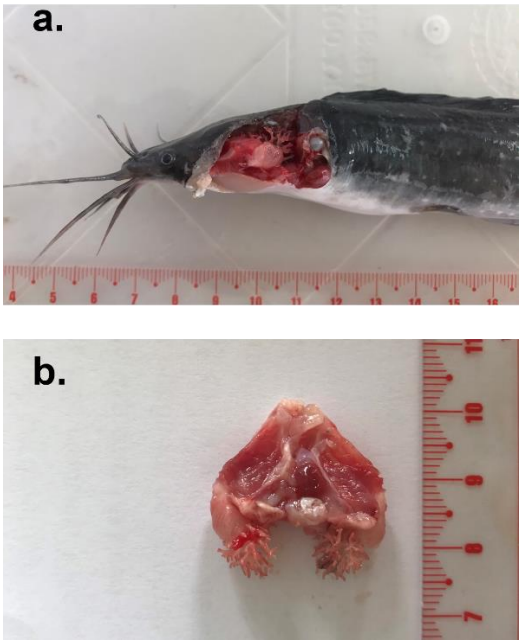

Figure 7. a. The gills and ABO of a juvenile bighead catfish (*Clarias macrocephalus*) produced in Can Tho University, Vietnam, **b.** An enlarged picture of the organ separated from the body of the fish

At 3 dpf, the gill and associated tissues were too delicate to locate the accessory air breathing organ (Fig. 9-a). However, those larvae showed a partial tolerance to hypoxic condition. Some fish were found to come up to the surface for a few minutes.

The structure of gills of 5 dpf larvae were like 3 dpf but with increased size (Fig. 9-b). The gill filaments were still very soft and fragile. Larvae continued to develop air breathing and during hypoxia challenge they came up to the surface moving slowly but did not die until 0.33 mg/L of dissolved oxygen. From the gene expression study, 169 genes were found showing a peak of expression at 5 dpf, which may relate the developmental fate determination of the air-breathing organ (Fig. 8-cluster 1).

At 13 dpf gills and accessory breathing organs expanded significantly in size (Fig.9-c). The gill arch and gill filaments became strong and more visible. The ability to sustain in hypoxia was

increasing gradually. During oxygen challenge in container 80% of the fish could survive at 0.36 mg/L of DO for first 80 min. The accessory air breathing organ- labyrinth organ, originates from the second and fourth gill arches. The tissues were still very soft, and detection of the organ was difficult. Some tissue around the posterior part of the second and fourth gill arch is indicative of the parts of the accessory breathing organ.

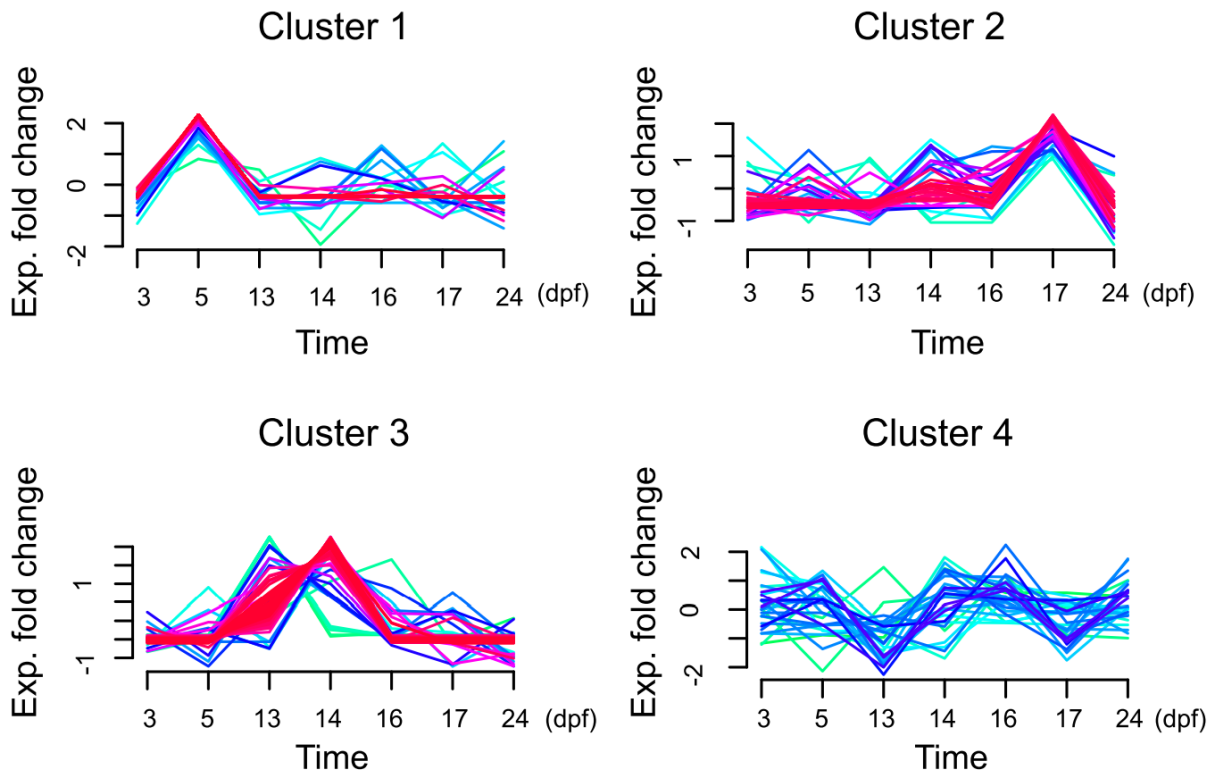

Figure 8. Expression clusters of 291 key genes across *Clarias macrocephalus* developmental stages (adapted from Ma et al (2021)).[83]

In 14-day-old larvae, the structure of gills and nearby tissues did not differ from 13 dpf but slightly increased in size and the gill arches and gill filaments became more distinct (Fig.7-d). Some broken tissues were also found as before at the second and fourth gill arches. These tissues were probably damaged due to fixation. In hypoxia, larvae at this stage had 6.7% survival at 0.11 mg/L of DO. In the petri dish experiment, all fish survived for the first 50 min but none of them could make it till the end. Expression cluster 3 also displayed a peak for 29 genes at 14 dpf (Fig. 8) and may have an important role in development of air-breathing function and structural development of accessory air-breathing organ.

At 16 dpf and 17 dpf the gills grew larger (Fig. 9-e, f). Some other tissues which were almost transparent, probably part of labyrinth organ, were also found. Likewise, the larvae had 36.7% and 43.3 % survival when exposed directly to the air. Gene expression profile revealed an unaltered expression of 35 genes at the first four developmental timepoints followed by a peak at 17 dpf (Fig. 8-cluster 2) which suggest the crucial transition between 16 and 17 dpf. The hypoxia challenge experiment also indicates the ability of surviving in aerial environment was obtained in this crucial window.

At 24 dpf, when exposed to an aerial environment, larvae could breathe air and had 56.7 % survival rate indicates the partial air breathing ability of some fish. At this age, the gills and other respiratory organs grew large and air breathing ability was also increased (Fig. 9-g).

At 26 dpf, the gills and associated tissue became more evident and increased in size. The membrane of the suprabrachial chamber was visible. The petri dish experiment obtained 100 % survival of the population which indicates that air-breathing ability of the ABO became fully functional at this age.

Based on the RNA sequencing, the expression patterns of key genes of air-breathing in bighead catfish over different developmental stages were clustered into four clusters as above (Fig. 8) which suggested that clusters 1–3 have the greatest potential to play a critical role in ABO development and air breathing in *C. macrocephalus*.[83] Twenty nine genes in cluster 4 which had variable expression across the developmental stages making them less likely to be involved in air-breathing function in *Clarias macrocephalus*.

Fras1 had peak expression at 13-14 dpf in cluster 3 along with other genes.[83] At this point gills were increased in size and the gill arches and gill filaments became more distinct. Hypoxia challenge experiment also suggest that the gradual development of air-breathing ability at this age becomes more efficient within 16 dpf to 17 dpf. Thus, *Fras1* might be important in ABO development. This gene was also considered for transgenesis into channel catfish to make potential phenotypic changes.

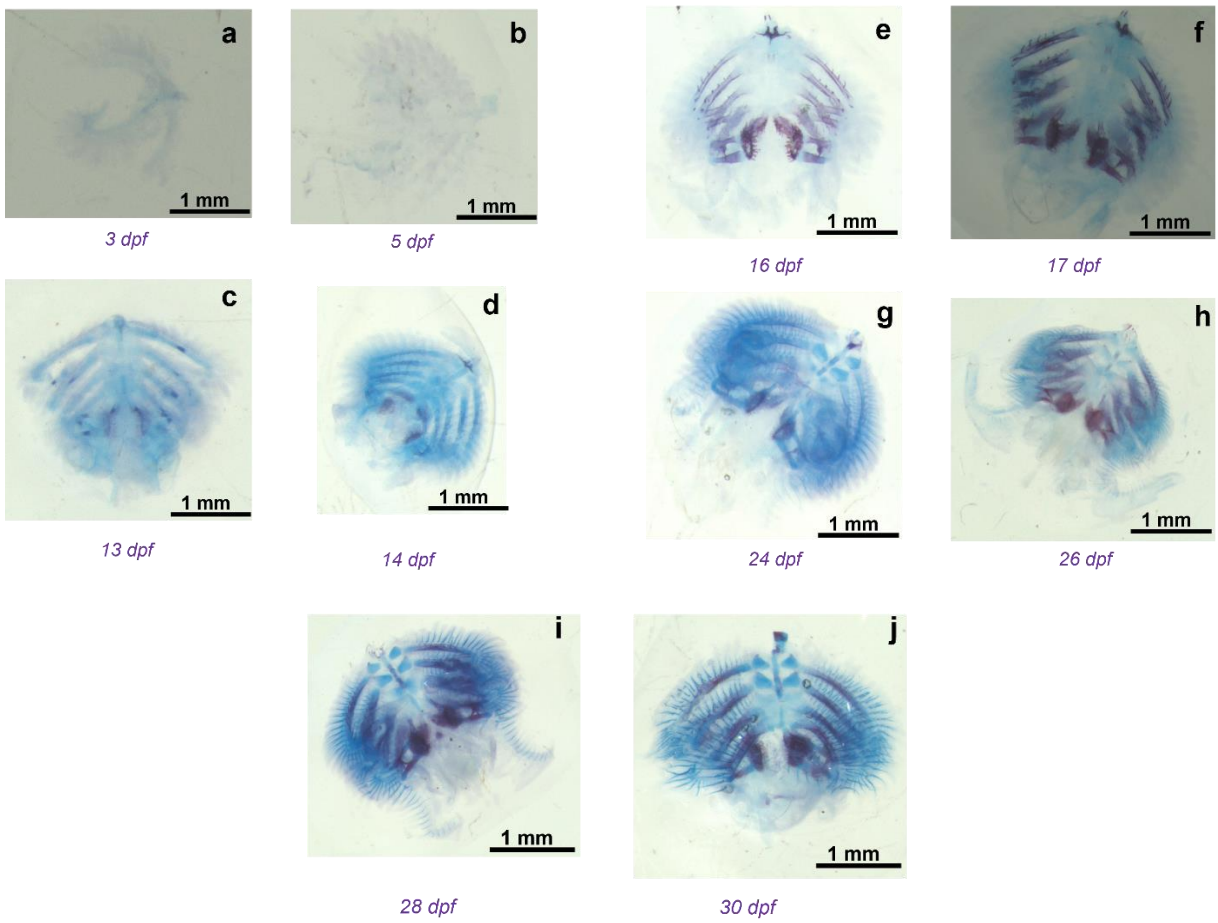

Figure 9. Gradual development of gills and other respiratory tissues of bighead catfish *Clarias macrocephalus* during ten early developmental stages (a-i, 3 dpf-30 dpf)

2.5 Discussion:

For millions of years, gills served as the primary gas exchangers for fish in water. Afterwards fish ancestors were forced to develop air-breathing organs when oxygen levels in water started to decrease.[190] *Pangasianodon hypophthalmus* is one the descendants which is a good example of adaptation for partial aerial breathing.[191] *C. macrocephalus* can use an accessary air-breathing organ to breathe in air, allowing them to survive extreme environments, such as hypoxia and high ammonia.[192] Therefore, *P. hypophthalmus* and *C. macrocephalus* are informative models to explore the mechanisms of formation of air-breathing function.

In this study, low to zero oxygen challenge experiment and anatomical observations were blended to reveal the development of air-breathing ability along with the formation of accessory air-breathing organ (ABO) in tra catfish (*P. hypophthalmus*) and (*C. macrocphalus*). Tra catish use the modified swimbladder and bighead catfish use modified gills, otherwise known as labyrinth organ, for air-breathing. 2 dpf to 12 dpf tra catfish larvae were challenged daily to assess their ability to survive in hypoxic water until 100% survival was observed at 0 mg/L of dissolved oxygen for 11 dpf larvae. In addition to the hypoxia challenge in water, bighead catfish larvae of 3 dpf to 30 dpf were also exposed directly into the air to test the development of air-breathing capability. They were kept in petri dishes without water and forced to breathe air and at 26 dpf the larvae obtained 100 % survival for the first time. These experiments revealed the critical time points of the development of the ability to endure hypoxia as well as air-breathing, which were correlated with the structural development of the ABO of the corresponding species. Gradual development of the ABO throughout the early stages of life further correlated with RNA-seq and gene expression analysis which was accomplished previously as a part of this study. The peak expression of genes at particular time points and the subsequent changes in structural development of the organ and behavioral response to hypoxia, indicates the potential involvement of these genes with the development of air-breathing capability and formation of air-breathing organ.

During this experiment, the temperature was maintained between 27 to 29 ℃. Tra catfish larvae of 6 dpf demonstrated behavior indicative of stress at DO concentration less than 2 mg/L for 15 min but all fish died when the anoxic (0 mg/L) condition was continued for 25 min. However, some larvae of this age displayed air gulping as a mechanism to survive hypoxia. It indicates that for a portion of the tra catfish larvae, air breathing was possible or that behavior to gulp air was now in place. Similar results have been found for *Pangasianodon sutchi*, an Asian commercial catfish species, which is an obligatory air-breather also using the swimbladder as air-breathing organ.[193] These fish die in 50 min to 18d if they do not have access to air.[194] Interestingly, these two closely related species appear to represent stepping stones toward terrestrial living. In the case of *P. sutchi*, the yolk sac is fully absorbed at about 3 dpf like *P. hypophthalmus.* Their swimbladder also develops within 8 to 10 dpf. At 12 dpf the lumen of the swimbladder is constructed, and the inner surfaces of alveoli are covered with a network of capillary, indicating aerial breathing ability at this stage for *P. sutchi.*[193, 195] In the case of tra, at 8 dpf, larvae had 100% survival rate at 0.6 mg/L dissolved oxygen in water and could survive for a while at of 0 mg/L

(anoxia). The survival rate of tra catfish larvae was 60%, 80%, 100% in 9, 10, 11 dpf respectively when the challenge was terminated after 120 minutes, indicating that at 9 and 10 dpf, the air breathing ability was relatively complete. At 11 dpf, the swimbladder in tra catfish was fully developed and they acquired complete air breathing capability. In conclusion, from 6 dpf, the tra catfish swimbladder served as an ABO, and aerial breathing ability was fully efficient 5 days later, at 11 dpf. Significant increase in size of the swimbladder was observed during anatomy observation at 11 dpf. Histology observation of tra catfish swimbladder proved that the fish did not possess full air-breathing ability until 11 dpf.[188] Gene expression profile also showed 9 genes with peak expression values at 9 dpf which might have potential contribution in expansion of the swimbladder (Fig. 4-cluster 6).

Comparative transcriptome analysis between tra and channel catfish detected 76 tra catfish-specific genes. Excluding the genes with unidentified function, 14 genes were picked as the best candidates of air-breathing in tra catfish.[188] From that list of key genes, *Grp* (gastrin-releasing peptide-like isoform X2), *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) and *Hrg* (histidine-rich glycoprotein) were the most likely genes associated with the formation of air-breathing ability in tra catfish as they were found to be essential for the formation and function of human lung and angiogenesis.[188] *Grp* and *Cx3cl1* were found to show peak expression in cluster 3 and *Hrg* in cluster 5 at 4 dpf.[188]

Grp was reported to be upregulated in human embryonic pulmonary system during 16 to 30 weeks post fertilization for canalicular phase of the lung development.[90] Increased expression of *Grp* at 12 weeks indicates its potential involvement in lung development in human as well as the formation of primitive air saccules and respiratory bronchioles and extension of airway epithelium.[90] Additionally, this gene also regulates local blood flow, glandular secretion, and the activity of smooth muscle.[91] It increases endothelial cell migration in human and cord formation and induces angiogenesis *in vitro*.[92] In mice, at embryonic day 12, related evidence is found in fetal lung branching during morphogenesis.[93] Since the swimbladder of air-breathing fish species is thought to be the origin of primitive lung, *Grp* might be one of the most important candidate genes that are responsible for air breathing function. *Cx3cl1* is another gene that is potentially important in the development of the swimbladder and air-breathing function. Although there is not enough literature about *Cx3cl1* in fish, it is reported that in response to hypoxia, excessive *Cx3cl1*

is produced by human lung microvascular endothelium to stimulate phenotypic switching, proliferation and muscle expansion in smooth muscle cells etc.[94] Genes related to angiogenesis are considered as one of the adaptations for the air-breathing organ to maintain efficient gas exchange.[98] *Hrg*, another important candidate gene for air-breathing in tra catfish, plays a variety of roles like angiogenesis, vascularization, coagulation etc. in human blood.[95] *Hrg* binds to thrombospondin (TSP) and TSP-1 to inhibit antiangiogenetic effect of TSP-1.[96-97]

In the current study, *C. macrocephalus* larvae absorbed all the yolk sac at 3 dpf and were found to be tolerant of low oxygen even at 3 dpf in the tank experiment. The survival rate was low until 16 dpf but increased gradually from 17 dpf. However, when they were exposed directly into the air and forced to breathe air, from 3 dpf to 14 dpf larvae could not survive. At 16 dpf, 36.7 % of the population was found to develop air-breathing capability. Air-breathing was increased up to 83.3 % by 24 dpf. Although 100 % survival was not attained until 26 pdf, a significant increase in air-breathing was observed by 17 dpf and gradually increased afterwards.

The Indian catfish *Heteropneustes fossili*, also known as stinging catfish, inhabits ponds, swamps and marshes and able to survive in high density stocking due to its aerial respiration ability. air breathing behavior of larvae of this species was not observed until 10 days after hatching.[196-197] In climbing perch (*Anabas testudineus*), another freshwater species, the labyrinthine organs differentiated at 51 hours after fertilization, but the hatchlings did not start aerial breathing until 13-14 days.[198]

In the bighead catfish, *Clarias macrocephalus*, the gill was observed shortly after hatching and when the larvae are about 11 mm in length the suprabranchial organ begins to develop and considered as the point of initiation of aerial breathing.[199] Similar results were also found in the current study. Significant air-breathing was observed in 17 dpf larvae when they were 11.81 mm in length. By 26 dpf, they grew more than 16 mm when air breathing was fully functional. In case of the survival rate, large individuals had a better ability of air-breathing and thus, better survival. Around 50% of bighead larvae was 85 to 97 % smaller than the average length of the population. Most of the dead individuals in the petri dish experiment were found to be the smaller ones.

For *Clarias* larvae, the ability to survive in hypoxia was observed to increase gradually from 13 dpf and subsequent changes in gills and ABO were also detected as they had a significant expansion of size. The membrane of suprabranchial chamber became visible which was more developed around 17 dpf since the larvae exhibited air-breathing to some extent and a significant

increase in survival rate. The gills and ABO became more evident and increased in size over 24 to 26 pdf. The larvae became more efficient in air-breathing and 100 % survival rate was obtained in petri dish at 26 pdf. This indicates the complete development of air-breathing organ in bighead catfish larvae. Based on the type of species, the dependency on water as a source of oxygen decreases with age and complete air-breathing ability is accomplished approximately within 8 to 20 days after hatching.[18, 200-201]

Through comparative genomic analysis between bighead and channel catfish, 291 genes for *Clarias* were identified to be served as candidate genes for air-breathing function development which were categorized into four expression clusters (Fig. 6). Considering the association with oxygen carrying and binding and heme-binding activities, 26 key genes were selected from that list.[83] Among them, Hbae, Pc, Pleca, Ezrb and Lrfn4b were found strictly in *C. macrocephalaus* while the remaining 21 genes at least one paralog in *I. punctataus* with less than 70 % similarity.[83] Mb, Ngb and Hbae3 and other 23 hub genes showed variable expression during the critical developmental time points.[83] All these three genes were found to be critical in bighead catfish larvae in terms of direct relation to oxygen binding and transport. [98, 202] Mb was found in cluster-1 with an increasing expression through 3-5 dpf (Fig. 6) indicating the onset of blood circulation during embryonic stages. Ngb was also found to co-express with Mb around the same time. Hbae3 was first detected at 3 dpf. By 13 pdf, expression of Mb and Ngb was steadily decreasing and *Fras1* expression was detected for the first time when Hbae3 expression was extremely high and survival time of the larvae was also increased significantly.[83] Moreover, distinct changes in the size and dimension of gills and ABO were also observed. Another key gene, Sars2, was found to have peak expression at this stage followed by a second peak at 17 dpf when larvae showed significant air-breathing and survival rate was increased to 43.3 %. *Fras1* and Sars2 might play important role for the development of ABO in bighead catfish larvae.

