

**EVOLUTIONARILY ADAPTIVE MECHANISMS TO BILIARY ATRESIA IN
THE SEA LAMPREY**

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ABSTRACT

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Lamprey species appeared over 500 million years and are therefore important evolutionary models for research interests ranging from neuronal signaling, genetics, to organ development. Among this group of jawless vertebrates, the sea lamprey is the largest and most widely distributed. It experiences a drastic life history, which includes a complete metamorphosis. During sea lamprey metamorphosis, the biliary tree and the gallbladder degenerate, in a process referred as biliary atresia. Atresia of the biliary system occurs as a rare disease in human while it is a programmed developmental event in the sea lamprey. The sea lamprey can be used as a model to study the etiology and the adaptive mechanisms of biliary atresia, and the compensatory and adaptive mechanisms in cholestasis based on its various life events including biliary atresia. In this dissertation, I hypothesized that the sea lamprey had evolved unique mechanisms in both liver and intestine in adaption to biliary atresia. To test this hypothesis, I examined sea lamprey liver throughout metamorphic stages at the levels of morphology, histology, mRNA transcripts, and bile salt composition. My results indicate that the sea lamprey has evolved several possible adaptive mechanisms in coping with its developmental biliary atresia. As expected, the enterohepatic circulation is conserved in this basal vertebrate before its metamorphosis. However, the intestine synthesizes and secretes bile salts during and after metamorphosis, or biliary atresia. It is further elucidated that the metamorphic sea lamprey reversed enterohepatic circulation by *in vivo* perfusion and *ex vivo* intestine transport assays. It

may be an adaptation to the lack of biliary system. Another adaptive mechanism in coping with the aductular life appeared to be dramatic changes in bile salt composition. Also, the down-regulation of *cyp7a1*, which encodes the rate limiting enzyme of bile acid synthesis, in liver during lamprey biliary atresia resembles the compensatory mechanism in many cholestatic animal models. All of the findings show that the sea lamprey has evolved to cope with biliary atresia and cholestasis. Understanding these mechanisms can shed light on developing treatment and management for patients suffering from biliary atresia and cholestasis.

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KEY TO ABBREVIATIONS

3-dehydro-allocholic acid (3k-ACA)

3-dehydro-petromyzonol sulfate (3k-PZS)

allocholic acid (ACA)

large parasite (LP)

disintegrations per minute (DPM)

liquid chromatography-mass spectrometry (LC-MS)

metamorphic stages 0-7 (M0-M7)

methanol (MeOH)

Michigan State University (MSU)

newly transformed (T)

ovulatory female (OF)

petromyzonamine disulfate (PADS)

petromyzonol sulfate (PZS)

petromyzonsterol disulfate (PSDS)

preparative thin-layer chromatography (PTLC)

small parasite (SP)

spermiating male (SM)

taurochenodeoxycholic acid (TCDCA)

taurocholic acid (TCA)

taurodeoxycholic acid (TDCA)

Chapter 1:

Using the sea lamprey as a model to study biliary atresia and cholestatic diseases

Abstract

The sea lamprey undergoes a developmental biliary atresia, progressive obliteration of the biliary tree, during metamorphosis. While biliary atresia is the most common reason for pediatric liver transplantation in human, it is a programmed and tolerable process in the sea lamprey. Over decades, various animal models have been developed to study the etiology and the compensatory mechanisms of biliary atresia. However, management is still limited to surgical treatments because very little is known about the etiology. In this review, I highlight areas for which the sea lamprey can be used as a model in biliary atresia and cholestasis research. Understanding the genetic network behind lamprey biliary atresia may provide insights to the etiology in human biliary atresia. In addition, information during lamprey biliary atresia may be used to further develop adjunct treatments for patients awaiting or receiving surgical treatments. At the end of its reproductive season, the cholestatic adult lamprey has unique adaptive mechanisms and therefore can be used to explore potential treatments for cholestatic patients. Upon elucidation of the adaptive mechanisms that the sea lamprey has evolved in coping with biliary atresia and cholestasis, treatment and management may be developed for patients suffering from these conditions.

Introduction

Human biliary atresia is a rare liver disease in infants without distinct etiology (1). It is an obliteration of bile ducts with progressive inflammation, which leads to liver failure if not treated (2). The Kasai operation (3) is often used to restore bile flow, and currently liver transplantation still remains the most effective way to treat biliary atresia (1). However, patients treated with these methods still bear high mortality and have significant complications with reduced quality of life (1, 2). Therefore, physicians and clinical researchers are still in search of better measures to manage patients who suffer from biliary atresia.

Studies in human biliary atresia are very limited in understanding the etiology and elucidating the compensatory mechanisms because of small sample size and variable uncertain factors. Animal models have been used in the past decades without much success because no animal model can represent both predictable and natural processes of biliary atresia, accurately reflecting its unpredictability and sporadic nature in the human disease (4). Sea lamprey is an exceptional animal model that can address the above-mentioned problems, and will be summarized in this review. Most studies for lamprey biliary atresia were performed in the 1980s when very few tools were available in terms of molecular biology and genetics. Nevertheless, Youson and his colleagues have set a foundation in morphology, anatomy and histology in lamprey biliary atresia (5, 6). As indicated in a recent review, no good animal model can simulate biliary atresia in humans (4). While lamprey biliary atresia is labeled as a developmental process (4, 5), not a disease, its value of being the only natural and predictable example of biliary atresia is certainly unequivocal. This process seems to be programmed at the onset of metamorphosis. The space held by the biliary tree in the larva is filled by hepatocytes in juveniles without apparent inflammation or fibrosis (5). This feature of hepatocyte proliferation

resembles that in cirrhotic biliary atresia patients (7). Hepatocyte regeneration is similar between human and lamprey biliary atresia.

Recently, major advances in tools and technologies have promoted lamprey research in various areas. A review on existing animal models used in biliary atresia research in past decades concluded that we have not obtained critical information to treat biliary atresia (4). However, two recent studies in sea lamprey that showed unique adaptive and compensatory mechanisms in coping with biliary atresia and cholestasis (8, 9), prompted interesting hypotheses on the synthesis, modification, and circulation of bile salts. Elucidation of such proposed mechanisms can help generate preventive and ameliorative measures for use clinically if the information is translated into human medicine.

In this review, I summarize three potential areas that the sea lamprey, as a model, may provide further insights into biliary atresia and cholestasis. I will highlight several areas of future research on biliary atresia based on lamprey biology and its drastic metamorphosis and development. Because the sea lamprey undergoes biliary atresia during metamorphosis, the metamorphic lamprey is therefore a potential model to study the etiology of biliary atresia, suggesting preventive measures in human biliary atresia. In addition, the metamorphic lamprey has adaptive mechanisms to cope with biliary atresia and cholestasis; understanding these can provide information to generate potential adjunct treatments to ameliorate liver damage in patients waiting for or receiving surgical treatments. In a different scope, studies in mature aductular cholestatic lamprey can provide crucial information on management of late-stage cholestatic patients. With the recently available lamprey genome assembly (10), siRNA technology (11), and development of the CLARITY histological method (12), mechanistic

studies in lamprey biliary atresia on the etiology or adaptive strategies (or both) are now more feasible than ever.

During vertebrate evolution, natural selection or genetic drift has resulted in populations with phenotypes that mimic human disease, but are nevertheless adapted to their environment (13). These extant assemblages of related organisms can serve as “evolutionary mutant models” that compliment induced laboratory mutant models in elucidating human disease processes or identifying genetic predisposition of diseases. Sea lamprey, the largest living jawless vertebrate, is an exemplary “evolutionary mutant” for biliary atresia and cholestasis because it undergoes a developmental biliary atresia immediately before the most voracious feeding and growing period in its life cycle as it parasitizes on fish blood and sometimes even flesh.

How does the sea lamprey prevent or ameliorate progressive cholestasis, necroinflammation, fibrosis and eventually cirrhosis, the complications that inevitably inflict humans with biliary atresia or any other factor that obstructs the bile flow? And what are the genetic programs that initiate biliary atresia and then prevent the expected pathogenesis within hepatic parenchyma? Answers to these questions should be informative in developing prevention methods and treatment for human biliary atresia and cholestatic injuries. On an evolutionary scale, lamprey has had more than 500 million years (10) to evolve mechanisms to cope with obstructed bile flow.

Although the sea lamprey is far removed from the *Homo sapiens* on the vertebrate phylogenetic tree, data collected from lamprey liver should be directly relevant in understanding the physiology and pathology of human liver (5). Virtually all major endocrine and exocrine functions known to mammalian livers have been confirmed in lamprey livers (with the exception

of the lack of storage and drainage of bile products in post metamorphic lampreys). The functional unit of larval hepatic parenchyma is composed of hepatocytes arranged into tubules surrounding canaliculi, which is continuous with bile ductules leading to bile ducts. Hepatocytes have access to tortuous sinusoids that receive blood from a hepatic artery and the hepatic portal vein; the specific hepatic circulatory patterns have been evolved. The hepatocytes are polarized (with microvilli on their apical membrane and an unfolded basal membrane) and have organelles characteristic of vertebrate hepatocytes. The fine structures of epithelial cells of the biliary tree are basically similar to those in mammals. Nonparenchymal cells, such as endothelial and Kupffer cells, are also similar to those in other vertebrates. The presumed utility of sea lamprey in biliary atresia research is based on the premise that the programmed degeneration of the biliary tree in metamorphic sea lamprey resembles progressive obliteration of biliary ducts in infant biliary atresia. This assumption appears to be valid because cellular features of degenerating bile ducts in lamprey and atresia of interlobular bile ducts in infants are remarkably similar (5, 14). While detailed studies have been described about lamprey liver at the level of histology, more detailed investigation into the exact mechanisms that the sea lamprey employ during biliary atresia and cholestasis have promise to contribute to future therapeutics based on advances in our understanding of the etiology of biliary atresia, adaptive mechanisms used during biliary atresia, and those employed during late-stage cholestasis. All of these have to be studied according to the various life stages of the sea lamprey.

Model for the Etiology of Biliary Atresia

Lacking an efficient animal model to study biliary atresia is an obstacle to understand its etiology. Mice and rats with induced biliary obstruction (15-18) allow studies to uncover mechanisms of cell injury caused by abnormal human genes and cholestasis, but have failed to elucidate the pathogenesis and develop treatment for biliary atresia (4). The lack of efficient animal models is certainly not a lack of effort devoted to this problem. In 2004, NIH listed “pathogenesis and management of biliary atresia” as its first research goal for pediatric liver diseases, with the development of new animal models as a priority (*Action Plan for Liver Disease Research*). Despite this interest, “our understanding of the cause and pathogenesis of biliary atresia has remained unchanged for several decades” (19). Another decade has passed since NIH issued their action plan in 2004; scientists and clinicians are still seeking the answer for biliary atresia after more than a dozen experimental models have failed to generate therapeutic breakthroughs. One example is the rhesus rotavirus model (20, 21), which induces bile obstruction and biliary atresia-like features in mice. However, potential clinical trials based on virus infection and the accompanying immune response (22) in this model do not look promising currently. In fact, one must reconsider the nature of such a rare disease for which no experimental model adequately recapitulates the associated pathological process. A natural model may have answers that may help to better inform successful therapeutic intervention.

