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Microscopic morphology of the liver of the Guppy
(Poecilia reticulata)
Sisandra Sousa

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2018

SISANDRA SOUSA

MICROSCOPIC MORPHOLOGY OF THE LIVER OF THE GUPPY (*Poecilia reticulata*)

Thesis Application to a Doctor degree in Marine and Environmental Sciences

Doctoral Program of University of Porto (Institute of Biomedical Sciences Abel Salazar), University of Aveiro and University of Algarve

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Affiliation – Institute of Biomedical Sciences Abel Salazar, University of Porto

Porto, November 2018

À minha Mãe.

Male guppies

Nota prévia

Para informação do papel da candidata na execução da Tese declara-se que a autora participou na concepção e na execução do trabalho laboratorial que deu origem aos resultados – em cooperação com os orientadores e outros investigadores – assim como na análise, interpretação e redacção dos manuscritos que foram inseridos na Tese.

Especificamente, a candidata foi autora única do material introdutório bem como das considerações finais da Tese. Redigiu ainda integralmente os Capítulos III e IV da Tese, tendo discutido previamente os resultados com o orientador. No futuro, pretende que tais Capítulos sejam revistos pelos orientadores e coautores, no sentido de seu apuramento de conteúdos, para efeitos de publicação em revistas internacionais da especialidade.

Esta Tese inclui um artigo científico original (Doi:/10.1016/j.tice.2017.12.009) publicado como 1ª autora em revista internacional indexada (Journal Citation Reports - Clarivate Analytics), resultante de uma parte do trabalho laboratorial, o qual se encontra incluído na Tese como Capítulo II. A candidata dispõe – enquanto autora do artigo – de autorização expressa publicamente (*online*) por parte da Editora para o poder incluir na presente Tese (https://www.elsevier.com/about/policies/copyright#Author-rights). O artigo em causa é:

Sousa, S., Rocha, M.J., Rocha, E., 2018. Characterization and spatial relationships of the hepatic vascular–biliary tracts, and their associated pancreocytes and macrophages, in the model fish guppy (*Poecilia reticulata*): A study of serial sections by light microscopy. Tissue and Cell, 50, 104–113.

Parte dos resultados do artigo foram divulgados numa apresentação em congresso:

Sousa, S., Azevedo, J., Rocha, M.J., Rocha, E. (2016). Intrahepatic distribution of the vascular and biliary tracts of the guppy (*Poecilia reticulata*): Some observations at light microscopy from serial sectioning the liver. Poster presented at the 50th Meeting of the SPMicros - Microscopy and Microanalysis in Materials and Life Sciences. Porto, Portugal.

Preliminary note

It is hereby declared, as communication of the candidate's role in the performance of the Thesis, that the author participated in the design and execution of the laboratorial work that gave rise to the results – in cooperation with supervisors and other researchers – as well as in the analysis, interpretation and drafting of the manuscripts that have been inserted in the Thesis.

Specifically, the candidate was the sole author of the introductory material as well as the final considerations of the Thesis. She further elaborated Chapters III and IV of the thesis, having previously discussed the results with the supervisor. In the future, these Chapters should be revised by both supervisors and coauthorsto improve their contents, for the purpose of publication in international journals of the specialty.

This Thesis includes one original article (Doi:/10.1016/j.tice.2017.12.009) published as 1st author in an international journal indexed in the Journal Citation Reports (Clarivate Analytics). The article has part of the data obtained in the laboratorial work. The candidate included the article in the Thesis under the public explicit permission of the publisher's to do so (https://www.elsevier.com/about/policies/copyright#Author-rights). The article is:

Sousa, S., Rocha, M.J., Rocha, E., 2018. Characterization and spatial relationships of the hepatic vascular–biliary tracts, and their associated pancreocytes and macrophages, in the model fish guppy (*Poecilia reticulata*): A study of serial sections by light microscopy. Tissue and Cell, 50, 104-113.

Parts of the results of the article were announced in a conference presentation:

Sousa, S., Azevedo, J., Rocha, M.J., Rocha, E. (2016). Intrahepatic distribution of the vascular and biliary tracts of the guppy (*Poecilia reticulata*): Some observations at light microscopy from serial sectioning the liver. Poster presented at the 50th Meeting of the SPMicros - Microscopy and Microanalysis in Materials and Life Sciences. Porto, Portugal.

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Abstract

The liver is the central study object of this Thesis. The organ is the largest gland of vertebrates with a crucial role in maintaining life. For fish, in addition to its role as a biotransformer of harmful substances in others that can be excreted or to store those that when in excess can be harmful, it also plays an important role as a bioindicator of the quality of the aquatic environment in which fish live.

This role comes from the fact that this organ often undergoes gross anatomical and histological changes (in parallel/addition to other) when subjected to the action of chemical pollutants from the environment. Thus, from the gross morphology and especially histological analysis of the liver, it is possible to infer about water quality statuses and prevent more serious situations caused by pollutants in aquatic ecosystems. However, to be possible to identify abnormal alterations of this organ is indispensable the detailed knowledge of its normal anatomy and histology. Although there are generic similarities between the liver belonging to animals of different taxonomic groups, there are however variations between close groups which implies the individualized study of this organ in the different species of fish. Besides, the liver of embryos, newborns, juveniles and adults are likely to differ.

The choice of guppy fish as a model for this work is related to the fact that this fish has very particular characteristics that make it is a privileged model of study, used in research in various areas of scientific knowledge over time. This analysis is done in Chapter 1, giving an account of the number of scientific articles from 1900 until January 31, 2018, using the guppy fish as the organism of study: 1453 studies have been indexed in Web of Science.

The first efforts of this work were focused on the microscopic analysis of adult guppy liver, and particularly the intra-hepatic distribution of blood vessels and bile ducts. The follow - up of the vessels' path from the observation of serial sections of the liver, 7 μm thick, allowed to observe associations between the vascular and bile elements and the different types of vessels and ducts, and to follow its course and evolution since from the afferent hilar areas of the liver to the efferent vascular points of the organ. The guppy liver presented intra-hepatic pancreatic tissue and its

association with the vascular and biliary elements. We observe that the input of afferent vessels (i.e., bringing blood into the liver) occurs not only in the hilum but pierce and enter the organ at various points. This observation was common both in adult and in subsequently analyzed fish of different ages, 7, 17 and 28 days old.

Within the liver, of the adult fish venous vessels and bile ducts are seen, isolated or associated as venous–arteriolar tracts (VAT), and venous-biliary- arteriolar tracts (VBAT). Sometimes, pancreocytes appear within the liver surrounding isolated veins, forming venous tract with pancreatic acini (VT-P), or dual associations with afferent vessels, forming venous–arteriolar tracts with pancreatic acini (VAT-P). Intrahepatic pancreatic ducts were tiny and rare, putting in question the functional role of the acini. Contrary to other fish species, we did not spot isolated arterioles and associations between these and biliary ducts (BAT). We found aggregates of macrophages, namely associated with afferent and efferent (i.e., draining blood out) venous vessels. There was a reduced arterialization of the organ (as arterioles were extremely rare), contrasting with an over predominance of a random distribution of the venous vascularization.

In order to contribute to the knowledge of the ontogeny of the liver of the guppy, a histological analysis of the liver of fish with different ages: 7, 17 and 28 days of age, was carried out. The follow up of the observation of serial sections of 7μm and 25 μm thick allowed us to follow the evolution of the histological development of this organ over time and establish a comparison with the histological study of the liver in the adult. In general, in 7-day-old fry, no exocrine pancreas was observed within the liver; however, their presence at the periphery of this organ was frequent, in the vicinity of the entry sites of the afferent vessels; in fish with subsequent ages (17 and 28 days), exocrine pancreas was observed within the liver and in some cases next to venous vessels and bile ducts. This finding allows us to infer that the penetration of pancreocytes into the liver possibly happens as the post-embryonic development of the fish occurs until it reaches the adult state. As in adult fish, several entry sites of venous vessels were identified in the liver, generally four, confirming the dominance of the venous circulation in this gland. No arterioles were observed within the liver, which is in agreement with the extremely reduced arterialization of the liver in adult fish. The presence inside the liver of macrophage

aggregates (MAs), defense cells observed adults, was registered in any of the guppy fry or juvenile. At the same time a stereological study, relaying on various so-called "design-based" techniques (with no shape, size or orientation assumptions) was made used the same fish. The aim was to know if the initial liver post-embryonic development was based on hepatocellular hyperplasia and/or hypertrophy. The targeted cells of this stereological study were the hepatocytes, to estimate some parameters that characterize them quantitatively: cell and nuclear volume, and total number. Total liver volume and hepato-somatic index (HSI) were also estimated. The differences between the sexes in two ages were investigated.

Within the same age, and from body morphometry to stereological estimates, it was verified in all the parameters that differences between the sexes do not exist. However, the age factor influenced the variation of some of the parameters. The increase in the number of cells and the reduction of their volume from 7 to 17 days is emphasized. This is important because it allows us to identify a phase of intense cell multiplication without a corresponding increase in liver volume which one will be at a higher age. This is a new exciting finding, implying that initially, at least in certain species as the guppy, the fish liver “needs” periods of increased cellularity without changes in mass. We wonder if liver growth needs waves of hyperplasia that would generate many small cells that could later hypertrophy to increase the liver mass. If so, liver enlargement could be made in steps instead of continuously.

In short, the work developed in this Thesis is a contribution to the knowledge of the guppy liver. The results from the descriptive histological studies complemented by the stereological analysis offered new insights not only of the microanatomy of the guppy liver but also for fish in general, while opening research perspectives.

Keywords: liver; Guppy; histology; microscopy.

Resumo

O Fígado é o objeto de estudo central desta Tese.

É a maior glândula dos animais vertebrados com um papel crucial para a manutenção da vida. Para os peixes, para além da sua função como órgão biotransformador de substâncias nocivas em substâncias passíveis de serem excretadas ou também de armazenar aquelas que em excesso podem ser prejudiciais, tem também um papel importante como bioindicador da qualidade do ambiente aquático em que os peixes vivem. Este papel advém do facto de este órgão sofrer alterações morfológicas e histológicas quando submetido a ação de poluentes químicos do ambiente. Assim, a partir da análise morfológica e histológica do fígado é possível avaliar a qualidade da água e prevenir situações mais graves provocadas pelos agentes poluentes nos ecossistemas aquáticos. No entanto para ser possível identificar possíveis alterações deste órgão é indispensável o conhecimento pormenorizado da sua normal morfologia e histologia. Apesar de existirem semelhanças genéricas entre o fígado pertencente a animais de diferentes grupos taxonómicos, existem, no entanto, variações entre grupos próximos o que implica o estudo individualizado deste órgão nas diferentes espécies de peixe.

A escolha do peixe Guppy como modelo para este trabalho está relacionada com o facto deste peixe possuir características muito particulares que o tornam um modelo privilegiado de estudo utilizado em pesquisas em diversas áreas do conhecimento científico ao longo do tempo. Esta análise é feita no capítulo 1 dando conta do número de artigos científicos produzidos desde 1900 até 31 de janeiro de 2018 usando o peixe Guppy como um modelo experimental, 1453 estudos foram indexados no Web of Science.

O primeiro trabalho realizado centrou-se na análise microscópica do tecido hepático do Guppy adulto e na distribuição dos vasos sanguíneos e ductos biliares no parênquima hepático. O acompanhamento do trajeto desses vasos a partir da observação de cortes seriados do fígado, com 5μm de espessura, permitiu observar associações entre os elementos vasculares e biliares e os diferentes tipos de vasos

e ductos e seguir seu trajeto e evolução desde sua entrada nesse órgão até o local de saída.

O fígado de Guppy apresenta tecido pancreático intra-hepático por vezes em associação com os elementos vasculares e biliares. Observamos que a entrada de vasos aferentes (isto é, que levam o sangue para o interior do fígado) ocorre não apenas no hilo, mas também em outos pontos de entrada. Essa observação foi comum quer em peixes adultos quer nas diferentes idades de peixes (em idade muito precoce, larvas de 7 dias, bem como com 17 e 28 dias).

Dentro do fígado do peixe adulto os vasos venosos e os ductos biliares são vistos, isolados ou associados como tratos venosos-arteriolares (VAT), e tratos venosos-biliares-arteriolares (VBAT). Às vezes, os pancreócitos aparecem dentro do fígado em redor de veias isoladas, formando tratos venosos com ácinos pancreáticos (VT-P) ou associações duplas com vasos aferentes, formando tratos veno-arteriolares com ácinos pancreáticos (VAT-P). Os ductos pancreáticos intra-hepáticos eram minúsculos e raros, colocando em questão o papel funcional dos ácinos. Ao contrário de outras espécies de peixes, não encontramos arteríolas isoladas e associações entre estas e os ductos biliares (BAT). Encontramos agregados de macrófagos, nomeadamente associados a vasos venosos aferentes e eferentes (transportam o sangue para o exterior do fígado). Verificou-se uma reduzida arterialização deste órgão (as arteríolas foram extremamente raras), contrastando com um predomínio excessivo de uma distribuição aleatória da vascularização venosa.

Com o objetivo de contribuir para o conhecimento da ontogenia do fígado do peixe Guppy foi efetuada a análise histológica do fígado total de peixes com diferentes idades: 7, 17 e 28 dias de idade. O acompanhamento da observação de cortes seriados com 7 μm e 25 μm de espessura, possibilitou acompanhar a evolução do desenvolvimento histológico deste órgão ao longo do tempo e estabelecer uma comparação com o estudo histológico do fígado no adulto. Em geral, nas larvas com 7 dias de idade, não se observou pâncreas exócrino dentro do tecido hepático, no entanto, a sua presença na periferia desse órgão foi frequente nas proximidades dos locais de entrada dos vasos aferentes; nos peixes com as idades subsequentes (17 e 28 dias) observou-se pâncreas no interior do tecido hepático e nalguns casos junto a vasos venosos e ductos biliares. A frequência desta observação aumentou com a idade das larvas. Esta evidência permite-nos inferir que a penetração de pancreócitos no fígado possivelmente acontece à medida que o desenvolvimento do peixe ocorre atingindo o máximo no estado adulto. A observação de vários pontos de entrada de vaso venosos para o interior do fígado, geralmente quatro locais, é comum aos peixes com diferentes idades, confirmando a dominância da circulação venosa nesta glândula. Não foram observadas arteríolas no interior do tecido hepático, o que está de acordo com a redução da arterialização do fígado em peixes adultos.

A presença no interior do parênquima hepático de agregados de macrófagos (MAs), conjuntos de células de defesa que estão associadas a idades mais avançadas, não foi registada em nenhuma das larvas de Guppy. Ao mesmo tempo, um estudo estereológico, "estereologia baseada no design" (sem hipóteses de forma, tamanho ou orientação), foi usado com o objetivo de aumentar o conhecimento sobre o fígado usando as mesmas larvas. O alvo deste estudo estereológico foram os hepatócitos para estimar os valores dos parâmetros biológicos que os caracterizam: volume celular e nuclear, e número total. Foi também estimado o volume total do fígado e o índice histossomático (HSI). As diferenças entre os sexos e idade foram também investigadas.

Verificou-se em todos os parâmetros morfométricos ou estimados pela análise estereológica, que não existem diferenças entre sexos, dentro da mesma idade. No entanto, o fator idade influenciou a variação de alguns parâmetros biológicos estimados. Destaca-se o aumento no número de células e a redução do seu volume, nos peixes de 7 para 17 dias. Este dado é importante porque nos permite identificar uma fase de multiplicação celular intensa sem que se verifique um correspondente aumento do volume do fígado, que possivelmente ocorrerá numa idade posterior.

O trabalho desenvolvido nesta tese constitui uma contribuição para o conhecimento do fígado do guppy. Os resultados obtidos a partir dos estudos histológicos complementados pela análise estereológica realizada em dois grupos de peixes de diferentes idades, permitem-nos ter um conhecimento mais objetivo e assim mais real do peixe guppy.

Palavras-chave: fígado; Guppy; histologia; microscopia.

CHAPTER I

General Introduction and Objectives

1. The Fish Liver

1.1. Importance of fish liver

Fish liver is a digestive gland with relevant importance, as in all other vertebrates. This organ is responsible for controlling many of the vital functions and where reactions occur either of anabolism, leading to the production and storage of proteins, lipids and carbohydrates like glycogen, but also reactions of catabolism promoting the transformation of harmful substances into the body in compounds that can be excreted (detoxification), such as reactions leading to the production of substances essential for the production of energy by cells like glucose (glycogenolysis).

Due to its importance, the fish liver is a preferred model of study to evaluate the interactions between the environment and wild populations from injuries that can be caused in this organ by pollutants present in the environment. Thus, a histological study of this organ can provide indication on the state of health of a given population as well as the respective environmental quality of the aquatic ecosystem.

On the other hand, the histological study of the liver of the fish could make it possible to establish phylogenetic relationships between the different groups of vertebrates.

In our days, the increase of aquatic environments pollution may induce morphological and physiological changes of the fish organs. Liver has a major role in animal physiological and biochemical functions and some morphologic alterations can be observed under certain toxic circumstances. At the tissue level, histological biomarkers are powerful tools to detect and characterize the biological endpoints of a toxic compound. Fish can be used as sentinel organisms in environmental studies (bioindicators) and liver can be used as biomarker. The utility of histological analysis to access fish health has been reported in several studies (Stentiford et al., 2003; Bernet et al., 2004; Ramírez-Duarte et al., 2008; Samanta et al., 2016). It has been shown that different pollutants (eg. roundup, glyphosate) is toxic to fish and cause histological changes. Several investigators have reported histological alterations, such necrotic and proliferative lesions, aneurysms, and leukocyte infiltration in gills, fatty degeneration, multifocal necrotic processes, and infiltration of leukocytes have been registered in liver of fishes exposed to pollutants like glyphosate.

Biomarkers are measures of sub organismal responses in organisms or exposed biological systems which can demonstrate exposure to, or the effects of, environmental contaminants (Peakall, 1992). The study of biomarkers has intensified over time and the relevance of its use in the monitoring of aquatic pollution has been consensual in the scientific community (SETAC, 2018, ANZECC, 2018). There are different levels of response that organisms can give when environmental conditions are unfavourable, ranging from molecular, biochemical, physiological, histopathological changes to higher levels as responses as the level of the individual, population and community over which adverse conditions are exercised (**figure 1**). These answers provide information, on spatial and temporal changes in the concentration of contaminants and indicate environmental quality or occurrence of adverse ecological consequences. The responses at lower biological organization levels (e.g. molecular and biochemical responses) are more specific, sensitive, reproducible and easier to determine, but more difficult to relate to ecological changes. On the other hand, responses at higher biological organization levels (e.g. organism, population and community responses) are directly indicative of ecosystem health and hence, much more relevant to environmental management. However, they are more difficult to determine, less specific and only manifest at a late stage when environmental damages have already occurred. Histo-cytological responses are relatively easy to determine and can be related to health and fitness of individuals which, in turn, allows further extrapolation to population/community effects. In this sense, the study of fish liver with other biomarkers is very important and therefore wide range of histo-cytological alterations in fish liver have been studied and recommended as biomarkers for monitoring the effects of pollution.

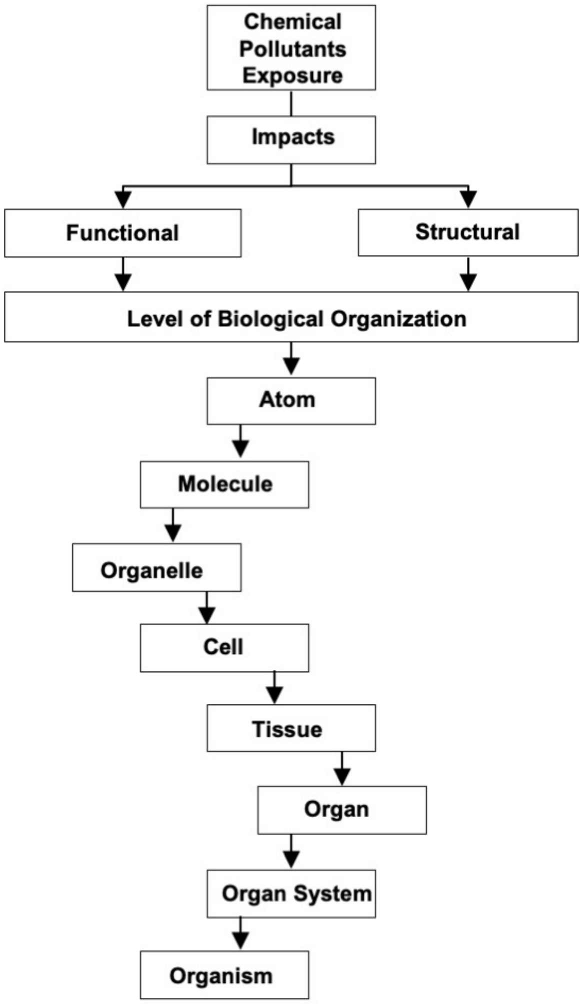

Figure 1 - Schematic representation of the impact of chemical pollutants at different levels of biological organization.

1.2. Normal structure of the liver

Fish liver is a dense organ located ventrally in the abdominal cavity. Its size, shape, and volume are adapted to the space available between other visceral organs. In many teleostei species the liver is divided into three lobes. However, no lobulation was recognized in some teleostei (Bruslé and Anadon, 1996).

Some species of fish have exocrine pancreatic tissue in the interior of the liver parenchyma named hepatopancreas (Rocha e Monteiro, 1999), such as *Ictalurus punctatus* (Kendall and Hawkins, 1975; Hinton and Pool, 1976), *Pimelodus maculatus* (Marconi Stipp et al., 1980), *Micropogon undulatus* (Eurell and Haensly, 1982), *Serranus cabrilla* (Gonzalez et al., 1993), *Oreochromis niloticus* (Figueiredo-

Fernandes et al., 2006); *Oreochromis mossambicus* (Carrola et al., 2018); Pancreatic tissue may be associated with blood vessels or bile ducts and is distinguished from hepatic tissue because it is organized in acini (Bruslé and Anadon, 1996; Rocha e Monteiro, 1999).

In order to make an evaluation of the pathological effects of environmentally adverse conditions on fish liver, a thorough knowledge of the normal morphology and histology of this organ is fundamental. The studies about ultrastructural features of normal fish liver are few. For the Salmonidae family, several light and electron microscopic studies on the normal structure of the liver have been undertaken, on the chum salmon, *Oncorhynchus keta* (Takahashi et al., 1977), coho salmon, *Oncorhynchus kisutch* (Leatherland, 1982), Atlantic salmon, *Salmo salar* (Robertson and Bradley, 1991, 1992) and rainbow trout, *Oncorhynchus mykiss* (Hacking et al., 1978; Chapman, 2006; Hampton et al., 1985, 1988, 1989; Schär et al., 1985; Hinton et al., 1988; Figueiredo-Fernandes et al., 2006). Although there are structural similarities of this gland between different orders, (Hampton et al., 1985) there are however differences within the same family and therefore it is important to study the morphology and physiology of the liver individually for each species (Schär et al., 1985; Robertson and Bradley, 1992). In general, the internal structure of the fish liver comprises two regions: the hepatic parenchyma, and the stroma, where different types of cells and structures can be found, as shown in **figure 2**.