2.6 Conclusion:

Tra catfish (*P. hypophthalmus*) is an aquatic species that can utilize its swimbladder to obtain oxygen directly from air, whereas bighead catfish (*C. macrocephalus*) uses accessory gills for aerial breathing. On the other hand, channel catfish (*I. punctatus*) can breathe only with gills in water but are unable to air-breathe and die of hypoxia at low or no oxygen in water. Therefore, these two species provide important and fundamental insights of the mechanism of air-breathing

activity. Through comparative genetic analysis species, specific genes of tra and bighead catfish that are important for air-breathing function were previously identified. Hypoxia challenge experiments revealed the critical time points for the air-breathing ability and development of swimbladder and accessory gills in these two species. In this experiment, fish larvae were dissected to observe the anatomy of tra catfish swimbladder and accessory breathing organ of bighead catfish to correlate the formation and development of ABOs with the response of fish to hypoxic environment and the previously conducted time series of gene expression profile. The change in structure and size of the respective ABO during the critical time points was found to be in accordance with the fish behavior in hypoxia challenge and gene expression profile. In future, overexpression and knock-out of selected genes could be performed to further investigate the cellular and molecular mechanisms air-breathing functions in tra and bighead catfish.

Chapter Three: In situ Hybridization for Cx3cl1 Expression in Swimbladder of Tra Catfish (Pangasianodon hypophthalmus) Embryos and Larvae

3.1 Abstract:

Air-breathing in tra catfish (*Pangasianodon hypophthalmus*) is a crucial example of the transition of life from the aquatic to terrestrial environment. Being a facultative air-breather, tra can use the swimbladder as an air-breathing organ (ABO) whereas channel catfish (*Ictalurus punctatus*) breathes only with gills and cannot breathe in the air. Hypoxia challenge coupled with histology and anatomy observation revealed critical time points for the development of the air-breathing function and swimbladder of tra catfish. Comparative genomic analysis between these species identified species specific genes for tra catfish and RNA-seq analysis based on the transition towards survival during anoxia revealed *Cx3cl1* as one of the most important candidate genes to be likely related to air-breathing ability in tra catfish. The mRNA of *Cx3cl1* was detected for the first time in the esophageal tissue of 1 day post fertilization (dpf) larvae which was significantly increased at 2 dpf over the entire swimbladder region. The expression was gradually decreasing during the later stages (5 to 6 dpf) and detected in the mid portion and posterior end of the swimbladder. Histological examination also confirmed the detection of swimbladder at 6 dpf for the first time. These results provide important information to understand the function of *Cx3cl1* during the development of swimbladder and air-breathing of tra catfish and confirm that this gene is related to the development of air breathing organ in tra catfish.

3.2 Background:

In situ hybridization (ISH) is a method of detecting specific nucleic acid sequences in morphologically preserved cells or embryos. It conveys microscopic topological information to gene activity at the DNA or mRNA level. ISH is being extensively used to describe the expression

pattern of developmentally regulated genes. Initially ISH was accomplished with radio-actively labeled DNA probes to detect gene expression on histological sections followed by autoradiographic anlyses.[174] Later, chemically labeled RNA probes were used instead of radio labeled ones, offering easier and quicker analysis of gene expression patterns from whole-mount tissues or embryos.[175-177] Use of in vitro synthesized RNA tagged with digoxygenin uridine 5′-triphosphate made ISH protocols very robust and reliable for determination of gene expression patterns. After hybridization the transcript was visualized immunohistochemically using an anti-digoxygenin (DIG) antibody conjugated to alkaline phosphatase on a chromogenic substrate.[178] This protocol has been used to establish the expression pattern of more than 10,000 zebrafish genes.[180]

Tra catfish (*Pangasianodon hypophthalmus*), otherwise known as striped catfish, is a facultative air-breather, which uses the swimbladder as an air-breathing organ (ABO). On the other hand, channel catfish (*Ictalurus punctatus*) breathes only with gills and does not have air-breathing capabilities. These two silurid species serve as great comparative models for understanding genes that are important for the development of the swimbladder and the air-breathing function in tra catfish. As a part of this study, previously conducted hypoxia challenge and microtomy experiments in conjunction with anatomical observation revealed the crucial time points for the development of air-breathing and swimbladder in tra catfish. Seven developmental stages between 2 to 11 dpf were selected for RNA-seq analysis based on the transition towards survival in anoxic (0 mg/L) condition. Genome comparison between tra catfish (*P. hypophthalmus*) and channel catfish (*Ictalurus punctatus*) revealed 76 tra catfish specific genes that are absent in channel catfish. From this list, 14 genes were selected which could be the key genes involved in the morphogenesis of the swimbladder and differences in aerial breathing ability.[188] Among them *Grp* (gastrin-releasing peptide-like isoform X2), *Hrg* (histidine-rich glycoprotein) and *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) were identified to be the most likely genes related to air-breathing function in tra catfish.

In this study ISH of DIG-labeled anti sense RNA probes were used with few modifications to detect the tissue specific expression of *Cx3cl1* in developing tra catfish (*P. hypophthalmus*) embryos.[178] This gene was found to be potentially important in the development of swimbladder and air-breathing function of tra catfish.

3.3 Materials and methods:

3.3.1 Ethics statement

All experimental procedures involving animal care and tissue collection were approved by the Auburn University and Can Tho University Institutional Animal Care and Use Committee. All animal related procedures were performed following the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the United States and in Vietnam.

3.3.2 Experimental animals and tissue collection

Tra catfish embryos were produced at Can Tho University, Vietnam. Embryos were hatched approximately within 1-day post fertilization (dpf) and samples were collected every 24 hours over a 30-day period post-hatch simultaneously with a hypoxia challenge where DO concentration was gradually reduced from 4 to 0 mg/L. A total of 20-50 eggs/embryo/fry from both species were collected at each sampling. Three sets of samples were taken at each time point and later used for anatomy and gene expression analysis. Eggs/embryo/fry from the respective time points were euthanized with 200 ppm buffered MS-222, and then stored as three separate sets. The first set was placed in 4 % paraformaldehyde and sealed with screw top lids[187] and the second and third set were preserved in 70% methanol and RNA-later solution (Thermo Fisher Scientific), respectively. Samples were shipped to the US on dry ice and immediately preserved at the appropriate temperature upon arrival. Formalin-fixed samples were kept at room temperature, whereas methanol and RNA-later samples were kept at -20°C and -80°C, respectively.

3.3.3 Primer design for amplifying probe templates containing an RNA polymerase

In addition to the antisense (experimental) RNA probe, a sense (control) RNA probe of the corresponding gene was used to provide information of nonspecific signals that may appear. Primers were designed by using Primer 3 software[203] for the amplification of probe templates. T7 and SP6 RNA polymerase promoters were used to synthesize sense and antisense probe, respectively (Table 1). RNA polymerase promoters were located at 3′ (for antisense probe) and 5′ end (for sense probe) of the sequence of interest. The detailed sequence of the gene of interest (*Cx3cl1*) is found in Appendix D.

Table 1. The sequences of RNA polymerase promoters and primers used for the amplification of probe template for *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) of tra catfish (*Pangasianodon hypophthalmus*)

T7	5′-TAATACGACTCACTATAGGG-3′
SP6	5′-ATTTAGGTGACACTATAG-3′
Forward primer	5′-TATCTCGTGGACTGGGAAGG-3′
Reverse primer	5′-CTGGCCTCCTATGGTTTTCA-3′

3.3.4 DNA template preparation

Genomic DNA from preserved tra catfish larvae was extracted by using a standard protocol.[204] For each probe, separate PCR reactions were conducted. PCR amplifications were performed as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with final extension at 72 °C for 7 min. PCR products were purified with QIAquick® PCR purification kit following the manufacturer's protocol.

3.3.5 Synthesis of antisense RNA probe

Digoxigenin (DIG)-labeled antisense RNA probe and sense probe for *Cx3cl1* were prepared with DIG RNA Labeling Kit (SP6/T7) (sigma-aldrich.com) according to the manufacturer's instructions. RNA sense (T7) and antisense (SP6) probe were purified by the phenol chloroform RNA extraction method.

3.3.6 *In situ* hybridization

Tra catfish larvae were fixed in 4% paraformaldehyde in 1xPBS at 4℃ overnight. Then the larvae were incubated in a 3% H_2O_2/0.5% KOH medium for removal of pigmentation. This step took 30 min to 1 h and progress was checked by observing the larvae on a white background under a dissecting scope, lit from above. After that larva were dehydrated in 100% methanol for 15 min at room temperature and they were kept at -20℃ overnight. The larvae were rehydrated

with successive dilutions of methanol in 1xPBS (75% methanol, 50% methanol, and 25% methanol: vol/vol), each for 5 min. Then the larvae were washed four times with 1 × PBT for 5 min per wash. The rehydrated larvae were digested with proteinase K (10 µg/ ml^{-1}) at room temperature for 30 min. The digestion reaction was stopped by incubating the larvae in 4% (wt/vol) paraformaldehyde in 1xPBS for 20 min followed by four washes in 1xPBT for 5 min per wash to remove residual paraformaldehyde.

Prehybridization was performed by incubating larvae with 700 µl of hybridization mix (HM) (50 % deionized formamide, 5 x SSC (saline sodium citrate), 0.1 % Tween 20, 50 µg ml^{-1} of heparin, 500 µg ml^{-1} of RNase-free tRNA adjusted to pH 6.0 by adding citric acid) for 2–5 h in a 70°C water bath. Next, the prehybridization solution was discarded and incubated overnight in 200 µl HM containing 30-50 ng of antisense DIG-labeled RNA probe at 70℃ in a water bath.

The following day, HM containing probe was discarded and the larvae were transferred into prewarmed (70°C) hybridization mix for 20 min. Then the HM was gradually changed to 2xSSC through a series of 10 min wash in HM diluted with 2xSSC (75% HM, 50% HM and 100% 2xSSC). Washes were performed at 70℃ in a hybridization oven with gentle agitation. Then washed twice, for 30 min per wash, in 0.2xSSC at 70℃.

The 0.2xSSC was progressively replaced with PBT through a series of 10 min washes in 0.2xSSC diluted with 1xPBT (75% 0.2xSSC, 50% 0.2xSSC, 25% 0.2xSSC and 1xPBT) at room temperature on a horizontal shaker (40 rpm). Then the larvae were incubated in blocking buffer (1xPBT, 2 % sheep serum (vol/vol), 2 mg ml^{-1} bovine serum albumin (BSA)) for 3-4 h at room temperature. Later, they were incubated with a solution of anti-DIG antibody solution in blocking buffer (1:10000) overnight at 4 ℃ with gentle agitation on a horizontal shaker (40 rpm).

On the third day, larvae were washed six times, 15 min per wash, in PBT at room temperature with gentle agitation (40 rpm on horizontal shaker). Then they were briefly dried on absorbent paper. Lastly, the larvae were incubated in alkaline Tris buffer (100 mM HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1 % Tween 20 (vol/vol)) three times (5 min per wash) with gentle agitation (40 rpm).

Larvae were then transferred to freshly prepared staining solution (Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate) and kept in the dark. The staining reaction

was monitored every 15 min using a microscope. When the desired staining intensity was reached, the larvae were immediately washed three times with 1 × PBT. The reaction was subsequently stopped by the stop solution (1x PBS, pH 5.5, 1mMEDTA, 0.1% Tween 20 (vol/vol)). Finally, the reaction was observed and imaged with an Olympus R BHS fluorescence binocular microscope equipped with a 3.4-megapixel color digital camera (Qimaging R model Micropublisher 3.3 RTV).

3.4 Results:

The whole mount *in situ* hybridization of tra catfish (*Pangasianodon hypophthalmus*) embryo revealed that *Cx3cl1* mRNA expression was not detected before hatching (Fig. 10-a). A low signal of *Cx3cl1* mRNA was detected at 1 dpf near the anterior portion of the esophagus (Fig. 10-b). The transcript of *Cx3cl1* increased significantly at 2 dpf and it was present from the esophagus to anus outside the peritoneal lining that should be the location of swimbladder (Fig. 10-c). At 5 dpf, the *Cx3cl1* mRNA signal decreased and was found on the posterior part of the swimbladder (Fig. 10-d). However, muscle degradation due to poor preservation made its detection difficult at 5 dpf. *Cx3cl1* transcript was also found at 6 dpf in the swimbladder (Fig. 10-e). In the previous histology experiment, the swimbladder could be observed in tra catfish larvae for the first time from 6 dpf.[188] Moreover, the 0 mg/L oxygen challenge experiment showed that tra catfish larvae had partial ability to survive in hypoxia even before 6dpf. However, both the hypoxic challenge experiment and histology experiment demonstrated that before 11 dpf, the fish did not possess full air-breathing ability. The gene *Cx3cl1* was found to be upregulated at 4 dpf during the developmental time series expression profile analysis of tra catfish specific genes.[188] Therefore, *Cx3cl1*, which is potentially responsible for swimbladder development and air-breathing would be expected to be upregulated before 6 dpf, approximately around 2 to 4 dpf.

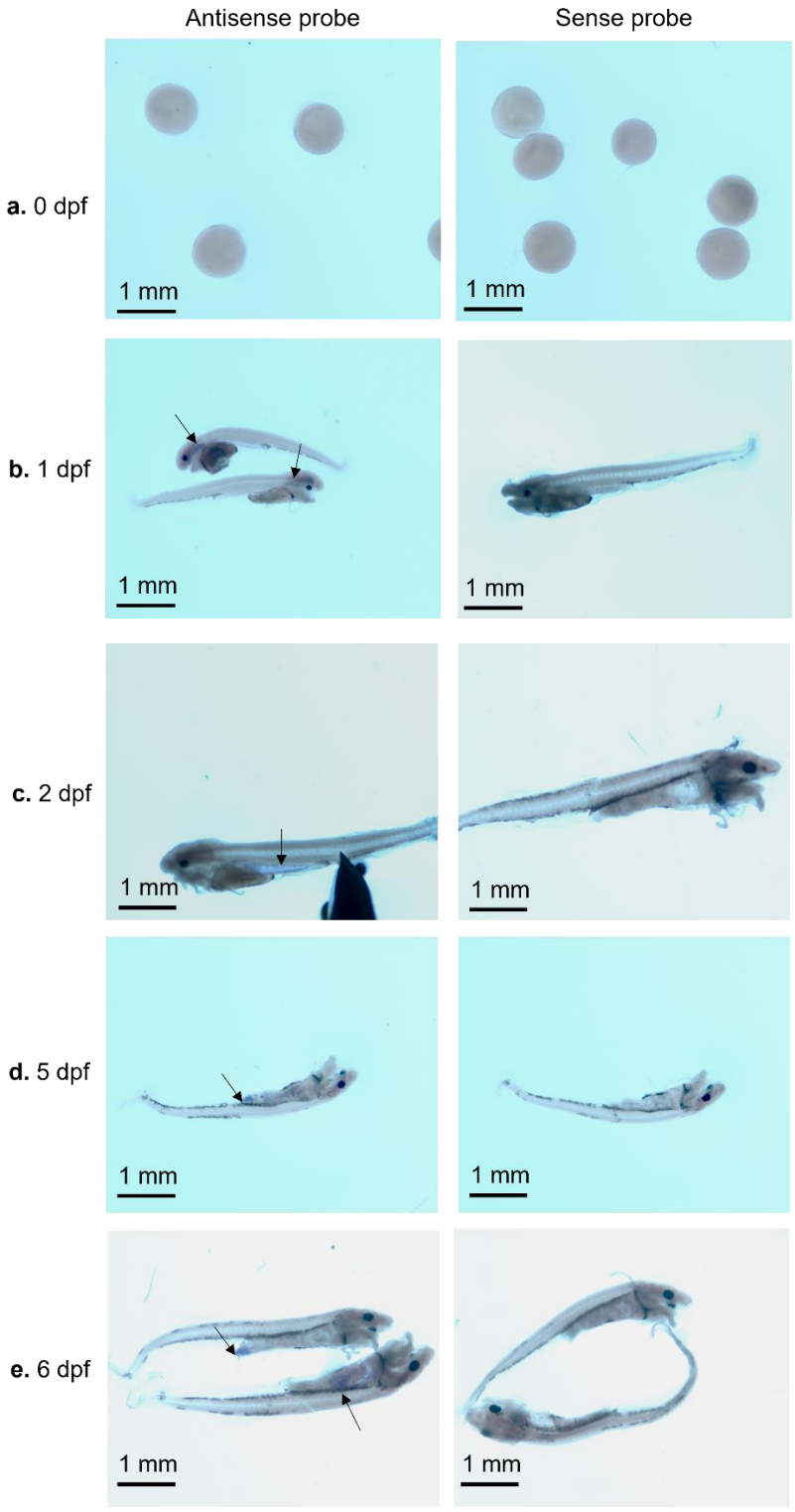

Figure 10. Whole mount *in situ* hybridizaton analysis of *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) mRNA in tra catfish (*Pangasianodon hypophthalmus*) unhatched embryo and larvae of 1, 2, 5 and 6 days post fertilization (dpf). a) The transcript of *Cx3cl1* was not

expressed in unhatched embryo (0 dpf); b) The *Cx3cl1* mRNA was present at the anterior esophagus of 1 dpf larvae; c) *Cx3cl1* expression was increased significantly at 2 dpf through the entire swimbladder region; d) *Cx3cl1* mRNA signal was decreased at 5 dpf and found on the posterior part of swimbladder; e) *Cx3cl1* transcript was also found at 6 dpf on swimbladder; The sense probe served as a negative control.

3.5 Discussion:

In the developmental time series analysis, *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) gene was observed to be potentially important in the development of swimbladder and air-breathing function in tra catfish (*P. hypophthalmus*).[188] The 76 species specific genes were classified into six different clusters. Excluding the genes with unknown function, 14 genes were identified as the best candiadtes for air-breathing function related genes in tra catfish which contribute to structural formation of swimbladder and air-breathing function. According to the findings from the developmental time series expression profile, these genes would be expected to be turned on before 6 dpf. They may start expressing at 4 dpf or as early as 2 dpf and increase expression with the formation of air-breathing functon over time.[188] *Cx3cl1* was found to have peak expression at 4 dpf along with other genes such as *Grp* (gastrin-releasing peptide-like isoform X2), Ly6d (lymphocyte antigen 6D), Shkd (dual-specificity protein kinase shkd-like isoform X2) and Ncmap (non-compact myelin-associated protein) etc. follwed by a decrease in the following days.[188] Whole mount *in situ* hybridization demonstrated that the mRNA of *Cx3cl1* was detected at 1 pdf larvae for the first time near the anterior part of the esophagus. The signal of *Cx3cl1* was significantly increased on the following day (2 dpf) in the swimbladder which is expected to be increasing through 4 dpf. The expression was gradually decreased at 5 to 6 dpf as development continued.

In the hypoxia challenge, the 4 dpf tra catfish larvae demonstrated the ability to endure hypoxic condition (1.1 mg/L) for around 15 min. At 6 and 8 dpf, the larvae showed similar behavior but the ability to survive in low oxygen was increasing gradually.[188] These results are in accordance with the observation of *Cx3cl1* expression in tra catfish larvae. *Cx3cl1* was expressed at first near esophagus at 1pdf and substantially increased at 2dpf and then gradually decreased at 5 to 6 dpf. However, in the hypoxia challenge, tra catfish larvae displayed 100 % survival at 11 dpf. In the histology experiment, the swimbladder was observed at 6 dpf for the first time and the

morphology of the swimbladder was changed gradually with the development. The inner layer made of cuboidal epithelium as well as elastic collagenic fibrosa on the outer layer was detected at 9 dpf. The musculature has also become slightly thicker on the following day when a significant increase in survival rate (80 %) was obtained. At 11 dpf, the swimbladder was significantly increased in size occupying a large portion of the body cavity and fully developed for air-breathing.

Cx3cl1 has been reported to be involved in respiratory activity and pulmonary circulation in human. The distal arterioles with limited smooth muscles in human pulmonary circulation preserved the high blood flow and low pressure. The pulmonary alveolar in human lung compensate the situation by increasing the air exchange area and capillary length. [94] Moreover, in response to hypoxia, excessive *Cx3cl1*is produced by lung microvascular endothelium. It stimulates different activities such as phenotypic switching, proliferation, and muscle expansion in smooth muscle cells.[94] The inner layer of the swimbladder of tra catfish is made up of cuboidal epithelium with an outer layer of elastic collagenic fibrosa (Ma 2021).[188] Additionally, smooth muscles are present in part or all of the swim bladder wall or pneumatic duct of the most physostomes.[205-206] These structural similarities between fish swimbladder and mammalian lung indicate the association of *Cx3cl1* with the ability of tra catfish to survive in hypoxia and expansion of its swimbladder.

Cx3cl1 was observed to be present at a high level in multiple myeloma (MM) patients due to MM-induced angiogenesis.[207] The phylogenic and genomic organization analyses of zebrafish suggest that a substantial number of chemokine genes in this fish may reflect the adaptation of the individual species to their respective biological environment.[208] In the current study, *Cx3cl1* was found as potentially important in the development of swimbladder and air-breathing function in tra catfish in developmental time series analysis. Although most likely related to hypoxia tolerance, there is little information available about this gene in other fish.