The sea lamprey model provides different and significant insights into research of biliary atresia. From laboratory models, no prevention for biliary atresia is resolved because little is known about the etiology and the timing of the disease process (1). As pointed out by Suemizu *et al.*, hepatocytes from cirrhotic biliary atresia patients still retain proliferating potential if the etiological agent can be removed (7). While human biliary atresia may be the outcome of multiple factors including genetic predisposition and environmental factors (23), understanding

the genetic predisposition is extremely difficult because of its sporadic nature. The sea lamprey is the only programmed and hence predictable vertebrate model. Because understanding the etiology is always difficult in other models due to its sporadic or artificial nature (4), having the opportunity to capture the genetic network behind biliary atresia in the sea lamprey is certainly a treasure to mine. One must understand that biliary atresia in the sea lamprey is not the same as human biliary atresia in terms of the pathological outcome, just as other models that are not perfect in simulating human biliary atresia (4). In fact, lamprey biliary atresia does not lead to liver fibrosis, or cirrhosis, as in infants (Table 1); this is self-explanatory in the differences between these two processes. Nevertheless, it is the only natural model to date that allows us to understand the etiology of biliary atresia in depth because of its predictable and robust nature, and high availability. Obviously, understanding the etiology of biliary atresia is the ultimate answer to treat and prevent biliary atresia in infants.

Although no etiology has been unequivocally proven, it is recognized that biliary atresia in infants is caused by the interaction of genetic predisposition with environmental factors (23). Laboratory and patient-based examinations have led to five possible mechanisms (24): “(1) defect in morphogenesis of the biliary duct, (2) defect in fetal/prenatal circulation, (3) environmental toxin exposure, (4) viral infection and (5) immunologic/inflammatory dysregulation.” Evidence for each etiology is conflicting and enigmatic. Desmet and Roskams (25) pointed out that etiologies (4) and (5) are less plausible because similar lesions of biliary atresia do not recur after liver transplantation. Nakamura and Tanoue (23) suggested that human biliary atresia is a developmental anomaly with various predispositions including the Notch signaling pathway (26, 27), congenital left-right patterning, and some other genes. Recent studies

have proposed that microRNAs (28, 29) and DNA methylation (30) are also involved in biliary atresia pathogenesis.

The lamprey model should be particularly useful for examining etiologies (1) and (2). Many types of malformation have been described for the fetal form of biliary atresia (which account for ~30% of total biliary atresia patients), and to a lesser extent, for perinatal biliary atresia. Mutations in genes that establish left-right patterning have been described for fetal biliary atresia (31-34), so have disruption of factors that regulate apoptosis and cell cycle (35). Intriguingly, previous evidence suggests that biliary atresia in the sea lamprey is largely an apoptotic process (5, 14). Such a programmed apoptotic event without liver complications indicates well-adapted genetic networks and circulation of bile products underlying this process. Understanding the genetic network behind lamprey biliary atresia can thus offer great potential to elucidate the etiology of human biliary atresia (36).

Model for Adjunct Treatment in Biliary Atresia

Another layer of interest comes from the adaptive mechanisms that are at play when the sea lamprey transitions from ductular to aductular stages. The lamprey liver experiences transient cholestasis immediately after the onset of biliary atresia (8). While regulation of *cyp7a1* is a key compensatory mechanism in many cholestatic systems (37), cholestasis leads to liver damages even when its transcript level is decreased. Down-regulation of *cyp7a1* is certainly not the sole answer to the alleviation of cholestasis in the sea lamprey because the outcomes are quite different despite a similar pattern of transcript changes in sea lamprey (8) and human (37). Must there be other protective measures in lamprey liver that have not been found? Such agents, upon

elucidation of how they work, can be further used in infants that are in awaiting the Kasai operation or liver transplantation, and grant better prognosis for these infants if advanced fibrosis can be prevented. It is indicated that the development of an adjunct treatment may be necessary to increase the survival rate on top of surgical treatments in biliary atresia (38). Unfortunately, use of steroids may not provide significant protection based on the START trial (38) even though abnormal immune responses are thought to be key to injuries in biliary atresia (39). Most biliary atresia models are currently induced to simulate the pathological conditions without discrimination between secondary and primary reactions or agents (4). It is therefore difficult to develop useful adjunct treatments. The sea lamprey can provide an answer to such treatment because the liver undergoes transient cholestasis and recovers from it during biliary atresia (8), and also because lamprey biliary atresia is a programmed process. Although biliary atresia accounts for the highest proportion among neonatal cholestatic patients, other triggers such as infections, Alagille syndrome, and idiopathic causes make up about 50 percent of total neonatal cholestasis cases (40). Understanding the mechanism underlying the transient cholestasis in sea lamprey may provide new insights into management of other neonatal cholestatic diseases as well.

The ability of the sea lamprey to survive and grow after developing complete biliary atresia implies that unique hepatic and extrahepatic adaptive responses have been developed as alternative pathways to minimize bile acid toxicity and to eliminate bile acids, bilirubin (biliverdin) and other toxic substances normally excreted into bile. Studies in patients with biliary atresia have documented significant compensatory changes in bile acid metabolism and transporters that are generally similar to those observed following bile duct ligation in rodents and other chronic cholestatic disorders (37). In a recent study, Yeh *et al.* demonstrated that the

lamprey has unique adaptive mechanisms in addition to the down-regulation of bile acid synthetic enzyme, Cytochrome P450 7A1 (8), whose transcript level is reduced at both early- and late-stage cholestasis in many animal models and human patients (37). Lamprey intestine not only has the potential to synthesize bile salts, but also has the capacity to excrete bile salts into the intestinal lumen from blood during its aductular feeding life stage (8). In their study, lamprey liver goes through a transient cholestasis during metamorphosis, yet recovers from it after biliary atresia occurs. This raises questions about what specialized protective mechanisms might exist to protect animals from the toxic effects of cholestasis. Could this intestinal excretory route account for the elimination of the transient cholestasis? What are the transporters involved in this reversed enterohepatic circulation? Are there additional cyto-protective agents present in lamprey liver during this process? Elucidating the exact protective mechanisms in lamprey liver during biliary atresia and the transient cholestasis may uncover new therapeutic targets for patients that suffer from biliary atresia and receive surgical treatments.

Model for Treatment in Late-Stage Cholestasis

A third area that may be of interest concerns the adaptive mechanisms during severe cholestasis when adult sea lamprey reach their final maturation before they spawn and die. It has been shown that bile salt composition shifts dramatically when life stage advances from juvenile to mature adult (8, 9). Understanding the mechanisms involved in these shifts may have impact on management in late-stage cholestatic patients. More specifically, it was pointed out that the less toxic keto-form of petromyzonol sulfate may be another adaptive mechanism on top of renal excretion of bile salts (9). Whether this transformation of petromyzonol sulfate to 3-keto

petromyzonol sulfate can be attributed to its ligand-receptor specificity as a pheromone (41), to its lower toxicity, or both, still requires further investigation. Nevertheless, the sea lamprey liver does not develop fibroncrosis even at advanced stages of cholestasis. Could there be other protective agents such as antioxidants (42) that have yet to be found in this system? The lamprey model during its reproductive stage provides a tool to study late-stage cholestasis, and bile salt composition and circulation (9, 36).

Two recent studies have shown that the sea lamprey has very unique adaptive mechanisms in surviving biliary atresia and cholestasis (8, 9). In the study by Yeh *et al.*, lamprey intestine was shown to excrete taurocholate in both *in vivo* and *ex vivo* settings in animals experiencing post-biliary atresia during its vigorous feeding stage (8). In the same study, bile composition was shown to shift from sulfated bile salts to taurine-conjugated ones immediately after biliary atresia , and from taurine-conjugated bile salts back to sulfated ones when lamprey reach sexual maturity (8) and release one of the sulfated bile salts as a sex pheromone (41). It is unknown why bile salt composition shifts twice in the sea lamprey during different life stages. Enterohepatic circulation in lamprey is different from that in human (8), and lamprey bile salt transporter ASBT does not have affinity for taurocholate (43). Taurine conjugation may be an adaptation to lamprey biliary atresia. Kidneys have also been shown to have increased excretion of bile salts in mature lamprey during its non-feeding stage when severe cholestasis is observed (9). The sea lamprey has evolved to have adaptive changes to cope with both biliary atresia and cholestasis. Understanding the mechanisms behind these bile salt modifications and their circulation may provide information to treatments for cholestatic patients.

Discussion

Sea lamprey liver shares essential anatomy and physiology with human liver. Sea lamprey biliary atresia resembles the apoptotic process seen in human biliary atresia (14, 35). Although there is an emerging hypothesis of a virus-induced immune response (44), developmental abnormality has not been ruled out as an etiology of biliary atresia. When the general consensus of the etiology of biliary atresia is the interaction of genetics and environmental factors, sea lamprey biliary atresia should be particularly useful in determining the underlying genetic networks, and it is probably the best model to do so because it is the only example of programmed biliary atresia without the sporadic nature of other models.

Common bile duct ligation (15), bile acid feeding assay, and virus-induced biliary atresia (20) are manipulations that provide critical information of compensatory mechanisms on cholestasis. The sea lamprey, already adapted to biliary atresia, can provide pivotal information that experimental manipulations cannot. Specifically, answers to why bile salt composition shifts dramatically at the stage when biliary atresia occurs may lead to therapeutics. Could taurine be an antioxidant that protects liver from transient cholestasis (42)? Or, could taurine conjugation be hepatoprotective (45)? Studies to address these questions may provide answers to management of biliary atresia.

Even at the severe cholestatic state, the sea lamprey liver does not show signs of necrosis or fibrosis (9, 36). Is this striking outcome solely due to bile salt transformation and regulation of transporters in liver and kidneys (9)? The sea lamprey model deserves further investigation because of the adaptation to cholestasis that others have not developed. Further studies on nuclear receptors in the sea lamprey system can provide vital information that other systems

cannot. The abundance of adult lamprey in the Great Lakes, in particular, renders such studies feasible without having to induce cholestasis.

Conclusion

In this dissertation, I hypothesize that developmental modulations of hepatic and intestinal biosyntheses and transport of bile salts occur in the sea lamprey biliary atresia, resulting in adaptive changes in bile salt enterohepatic circulation and homeostasis in the post-metamorphic feeding lamprey. I describe possible adaptive mechanisms that the sea lamprey has evolved to cope with biliary atresia. While sea lamprey and human biliary atresia share many similarities at the histological level, I demonstrate that the sea lamprey has adapted to promote normal physiological functions in aductular life. The sea lamprey not only has mechanisms such as hepatic down-regulation of bile salt synthesis during biliary atresia, it also has a unique pattern of extrahepatic excretion of bile salts. More interestingly, the sea lamprey intestine can synthesize bile salts after biliary atresia. This is the first observation of bile salt synthesis in intestine of vertebrates that already have well-defined liver development. Furthermore, the exciting result of intestine being an excretory organ strikes the current dogma on the enterohepatic circulation in which the intestine serves an absorptive organ to recover bile salts and initiate their transport back to liver. Moreover, I showed that the sea lamprey displays dramatic shifts in bile salt conjugation at the stages of metamorphosis, when biliary atresia occurs, and during reproductive maturation. These shifts of bile salt functional groups may be due to reduced toxicity, changes in food source and metabolism, and/or pheromone signaling.

Taken together, I tested the hypothesis from the perspectives of histology, genetics, chemistry, and physiology. While this dissertation does not focus on studying the etiology of

biliary atresia, it provides the groundwork in staging the animals and making observations of liver changes at the onset of biliary atresia. This information can be further extended in future research on lamprey biliary atresia, and hence leads to potential prevention of human biliary atresia. Results presented in this dissertation have a major focus on the adaptive changes that occur during and after biliary atresia in the sea lamprey. Implications based on these results may be used in generating potential adjunct treatments for use in biliary atresia patients waiting for surgical treatments. Comparisons of adaptive mechanisms of the aductular parasitic lamprey and those of aductular non-feeding adults have indications on the differences between transient and late-stage cholestasis. These results may be considered to generate future strategies for dealing with patients that are in different stages of cholestasis. This dissertation fills in some knowledge gaps in our current understanding of biliary atresia, which still requires more research for therapeutic development and better patient management.