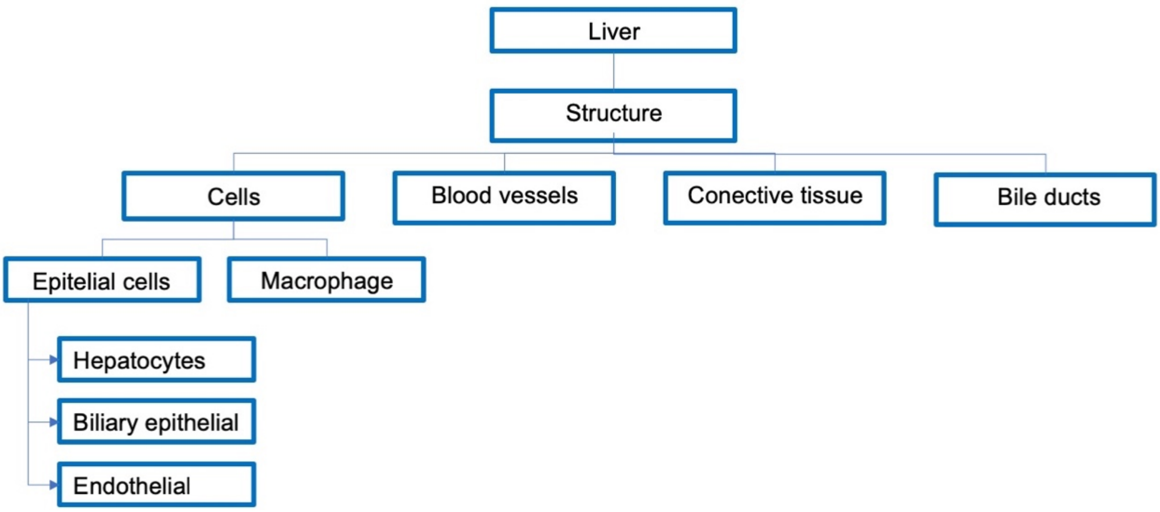

Figure 2 – Representation of structural components of the teleost liver.

Of the different types of cells present, hepatocytes are the most abundant (Rocha et al., 2003).

An analytical study by Elias and Benelsdorf (1952), combining the three-dimensional representations made by several authors, with the three-dimensional characteristics of the tissues allowed to conclude that the vertebrate liver does not present as a tubular gland, consisting essentially of masses of hepatocytes separated by gaps more or less tubular where are the vascular elements (sinusoids). The different sinusoids are separated by walls consisting generally of two layers of cells - hepatic plates.

Hepatocytes are organized into hepatic cords (Hampton et al., 1988) and communicate with the sinusoids (Hinton et al., 1988; Robertson and Bradley, 1991). The hepatocyte clusters are separated by the sinusoids and bile ducts (Bruslé and Anadon, 1996; Bombonato et al., 2007) found in the hepatic stroma.

Blood supply to the liver is done through the portal vein and the hepatic artery which branch into smaller vessels within the liver (Damjanov and McCue, 1996). From these small vessels, the blood enters the sinusoids. It is in the sinusoids that tissue liver establishes the exchanges between the hepatocytes and the blood that later is collected in the hepatic vein (Ross et al.,1989). The base of the hepatocytes is directed towards the sinusoids, for absorption and the top to the bile canaliculi, for excretion (Hampton et al., 1985; Schär et al., 1985; Robertson and Bradley, 1991).

Studies on fish liver ultrastructure show that hepatocytes are cells poor in organelles, which may indicate that protein synthesis is low (Gonzalez et al., 1993; Bruslé and Anadon, 1996). On the other hand, it is generally observed that hepatocytes accumulate large amounts of glycogen in the cytoplasm (Moon et al., 1985; Hampton et al., 1985; Gonzalez et al., 1993; Vicentini et. al., 2005).

The macrophages are other type of cells that may be present in the liver, constituting the melano-macrophage centers (MMC) (Hartley et al., 1996) – cells that among other compounds concentrate in their interior melanin that gives them a yellow-brown color (Munshi and Dutta, 1996). This melanomacrophage centers, are usually near to blood vessels or bile ducts. The size, number and intensity of MMC staining varies with fish species and depends on the age and individual health status, increasing their frequency in oxygen-poor environments and polluted by different chemical agents, and can therefore be considered as biomarkers of water quality (Agius and Roberts, 2003). Another type of cells that can be found inside the liver are pancreocytes. The liver which presents exocrine pancreatic tissue inside is called hepatopancreas (Rocha and Monteiro, 1999). Pancreatic tissue may be associated with blood vessels and bile ducts present in the hepatic stroma.

1.3. Histopathology of liver

Teleost liver is the primary organ for biotransformation including organic xenobiotics, and is crucial for the excretion of harmful trace metals, food digestion and storage, and metabolism of sex hormones (Hinton et al., 2001), as it is the major detoxifying organ for the excretion of toxic substances in fish. There have been numerous reports of liver histo-cytopathological changes of fish exposed to a wide range of synthetic compounds and heavy metals (Hinton et al., 1988; GlobalTox, 1997; Braunbeck, 1999; Carrola et al., 2009). The source and pathway of different chemical pollutants in the environment is diagrammed in **figure 3**.

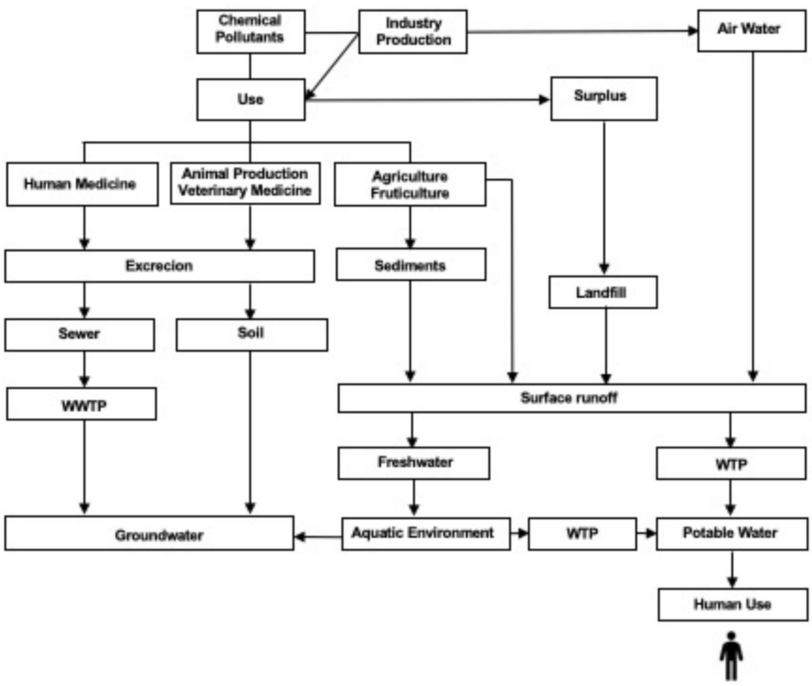

Figure 3 - Source and pathway of chemical pollutants in the environment.

Fish liver is sensitive to environmental contaminants because many contaminants tend to accumulate there, making this organ exposed to a much higher level (several orders of magnitude) than in the environment, or in other organs. Several studies had conducted to determine the relationships between toxicohepathic lesions in livers and exposure to chemical contaminants, to assess the impact of environmental contaminants on fish health. These survey data generally show a good correlation between the concentrations of various persistent chlorinated hydrocarbons, heavy metals, herbicide (glyphosate) and liver lesions (Malins et al., 1987; Carrola et al., 2009; Myers et al., 2016).

In general, liver histopathological lesions are not specific to pollutants. Furthermore, not all hepatic lesions identified in fish can be used as biomarkers since certain liver lesions appear to be species specific. For example, exposure to PAHs, PCBs, DDTs, chloranes and dieldrin increases the prevalence of a wide range of liver lesions in English sole (*Pleuronectes vetulus*) (i.e. neoplasms, foci of cellular alteration (FCA), megalocytic heptosis (MH), hepatocellular nuclear pleomorphism (NP), hydropic vacuolation (HV); while in winter flounder (*Pleuronectes americanus*), exposure to PAHs, DDTs or chlordanes significantly increased the risk for HV, non-neoplastic proliferative lesions, non-specific necrotic lesions, but not neoplasms and FCA (Johnson et al., 1993; Myers et al., 1998). Species-specific differences in sensitivity to chemicals, and differences in regional distribution of chemical contaminants may explain some of the variations of chemical risk factors observed among species, lesions types and between studies (Stehr et al., 2004).

Other confounding factors also have a significant influence on the prevalence of hepatic lesions in winter flounder. For example, a higher prevalence was found in spring than in winter, when spawning migration was taking place (Johnson et al., 1993). Fish age also affected the occurrence of certain hepatic lesions including, neoplasms, non-neoplastic proliferative lesions in several fish species (Myers et al., 1998). Hepatic neoplasms did not appear at appreciable prevalence in free-living English sole until about 3 years of age, and the mean age of sole with neoplasm was 5,6 years (Landahl et al., 1990). Indeed, it has been shown that liver neoplasms were rare in young wild fish, and the risk of hepatic disease increased with fish age

(Johnson et al., 1993; Myers et al., 1998), this may be due to a long period of exposure. Surveys conducted in the Black River, Ohio also affirmed that PAH exposure and liver cancer occurred only in wild brown bullhead of over 3-year-old (Baumann and Harshbarger, 1998). Sex-specific differences in cellular stress responses were repeatedly demonstrated in hepatocytes of European flounder, which may explain differences in the susceptibility of fish to toxic and carcinogenic compounds in polluted environment.

1.4. Evaluation of liver histo-cytological biomarkers to monitor freshwater pollution

Although it has not been completely demonstrated, it is likely that histopathological symptoms described in liver fish may decrease individual fitness through disturbing the homeostasis and proper functioning of vital biological processes (e.g. detoxification, storage, glycogenolysis) these histopathological symptoms are highly ecological relevant.

A major advantage in employing fish “gross lesions”, including liver tumours, is that they are easy to observe and therefore offer a cost-effective monitoring tool.

Cost effectiveness can be further enhanced by incorporating this as an on-board observations/examinations program in normal commercial fishing. Moreover, the symptoms can be easily understood by the general public, laymen and legislators, and hence can be easily interpreted in term of the *“health status”* of the freshwater environment.

Certain hepatic lesions in fish have been well correlated with contaminant exposure. Yet, the relevance of a certain lesion for the fish depends on its pathological importance, which indicates how the lesion affects the function of the organ and the ability of the individual to survive. An importance factor (W) (range 1–3) was assigned to each alteration (**table 1**). A high importance factor represents a greater potential of an alteration to impact fish health (Bernet et al., 2004).

Table 1 - Importance factor assigned to liver histological alterations for each reaction pattern. W - importance factor (Bernet et al., 2004).

Liver reaction pattern	Histological alteration	W (importance factor)
Circulatory disturbances	Haemorrhage	1
	Sinusoid dilation	1
	Blood congestion	1
Regressive changes	Hydropic degeneration	1
	Lipid degeneration	2
	Hepatocyte picnosis	2
	Fibrosis	3
	Necrosis	3
Progressive changes	Hepatocyte hyperplasia	2
	Hepatocyte hypertrophy	2
Inflammation	Leukocyte infiltration	2

The value of w (importance factor) allows to evaluate the histological alterations of the liver that can be used to evaluate the effect of the different contaminating agents present in the aquatic environment, as a result of the human activity, on the fish populations. Given the importance of the liver to maintaining of the body vital functions, these changes are a pre-determined indicator of fish health status (Hinton et al., 2001; Stentiford et al., 2003).

1.5. Conclusion

Fish liver is a gland of special relevance on the control of several vital functions and is therefore used as a study model to evaluate the health status of natural populations. As far as liver morphology and histology are concerned, they can be used as a sensitive bio-monitoring tools about toxicant impact assessment to indicate the effect of toxicants on fish health in polluted aquatic ecosystems. Histopathological assessment of fish tissue allows for early warning signs of disease and detection of long-term injury in cells, tissues, or organs. The analysis of blood constituents, fish necropsy, calculation of condition factors, and hepatosomatic indices were employed to support the findings of the qualitative and quantitative histological assessment of liver tissue. The assessment revealed marked histopathological alterations including: structural alterations of wild specimens of liver; vacuolization of the hepatocytes cytoplasm; hepatocyte nuclear alterations; an increase in melanomacrophage centers; and necrosis of liver tissue.

We highlight the role of the liver as an essential gland for life. It was also emphasized the role of the liver as a bioindicator of the quality of freshwater and marine ecosystems. The various lesions in the liver caused by different types of pollutants in different species, allow us to conclude that liver histopathology is indicative of general health condition of fish and levels of toxic xenobiotics, carcinogens and urban pollutants present in the environment. The histopathology presented by the liver fish were induced by different types of pollutants and could be used as indicators of environmental quality. Thus, the histological condition of the liver allows to establish the correlation between the state of population health and the quality of the environment where the population lived. However, it was clear that this use can only be made if the structure and histology of the normal liver of this species is well defined. For this reason, studies that lead to the knowledge of the structure, physiology and histology of the fish liver are increasingly important to enable an early assessment of environmental quality and thus prevent the emergence of serious fish health problems and trofic food web.

Although the importance of this studies, *early life stage* tests using fish embryos have been suggested to be one of the most promising and viable animal alternative tests in environmental toxicology (Braunbeck et al., 1999; Embry et al., 2010). The embryos provide an ethically acceptable model according to European legislation which only considers fish as animals from the time they reach the stage of exogenous feeding (EC, 2010).

2. Biology of the Guppy and its Use in Animal Experimentation

2.1. Introduction

Tropical guppy (*Poecilia reticulata*) is an ecologically significant species that is very abundant year-round in shallow waters of the canals, rivers, lagoons, reservoirs and estuaries of Northeastern regions of Venezuela. Guppies have a large intra-specific diversity being an ornamental species widely used in aquarofilia (Monticini, 2010).

Guppy fish are small in size and therefore do not have great ability to travel on watersheds during their lifetime (Castro, 1999). Moreover, geomorphological characteristics of the habitat influence the distribution of these organisms, increasing the adaptive pressure, thereby making the degree of endemism to become high in isolated populations (Winemiller and Willis, 2011). The great diversity of external morphology enables them with a high degree of adaptability to the environment. For this reason, variations in pressure, temperature, light, space, water flow, dissolved oxygen in water and food resources can be supported by guppies. Thus, the presence of the exotic species *Poecilia reticulata* (Peters, 1859) in natural environments may indicate negative environmental disturbances, since the ecological adaptations that this species possesses allows them to live in harsh environmental conditions. If we associate with this adaptability, the absence of endemic predators in the environment by notwithstanding the harsh environmental conditions, then guppy may have favorable conditions for colonization of habitat, (Reznick et al., 2012).

The possible carelessly introduction of this species in the natural environment, allowed it a wide dispersion being now regarded as an invasive species. The introduction of non-native species into new habitats poses a major threat to native populations. Invasive species now represent one of the greatest threats to biodiversity (Mack et al., 2000).

Throughout this chapter will be presented the main features of guppy which allow them to be used in many researches as a model of animal experimentation (**table 2**) over time, contributing with great relevance for the evolution of scientific knowledge, especially in the areas of toxicology and health.

2.2. Taxonomy, distribution and habitat

The guppy (*Poecilia reticulata*), is a tropical fish and is one of the most popular freshwater aquarium fish species. This specie is a member of class Actinoperygii order Cyprinodontiformes, and Poeciliidae family. This family contains about 200 species in 16 genera and is widely distributed occurring in America and Africa (Parenti, 1981). The name assigned to the species changed over time. The first name given to it was *Poecilia reticulata, Peters,* a new species identified in 1859. Later in 1861 it was changed to *Lebistes poeciloides*, based on the formation of the gonopodium. Then in 1866 it was changed to *Gyrardinus guppyi*, in honor of the Reverend Robert John Lechmere Guppy.

The natural range of the guppy is Central America and northeast South America, more specifically Venezuela, Trinidad and Tobago (Endler, 1978; Reis et al., 2003). The first population of guppies appeared in the native environment in 1970 (Milenković et al., 2014). The current knowledge on the distribution of guppies in natural environment suggests that this is certainly the result of improper handling and inadequate release by aquarists. On the other hand, its accidental or intentional release into natural aquatic environments of different countries has contributed to this species to acquire the status of invasive species in some locations, by being associated with the competition and the elimination of native species (Deacon et al., 2011). This insertion of the guppies in different habitats makes them be currently found in various regions of the Earth and a species well adapted to different environmental conditions. The wild guppies are fed on benthic organisms, like insect larvae and algae. For this reason, they were introduced into Brazil and other Latin American countries in order to eat mosquito larvae reducing the natality rate of these insects and thus contributing to the eradication of malaria. This insertion allowed the guppies to persist successfully in environments with unstable limnologic conditions (Reis et al., 2003). Guppies can rather be found in tropical rivers and their effluents. They prefer murky waters. It is a non-migratory benthopelagic species, being well adapted to polluted environments (Lucinda and Costa, 2007; Araújo et al., 2009). Regarding the population distribution, it was found, in a study with guppies which has occurred in a natural environment in the thermal waters of Serbia, (Milenković et al., 2014), that the younger fish, sub-adults and juveniles, express preference for locations where there is an abundance of vegetation which is a protection for newborns that develop around. Adults show a preference for open habitats with low water flow. The favorable conditions of the current allowed the establishment of a stable population of guppies for a long period of time. Although this study has been done during the winter, the results should be identical in the summer, once the annual temperature is very steady, 24-25ºC.

2.3. Tolerance to abiotic factors

The species from the Poeciliidae family, which guppies are a good example of, are found mainly in tropical regions showing a relatively high degree of tolerance to the environmental conditions (Britski, 1972; Vazzoler, 1996; Machado, 2002; Reis et al., 2003) and being able to use a minimum concentration of oxygen to live (Pereira and Andreata, 2003; Rocha et al., 2009).

The fact that they are viviparous animals allows them some independence regarding the environmental conditions (Aranha and Caramaschi, 1999).

The guppies can live whithin the temperature range between 12.8ºC and 37.8.ºC. It is clear that this species is very tolerant, despite the normally accepted range for culturing guppies were between 23.3°C and 26.7°C.

One of the consequences of temperature in guppies, is the variation of its longevity. Guppies kept at a temperature of 26.7ºC live for 18 to 21 months, if kept at a temperature of 23.3ºC live between 30 to 48 months. Between 20.6ºC to 21.7ºC they can live up to 60 months (Larr, 1972).

Regarding variation in size, studies carried out with guppies have showed that the fish bred at higher temperatures and those bred at lower ones do not show variations in their size. Nevertheless, it appears that at higher temperatures, they grow faster than when they are created at lower temperatures, where they grow slowly (Larr, 1972). It was also found high mutation rates in the offspring, when raised to high temperatures (Larr, 1972).

A study was made with guppies, *P. reticulata*, collected from the canal of La Laguna Los Patos (Venezuela) to verify the influence of acclimation temperature in determining the value of critical thermal maxima (CTM) and death points (DP), as criteria of thermal tolerance (Chung, 2001) The CTM was originally defined by Cowles and Bogert (1944) as "*the thermal point as which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death*". This has been modified to use statistical variation and standardized methods (Hutchison, 1976). The criterion of fish death was defined as the cessation of opercular motion (DP).

The results of this study (Chung, 2001) allowed to determine a positive relationship between thermal tolerance and acclimation temperatures, and thus heat tolerance criteria were significantly different among acclimation temperatures. It was also found that the individual tolerance time to heat when the fish are transferred to higher acclimation temperatures (eg.: from 24ºC to 30ºC) is higher than the tolerance time when transferred to lower acclimatization temperatures (eg.: 30ºC to 24ºC). The results confirm that acclimation temperature is a determining factor for thermal adaptability of the tropical guppy. The acclimation rate of the guppies is faster in increasing temperature than in decreasing temperature. This phenomenon is very favorable for tropical fish because they will acclimate very rapidly during the heat of the day and will not lose this level of acclimation during the cool night; thus, they will survive in the abrupt temperature alterations in their fluctuating natural environments (Chung, 2001; Reeve et al., 2014).

The high tolerance to environmental factors of the *P. reticulata* (Peters, 1859), makes it possible to this species when introduced in native environments to be used as a bioindicator of negative environmental disturbances where it occurs, since this species has ecological adaptations which gives it a great success in colonizing in many types of environments such as high competence in inter-and intraspecific competition (they use the same resources of native species) and the ability to withstand extreme variations in the environment (Reznick et al., 2012).

These facts may result in the elimination of native species and cause drastic changes in the community structure in aquatic environments. Moreover, this species can also be seen as causing some environmental problems due to the damage it can cause in small aquatic communities. This is mainly due to their intrinsic characteristics: a high rate of reproduction (Barlow, 1992) and renewal of offspring;

a high ability to compete and operate in several habitats**.** However, studies focusing on the ecological consequences of the invasion of this species can generate on the environment need to be conducted so that we can establish a correlation between environmental quality and the presence of this species (Souza and Tozzo, 2013).

Studies performed with guppies in natural environment allow us to conclude that the most important factors limiting their growth and survival are the water temperature (if lower) and the existence of predatory fish.

2.4. Morphology

2.4.1. Macroscopic anatomy and phenotypic variations

The species of Poeciliidae family which can be found mainly in tropical regions are predominantly viviparous and small (Britski, 1972; Vazzoler, 1996; Machado, 2002; Reis et al., 2003).

The guppies show sexual dimorphism: males are smaller than female (male with approximately 5 cm in length, female 7 cm) and have colorful caudal and dorsal fins, while the females have a more uniform color (**figure 4**).