3.6 Conclusion

Tra catfish uses gills for regular exchange of respiratory gases. They can use the modified swimbladder to breathe air when the concentration of dissolved oxygen in water is low for facultative air breathing. In this experimen*Grp*t, *Cx3cl1* expression was detected with *in situ* hybridization in the swimbladder of tra catfish larvae. *Cx3cl1* is one of the most important candidate genes which was identified previously as a part of this study through a comparative

genomic analysis between tra catfish (*P. hypophthalmus*) and channel catfish (*I. punctatus*). *Cx3cl1* was found to play crucial roles in development of air-breathing ability in tra catfish. *Cx3cl1* was shown to be essential for the growth and maintenance of human lung function, angiogenesis and respiratory efficiency. Therefore, this gene may contribute to functioning of air-breathing ability of tra catfish. The ISH showed that *Cx3cl1* expresses in the early development of the tra swimbladder, but not in surrounding tissues, providing evidence that this gene is indeed involved in the ABO structure development. Further study should include investigation of other genes that have been co-expressed with *Cx3cl1* during the same developmental stages to identify their roles in air-breathing of this species. Knock out of *Cx3cl1* gene should be done in future to verify its cellular, structural, and molecular impacts and to determine whether it is essential for air-breathing in tra catfish.

Chapter Four: Transfer of Putative ABO Transgenes into Channel catfish (Ictalurus punctatus) Using a CRISPR/Cas9 Knock-in System

4.1 Abstract:

When exposed to a hypoxic environment, certain catfish species can survive for a substantial amount of time by means of air-breathing organs (ABO), which enables them to take oxygen from the air. Tra catfish (*Pangasianodon hypophthalmus*) and bighead catfish (*Clarias macrocephalus*) are two such Asian species capable of air-breathing. Tra catfish possess modified swimbladder while bighead catfish has modified gills that serve as ABO. Channel catfish (*Ictalurus punctatus*), one of the leading aquaculture species in the US, breathes only with gills, but has no air-breathing ability and die at low dissolved oxygen levels. To achieve modification in their response to hypoxic water and to improve survival, *Grp*, *Hrg* and *Cx3cl1* transgene isolated from tra catfish and *Fras1* transgene from bighead catfish, along with their native promoters were knocked in into the channel catfish genome through the CRISPR/Cas9 system. The integration rate of these four transgenes varied among different life stages of transgenic channel catfish, but a significant difference was observed only among the live fingerlings from four types of transgenic groups ($P < 0.05$). *Grp* had the highest integration rate in live fingerlings (7.32%). A significant negative correlation ($r = -1.0$) was observed between the integration rates and the size of the transgene constructs ($P < 0.001$). Sequencing results revealed the precise integration of ABO transgenes along with insertion of the complete sequence of *Grp* and *Cx3cl1* transgene in several individuals despite their large size. *Hrg* and *Cx3cl1* transgenic individuals had significantly lower mean body weight (35.53 to 36.02 % for *Hrg* and 22.25 to 25.65 % for *Cx3cl1*) than the corresponding non-transgenics while *Grp* had no pleiotropic effect on growth. *Fras1* transgenic fish displayed body weight 22.24 to 23.92 % larger than the non-transgenics, but this was not statistically significant ($P > 0.05$). Transgenic individuals were observed to be more vulnerable to enteric septicemia of catfish (ESC) but more resistant to 'Ich'. Alterations in swimbladder

morphology for a sample of *Grp* transgenic individuals supports the hypothesis that this gene could be related to ABO structure. Optimization of sgRNA design to increase activity and lower off-target effects of CRISPR/Cas9 should be examined in future transgenic research. This study will provide preliminary insights of production of ABO transgenic channel catfish capable of surviving in low oxygen water.

4.2 Background:

Catfish farming is one of the major aquaculture industries in the United States and Alabama, Arkansas and Mississippi are the three leading states in catfish production.[4] The catfish industry peaked at 350 million kilograms in the year 2003 followed by a drastic decline due to economic recession, increased costs, and competition from imported products.[4] Despite all of these, in 2010 about 214 million kg of catfish was processed, and it has ranked as the sixth position in 'Top 10' of fish and seafood consumption in the United States.[4] However, the production dropped to 138 million kg in 2011.[5] During 2015-2017, catfish production increased slightly and reached 150 million kg in 2017 and continued to grow slowly with 158 million kg catfish produced in 2019.[5-6]

Channel catfish (*Ictalurus punctatus*) aquaculture began in the United States, and later they were extensively introduced to Europe, Russian Federation, Cuba and portions of Latin America as well as Thailand and China. In 2016, global production of channel catfish was 432.93 million kg.[7] Likewise Asian catfish such as tra (*Pangasianodon hypophthalmus*) and walking catfish (*Clarias batrachus*) and bighead (*Clarias macrocephalus*) are being produced in a substantial amounts. African catfish (*Clarias gariepinus*) is another popular catfish species. During late 80s, the establishment of hybrid between female bighead and male African catfish was a revolutionary milestone in the catfish industry of Thailand.[209]

Several catfish species have some interesting evolutionary features for adaptation to a variety of environmental conditions. For instance, bighead catfish (*C. macrocephalus*) are well adapted, to hypoxic environments[16] and tra catfish (*P. hypophthalmus*) can breathe air in low oxygen water as well.[17] With the help of accessory breathing organs, they can take oxygen from air and combat and survive in the hypoxic environment. The bighead catfish, *Clarias macrocephalus,* has both

gills and modified gill structures that help air-breathing. In a terrestrial environment, they can survive and move for a significant time and distance as long as the skin is moist.[18-19] The tra catfish (*Pangasianodon hypophthalmus*) can initiate facultative air breathing with adapted swim bladder in oxygen-depleted waters.[17] On the other hand, channel catfish does not possess such air breathing organ (ABO) and neither can breathe air.[20] Sometimes the North American channel catfish industry faces catastrophic mortality and economic loss since the fish do not tolerate low oxygen levels. The transfer of putative structural and functional ABO genes from tra and bighead catfish into channel catfish could be a partial solution to improve air-breathing ability.

In recent decades, a new gene editing technique known as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), is being applied in some fish species to produce transgenics.[135, 137, 210] In this method the genome is edited by stimulating a double-strand break (DSB) at the desired site(s) with endonuclease Cas9 combined with a synthetic small guide RNA (sgRNA) targeting certain gene(s) into eukaryotic cells.[211] It became popular for being precise, efficient and inexpensive. A lot of researchers have used this technique to modify various genes in model fishes such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*).[131, 212] Now a days it is being successfully used in aquaculture to create a variety of phenotypes in terms of reproduction, fertility, muscle growth and disease resistance in Atlantic salmon (*Salmo salar*),[213] Nile tilapia (*Orechromis niloticus*),[158] common carp (*Cyprinus carpio*),[137] channel catfish (*Ictalurus punctatus*),[138, 210] red sea bream (*Pagrus major*)[135] and rainbow trout (*Oncorhynchus mykiss*).[214]

Eukaryotic genomes include regions comprised of protein coding genes as well as noncoding DNA that are integral to the function of cells and control of gene activity.[153-154] It is now known that the channel catfish genome has 100 % function.[157] Protein coding sequences work as ready targets for many CRISPR/Cas9 applications to create small insertion and deletion (indel) mutations to interrupt the open reading frames of coding genes.[125, 136] However, mutation of non-coding sequences using CRISPR/Cas9 system is difficult because small indels by a single mutation may not lead to a detectable loss of function.[158] Since the noncoding regions are known to affect the expression of neighboring or distant genes by acting as signaling, guiding, sequestering or scaffolding molecules, targeting non-coding sequences either by means of loss-of-function or gain-of-function approaches can be beneficial.[159-160] It may affect multiple genes resulting in off-target

effects too. Several experiments have successfully produced large genomic deletions with dual guide RNAs (gRNAs) in mammalian cells and animal models such as mouse and zebrafish.[161-162] A recent advancement in channel catfish is the deletion mutations of up to 900 bp targeting the toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) by a single guide RNA.[138] Additionally, CRISPR/Cas9 system effectively generated desirable non-coding sequence mutants in Nile tilapia (*O. niloticus*).[158] There has been no attempt of targeted gene insertion in non-coding regions that can create phenotypic changes for air-breathing ability in fish. Therefore, CRISPR/Cas9 directed insertion of ABO genes has the potential to develop transgenic channel catfish capable of survival in low oxygen water.

Previously conducted comparative genomic and transcriptomic analyses of early developmental stages of channel (*I. punctatus*), tra (*P. hypophthalmus*) and bighead catfish (*C. macrocephalus*) revealed twenty-six and fourteen species specific genes putatively responsible for development of the air breathing organ (ABO) and air-breathing function in bighead and tra catfish, respectively.[83, 188] Since these genes are absent from channel catfish genome, CRISPR/Cas9 mediated knock-in of ABO transgenes may potentially create phenotypic changes in terms of survival in low oxygen water. From the list of candidate genes, *Grp, Hrg, Cx3cl1* from tra catfish (*P. hypophthalmus*) and *Fras1* from bighead catfish (*C. macrocephalus*) were selected for CRISPR/Cas9 mediated insertion. The overarching objective of this project was to initiate the process of generation of low oxygen tolerant transgenic lines of channel catfish carrying actively expressing ABO genes with positive phenotypic changes that can be inherited by subsequent generations. The primary specific objectives were to determine the feasibility of the insertion of these ABO transgenes into channel catfish and to determine any effect on growth and survival.

4.3 Materials and methods:

4.3.1 Ethics statement

Channel catfish were reared at the Fish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences at Auburn University, Alabama, USA. The Institutional Animal Care and Use Committee (IACUC) approved all the experiments in this study.

4.3.2 Design of donor DNA templates for knock-in

Plasmid DNA, pUC-GW-Amp (2.6 kb), was used for four different donor DNA constructs (Figure 11). The selected ABO genes were *Grp* (gastrin-releasing peptide-like isoform X2, Gene ID. 113535796), *Hrg* (histidine-rich glycoprotein, Gene ID. 113547643), *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2, Gene ID. 113533685) from tra catfish (*P. hypophthalmus*) and *Fras1* (fraser extracellular matrix complex subunit 1, unpublished annotation)[98] from bighead catfish (*C. macrocephalus*). The coding sequences for *Grp*, *Hrg*, *Cx3cl1* and *Fras1* are 441 bp, 1497 bp, 324 bp and 5145 bp long respectively and they were driven by their native promoters. The total size of the entire transgene construct is 3353 bp for *Grp*, 4874 bp for *Hrg*, 3692 bp for *Cx3cl1* and 6467 bp for *Fras1*. The detailed sequences of the constructs are found in Appendices E, F, G and H. The constructs also included left and right homology arms (323 bp each) derived from chromosome 1 (Database ID. NC_030416.1) and chromosome 7 (NC_030422.1) of channel catfish. The plasmid DNA constructs were synthesized by GENEWIZ from Azenta Life Sciences (Chelmsford, MA, USA).

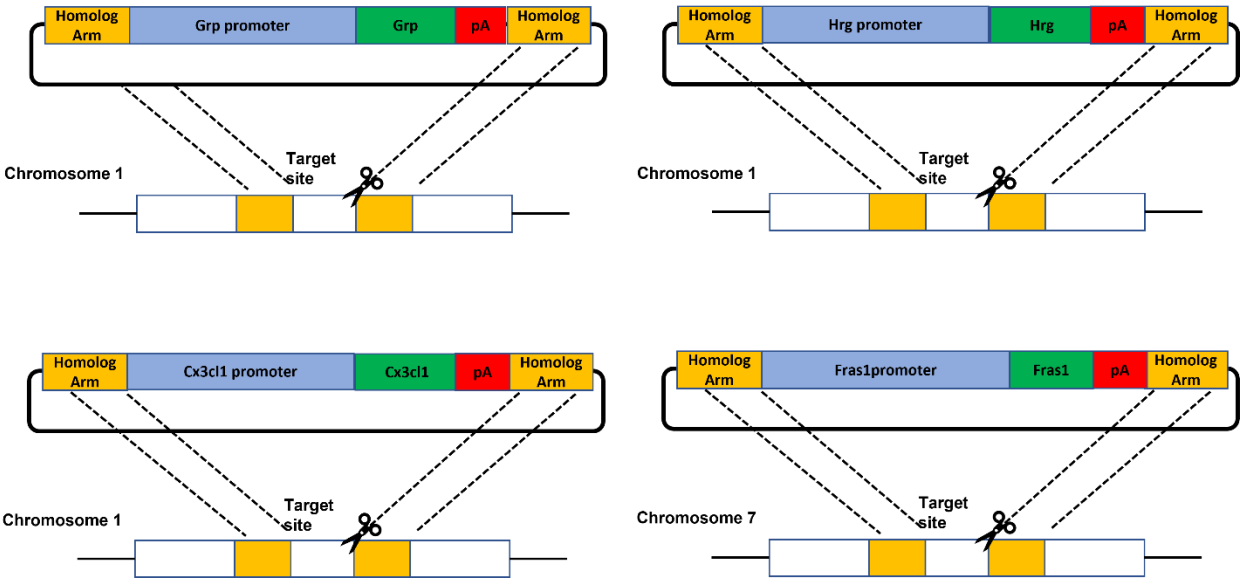

Figure 11. Schematic representation of donor DNA constructs used in CRISPR/Cas9 knock-in of channel catfish (*Ictalurus punctatus*). All the constructs were driven by their native promoters. The origin of species for *Grp*, *Hrg* and *Cx3cl1* is tra catfish (*Pangasianodon hypophthalmus*) and for

Fras1 is bighead catfish (*Clarias macrocephalus*). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1 and pA = poly A tail region.

4.3.3 Design of sgRNA and preparation of CRISPR/Cas9 system

The CRISPR Design online tool (https://zlab.bio/guide-design-resources) was used to design the small guide RNA (sgRNA) that targeted the channel catfish chromosome 1 (Database ID. NC_030416.1) and chromosome 7 (Database ID. NC_030422.1). A cloning-free (PCR-based) method was used to create sgRNA. The sequences of the universal primer and sgRNA used in this study and the upstream and downstream of the target are listed in Table 2. The sgRNAs were produced by T7 run off.[210, 215] The universal primer and ssDNA templates were annealed and filled by Platinum™ Taq DNA Polymerase (Invitrogen, Waltham, MA). The subsequent dsDNA was used as the template for *in vitro* transcription to make sgRNA using the Maxiscript T7 Kit (Thermo Fisher Scientific, Waltham, MA). The sgRNA was purified by RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The Cas9 protein, the RNA-guided DNA endonuclease enzyme, was taken from PNA BIO Inc. (Newbury Park, CA). The concentration of each of the four donor DNA constructs was 50 ng/µl. The CRISPR/Cas9 system used in microinjection was a mixture of sgRNA, Cas9 protein and donor DNA in the ratio of 1:1:1 along with one component of phenol red (60%) that helps to track microinjected eggs visually. The final concentrations of sgRNA and Cas9 protein were 150–200 ng/µl and 300–350 ng/µl, respectively. The sgRNA and Cas9 protein mixtures were incubated on ice for 8 min followed by adding donor DNA and phenol red. The mixture was delivered into fertilized eggs after loading into the microinjection needle.[216]

Table 2. The sequences of small guide RNA (sgRNA) and the universal (common) primer used to target chromosome 1 and 7 of channel catfish (*Ictalurus punctatus*). Underlined sequences represent the protospacer adjacent motif (PAM). Numbers in parenthesis represent the physical location of the target in the channel catfish genome.

Guide RNA	Oligo sequence (5'-3')
Chromosome 1 sgRNA	TCGACATCAGTAATCGAACAAGG (21472187 - 21472207 bp)
	CATCCAGACGAGCTTAGCCAAGG (21518761 – 21518780 bp)
	GTGCTCCTGCTGCTGTTGTATGG (18501052 – 18501071 bp)
Chromosome 7 sgRNA	CCCATTGGAGGAGCTTTCGGTGG (20449053 – 20449072 bp)
Universal primer	TTTTGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT
	TATTTTAACTTGCTATTTCTAGCTCTAAAAC

4.3.4 Egg collection, sperm preparation and fertilization

Brood stock preparation and artificial spawning were performed following a standard protocol with few modifications.[216] Sexually mature channel catfish males and females from different strains were chosen for artificial spawning. The female broodfish were implanted with 75 μg/kg of luteinizing hormone releasing hormone analog (LHRHa) to induce ovulation. Eggs were stripped into a 20-cm greased spawning pan. The males were euthanized followed by collection of testes. The sperm was prepared by crushing the testes in 0.9% saline solution. Then 1-2 mL of sperm suspension was added to the eggs and mixed by rotating gently. Fresh water was added to activate the sperm and to cover the egg mass. The sperm-egg mixture was gently swirled nearly for 30 s. The eggs were allowed to harden for 10–15 min prior to microinjection by adding more fresh water.

4.3.5 Microinjection and hatching of embryos

The one-cell stage embryos were microinjected with sgRNA, Cas9 protein and donor DNA mixture using a microinjection system from Applied Scientific Instrumentation (Eugene, OR). Around 50 nl of the solution was directly injected into the yolk sac of each embryo with a 1.0 mm

OD borosilicate glass capillary that was previously pulled into a needle by a vertical needle puller (David Kopf Instruments, Tujunga, CA). Embryos were injected approximately within 15-90 min post-fertilization. The number of embryos injected and their sham injected controls for each trial can be found in Appendix I. The injected and control embryos were incubated in 8 L plastic tubs loaded with Holtfreter's solution (59 mmol NaCl, 0.67 mmol KCl, 2.4 mmol $NaHCO_3$, 0.76 mmol $CaCl_2$, 1.67 mmol $MgSO_4$) containing 10 ppm doxycycline.[217] The embryos were incubated at a density of 250-260 embryo/tub with uninterrupted aeration at 27 °C for 6–8 days until hatching is completed. Dead embryos were removed twice daily and recorded. The hatched ones were transferred to a Holtfreter's solution without doxycycline until they were capable of free swimming. Once their yolk sac was absorbed, the larvae were fed with Artemia nauplii three to four times a day. Early fry survival was calculated at 15 days post-fertilization and the live fry were transferred into 60-L recirculating aquaria with 70 to 80 individuals in each tank.

4.3.6 Fish culture and sampling

At 3 months of age, the fish were transferred to additional tanks to reduce the density to 40 to 50 individuals per tank. Transgenic, non-transgenic and sham injected control (iCntl) channel catfish were weighed at approximately 4 months of age and pit-tagged. The transgenic and non-transgenic fish in each treatment were mixed in the same tank to ensure that they were exposed to the same feeding and environmental conditions. The sham injected controls were kept in separate tanks. Fish were fed ad libitum three times daily (50 % crude protein, Purina AquaMax® Fingerling Starter 300) with the size of pellets appropriate for the body size (developmental stage) of the fish. After another 4 months of growth (8 months of age), 46 transgenics, 65 non-transgenics and 26 sham injected controls were selected and weighed. The water quality parameters such as temperature, pH, salinity, ammonia, dissolved oxygen, nitrite, alkalinity, and hardness were measured at weekly intervals. Dissolved oxygen level was maintained above 5 ppm and ammonia below 0.4 ppm. Hardness was kept at more than 30 ppm and nitrite was 0 ppm. Salinity was maintained above 3 ppt throughout the experiment. The mortality data were also recorded.

4.3.6 Integration analysis

Fingerlings were tagged with PIT tags. Genomic DNA from dead fry and fin-clip samples of 4-month-old fingerlings were extracted via proteinase K digestion and iso-propanol precipitation.[204] A sample of 1026 individuals from the four treatments were subjected to PCR amplification for genotyping. Primer pairs that amplified the ABO transgene regions for all the four different constructs are listed in Table 3. Later, the positive samples were tested for PCR amplification of 5′ (promoter end) and 3′ (terminal end). A positive band of the right size implied a correctly placed knock-in at the targeted locus. Polymerase Chain Reaction (PCR) products from individual fry were verified by sequencing (Sequetech Corporation, Mountain View, CA). Integration rates were calculated as the number of positive individuals identified by PCR in a treatment divided by the total number of individuals in the same treatment multiplied by 100.

Table 3. Oligonucleotide primers used for CRISPR/Cas9 knock-in for putative ABO transgenes *Grp*, *Hrg*, *Cx3cl1* and *Fras1* in channel catfish (*Ictalurus punctatus*). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1.

Target gene	Purpose	Name	Nucleotide sequence (5′-3′)
Grp	PCR: *Grp* gene region	*Grp*-F1	GCTGTTTTTGCGTTGATTGA
		Grp-R1	CTGTTCGTTCTGCACTGCAT
	PCR: Promoter end	*Grp*-F2	TGGGTTCTTTGAGGAACAGG
		Grp-R2	GTCATTAGGCTTCCAGGCTG
	PCR: Terminal end	*Grp*-F3	GGAAGAACAGCTGGCAAAAG
		Grp-R3	CAGGAACACTGGGTGTGATG
Hrg	PCR: *Hrg* gene region	*Hrg*-F1	GAGGTTACGCGATCCATGTT
		Hrg-R1	GCGTAACACGTATGCTGAGC
	PCR: *Hrg* gene region	*Hrg*-F2	GTTCCAACACACGCTCACAC
		Hrg-R2	CAAAGAGCAGTGACAAACGC
	PCR: Promoter end	*Hrg*-F3	TGGAATTAAACGCAGCAACA
		Hrg-R3	CGTTACTACGGAGACTCGCC
	PCR: Terminal end	*Hrg*-F4	ACACAAGCTGCCACACAAAG
		Hrg-R4	CTATGAGGAGTCAGCCTGCC

Cx3cl1	PCR: *Cx3cl1* gene region	*Cx3cl1*-F1	CCCGAAAATGAGGGTCTGTA
		Cx3cl1-R1	GAAGCCTGGCACTACCTCTG
	PCR: Promoter end	*Cx3cl1*-F2	GGTTTGGTGGACAGGGTCTA
		Cx3cl1-R2	GCCTACACAGTCCACCGAAT
	PCR: Terminal end	*Cx3cl1*-F3	TATCTCGTGGACTGGGAAGG
		Cx3cl1-R3	AAGAAGGATGTGTCCCAGGA
Fras1	PCR: *Fras1* gene region	*Fras1*-F1	AGGGATTTATCACGCACCTG
		Fras1-R1	AGTCCACCGTTCCTCATCAC
	PCR: *Fras1* gene region	*Fras1*-F2	AGTGGAAGGTGGAGGTTGTG
		Fras1-R2	GTCTAAGGCCTGCACTCCAG

4.3.8 Statistical analysis

Hatchability (%) of embryos was calculated as the total number of fry that completed hatching divided by the total number of embryos followed by multiplication by 100. Hatching was completed approximately after 6 or 7 days post fertilization (dpf). Embryo mortality (%) was calculated as the number of dead embryos in a treatment divided by the total number of embryos and multiplied by 100. Fry survival was calculated as the total number of fry that survived 15 days post hatch (dph) divided by the total number of hatched embryos and multiplied by 100. Integration rates for each treatment were determined as the total number of positive fish divided by the total number of fish analyzed multiplied by 100. One-way ANOVA and Tukey's multiple comparisons test were used to analyze data for significant differences among treatments in terms of hatchability (%), embryo mortality (%) and fry survival (%). Histograms were made using Microsoft Excel 2016. Statistical analyses were performed using R software.[218] Statistical significance was set at $P < 0.05$, and data were presented as the mean ± standard error (SEM).