APPENDIX

Table 1. Comparison between human and sea lamprey biliary atresia.

	Human	Sea Lamprey
<i>Bile Formation</i>	Yes	Yes
<i>Cholestasis</i>	Yes	Yes
<i>Inflammation</i>	Yes	No
<i>Fibrosis</i>	Yes	No
<i>Cirrhosis</i>	Yes	No
<i>Liver Failure</i>	Yes	No
<i>Down-Regulation of CYP7A1 Transcript</i>	Yes	Yes
<i>Extrahepatic Excretion Sites of Bile Salts</i>	Kidneys	Intestine, Kidneys, and Gills

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Chapter 2:

Intestinal synthesis and secretion of bile salts as an adaptation to developmental biliary atresia in the sea lamprey

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Abstract

Bile salt synthesis is a specialized liver function in vertebrates. Bile salts play diverse roles in digestion and signaling, and their homeostasis is maintained by controlling input (biosynthesis) and intestinal conservation. Patients with biliary atresia (i.e., obliteration of the biliary tree) suffer liver fibrosis and cirrhosis. In contrast, sea lamprey thrives despite developmental biliary atresia. We discovered that the sea lamprey adapts to biliary atresia through a unique mechanism of *de novo* synthesis and secretion of bile salts in intestine after developmental biliary atresia, in addition to known mechanisms, such as the reduction of bile salt synthesis in liver. During and after developmental biliary atresia, expression of *cyp7a1* in intestine increased by more than 100-fold ($p < 0.001$), whereas in liver it decreased by the same magnitude ($p < 0.001$). Concurrently, bile salt pools changed in similar patterns and magnitudes in these two organs and the composition shifted from C24 bile alcohol sulfates to taurine-conjugated C24 bile acids. In addition, both *in vivo* and *ex vivo* experiments showed that aductular sea lamprey secreted taurocholic acid into its intestinal lumen. Our results indicate that

the sea lamprey, a jawless vertebrate, may be in an evolutionarily transitional state where bile salt synthesis occurs in both liver and intestine. Understanding the molecular basis of these mechanisms may shed light on the evolution of bile salt synthesis and possible therapy for infant biliary atresia.

Introduction

Bile salt synthesis is a unique and vital function of the liver for all vertebrates. Bile salts are excreted into the intestinal lumen, where they solubilize fatty acids in mixed micelles and thus facilitate the absorption of lipid-soluble vitamins (1). Recently, bile salts have been implicated in a wide array of signaling functions, from their own homeostasis to glucose metabolism and cardiovascular functions (2, 3), by interacting with nuclear and G-protein coupled receptors that regulate gene expressions (4-10). However, bile salts also have several pathologic effects, such as carcinogenicity and cellular toxicity (11). Through enterohepatic metabolism and circulation, vertebrates maintain bile salt homeostasis essential for physiological functions and detoxification (12-14).

Disruption of bile secretion into the intestine results in liver dysfunctions. Obstruction of bile ducts often causes cholestasis or disturbance of bile formation. Without clinical intervention, cholestasis results in liver toxicity, cirrhosis, and eventually liver failure. In infant biliary atresia, a rare disease in newborns, patients often die within the first few years without treatment (15). A striking contrast is found in lampreys, a group of extant jawless vertebrates that go through developmental biliary atresia during metamorphosis (16). The sea lamprey (*Petromyzon marinus*), the largest and most widely distributed lamprey species, loses the entire biliary tree when their larvae metamorphose into parasitic juveniles (17). The aductular juveniles feed ferociously and grow exponentially (up to 500-fold increase in body mass within two years) into fecund adults (FIGURE 1) without complications or liver failure that afflict patients with biliary atresia or other forms of cholestasis.

We reasoned that unique hepatic or extrahepatic mechanisms (or both) have evolved in sea lamprey to promote physiological functions yet minimizing cytotoxic effects of bile salts in an aductular life. In particular, we hypothesized that sea lamprey bile salts are mainly synthesized in different digestive organ at different life stage. Here we present evidence for *de novo* synthesis and secretion of bile salts in sea lamprey intestine.

Results and Discussion

Bile Salt Production During Sea Lamprey Developmental Biliary Atresia

Sea lamprey and human infant biliary atresia share similarities in cellular and histological morphology (18). The main event during biliary atresia is the apoptotic obliteration of bile ducts in both human (19) and sea lamprey (20). However, the cholestatic features commonly found in human liver with biliary atresia have not been documented in sea lamprey. We examined possible signs of cholestasis in sea lamprey liver during metamorphosis, which is divided into seven metamorphic stages, namely M1-M7 (21). We noticed that liver color altered from orange to green at stages M3 to M6, returned to orange when sea lamprey became trophic and parasitic, and changed again to green when adults reach final maturation (FIGURES. 1 and 2). Green liver is often caused by the accumulation of the bile pigment biliverdin, and is indicative of cholestasis in human biliary atresia and other cholestatic diseases (22). Therefore, sea lamprey seems to experience transient cholestasis during biliary atresia, but recover from this pathologic condition at the end of metamorphosis.

We further hypothesized that bile salt production in liver was reduced during and after developmental biliary atresia in sea lamprey. To test this hypothesis, we examined the bile salt

profile and mRNA expressions of *cyp7a1* and *cyp27a1*, which encode the initial enzymes in the classic and alternate pathways, respectively (23, 24). The *Cyp27a1* expression was not changed in *Cyp7a1* knockout mice (25). We found that hepatic *cyp7a1* mRNA and bile salt concentrations decreased dramatically during early developmental biliary atresia (FIGURE 3). As metamorphosis progressed, the gallbladder shrank at M2 (FIGURE 2), and liver *cyp7a1* mRNA level decreased by fivefold compared with M0 ($p = 0.01$) (FIGURE 3A). By M4, the gallbladder and most bile ducts disappeared (FIGURE 2 and FIGURE S1), and liver *cyp7a1* expression reached the lowest level, about 100-fold less than M0 ($p < 0.001$) (FIGURE 3A). *Cyp27a1* expression, although not as dramatic, showed similar patterns with a decrease of about 10-fold from M0 to newly transformed (T) in liver ($p < 0.001$) (FIGURE S3A). *Cyp7a1* expression levels in kidneys remained the same across all stages ($p > 0.05$) (FIGURE 3A). These results implied that both bile salt synthetic pathways were suppressed in liver during developmental biliary atresia.

Liver bile salt concentrations decreased in similar patterns as *cyp7a1* and *cyp27a1* expressions during metamorphosis (FIGURE 3). Sea lamprey is known to synthesize unique 5 α -bile acids and 5 α -bile alcohols: allocholic acid (ACA) (26) and its derivative 3-dehydro-ACA (3k-ACA) (27), petromyzonol sulfate (PZS) (28) and its derivative 3-dehydro-PZS (3k-PZS) (29), petromyzonsterol disulfate (PSDS) and petromyzonamine disulfate (PADS) (30). We analyzed their concentrations throughout life stages. C24 bile alcohols, PZS and 3k-PZS, have been found in nature only in lamprey species. Notably, 3k-PZS was one of the major bile salts in larval liver, and its concentration in liver decreased by more than 10,000-fold from M0 to T and remained low throughout the parasitic stage (FIGURE 3B). To our surprise, taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), and taurodeoxycholic acid (TDCA) were detected in

larval and metamorphic livers (Fig. 3C and D). TDCA had not been identified in fish or lamprey. Here its identification was based on the retention time of liquid chromatography and fragmentation patterns of tandem mass spectrometry (FIGURE S2). TCDCA concentrations did not vary among tissues and stages. These data demonstrate that hepatic production and concentrations of bile salts were minimized during developmental biliary atresia.

Cyp7a1 down regulation in sea lamprey biliary atresia is strikingly similar to the phenomenon in human with biliary atresia or other cholestatic diseases. In particular, *cyp7a1* transcripts decreased by more than 99% in sea lamprey (FIGURE 3A), similar to the decrease seen in human biliary atresia (31, 32). However, *cyp7a1* down regulation does not prevent cholestatic injuries to the liver in many mammalian models and human biliary atresia.

***De Novo* Synthesis of Bile Salts in Sea Lamprey Intestine**

After developmental biliary atresia, sea lamprey no longer maintains direct bile flow from the liver to intestine. Secretion into the systemic circulation is the only alternative route to transport hepatic bile salts. Although bile salts are often sulfated before urinary excretion in mammals with biliary atresia and other forms of cholestasis (33), renal excretion of bile salts in parasitic sea lamprey is highly unlikely because it must minimize or stop urine production to retain water in the hyperosmotic ocean. We found that bile salt production in liver remained minimal during parasitic stage (Figs.1 and 3A and B), prompting the question whether extrahepatic bile salt synthetic mechanisms exist in parasitic sea lamprey.

We focused our search on the intestine, where bile salts exert their digestive function. We determined bile salt profiles and *cyp7a1* and *cyp27a1* mRNA levels in the intestines of larvae

before, during and after biliary atresia, and of feeding parasites. Interestingly, the intestine of parasites showed increase in taurine-conjugated bile salts (FIGURE 3C and D). Intestinal *cyp7a1* expression increased more than 100-fold from pre- (M0, M2 and M4) to postdevelopmental biliary atresia stages [T, small parasite (SP), and large parasite (LP)] (FIGURE 3A). The increase in *cyp7a1* expression also correlated with the increase in TCA and TDCA in the intestine of parasites (FIGURE 3A, C and D). In contrast, *cyp27a1* mRNA levels among M0, T and SP were not different ($p > 0.05$), and increased by two- to fivefold from M2 and M4 to T (FIGURE S3A). In addition, bile salt synthetic intermediates (7α -hydroxycholesterol, 7α -hydroxy-4-cholesten-3-one, 7α -hydroxy- 5β -cholestan-3-one, 5β -cholestane- 3α , 7α , 12α , 27α -tetrol, and 3α , 7α , 12α -trihydroxy- 5β -cholestanoic acid) were also detected in the intestine of parasites (Table S1). Furthermore, the slower enterocyte turnover rate (9-14 days) in parasitic sea lamprey (34) compared to that of mammals (2-3 days) may favor bile salt synthesis in intestine. These data indicate that bile salt *de novo* synthesis occurs in the intestine of parasitic sea lamprey.

We also examined whether the increase in intestinal bile salt pool is corroborated with *de novo* synthesis of cholesterol in intestine. We measured mRNA concentrations of *HMG-CoA reductase*, the rate-limiting enzyme in cholesterol biosynthetic pathway. Although the decrease in *HMG-CoA reductase* mRNA concentrations corresponded to the decrease in *cyp7a1* and *cyp27a1* mRNA concentrations from M0 to SP and LP in liver (FIGURE 3A and FIGURE S3A and B), no difference was found in intestine from M0 to LP ($p > 0.05$) (FIGURE S3B). The cholesterol precursor for bile salt synthesis in intestine may be from either the *de novo* synthesis in tissues or diet, or both. Notably, in the non-trophic adults, their hepatic or intestinal (or both) *HMG-CoA reductase* mRNA levels increased dramatically ($p < 0.05$) (FIGURE S3B). This increase in spermiating males may facilitate the synthesis of a bile acid pheromone with

secretion rate about 0.5 mg per hour (35). Interestingly, lipolysis and fatty acid synthesis in liver and intestine vary significantly among life stages (36). Further examination of cholesterol homeostasis may elucidate the adaptive value of bile salt *de novo* synthesis in lamprey intestine.