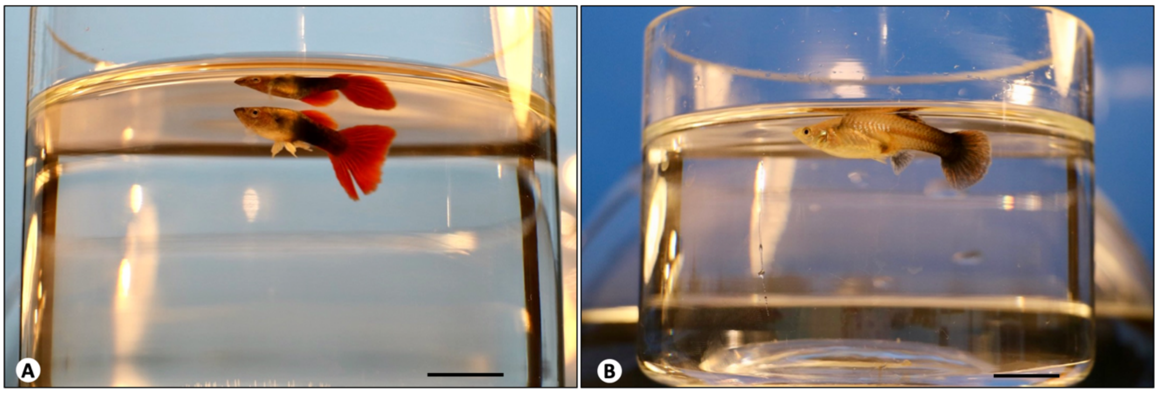

Figure 4 - Sexual dimorphism of guppy. A – male guppy; B – female guppy. Scale Bar: 2 cm.

One of the morphological variations of these organisms are related to their capacity to detect and respond to environmental stimuli. This is essential to an ability of the organism to survive and reproduce. Thus, in general the organisms have sensory systems that are adapted to the environments in which they live (Fischer et al., 2013).

One of the characteristics of the fish that allows them to sound movements in the water is its mechanosensory system – lateral line - which allows them to detect minor variations in water pressure (Bleckmann, 1986).

Fish and other aquatic vertebrates use their mechanosensory lateral line to detect objects and motion in their immediate environment.

The sensory end organs of the lateral line are the neuromasts which are derived from epithelial placodes on the head and function in the detection of water movements (Bleckmann, 1986). A neuromast is composed of a collection of hair cells. The cell bodies of the hair cells are surrounded by support cells and the ciliary bundle on the apical surface of each hair cell is embedded in a gelatinous cupula (Coombs et al., 1992).

Guppys do not have differences in the total number of neuromasts between sexes. However, it was demonstrated by a recent study (Fischer et al., 2013) that wild-caught guppies exhibited differences in superficial neuromasts based on predation level and variation of the environmental conditions. These differences were the consequence of both genetic and environmental variation in laboratory-reared fish. This study was the first to demonstrate variation in lateral line morphology based on environmental exposure to an ecologically relevant stimulus, (Fischer et al., 2013).

Rodd et al., (1997) have also demonstrated that guppies living in the streams and rivers of Trinidad show significant interpopulation variation in life history traits. Some of the greatest divergence is among guppies that occur with different assemblages of predators. These differences relate to the size and degree of maturation of individuals.

Another example of phenotypic variation in guppies refers to the morphology of the male organ of copulation (gonopodium). A study done by Evans et al., (2011) using natural populations of guppies showed that these fishes exhibit divergent patterns of male sexual behavior among populations. In this study, the correlation was made between the morphology of the gonopodium and forced mating success. Observed that variation in the length and shape of the gonopodium predicted the success of forcing matings in terms of the rate of genital contacts and inseminations success. This showed consistent patterns of variation in male genital size and shape in relation to the level of predation and corresponding patterns of variation in female genital morphology. The data shows that exists selective pressure causing correlated patterns of divergence in male and female genital trait which point to a role for sexual selection.

Phenotypic variability on coloring in male guppies can also be influenced by the presence of predators. It was found that in places where the number of predators is smaller, male fish are more colorful probably due to sexual selection.

In places where the presence of predators is most frequent, males exhibit a phenotype which is characterized by a near absence of intense color on the body and fins (Milenković et al., 2014), probably to protect themselves from predators.

Guppies can be considered an invasive species with a high success, and a contributing factor to this success may be a high degree of phenotypic plasticity (Torres-Dowdall et al., 2012). This high phenotypic plasticity may have been caused by the large variation in environmental conditions to which the ancestral guppies were submitted, as happens in the streams of Trinidad (Reeve et al., 2014).

2.4.2. Microscopic anatomy

Histological study of *Poecilia reticulata* gonoducts made during previtelogenese, vitellogenesis and gestation, has observed that gonoducts lack germ cells, cells of the immune system are found in the lumen and mucosa.

The gonoduct is divided into three regions: the head, middle and tail. In the caudal region, there are the exocrine glands and the smooth muscle layer is thicker than in other areas.

During pregnancy cells of the immune system are abundant, the melano-macrophage centers become larger and glands have sloughed cells.

These observations allow us to suggest the following functions for the gonoduct: reduction in lumen diameter; receive and maintain sperm during vitellogenesis; production of secretions more abundant during vitellogenesis; immunological activity throughout the reproductive cycle. The ciliary epithelium and the thick muscle of the caudal region may be involved during delivery (Campuzano-Caballero and Uribe, 2014).

2.5. Lifecycle

Fish present different reproductive strategies whose mechanisms aim the perpetuation of the species, even under adverse conditions.

The viviparity occurs when the fertilization and embryo development are carried out inside the female's body and the embryo has a dependency ratio of the female to nutrition and elimination of metabolites.

The Poeciliidae family shows a direct development and the females have internal fertilization and development where the release occurs only when individuals are able to live independently (Vazzoler, 1996) by assigning the newborn fingerlings**.** The viviparity as a reproductive strategy *Poecilia reticulata* allows them to be a species with reproductive success even in environments altered by Man. The fact that they are viviparous animals also allows them some independence to the environmental conditions (Aranha and Caramaschi, 1999).

The study of the morphology of the reproductive system of fish reveals that females have no oviducts. The ducts of Muller have not developed the caudal region in the ovary - gonoduct, connected to the outside. Due to this absence of oviducts in viviparous fish, embryos develop in the ovary, being this a single example of intra ovarian pregnancy among vertebrates (Campuzano-Caballero and Uribe, 2014).

A particularity of the fish of the Poeciliidae family consists in the possibility of the female being able to store sperm in the ovary wall for about 10 months which allows them fertilizations that can give 8 - 9 consecutive spawns. This feature allows to enlarge the playback rate of these fish.

The phenomenon of "superfetation" was observed in guppy female - females show various stages of development of the eggs and embryos in same gestation, or while some eggs are ready to hatch, other are in early stages, maintaining a continuous cycle (Matthews and Magurran, 2000). This contributes to the reproductive success of guppies, as it reduces the risk of exposure of baby fish to predators. Another contribution to reproductive success in guppies is that the reproductive cycle of the females was continuous throughout the year.

The reproductive tactics of guppy, as fertilization and internal development, parental care, the largest number of females, sexual dimorphism and reproduction throughout the year, may have been the target of natural selection over time due to unstable environmental conditions imposed throughout the evolution of the species. These characteristics enable its survival even in degraded environments (Vazzoler, 1996).

2.6. Economic importance

Guppy is a kind of very popular ornamental fish in aquarofilia all over the world. It is a species that can be easily reproduced in captivity with a length of reproductive cycle between 25 to 30 days.

The main producers worldwide of this kind of fish for aquariophily are China Indonesia and India (FAO, 2016). The report published by FAO in 2005 reported that the average annual value of imports of ornamental fish and invertebrates of its members during the period 1994-2003 was 278 million U.S. dollars (FAO, 1996-2005).

The United States was the largest importer of ornamental fish in the world in 2006, buying $48.3 million, followed by the UK with imports of $30.8 million and Japan with $27.2 million (Ribeiro, 2008).

Guppies (*P. reticulata*) are the species preferentially imported by the United States (Ribeiro, 2008).

This tropical fish is considered the most popular due to its beauty, easy maintenance and reproduction, especially for beginners of fishkeeping. Guppies are able to tolerate large populations, high temperature variations, water pH, and salinity. There are a large number of varieties of guppies (greater than 40), which are marketed as a result of hybridization of the species and artificial selection. They are also called rainbow fish because of the numerous colors and patterns of the tail and body (Watson and Shireman, 2002). Because of their wide dispersal in natural habitats and their generalized knowledge in different continents, guppies are, for the reasons stated above, a species of high economic value.

2.7. The use of guppies in research

The guppies are used as a model in animal experiments in different contexts: ecological, toxicological, genetic, evolutionary, cytological, cytochemical reproductive and behavioral studies and comparative anatomy, etc.

The **table 2** shows the quantified work performed with guppies since the early 20th century to the present year (January 30, 2018).

The **figure 5** represent the articles by subject area (% of total) published since 1950 until January 30, 2018, about different thematic areas of knowledge using guppy.

The **Table 3** shows the number of articles included in major Web of Science (WOS) categories published since 1950 until January 30, 2018. The variation of the number of articles in each thematic area over time are shown in **figure 6**.

The research in WOS was done using the designations Guppy or *Poecilia reticulata* or *Lebistes reticulatus.*

Table 2 - Number of articles published over time, using the Guppy fish as a study target. Data collected from the main collection of Web of Science (A) and all databases (B).

Period	Web of Science (A)	All databases (B)
1900-1950	43	54
1951-1960	38	72
1961-1970	100	151
1971-1980	99	147
1981-1990	180	218
1991-2000	236	252
2001-2010	383	423
2011-2017	358	390
Total	**1437**	**1707**

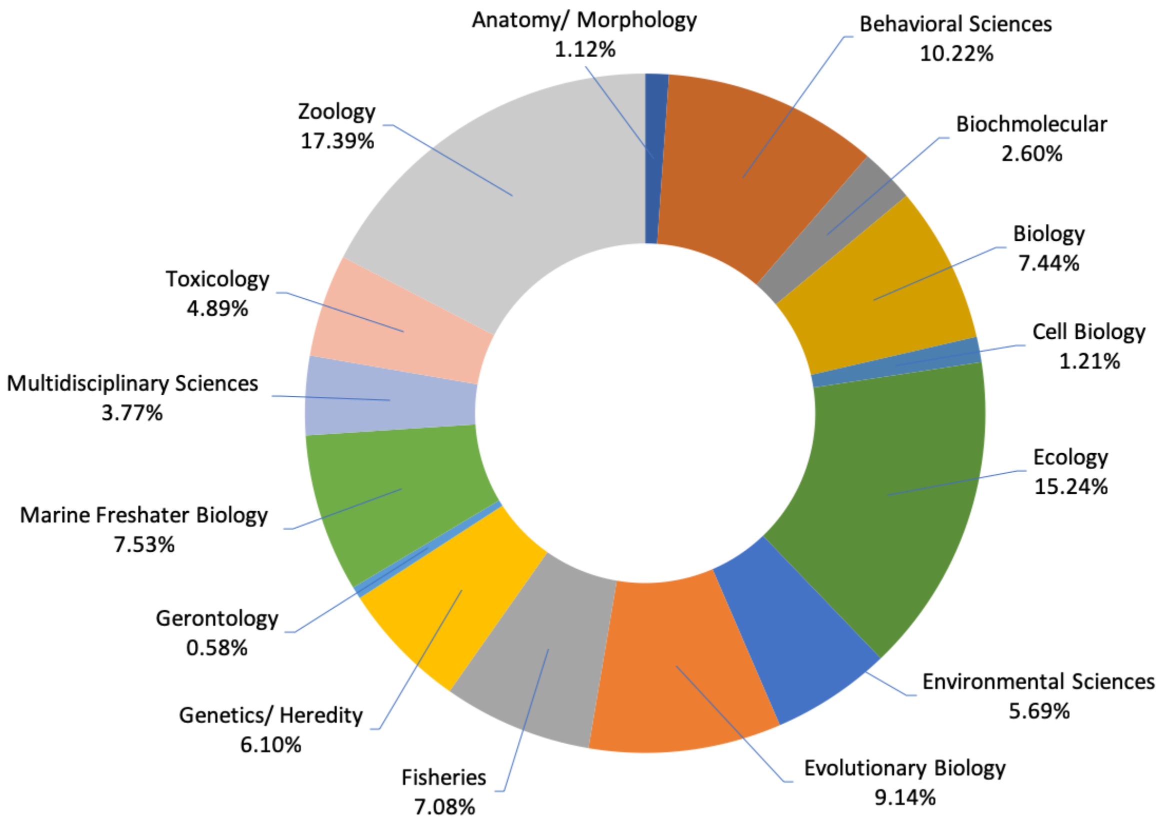

Figure 5 – Articles by subject area (% of total) published since 1950 until January 30, 2018, about different thematic areas of knowledge using guppy.

Table 3 - Number of articles (NA) using the Guppy fish as a model, distributed by major categories of research, over time. Data indexed in Web of Science and collected on January 30, 2018.

Period (NA)	Biology/ Environmental Sciences	Genetics/ Heredity	Zoology	Anatomy/ Morphology/ Cell Biology	Multidisciplinary Sciences	Evolutionary Biology	Ecology	Marine Freshater Biology	Gerontology/ Behavioral Sciences	Biochmolecular	Fisheries	Toxicology
1900-1950 **(43)**	9	7	7	5	5	4	3	3	-	-	-	-
1951-1960 **(38)**	17/-	2	3	-/1	8	3	2	3	1	-	-	-
1961-1970 **(100)**	21	5	24	6	16	1	2	3	6	11	7	1
1971-1980 **(99)**	5/14	12	22	1/10	5	4	7	11	1	7	12	9
1981-1990 **(180)**	3/29	17	43	5/12	3	13	28	35	3	9	16	31
1991-2000 **(252)**	26/26	14	80	3/1	8	34	61	30	2/54	5	33	25
2001-2010 **(383)**	46/32	42	109	3/1	9	81	129	52	/90	17	60	21
2011-2017 **(358)**	39/26	37	100	2/2	30	64	108	31	/84	9	30	22
Total NA (1453)	**166/127**	**136**	**396**	**25/27**	**84**	**204**	**340**	**168**	**13/228**	**58**	**158**	**109**

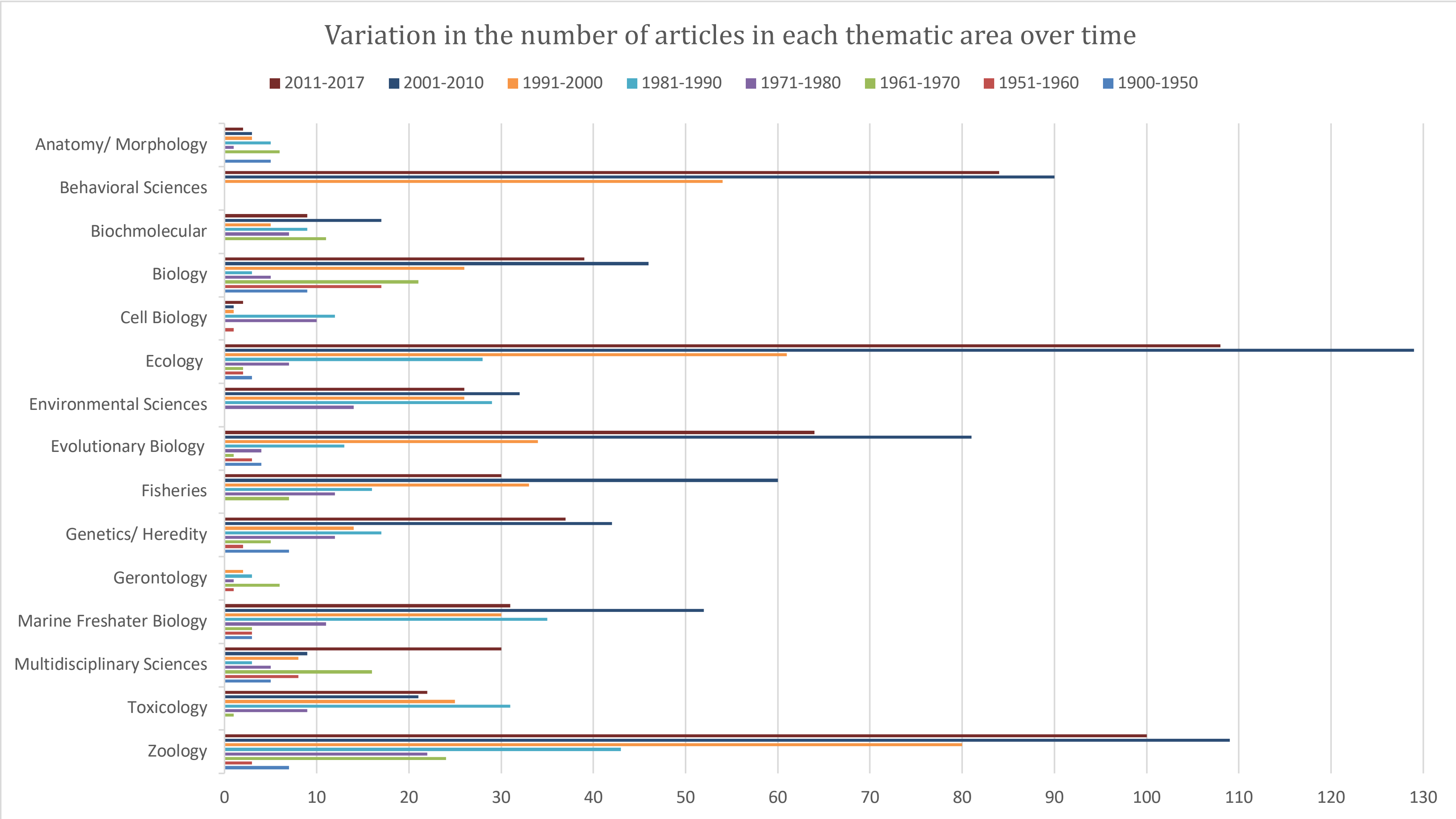

Figure 6 - Variation in the number of articles in each thematic area over time.

The analysis of the data shown in **Fig. 5** and **Fig. 6** allows us to verify that the guppy fish has been used as an experimental model in several areas of scientific knowledge. Highlighting the areas of Ecology and Zoology whose number of articles was greater than 100 in the two last mentioned time intervals. The data also show that the studies in the areas of Anatomy and Morphology are the smallest in the number of articles published. The number of articles suffered a significant increase from the decade 80 of the XX century for the majority of the study areas referenced. It was also noted that the maximum number of publications for most of the thematic areas referred is in the time span from 2001 to 2010.

The criterion used to choose the articles in each decade was the number of citations, and so the 3 articles with the highest number of citations were selected independently of the thematic area to which they referred. This criterion allows observing the diversification of thematic areas that use guppy fish in their research, thus reinforcing the importance of this species to contribute for the development of scientific knowledge.

Time interval: 1900-1950

Although it is not one of the three most cited articles, it is important to point out that the first work using the guppy as an experimental model was made in 1919 (Schmidt, 1919). This was a research work in genetics and the importance of temperature as an external factor that may influence the manifestation of characteristics of fish. The main subject of this research was to know to what extent the quantitative racial characteristics are heritable, using the number of dorsal rays. Another aspect of the research work discussed in this article was to identify the influence of external factors, using the temperature on quantitative characteristic in this study (number of dorsal rays). It is important to note that these research themes will continue to be explored, verifying the existence of work whose object of research is genetics, focused on the research of the influence of environmental factors on this species of fish as well as correlation research works between both, genetic versus environment, over time such as in (Fujio et al., 1990).

In this time span, genetic studies prevailed, using the guppy fish as a model.

The most cited articles in this period were: The location of eighteen genes in *Lebistes reticulatus* (Winge, 1927), number of citations – 143, inserted in the Categories of Web of Science: Genetics and Heredity. One-sided masculine and sex-linked inheritance in *Lebistes reticulatus* (Winge, 1922), number of citations - 82, inserted in the Categories of Web of Science: Genetics & Heredity. The spermatogenesis of *Lebistes reticulatus* (Vaupel, 1929), number of citations - 50, inserted in the categories of the Web of Science: Anatomy & Morphology.

In the most cited article in this time interval, 9 of the eighteen previously discovered genes are located, responsible for transmitting the color in the guppy fish. Of the 18 genes, 9 are located on the Y chromosome and 3 on the X chromosome. One of the genes is located in autosomes. In both sexes, crossing-over phenomena are observed.

In the second most cited article above mentionated (Winge, 1922) an investigation was made to determine how male secondary sex characteristics are transmitted in guppy. In previous work, it was not possible checked any influence of the mother in the transmission of secondary sexual characters of the male children, for this reason it was supposed that the transmission of this characters was made unilaterally of the father to the son. Thus, establishing a relationship between the Y chromosome and the transmission of secondary male sexual characteristics. In this work, the karyotype of the fish was determined, being 46 chromosomes in the somatic cells (44XX female and 44XY male) and 23 in the reproductive cells. No morphological or other differences were observed between the two sexes, allowing the distinction between the sex chromosomes of the autosomes.

In the third most cited article above mentionated (Vaupel, 1929), a study was made on spermatogenesis in guppy. It has been found that germ cells are found in cysts that are younger in the region of the testicular cortex. The number of spermatocyte chromosomes is 23 and may have an X-Y pair. After completed maturation the spermatozoa are stored in the testicular canal and conducted into the female with the help of the modified anal fin, the gonopodium.

Time interval: 1951-1960

The most cited articles in this period were: Observations on Reproduction of the Poeciliid *Lebistes reticulatus* (Peters), (Rosenthal, 1952), number of citations – 43; inserted in the Categories of Web of Science: Marine & Freshwater Biology. Intraspecific sperm competition in *Lebistes reticulatus*, (Hildemann and Wagner, 1954), number of citations – 37; inserted in the Categories of Web of Science: Ecology; Evolutionary Biology. The Inheritance of certain color patterns in wild populations of *Lebistes reticulatus* in Trinidad, (Haskins and Haskins, 1951). number of citations – 34; inserted in the Categories of Web of Science: Ecology; Evolutionary Biology; Genetics & Heredity.

In the most cited article in this decade, aspects related to the reproduction of guppy were studied. The cyclic production of oocytes by females was verified; the time interval required for copulation to occur after joining females and males in the same aquarium was determined (12 to 24 hours). The fertilization time of the eggs was also determined.

The second most cited article above mencionated (Hildemann and Wagner, 1954) quoted the subject under study is the intraspecific competition between sperm for the reproductive success of virgin females. It has been found that reproductive success is not always due to the more recent sperm. The sperm competition depends on the potency of the individual males and also seems to be due to the different receptivity of the female to each male.

In the third most cited article (Haskins and Haskins, 1951). an analysis is made on how the color transmission of the male fish is made in wild populations of guppy in Trinidad. It has been found that the genetic transmission of the different color patterns appears to be linked to genes located on the Y or X chromosomes or in the autosomes. Complex patterns appear to be controlled by a single pair of genes or by several linked together.