Integration rates were analyzed with Fisher's exact test. Integration rates were calculated and compared for different life stages such as dead embryos, dead fry and live fingerlings injected with four types of above-mentioned transgene constructs and also for each type of transgene construct at different developmental stages. Paired t-tests were done to compare the mean body weights between transgenic and non-transgenic individuals of each transgene treatment. The overall integration rates were also calculated for each type of transgene construct combining all the developmental stages such as dead embryo, dead fry and live fingerling as a percentage of the

total number of individuals tested. Spearman's rank correlation was used to determine the correlation between the overall integration rate (%) and the corresponding size of the transgene construct (bp).

4.4 Results:

4.4.1 Embryo hatchability and mortality, fry survival rates

No significant difference ($P > 0.05$) was found among the hatchability of embryos microinjected with four different donor DNA constructs (Table 4, Fig. 12-a). However, observed hatch rate was low for *Hrg* (20.24 %) compared to the other three treatments. The injected control (iCntl) had the highest number of embryos hatched (42.19 %) compared to other groups ($P > 0.05$).

Mortality of embryos ranged from 57.82 % in iCntl to 79.76 % in *Hrg* (Table 4, Fig-12-b). Similar mortality patterns were observed in the rest of the three treatments. No significant difference was detected in embryo mortality among embryos microinjected with all four different donor DNA constructs and their controls ($P > 0.05$).

A significant difference in fry survival was detected between *Grp* and iCntl ($P < 0.05$) (Table 4, Fig. 12-c). However, the four transgene treatments did not vary among each other. Highest survival rate was observed in *Hrg* (51.99 %) compared to other three treatments. *Grp* has the lowest survival rate (4.47 %) but is not significant when compared to *Fras1*, *Cx3cl1* and *Hrg* ($P > 0.05$).

Table 4. The hatchability, embryo mortality and fry survival of channel catfish (*Ictalurus punctatus*) microinjected with four transgene constructs carrying ABO transgenes with CRISPR/Cas9 system. All data are presented as mean ± standard error (SEM) and analyzed by one-way ANOVA followed by Tukey's test. Means in the same column followed by different superscripts are significantly different ($P < 0.05$). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1; iCntl= injected control (60% phenol red solution); N = total number of individuals.

Treatment	Donor DNA	Embryos injected	Hatchability		Embryo mortality		Fry survival	
	Conc. (ng/µl)		N	%	N	%	N	%
Grp	50	3567	1056	26.16 ± 8.17[a]	2511	73.84 ± 8.17[a]	62	4.47 ± 1.12[b]
Fras1	50	3647	1188	32.89 ± 17.90[a]	2429	66.27 ± 18.74[a]	550	29.62 ± 15.84[ab]
Cec1	50	2641	1177	37.02 ± 14.58[a]	1464	62.98 ± 14.58[a]	417	20.94 ± 11.62[ab]
Hrg	50	3082	617	20.24 ± 12.46[a]	2465	79.76 ± 12.46[a]	319	51.99 ± 0.47[ab]
iCntl		1968	815	42.19 ± 8.43[a]	1153	57.82 ± 8.43[a]	515	65.96 ± 29.50[a]

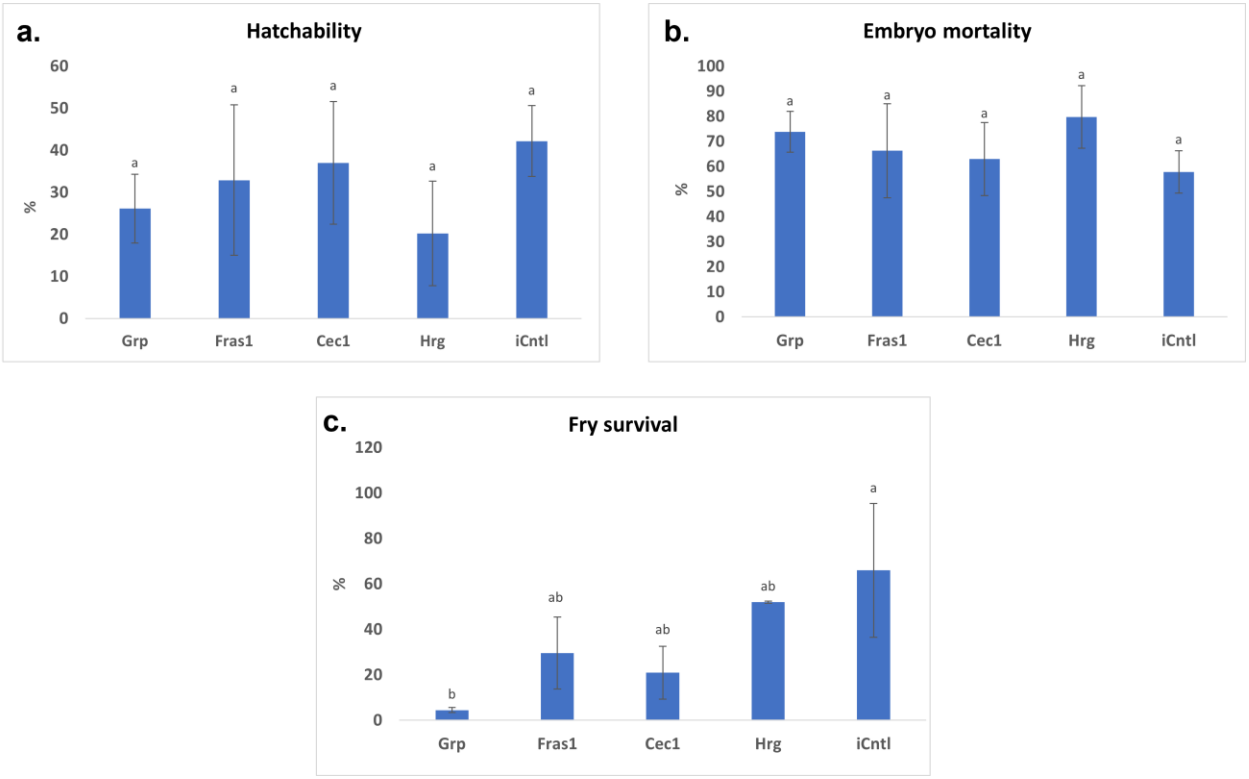

Figure 12. Plots of a) hatchability, b) embryo mortality, and c) fry survival of channel catfish (*Ictalurus punctatus*) microinjected with four ABO transgene constructs: *Grp*, *Fras1*, Cec1 and *Hrg* and iCntl. The values represent mean ± SEM and analyzed by one-way ANOVA followed by

Tukey's test. Means with different letters are significantly different ($P < 0.05$). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1; iCntl= injected control (60% phenol red solution).

4.4.2 Analysis of integration

Genotyping of fish was done in two steps. First the amplification of the ABO transgene region of the donor DNA constructs were tested to confirm the insertion of the gene. Then, amplification of the 5′ and 3′ junctional regions were tested to ensure that the promoter and terminal regions were also inserted.

Grp

Figure 13-c shows the amplification of the *Grp* region. Using primer pair 1, digested PCR products for the *Grp* gene exhibited one distinct band (597 bp) comparable to the band seen in the plasmid control (C). In contrast, wild type (WT) fish displayed no band. Sequencing results also confirmed the integration of a 597-bp *Grp* gene in the positive fish (Fig.13-d). After amplifying the *Grp* region of the transgene constructs, the 5′ and 3′ junctional regions were also amplified. Two sets of primer pairs amplified this transgene construct. Distinct bands amplified the different regions of the constructs. The promoter region was amplified by primer pair 2 (*Grp*-F2 & *Grp*-R2) while primer pair 3 (*Grp*-F3 & *Grp*-R3) amplified the terminal end (Figures 13-c).

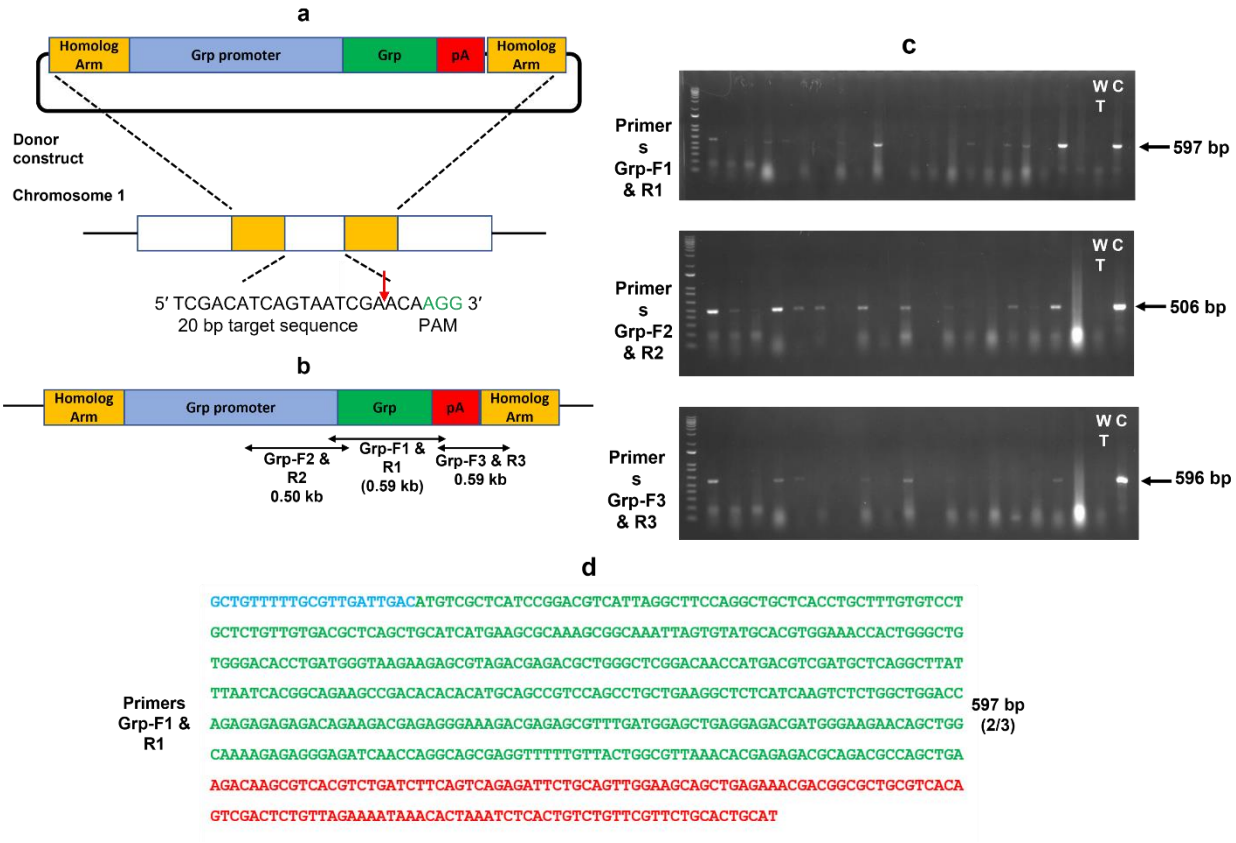

Figure 13. a) Schematic representation of the 20-bp guide RNA containing the PAM and the cut site (red arrow) which aided the targeted insertion of the donor construct, b) Schematic representation of *Grp* (gastrin-releasing peptide-like isoform X2) transgene construct showing the region of amplification, c) Representative gels of PCR amplification of the insert-specific region for *Grp* gene (primer set *Grp*-F1 & R1) and the 5′ and 3′ junctional regions (primer sets *Grp*-F2 & R2 and *Grp*-F3 & R-3) form positive fish, d) Representative sequences derived from channel catfish (*Ictalurus punctatus*) positive for *Grp* transgene construct. Sequences in blue are partial sequences from 5′ UTR (untranslated region); green are the sequences of *Grp* gene. Red sequences belong to the poly A terminator sequence. Numbers on the right side of each sequence indicate the number of base pairs in the sequence that revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which produced positive transgene integration over the total number of sequencing reactions. WT = wild type and C = plasmid control.

Hrg

The digested PCR products showed distinct bands (588 bp and 603 bp) for the individuals carrying the *Hrg* gene in their genome while wild type control did not show any band (Fig.14-c). Primer pair *Hrg*-F1 & R1 and *Hrg*-F2 & R2 were used to amplify these regions. Sequencing results also indicated successful insertion of this gene (Fig.14-d). Figure 14-c shows the amplification of the *Hrg* region, and examples of positive individuals. After amplifying the *Hrg* region of the transgene construct, the 5′ and 3′ junctional regions were also amplified. Distinct bands were amplified from different regions of the construct (Fig.14-c). The promoter region was amplified by primer pair 3 (*Hrg*-F3 & R3) while primer pair 4 (*Hrg*-F4 & *Hrg*-R4) amplified the terminal end.

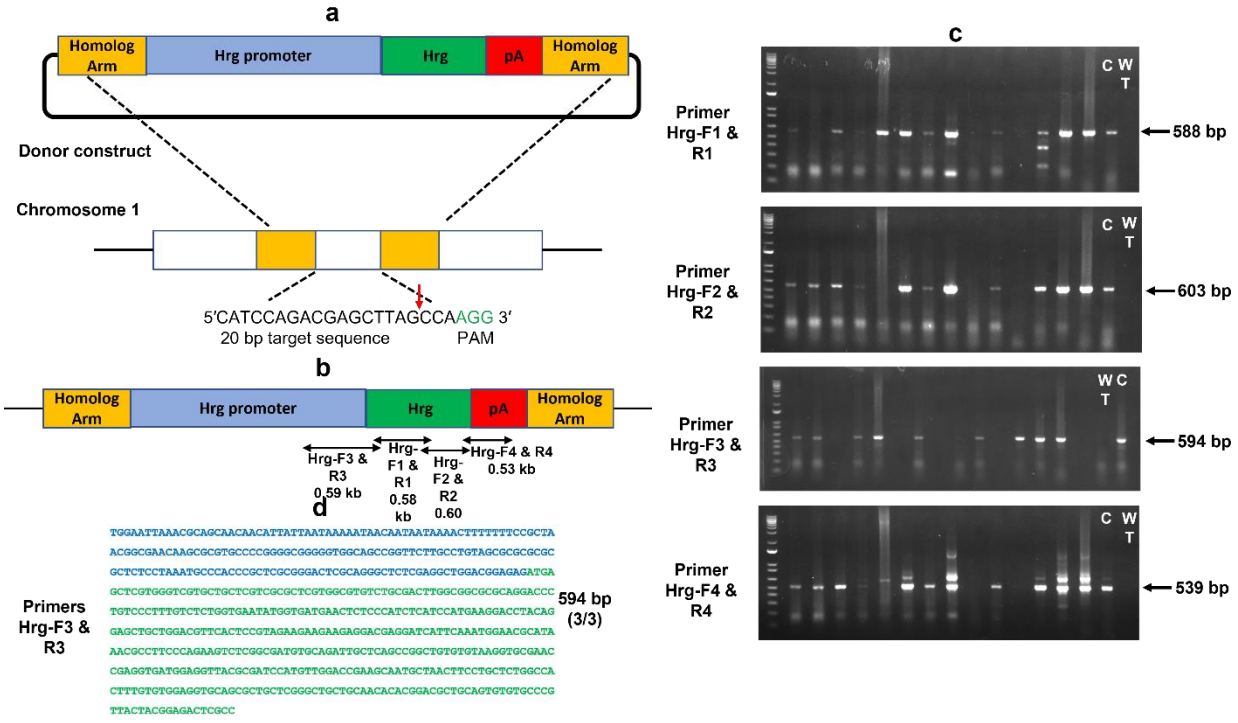

Figure 14. a) Schematic representation of the 20-bp guide RNA containing the PAM and the cut site (red arrow) which aided the targeted insertion of the donor construct, b) Schematic representation of *Hrg* (histidine-rich glycoprotein) transgene construct showing the region of amplification, c) Representative gels of PCR amplification of the insert-specific region for *Hrg* gene (primer set *Hrg*-F1 & R1; *Hrg*-F2 & R2) and the 5′ and 3′ junctional regions (primer sets *Hrg*-F3 & R3 and *Hrg*-F4 & R4) form positive fish, d) Representative sequences derived from channel catfish (*Ictalurus punctatus*) positive for *Hrg* transgene construct. Sequences in blue are

partial sequences from 5′ UTR (untranslated region); green are the sequences of *Hrg* gene. Numbers on the right side of each sequence indicate the number of base pairs in the sequence that revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which produced positive transgene integration over the total number of sequencing reactions. WT = wild type and C = plasmid control.

Cx3cl1

A distinct band (502 bp) was observed for the individuals carrying the *Cx3cl1* gene (Primer pair *Cx3cl1*-F1 & R1) in their genome while wild type controls did not exhibit any band. Sequencing results also indicated successful insertion of this gene (Fig.15-d). Figure 15-c shows amplification of the *Cx3cl1* region along with examples of positive individuals. After amplifying the *Cx3cl1* region of the transgene construct, the 5′ and 3′ junctional regions were also amplified. Distinct bands were amplified from the different regions of the construct (Figures 15-c). The promoter region was amplified by primer pair 2 (*Cx3cl1*-F2 & R2) while primer pair 3 (*Cx3cl1*-F3 & R3) amplified the terminal end.

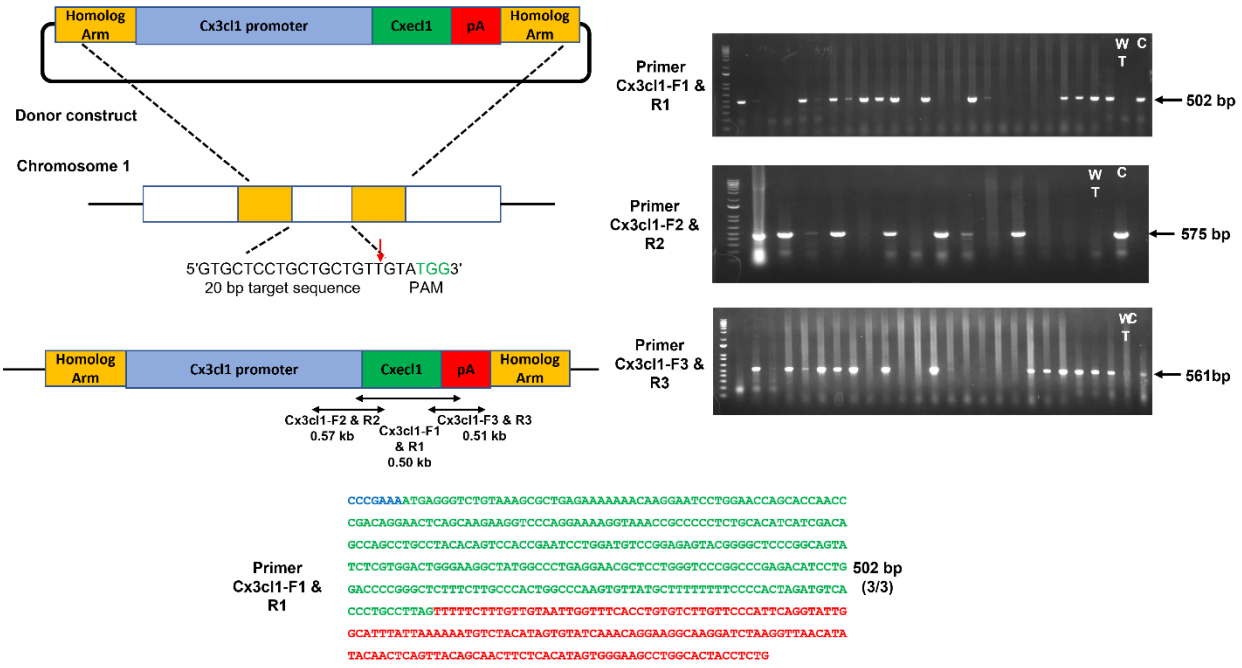

Figure 15. a) Schematic representation of the 20-bp guide RNA containing the PAM and the cut site (red arrow) which aided the targeted insertion of the donor construct, b) Schematic

representation of *Cx3cl1* (chromo domain-containing protein cec-1-like isoform X2) transgene construct showing the region of amplification, c) Representative gels of PCR amplification of the insert-specific region for *Cx3cl1* gene (primer set *Cx3cl1*-F1 & R1) and the 5′ and 3′ junctional regions (primer sets *Cx3cl1*-F2 & R2 and *Cx3cl1*-F3 & R3) form positive fish, d) Representative sequences derived from channel catfish (*Ictalurus punctatus*) positive for *Cx3cl1* transgene construct. Sequences in blue are partial sequences from 5′ UTR (untranslated region); green are the sequences of *Cx3cl1* gene. Red sequences belong to the poly A terminator sequence. Numbers on the right side of each sequence indicate the number of base pairs in the sequence that revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which produced positive transgene integration over the total number of sequencing reactions. WT = wild type and C = plasmid control.