Bile salt *de novo* synthesis had been recognized as a crucial function limited to the liver, a specialized digestive organ in vertebrates although some hepato-pancreas- like functions have been found in invertebrates such as crabs (37). In two invertebrate species, *Sollasella moretonensis* (38) and *Ciona intestinalis* (39), where intestine is the most developed digestive organ, bile salt-like compounds have been discovered. *C. intestinalis* does not have a liver but contains a *cyp7a1* ortholog (E-values of 1E-64 and 2E-64 compared with mice and human sequences, respectively, in a tBLASTn search; Basic Local Alignment Search Tool, National Center for Biotechnology Information). The notion that evolution of bile salt synthesis may have preceded that of liver is similar to the example where evolution of insulin is believed to have preceded that of the pancreas (40-42). The *de novo* bile salt synthesis in sea lamprey intestine suggests that bile salt synthesis is a function less specialized in jawless vertebrates compared to jawed vertebrates. Sea lamprey is one of the most basal vertebrates to contain both intestine and liver, both of which support bile salt synthesis. It seems that the site of bile salt synthesis has shifted from the digestive tract to liver as the digestive system became more complex during animal evolution.

Another surprising, but likely adaptive, change in lamprey bile salt synthesis after metamorphosis is the shift in bile salt conjugation. Larval and adult lamprey are known to produce bile alcohol sulfates (26, 28, 43). Sulfation renders bile alcohols soluble, and enables their secretion. Sulfation of taurine-conjugated bile acids is considered an adaptive detoxification mechanism (44). In larval and adult lamprey, bile alcohol sulfates are released into water in large

quantities as putative pheromones (29, 30) and sulfation facilitates this process (29). In contrast to the dominance of bile alcohol sulfates in larvae and mature adults, taurine-conjugated bile salts were prominent in parasites (FIGURE 3C and D, and FIGURE S4). These taurine-conjugated bile salts may facilitate lipid digestion in the absence of a biliary tree. Alternatively, synthesis of these bile salts may simply serve to eliminate cholesterol. The dramatic changes in the location and the type of bile salt synthesis throughout sea lamprey life history represent a striking example for the adaptation of bile salts to specific functions in particular life stages.

Intestinal Secretion of Taurine-Conjugated Bile Salts

Because TCA was the most abundant bile salt (FIGURE 3C) and *cyp7a1* mRNA level was high (FIGURE 3A) in the intestine of parasites, we predicted that TCA was secreted through the intestine. To test this hypothesis, we designed both *in vivo* and *ex vivo* transport assays to measure [^3H]TCA transport in the intestine. *In vivo* tissue distribution of radioactivity after intravenous injection of [^3H]TCA showed that intestinal lumen content had 10-times higher tritium concentrations than the plasma in parasites ($p < 0.001$) after a 12-hr incubation (FIGURE 4A). Total tritium activity in intestine was at least two-times higher than in plasma (FIGURE 4B). Most tritium activity was trapped in the mixture of muscle and fat because of their mass. A minimal amount of tritium was found in kidneys and ureter washings, indicating that kidneys were neither the major storage nor excretion organ for TCA in parasitic lamprey. Whether this is the case in adults when they stop feeding and start their spawning migration remains to be determined.

To further elucidate the dynamics of TCA secretion by intestine, we examined TCA transport by *ex vivo* intestinal sacs over a 24-h time course (FIGURE 5). The intestinal tissue did not degenerate after 24-h incubation (FIGURE S5) and the tritium counts from lumen solution were contributed by [^3H]TCA (FIGURE 5B). Intestinal transport of [^3H]TCA was much higher in secretion than in absorption ($p = 0.0001$) (FIGURE 6), indicating that TCA transport is a directional and active process. A three-way ANOVA showed no interaction among intestinal section, intestinal orientation, and the sex of animals. The sac orientation is the only factor that affected the transport activity ($p = 0.0002$, power = 0.988). Taken together, our data implicate an active transport mechanism in the intestine that secretes taurine-conjugated bile salts into the lumen in parasitic sea lamprey. This mechanism is distinct from the enterohepatic circulation where bile salts are reabsorbed from the intestinal lumen to circulation in mammals.

In patients with biliary atresia, bile pigments are not delivered to the intestine, resulting in pale or white stool and green liver. In contrast, intestinal secretion of bile pigments may exist in metamorphic and parasitic sea lamprey where bile salt synthesis occurred. Lamprey intestinal color altered from pink to green during metamorphosis (M2-M6 in FIGURE 2). Biliverdin, a bile pigment (from hemoglobin) responsible for this color change, was highly concentrated in intestine at M6, the last stage of metamorphosis. The concentration of biliverdin in intestine at M6 was about 10,000-times higher than M2 ($478 \pm 109 \mu\text{g/g}$ in M6, $51.3 \pm 18.7 \text{ ng/g}$ in M2). Biliverdin was also concentrated in the mucosal content collected from the intestine at M6. By M4 (FIGURE S1), most bile ducts were obliterated and no longer available for bile flow from liver to intestine. Although the liver began to turn green at M3, a sign of biliverdin accumulation, the intestine had a delayed color change to green at M5, and this phenomenon lasted until M6 in

both organs (FIGURE 2). The corresponding high concentrations of biliverdin ($478 \pm 109 \mu\text{g/g}$) and 3k-PZS ($6.65 \pm 4.01 \mu\text{g/g}$) in the intestine at M6 indicated an alternative secretory route for bile products, and most likely occurred through systemic circulation during non-trophic metamorphosis. Contrary to the absence of bile pigments or bile salts in the intestine of patients with biliary atresia, both bile products were secreted into sea lamprey intestine after developmental biliary atresia.

In contrast to the increase in liver bile salts in patients with biliary atresia (31), bile salt levels in sea lamprey liver decreased and intestinal bile salt levels remained the same during developmental biliary atresia (FIGURE 3). The disappearance of tight junctions (45) during and after metamorphosis in sea lamprey liver may promote regurgitation into sinusoids followed by secretion through the intestinal epithelium. The clearance of 3k-PZS and biliverdin in intestine was not apparent until post biliary atresia in T (Figs. 2 and FIGURE 3B). The alternative excretion through kidneys, adopted by mammals under cholestatic conditions (46), may be less important in sea lamprey as their kidneys completely degenerate and new kidneys regenerate during metamorphosis (17). Taken together, our data show that intestinal secretion of bile salts in sea lamprey is a unique pathway that is likely evolutionarily adaptive, and differs from the pathologically adaptive mechanisms in human biliary atresia.

During evolution, natural selection or genetic drift has resulted in mutant species with phenotypes that mimic human diseases, but are nevertheless adapted to specific environments (47). Sea lamprey biliary atresia is a developmental process that resembles human pathogenic biliary atresia in many cellular features. Aductular parasites secrete bile salts during their rapid growth but slow-growing larvae and atrophic mature adults utilize bile alcohol sulfates as pheromones (29, 30, 48). The complex life style and bile salt utilization are facilitated by two

organs, the liver and the intestine, synthesizing different conjugated bile salts throughout sea lamprey life cycle.

In summary, our data suggest that sea lamprey has evolved adaptive mechanisms to survive developmental biliary atresia through minimizing bile salt synthesis in liver and relocating bile salt synthesis and secretion to the intestine, but other cholestatic models and humans with biliary atresia suffer from cholestatic liver injuries. Further investigations of the molecular mechanisms underlying these observations may lead to therapies for human biliary atresia.

Materials and Methods

Animals and Tissue Collection

Experimental procedures were approved by the Michigan State University (MSU) Institutional Animal Use and Care Committee. Animals were held at the US Geological Survey Hammond Bay Biological Station (Millersburg, MI) until shipping to MSU or sampling. Parasites were used in whole animal perfusion of [H^3]taurocholic acid and isolated intestine experiments within 2 months after detached from hosts. Large larvae (>12 cm) were screened in 2009-2011 to obtain metamorphic animals (21). Randomly selected metamorphic livers were fixed in 4% paraformaldehyde (PFA) for histological examination (FIGURE S1). Mature adults were collected during upstream spawning migration by personnel of US Fish and Wildlife Service Marquette Biological Station and Canada Department of Fisheries and Ocean Sea Lamprey Control Center, Sault Ste. Marie, Ontario. Actively feeding parasites were collected from fishermen by US Geological Survey personnel, immediately transported to MSU and held at 5-8

°C until sampling. Larvae were obtained by Hammond Bay Biological Station (Millersburg, MI) staff and kept in tanks with sand and flow-through lake water. Newly transformed (T) animals were caught during downstream migration before extensive feeding started. Animals were euthanized with 0.1% MS-222 (Sigma; St. Louis, MO, USA). Tissues were fixed in 4% PFA or snap frozen in liquid nitrogen. Frozen tissues were stored at -80°C until use. Fixed tissues were processed by staff in the Investigative Histopathology Laboratory at MSU.

Real-time Quantitative PCR (QRT-PCR)

Real-time quantitative PCR was performed using the TaqMan MGB system (Applied Biosystems) as described previously (49). Synthetic oligos were used as standards. The amplicon sequence of *cyp7a1* was chosen based on a 771 bp fragment PCR amplified from total cDNAs of sea lamprey tissues. This fragment contained a reading frame of 257 amino acid residues and was verified by tBLASTn (National Center for Biotechnology Information) with an E value of 1E-97 compared with human *CYP7A1*. Full-length sequences of *cyp27a1* and *HMG-CoA reductase* were obtained from Sea Lamprey Draft Genome 6.0, and both sequences had E values < 1E-150 compared with multiple species including *Xenopus laevis*, *Homo sapiens*, and *Mus musculus*. Sequence information used for real-time quantitative PCR of *cyp7a1*, *cyp27a1*, and *HMG-CoA reductase* is listed in Table S2.

Bile Salt Distribution Measured by Liquid LC-MS/MS

Frozen tissues were homogenized in 75% (vol/vol) ethanol containing [$^2\text{H}_5$]3k-PZS and [$^2\text{H}_4$]TCA as internal standards and centrifuged at 13,000 x g for 10 min. Supernatants were freeze-dried and reconstituted in 50% methanol in water (vol/vol) and subjected to LC-MS/MS. Mass spectra were acquired using electrospray ionization in negative or positive (ES+ or ES-) ion mode with multiple reaction monitoring. Data were acquired with MassLynx 4.1 and calibrated and quantified with Quanlynx. Conditions for LC-MS/MS are listed in Table S3.

***In Vivo* Bile Acid Uptake and Distribution**

Parasites were injected with 25 μCi of [^3H]TCA in 400 μL PBS via caudal vein and placed in a bisected aquarium (35) filled with aerated water. About 12 h after the injection, animals were anesthetized with 0.02% MS-222 for blood drawing, and then euthanized with 0.1% MS-222. The ureter was cannulated and washed with PBS to collect washings. Intestinal content was collected directly after opening the abdomen. Liver, intestine, kidneys, gills, and muscle were collected and weighed. Mass of muscle with fat was estimated by total body mass after subtractions of major organs described above. Blood was centrifuged at 1000 x g with EDTA for 10 min to obtain plasma. All tissues were homogenized in PBS, and then added with triton X-100 (1%), incubated at room temperature for 20 min, and centrifuged at 13,000 x g for 10 min. Tritium activity of the supernatant was measured as disintegrations per minute (DPM). Amount of [^3H]TCA was calculated from DPM detected based on a standard curve. Tritium level was normalized to tissue mass or collected volume.

Whole Intestinal Sac Time Course

Whole intestine was isolated from parasitic lamprey and sutured into a sac containing 10 mL of lamprey's Ringer solution (130 mM NaCl, 2.1 mM KCl, 1.8 mM MgCl₂, 4 mM HEPES, 4 mM Dextrose, 1 mM NaHCO₃, 2.6 mM CaCl₂). Sacs were incubated in 700 ml aerated ice cold Ringer containing 20 µCi of [³H]TCA in ice-water bath (0-4°C). At 30 min, 1, 2, 4, 8, 12, and 24 h, about 100 µL of lumen fluid was collected by a syringe. The incubation solution was also measured simultaneously and the tritium count decreased by less than 10% at 24 h. Intestine was fixed in 4% PFA, examined by H&E staining, and no morphological difference was found from intestinal tissues fixed directly from euthanized parasites.