The bright male patterns seem to have an advantage in selection for copulation, however they make these males more vulnerable to predation, thus constituting a negative factor. It may be predicted that fish exhibiting X-linked or autosomal patterns tend to be dominant in environments with high predation, while those attached to the Y chromosome are dominant patterns in populations that are protected from predators.

Time interval: 1961-1970

The most cited articles in this period were: An Electron Microscope Study of Yolk Formation During oogenesis in *Lebistes reticulatus* Guppy (Droller and Roth, 1966), number of citations – 164; inserted in the Categories of Web of Science: Cell Biology. Effect of delayed and resumed growth on longevity of a fish (*Lebistes reticulatus*, Peters) in captivity, (Comfort, 1963), number of citations – 75; inserted in the Category of Web of Science: Gerontology. Effects of food supply on fecundity in the female Guppy, Lebistes-Reticulatus (Peters), (Hester, 1964); number of citations – 68; inserted in the Categories of Web of Science: Fisheries; Oceanography.

The article with the largest number of citations (Droller and Roth, 1966) refers to an investigation of the structural changes that occur in the development of Guppy oocytes. The alterations seem to be related to a set of different processes that occur inside and outside the oocytes and explain the synthesis and deposition of the protein yolk in the oocytes.

In the second most cited article above mencionated (Comfort, 1963) a study was done on the effect of slow growth on the longevity of Guppy in captivity. The fish were fed time apart, that is, once every 15 days, compared to the control group receiving the same amount of feed once a day. It was found that the females belonging to the experimental group had a life expectancy greater than 200 days at birth compared to the control group. These results have shown that the spaced feeding of these fish has a positive effect on their longevity.

In the third article, most cited listed above (Hester, 1964) a study was made on the influence of the amount of food available on the fecundity of Guppy females. Over three consecutive periods different amounts of feed were provided to 27 females. The amounts of food ranged from almost starvation to a normal amount. It was found that the number of oocytes that grew to reach larger size as well as the number of offspring was influenced by the amount of feed. Thus, reduced rations resulted in a reduced number of oocytes reaching larger sizes as well as a reduced number of offspring.

Time interval: 1971-1980

The most cited articles in this period were: Natural-Selection on color patterns in *Poecilia reticulata*, (Endler, 1980); number of citations – 837; inserted in the Categories of Web of Science: Evolutionary Biology; Genetics & Heredity. Toxico-Kinetics in fish – accumulation and elimination of 6 chlorobenzenes by guppies (Könemann and van Leeuwen, 1980), number of citations – 170; inserted in the Categories of Web of Science: Environmental Sciences. Social-Behavior patterns as determinants of reproductive success in the Guppy, *Poecilia reticulata* Peters (Pisces, Poecilidae) – an experimental-study of the effects of intermale competition, female choice, and sexual selection (Farr, 1980), number of citations – 145; inserted in the Categories of Web of Science: Behavioral Sciences; Zoology.

In the most cited article (Endler, 1980), experiments were performed to verify the relationship between predation intensity and coloring patterns. Populations living on coarser substrates have patterns with larger dimensions than those living on thinner substrates within the same level of predation. It has been observed that guppies have lower color intensity patterns in habitats with higher predation intensity, showing higher color intensity when predation is minimal. Sexual selection increases color standards. The complexity of guppy fish color patterns can have diverse origins. Natural selection acts differently on different components of color patterns.

In the second most cited article of this period (Könemann and van Leeuwen, 1980) a study was made on the kinetics of six chlorobenzenes in guppies. The constants of the rates of absorption, elimination and bioaccumulation were determined. A parabolic curve seems to be the best way to describe the ratio of the rate of absorption as well as the bioaccumulation.

In the third article quoted above mentionated (Farr, 1980) a study was made on the influence of several characteristics of the social behavior as well as the coloration of the male guppies on their reproductive success. It was verified that the most important determinant was the female courtship rate for males. The higher the display rate, the greater the likelihood of finding a female subject. The color of males is also a feature that influences the choice of females. These choices determine greater heterogeneity of offspring leading to polymorphous populations.

Time interval: 1981-1990

The most cited articles in this period were: The impact of predation on life-history evolution in Trinidadian Guppies (*Poecilia reticulata*) (Reznick and Endler, 1982), number of citations – 575; inserted in the Categories of Web of Science: Ecology; Evolutionary Biology; Genetics & Heredity. Correlated evolution of female mating preferences and male color patterns in the Guppy *Poecilia reticulata*, (Houde and Endler, 1990); number of citations – 329; inserted in the Category of Web of Science: Multidisciplinary Sciences. Predation light-intensity and courtship behavior in *Poecilia reticulata* (Pisces, Poecilidae), (Endler, 1987); number of citations – 323; inserted in the Categories of Web of Science: Behavioral Sciences; Zoology.

In the first most cited article (Reznick and Endler, 1982), of this decade, a study was undertaken to explore the implications of predation in the evolutionary history of Guppy. Thus, they were submitted to three different levels of predation: high intensity, in the locality of Crenicichla, medium intensity, in the locality of Rivulus and low intensity, in the locality of Aequidens. It was found that Guppies subjected to the highest level of predation increased their reproductive investment relative to the others, devoting a greater percentage of their body weight to the development of the offspring. The Guppies of the localities of Aequidens present intermediate characteristics between the localities of Crenicichla and Rivulus or similar to those of the last-mentioned locality in the various statistics of the history of the life. The results show that predators play a decisive role in modeling the evolutionary history of guppies.

The second article most cited in this period (Houde and Endler, 1990) investigates the correlation between females' preferences and male color patterns. Sexual selection may explain the development of some secondary sexual characteristics of males. The female`s preference favors the evolution of more intense colors of the males, however the color patterns present a great variability among the populations. This preference in mating by females will also have led to some of the less preferred male traits not having evolved. Thus, there seems to be a positive correlation between the evolution of some of the characteristics of males with the degree of preference manifested by females.

In the third work, most cited above mencionated (Endler, 1987) the influence of predation and light on male behavior is investigated. The color patterns of the guppies, *P. reticulata*, result from a balance between selection by predators and selection by females. This study investigated the effect of predation and light on male courtship behavior. The courtship behaviors are smaller in the presence of predators and in bright light. Thus, males intensify their cutting behaviors when the period of the day presents less intensity of light, being also minimal in this period the visual predation. When light intensity is greater and the risk of predation also males use less visible courtship strategies. The visibility of color patterns in males results from an evolutionary balance between color patterns that reduce the risk of predation and those that increase visibility for females.

Time interval: 1991-2000

The most cited articles in this period were: Evaluation of the rate of evolution in natural populations of guppies (*P. reticulata*), (Reznick, 1997); number of citations – 533; inserted in the Categorie of Web of Science: Multidisciplinary Sciences. Geographic-variation in female preferences for male traits in *Poecilia reticulata*, (Endler and Houde, 1995); number of citations – 468; inserted in the Categorie of Web of Science Ecology; Evolutionary Biology; Genetics & Heredity. Variation in the appearance of Guppy color patterns to guppies and their predators under different visual conditions, (Endler, 1991); number of citations – 322; inserted in the Categorie of Web of Science: Neurosciences; Ophthalmology; Psychology.

In the most cited article of this period (Reznick, 1997) the research aimed to evaluate the rate of evolution in natural populations of guppies. It was found that the evolution rates were similar in natural and artificial environment for the same characteristics under study. However, they were significantly higher than the rates inferred from the fossil record. Another conclusion was that the rate of evolution of the male characteristics was superior to that of the female characteristics, perhaps because the males present a greater number of characteristics on which natural selection can act. These results came to show the importance of natural selection in the process of evolution.

In the second work above mencionated (Endler and Houde, 1995) it was done a study in 11 localities of Trinidad to evaluate the preferences of female guppies in relation to some of the characteristics of males: pattern of color, shape and size of the body and contrast between color and brightness. It was verified that females prefer males belonging to their own population and that among the different populations the selection criteria used by females differ not only in number but also in intensity. It was also found that the color of water has a possible effect on the intensity of predation. The discussion of the results is made based on the models of sexual selection and with reference to the first stages of speciation.

In the third article (Endler, 1991) the investigation had as objective to verify if the variation in the color patterns of the guppy fish is influenced by the presence of its predators. Field work was done to measure the visibility of guppy fish under different ambient light conditions. The results suggest that fish have more visible color patterns at courtship sites and have less visible color patterns in places where the risk of predation is highest. These results will have implications for the evolution of the guppy vision and behavior.

Time interval: 2001-2010

The most cited articles in this period were: Life-history evolution in guppies. VII. The comparative ecology of high- and low-predation environments, (Reznick et al., 2001); number of citations – 253; inserted in the Categories of Web of Science: Ecology; Evolutionary Biology. Effect of extrinsic mortality on the evolution of senescence in guppies, (Reznick et al., 2004); number of citations – 242; inserted in the Category of Web of Science: Multidisciplinary Sciences. Female guppies agree to differ: Phenotypic and genetic variation in mate-choice behavior and the consequences for sexual selection, (Brooks and Endler, 2001). number of citations – 192; inserted in the Categories of Web of Science: Evolutionary Biology; Genetics & Heredity

In the most cited article above mencionated (Reznick et al., 2001) a study is made to evaluate the indirect influence of several environmental parameters on the rate of predation of guppies. This work is based on the results of previous studies associating the evolutionary history of guppies with the presence of predators. In the present work, different physical, chemical and biological parameters were quantified in seven locations with high and low levels of predation. It was verified that the environments with greater intensity of light, greater primary production and therefore with greater availability of food, are associated with higher rates of predation. In these environments, there is a greater number of small individuals and smaller numbers of larger individual’s due to a higher birth rate and a higher mortality rate. Thus, the difference in food availability that is directly related to environmental factors, influences the rate of predation and consequently condition the evolutionary history of the guppies.

In the second most cited article of this period (Reznick et al., 2004) a study is carried out to evaluate how populations of guppies that have low mortality rates associated with external factors such as diseases or predation can evolve towards the beginning of cellular aging. Previous studies have shown that in environments with higher rates of predation there is a higher birth rate, because individuals invest more in reproduction, but have higher mortality rates. In this work, it was verified that these populations do not initiate aging earlier but manifest an early onset of the neuromuscular function responsible for the movement of fish. These results are not in agreement with the generalization that a lower mortality caused by extrinsic causes leads to a late senescence of the individuals.

In the third most cited article listed above (Brooks and Endler 2001) a study was made to evaluate if the variation in the selection of the males by the females influences the evolution by sexual selection. The repeatability and heritability were chosen in the choice of females of two components of feminine variation, responsiveness and discrimination, and the preferences of the females in relation to the ornaments of the males. It has been found that there appear to be several male ornaments that females generally prefer and others whose preference differs among females. It has been found that there is no universally attractive male phenotype. The variation in the response capacity of the females seems to be the main biological source of the phenotypic and genetic variation in the behavior of the male selection. The models of sexual selection that incorporate the individual characteristics of the females in the choice of the male do not improve the prediction of the results in relation to the models that do not make this incorporation.

Time interval: 2011-2017

The most cited articles in this period were: Artificial Selection on Relative Brain Size in the Guppy Reveals Costs and Benefits of Evolving a Larger Brain, (Kotrschal et al., 2013); number of citations – 127; inserted in the Categories of Web of

Science: Biochemistry & Molecular Biology; Cell Biology; Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy, (Gasparini and Pilastro, 2011); number of citations – 71; inserted in the Categories of Web of Science: Biology; Ecology; Evolutionary Biology; Evidence for Two Numerical Systems That Are Similar in Humans and Guppy, (Agrillo et al., 2012); number of citations – 67; inserted in the Category of Web of Science: Multidisciplinary Sciences.

In the first most cited article of this period (Kotrschal et al., 2013) a study was made to evaluate the costs and benefits of increasing brain size using guppy fish as a model. Live guppy fish were artificially selected with large and small brain size relative to body size and it was found that relative brain size evolved rapidly in response to divergent selection in both sexes. Tests to test cognitive ability have shown that large brain females outweigh small brain females; especially in males, it is found that those with large brains develop smaller intestines and produce fewer offspring. Following these results, the authors propose that the evaluation of brain size evolution be evaluated by cognitive enhancement and reproductive capacity and performance with further discussion on the implications of these results on vertebrate brain evolution.

In the second article above mencionated (Gasparini and Pilastro, 2011) the preference of females for males genetically unrelated to ovum fluid was measured. It was hypothesized that polyandry evolves as a way to reduce the negative physical consequences resulting from mating with genetically related males. Experimental work showed that when female guppies was artificially inseminated with the same number of spermatozoa from related and unrelated males, the success of sperm competition in the first case was on average 10% lower than in the second. Sperm mobility, a factor for sperm competition success in guppy, was also found to be much lower when measured in a solution containing ovarian fluid of a sister female compared to the rate measured in a solution with ovarian fluid of a female not genetically related. This work showed the importance of sperm interaction in the measurement of the trend of sperm competition for unrelated males, giving importance to post copulatory factors to reduce the costs of mating with related males.

The third most cited article (Agrillo et al., 2012) in this time interval is based on the previous assumption that humans and nonhumans share a near nonverbal system to represent and compare numbers with each other, whose accuracy does not depend on numerical reason. Thus, a study was made to compare the ability to discriminate the same numerical indices in a small range of numbers and beyond, between a group of students and the guppy fish. Performance was found to be independent of the index within the range of 1-4, steadily increasing with the numerical distance. The results show that quantitative discrimination is common to humans and fish leading to the belief that human mathematical abilities may have resulted from a long-standing evolution that originated prior to the phylogenetic divergence between bonefish and tetrapod. This study showed evidence that the two numerical systems, for humans and fish, are similar.

2.8. Conclusion

The guppy (*P. reticulata*) is a flagship tropical fish that is native from Central America and northeast South America. It is very abundant year-round in shallow waters of the canals, rivers, lagoons, reservoirs and estuaries of northeastern regions of Venezuela, Trinidad and Tobago. It became one the favorite (often the most) ornamental species for hobbyists.

Due to their morphological, physiological, behavioral and ecological characteristics, guppies have been used over time in many studies as an experimental model in various fields of research. One emphasizes its importance in many research areas such Biology and environmental Sciences, Genetics, Zoology, Anatomy, Morphology, Cell Biology, Multidisciplinary Sciences, Evolutionary Biology, Gerontology and Behavioral Sciences.

Over time, since the early twentieth century to the present day, 1453 studies have been indexed in Web of Science as using guppies as a target organism, in several research areas, until January 30, 2018. Of note the areas of Ecology and Zoology which include a higher percentage of works. Over time, the use of guppy suffered a significant increase from the 80's decade of the XX century for the majority of the study areas referenced. Advances in technology and growing concerns with social welfare and ecosystems health in our days, are likely to be the reasons behind the growing use of guppy as a model in scientific reseIt is also noted that the maximum number of publications for most of the thematic areas referred is in the time span from 2001 to 2010. This data makes it possible to establish a positive correlation between advances in technology and the consequent advances in scientific research that have been felt since the beginning of the 21st century.

This fact points out the importance of the work done with guppies specially in ecology and health areas. Intrinsically, this is a well-adapted species not only in its natural habitat but also in innumerous locations where it was successfully introduced, enabling it to become widely available fish worldwide (Reznick et al., 2012).

Guppies introduced into the environment can become an invasive species causing changes in the composition of ecosystems. Due to its high level of tolerance to environmental factors and of survival in adverse conditions (Chung, 2001; Reeve et al., 2014) may win in the competition with native species that share the same ecological requirements, leading to their decline over time. This is an issue that should be investigated in the future: the reasons that lead the guppy to be a species very resistant to unfavorable environmental conditions, as well as studies which may be able to establish the correlation between the presence of this species in natural environments as a possible bioindicator of poor environmental quality.

This species has a high tolerance to adverse environmental factors of which the temperature was highlighted since the tolerance to this environmental factor is especially important in the global warming era.

3. Objectives

Fish liver is a gland of special relevance on the control of several vital functions and is therefore used as a biological target to evaluate the health status of wild and cultivated populations. To understand liver changes induced by exogenous factors, the normal development, structure and function of the organ must be well-known. However, there are few studies regarding many aspects of the microscopic morphology of this fish gland, in particular about the spatial structural organization of non-hepatocytic components and quantitative perspectives on how the organ grows and regenerates.

The guppy fish (*Poecilia reticulata*) was chosen as our model of study because it is one of the model organisms most used in several scientific research areas. Accordingly, there is some good bibliographic background about the species and an interested research community, spanning from ecology to the biomedical field. Additionally, the guppy is of economic importance in the ornamental fish industry.

Overall, the general objective of this Thesis was to contribute to a more detailed histological knowledge of the guppy liver, allowing not only the disclosing of new fundamental data but also the comparison of the guppy with other fish species; eventually identifying variations of the liver structure in different teleost species. We were particularly interested in shedding more light on the spatial distribution of blood vessels and bile ducts, starting from portal vessels and up to hepatic veins.

One of the specific objectives of this study was to understand certain aspects of the ontogenic development of the guppy liver, from a qualitative and quantitative perspective, by making a histological analysis of this fish organ with different ages: 7, 17 and 28 days and in adult fish. The qualitative evaluation was made from the microscopic observation of serial sections of the whole liver, with thicknesses of 7 and 25 μm. The structural targets were the distribution of the venous and arterial vessels and the bile ducts, an issue that is still very poorly understood in fish. The quantitative evaluation tackled another fundamental problem: how the liver grows, particular at young ages. Does it enlarge soon after birth or later on? Is there hyperplasia and/or hypertrophy of hepatocytes? To start answering these questions a study was made in fish with 7 and 17 days old, using design-based stereological analysis. This estimated the liver volume and the number and cellular volume of the most abundant constituents of the hepatic parenchyma - the hepatocytes.

The liver of the adult guppy fish presents in its interior exocrine pancreas, being often designated by this reason as hepatopancreas. Taking into account this fact, it was also a specific objective of this work to know the spatial distribution of the pancreas and to understand when it appears inside the liver. Does it already exist immediately after the liver is formed? Does it appear inside the liver by migration from the exocrine pancreas? Is it possible to have intra-hepatic cell differentiation in pancreocytes either during the embryonic development and/or later after birth?

In many fish species, including the guppy, juveniles and adults normally present intrahepatic aggregates of pigmented macrophages, usually in the vicinity of blood vessels and bile ducts. Even so, the exact age of appearance of those aggregates is unknown, both in guppy and other fish species. As such it was also one of the specific objectives of this study to determine whether or not these defence cells are found in very early life stages of the guppy. The conglomerations of macrophages are expected to appear during the fish life, namely as one defence mechanism against bacteria or parasites that cause chronic infections, or as adaptive changes when environmental conditions are not favourable and impose functional stresses.

Finally, for every main objective, it was our parallel (sub-) objective to determine if there were differences between the sexes in the qualitative and quantitative analyses in the liver of the guppy. This seemed opportune taking into account that males and females of other fish display morphofunctional hepatic differences, at least at points of their life where they have to deal with functional reproductive needs. Anyway, there is no knowledge if there are differences in early life stages.

Once the above-mentioned objectives are achieved they will lead to a more detailed knowledge of the microanatomy of the guppy liver, allowing establishing better foundations for future evaluations of histological and functional alterations that this organ may present, particularly in view of the range of changes that can be triggered either by natural biotic-abiotic factors or by environmental pollutants.

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CHAPTER II

Characterization and spatial relationships of the hepatic vascular–biliary tracts, and their associated pancreocytes and macrophages, in the modelfish guppy (*Poecilia reticulata*): A study of serial sections by light microscopy

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Characterization and spatial relationships of the hepatic vascular–biliary tracts, and their associated pancreocytes and macrophages, in the model fish guppy (*Poecilia reticulata*): A study of serial sections by light microscopy

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A B S T R A C T

The guppy is a tropical fish that has been used as an experimental model organism in science. It is a species well adapted to the natural environment and that can support adverse environmental conditions, and so, at occasions, its presence can be indicative of environmental disturbances. Moreover, as the liver is very important when studying fish diseases, the knowledge of normal microanatomy is essential to assess histological changes, e.g., related to environmental change or toxic pollutants. The target organ of this histological study is the liver. The main objective is to contribute to the identification of anatomical and structural variations of this organ in different teleost species. We studied the distribution and spatial organization of the different types of blood vessels and biliary ducts and the relationships between them are established. For this, each liver was totally sectioned and the serial sections inspected in detail. The guppy liver presented intra-hepatic pancreatic tissue and so reported its association with the vascular and biliary elements. We observed that the input of afferent vessels (i.e., bringing blood into the liver) occur not only in the hilum but pierce and enter the organ at various points. Within the liver, venous vessels and bile ducts are seen, isolated or associated as venous–arteriolar tracts (VAT), and venous-biliary- arteriolar tracts (VBAT). Sometimes, pancreocytes appear within the liver surrounding isolated veins, forming venous tract with pancreatic acini (VT-P), or dual associations with afferent vessels, forming venous–arteriolar tracts with pancreatic acini (VAT-P). Intrahepatic pancreatic ducts were tiny and rare, putting in question the functional role of the acini. Contrary to other fish species, we did not spot isolated arterioles and associations between these and biliary ducts (BAT).We found aggregates of macrophages, namely associated with afferent and efferent (i.e., draining blood out) venous vessels; the latter fact not commonly reported in other fish species. There was a reduced arterialization of the organ (as arterioles were extremely rare), contrasting with an over predominance of a random distribution of the venous vascularization. The guppy differs to some extent from other previously studied models, highlighting the importance of making this kind of study to offer specific frameworks that can explain specific physiological processes or avoid misinterpretations; for instance about gene expression, as the whole liver specific expression will reflect the presence of hepatocytes and pancreocytes as well.

1. Introduction

The guppy (*Poecilia reticulata*, Peters, 1859) is a tropical fish and is one of the most popular freshwater aquarium fish species in the world (Monticini, 2010). The natural original range of the guppy is Central America and northeast South America, more specifically Venezuela, Trinidad and Tobago (Endler, 1978; Reis et al., 2003). Due to their morphological, physiological, behavioural and ecological characteristics, the guppy has been targeted in field studies, for instance, related to speciation and environmental adaptations (Alexander and Breden, 2004; Milenković et al., 2014).

Additionally, the guppy is one of the widely used model fish in laboratories (e.g. Bayley et al., 2002; Russell and Magurran, 2006), and has been used as a strategy of biological control agent against mosquitoes (Elias et al., 1995; Kusumawathie et al., 2008). This is a species well adapted to the natural environment and that can tolerate adverse

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environmental conditions, so its presence can be indicative of environmental disturbances (Reznick et al., 2012). In accordance with the cited good tolerance, some studies have demonstrated that despite guppies are useful in carcinogenicity evaluations (Hawkins et al., 2003) – which mainly (but not only) exposed liver neoplasms, particularly hepatocellular adenomas or carcinomas, and cholangiomas or cholangiocarcinomas – the cited species is less sensitive to carcinogens than other models, such as the medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). This was demonstrated, e.g., for the carcinogens dimethylnitrosamine, diethylnitrosamine, and nitrosomorpholine (Khudoley, 1984), for methylazoxymethanol acetate (Hawkins et al., 1985), and nitromethane, 1,2,3-trichloropropane, and for propanediol (Kissling et al., 2006). Overall, such its intrinsic characteristics make the guppy prone for diverse uses.