Fras1

Figure 14-c shows the amplification of portions of the *Fras1* gene region. Using primer pairs *Fras1*-F1 & R1 and *Fras1*-F2 & R2, digested PCR products for the *Fras1* gene exhibited distinct bands (510 bp and 553 bp) comparable to the band seen in the plasmid control (C). In contrast, wild type (WT) fish displayed no band. Sequencing results also confirmed the integration of a 510-bp *Fras1* gene in the positive fish (Fig.16-d).

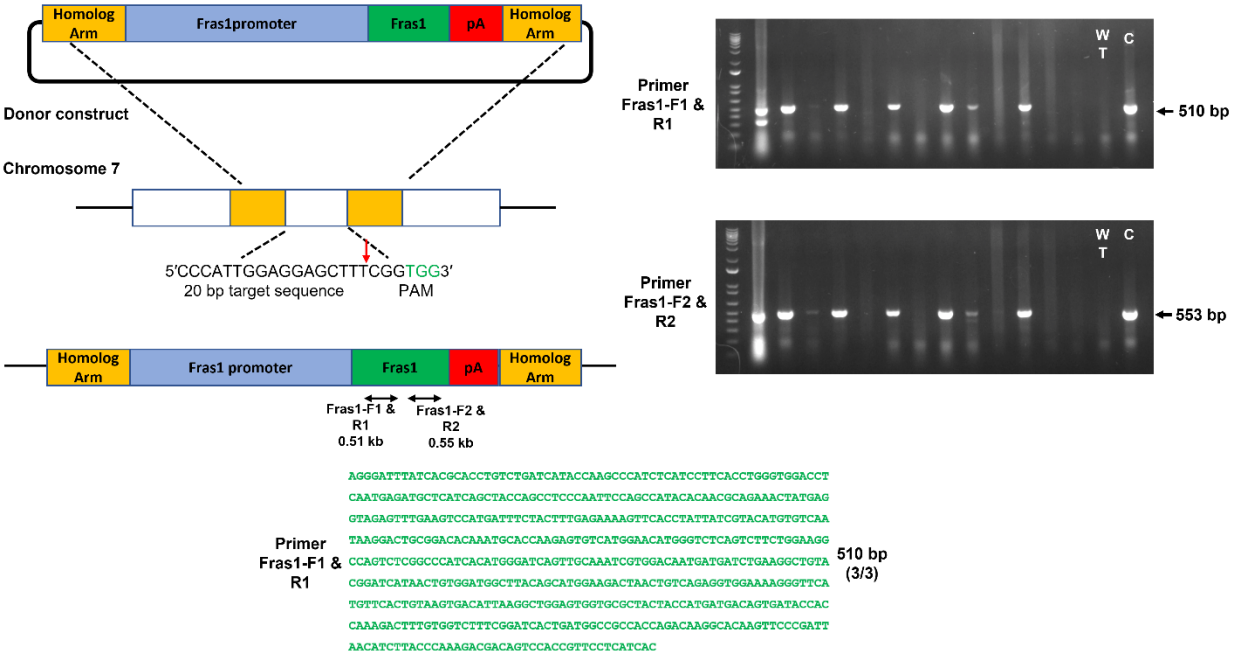

Figure 16. a) Schematic representation of the 20-bp guide RNA containing the PAM and the cut site (red arrow) which aided the targeted insertion of the donor construct, b) Schematic representation of *Fras1* (fraser extracellular matrix complex subunit 1) transgene construct showing the region of amplification, c) Representative gels of PCR amplification of the insert-specific region for *Fras1* gene (primer set *Fras1*-F1 & R1 and *Fras1*-F2 & R2) form positive fish, d) Representative sequences derived from channel catfish (*Ictalurus punctatus*) positive for *Fras1* transgene construct (primer set *Fras1*-F1 & R1). Sequences in green are the partial sequences of *Fras1* gene. Numbers on the right side of each sequence indicate the number of base pairs in the sequence that revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which produced positive transgene integration over the total number of sequencing reactions. WT = wild type and C = plasmid control.

4.4.3 Integration rates

Integration rates were calculated and compared for different life stages such as dead embryos, dead fry and live fingerlings, injected with four types of putative ABO transgene construct at (Table 5). Fisher's exact test was used to analyze these data since the sample size is small. No significant difference was observed among dead embryos microinjected with different transgene constructs ($P > 0.05$). *Hrg* showed the highest observed integration rate (16.67 %) in

dead embryos followed by *Grp* (11.43%) and *Cx3cl1* (5.88%) while *Fras1* had no integration at all.

Dead fry microinjected with different putative ABO transgene constructs also showed no significant difference ($P > 0.05$). *Cx3cl1* had the highest integration rate (12.20%) in dead fry whereas *Fras1* had the lowest (4.16%).

However, a significant difference was observed among the live fingerlings from four types of transgenic groups ($P < 0.05$). The highest integration rate was observed in *Grp* (7.32%) followed by *Cx3cl1*(7.07%) and *Hrg* (4.31%). *Fras1* showed the lowest integration rate (2.49%).

Integration rates were also calculated and compared for each type of transgene construct at different developmental stages with same statistical test as mentioned above. No significant difference in integration rates among dead embryo, dead fry and live fingerlings was observed for *Grp* ($P > 0.05$). Similar results were also found for *Hrg*, *Cx3cl1* and *Fras1* ($P > 0.05$).

Table 5. Integration rates of *Grp*, *Hrg*, *Cx3cl1* and *Fras1* transgene constructs in dead embryo, dead fry and live fingerlings of microinjected channel catfish (*Ictalurus punctatus*). Fisher's exact test was used to analyze the integration rates. *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1; N = total number of individuals analyzed.

	Dead embryo			**Dead fry**			**Fingerling**		
Treatment	**Tested (N)**	**Positive (N)**	**Integration rate (%)**	**Tested (N)**	**Positive (N)**	**Integration rate (%)**	**Tested (N)**	**Positive (N)**	**Integration rate (%)**
Grp	140	16	11.43	26	2	7.69	41	3	7.32
Hrg	6	1	16.67	50	5	10.00	232	10	4.31
Cx3cl1	17	1	5.88	41	5	12.20	311	22	7.07
Fras1	27	0	0	48	2	4.16	442	11	2.49

The overall integration rates were also calculated for each type of transgene construct combining all the developmental stages such as dead embryo, dead fry and live fingerling as a percentage of the total number of individuals tested and assessed to correlate the respective transgene construct size. Spearman's rank correlation was used to calculate the correlation coefficient. There was a statistically significant ($P < 0.001$) negative correlation ($r = -1.0$) between

the overall integration rate and the size of the transgene construct which means the overall integration rate decreased with the increasing size of the construct (Fig. 17).

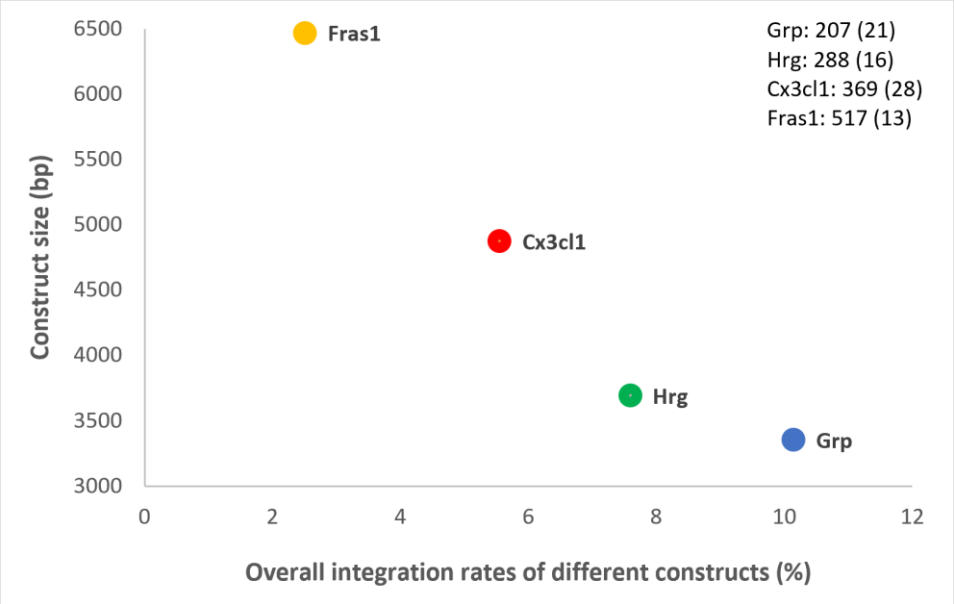

Figure 17. The scatter plot of the correlation between the overall integration rates (%) and the size of the transgene constructs (bp) among the transgenic channel catfish (*Ictalurus punctatus*). The overall integration rate for each treatment was calculated for the total number of positive dead embryo, dead fry and live fingerling as a percentage of the total number of individuals analyzed. Spearman's rank correlation coefficient, r = -1.0 and P < 0.001. *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1. The numbers listed on the righthand top indicate the total number of individuals tested and the subsequent positive individuals (in parenthesis) for their corresponding transgene construct.

4.4.4 Body weight measurement

The body weights were measured at two sampling periods: 4 months post hatch and 8 months post hatch to evaluate the potential pleiotropic effects of putative ABO transgenes on the growth performance of channel catfish (*I. punctatus*). No significant difference in mean body

weight was found between the transgenic and non-transgenic fish of *Grp* and *Fras1* during the first and the second sampling (P > 0.05) (Table 6). However, for both *Hrg* and *Cx3cl1* the mean body weight was significantly different between transgenics and non-transgenics during both samplings (P < 0.05). During the second sampling, four months apart from the first one, only *Cx3cl1* was observed to have a significantly different average weight gain from the non-transgenic individuals. Among the four transgene treatments *Hrg* had the highest average weight gain of 15.2 g.

Table 6. Body weight of channel catfish (*Ictalurus punctatus*) microinjected with one of four transgene constructs- *Grp, Hrg, Cx3cl1* and *Fras1* and the sham injected control (60% phenol red solution) at 4 and 8 months post hatch. Transgenic and non-transgenic full-siblings were cultured communally in 60-L tanks initially with 70-80 individuals per tank and later reduced to 40-50 individuals per tank. Data are presented as mean ± standard error (SEM) and analyzed by paired t-test. Means of different groups under each treatment followed by different superscripts are significantly different (P < 0.05). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1 and iCntl = injected control; N = total number of individuals sampled.

Treatment		N	Mean weight (g)		
			First sampling	Second sampling	Weight gain
Grp	Transgenic	3	22 ± 0.58^{a}	27 ± 1.04^{a}	5 ± 0.76^{a}
	Non-transgenic	3	22 ± 3^{a}	27.33 ± 2.91^{a}	5.33 ± 1.20^{a}
Hrg	Transgenic	10	24.15 ± 3.08^{b}	39.35 ± 6.03^{b}	15.2 ± 3.15^{a}
	Non-transgenic	10	37.46 ± 1.91^{a}	61.5 ± 4.33^{a}	24.04 ± 3.89^{a}
Cx3cl1	Transgenic	21	21.10 ± 1.36^{b}	26.52 ± 1.77^{b}	5.43 ± 0.58^{b}
	Non-transgenic	21	27.14 ± 3.06^{a}	35.67 ± 4.48^{a}	8.52 ± 1.50^{a}
Fras1	Transgenic	12	25.08 ± 3.66^{a}	29.58 ± 4.54^{a}	4.50 ± 1.07^{a}
	Non-transgenic	12	19.08 ± 1.25^{a}	23 ± 1.78^{a}	3.92 ± 0.68^{a}

4.4.5 Fish behavior and deformity

In most cases, no obvious differences in behavior were observed between the transgenic groups and the control. However, *Fras1* fishes were often found to huddle together around the

corner at the bottom of the tank. This might be a family effect as the different groups were made with different spawns. Since only a small fraction of these fish were transgenics, they also may have influence on the rest of the stock.

Hrg, *Fras1* and *Cx3cl1* transgenic did not exhibit any noticeable deformities. In *Grp*, a deformed swimbladder was observed in three fish (Fig. 18). Immediately after hatching, large number (540 hatchlings, 92% of total hatched) of *Grp* injected fish died within a short window of approximately 12 hours. As this treatment had significant mortality at early stage of life, the transgene may have some deleterious effect on swimbladder formation, and perhaps other traits resulting in mortality.

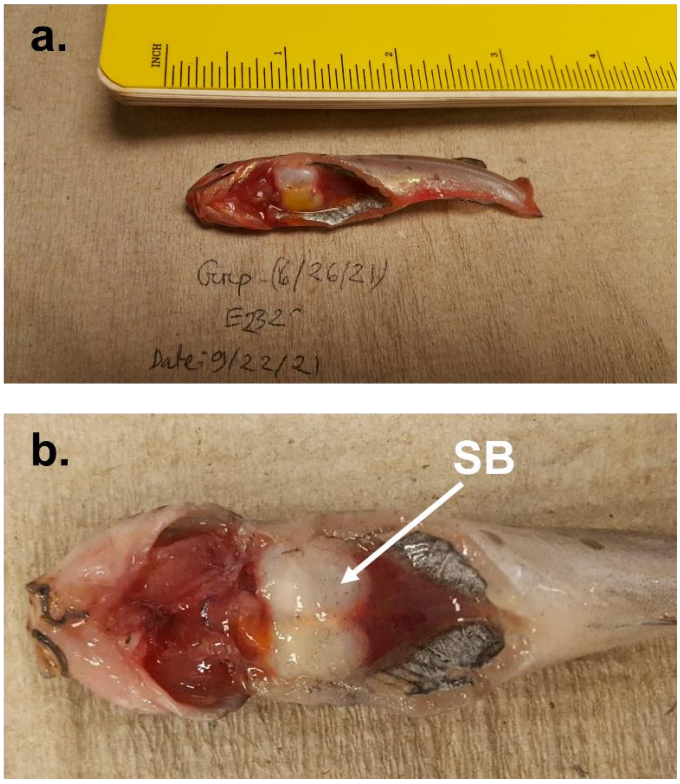

Figure 18. a. A 3-month-old *Grp* transgenic positive channel catfish (*Ictalurus punctatus*) with a deformed swimbladder; **b.** An enlarged image of the deformed swimbladder (SB) marked with an arrow. *Grp* (gastrin-releasing peptide-like isoform X2) was isolated from tra catfish (*Pangasianodon hypophthalmus*).

4.4.6 Disease and survival

An outbreak of enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri*, occurred when the fish were approximately 2.5 months of age. Some fish were co-infected with white spot disease (caused by *Ichyophthirius multifilis*). They were treated with 3.5 ppt salt and 20 ppm formalin to reduce the spread of the disease. During that time, fish stopped eating until the infection subsided. Table 7 shows the number of fry stocked into the system and their approximate survival after the disease events.

For the above-mentioned treatments in recirculatory tanks, the survival of transgenic fish was not affected by the cold temperature in winter although some other ABO treatments in flow through system had large number of fish died at that time. The salinity of the recirculatory system was kept above 3 ppt which might have been advantageous to the fish whereas flow through water had no salinity.

Table 7. The number of transgenic positive channel catfish (*Ictalurus punctatus*) and full-sibling negative controls stocked for the four putative ABO transgene constructs and lost due to ESC and white spot disease. No significant differences were found between positive individuals and negative individuals within each transgene type (Fisher's Exact Test). ESC = enteric septicemia of catfish; *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1 and iCntl =sham injected control.

Treatment	Fry stocked	ESC	% ESC mortality	White spot disease	% White spot disease mortality
Grp (-)	77	24	31.2	0	0.0
Grp (+)	5	2	40.0	0	0.0
Hrg (-)	352	33	9.4	15	4.3
Hrg (+)	10	2	20.0	0	0.0
Cx3cl1 (-)	392	29	7.4	11	2.8
Cx3cl1 (+)	22	1	4.5	0	0.0
Fras1 (-)	505	40	7.9	6	1.2
Fras1 (+)	11	2	18.2	0	0.0

4.4.7 Discussion:

The efficiency of insertion of dsDNA donor templates in CRISPR/Cas9 system is generally poor[219] as compared to single-stranded DNA (ssDNA) donors.[220-222] Despite the low integration rates, dsDNA donor templates of putative ABO transgenes, *Grp*, *Hrg*, *Cx3cl1* and *Fras1* were successfully knocked in using CRISPR/Cas9 system. The design of homologous donor construct may affect the integration rate. The length of homologous arm at each end of the construct plays vital role in precise integration. Several studies have shown that homology directed repair (HDR) is more efficient with longer homology arms.[136, 145, 151] In this study, 323-bp homology arm on both sides of the construct, identical to the sequences neighboring the target site in channel catfish chromosome 1 (for *Grp, Hrg* and *Cx3cl1*) and chromosome 7 (for *Fras1*) was used to design the transgene constructs and the observed integration rates in live fingerlings for *Grp, Hrg, Cx3cl1* and *Fras1* were 7.32 %, 4.31 %, 7.07 % and 2.49 % respectively. In an experiment involving T cells, 77 % precise integration rate was achieved using a 330 bp homology arms while 50 bp homology arms had only 63 % integration.[151] Variable results were found from 12-58 % HDR rate when 1 kb or more homology arms were used.[145, 152] However, shorter homology arms would be expected to be more available to be inserted into the target site throughout the concurrent cleavage of genomic and plasmid DNA.[151]

In the current experiment, integration rates were also found to decrease with the increasing size of transgene constructs. The length of the insert between the homology arms normally ranges from 1-2 kb.[223] Although longer inserts are possible, the efficiency of integration might decrease with the increasing insert size. In an experiment with murine embryonic stem cells at the *Mef2c* locus, changing the insert length from 99 bp to 720 bp reduced modification frequency 9-fold (36.3% to 4.3%).[224] Although CRISPR/Cas9 mediated knock-in by HDR remains low for large fragment integration,~20 kb multiple digestion enzyme genes were integrated in the CEP112 locus in pig fetal fibroblasts by optimization of the length of homology arms.[225] However, unexpected phenotypic effects such as less body mass and fat percentage were observed in mice due to integration of 6 kb transgene construct coding for human granulocyte-macrophage colony-stimulating factor (hGMCSF), which replaced 170 bp of Cntn5 gene.[226]

No significant difference was found among the integration rates of dead embryos, dead fry and live embryos within each type of four transgene constructs. However, the observed trend was a decrease in the percentage of transgenic individuals as the fish transitioned from one life stage to another. For example, *Hrg* showed the highest integration rate (16.67 %) in dead embryos and 10% and 4.31% integration in dead fry and live fingerlings, respectively. The decline in integration rate of *Hrg* from embryo to fingerling stage may indicate an off-target effect for this construct. Alternatively, the construct may be negatively interacting with other gene functions, or the large size of the construct could affect the survival and early development of the transgenic individuals. *Grp* transgenic individuals had the highest integration rate (7.32%) in live fingerlings and *Fras1* the lowest (2.49%). *Grp* was 3.3 kb long, the shortest among the four constructs whereas *Fras1* was the longest (6.5 kb).

Channel catfish embryos injected with different transgene constructs did not present any significant difference in terms of the integration rate at a particular life stage except in live fingerlings. The highest integration rate was observed in dead embryos microinjected with *Hrg* (16.67 %) followed by *Grp* (11.43%) and *Cx3cl1* (5.88%) while *Fras1* had no integration in dead embryos at all. In dead fry, *Cx3cl1* showed the highest integration (12.20%). Although the survival of *Grp* transgenic channel catfish was low, this treatment had the highest integration rate (7.32%) in live fingerlings. Insertion through homologous recombination of dsDNA donor template in mouse models produced a knock-in efficiency of ~10% or less.[227-229] A 3.5 % of knock-in efficiency was reported in zebrafish using a dsDNA donor oligonucleotide targeting C13H9orf72 genomic locus but only 1.7 % produced correct knock-in without additional mutations.[230] In this study, sequencing results revealed precise integration of ABO transgenes and several individuals contained the complete sequence of *Grp* and *Cx3cl1* transgene.

In an experiment with medaka (*Oryzias latipes*), 25 to 27 % integration rate was obtained via homology directed repair (HDR) using plasmid donor.[231] Another study found 26 to 46 % integration rate in zebrafish (*Danio rerio*) embryos using the same technique.[232] However, some studies reported very low efficiency of homologous recombination in transgenic fish. In an experiment of targeted insertion of plasmid by homologous recombination in zebrafish embryonic stem cell culture, only two colonies of homologous recombinant cells were found out of approximately 1 x 10^6 cells. [233] Similarly, the probability of homologous recombination was only

10^{-5} in a carp epithelioma papulosum cell line.[234] The key reasons behind the very low rates of homologous recombination of exogenous genes are not fully understood and research is being continued in this area.

The CRISPR/Cas9 transfection have some drawbacks such as random integration of all or part of the plasmid DNA into the host genome as well as undesirable insertions of plasmid DNA sequences at on-target and off-target sites. It may also trigger cGMP-AMP synthase activation, which is stressful to cells.[169, 235-236] However, in the current study, sequencing results obtained for positive samples for the transgene revealed no unwanted integration of plasmid sequences into the target site.