Preparative Thin-Layer Chromatography Analysis

Intestinal solution recovered from lumen and incubation solution were freeze-dried and reconstituted in methanol. Samples were loaded on a preparative thin-layer chromatography (PTLC) plate (20×20 cm; Analtech) along with unlabeled TCA. PTLC plate was developed in 2:1 of CHCl₃/MeOH. Lanes loaded with TCA were sprayed with 5% sulfuric acid and heated to 110 °C to develop a blue color on TCA. Sample lanes were divided into 15 individual zones and each zone was scraped separately into MeOH. All scraped samples were counted by scintillation. Each measurement was normalized to the total recovered count of the corresponding lane.

Regular and Everted Intestinal Sac Transport Assay

The intestine of each parasite was isolated and cut into three sections (proximal, middle, and distal) and placed in aerated sea lamprey Ringer's solution at 4°C. Each intestinal section was cut into halves and randomly sutured into a regular and an everted sac. One segment of each section was everted with a p-1000 pipette tip. Each intestinal sac was injected with 1-2 ml aerated cold lamprey Ringer's solution. Sacs from each animal were incubated in 500 mL aerated Ringer's solution containing 15 μCi of $[\text{H}^3]\text{TCA}$ at 4°C for 4 h. The initial background $[\text{H}^3]$ reading of intestinal sacs was ~40 DPM. The solution in each intestinal sac was collected by a syringe and its H^3 activity was counted after 4 h. The effects of intestinal section (proximal, middle, and distal), intestinal sac orientation, and the sex of animals on DPM were analyzed with a three-way ANOVA. To determine the effect of sac orientation when no effect of other two factors was found, a paired t-test was used to compare DPM between the regular and the everted sacs within each section of each animal.

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APPENDIX

FIGURE 1. Sea lamprey life history characterized by a dramatic metamorphosis and subsequent migration between fresh and oceanic waters. Sea lamprey larvae filter feed in freshwater until metamorphosis. Biliary atresia occurs during metamorphosis. The postmetamorphic juveniles migrate downstream to the ocean or the Great Lakes (if landlocked) to feed on fish until they reach reproductive stage. The non-trophic adults migrate back to freshwater to spawn.

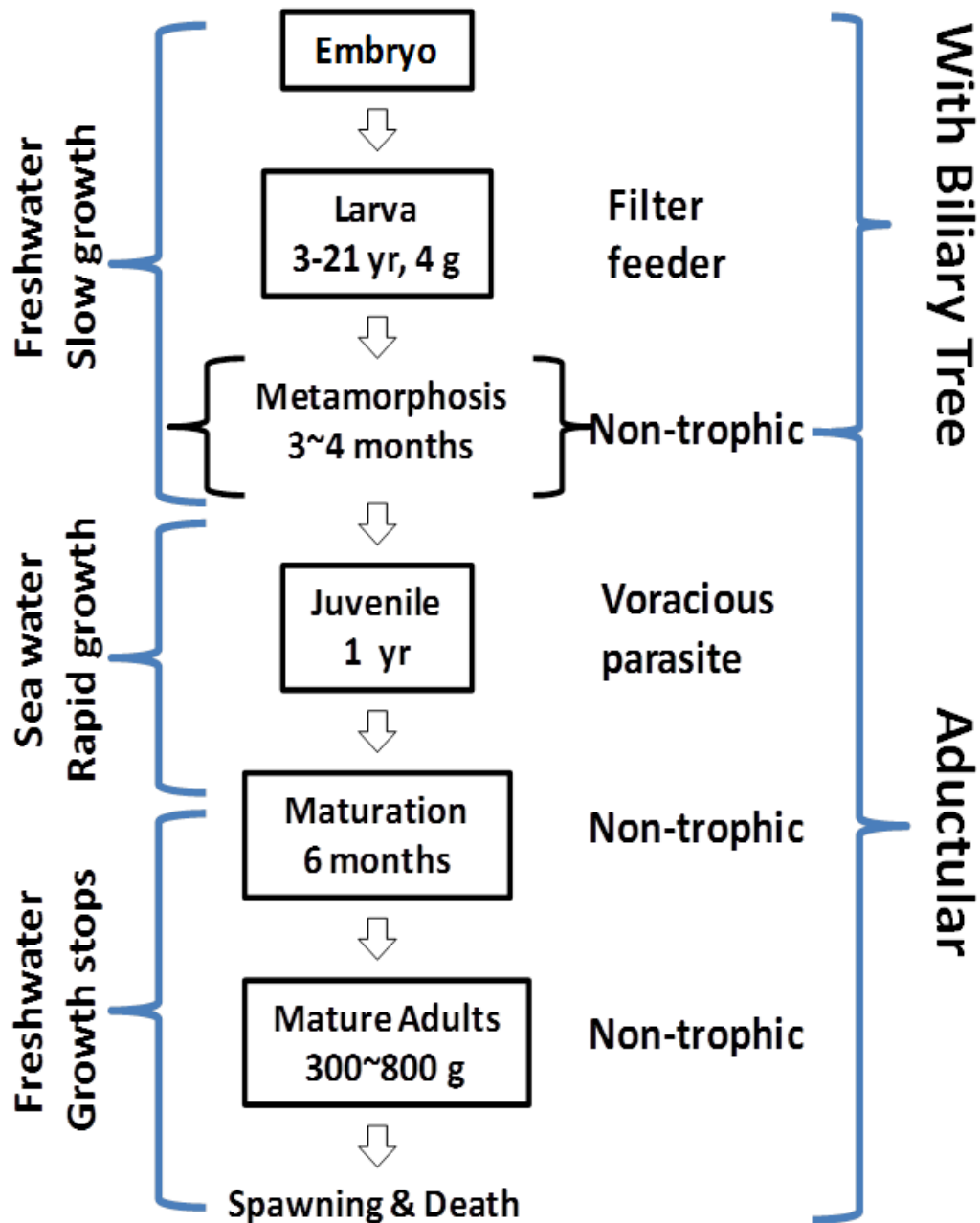


FIGURE 2. Sea lamprey liver and intestine in selected life stages. Selected metamorphic stages (M2, M3, M5, and M6) are shown with larval (M0), newly transformed (T), large parasite (LP), and adult stages. Liver in M5 is not shown in full-size for the purpose of presenting more proportion of the intestine. L: liver; I: intestine. Black arrows indicate the gallbladder in M0 and M2. (Scale bar, 1 cm.)

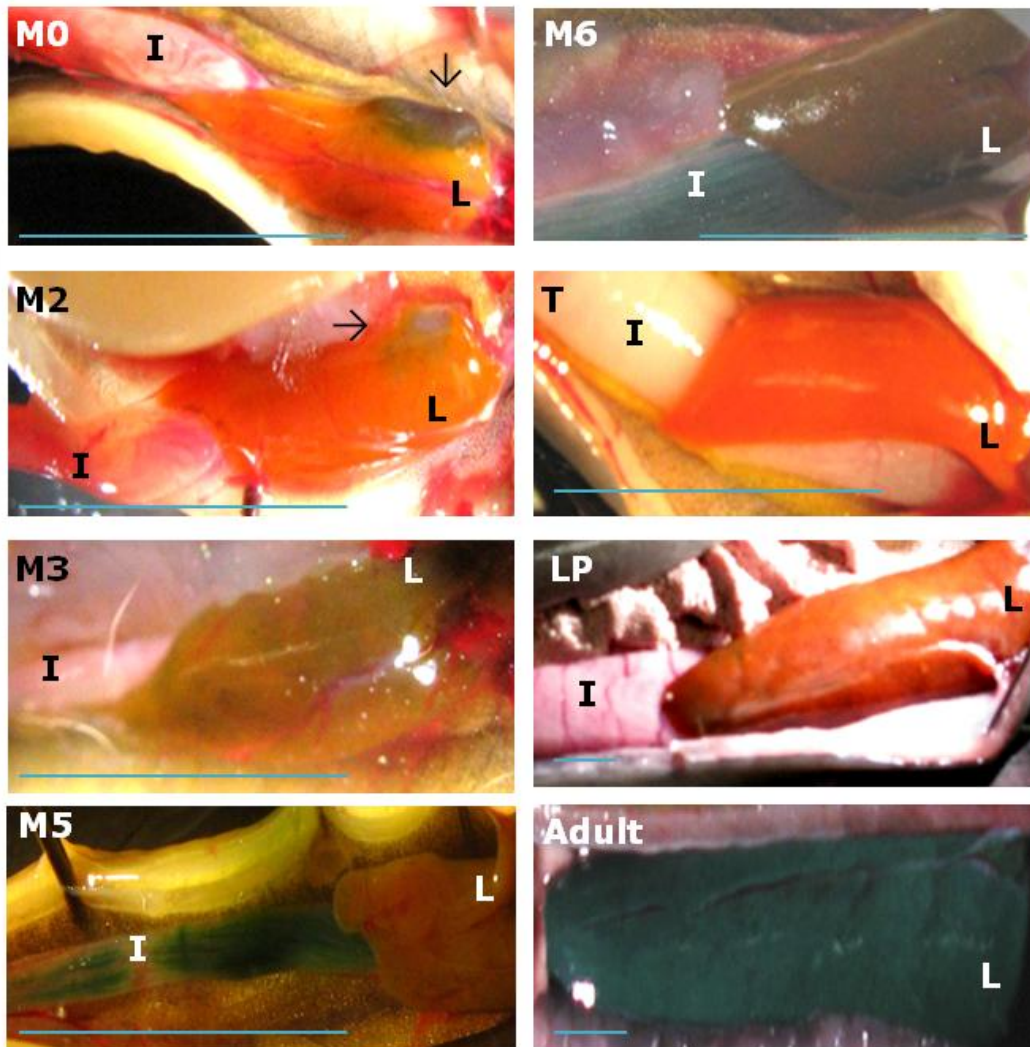


FIGURE 3. Bile salt and *cyp7a1* in sea lamprey liver and intestine during and after biliary atresia. Concentrations of (A) *cyp7a1* mRNA, (B) 3k-PZS, (C) TCA, (D) TDCA. M0, larval; M2-M4, metamorphic stages 2-4; T, newly transformed; SP, small parasite; LP, large parasite; SM, spermiating male; OF, ovulatory female stages. The y axes are in log scale. Solid bars: liver; open bars: intestine; gray bars: kidney. Vertical lines represent mean \pm 1 SEM ($n \geq 3$ for all datapoints). Two-way ANOVA showed that both stage and tissue type had effects in A, B, and D ($p < 0.001$), and an interaction between stage and tissue type in A ($p < 0.001$), B ($p < 0.001$), C ($p = 0.017$), and D ($p < 0.001$); Stage, but not tissue type, had effects in C ($p < 0.001$). Within each panel, bars with same lower case labels indicate $p > 0.05$.