The target organ of this study is the liver, in a perspective that was not evaluated yet in the species. This work aims to be another contribution to the identification of the anatomical and structural variations of the liver of fish species, now known to consist of 31,000 (Eschmeyer et al., 2010). In fact, previous studies with other species (e.g., Nile tilapia, *Oreochromis niloticus*, and Indian flying barb, *Esomus danricus*) had shown that these variations are not only interspecific but also may exist within the same species, varying with gender, age, water temperature, or hormonal changes related to the period of life cycle (Rocha and Monteiro, 1999; Rocha et al., 2003, Figueiredo-Fernandes et al., 2007; Das et al., 2013). This knowledge is very important to be able to identify liver changes, such as those caused by chemical pollutants, and structural adaptations of that organ to the environmental pressures. Accordingly, it is established that histopathology offers useful data in characterizing toxic effects in fish (Wester and Canton, 1991; Huggett et al., 1992; Watiporn et al., 2011; Dogan et al., 2012; Das et al., 2013).

The liver has a crucial importance in the metabolism of virtually all substances that reach it through the bloodstream and can offer excellent histological biomarkers (Giari et al., 2007), through analysis of microanatomical and immunophenotypic changes, namely those induced by environmental contaminants, such as heavy metals, pesticides, pharmaceuticals and other (Amaral et al., 2002; Dogan et al., 2012; Das et al., 2013). Importantly, exposed guppy may exhibit gender-specific histopathological responses (Antunes et al., 2017), and this justifies studies offering fundamental data for both sexes.

The fish liver can make not only hepatic but also pancreatic functions (Rocha and Monteiro, 1999; Torres et al., 2010). Accordingly, fish can present two liver types: either associated with exocrine pancreas – hepatopancreas – or not (Rocha and Monteiro, 1999). In general, knowledge about the fish intrahepatic spatial distribution of blood vessels is much reduced, and there seem to be no published studies of this distribution in guppies. About such structural aspects, we found in depth studies in Nile tilapia (Figueiredo-Fernandes et al., 2007) and brown trout, *Salmo trutta fario* (Rocha et al., 1997). The spatial distribution of intrahepatic vascular–biliary tracts of guppy was designed to identify the efferent and afferent areas of the liver as well to evaluate the possible spatial interaction between the different types of vessels. Tracking sequences of serial sections covering the whole liver allowed us to follow the bile ducts and blood vessels inside the organ, and have a 3D perspective of the relationships among tracts.

Knowledge of the spatial distribution of intrahepatic vascu- lar–biliary tracts of the guppy not only adds to our knowledge of microanatomical features and their contribution to hepatic architecture, but also helps to understand the peculiar species environmental and physiological adaptations, including adaptability to different types of environmental contaminants and tolerance to water temperature (Chung, 2001; Reeve et al., 2014). Our aim here was to serially track venous and arterial blood vessels and biliary ducts across histological sections, to realize their spatial relationships, including convergence and divergence in 3D, and their eventual association with pancreatic acini and macrophages.

2. Material and methods

2.1. Animals

Adult male (n = 6) and female (n = 6) guppy were obtained from a local supplier of specimens raised in an Israel farm. The fish were acclimatized for 3 weeks to laboratory conditions, being kept grouped in glass aquaria, with dechlorinated and continuously filtered and aerated tap water, at a temperature of 24 ± 1 °C, and under a 12:12 h light- dark photoperiod. Animals were fed once a day with TetraMin Tropical Fish Flakes. The studied males measured, on average, 3 cm in total length, and weighed 240 mg. Females were bigger, and had, on average, 4 cm in total length, and weighed 700 mg. Housing and sacrifice of the fish followed the European directive of animal welfare. 2010/63/EU, as managed by the Directorate-General of Veterinary (Law 113/2013).

2.2. Fixation and tissue processing

Animals were euthanized by an anaesthetic overdose, via immersion in a 0.2 ml/l aqueous solution of ethylene glycol monophenyl ether (Merck, Germany). Under a stereo microscope, each liver only was easily and rapidly dissected for fixation *in toto* and subsequent histological analysis. Fixation was made in Bouin's fluid, for 24 h, at room temperature. Then after, the fixed livers were washed in 70% ethanol and then dehydrated through 90% and 100% ethanol, cleared in xylene, and impregnated in high quality paraffin (Paraplast Plus, Sigma-Aldrich); the processing was made using an automatic tissue processor (Leica TP1020, Germany). The samples were then embedded in plastic cassettes using a modular tissue embedding centre (Leica EG 1140H, Germany). The final blocks with the entire liver were totally serially cut into 5 μm thick sections using a fully automatic rotary microtome (Leica RM2155, Germany). Sections were picked in slides coated with aminopropyltriethoxysilane (Sigma A3648). Male livers produced ~400 sections and those of females provided ~900 sections. For an easier detection of the connective tissue associated with blood vessels or biliary ducts, the sections were stained with Masson's trichrome. Slides were mounted with DPX (Sigma-Aldrich), and studied with a light microscope (Olympus BX50, Japan). High resolution images (2560 × 1920 pixels) were taken with a digital camera (Olympus Camera C-5050, Japan).

2.3. Gross and histological aspects and serial section tracking of selected structures

Accordingly with the aims, from the hilum, or other vascular afferent points, we tracked along the serial sections the small venous and arteriolar blood vessels, and the bile ducts. Also, we reversely tracked down isolated veins and smaller starting bile ducts, up to the point they joined other afferent elements or left the organ (as efferent structures). As such, afferent vessels were all those that entered the organ by the hilum or hilar area (recognized by the presence of bile ducts, arterial vessels and pancreatic tissue), and efferent (venous) vessels where all those that emerged from the capillary network, that were not connected with hilar vessels and emerged from the liver at a non-hilar zone. According with the situations of seeing either isolated vascular and/or biliary elements, or associations between them, we named each structure according to the nomenclature established in studies with other fish, namely with rainbow trout, *Oncorhynchus mykiss*, brown trout and Nile tilapia (Hampton et al., 1988; Rocha et al., 1994; Figueiredo- Fernandes et al., 2007). Each vascular and/or biliary element was classified after concluding the visualization of all the series of sections that covered the particular type of structure/association of element(s) being serially tracked. Typically, from the entire series of sections that covered the whole organ, we studied sub-sequences of slides that had the structure(s) of interest, following the path from the start to the end

Fig. 1. Guppy liver isolated after necropsy. The macroscopic observation shows that the organ is devoid of lobation, that is, there are no anatomically distinct lobes.

of the target.

3. Results

Under visual inspection, the liver does not present anatomically distinct lobes (Fig. 1). At light microscopy, the sections were essentially made of hepatocytic parenchyma, being the blood vessels (other than those of the sinusoidal network) and biliary channels disorderly scattered (Fig. 2A–B). Depending on the animal, hepatocytes were either basophilic or almost unstained and very vacuolated (which is typical of liver cells heavy loaded with lipid droplets). About 70% of the animals had a parenchyma that was very rich in the vacuolated hepatocytes. There was variability among individuals as to the hepatocyte cytology, but, overall, no differences could be demonstrated between sexes.

Other structural elements that commonly appeared in the sections, and always positioned in the close vicinity of blood vessels, were "pockets" of serous acini composed of exocrine pancreocytes (Fig. 2C). These could be attached to the adventitia so that they either completely surrounded the adjoining blood vessel(s), typically veins, or encircled only part of it/them. When following such veins by serial sectioning, we noted that almost all of them were afferent, but, very occasionally, some were efferent. So, adventitial pancreocytes were not only collated to afferent but also to efferent veins. The pancreocytes are organized in acini of various sizes. Small and roundish basophilic nuclei are often seen at the centre of acini (Fig. 2C., inset), corresponding to nuclei of centroacinar cells. Extremely rarely, amidst the acini, we observed very tiny channels (with simple low cuboidal epithelium) that are interpreted as pancreatic ducts (Fig. 2C., inset). Despite some of the ducts were tracked in serial sections, we could not confirm that they established a continuous network that would reach the hilar region and far beyond. Additionally, many acini did not seem to connect with any emerging duct. Anyway, the diminutive size of the ducts, coupled with the not excellent resolution offered by paraffin embedding, made those structures extremely hard to trace serially.

Commonly associated to the adventitia of both blood vessels and bile ducts there were also aggregates of pigmented macrophages, of various dimensions. Despite some of these cells occasionally exhibited the cytoplasm with dark/black melanin deposits (Fig. 2D), most of the aggregates showed cells with slightly stained cytoplasm (Fig. 2E).

When tracking the afferent blood vessels and biliary channels it was noticed that despite there was a hilum (Fig. 2F), and so a preferential zone for entrance of both venous and arteriolar vessels, that area was not the only admission zone for such vessels into the organ. Indeed, we registered that extrahepatic afferent blood vessels branches – these being often associated with "sleeves" of pancreocytes – could pierce the very thin Glisson's capsule and enter the organ at extrahilar regions (Fig. 3A–B). Also, biliary channels could emerge out of the organ not as a single hepatic duct but rather as several smaller ducts. Converging and anastomosing of such extrahepatic ducts continued until they en- tered the common bile duct near its entry to the gallbladder (Fig. 3C–D).

Within the liver, we identified isolated vessels or bile ducts and of associations of two to three kinds of vascular-biliary structures, as follows: 1) venous-biliary-arteriolar tracts, (VBAT) (Fig. 3E); 2) venous–arteriolar tracts, with associated pancreatic acini (VAT-P) (Fig. 3F); 3) venous–arteriolar tracts (VAT) (Fig. 4A); 4) isolated bile ducts, named as biliary tracts (BT) (Fig. 2E); 5) isolated veins, named as venous tracts with associated pancreatic acini (VT-P) (Fig. 4B); 6) or without them, named as venous tracts (VT) (Fig. 4C–D). We could neither observe to this date 3D associations of biliary tracts with veins or with arterioles, which would form venous-biliary tracts (VBT) and biliary arteriolar tracts (BAT), respectively, nor did we see isolated arteriolar tracts (AT). As to VBAT, they were very rarely observed (Fig. 3E). Furthermore, the association of the three elements rapidly ended – at most, the triple associations were preserved in the 3D space for ~30 μm in length, after which the arterioles end as capillaries; in vivo the distance should be longer, as paraffin embedment induces shrinking. The VBAT type of tract typically is in continuity with VT – both when arterioles end, as cited, and before the attachment of bile ducts. However, VBAT could also become a VAT (Fig. 4A).

The VAT-P (Fig. 3F) were found much more frequently than VBAT. In VAT-P the pancreatic tissue was seen partially around the vessels. In the same way as for the VBAT, arterioles and veins in VAT-P are seen together for short distances in 3D (~30 μm). When the arteriole disappears from VAT-P, the vein continues as VT-P (Fig. 4B) or VT, for long paths. A VAT-P entered the liver already as such kind of association/tract (Fig. 3A–B). Once losing the pancreatic sleeve, the VAT-P becomes a VAT (Fig. 4A). Despite in singular sections we could spot profiles of what seem to be various arterioles, when making the serial section-tracking both VAT-P and VBAT had a single arteriole.

The BT were quite frequent, either seen in longitudinal (Fig. 2E) or in cross sections (Fig. 4E); individual biliary ducts rapidly merged into tracts with several of those channels. In contrast, we did not encounter VBT (and VBT-P), but, despite this fact, we saw very occasionally that a vein and a bile duct could cross almost orthogonally in 3D space, forming a "fugacious VBT" (Fig. 4F). In these, the vein and bile duct become isolated again (running as single tracts) after the "crossing zone". Because veins of the "fugacious VBT" emerged from the capillary network they were efferent in what regards intrahepatic blood flow.

For summarizing the observations made in this study, a stylized model of the 3D spatial distribution of the vascular–biliary tracts, and "sleeves" of associated pancreatic acini, was created (Fig. 5). No differences existed between sexes, and so the model is unique.

4. Discussion

As far as we observed, the liver of the guppy does not present anatomically distinct lobes, contrarily to the liver of other fishes, in which clear divisions into lobes are seen, such as the three lobes reported in Teleostei, for instance in the characid lambari, *Astyanax altiparanae*, and in the cyprinid zebrafish (Bertolucci et al., 2008; Yao et al., 2012). Anyway, there are species with only two liver lobes, such as in the cichlid Nile tilapia (Vicentini et al., 2005). However, the absence of lobation is verified in other species of fish (Truslé and Anadon, 1996), including members of quite diverse families, such as the Japanese medaka *Oryzias latipes* (Okihiro and Hinton, 1999), of the Oryziinae family, the tropical catfish *Trichomycterus brasiliensis* (Ribeiro and Fanta, 2000), of the Trichomycteridae family, or the platyfish, *Xiphophorus hellerii* (Ferreira, 2011), of the Poecilidae family, like the guppy. A propos, detailed search on literature reveals that no specific information is available regarding the other common species of Poecilidae. Such lack of information about poeciliids hampers interspecific comparisons regarding the detailed liver anatomy and microanatomy.

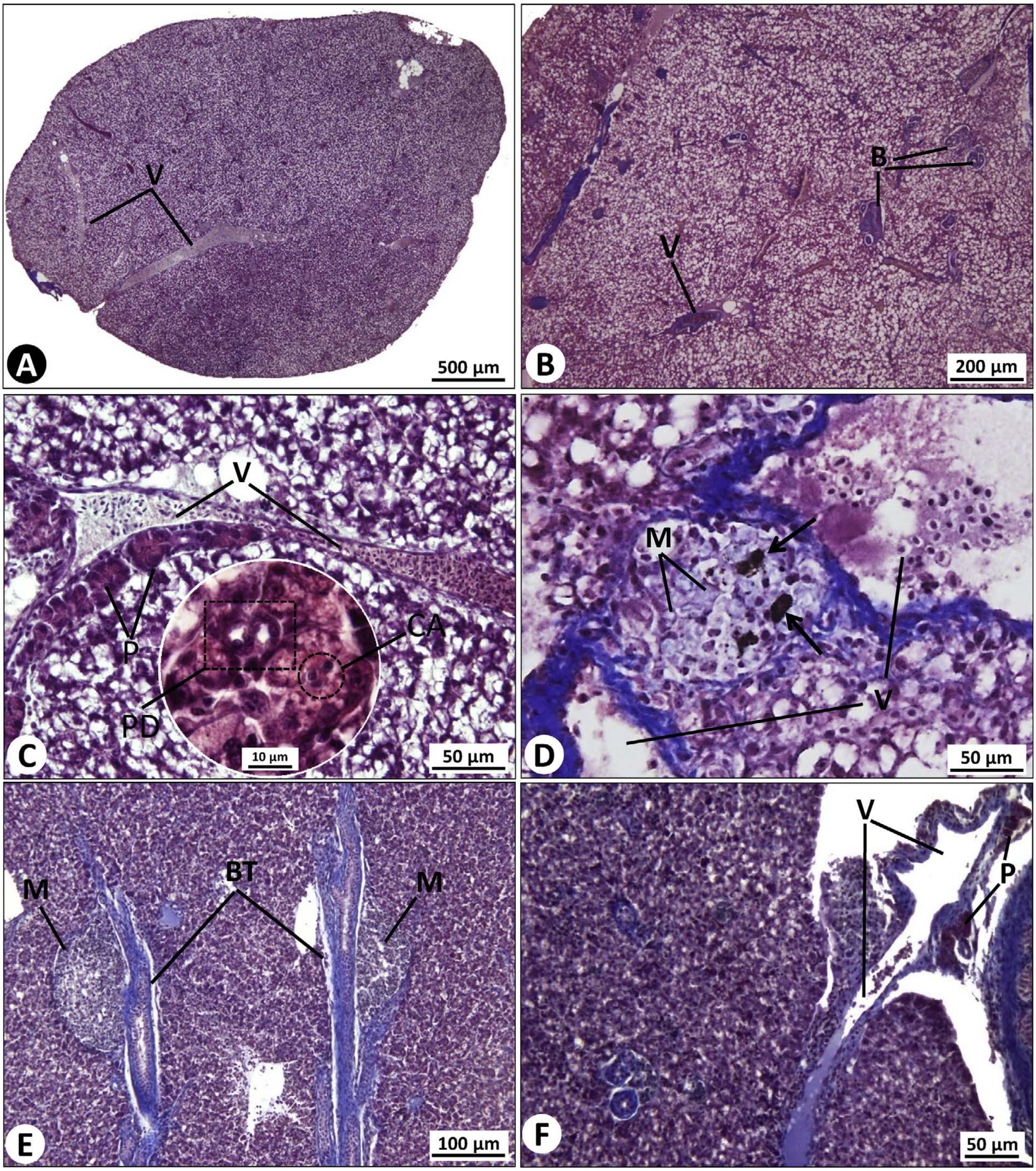

Fig. 2. Histological aspects from the liver of female guppies. A) General aspect in low magnification, showing that the structural compartments do not exhibit lobulation. The parenchyma reveals moderate basophilia, due to the hepatocytes cytoplasmic contents. Stromal elements, and particularly veins (V), are seen perceiving the parenchyma, seemingly at random. B) Higher magnification image taken from the liver shown in A), to illustrate the multitude of scattered stromal tracts, with blood vessels (V) and/or bile ducts (B). C) Acini of pancreocytes (P) adjoining to the adventitia of an isolated vein (V). Inset: Higher magnification of acini, where two pancreatic ducts are seen (PD). The tiny nuclei of a few centroacinar cells (CA) are well visible at the center of one acinus. D) Isolated vein (V) associated with an aggregate of macrophages (M), some of them exhibiting cytoplasmic dark brown to black pigments (arrows). E) Longitudinal section of several merging bile ducts that make two biliary tracts (BT), which in this case are associated with faintly pigmented macrophages (M). F) Afferent venous tracts (VT) in the hilum area, with attached pancreatic acini (P). Staining: Masson trichrome.

Nevertheless, in the paragraphs below, whenever there are data available we use them to stress particular comparisons.

The liver of the guppy presents intrahepatic pancreatic acini; a feature found in all the studied specimens. This feature happens in many other freshwater and marine fish (Ferguson, 1989; Rocha and Monteiro, 1999), and in those cases the whole liver is said to form a hepatopancreas (Hundet and Prabhat, 2014). Alternatively, the term hepatopancreas is used to specifically refer to the pancreatic elements within the liver (Nejedli and Gajger, 2013). Irrespective of the nomenclature, such intrahepatic pancreatic acini contrasts with the picture seen in other species, such as members of the Salmonidae family (trouts and salmons) studied so far, whose liver does not contain pancreocytes (e.g., Hampton et al., 1988; Rocha et al., 1994; Speilberg et al., 1994; Jordanova et al., 2013). As in other fish with hepatopan- creas, the pancreatic elements of the guppy appear both in the extra- hepatic zone and within the liver, where it presents a distribution

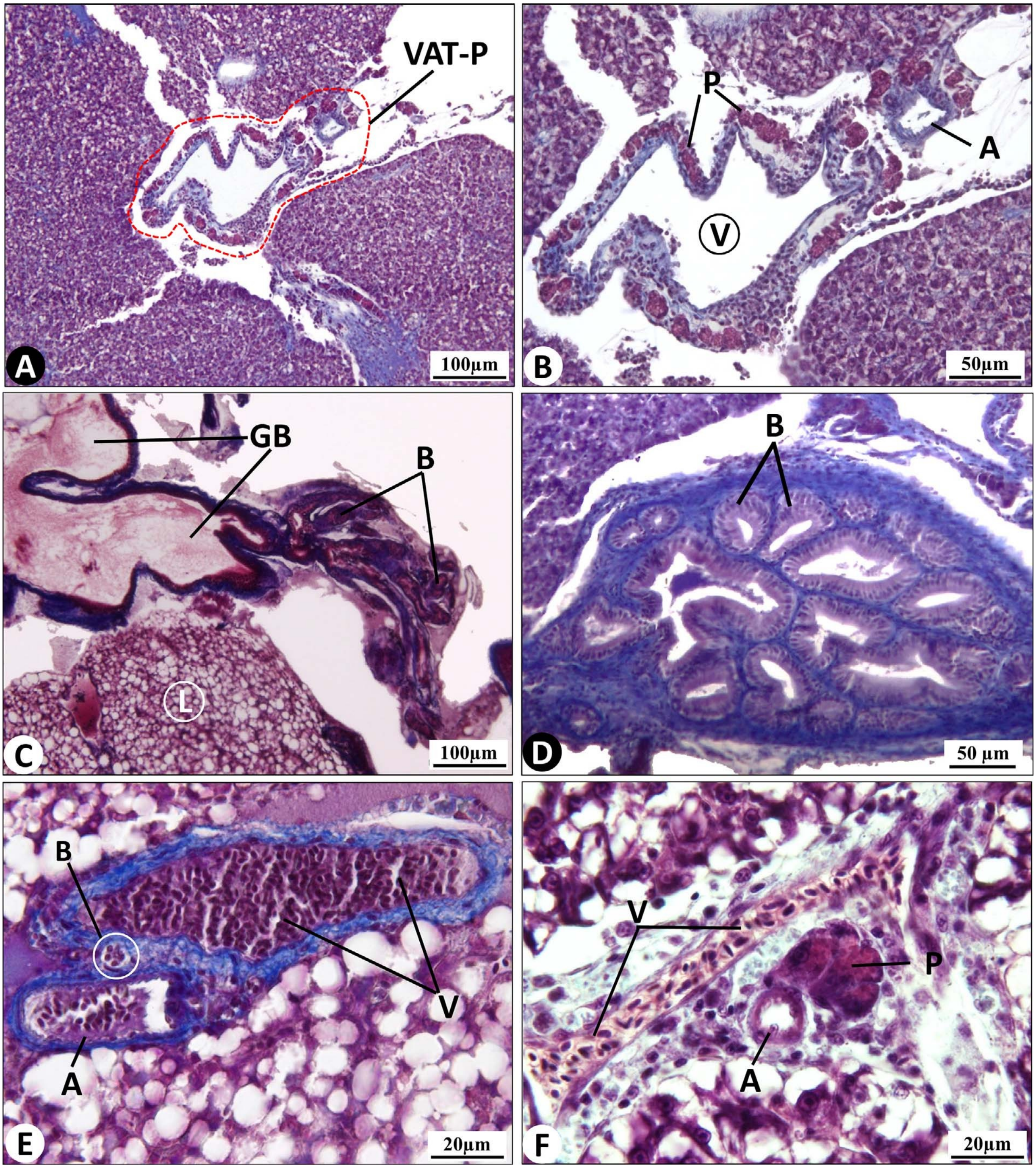

Fig. 3. Histological aspects from the liver of female and male guppies. A) Guppy female, venous–arteriolar tracts with pancreatic acini (VAT-P); this afferent vein is seen entering into the liver in an area which is not the hilum. The arterioles do not enter the liver. B) Guppy female, detail of VAT-P association, described in A, where the vein (V), surrounded with pancreatic acini (P), and the arteriole (A) stand out. C) Guppy female, one extrahepatic biliary tract, where several bile ducts (B) come together to reach the gall bladder (GB); illustrating that there are various liver exit points for ducts. D) Guppy female, detail of another (bigger than in C) extrahepatic biliary tract with anastomosing ducts (B). E) Guppy male, association between vein (V), arteriole (A) and bile duct (B), forming a VBAT. F) Guppy male, one venous–arteriolar tract, where the vein (V), arteriole (A) and associated pancreatic acini (P) are distinctively observed.