The design of the sgRNA can also influence precise integration as the sgRNA is designed to cleave at the exact insertion site, which is three nucleotides upstream of PAM. The further the guide RNA from the target site, the poorer will be the correct insertion.[220] In another experiment, DSBs were induced by Cas9 protein at the exact target cut site which led to HDR and precise insertion of the transgene constructs.[145] However, the induction of DSBs on sgRNA target sequences next to homologous arms using the CRISPR/Cas9 system in circular donor plasmid has been reported to increase targeted integration events.[232, 237]

The different plasmid constructs did not affect the hatchability and mortality of the microinjected embryos. Although there was no significant difference, the lowest hatchability was observed in embryos microinjected with *Hrg* (20.24%) among the four treatments. During microinjection, increase in the gRNA and Cas9 protein concentration can lead to decreased hatchability and increased mortality of embryos in channel catfish.[216] However, in this experiment, concentration of gRNA and Cas9 were the same for all the treatments but with different sizes of transgene constructs. The concentration of donor DNA for the four different treatments was 50 ng/µl. The high concentration of donor DNA could have a toxic effect on the cells resulting increased mortality of embryos as well as large transgene construct may also have a negative effect on survival. Higher mortality was observed in microinjected embryos than sham injected controls. A similar effect was also observed in development and hatchability of channel catfish embryos microinjected with TICAM1 through CRISPR/Cas9 at one cell stage.[138] Moreover, microinjection may cause physical damage to the embryo which reduces the viability of cells.

The survival of fry was significantly lower in the *Grp* than the sham injected control. However, survival rate did not vary among the fry from the four different treatments while *Hrg* had the highest rate of fry survival. Overall, although the fry survival rate of the controls was not significantly different form *Hrg*, *Cx3cl1* and *Fras1*, had better survival than all the transgenic groups. The components of plasmid DNA construct may have negative effects on survival of fry as previously described for embryo development and mortality. Sham injected controls had higher survival of fry than the microinjected fry. Physical stress during the delivery of constructs into the embryos and low quality of eggs may also decrease the survival.[238] Overexpression or expression in a sensitive tissue or cell, might also be a reason for embryo death that integrated these constructs.

Evaluation of body weights at 4 months and 8 months post-hatch showed that *Grp* had no effect on the growth of the transgenic channel catfish since there was no significant difference in mean body weight between the transgenic and non-transgenic groups. The fish from *Hrg* and *Cx3cl1* transgenics had significantly lower mean body weight than the corresponding non-transgenic full-siblings during both samplings. These two transgene constructs may have been responsible for severely decreasing growth. Although not statistically significant, the observed mean weight of the *Fras1* transgenic individuals was higher than controls. The huge variation in mean body weights among the different non-transgenic groups suggests possible strain or family effects on growth since three to four batches of eggs from different strains were used during microinjection for different constructs. Additional investigations are needed to check if the pleiotropic effects are impacting the growth rate with transgene insertion. An additional adverse effect of microinjection may include slow growth.

During the experiment, some fish became infected with ESC and white spot disease (otherwise known as 'Ich'). Fish from non-transgenic groups tended to be more resistant to ESC than their transgenic counterparts. The transgenic individuals seemed to be Ich resistant. Pleiotropic effects have been observed from the transfer of other transgenes such as growth hormone genes on disease resistance.[239]

4.6 Conclusion:

ABO transgenes from tra and bighead catfish were successfully inserted into channel catfish using a CRISPR/Cas9 knock-in system. The constructs driven by their native promoters and a sgRNA designed to cleave at the exact insertion site led to precise integration of the transgene into the channel catfish genome with no unwanted integration of plasmid sequences into the target site. To the best of our knowledge, this study represents the first demonstration of targeted exogenous putative air-breathing genes in a non-coding region of channel catfish. A sample of individuals from one transgenic group had altered swimbladder morphology providing additional evidence that these are indeed genes related to air breathing structure.

Because of low integration rates and thus, low fish numbers, testing the air breathing ability of these ABO transgenic channel catfish was not conducted and will need to be done when the lines are expanded. However, pleiotropic effects of these transgenes were measured. Even if air breathing is accomplished, its benefits could be negated if disease resistance, growth or other important traits are adversely affected. Overall, survival was similar between the various ABO transgenic channel catfish and their full-sibling controls. However, there was a trend of the transgenic individuals being more vulnerable to the bacterial disease, ESC, and more resistant to the parasite, *Ichthyophthirius multifiliis.* One transgenic line exhibited increase growth rate, a second had no change in body weight compared to non-transgenic controls and the other 2 lines experienced severe decreases in growth rate.

Future research also needs to include crossing and combining these lines (including 5 other lines not reported here). The air breathing of the transgenic fish will more likely be impacted when the fish possess multiple ABO transgenes addressing the entire system rather than a single ABO locus, and this hypothesis should be tested.

When multiple transgene loci are present, some of the negative pleiotropic or epistatic effects could be negated or might become worse. If the negative effects on other economic traits persist, then this approach of genetically overcoming low oxygen tolerance would not benefit aquaculture. Whether aquaculture application is feasible or not, tra catfish and bighead catfish still provide excellent contrasting models to study the genetic basis for the transition from aquatic to

terrestrial living. Future hypoxia challenges when these transgenic lines are expanded and crossed should allow progress in studying this transition to air breathing.

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Appendix A

Results of hypoxia challenge for tra catfish (*Pangasianodon hypophthalmus*) at 7 early developmental stages[188]

Date	Stage	Time (minute)	Oxygen concentration (mg/L)	Survival Rate %	Fish behavior
19/11/2016	2 dpf	0	5.7	100	Active, move around bottle
19/11/2016	2 dpf	15	2.1	100	moving fast on the surface
19/11/2016	2 dpf	30	1.3	100	move slower
19/11/2016	2 dpf	45	0.7	0	not moving, stay on the bottom and all fish died
21/11/2016	4 dpf	0	5	100	Active
21/11/2016	4 dpf	15	1.5	100	Slow moving
21/11/2016	4 dpf	30	1.1	100	moving fast on the surface
21/11/2016	4 dpf	45	0	100	50% drop to the bottom
21/11/2016	4 dpf	55	0	0	not moving, stay on the bottom and all fish died
23/11/2016	6 dpf	0	4.7	100	active
23/11/2016	6 dpf	15	1.2	100	Moving on the surface
23/11/2016	6 dpf	30	0.7	100	moving fast on the surface
23/11/2016	6 dpf	45	0	100	50% drop to the bottom
23/11/2016	6 dpf	60	0	0	not moving, stay on the bottom and all fish died
25/11/2016	8 dpf	0	4.6	100	Active
25/11/2016	8 dpf	15	0.9	100	moving on the surface
25/11/2016	8 dpf	30	0.6	100	moving fast on the surface
25/11/2016	8 dpf	45	0	100	30% drop
25/11/2016	8 dpf	60	0	30	70% died
25/11/2016	8 dpf	75	0	0	not moving, stay on the bottom and all fish died
26/11/2016	9 dpf	0	5.4	100	Active, swim on the bottom
26/11/2016	9 dpf	15	1.6	100	Active, swim on the surface
26/11/2016	9 dpf	30	0.8	100	Active, swim on the surface
26/11/2016	9 dpf	45	0.4	100	swim on the surface
26/11/2016	9 dpf	50	0	100	swim on the surface
26/11/2016	9 dpf	65	0	100	start to skin on the bottom
26/11/2016	9 dpf	75	0	73	27% died
26/11/2016	9 dpf	85	0	73	40% stay on the bottom
26/11/2016	9 dpf	90	0	73	40% stay on the bottom
26/11/2016	9 dpf	120	0	60	not moving, stay on the bottom and 40% fish died
27/11/2016	10 dpf	0	4.6	100	swim on the bottom
27/11/2016	10 dpf	15	0.9	100	Active
27/11/2016	10 dpf	30	0.5	100	50% on the surface
27/11/2016	10 dpf	45	0	100	100% on the surface
27/11/2016	10 dpf	55	0	87	13% died
27/11/2016	10 dpf	70	0	80	20% died

27/11/2016	10 dpf	120	0	80	20% died
28/11/2016	11 dpf	0	4.6	100	Active, swim on the bottom
28/11/2016	11 dpf	15	1	100	Active, swim on the bottom
28/11/2016	11 dpf	40	0.5	100	Active, swim on the bottom

Appendix B

Results of hypoxia challenge for bighead catfish (*Clarias macrocephalus*) at 12 early developmental stages

Date	Stage	Time (m)	Oxygen concentration (mg/L)	Survival rate (%)	Fish behavior
7/1/2020	3 dpf	0	4.15	100.0	active, move round the bottom
7/1/2020	3 dpf	15	0.39	100.0	some move to the surface, some in bottom
7/1/2020	3 dpf	30	0.35	100.0	Most fish try to move to the surface
7/1/2020	3 dpf	45	0.28	100.0	Most move to the surface, some move up and down
7/1/2020	3 dpf	50	0.26	93.3	Most move to the surface, few in bottom
7/1/2020	3 dpf	60	0.22	80.0	Some weak, passive movement, fell in bottom
7/1/2020	3 dpf	65	0.2	53.3	most weak, fell in the bottom
7/1/2020	3 dpf	70	0.19	33.3	no movement, weak and lie at the bottom
7/1/2020	3 dpf	72	0.18	13.3	no movement, weak and lie at the bottom
7/1/2020	3 dpf	74	0.18	6.7	no movement, weak and lie at the bottom
7/1/2020	3 dpf	78	0.17	0.0	Died all
7/3/2020	5 dpf	0	4.3	100.0	active, move round the bottom
7/3/2020	5 dpf	15	2.02	100.0	active, move round the bottom
7/3/2020	5 dpf	30	0.8	100.0	slower, some laid in the bottom
7/3/2020	5 dpf	45	0.57	100.0	one fish move to the surface and some laid in the bottom
7/3/2020	5 dpf	55	0.5	100.0	2 fish move to the surface, erratic swimming in the middle, some laid and breath in the bottom
7/3/2020	5 dpf	65	0.45	100.0	4 fish move to the surface, erratic swimming in the middle, some laid and breath in the bottom
7/3/2020	5 dpf	75	0.43	100.0	4 fish move to the surface, erratic swimming in the middle, some laid and breath in the bottom
7/3/2020	5 dpf	85	0.39	100.0	8 fish move to the surface, erratic swimming in the middle, some laid and breath in the bottom

7/3/2020	5 dpf	95	0.35	100.0	all fish move to the surface, very weak, erratic swimming
7/3/2020	5 dpf	100	0.33	80.0	fish died, erractic swimming, fell down and stop moving
7/3/2020	5 dpf	105	0.33	66.7	fish died, fell and laid in the bottom
7/3/2020	5 dpf	110	0.32	20.0	fell and laid in the bottom, stopped moving and only breath
7/3/2020	5 dpf	115	0.31	13.3	fell and laid in the bottom, stopped moving and only breath
7/3/2020	5 dpf	120	0.31	13.3	fell and laid in the bottom, stopped moving and only breath
7/3/2020	5 dpf	125	0.3	0.0	Died all
7/11/2020	13 dpf	0	3.85	100.0	Active, moved round the bottom
7/11/2020	13 dpf	15	1.94	100.0	Active, moved round the bottom somtime in middle
7/11/2020	13 dpf	30	1.2	100.0	Active, moved round the bottom somtime in middle
7/11/2020	13 dpf	45	0.67	100.0	6 fish moved to surface, slower, some laid in the bottom
7/11/2020	13 dpf	60	0.52	100.0	10 fish moved to the surface & middle, some laid in the bottom
7/11/2020	13 dpf	70	0.41	100.0	14 fish moved to the surface & middle, some laid in the bottom
7/11/2020	13 dpf	80	0.36	86.7	All fish swam on surface, some began to die
7/11/2020	13 dpf	90	0.34	80.0	Some started to fall & turned back to surface, slow movement. Some died
7/11/2020	13 dpf	100	0.33	80.0	Gathered on the surface far from the air stone, some fell down to the bottom
7/11/2020	13 dpf	115	0.28	73.3	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/11/2020	13 dpf	120	0.24	73.3	Some fell & turned back to surface, very weak
7/11/2020	13 dpf	125	0.2	73.3	Some fell & turned back to surface, very
7/11/2020	13 dpf	130	0.18	60.0	Passive, some fell & died, some still on surface, stop movement and died
7/11/2020	13 dpf	135	0.16	40.0	Passive, some fell down, stop movement, laid in the bottom and died
7/11/2020	13 dpf	140	0.14	13.3	Passive, too weak, some fell down, stop movement, laid in the bottom and died
7/11/2020	13 dpf	145	0.12	6.7	Passive, too weak, some fell down, stop movement, laid in the bottom and died
7/11/2020	13 dpf	150	0.11	6.7	Passive, too weak, some fell down, stop movement, laid in the bottom and died
7/11/2020	13 dpf	155	0.1	0.0	All fish died
7/12/2020	14 dpf	0	4.55	100.0	Active, moved round the bottom
7/12/2020	14 dpf	15	2.31	100.0	Active, moved round the bottom somtime in middle

7/12/2020	14 dpf	30	0.95	100.0	2 fish moved to surface, slower, some laid in the bottom
7/12/2020	14 dpf	45	0.68	100.0	10 fish moved to surface, slower, some laid in the bottom
7/12/2020	14 dpf	60	0.55	100.0	13 fish moved to the surface & middle, some laid in the bottom
7/12/2020	14 dpf	70	0.49	100.0	14 fish moved to the surface & middle, some laid in the bottom
7/12/2020	14 dpf	80	0.38	100.0	All fish swam on surface
7/12/2020	14 dpf	90	0.32	80.0	Some started to fall & turned back to surface, slow movement
7/12/2020	14 dpf	100	0.25	80.0	Gathered on the surface far from the air stone, some fell down to the bottom
7/12/2020	14 dpf	115	0.2	73.3	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/12/2020	14 dpf	120	0.18	53.3	Some fell & turned back to surface, some died, so weak
7/12/2020	14 dpf	125	0.16	40.0	Some fell & turned back to surface, some died, so weak
7/12/2020	14 dpf	130	0.14	20.0	Passive, some fell & died, some still on surface, stop movement
7/12/2020	14 dpf	135	0.13	20.0	Passive, some fell down, stop movement & laid in the bottom
7/12/2020	14 dpf	140	0.11	6.7	Passive, too weak, some fell down, stop movement & laid in the bottom
7/12/2020	14 dpf	145	0.09	0.0	All fish died
7/14/2020	16 dpf	0	4.41	100.0	Active, moved round the bottom
7/14/2020	16 dpf	15	2.22	100.0	Active, moved round the bottom somtime in middle
7/14/2020	16 dpf	30	0.98	100.0	3 fish moved to surface, slower, some laid in the bottom
7/14/2020	16 dpf	45	0.54	100.0	11 fish moved to surface, slower, some laid in the bottom
7/14/2020	16 dpf	60	0.48	100.0	14 fish moved to the surface & middle, some laid in the bottom
7/14/2020	16 dpf	70	0.42	100.0	All fish swam on surface and some started to die
7/14/2020	16 dpf	80	0.37	93.3	Some started to fall & turned back to surface, slow movement, 1fish died.
7/14/2020	16 dpf	90	0.31	86.7	Some started to fall & turned back to surface, slow movement, 1 fish died
7/14/2020	16 dpf	100	0.26	86.7	Gathered on the surface far from the air stone, some fell down to the bottom
7/14/2020	16 dpf	110	0.24	66.7	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/14/2020	16 dpf	120	0.21	40.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/14/2020	16 dpf	130	0.17	26.7	Gathered on the surface far from the air stone, some fell down to the bottom & died

7/14/2020	16 dpf	135	0.16	26.7	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/14/2020	16 dpf	140	0.14	26.7	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/14/2020	16 dpf	145	0.12	20.0	Some fell & turned back to surface, some died, so weak
7/14/2020	16 dpf	150	0.11	13.3	Some fell & turned back to surface, very weak
7/14/2020	16 dpf	155	0.1	13.3	Passive, some fell, some still on surface, stop movement
7/14/2020	16 dpf	160	0.09	6.7	Passive, some fell down, stop movement & laid in the bottom
7/14/2020	16 dpf	165	0.08	0.0	All fish died
7/15/2020	17 dpf	0	4.38	100.0	Active, moved round the bottom
7/15/2020	17 dpf	15	2.28	100.0	Active, moved round the bottom somtime in middle
7/15/2020	17 dpf	30	1.13	100.0	Active, moved round the bottom somtime in middle
7/15/2020	17 dpf	45	0.57	100.0	2 fish moved to surface, slower, some laid in the bottom
7/15/2020	17 dpf	60	0.47	100.0	6 fish moved to the surface & middle, some laid in the bottom
7/15/2020	17 dpf	70	0.4	100.0	14 fish moved to the surface, some moved in the bottom
7/15/2020	17 dpf	80	0.36	100.0	All fish swam on surface
7/15/2020	17 dpf	90	0.34	100.0	Some started to fall & turned back to surface, slow movement
7/15/2020	17 dpf	100	0.29	100.0	Some started to fall & turned back to surface, slow movement
7/15/2020	17 dpf	110	0.28	93.3	Gathered on the surface far from the air stone, some fell down to the bottomc& died
7/15/2020	17 dpf	115	0.24	93.3	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/15/2020	17 dpf	120	0.23	93.3	Weaker, gathered on the surface far from the air stone, some fell down to the bottom & died
7/15/2020	17 dpf	125	0.2	80.0	Weaker, gathered on the surface far from the air stone, some fell down to the bottom & died
7/15/2020	17 dpf	130	0.18	80.0	Weaker, gathered on the surface far from the air stone, some fell down to the bottom & died
7/15/2020	17 dpf	135	0.17	73.3	Weaker, gathered on the surface far from the air stone, some fell down to the bottom & died
7/15/2020	17 dpf	140	0.15	46.7	Passive & stop movement, some fell & died, some turned back to surface, very weak
7/15/2020	17 dpf	145	0.13	20.0	Passive, some fell & died, some turned back to surface, very weak

7/15/2020	17 dpf	150	0.1	6.7	Passive, some fell & died, some turned back to surface, very weak
7/15/2020	17 dpf	155	0.09	6.7	Passive, fell down & died, suspend in the water
7/15/2020	17 dpf	160	0.08	6.7	Passive, fell down & died, suspend in the water
7/15/2020	17 dpf	162	0.08	0.0	All fish died
7/19/2020	21 dpf	0	5.03	100.0	Active, moved round the bottom
7/19/2020	21 dpf	15	2.38	100.0	Active, moved round the bottom somtime in middle
7/19/2020	21 dpf	30	0.95	100.0	Active, moved round the bottom somtime in middle
7/19/2020	21 dpf	45	0.61	100.0	Active, moved round the bottom somtime in middle
7/19/2020	21 dpf	60	0.44	100.0	2 fish moved to surface, slower, some swam in the bottom
7/19/2020	21 dpf	70	0.39	100.0	5 fish moved to surface, slower, some swam in the bottom
7/19/2020	21 dpf	80	0.33	100.0	10 fish moved to surface, slower, some swam in the middle
7/19/2020	21 dpf	90	0.26	100.0	12 fish moved to surface, slower, some swam in the middle
7/19/2020	21 dpf	100	0.22	100.0	14 fish moved to surface, slower, some swam in the middle
7/19/2020	21 dpf	110	0.19	100.0	14 fish moved to surface, slower, some swam in the middle
7/19/2020	21 dpf	115	0.17	100.0	All fish swam on surface
7/19/2020	21 dpf	120	0.15	100.0	Some started to fall down & turned back to surface
7/19/2020	21 dpf	125	0.14	100.0	Some started to fall down & turned back to surface
7/19/2020	21 dpf	130	0.12	100.0	Some started to fall down & turned back to surface
7/19/2020	21 dpf	135	0.12	100.0	Some started to fall down & turned back to surface
7/19/2020	21 dpf	140	0.11	93.3	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/19/2020	21 dpf	145	0.1	93.3	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/19/2020	21 dpf	150	0.08	86.7	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/19/2020	21 dpf	155	0.08	73.3	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/19/2020	21 dpf	160	0.07	73.3	Passive & slow movement, some fell & died, sometime jumped out of surface

7/19/2020	21 dpf	165	0.07	66.7	Passive & slow movement, some fell & died, sometime jumped out of surface
7/19/2020	21 dpf	170	0.06	46.7	Passive & slow movement, some fell & died, sometime jumped out of surface
7/19/2020	21 dpf	175	0.05	26.7	Passive & slow movement, some fell & died, sometime jumped out of surface
7/19/2020	21 dpf	180	0.05	13.3	Passive, fell down & died, some suspend in the water or floated in surface
7/19/2020	21 dpf	185	0.04	13.3	Passive, fell down & died, some suspend in the water or floated in surface
7/19/2020	21 dpf	190	0.04	6.7	Passive, fell down & died, some suspend in the water or floated in surface
7/19/2020	21 dpf	195	0.03	0.0	All fish died
7/22/2020	24 dpf	0	4.34	100.0	Active, moved round the bottom
7/22/2020	24 dpf	15	2.32	100.0	Active, moved round the bottom somtime in middle
7/22/2020	24 dpf	30	1.06	100.0	Active, moved round the bottom somtime in middle
7/22/2020	24 dpf	45	0.66	100.0	2 fish moved to surface, slower, some swam in the bottom
7/22/2020	24 dpf	60	0.42	100.0	4 fish moved to surface, slower, some swam in the bottom
7/22/2020	24 dpf	70	0.37	100.0	7 fish moved to surface, slower, some swam in the bottom
7/22/2020	24 dpf	80	0.32	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	90	0.29	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	100	0.26	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	115	0.19	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	120	0.17	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	125	0.14	100.0	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	130	0.12	86.7	Some swam quickly to surface & turned back to the bottom
7/22/2020	24 dpf	135	0.12	86.7	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/22/2020	24 dpf	140	0.11	86.7	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/22/2020	24 dpf	145	0.1	86.7	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/22/2020	24 dpf	150	0.1	80.0	Passive & slow movement, some fell & died, sometime jumped out of surface