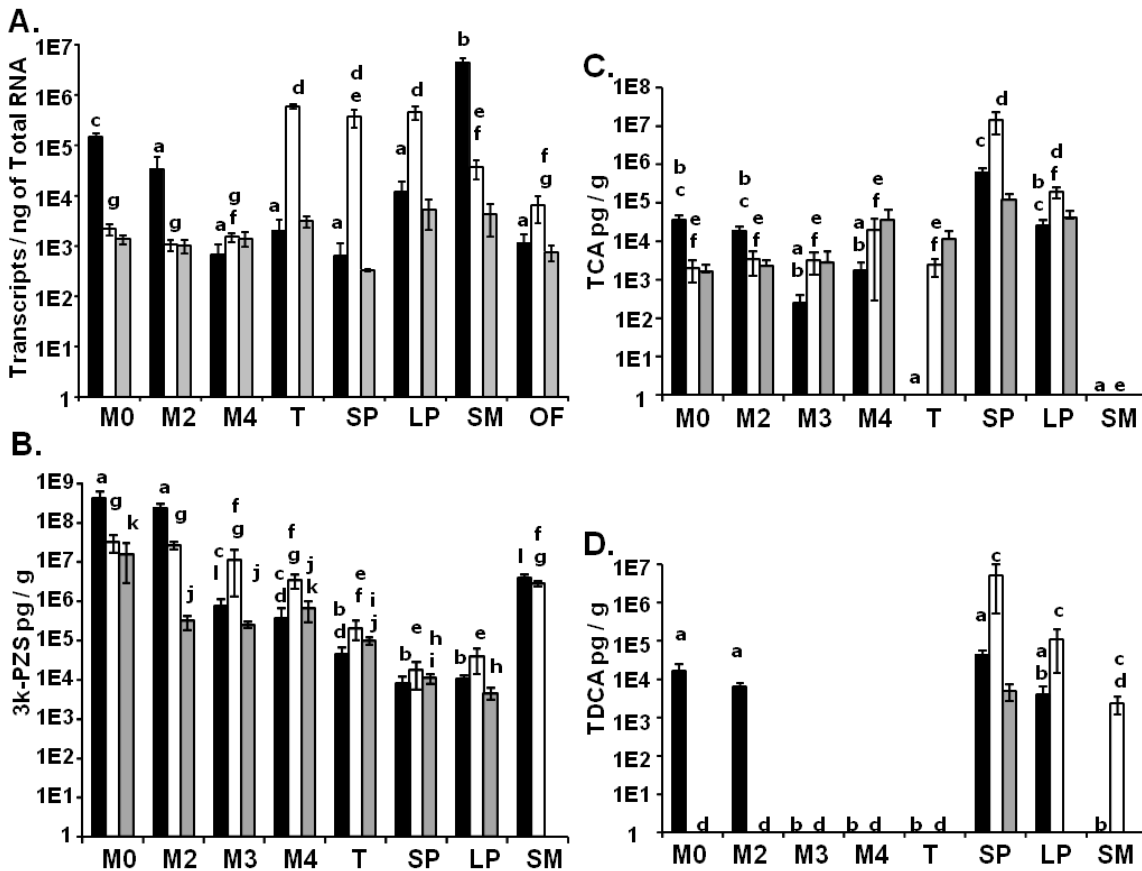


FIGURE 4. *In vivo* whole animal perfusion of [H^3]TCA showing intestine as a major bile salt secretory organ. Animals used in this experiment were actively feeding parasites. (A) A distribution of tritium concentration in each tissue at 12 h after intravenous injection of [H^3]TCA. (B) A percent distribution of the total recovered [H^3]TCA in each tissue. **P* value < 0.001 compared with any other tissue. Dist., distal ; Prox., proximal. Vertical bars represent mean + 1 SEM.

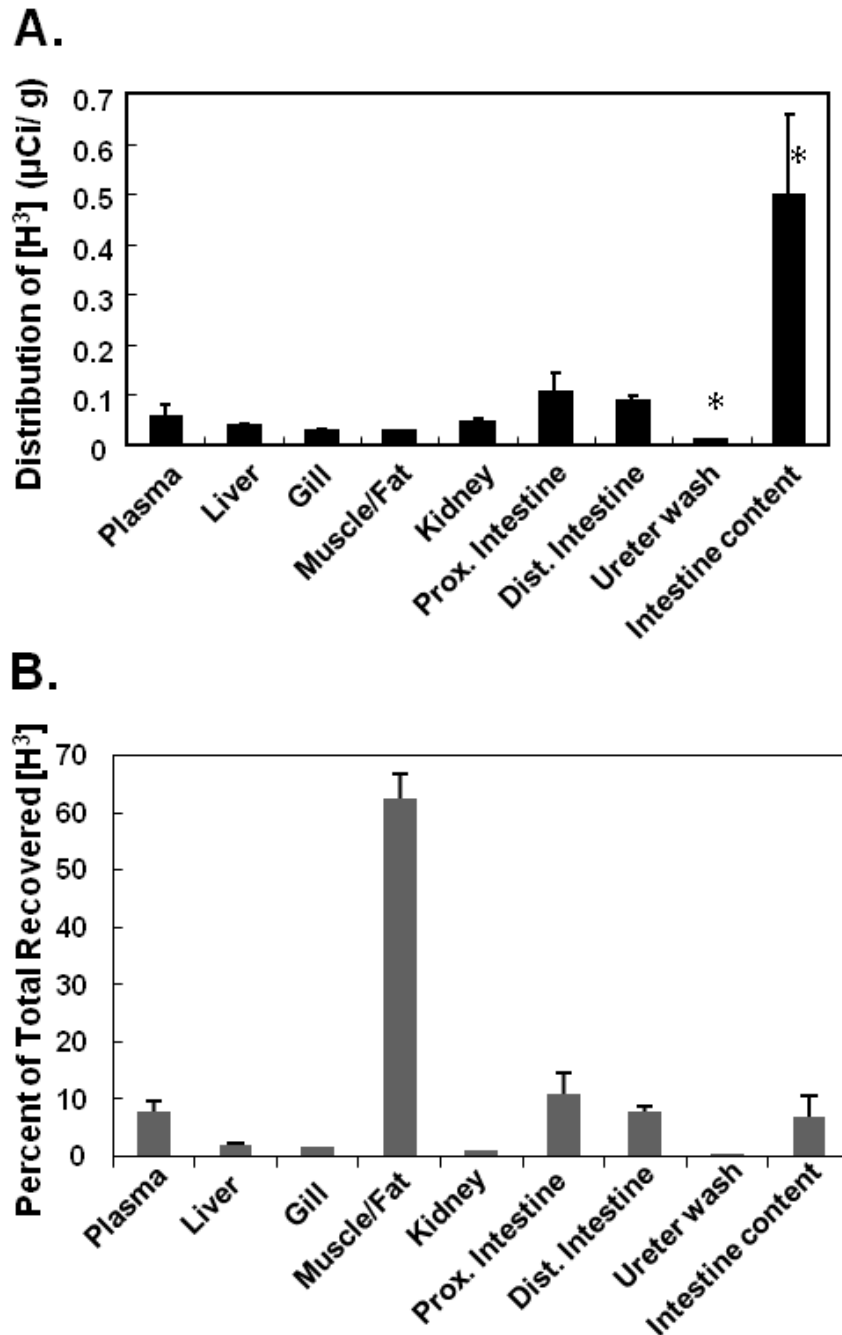


FIGURE 5. *Ex vivo* time course of TCA transport assay in whole intestinal sac. (A) A 24-h time course of parasitic intestinal sacs incubated in $[H^3]$ TCA in Ringer's solution; $n = 4$; vertical lines indicate mean ± 1 SEM. (B) PTLC separation and identification of TCA. A PTLC plate was loaded with samples (upper); TCA, TCA loaded; 5% sulfuric acid solution was used to detect TCA (blue staining); lumen, extracts of intestinal contents from lumen after incubation; incubation solution containing sea lamprey Ringer's solution and $[H^3]$ TCA was loaded in parallel with lumen sample; both lumen sample and incubation solution were scraped into MeOH based on grids 1-15 for scintillation counting; diamonds show counts from intestinal lumen solution after incubation in $[H^3]$ TCA Ringer's solution; squares show counts from incubation solution.

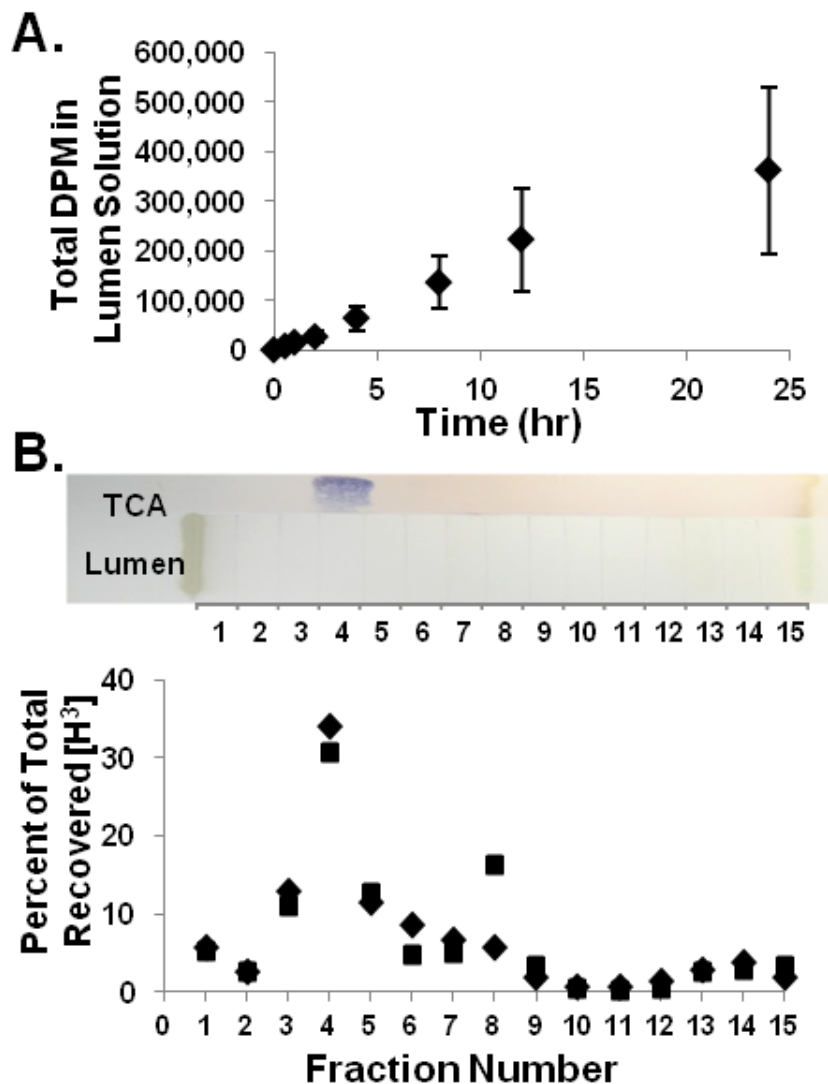


FIGURE 6. Serosal to mucosal transport of $[H^3]$ TCA in the intestinal sac. Both regular and everted sacs from three (proximal, middle, and distal) sections of the intestine of each lamprey (six sacs per animal). All six sacs were incubated in sea lamprey Ringer's solution containing $[H^3]$ TCA in an ice-water bath for 4 h. Total DPM was reported after normalized to the surface area of the connective tissue. Closed bars: regular sac readings represent $[H^3]$ TCA transport from serosa to mucosa. Open bars: everted sac readings represent $[H^3]$ TCA transport from mucosa to serosa. A three-way ANOVA shows that only the sac orientation had an effect on DPM ($p = 0.0002$, power = 0.988) but not the sex or the sections of the intestine. A one-tailed paired t-test showed that regular sacs had greater effect on DPM than everted sacs ($p = 0.0001$). Vertical lines represent mean ± 1 SEM ($n = 12$, seven females & five males).