essentially close to blood vessels, almost often associated with afferent veins. These facts have been observed in other fish species, such as of the families: Ictaluridae – catfish, *Ictalurus punctatus* (Kendall and Hawkins, 1975; Hinton and Pool, 1976); Characidae – lambari, *Astyanax altiparanae* (Bertolucci et al., 2008) and dogfish *Oligosarcus jenynsii* (Petcoff et al., 2006); Cichlidae – Nile tilapia (Vicentini et al., 2005; Figueiredo-Fernandes et al., 2007); and Channidae – snakehead, *Channa punctatus* (Devi and Mishra, 2013). Despite our aim was not to study in detail the guppy intrahepatic pancreatic acini, from our data it is unclear if they are all physiologically relevant, in the sense that each acini would liberate enzymes that reach the intestine. It is clear that the acini have typical pancreocytes, full of granules that accumulate at the centre of the acini, where centroacinar cells are seen. However, finding ducts looking as of pancreatic nature was extremely rare, and we did not prove a continuous duct system network. We could not find studies in fish that detail the intrahepatic pancreatic acini in terms of their excretory duct system, as typically only the secretory acini are mentioned. Even in the most complete studies in fish with intrahepatic and

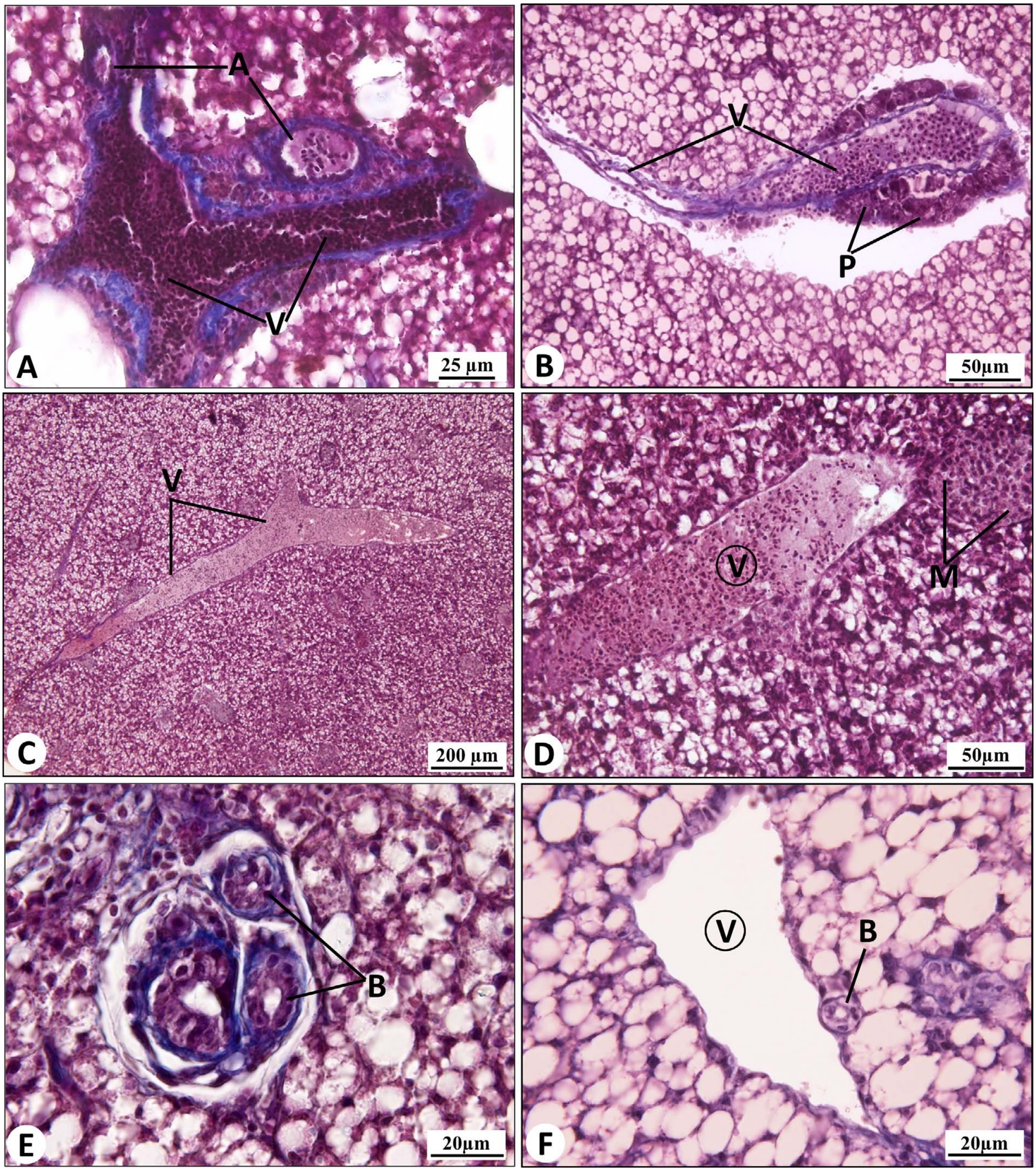

Fig. 4. Histological images from the liver of female and male guppies. A) Guppy male, association between a vein (V) and three small arterioles (A), forming a VAT. B) Guppy male, isolated vein (V) with a dense sleeve of associated pancreatic acini (P), forming a VT-P. C) Guppy female, an intrahepatic isolated vein (V) of a greater dimension. D) Guppy female, efferent large vein (V), associated with macrophage agglomerates (M). E) Three isolated bile ducts (B) forming a BT. F) Guppy female, "rendez-vous" of and efferent large vein (V) that at a certain point intersects a bile duct (B), giving rise a punctual VBT that quickly disappeared once tracked in serial sections.

extrahepatic pancreas we can see details of the duct system only regarding the extrahepatic portions. This fact is well illustrated in a study that used multiple techniques for an in depth description of the pan- creas of the grass carp (*Ctenopharyngodon idella*) (Mokhtar, 2015). For the guppy, and likely other fish, to definitively conclude about the extent of the intrahepatic pancreatic duct system, the use of plastic resins – offering minimal shrinkage and better resolution – coupled with serial sectioning (at least up to the hilar region) is critical.

A type of cell that is always present in the fish liver is the macrophage. The cell type is typically located either amidst the hepatocytes or in the connective tissue of the stromal areas (Rocha et al., 2003). However, at least in some species, there are macrophages positioned inside the lumen of the peri-hepatocytic capillaries (as the mammalian Kupffer cells do), such as seen in the spotted pimelodus, *Pimelodus maculatus*, of the family Pimelodidae (Ferri and Sesso, 1981). In the guppy liver, macrophages are found in aggregates amidst hepatocytes and mainly in association with the adventitia of blood vessels and of bile ducts, as detailed described for other species of fish, and irrespective of whether or not there is intrahepatic pancreatic tissue (Rocha and Monteiro, 1999; Figueiredo-Fernandes et al., 2007; Coimbra et al., 2007; Devia and Mishra, 2013). In some species, it seems that the macrophages are more commonly found in association with the bigger

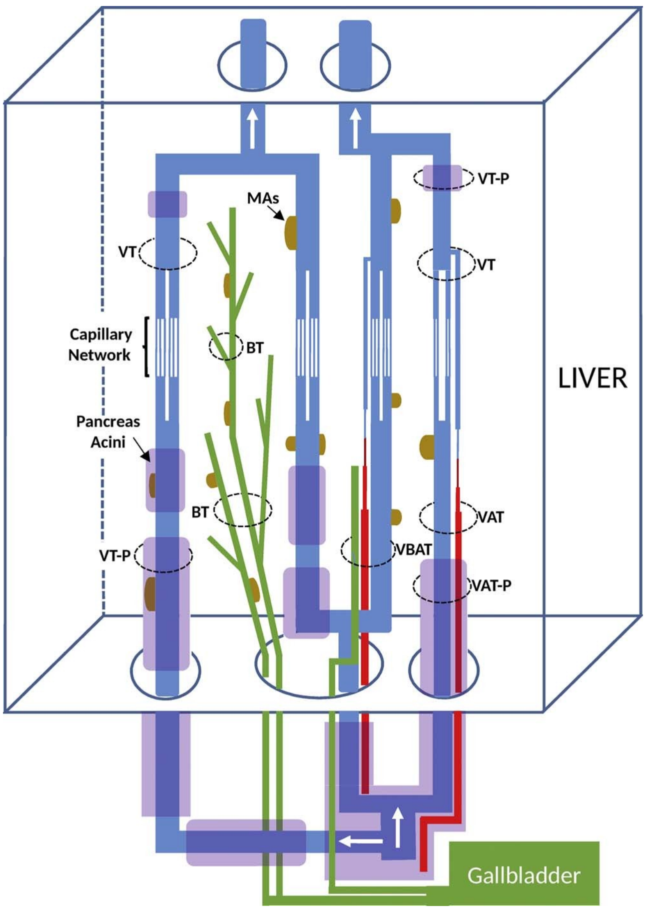

Fig. 5. Stylized 3D model of the intrahepatic vascular–biliary tracts spatial distribution in the guppy. Blue color represents the venous blood vessels and capillary network. Red is used for arterial blood vessels. Green is used for the biliary ducts. The (occasionally discontinuous) pancreatic tissue "sleeves" (of acini of exocrine pancreocytes) around blood vessels are shown in purple; they were only sporadic in efferent veins. Macrophage aggregates (MAs) are colored in brown. The white arrows indicate the direction of the blood flux. There is not a unique portal vein branch entering the liver into a single hilar point, and efferent blood leaves the organ by more than one vein. The different vascu- lar–biliary tracts observed in this study are represented and indicated with circles: BT (biliary tracts); VT (venous tracts); VT-P (venous tracts with pancreatic acini); VAT (ve- nous–arteriolar tracts); VAT-P (venous–arteriolar tracts with pancreatic acini); VBAT (venous-biliary-arteriolar tracts). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pancreatic–venous complexes, such as reported for the cichlids Nile tilapia (Figueiredo-Fernandes et al., 2007) and pearlscale eartheater, *Geophagus brasiliensis* (Sales et al., 2017). However, in the guppy we did not observe this preferential localization pattern. Whatever the exact location, the (usually encapsulated) groups of phagocytes are named melanomacrophage centers or macrophage aggregates (MAs), because their cells contain a variety of pigments, namely melanins, lipofuscin, ceroid, and hemosiderin, conferring colors from light yellow to deep black (Agius and Roberts, 2003). In the case of the guppy, the color with the trichrome was typically light bluish (at times slightly yel- lowish). Rarely, the MAs had a few cells with dark black deposits. The physiological meaning of the type of pigment and color of the MAs is still open to interpretation, but at least it is known that, on the one hand, the content in pigments increases in range and volume in both older and in cachectic diseased fish (Aigus and Roberts, 2003), and, on the other hand, that pigment composition, particularly in liver, differs among fish (Jordanova et al., 2008). The amount of MAs we found in the guppy can be considered scarce, which is not surprising because the fish were healthy and kept in disinfected water with regular main- tenance. In this context, it is relevant to stress that MAs are important elements of defense against resistant bacteria that can cause chronic infections and increase in size and frequency with the fish age (Schwindt et al., 2006). They are biomarkers of water quality because those increases also occur under conditions of environmental stress caused by oxygen-poor water and chemical pollution (Agius and Roberts, 2003; Passantino et al., 2014).

As to the intrahepatic vascular network of venous and arterial blood vessels, a striking characteristic was the near absence of the latter. Indeed, other than the capillaries, nearly all the sectioned blood vessels were small veins or venules. These were either isolated or associated with other vascular or biliary elements, with pancreatic tissue, much as earlier seen in the Nile tilapia (Figueiredo-Fernandes et al., 2006, 2007). Notably, and in contrast with the latter species, no eosinophilic granular cells (Reite and Evensen, 2006) were seen in guppy, suggesting that their normal existence in liver is species-specific.

The unequivocal identification of afferent and efferent vasculature was possible only via the follow-up of the vessel path in 3D through serial cuts, a procedure previously found also necessary to characterize the spatial distribution of blood vessels and bile ducts in brown trout (Rocha et al., 1995) and Nile tilapia (Figueiredo-Fernandes et al., 2007).

During the 3D follow-up, it was possible to observe herein, in some cases, associations of efferent veins with MAs. This observation is comparable to that made in Nile tilapia (Figueiredo-Fernandes et al., 2007), corresponding to an aspect that seems not to have been reported in other species. In view of the present data and that of Nile tilapia, we should view with great caution, and eventually exclude, the criterion of an association with MAs for the identification of afferent veins (Hampton et al., 1985; Robertson and Bradley, 1992). Rocha et al. (1995) warned that in brown trout some afferent veins were also not associated with MAs, which strengthens our concern to use the asso- ciation of MAs with veins as a criterion for classifying them as afferent. Eventually, this aspect may be also species-specific. Other studies have confirmed the presence of hepatic MAs mainly near blood vessels (Coimbra et al., 2007; Passantino et al., 2014), without, however, identifying whether they were afferent or efferent. In short, it may be concluded that, at least in the guppy, and likely in other species, in- trahepatic MAs may be associated with both afferent and afferent veins. Therefore, in histology and histopathology, their presence vs. absence should not be used lightly (i.e., without a full study in every species) as the differential criterion between those two types of veins.

As mentioned above, according to the serial tracking done in the guppy, the intrahepatic pancreatic acini were more often associated with afferent veins. However, our data confirms that in the guppy this is not an absolute criterion since time to time efferent isolated veins had pancreatic acini in the adventitia. Again, such veins were definitively classifiable as efferent by means of their follow-up along the serial sections, back from their genesis as small venules in direct connection with the capillary network up to their terminus, in bigger efferent or in terminal efferent veins that finally pierced the capsule. So, our study in the guppy backs the conclusions originally drawn from the brown trout liver, for which it was proposed that a definitive identification of af- ferent vs. efferent venous vessels is only possible from the tracking of serial sections (Rocha et al., 1995). An interesting fundamental ques- tion worth exploring is from where the pancreocytes occasionally at- tached to efferent cells come from. In addition to the partially common liver and pancreas embryological origin, and despite peculiarities de- picted in fish, such as that it is not yet clear where exactly exocrine pancreatic cells originate, we could envisage other ways how pancreas may appear attached to both afferent and efferent tracts (Ober et al., 2003). Indeed, there have been rare cases of pancreatic acini seen in the liver of humans that may shed some light (Mobini et al., 1974; Wolf et al., 1990; Lawrence et al., 2015). Metaplastic transformation of biliary epithelium into pancreatic tissue was early proposed (Wolf et al., 1990) but a heterotopic nature is also considered (Lawrence et al., 2015). Yet, there are data backing a metaplastic origin from a hepatic progenitor lineage (Kuo et al., 2009). If we recall that the fish liver has a notorious widespread network of biliary passages called pre-ductules (Hampton et al., 1988; Rocha et al., 1994; Hardman et al., 2007), much longer than the equivalent Hering channels of humans, and that those passages are rich in bile preductular epithelial cells, with bipotential lineage capabilities (Wettere et al., 2013), such cells may well generate pancreatic tissue anywhere in the liver depending on local signaling (Horb et al., 2003). Whatever the origin, pancreocytes in efferent veins would not be functional, in the sense that they surely do not be connected to a pancreatic duct network reaching the intestine.

As mentioned, when venous vessels did not appear isolated, they could be seen associated either with bile ducts and/or with arterioles. The existence of intrahepatic triple associations, i.e., associations between a small vein, a biliary canal and an arteriole (VBAT) were extremely rare. These types of associations have been observed in detail in brown trout (Rocha et al., 1994) and in Nile tilapia, and in the latter case with pancreatic tissue associated to the "triplet" (VBAT-P) (Figueiredo-Fernandes et al., 2006). In those species, the VBAT were clearly not as rare as we found in the guppy. Anyway, it could be ex- pected that this type of association would not be restricted to a couple of fish species. In a comparative study involving 200 teleost species, and despite the observations were not primarily based on exhaustive serial sectioning, VBAT were found (Akiyoshi and Inoue, 2004). Yet, in the cited study the structures were referred to in the text as "portal-tract type" or "portal triads", despite another type of nomenclature, viz. BAT, were similar to terminology used herein. In their survey, Akiyoshi and Inoue (2004) did not particularly mention the frequency of VBAT in a species, but they did not found this tract in all fishes; e.g., almost all Gobioidei and Tetraodontiformes did not have VBAT. They did not study fish from the Order the guppy belongs to, the Cyprinodonti- formes. Our results point that the guppy is a species with very rare or even no VBAT at all; it is important to stress that we did not find the triads in all studied specimens and when found, the arteriole of the triple association ended rapidly in length. Accordingly, in histopatho- logical exams of a guppy, the absence of VBAT should be viewed as normal.

In the guppy, we did not find associations (tracts) made of only bile-ducts and arterioles, which would be named BAT. However, the BAT was earlier depicted in detail in both the rainbow and brown trout (Hampton et al., 1988; Rocha et al., 1995). In Nile tilapia, both BAT and BAT-P were found (Figueiredo-Fernandes et al., 2006). In the study of Akiyoshi and Inoue (2004), they wrote that the BAT was identified in "almost all species", but in their tabular summary (see Table 1 of the cited article) BAT are registered for only 104 out of the 200 species; fish wich no BAT is indicated to exist belong to Cupleiformes, Siluriformes, Gasterosteiformes, and Mugiliformes. So, the guppy stands now as a species belonging to what seems to be a minority of fishes with no BAT at all.

In the guppy, neither VBT nor VBT-P were registered, and very close spatial proximity between an isolated vein and a biliary duct was found very rarely (see next paragraph). The VBT association was illustrated earlier in the brown trout (Rocha et al., 1995), and in the Nile tilapia, despite in the latter species it the tract was always associated with pancreas (VBT-P) (Figueiredo-Fernandes et al., 2007). Akiyoshi and Inoue (2004) only alluded to the presence *vs* absence of BVT in every species of fish they studied, and not to the frequency of appearance of that type of tract in the sections, precluding further comparisons. However, of the species studied by those authors, only 34 presented VBT, thus a minority of the investigated species. Therefore, and as for the absence of VBT, the guppy is in line with what was seen for the majority of fishes studied to this date.

Still regarding VBT, we found in the guppy a type of such dyad we termed "fugacious VBT", highlighting specific situations in which the two types of structures intersected each other orthogonally, for short distances, and then diverging as VT and BT. This situation was not reported in the other detailed (serial-sectioning based) studies with fish liver (Rocha et al., 1994; Figueiredo-Fernandes et al., 2007). From our observations, we conclude that BT in guppy either reach the hilum as such or eventually end up integrated into the rare VBAT. The "fugacious VBT" may have no particular functional meaning, but stresses the quite complex and apparently unorganized spatial distribution of the veins and the biliary tree, not only in the guppy liver but in fish in general.

Another association detected in the guppy liver was that between a vein and an arteriole, partially/totally surrounded by pancreatic tissue (VAT-P). This type of tract was not mentioned in the study of Akiyoshi and Inoue (2004), and, to our best knowledge, specific references to intra-hepatic associations of arteries and veins in fish are restricted to the brown trout (Rocha et al., 1995) and Nile tilapia (Figueiredo-Fernandes et al., 2007). While in the brown trout the VAT are not associated with pancreatic acini, in the Nile tilapia the VAT could either display pancreocytes or not in the tract's adventitia. In addition, and in opposition to the guppy, in Nile tilapia the P-VAT are more frequent than the P-BVT, highlighting once more the interspecies differences in the spatial distribution of both the intrahepatic vascular–biliary tracts and of the pancreocytes. A propos, in the guppy we never saw intrahepatic arterioles isolated from veins/venules, which contrasts with the pure (despite very rare) arterial tracts described in the brown trout (Rocha et al., 1995). The functional effects of such disparities between species remain unknown, but may be connected with environmental/ physiological adaptations that may implicate different liver needs as to receiving lower/higher arterialized blood.

In the guppy, the entrance of venous vessels in the liver occurred at points not located within the hilar area, suggesting branching of the portal vein prior to its splitting at the hilum. The phenomenon of the ramification of the portal vein and artery before reaching the "hilum-liver border" was previously reported in Nile tilapia (Figueiredo-Fernandes et al., 2007). However, the condition does not happen in all species, as for example in the zebrafish, in which the hepatic artery is said not to exist and the venous blood enters the liver at the hilum, through the portal vein or branches at the hilum (Yao et al., 2012). The concept of an absence of a hepatic artery as advanced for the zebrafish is somewhat in line with our data. Indeed, in the guppy, from the various extrahepatic presumptive afferent blood vessels (with or without associated pancreocytes), while most often the veins pene- trated into the liver, the small arteries did not. We cannot find this situation in the descriptions made in trout (Rocha et al., 1995) or Nile tilapia (Rocha et al., 1997). Because the length of zebrafish and guppy are much smaller than that of trout or tilapia, we hypothesize that having or not a well-defined hepatic artery and arterial supply may be connected with body mass and size-specific functional demands. In- deed, in the guppy (and in zebrafish too), intra-hepatic arteries are rarer. That hypothesis is worth exploring by studying the afferent ar- terial supply of more fishes, namely because it may explain functional hepatic differences among species. The idea is supported by recalling the intrahepatic differences as to arterial supply in mammals, namely in the isolated artery (e.g. seen in human and not in rat) and its arterial pathway (Ekataksin, 2000).