7/22/2020	24 dpf	155	0.09	73.3	Passive & slow movement, some fell & died, sometime jumped out of surface
7/22/2020	24 dpf	160	0.08	53.3	Passive, fell down & died, some suspend in the water or floated in surface
7/22/2020	24 dpf	165	0.06	40.0	Passive, fell down & died, some suspend in the water or floated in surface
7/22/2020	24 dpf	170	0.05	13.3	Passive, fell down & died, some suspend in the water or floated in surface
7/22/2020	24 dpf	175	0.05	13.3	Passive, fell down & died, some suspend in the water or floated in surface
7/22/2020	24 dpf	180	0.04	6.7	Passive, fell down & died, some suspend in the water or floated in surface
7/22/2020	24 dpf	185	0.03	0.0	All fish died
7/23/2020	25 dpf	0	4.31	100.0	Active, moved round the bottom
7/23/2020	25 dpf	15	1.86	100.0	Active, moved round the bottom somtime in middle
7/23/2020	25 dpf	30	0.97	100.0	Active, moved round the bottom somtime in middle
7/23/2020	25 dpf	45	0.6	100.0	1 fish moved to surface, slower, some swam in the bottom
7/23/2020	25 dpf	60	0.48	100.0	3 fish moved to surface, slower, some swam in the bottom
7/23/2020	25 dpf	70	0.38	100.0	6 fish moved to surface, slower, some swam in the bottom
7/23/2020	25 dpf	80	0.33	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	90	0.26	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	100	0.21	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	110	0.19	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	120	0.14	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	125	0.12	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	130	0.11	100.0	Some swam quickly to surface & turned back to the bottom
7/23/2020	25 dpf	135	0.1	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/23/2020	25 dpf	140	0.09	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/23/2020	25 dpf	145	0.08	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/23/2020	25 dpf	150	0.07	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface

7/23/2020	25 dpf	155	0.07	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/23/2020	25 dpf	160	0.05	100.0	Passive, fell down, some suspend in the water or floated in surface
7/23/2020	25 dpf	165	0.05	100.0	Passive, fell down, some suspend in the water or floated in surface
7/23/2020	25 dpf	170	0.04	100.0	Passive, fell down, some suspend in the water or floated in surface
7/23/2020	25 dpf	175	0.04	93.3	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	180	0.03	93.3	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	210	0.03	80.0	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	300	0.04	73.3	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	320	0.03	66.7	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	360	0.04	60.0	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	370	0.04	53.3	Passive, fell down & died, some suspend in the water or floated in surface
7/23/2020	25 dpf	420	0.03	53.3	Remaining fish are passive, suspending in the water or floated in surface, sometime actively go up and down
7/24/2020	26 dpf	0	4.57	100.0	Active, moved round the bottom
7/24/2020	26 dpf	15	1.89	100.0	Active, moved round the bottom somtime in middle
7/24/2020	26 dpf	30	0.95	100.0	Active, moved round the bottom somtime in middle
7/24/2020	26 dpf	45	0.62	100.0	1 fish moved to surface, slower, some swam in the bottom
7/24/2020	26 dpf	60	0.42	100.0	2 fish moved to surface, slower, some swam in the bottom
7/24/2020	26 dpf	70	0.4	100.0	4 fish moved to surface, slower, some swam in the bottom
7/24/2020	26 dpf	80	0.35	100.0	Some swam quickly to surface & turned back to the bottom
7/24/2020	26 dpf	90	0.29	100.0	Some swam quickly to surface & turned back to the bottom
7/24/2020	26 dpf	100	0.27	100.0	Some swam quickly to surface & turned back to the bottom
7/24/2020	26 dpf	110	0.24	100.0	Some swam quickly to surface & turned back to the bottom
7/24/2020	26 dpf	120	0.19	100.0	Some swam quickly to surface & turned back to the bottom
7/24/2020	26 dpf	125	0.16	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died

7/24/2020	26 dpf	130	0.15	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/24/2020	26 dpf	135	0.14	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/24/2020	26 dpf	140	0.12	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/24/2020	26 dpf	145	0.11	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/24/2020	26 dpf	150	0.09	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/24/2020	26 dpf	155	0.06	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/24/2020	26 dpf	160	0.05	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/24/2020	26 dpf	165	0.03	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/24/2020	26 dpf	170	0.04	100.0	Passive & slow movement, some fell & died, sometime jumped out of surface
7/24/2020	26 dpf	305	0.02	93.3	Passive, fell down & died, some suspend in the water or floated in surface
7/24/2020	26 dpf	315	0.03	80.0	Passive, fell down & died, some suspend in the water or floated in surface
7/24/2020	26 dpf	325	0.03	66.7	Passive, fell down & died, some suspend in the water or floated in surface
7/24/2020	26 dpf	355	0.03	53.3	Passive, fell down & died, some suspend in the water or floated in surface
7/24/2020	26 dpf	375	0.02	46.7	Passive, fell down & died, some suspend in the water or floated in surface
7/24/2020	26 dpf	420	0.03	46.7	Remaining fish are passive, suspending in the water or floated in surface, sometime actively go up and down
7/26/2020	28 dpf	0	4.43	100.0	Active, moved round the bottom
7/26/2020	28 dpf	15	2.08	100.0	Active, moved round the bottom somtime in middle
7/26/2020	28 dpf	30	0.92	100.0	Active, moved round the bottom somtime in middle
7/26/2020	28 dpf	45	0.67	100.0	1 fish moved to surface, slower, some swam in the bottom
7/26/2020	28 dpf	60	0.44	100.0	5 fish moved to surface, slower, some swam in the bottom
7/26/2020	28 dpf	70	0.39	100.0	Some swam quickly to surface & turned back to the bottom
7/26/2020	28 dpf	80	0.32	100.0	Some swam quickly to surface & turned back to the bottom
7/26/2020	28 dpf	90	0.28	100.0	Some swam quickly to surface & turned back to the bottom

7/26/2020	28 dpf	100	0.26	100.0	Some swam quickly to surface & turned back to the bottom
7/26/2020	28 dpf	110	0.2	100.0	Some swam quickly to surface & turned back to the bottom
7/26/2020	28 dpf	120	0.17	100.0	Some swam quickly to surface & turned back to the bottom
7/26/2020	28 dpf	130	0.15	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/26/2020	28 dpf	140	0.14	100.0	Gathered on the surface far from the air stone, some fell down to the bottom & died
7/26/2020	28 dpf	150	0.12	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/26/2020	28 dpf	160	0.09	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/26/2020	28 dpf	170	0.09	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/26/2020	28 dpf	180	0.07	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/26/2020	28 dpf	190	0.07	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/26/2020	28 dpf	200	0.06	100.0	Passive & slow movement, sometime jumped out of surface
7/26/2020	28 dpf	210	0.06	100.0	Passive & slow movement, sometime jumped out of surface
7/26/2020	28 dpf	220	0.04	100.0	Passive & slow movement, sometime jumped out of surface
7/26/2020	28 dpf	255	0.03	86.7	Passive, fell down & died, some suspend in the water or floated in surface
7/26/2020	28 dpf	270	0.03	80.0	Passive, fell down & died, some suspend in the water or floated in surface
7/26/2020	28 dpf	340	0.02	73.3	Passive, fell down & died, some suspend in the water or floated in surface
7/26/2020	28 dpf	360	0.04	66.7	Passive, fell down & died, some suspend in the water or floated in surface
7/26/2020	28 dpf	410	0.02	60.0	Passive, fell down & died, some suspend in the water or floated in surface
7/26/2020	28 dpf	420	0.03	60.0	Remaining fish are passive, suspending in the water or floated in surface, sometime actively go up and down
7/28/2020	30 dpf	0	4.53	100.0	Active, moved round the bottom
7/28/2020	30 dpf	15	1.95	100.0	Active, moved round the bottom somtime in middle
7/28/2020	30 dpf	30	0.97	100.0	Active, moved round the bottom somtime in middle

7/28/2020	30 dpf	45	0.62	100.0	Active, moved round the bottom somtime in middle
7/28/2020	30 dpf	60	0.45	100.0	2 fish moved to surface, slower, some swam in the bottom
7/28/2020	30 dpf	70	0.38	100.0	7 fish moved to surface, slower, some swam in the bottom
7/28/2020	30 dpf	80	0.35	100.0	9 fish moved to surface, slower, some swam in the bottom
7/28/2020	30 dpf	90	0.3	100.0	Some swam quickly to surface & turned back to the bottom
7/28/2020	30 dpf	100	0.26	100.0	Some swam quickly to surface & turned back to the bottom
7/28/2020	30 dpf	110	0.23	100.0	Some swam quickly to surface & turned back to the bottom
7/28/2020	30 dpf	120	0.18	100.0	Some swam quickly to surface & turned back to the bottom
7/28/2020	30 dpf	130	0.16	100.0	Some swam quickly to surface & turned back to the bottom
7/28/2020	30 dpf	140	0.13	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/28/2020	30 dpf	150	0.11	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/28/2020	30 dpf	160	0.07	100.0	Slow movement, gathered on the surface far from the air stone, some fell down to the bottom
7/28/2020	30 dpf	170	0.06	100.0	Passive & slow movement, sometime jumped out of surface
7/28/2020	30 dpf	180	0.06	100.0	Passive, fell down, some suspend in the water or floated in surface
7/28/2020	30 dpf	190	0.05	100.0	Passive, fell down & swam up, some suspend in the water or floated in surface
7/28/2020	30 dpf	200	0.03	100.0	Passive, fell down & swam up, some suspend in the water or floated in surface
7/28/2020	30 dpf	280	0.04	93.3	Passive, fell down & died, some suspend in the water or floated in surface
7/28/2020	30 dpf	360	0.04	86.7	Passive, fell down & died, some suspend in the water or floated in surface
7/28/2020	30 dpf	420	0.03	86.7	Remaining fish are passive, suspending in the water or floated in surface, sometime actively go up and down

Appendix C

Time of first death, last death, and survival of larval bighead catfish (*Clarias macrocephalus*) at various times day post-fertilization (dpf) in an aerial environment

dpf	First death (min)	Last death (min)	Total survival (%)
3	20	80	0
5	30	135	0
13	40	135	0
14	60	150	0
16	90	410	36.7
17	200	420	43.3
21	220	400	56.7
24	380	410	83.3
25	390	390	96.7
26	-	-	100
28	-	-	100
30	-	-	100

Appendix D

Sequence of *Cx3cl1* gene (>XM_026925946.2 PREDICTED: *Pangasianodon hypophthalmus* chromo domain-containing protein cec-1-like (LOC113533685), transcript variant X2, mRNA) used for *In situ* hybridization of air-breathing gene of tra catfish (*Pangasianodon hypophthalmus*). Sequence in green is *Cx3cl1*.

AGAGAGAGAGAGAGAGAGAGAGAGAGAGAAAGACACCTGAGCAAACCGCAGACTCGCGCTCA
CCCTGGCTTTCCCGAAAATGAGGGTCTGTAAAGCGCTGAGAAAAAAACAAGGAATCCTGGAACC
AGCACCAACCCGACAGGAACTCAGCAAGAAGGTCCCAGGAAAAGGTAAACCGCCCCCTCTGCAC
ATCATCGACAGCCAGCCTGCCTACACAGTCCACCGAATCCTGGATGTCCGGAGAGTACGGGGCT
CCCGGCAGTATCTCGTGGACTGGGAAGGCTATGGCCCTGAGGAACGCTCCTGGGTCCCGGCCCG
AGACATCCTGGACCCCGGGCTCTTTCTTGCCCACTGGCCCAAGTGTTATGCTTTTTTTTCCCCA
CTAGATGTCACCCTGCCTTAGTTTTTCTTTGTTGTAATTGGTTTCACCTGTGTCTTGTTCCCAT
TCAGGTATTGGCATTTATTAAAAAATGTCTACATAGTGTATCAAACAGGAAGGCAAGGATCTAA
GGTTAACATATACAACTCAGTTACAGCAACTTCTCACATAGTGGGAAGCCTGGCACTACCTCTG
AAGTCATGTATTTGAACCAACATTGTAATGCAATGTTACTCTTAATGAAATTGTATCATATTAG
ATGTGTTATTACTCTGTAAACACACCATTGTATCCAGAAGCTAATGGTCACACTCATGTATGTC
GTTTTTGATGTCTGCATGTTTGTTTTCATGTTTGTTTTCATTCATATCACACAGTTATTATAAG
TATTAAAGACAAACAAGCCCAAGTTACAATGGACATTAAGAAGGATGTGTCCCAGGAGGGGTCA
CATGCAAATTAGCTGGAGGACCAGGCAAACTGGCCACCAGTCGTCTCCAAGCCGACCCGTGGTT
GGATGAAAACCATAGGAGGCCAGCTCAGCTGGTGCAGTAACACTAAGAACTCTCTAACCAATTC
ACAATTTAATGCCGACATATTGATGGTTCACTCAGGTGAACTTTTGTCCAAGAACGCAACCTTC

TGATTGTTATCACTGGTTCTTCTCATGCCATATTCCTACATGTATGAGTGTGTATGTCTATGTG
TGTGTGTTAGTTGTATGTAGTAGTCTTAGTCTATGTATG

Appendix E

Detailed sequence of donor DNA construct for *Grp* driven by its native promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 2; sequences in blue = promoter; sequences in green = *Grp* gene from tra catfish (*Pangasianodon hypophthalmus*); sequences in red = poly-A terminator

TCGACATCAGTAATCGAACAAGGCCTATTTATTATGTTGTTTTGATTACTTGATTTGTTACTCTATTTACTGTGCATTGAATGTTC
TGGATGGGGGCAGTATTAGTACTCACCCCTTTTTAGTCAATATAGGATTTCACAGAGTTTTTATTTTGTGATTGTTTGCTCGATTT
TGCTGCGTCTTTATTTCAAAACTTTTTTCTGAAAAACTAATTAAATGGCACAATTGTAAGTGCACAAATTTGTTTAGCACAGACTT
TCACAGCGATGTTTGTTGGTAAATGAGACCTCTCAGCTGTATTCATGTTCGACATCAGTAATCGAATTGGGGTTGATACCAAGATC
GAGACCAAAAACCATCAAGGCCAAGGCTTTACCTCAGCTAGTCAGCTTCATTCGTGGATTGTGACATGGATTAGGCAGTTTTCTAG
AAGAATTCATTACTTCTTGGCAAATTTTGTGAATGCAAAAAAAAGAAAAGACAGCACAAACATTATTTTTACAAATGCTAACCAAG
ACTAAGACCTTGATTAAGGAGTACAAGATCAAATACAAACTCTAATCCAAGACCTACAGCCTTTAATCACATACAGTCTTCAGTAA
AATGTGATGTTTTACGGCAAAATTGGCCGTGCTGTCTGGGTGGGAGGGGCATACTCTCTCTGTTAATTACAGTGACACTAGCCAAT
CATGTGCGTCTGTGAGCTCAGGTATGTGGAAGAGGGCAGATAGTATTTTCTTCTGCTGCCCTCTGACGCAGCAGATGCAGCACCTC
GAGAACATGCAGTTGGTTTGCTTCATGTGTCTCAGAGGAAGCACGTGTTAACCTTCACCCTCCCTGGTGTCAGGGATTGGAGTCGG
ATGCAACCTGACCCCTGGTTTGGGAATACCTGGATTAGGTATATATTATAACGGTGTATTATCTTGTGATTTCACTTTTCACTGTT
TTCTGGATGATGAGTTTTAAGGTTTGGAGATATAAAAGAGCGCAAGATGTTAGGAGAACCTTAAATGTTTCCACTATAACCTTAAA
GTATTATATTCTCATTTGTGTTTCTGAAAGGCCCGTCCTTACAGAGAAATCACATTCAGACATACACACATACACATCACACGTGC
CACCAACTCTCAATTAGATTAAGGTCTGGGGTTTTTCTGGGTCACTCTAACACATCCACTGTAGCTGTGGTGGTACATTTAGGGTC
ATGGTCCTGTTGGAAGATGAAGCTAAGCCCCCAGTTTCAAGTCTCTTGCAGACTGGAACAGATTTTTCTTCTAGGAATGCCCAGTA
TTTGGCTTAAACCATCACACCTTCAACCCTGACCAGTTTCCCAGTCCCTGCTGATTAAACGCATCCCCACAACATGATGCTACCAC
CACCGTGCTTCACAGTGAGGATGCTGTTCTCAGGGTGATGTTGGGTTTCTGGCCACTTTGTGTCCCAAGGTGTACAATTTTTTGG
TCAGTTGTGGTCTCATCAGACCAGAGAACCTTTCCACACATGCATTTTCCCAAACTCCTCCATAAAGCCTGGCTTTGTGGATTACC
AGGGCTATGGTTATGGATCTCTCCAGCTTCTTCATGGTTGTCCTTGCTCTCTTGGTTGCTTCTATGGCTAAGATCCTCTTTACACA
TTCAATGACTTTTGATGGACGGCTTCCTCTTGTCACAGTTGTGCCATATTCTTTCCATTCTTTTAAAAAATGGATTTAATGACACT
TCACAGGACATTTAAAGTCTGGGATAAATTTTTTATATACAGTGTCCAAATAATACCTTTCCAGAACAAGTCCTGGACTTGTTTTG
ATCACTCCTTGGTCTTTTTATGATGATGTGTGTTTAGGTAACAAACTGTGGGTTCTTTGAGGAACAGGTGTATTTATACTGAGATC
ATGTGACACTTTAATTACACACAGAATGACTCCATTTAACTAATTATGTGACTTTTGGTGGTGACTAGGAAAGAAGGAAATTAGGA
CATTGTGACATTGGCAAGAAAGGAACAAGAAAACAAGCAAGCGCTTGTAAAACCAAGAAAGAAGGAAATTGTGAACACAAGAAAAA
CAAGAAAACAAAAAAATAAGGGACACTGGAAAGGGAACAGTGATAAATTTTGTAGATTGTGGAATGAAAAACCTGAAAAACCTGAA
CATGTATCAGTTCCATCAGTATAAAAGGTTCTTTGGTCATCAGGGTGATGGTGGGGGTTTGATGGCAGAGGGATGGGTGGCGGGGG
CAGGACTGGCCTTGATGATATTAATCACCATATTAAGCCTTTGACATTTTGCCATGAATGTTGAATGCTGTTTTTGCGTTGATTGA
CATGTCGCTCATCCGGACGTCATTAGGCTTCCAGGCTGCTCACCTGCTTTGTGTCCTGCTCTGTTGTGACGCTCAGCTGCATCATG
AAGCGCAAAGCGGCAAATTAGTGTATGCACGTGGAAACCACTGGGCTGTGGGACACCTGATGGGTAAGAAGAGCGTAGACGAGACG
CTGGGCTCGGACAACCATGACGTCGATGCTCAGGCTTATTTAATCACGGCAGAAGCCGACACACACATGCAGCCGTCCAGCCTGCT
GAAGGCTCTCATCAAGTCTCTGGCTGGACCAGAGAGAGAGACAGAAGACGAGAGGGAAAGACGAGAGCGTTTGATGGAGCTGAGGA
GACGATGGGAAGAACAGCTGGCAAAAGAGAGGGAGATCAACCAGGCAGCGAGGTTTTTGTTACTGGCGTTAAACACGAGAGACGCA
GACGCCAGCTGAAGACAAGCGTCACGTCTGATCTTCAGTCAGAGATTCTGCAGTTGGAAGCAGCTGAGAAACGACGGCGCTGCGTC
ACAGTCGACTCTGTTAGAAAATAAACACTAAATCTCACTGTCTGTTCGTTCTGCACTGCATCAGCTCACAAATAACTTCTATTTTT
TGTAAATTAATCTAATTGTATGCCATTGTTTTATAGGCCTACGTATATAATTATGTTCTTCTGAACTGTTTGTGTGACTGATGTTT
CGTCAAATGTATTCAATAAAACAAGGCTATGACTGAATGCAGGATCTGATGACATCAAATGATGCGTCTTGGCCCAAATCTGCAGA
AACTCTGCGGGAATTTTGAAAAGTGCCTTTCATAATGACTTAAATAATATGAAATGTGGTGACTTCACTCCAATTTACCACTCCTA
TATCATTCACTTATCTTCCATAAACGCTTTATCCTGGTCATGGTCACAGTGGATCCGGAGCCTATCCCAGGAACACTGGGTGTGAT
GTAGGAAAACATCATGCTATACACACACACACACACACACACACACACACATTCACATGAAGGGTCGACATCAGTAATCGAACAAGG

Appendix F

Detailed sequence of donor DNA construct for *Hrg* driven by its native promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 2; sequences in blue = promoter; sequences in green = *Hrg* gene from tra catfish (*Pangasianodon hypophthalmus*); sequences in red = poly-A terminator