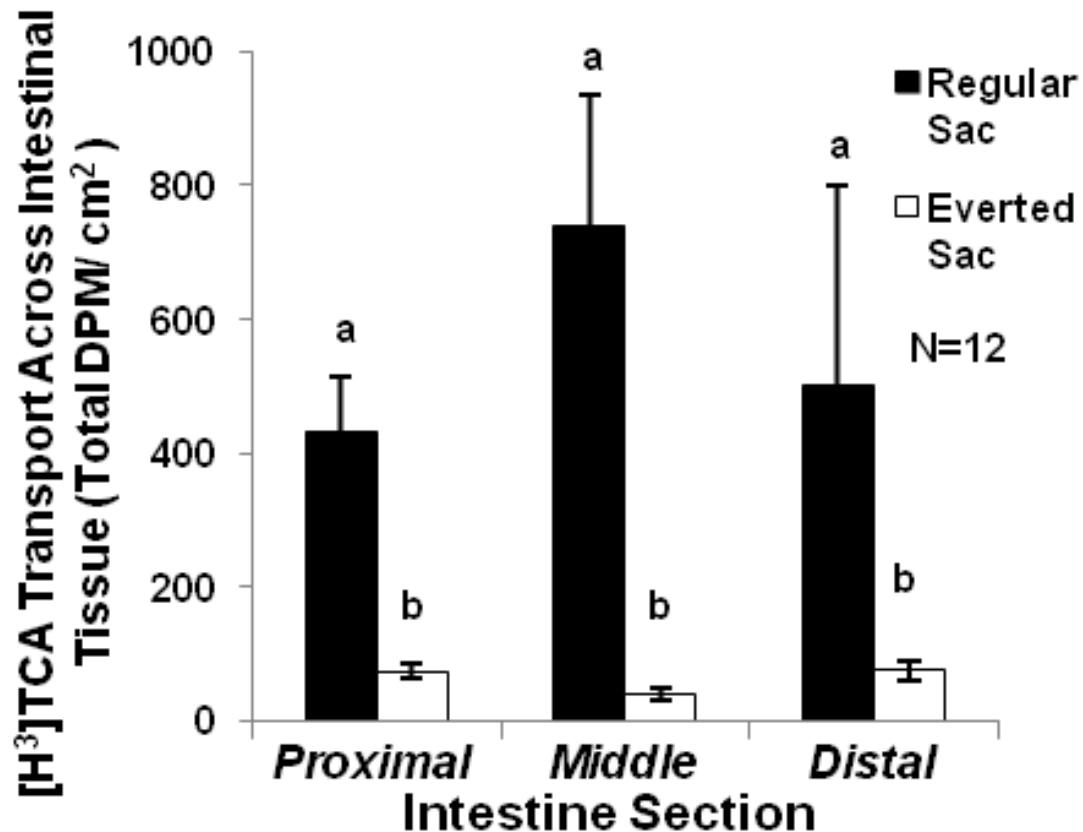


FIGURE S1. Biliary atresia in metamorphic livers. H&E staining on paraffined liver sections. Selected stages of metamorphosis (M1-M4) are shown along with larval stage (M0), and newly transformed stage (T). Red arrows point to hepatocytes. Green arrows point to cholangiocytes.

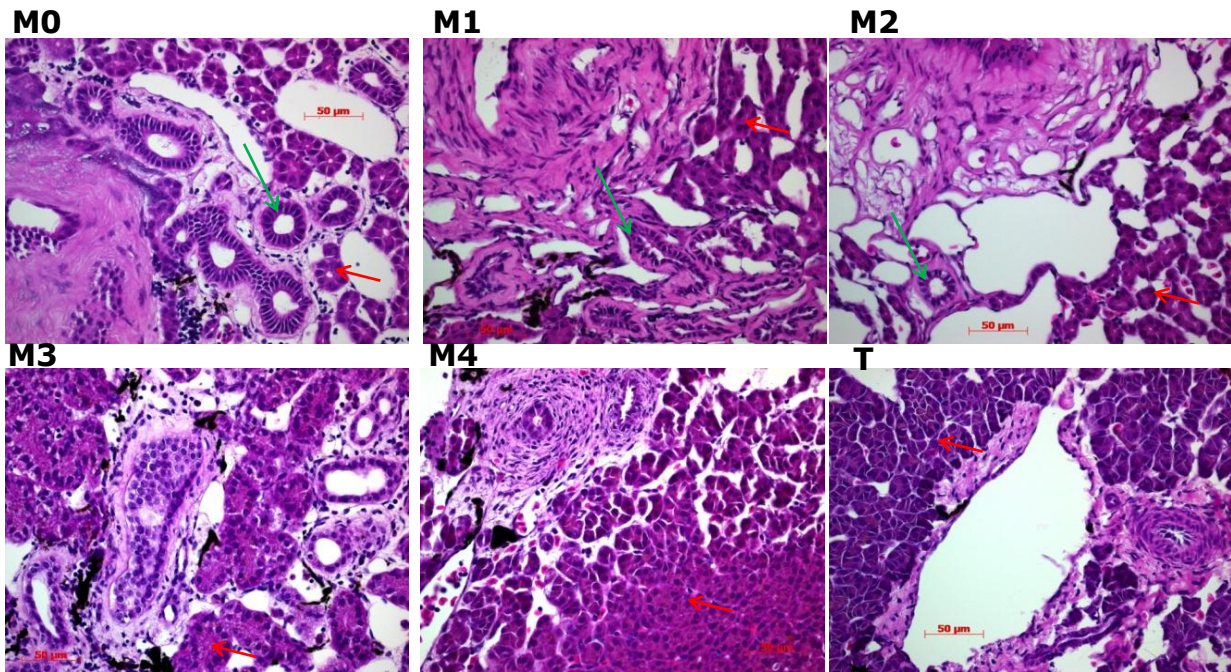


FIGURE S2. Representative LC-MS/MS chromatograms of taurodeoxycholic acid (TDCA).

A: sample from sea lamprey liver at SP stage, B: spiked standard solution containing TDCA. The best response of TDCA was observed in negative ESI MRM mode by monitoring the reaction m/z 498.44>123.85. The MRM transition as well as the cone voltage and collision energy voltage applied to the determination were displayed in Table S3. The peak observed in A has retention time comparable to the peak of standard in B. C: ion mass spectrum of TDCA separated from A. The product ion mass spectrum obtained showed parent/ daughter ion pattern that confirmed the compound separated from A is TDCA.

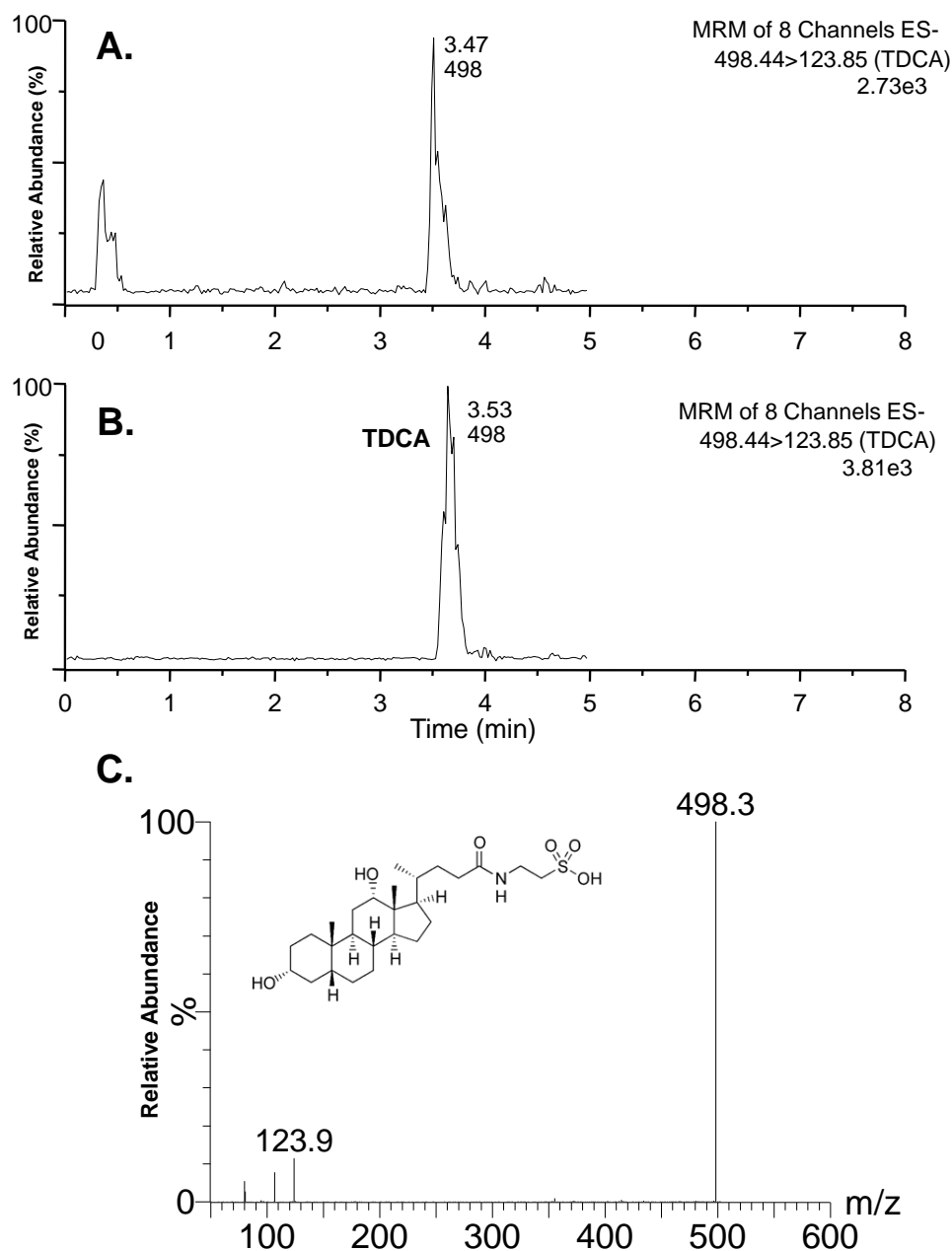


FIGURE S3. Expression level of *cyp27a1* and *HMG-CoA reductase* in sea lamprey tissues during and after biliary atresia. A: *cyp27a1* mRNA, y axis is in logarithmic scale, B: *HMG-CoA reductase* mRNA, y axis is in linear scale. M0: larval, M2-M4: metamorphic stages 2 to 4, T: newly transformed, SP: small parasite, LP: large parasite, SM: spermiating male, OF: ovulatory female stages. Solid bars: liver; open bars: intestine; gray bars: kidney. Vertical lines represent mean \pm 1 standard error ($n \geq 3$ for all data points). Within each panel, bars with same lower case labels indicate $p > 0.05$. In B, ANOVA showed $p > 0.05$ for kidney mRNA levels.