In connection with the above remarks, about the more or less degree of the ramification of venous and arterial blood vessels near the liver hilum, and as to the biliary tree, we noted that the guppy does not have a single hepatic duct. Several ducts come out from the liver separately and then merge consecutively along the way up to the very entrance of the gallbladder. The extrahepatic merging of bile ducts exists in other fishes too, as confirmed in Nile tilapia (Rocha et al., 1997), but we found no study with the "guppy pattern". In view of the bile ducts phylogeny, the system evolved from fish to human towards a structural pattern based on far less ductal branching outside the liver. The logic of the anatomical differences should be associated with functionality, namely in connection with how the bile acid composition greatly evolved (Moschetta et al., 2005; Hofmann et al., 2010).

Still regarding functionality, we may conjecture what the implications of our data are, namely in view of hypothetical differences among hepatocytes, as to metabolism and susceptibility to liver toxicants, depending on the cells proximity to the diverse types of vascular-biliar tracts. It is well known studied in detail that mammalian hepatocytes display marked metabolic heterogeneity according to their proximity to either afferent or efferent vascular tracts, i.e., depending on their position in lobular/acinar units (Colnot and Perret, 2011). In fish, and despite histological liver lobulation is not recognizable (Hardman etal., 2007), there are already a few evidences that support forms of parenchymal zonal metabolism and responses to toxicants (e.g., Ayadi et al., 2015; Schär et al., 1985). However, lack of studies in fish of the types of vascular-biliar tracts do not help the clear understanding of what are afferent versus efferent zones in the liver. In this regard, our work stands now as a baseline to future functional studies in guppy (and species alike) looking for eventual specific responses depending on the proximity versus distance of liver cells to the hilar area or to particular stromal tracts.

5. Conclusions

The histology of stromal compartments revealed in this study of the non-lobated liver of the guppy shows that pancreatic acini are contained within adventitia of afferent vascular tracts. The analyses exposed stromal (and non-stromal) macrophage aggregates, that were scarce, faintly pigmented, and with no location pattern. Intra-hepatic vessels (mostly venous) and bile ducts were randomly distributed, as confirmed by the study of serial-sections. These allowed the identification of isolated veins (VT) and bile ducts (BT) and of specific associations between blood vessels and/or bile ducts: VBAT (rare) and VAT-P (more common). The guppy does not seem to have BAT, VBT and AT. The 3D-tracking of VT was essential to conclude that they were afferent or efferent. The study of the hilar zone showed that afferent vessels ramify before entering the liver, and so the venous and arterial vessels could pierce the organ in various locations. Arterial vessels at the hilar area rarely entered the liver, making the concept of the existence of a hepatic artery not applicable, as reported in the zebrafish. The same applied to the “hepatic bile duct”, because many BT emerged from the liver at the hilar zone, and continued their merging for as far as to the gallbladder entrance. This detailed serial-sectioning study of the guppy liver, together with the two similar ones, made in brown trout and Nile tilapia, reveals that each species has specific features. Our study supports the view that, in the absence of definable lobules, and as proposed for the Nile tilapia (Rocha et al., 1997), the whole organ could constitute a single lobule, with the afferent zones being areas located near the hilum and the efferent ones being those far from it. It is yet to be determined the functional implications of our findings for the guppy, namely in view of hypothetical different metabolic responses of the liver cells to hepatotoxicants, in view of the cells specific distance (as proved in some mammalian model organisms) either from the afferent or efferent stromal tracts we exposed herein.

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CHAPTER III

Liver development in the guppy (*Poecilia reticulata*): A histological study by serial sectioning at the early organization of the vascular-biliary tracts and associated pancreocytes in 7, 17 and 28 days-old fish.

Liver development in the guppy (*Poecilia reticulata*): A histological study by serial sectioning at the early organization of the vascular-biliary tracts and associated pancreocytes in 7, 17 and 28 days-old fish

Abstract

The present study has main objective to contribute for the ontogenic knowledge of the histology of the guppy liver from histological analysis of serial cuts of three different fish age: 7, 17 and 28 days old. The observations made it possible to follow the histological development of this organ over time and establish a comparison with the histological study of the liver in the adult. In general, in the larvae of 7 days old, the presence of exocrine pancreas was not observed within the liver tissue, however, its presence on the periphery of this organ was frequent in the vicinity of the entrance sites of afferent venous vessels. The fish with 17 and 28 days present exocrine pancreas inside the liver, most often associated with blood vessels. This finding allows us to infer that the penetration of pancreocytes into the liver possibly happens as the development of the fish occurs until it reaches the adult state. As in adult fish, several entry points of venous vessels into the liver were observed, generally four, confirming the dominance of the venous circulation inside this gland. No arterioles were observed within the hepatic tissue, which is in agreement with the reduced arterialization of the liver in adult fish. None of the associations between different vessel types were observed. This is probably due to the very young age of the larvae-fish and consequently to the poor development of various types of blood vessels and bile ducts which have not yet established a defined biliary network. The presence inside the hepatic parenchyma of macrophage aggregates (MAs), defense cells that are associated with more advanced ages, has not been registered at any age of the studied fish. A particular type of cell - Rodlet cells was observed in some specimens. This study contributed to confirm and reinforce the specificity of some histological features observed in the guppy liver.

Keywords: histology; guppy liver, larvae; hepatic parenchyma.

1. Introduction

The importance of the study of fish liver has been increasing over the last decades. In fact, the more detailed knowledge of this gland allows to evaluate the state of health of the fish as well as the environmental quality of the aquatic ecosystems. Although few, there are already some scientific research papers whose target is fish liver, for example with medaka *Oryzias latipes* (Hinton et al., 1984; Braunbeck et al., 1999), rainbow trout *Oncorhynchus mykiss* (Hampton et al., 1989; Moutou et al., 1997), brown trout *Salmo trutta f. fario* (Rocha et al., 1997, 2001). On the other hand the study of this organ in fish can be useful to the understanding of disease risk in mammals, as for example in Man.
The growing interest in the study of liver in fish is related to the fact that it can be used as a model in several fields of study: for example carcinogen studies since it is a target organ of tumors (Sato et al., 1973; Nunez et al., 1991); studies in the field of environmental quality assessment (Könemann and van Leeuwen,1980; Doğan et al., 2013); studies to know the influence of feeding on the morphological structure and histological of the liver (Caballero et al., 1999; Figueiredo-Silva et al., 2005); studies in the area of reproduction to evaluate the influence caused by pollutants in the production capacity by the liver of vitellogenin, phosphoglycoprotein essential to the formation of oocytes (Yadetie et al., 1999; Madureira et al., 2012).

Thus, given its importance in the different fields of study identified, the detailed knowledge of the liver is indispensable in order to detect the morpho-histological changes it may present and thus to prevent early changes in fish health status as well as the progression of the aquatic environment quality alteration by the presence of pollutants that caused these changes. Chemical pollutants that may be present in the water sediments or food may be bioaccumulated by fish and thus affect not only the fish population but also the other links in the food chain present in the ecosystem (De Wolf et al., 1992; Winkaler et al., 2001), may also affect the human species (Barthem and Fabré, 2004). Histological studies allow the diagnosis of the acute and chronic effects of chemical agents on fish (Akaishi et al., 2004). Histological biomarkers allow visualizing the effects of exposure to various agents, biological and chemical, and are considered an important tool in the evaluation of pathological changes in fish (Van der Oost et al., 2003; Gernhöfer et al., 2001). Fish liver is thus considered a great histological biomarker since being an essential organ for the metabolism of nutrients and toxic substances undergoes gross lesions and histological changes when exposed to the various types of chemical pollutants present in the environment (Fanta et al., 2003; Au, 2004; Bombonato et al., 2007). However, given the great morphological and histological diversity of the liver among the various taxonomic groups of fish, the evaluation of eventual pathological conditions can only be possible if there was previous knowledge of the normal histology and structure of this organ. It is within this scope that this work is inserted: contribute to the knowledge of the histological development of the guppy liver at very young ages by comparison of the available knowledge of the liver histology in adult fish.

2. Material and methods

2.1. Animals

Adult guppies reared in Israel were purchased by the laboratory from a local importer. The fish were acclimatized for 3 weeks to laboratory conditions, being kept grouped in glass aquaria, with dechlorinated and continuously filtered and aerated tap water, at a temperature of 24 ± 1°C, and under a 12:12 h light- dark photoperiod.

For the experiments, pregnant adult female was transferred to an individual five-liter aquaria, inside standard ovoviviparous breeding floating boxes; with a bottom grid trap to prevent the mother from eating the offspring. The physical conditions referred to above were maintained. The female was fed once a day with TetraMin Tropical Fish Flakes (protein, lipids, vitamins).

After parturition, the juveniles were separated from the mother and left to grow until 7 days of age. At this time, they are all collected (25) for histological study. Two pregnant females were placed in a second glass aquaria with the same characteristics and maintaining the same conditions as the previously described above. After birth, the juveniles were separated from the mother and placed in another aquarium where they grew up. At the end of 17 days 14 of them were collected, remaining 4 growing until 28 days old.

2.2. Fixation and tissue processing

The guppy larvae were euthanized by overdose with immersion in a solution anaesthetic of ethylene glycol monophenyl ether (Merck, Germany), at a concentration of 1µL/mL. Then the total larvae (**figure 1**) was fixed in Bouin's solution, for 24h, at room temperature.

After, the fixed individuals were washed in 70% ethanol and then dehydrated with an ascending series of alcohol (70, 95, and 100%), followed by their clearing with xylene. Then the samples were embedded in plastic cassettes, impregnated in high quality paraffin (Paraplast Plus, Sigma- Aldrich); the processing was made using an automatic tissue processor (Leica TP1020, Germany).

Eleven of the total final blocks with the entry guppy larvae at the age of 7 days old, were totally serially cut according to a longitudinal plane into 25 µm thick sections and two of them were serially cut into 7 µm thick sections; ten of the total final blocks with the entry guppy fish at the age of 17 days old, were totally serially cut according to a longitudinal plane into 25 µm thick sections and two of them were serially cut into 7 µm thick sections; four blocks with the entry guppy larvae at the age of 28 days old, were totally serially cut according to a longitudinal plane into 7 µm; The serially cuts were made using a fully automatic rotary microtome (Leica RM2155, Germany). Sections were picked in slides coated with ami-nopropyl triethoxysilane (Sigma A3648). On average, each larvae of 7 days old produced ~56 sections with 25 µm of thickness and ~156 sections of 7 µm of thickness, each fish of 17 days old produced ~68 sections with 25 µm of thickness and ~175 sections of 7 µm of thickness, and each fish of 28 days old produced ~210 sections of 7 µm of thickness (only used for qualitative analysis of the liver histology). Histological sections were stained with hematoxylin-eosin (H&E).

Slides were mounted with DPX (Sigma-Aldrich), and studied with a light microscope (Olympus BX50, Japan). High resolution images (2560 × 1920 pixels) were taken with a digital camera (Olympus Camera C-5050, Japan).

Figure 1 – A) Guppy of 7 days old; B) Guppy of 17 days old; C) Guppy of 28 days old.

2.3. Histological observation and serial section

According with the aims proposed, the microscopic observations of the serial cuts sections were directed in order to establish a perspective of the histological development of the guppy liver since early stages of life until the adult state. Thus, we sought to find aspects already observed in adult fish, such as the presence inside the hepatic parenchyma of exocrine pancreas to try to understand the origin of this tissue present inside the liver of adult fish (Sousa et al., 2018). Another important aspect was the confirmation of the existence of various entry points in the liver of venous blood vessels (afferent veins), as had been observed in adult fish (Sousa et al., 2018). Still another point to be considered was the research of particular type of defense cell - melanomacrophage aggregates (MAs) found frequently in adult fish. It would be difficult to distinguish the different types of blood vessels due to their small size, as well as possible associations between them as observed in adult fish, for example venous-biliary-arteriolar tracts (VBAT), venous-biliary tracts (VBT), or venous-arteriolar tracts (VAT), with or without associated pancreas, however this was also an aspect to be considered during the observations.

The small size of the 7 days larvae of the guppy fish allows the serial cuts to show the whole of the individual and the relative position of the different organs within the animal body (Fig. 2A). The liver lies in the abdominal cavity positioned in the vicinity of the intestine and heart, presenting as a large gland compared to the remaining organs. A more detailed observation of the liver (Fig. 2B) shows us a compact organ, whose parenchyma consists essentially of hepatocytes, usually intensely stained, characteristic of basophilic cytoplasm. Among the hepatocytes, in the hepatic stroma, it is possible to see small blood vessels that establish the communication between the hepatocytes and the blood. This observation is common to both sexes.

The exocrine pancreas appears almost always at the periphery of the liver, usually near the entrance of a blood vessels-afferents veins, which were entering the liver at a ventral position - ventral 3 (Fig. 2C; 2E) and at a dorsal position – dorsal 4 (Fig. 2C; 2F). No pancreocytes were observed inside the hepatic tissue, except in three fish, in two of them the pancreas was already inside the hepatic parenchyma, although still close to the periphery (Fig. 3D; 5E) and in the other one the pancreocytes were located deep in the liver (Fig. 7E).

As in adult fish, several entry points of blood vessels were observed in the liver (Fig. 2C-F). In most fish besides the entrance of a blood vessel in the main hillum zone, two or three entry sites of afferent vessel were observed, making a total of 4 entry points observed. Generally, there are two entry points in the ventral region of the liver of small vessels near the intestine (position 1, 2) (Fig. 2C; 2F), the second in lower position compared to the first; a third entry point positionated more posterior and bigger, afferent vein that corresponds to ventral 3 (Fig. 2C) and is usually accompanied at the periphery of the organ by the presence of pancreatic tissue (Fig. 2C; 2E). This observation was common to both sexes at the three fish groups of different aged studied. In some cases, the ventral afferent vein 2 (Fig. 2F), whose entrance is located in the ventral region near the intestine, travels almost the entire length of the liver.

In most animals, it was common to observe a biliary canal wich may present one or two inta-hepatic branches (Fig. 3E; 3F). In most fish, the hepatic canal communicates with the exterior with a more dilated, globular zone, in some cases of large dimensions, which will be the future gallbladder (Fig. 2F; 3E).

A constant observation in all fish was the presence of one or two efferent intra-hepatic veins (V) merging into one hepatic vein (HV) (Fig. 5A; 5F). In one of the larvae the efferent hepatic vein had a single very long branch that almost crosses the whole liver (Fig. 5A). This observation was common in exemplars of animals with 17 and 28 days old.

None of the types of associations between different vessel types were observed as in adult fish into the liver (e.g. VAT, VBT, VBAT). This is probably due to the very young age of the animals and consequently to the inicial poor development of various types of blood vessels and bile ducts that have not yet established a defined biliary network.

The source of exocrine pancreas that accompanies the afferent veins into the liver is the intestine wall (Fig. 3A; 4B; 6B), pancreatic tissue together with the blood vessels come from the intestine wall to the liver; this observation is common to the three age groups of fish.

Finally, no arteriolar vessels were observed inside the liver. However, arterioles were observed in the outer zone of the liver, next to the main hilar zone normally surrounded by pancreocytes (Fig. 3E; 4F; 6D;). This reduced arterialization also occurred in adult fish. This fact was already observed in adult fish.

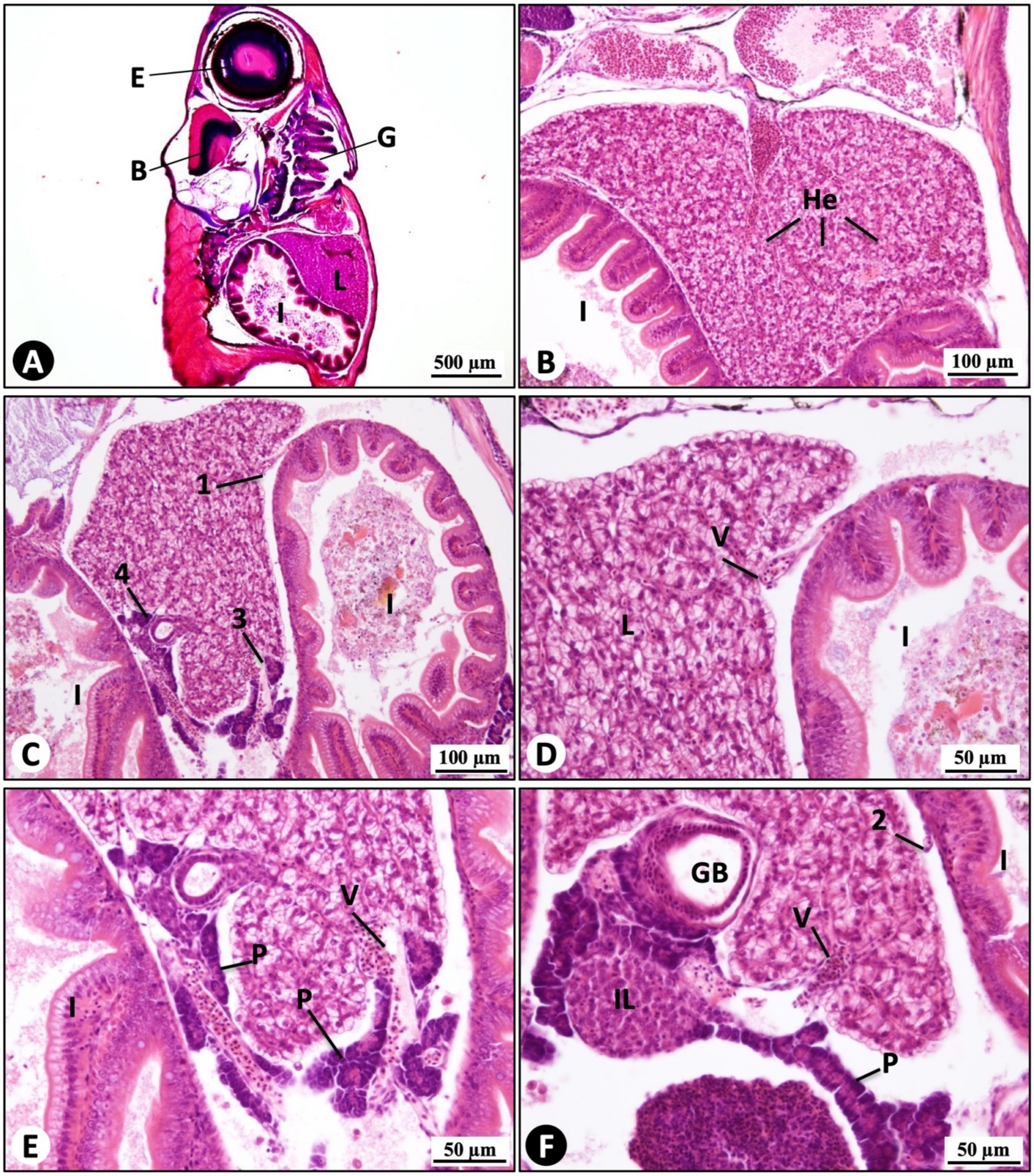

Figure 2 – Histological images from male (A, B) and female (C, D, E, F) guppy with 7 days old. A) General aspect in low magnification, showing the relative position of different organs within the animal (the ventral side is at the right side); B) Higher magnification of the liver, displaying as a compact aspect, and whose parenchyma consists essentially of hepatocytes (He). The stroma is yet scarce and barely perceivable at low magnification; C) General aspect showing three of the four entrance points of blood vessels in the liver (ventral 1, ventral 3, and dorsal 4); D) Closer observation of the most ventral and anterior entrance of a small afferent vein (V), originating from the intestinal wall (corresponds to ventral 1 in image C); E) Detail of another, more posterior and bigger, afferent vein (V) (that corresponds to ventral 3 in image C), tipically surrounded by exocrine pancreocytic acini (P); F) Section that captured the very small vein (V) entering the liver at ventral position 2. One islet of Langerhans (IL) appears, as a roundish cluster of cells in the vicinity of the liver and pancreocytic acini (P). B – Brain; E – Eye; GB – Gallbladder; G – Gills; I – Intestine, L – Liver. Staining: H&E.

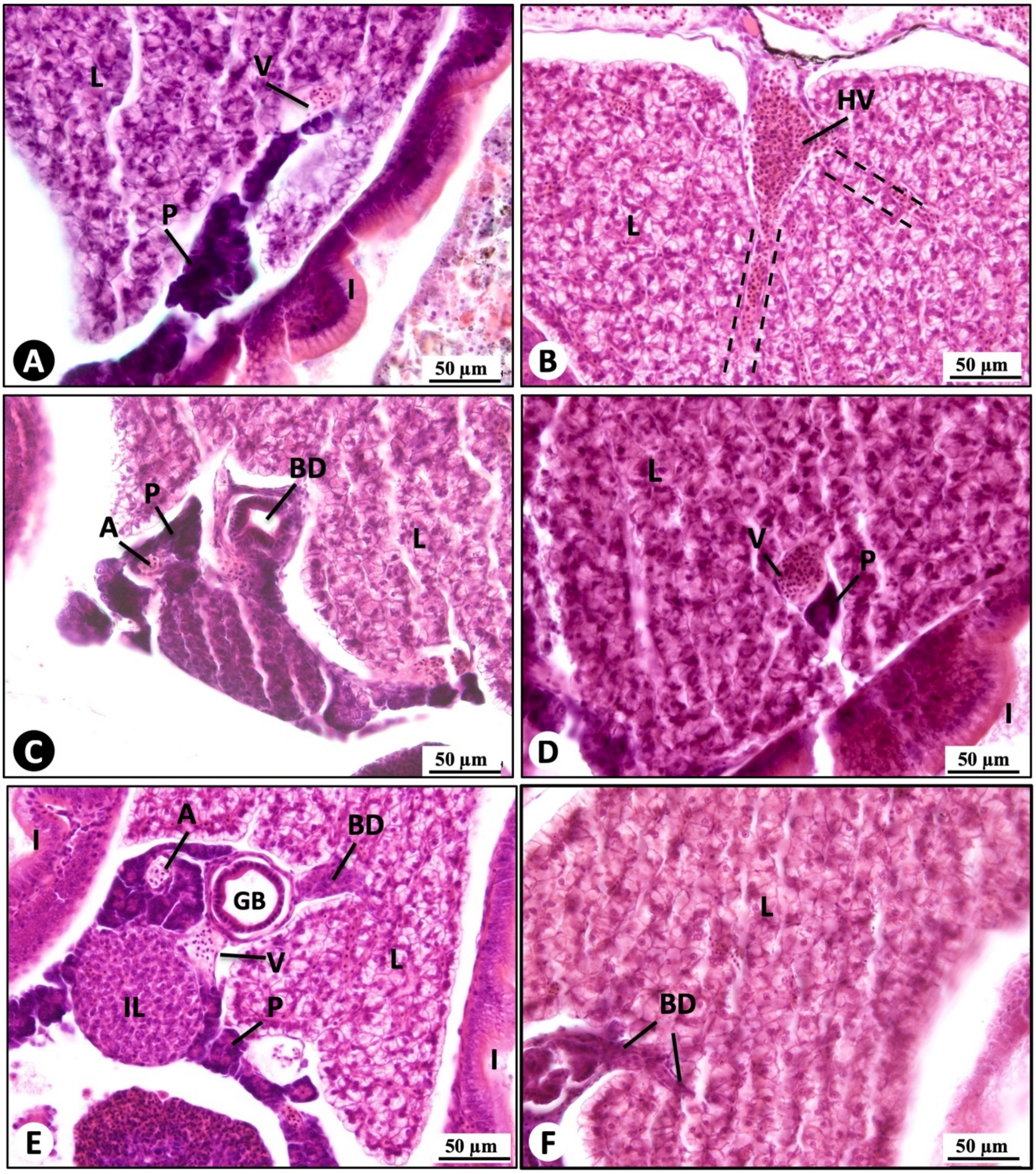

Figure 3 – Histological images from female guppy with 7 days old. A) Image showing the entry of exocrine pancreas (P) into the liver (L), coming from the intestine together with an afferent vein (V). B) One emerging hepatic vein (HV), presenting two intrahepatic branches (circumscribed areas); the one in the middle of the image ran antero-posteriorly, crossing almost the entire liver. C) Image of the main hilar zone of the liver, where one arteriole (A) is seen, outside the organ, and surrounded by exocrine pancreas (P). One extrahepatic bile duct (BD) is osberved. D) Image to illustrate exocrine pancreas (P) inside the liver, collate to a small vein, close to the organ periphery. E) Main hilar liver area, containing exocrine pancreas (P), endocrine pancreas in the form of islets of Langerhans (IL), one arteriole (A), one afferent vein (V), which would enter the liver. One bile duct emerges from the liver towards the forming gallbladder (GB). F) Detail of an emergin larger bile duct (BD) resulting from several smaller ducts. Staining: H&E.