CATCCAGACGAGCTTAGCCAAGGTAGCCTTTTTTTCTAGCATCAAATGCAGGCTGGGTTAAGAGTTAACAACCATACAATTCTTTT
TCAGACTTTCTTCTTACTCAGGCTCAGCCGTCTTTCTCTCTTTCTCTCTCCCCATCCAGAGTAAACTCCACAAACCATCCCCTGAG
GTCACAACCAAATGTCATGGCCATGTTAACCCCCATCCAAATTCACACCACTGTCCCTCTCCTTCATTTGGCCCTGCATACATTAT
GTTCTGCATGTCCTCCCATGTCAACCACAGTTCAGTGCCTGAAGAGGACATCCAGACGAGCTTAGTCACTCCCATCTCTCAGTACA
ACGTCTGTCATGTCAATCATTCATCGCACGCGGATATGAAAGGTCAGTGGGTTTATAAATAGCCCCCTAGAGACGCGCTATACACC
GCTGTCACTTCCTGCCTACTTGAACGGCAGTATGGAGGTTGTGGGTGGGGGTGGGGGGATGTCAAGGGGTATCAATGTAGGTACTA
GTGTGCCCTAATGATCTCTGACTTCCAGTGGCACATGTGATGATGAACTTGGACATGAGAGCGTACATTCCAAGATTTAAAAAAAA
AAAAAAGTCAAGTCGAAGTCCAAACAAGATGCTTCAGATCTAATGATTCTCTGTTCAGGGTAGCTACATAGACTTATTTCTAGTAA
CATTAACTAGCTAAAATTTGCTAATATAGCTAGCTAAGCAGGTCTAGCATTTAAAATGGGCCTGTATCAATATGATGCTAATCTCC
ATACTTGTGACTAGCATGGCACTTTCGTGACCTCCATTGGACTTAGCTAGCATTTTGGACTTTGTACAAAACTAGCTAGTGTTGCC
TTGAACAGATGGACTGTTCCCACCTCATGAAGCTCTTCAGGAATCCAGGAACTTTTCAGAAATTCAGGAACTTCAGGCCCCAGAAA
AACCAGGACTGGATTAGTTCCTGGGCAGGAACTATATTAGCTCCAGGATCTGTACTTTTGCTGTGGAGCAGAACACACAGGACTTG
GGAACTGATGTCACTGGACAACATTCTGATGGACAGCTCCATAACTTCGGAGTAGTAGCAATAACTTTATCACACCACCCCTTAAC
TGATTATTTTCCTATTTCCAGCACACCCCAAAATGATTTATTCCTTACTTAACAGACGCCTCCAAAGCCCAACGTCTACCCCGACT
CACACTTTCACACTGCACATTTTGAGATGGCATCATCTCAGACTGAAGCGTCCAATCAGACAGCTTTGGTGTTGTTTCTGCACCAT
CTAGGTCATGTAGTGTTACGTACAGGGAAATTTAGCAGAAACAACGGGAAATTGAGGTTTGGGGAAATTGTGGGAAATTGAGAAGT
GATACTGATTTAATTTCATCACGCAAGATCACAACCTTGATCATTCTGGTATACCGCAAATCTGCACAGCTCTCCACAAGCCTCAA
GCGGAAGCTCCTCCCACTAGAGCGCATGAACTTTGTAGAAAGGTCTCCAGACGCATGAGTAAGATTAGGGATGGGACAGTGGTGTG
GTGCCAGAACCGGAACAAGTATACTTATTGTGCTGTGAGTAATATAAAATATCACCAAATATTATGAAATGTAACATTCTGACTGC
ACCTTTAACAAATTTAAATAAAGGAAACTGATCTAAATATTAAACTGTAAACTTTATTACTTAAAACTGGAGCCAGCTCAGTTGCT
TCACAAAACACGAGACATCTCATTGTTAAAACTGATCAACTTACTTCAATCAGCAGAAGGAACCGCCCACTTCTTAAGAACAGTCG
TCCTTATATGAAACACAAAGCCCCGCCCCTTTATTCTCAGAGAGCCAATCAGAGCAAAGGGGGGAGGTTCTTTCTCTTTTGCATAC
AGCTCGGAGTCCCGGTGCAAGCAGAGCGGCGCGGAGCTCAGACCACAAGCACCTGAGCTCCCGTTCCAACCGAGAGACACGGATAC
ACTTCACAGCGTTATAGAGGTCATAAGACGCTTCATAACGGGCTGCACGCGAAAGGAACATTCATTTGTGCACTTGTCTTTCATCA
GCTTGGATACTTGTTGGGGTTTTGTTGTTGTTTTTGGTTTTTTGACTTCACGGGGTTGAGCACTTGGGACTGCTGCGATGCCGTTA
TTAACGGAACTCTAGTCAGCTTGCGGTTGACATTTCTCGGATAGCGCGCGAGCCTTTTAAGGAGCTCTGCTTTCACTTTGCGTGCA
ATGCCAAGCGCTCGAGACCCGACGCTCATTTGGAATTAAACGCAGCAACAACATTATTAATAAAAATAACAATAATAAAACTTTTT
TTCCGCTAACGGCGAACAAGCGCGTGCCCCGGGGCGGGGGTGGCAGCCGGTTCTTGCCTGTAGCGCGCGCGCGCTCTCCTAAATGC
CCACCCGCTCGCGGGACTCGCAGGGCTCTCGAGGCTGGACGGAGAGATGAGCTCGTGGGTCGTGCTGCTCGTCGCGCTCGTGGCGT
GTCTGCGACTTGGCGGCGCGCAGGACCCTGTCCCTTTGTCTCTGGTGAATATGGTGATGAACTCTCCCATCTCATCCATGAAGGAC
CTACAGGAGCTGCTGGACGTTCACTCCGTAGAAGAAGAAGAGGACGAGGATCATTCAAATGGAACGCATAAACGCCTTCCCAGAAG
TCTCGGCGATGTGCAGATTGCTCAGCCGGCTGTGTGTAAGGTGCGAACCGAGGTGATGGAGGTTACGCGATCCATGTTGGACCGAA
GCAATGCTAACTTCCTGCTCTGGCCACTTTGTGTGGAGGTGCAGCGCTGCTCGGGCTGCTGCAACACACGGACGCTGCAGTGTGTG
CCCGTTACTACGGAGACTCGCCATCTACAGATGACTAAGATCCAGTTCATCAACAGACAGCCCGTCTACGAGAAGGTCATCTTACC
TGTGGAGGACCATCTCAGCTGTAGCTGTAAATCACGCGCTGTCAGTCAAACCACTTCGACGAAGACCACAACGTCCCCGCCTCCTC
CCCCCAAACTCATCACCAAACCCCCCATCTCCAGGAGCCAATCAAAAGAGGAGCTGCATCGCCACGACGACCTAAAACACAACCAG
AGGTTCCAGCTGGAGGATCGAGAGACTCAGTGGCAATCGAAATACACATTGTCTCACACGCAGAGAGTGCCCGTACACACACCCGC
ACACACACCCGCACACACACAGGGCTACGGATCCGGTCCGTTCCAACACACGCTCACACACGCGCAGGGCTCAAGTACAGAAGACG
CCTCGACGAGGCACGCGAGCTTTGGCGTAACACGTATGCTGAGCGACACGACGCAACATGTCACACCAATACAAGAGCACCACACA
CGCCATTCAGACCCGGAGAAGGTTCGGGAACACCACACACACCCTGGCAGTGGCGACGACAACATAAAATCCACGGCGAGCGGAGA
ACACCCAGAACATCATCGGCATCACCATGACAACCACGAAGCAGCGACAAAACACCAACACGAGCCGAGCAAATCACACACCGCTG
AGCATCACCGCAGCCAATCAGACATGGCGCTTCAGCACAGCGGCCAACTGGAAGCCCCTGTTGTCAGCTTCAGCCACGTAGAGGTC
ATTGGCCAGTCAAGCGGCCAAAGCGAGCTGTCCAGCCAATCATCAACGCCCAGTTTTCACCAATCAGAAGCGAAGGACCAGCACCA
AAGCCAATCAGAGACAGACAAGCTATCAGAGTCGGGGCAGGAAAAAACAGAGCATCATCATCATCACCATCATCATCCTCATCATC
ATCCAACACAAGCTGCCACACAAAGAGCAGTGACAAACGCTCCCGCTGGCACATCTCCTGCGCCTCACACACCCCCTCCAGCCGTC
CCTCAGCGGAAACGAAGGAGAAAGCAAAAGAGGAGAATGAGCAAGTCTGCCATGAGGGCCATGATCATGGTCATGTCCTAAACGAA
CCGGCACTTTGCCATCACGGACCAGAAGAAACCCGGAGAGATGCCGGATACCGCACAGACACATACACTGACACACTGGAAATATC
GCTCACAATAACTGGAAATGGCTTTGATCTCTGAAACATAAACTCAGAATGACTACATTTCCCAGCAGCCAGCACGGCAAAGAAGC
TCCGACGCTCGGCATTATGGGATGTCCTTAGTGTTCACTGCACATGAAAGAAACACTGCTGACACAGCCTGCCAAATGATGAGACT

GCATGGAAACCAATGCAGGGAGAGTTTGCCAAATAATGAGATTGCATCTAATACAAAGCAGAAAGCCTGCCAGAAAATGAGACTGC
ATCTCAAACTATGAGGAGTCAGCCTGCCAGATAATGAGACTGCATCTAATACAAAGCAGACACAACCTGCCAAATAACGAGACTGC
ATGGAAATCAATGCACGGATGATTTGCCAAATAATGAGATCGCATCTAATACAAAGAGAAATACTGCCTGCCAAAAAATGAGACTG
CATCCCTAACTATGAGGAGTCAGCTTGCCGAAAAATGAGACTGCATCAAAATCAGTGCAGACACAACCTGCCAAATAATCCAAGGA
CAAGACATCCTGGGGTTCAAACAATGTTTTTTTTTTGTTGTTTTTGTTTTTTTTTAATATTACACACACACATATACTATAATTAT
TGCATTTACACACAAATTATTTGTAGTGCATATTTATCATAAAATTCCTTGAGCCAAAACCTGATGTAAACATCATATTTGTAAAA
CATGAAACAAATGATTGTCTTAGAGATCTATTTATTACACCTGGTTTCTGAATTGGATAAAAATATTGTCTGGAGGATCTTAGAGA
CTTTCTTTTTCCAAATCCCATATGCTGCTAGAACACATCCAGACGAGCTTAGCCAAGG

Appendix G

Detailed sequence of donor DNA construct for *Cx3cl1* driven by its native promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 2; sequences in blue = promoter; sequences in green = *Cx3cl1* gene from tra catfish (*Pangasianodon hypophthalmus*); sequences in red = poly-A terminator

GTGCTCCTGCTGCTGTTGTATGGGTGTACAGATCTTCAGGCTGTTGGTGTAGGGTTTCTGAACTGTCACATAATGCTGGTTCATCT
GATTCAAACTCTCTCTATCCTCTGTATGTGTGAGATTTCCTACTGTAGTACCTGTGCTGAACTCTGCTCCTTCACTCTCCTGCATC
ATAACAACATTCCCTGATTCACTGCAGTTTTTTGGTTTTCCTGATGGTTTTGCAATGTTAATTGTTTCCTTGCTTTCAGTTGTCAG
CTTTGCTGTAGTGTCCTATGCTTTATCATTGTTATGCAGTGCCTGTGAGTGCTCCTGCTGCTGTTTGAATCAAAATTGCCAACATA
GATAGACTTTCTTTGCACACCTCTATCATACTTTATTTGGTATCAGTATCAGCTTAAAAACATGAAGCCAGATAAACGTCAGTTAC
AGTAATACTCAAGTCACTCCCTGCATGCAGAGAAGTAGAGCTATTCTGGCCTGGGATGATTTAACATAAGAAAAACCCAAAGTAGG
AAGCAAACAAAAACGTGCTATTAAAACACACACTTAAAGTAAAGTATGTCTGGCTACTATCTGACTCTAAAATTCCCTTTCATTGC
GCCAGAACTTACCTGACCCATGCACATCAAACATATGTCAGCAACTTCAAACATTAAGGCGCATCCAATGAATGTTGCAACCTGCT
GCATGTAAATGAAGTTGTTTCATTCAGCTTACTTGAATGCTTGCTGTGGTATGTGTGCTTAGTGCAGCCATAGTGCACACAAAAAG
AAGGTATACCTCAGAGGTACTGTGTGTCTTAAATTACAACAATAACATTGCCTGACAGGATACTTGCTACACTGTGCTTTTCTCTA
TACTGTGGCTATAGACCTATTTTAACACCCGGCACACAAGAATATGTTTAAATGTTTACTGCATGTTGTGTATATTTATGGCACAG
CACAGGGGCTCAGTGGCTTGTGTGCATGTAACCATATCAGCCACATCATTCCACATCAGATTGTATAGCCACAGTAAACAATTGAT
GAATTATATCCAGTATCAGATGGCTCAAAGCAGTGCAATGTAGCCAAGTTAAACATTTTTTCACTGGATTTCAATGATGTAGTTCT
CTTGTACAGGCTAACAGTAATAACAGACAGGGACCGAAACACCAACAAACACGTTAAGGATAGCCTACCTGCTATAGGTGCTGACT
AAACAAAAACTAACTGCACAACACTAGGCGTAGTCTTGGAGTAACCTGGAGTGGCGGGATTCAGGATCGTCGTTGTTTTCATCCTA
GTCCTTGATAACCTTAGAATTTGTGTCATTAATTTTAGTCTTCATTGAAAGATTACTTGAAAAAAAAAATCTGATATTTTTCTGCT
TCCTGTTTGCCATTTTTGCTTGTAAAATGTATGGGTGGCATTTCTGTTTTAGAGACTTTAGTGTGATGTCCTTGATATTATTTTCA
TCACTTTACTCATTAACTAATTAATGAGTTATTAATGAGAATTAATTAATCTCTGTTTTGAGCAGGTACAACAGTGCAACATTTGG
CTGAAATATCCTGTTTCAAGATACAATGGCAGTGAGCAGAGGTAGCTTGTTTAAGCTCTAGTTCATTATCATTCCTAGTCACTACC
TGAAGATTAAGCTAAAAACCTCTCTCCTTTTCATAGGATCACTAACCAAAAGCATCTTGCCAAGAATATGTCAGGTTCCTACAACA
TCCTACACACTCAACTCTTATCCATGTGTGGTGGAGCAGAGGTGGCTAGATCAAGAGAGGACAAAGGAAGCAATGTAATGAAAGAG
AGACAACTAGATAATTATTATATTATTACATATATAATGACGTATCTATTTAATGCAAGACATGTTTACAATAAGCTATTCTATTA
TTAAGGGTTTGGTGGACAGGGTCTATTGAACATTCTGCAACACTGCTTCTCACATCCTGCATACAAAACACATAAAAACATATCTG
AAAAATTATTTTTATTTTTATTTTTATTATTATTATTATTATTATTATTATTATTATTATTATTTGAGAGAATGTGTTAAAGC
ATACAAGGTTCAGAGAGTGAAAAAGAGAAATTTAAAAAAGGAGAGAGAGAGAGAGAGAGAGTGATAGAAAGAGAGCAAGAGAGA
TATGGGGTGAAAGCAGTGAAAGCGGAAGTGGGGAGCGGATGTTTGGGTTTTTGAGCCCATTCATTTAAAAAGGACTTAAAAAAAAG
AGAAGAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAAGACACCTGAGCAAACCGCAGACTCGCGCTCACCCTGGCTTTCCCGAA
AATGAGGGTCTGTAAAGCGCTGAGAAAAAAACAAGGAATCCTGGAACCAGCACCAACCCGACAGGAACTCAGCAAGAAGGTCCCAG
GAAAAGGTAAACCGCCCCCTCTGCACATCATCGACAGCCAGCCTGCCTACACAGTCCACCGAATCCTGGATGTCCGGAGAGTACGG
GGCTCCCGGCAGTATCTCGTGGACTGGGAAGGCTATGGCCCTGAGGAACGCTCCTGGGTCCCGGCCCGAGACATCCTGGACCCCGG
GCTCTTTCTTGCCCACTGGCCCAAGTGTTATGCTTTTTTTTCCCCACTAGATGTCACCCTGCCTTAGTTTTTCTTTGTTGTAATTG
GTTTCACCTGTGTCTTGTTCCCATTCAGGTATTGGCATTTATTAAAAAATGTCTACATAGTGTATCAAACAGGAAGGCAAGGATCT
AAGGTTAACATATACAACTCAGTTACAGCAACTTCTCACATAGTGGGAAGCCTGGCACTACCTCTGAAGTCATGTATTTGAACCAA
CATTGTAATGCAATGTTACTCTTAATGAAATTGTATCATATTAGATGTGTTATTACTCTGTAAACACACCATTGTATCCAGAAGCT
AATGGTCACACTCATGTATGTCGTTTTTGATGTCTGCATGTTTGTTTTCATGTTTGTTTTCATTCATATCACACAGTTATTATAAG
TATTAAAGACAAACAAGCCCAAGTTACAATGGACATTAAGAAGGATGTGTCCCAGGAGGGGTCACATGCAAATTAGCTGGAGGACC
AGGCAAACTGGCCACCAGTCGTCTCCAAGCCGACCCGTGGTTGGATGAAAACCATAGGAGGCCAGCTCAGCTGGTGCAGTAACACT
AAGAACTCTCTAACCAATTCACAATTTAATGCCGACATATTGATGGTTCACTCAGGTGAACTTTTGTCCAAGAACGCAACCTTCTG
ATTGTTATCACTGGTTCTTCTCATGCCATATTCCTACATGTATGAGTGTGTATGTCTATGTGTGTGTTAGTTGTATGTAGTAGT
CTTAGTCTATGTATGGTATGGGGTCAGGATCATCCTTAGTGCAGACATTAGGGATAGGGTCACTGCTAGCGCTATCACTTGTAGCA
GCACCGGGTTTGGCGCTAATGCTATCTTCAACATGAGCGGTGTTTGTGTCAGTGTTAGCATCAGTGCAGGTGTTGGTGTTATTCCT
GGGTGCTGCGTTTTTTTCAGTGTTGCTGTTGTGTTTGTCACTGACTTCTTTCAGCTCTAGCACCGGCACGTCGTAGCTCTGTGAGT
CATTTTCTCTAGGCTCCTGTTCTCCGGTGTTTTCCTGTTGGCTGTCCATGCGCTGCTGTGCTCCTGCTGCTGTTGTATGG

Appendix H

Detailed sequence of donor DNA construct for *Fras1* driven by its native promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 2; sequences in blue = promoter; sequences in green = *Fras1* gene from bighead catfish (*Clarias macrocephalus*); sequences in red = poly-A terminator

CCCATTGGAGGAGCTTTCGGTGGTTCATGAACATTTGTTTTGCAGTGTGGCAGGGTGTACTTTGTCTGCAACAATGTTTATGTAGG
TGATACATGTAAAAGTAACATCCACATGAATGCCAAGAACCAAGGTTTCCCAGCAGAAAATTGCCCAGAGCATCACACTGCCTCTG
CTGGCTTGCCCTCCTCCCATAGTGCACCCTGGTGCCATCTCTTCCCCAGGTAAGTGACACACATGCATCTGGCTGTTCACATGGTG
TAGAAGAAAATGTGATTTAAAATGTGATCCTGTTCAGATGCTCATGTGCCCATTGGAGGAGCTTTTACAAACATGTCAGGGAATAA
TGCTGATGAAGTATTTTTTTAGAGACTAGAGGAAAGAATTGTAGTGTTGTAGTATTATATGTACAGTATTAAAAATCTGCACACAG
CACACAGCAAAATATGCTCTCTGCAAAAGTGTATACACTTTTCTTTTACTTACAGATCTTCCCAGAACATGTAACACGACAAAGAG
AAGAGGAGTATAAATCATCCAATGTCAAACAAACGGTCCAGCTGAACTGAAAATGCACGAGGGTCTTTAAATTCACGAACCAAGGA
TGGGTCTTCGAAAGTGGCCCTTTTGGTTCCTCTTTTTTCTGGGACATTTGCATGACTGCATGTGCTCTCTGGTGAAGACCAACCAC
GGCCTGCGGGTGAAGCGAGGCCAGTCGGCTTTCTTGCAGGAAGGAGACCTGCAGTTCCACATACCCAGAGAGAAGGATGCCTGCAA
GCTGGAAGTGGTTCTGAATGAGCCTATTACTCAACGTGTTGGCACACTGTCTCCTCAGGTTTTTGACTGCCACTATTTGGCTGATG
AGGTGAAATATACGCATAATGGTTGTCCTATTTTAAAAGAAGACACCGTCAGACTTCGATTGTACAGATTCACAGAGACAGAGACA
TATAGTGAATTCTTCTCACTTCAAATTGAGATCATGGAACCTGACTGTAATGTTATAAAGTTGGGACCACAGACATTGCACGTCCC
AGAGTTCTACGGCCTTTCCAATATTCTTGATGGTAATGTGATGTCCTTCCACTATGAGCACCGACCCAACATAGAGTGTACCATTA
GGGTGACAACACCAGACTCCGGTCTACCTGGACATGAAAATAAAGCCAGAGCCAGATGCAAAAGTGAAGACTGTTTGAAAGGACTT
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Appendix I

Total number of channel catfish (*Ictalurus punctatus*) embryos microinjected with four different ABO transgene constructs and their injected controls (iCntl). *Grp* = gastrin-releasing peptide-like isoform X2; *Hrg* = histidine-rich glycoprotein; *Cx3cl1* = chromo domain-containing protein cec-1-like isoform X2; *Fras1* = fraser extracellular matrix complex subunit 1.

	Donor DNA conc. (ng/ µl)	**Number of embryos injected**				**Total**
Treatment		**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	
Grp	50	936	687	666	1278	3567
iCntl		433	250	300	370	1353
Fras1	50	1338	1119	1190	-	3647
iCntl		433	250	345	-	1028
Cxecl1	50	648	650	1343	-	2641
iCntl		433	250	345	-	1028

Hrg	50	1568	1514	-	-	3082
iCntl		300	270	-	-	570