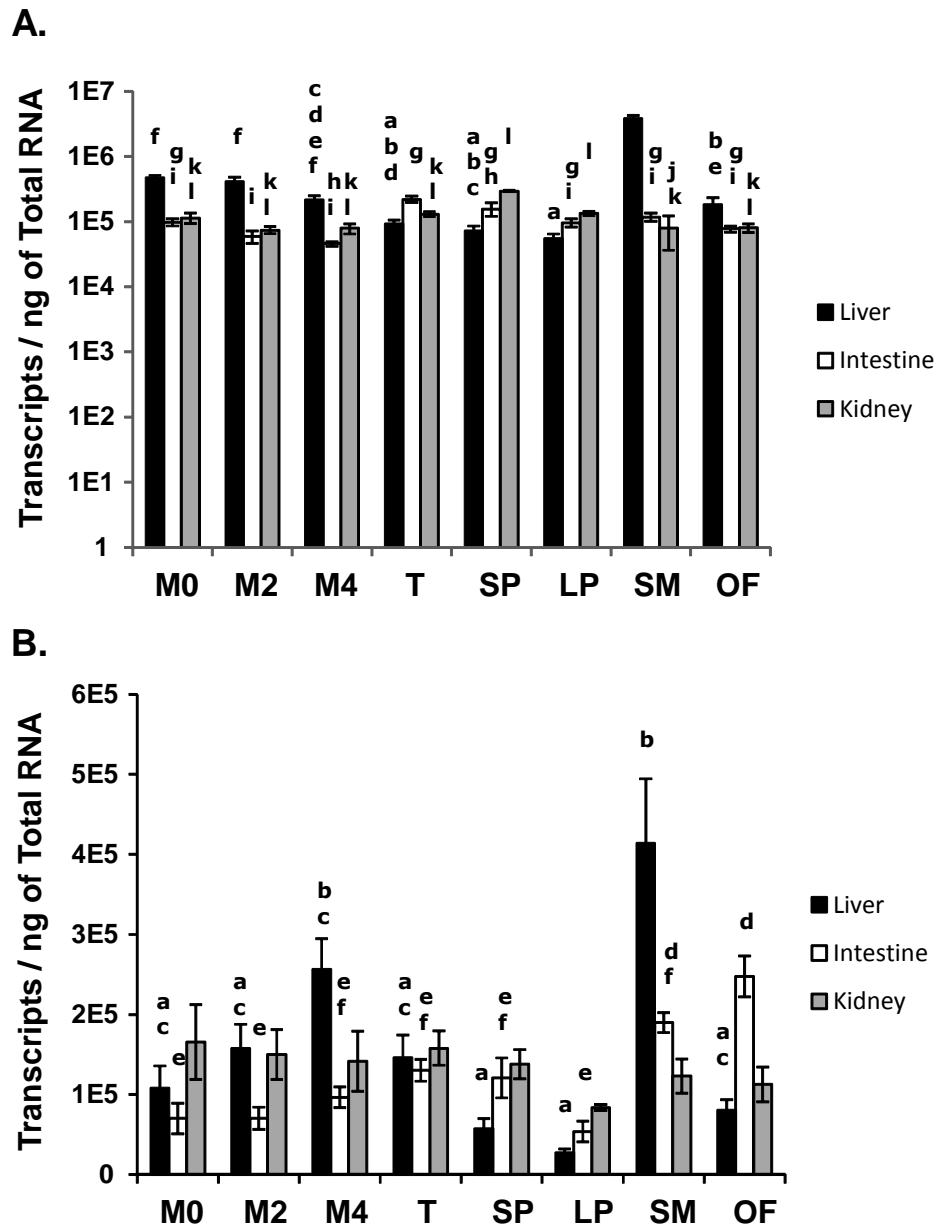


FIGURE S4. Relative bile salt composition in sea lamprey liver and intestine during and after biliary atresia. Relative composition of the four bile salts is presented to show shifts of bile salt types through life stages. A: Liver, B: Intestine. 3-dehydro- petromyzonol sulfate (3k-PZS), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA). M0: larval, M2-M4: metamorphic stages 2 to 4, T: newly transformed, SP: small parasite, LP: large parasite, SM: spermiating male stages.

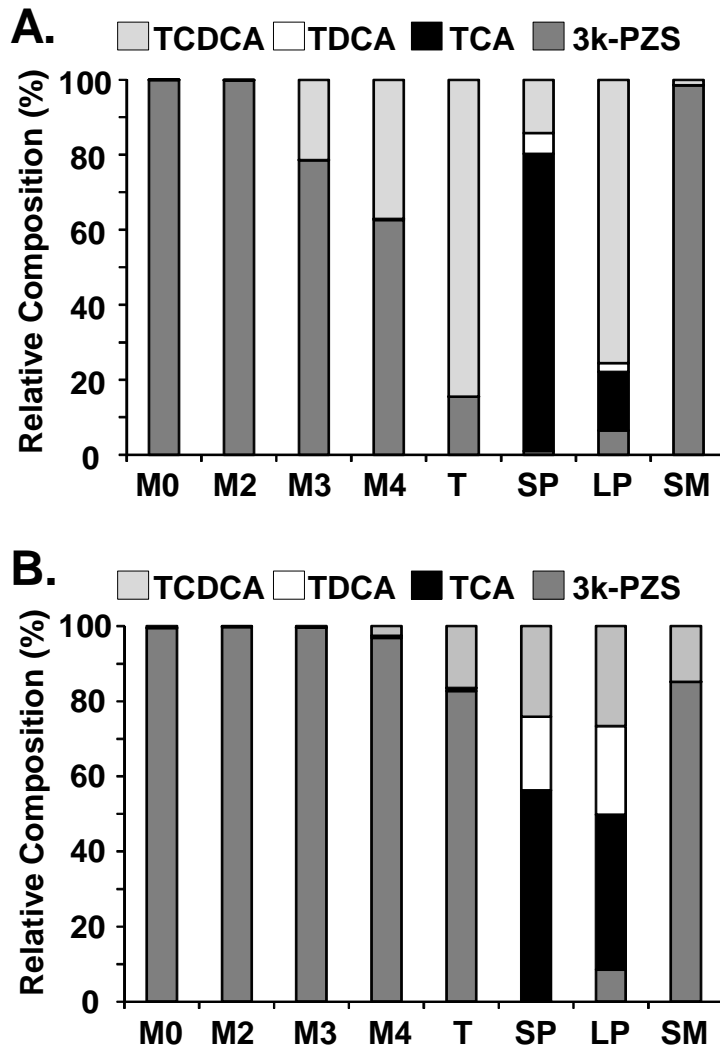
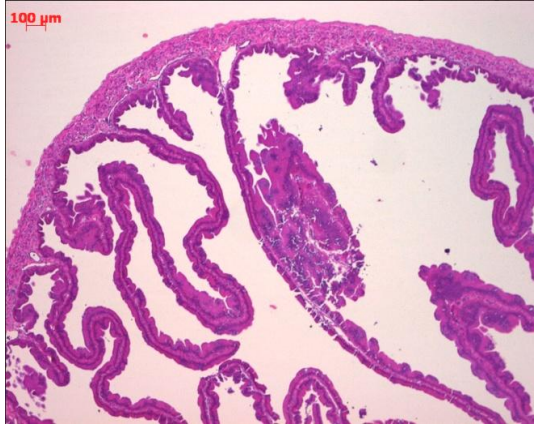


FIGURE S5. H&E of Intestine tissue after *ex vivo* incubation. Intestinal tissue was fixed in 4% PFA after 24 hours of incubation in whole intestine sac experiment. A, C, & E: 50X magnification of proximal, middle, and distal segment, respectively; B, D, & F: 400X magnification of proximal, middle, and distal segment, respectively.

A. Proximal Intestine (50X)



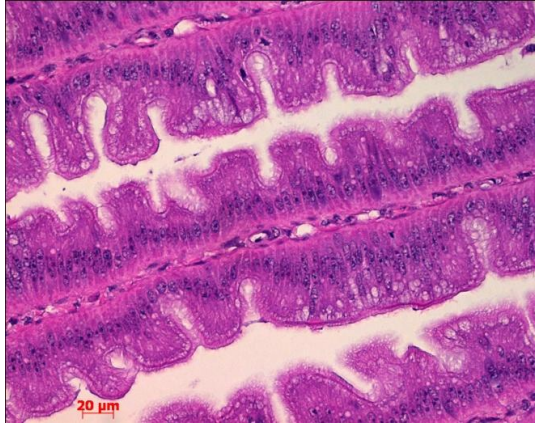
B. Proximal Intestine (400X)



C. Middle Intestine (50X)



D. Middle Intestine (400X)



E. Distal Intestine (50X)



F. Distal Intestine (400X)

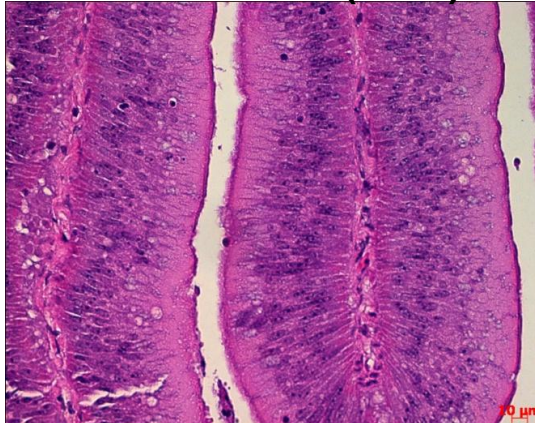


Table S1. Bile salt intermediates in intestine of parasites. Intestine extracts were submitted to full scan ES +/- for detection of bile salt intermediates. SP, small parasite (N=2); LP, large parasite (N=2). Total sample size of parasitic intestine is 4. +, detection of this molecular weight occurred in ES + mode; -, detection of this molecular weight occurred in ES – mode. ±, detection of this molecular weight occurred in both ES+ and – modes.

Bile Salt Intermediates	7α-hydroxycholesterol	7α-hydroxy-4-cholesten-3-one	7α-hydroxy-5β-cholestan-3-one	5β-cholestane-3α, 7α, 12α, 27α-tetrol	3α, 7α, 12α-trihydroxy-5β-cholestanoic acid
MW	402.66	400.64	402.66	436.67	450.33
SP	+	±	+	±	-
SP	+	±	+	+	-
LP	+	+	+	±	-
LP	+	+	+	±	-

Table S2. QRT-PCR related sequences. Sequences listed are partial sequences selected as amplicons in QRT-PCR experiments. The underlined sequences indicate 5' and 3' primers of each amplicon. Sequences used for probes are in both bolded and italic font.

Gene ID	Sequence for QRT-PCR
<i>cyp7a1</i>	<u>CAACATGTCGGCGCTCATCGCCCTCCGAATACA</u> <i>ACTCAATGACACGCT</i> <u>GTCTCGCATG</u>
<i>cyp27a1</i>	<u>TCTGGCCAAAATGTCATTCCTTAAGGCTGTCATCAAAGAGATTCTCAGA</u> <u>CTGTATCCAGTGGTGCC</u>
<i>HMG-CoA Reductase</i>	<u>AGGTGTCCGGCAGTTGGAGATCATGTGCTCATTG</u> <i>GGTTGCTTCTCCATC</i> <u>CTGGCC</u>

Table S3. Optimum of UPLC-MS/MS parameters for each analyte. A Waters Quattro Premier XE tandem quadrupole mass spectrometer (Milford, MA) coupled to a Waters Acquity ultra-performance liquid chromatography system was used. Separation was achieved by using a Waters C18 column (ACQUITY UPLC BEH 1.0 x 50 mm, 1.7 μ m particle size) with oven temperature at 50°C. The mobile phase was a gradient established between solvent A (10mM TEA in H₂O) and solvent B (MeOH) at a flow rate of 0.150 mL/min. Baseline separation was achieved by using the gradient started at 88% of A and 12% of B and followed by linear increasing of B to 50% in 3.0 min and 70% in 4.0 min. Data were collected in centroid mode with a scan range of 50-1000 *m/z*. MRM measurements of the analytes and internal standards were performed using individually optimized cone voltage and collision energy (see below).

Compound	MRM <i>m/z</i>	Cone (V)	Collision Energy (V)	Dwell (s)	Retention Time (min)
TCA	514.4 > 123.9	60	50	0.1	3.03
3kPZS	471.3 > 96.8	60	50	0.1	3.15
TDC	498.4 > 123.9	60	50	0.1	3.47
TCDC	498.0 > 123.0	50	50	0.1	3.68
5d-3kPZS	476.3 > 97.8	60	50	0.1	3.15
4d-TCA	518.4 > 123.6	58	46	0.1	3.03

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