The fish of 17 and 28 days old, present exocrine pancreas within the liver. This observation is common to both fish ages however is more frequent in 28-days old fish. This is the main difference found to the 7-day old larvae in which pancreocytes are only seen near the periphery of the liver (Fig. 3D). Usually the presence of pancreas appears associated with blood vessels. It is common to observe small veins from the intestine sorouded by pancreatic tissue that partially surrounds the vessel and accompanies it from the intestine wall to the interior of the liver, the pancreatic tissue partially encloses the vessel and traps it into the liver (Fig. 4B; 4C; 6B; 6C). This observation is common to the three fish groups of different aged (7, 17 and 28 days) as it had already occurred in adults. A common observation of the three fish ages was the presence of at least 4 entry points of afferent vessels. As in the 7-day larvae, in the other two ages fishs (17 and 28 days) the afferent veins positioned at ventral point 3 and dorsal point 4, closer to the main hilar area, are accompanied by pancreatic tissue, whereas normally the ones farther from the hilar zone, which are in the ventral position 1, do not present pancreas in the vicinity (Fig. 4B; 6A). Another observation common to the three ages of animals was the absence of melanomacrorphage centers within the liver. The vascularization intensifies with the age of the fish, being common the presence of a long vein which runs through of almost all the hepatic tissue (Fig. 5A; 7B). The bile ducts are difficult to observe in the larvae of younger ages, due to their small size, however fish aged 17 and 28 days presenting them (Fig. 5D; 6E; 6F; 7D) they are frequently observed, preferably in the region closest to the hilum.

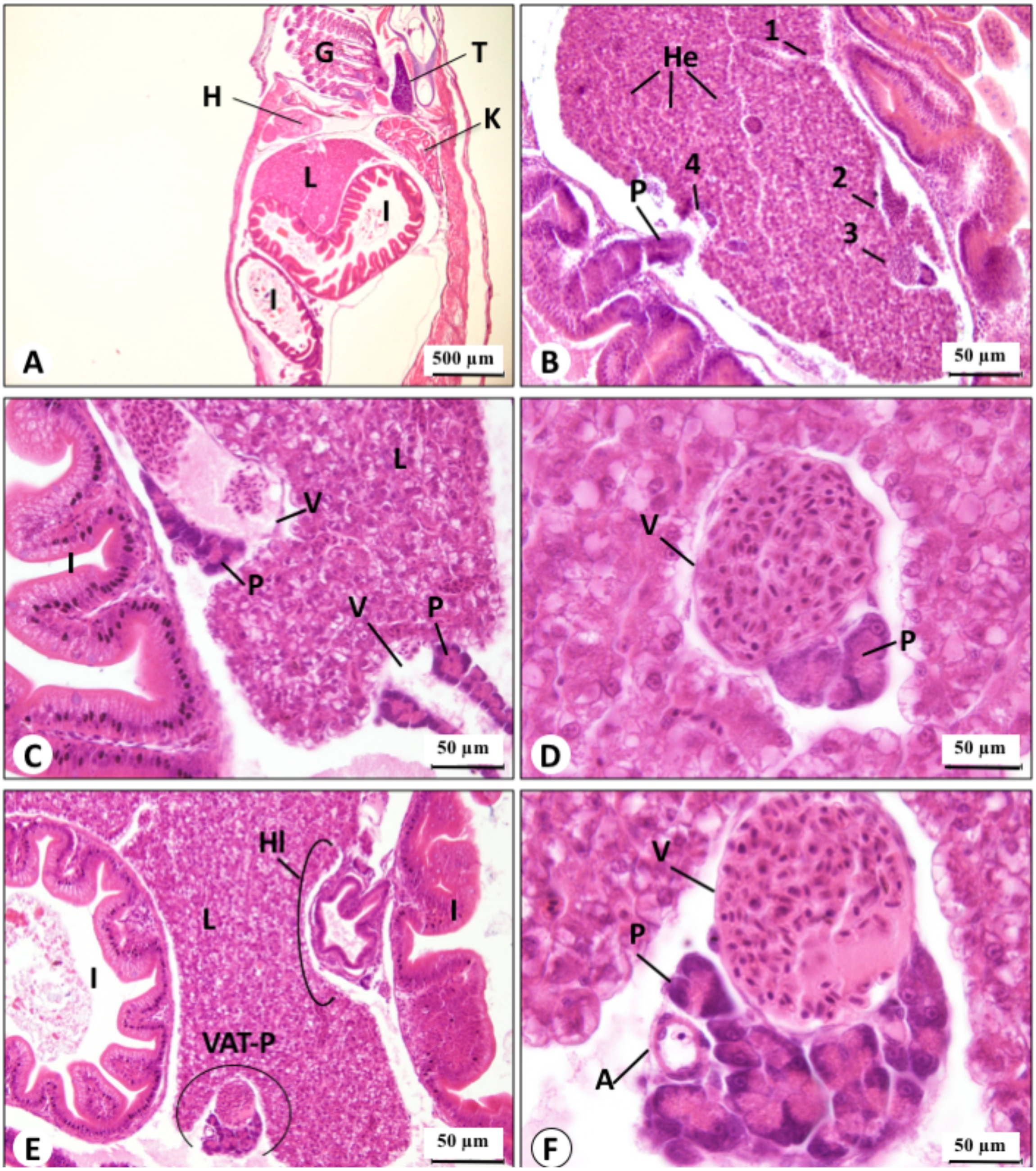

Figure 4 - Histological images from male (A, D) and female guppy (B, C, E, F) with 17 days old. A) General aspect in low magnification, showing the relative position of different organs within the animal (the ventral side is at the left side). B) General aspect in low magnification, showing several entry points of blood vessels into the liver (1, 2, 3, 4). Exocrine pancreatic acini (P) sorrouding a small vein coming from the intestinal wall. It is evident that the parenchyma consists essentially of intensily stained hepatocytes (He). C) Aspect of a more posterior hepatic zone, showing two small veins (V), with associated pancreatic tissue (P), piercing the organ at two positions (at left, dorsal 4, at right, ventral 3). D) Higher magnification of a vein (V) associated with pancreas (P) and surrounded by hepatic parenchyma. E) Image showing the main (bigger) hilum (Hl) and another (more posterior) zone of entrance of an afferent vein associated to pancreocytes and a small arteriole (VAT-P). F) Detail of the VAT-P in E. G – Gills, H – Heart, I – Intestine, K – Kidney, L – Liver, T – Thymus. Staining: H&E.

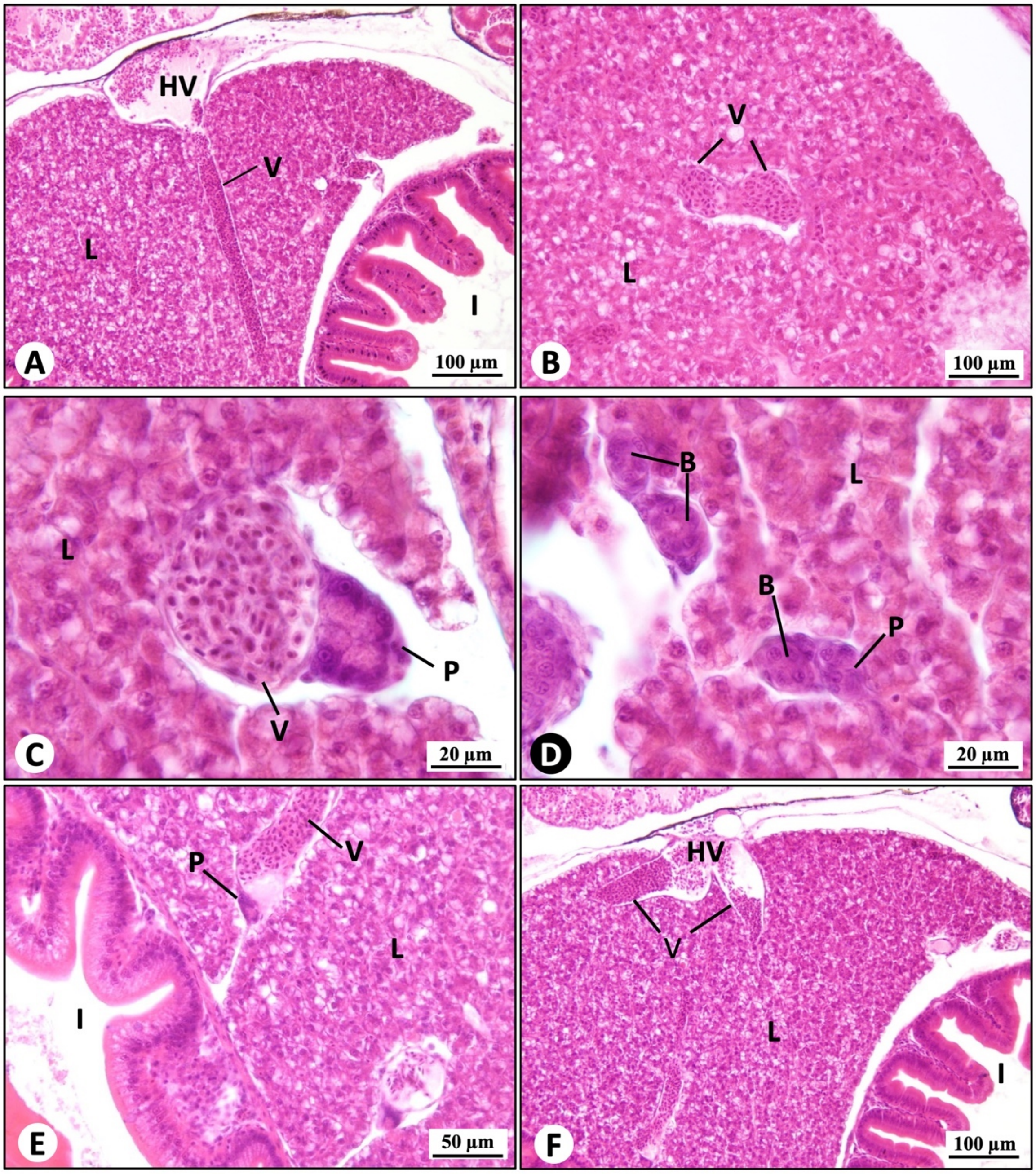

Figure 5 - Histological images from male (E, F) and female (A, B, C, D) guppy with 17 days old. A) Overall view of the liver (L), showing a long and straight intra-hepatic centrally positioned vein (V), which crosses almost the entire liver (L) postero-anteriorly to end in direct connection with a hepatic vein (HV). B) Intra-hepatic fusion of two (efferent) veins (V). C) Detail of an afferent vein (V) with associated pancreatic acini (P), piercing the liver (L) and starting to be encircled by parenchyma. D) Detail with three sections of small bile ducts (B), one of them in close contact with pancreocytes (P). E) Afferent vein (V) associated with pancreatic acini (P) observed already inside the liver (L). F) Two efferent intra-hepatic veins (V) merging into one hepatic vein (HV). Staining: H&E.

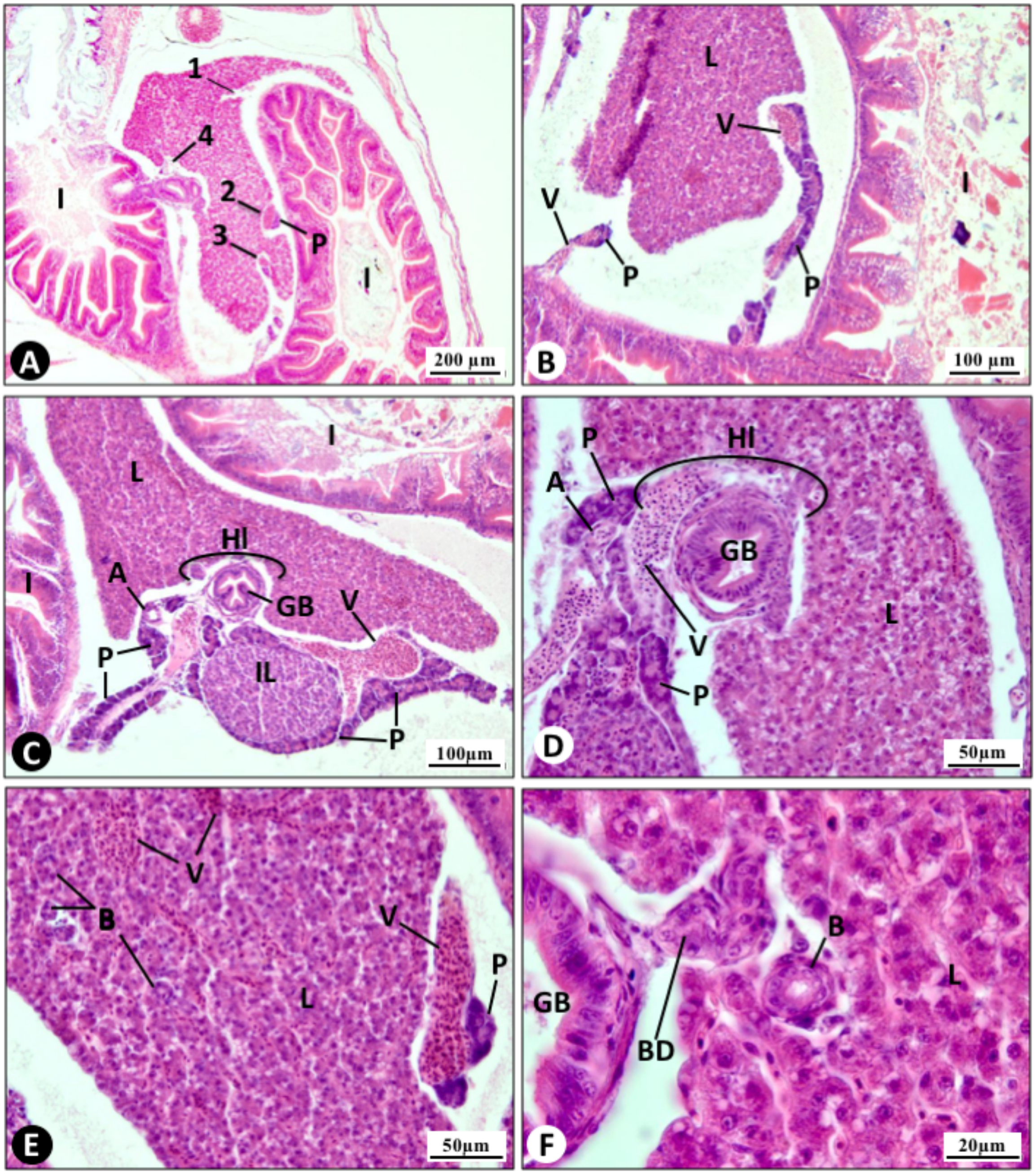

Figure 6 – Histological images from male (A, E, F) and female (B, C, D) guppy with 28 days old. A) General aspect at low magnification that shows four entry points (ventral 1, 2, 3, and dorsal 4) of afferent veins into the liver. Veins in positions 2 and 3 show adventitial pancreatic acini (P). B) Image at low magnification showing two small veins (V) coming from the intestinal wall and going towards the liver; they are ensheathed by pancreatic acini (P). C) Image at low magnification with two veins, attached to exocrine pancreas (P), piercing into the liver at different locations, including near the hilar zone (H); nearby, there is an islet of Langerhans (IL) and one small arteriole (A), associated to exocrine pancreas (P). D) Image detailing the hilum (Hl), where one vein (main portal vessel), associated with pancreatic acini (P), is entering the liver; one arteriole (A), also surrounded by pancreas, is also seen at the hilar zone. E) Image showing three small intrahepatic bile ducts (B) and one (afferent) vein partly ensheathed with pancreas (P), running parallel to the liver capsule. F) Detail of a biliary duct (BD) becoming extrahepatic close to small intrahepatic bile duct (B). GB – Galdbladder. Staining: H&E.

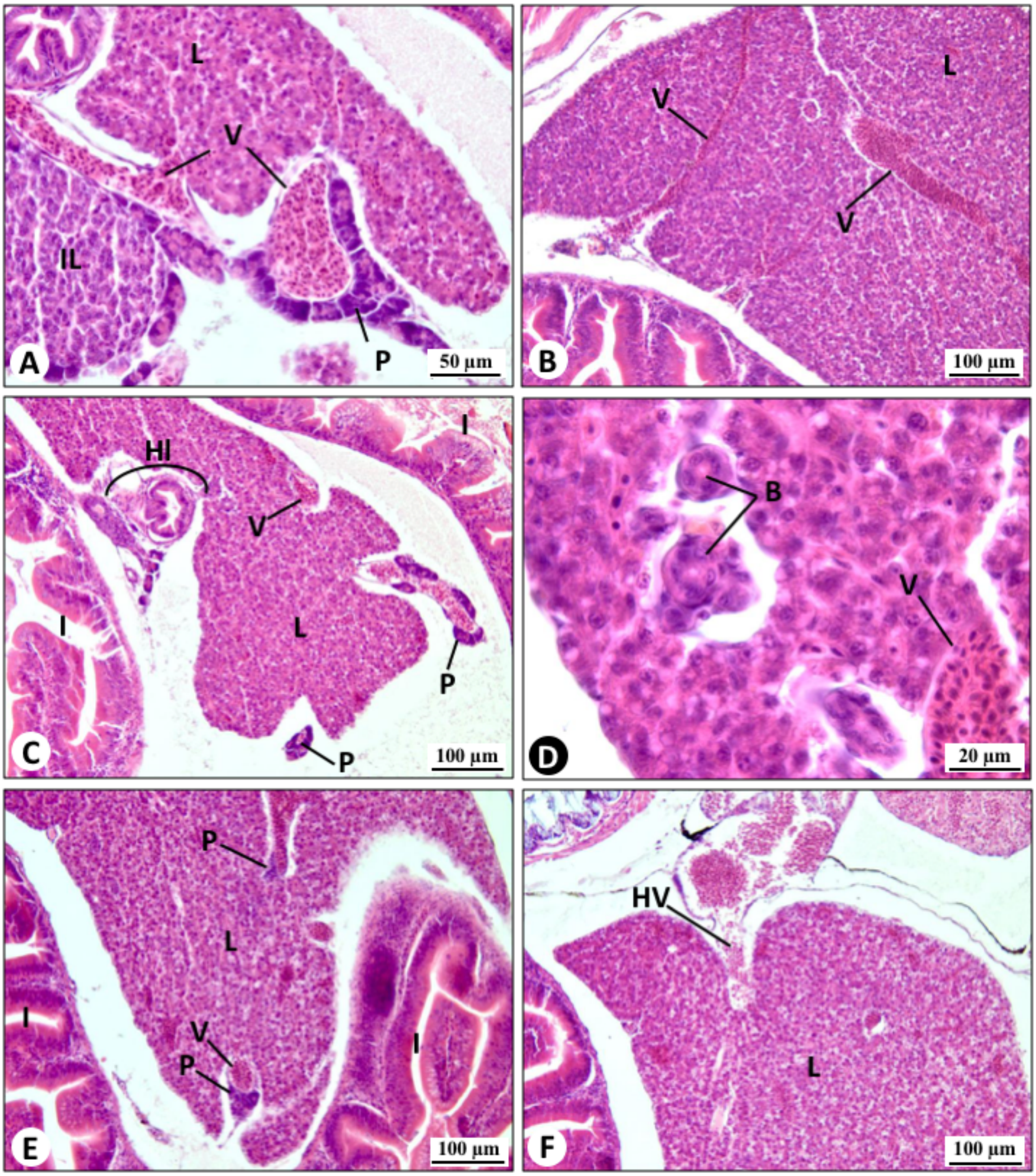

Figure 7 – Histological images with liver of male (A, B, C, E) and female (D, F) guppy with 28 days old. A) Image showing two more posteriorly located entry points of afferent veins (V), both of them with associated pancreatic acini (P). B) Image showing two long afferent veins (V) that went deep within the liver. C) Section showing the liver (L) at the level of the main hilum (HI) while allowing to visualize three additional afferent veins, two of them with associated pancreatic acini (P) and one without them (V). D) Detail of three small bile ducts (B), one of them side-by-side to an afferent vein (V). E) Two afferent veins (V) associated with pancreatic acini (P), one of them located deep in the liver (L). F) – Anterior most zone of the liver at the level of the single hepatic vein (HV). IL – islet of Langerhans. Staining: H&E

4. Discussion

The liver of adult guppy is characterized by the presence of exocrine pancreas within the liver - hepatopancreas, as in other fish such as in *Oreochromis niloticus* (Figueiredo-Fernandes et al., 2007), often associated with blood vessels and bile ducts. According to Bruslé and Anadon (1996) and also Bombonato et al. (2007), the development of exocrine pancreatic tissue around the vessels occurs during ontogenesis and this tissue may remain extrahepatic or penetrate the liver. From the observations made on larvae of different ages it was found that in the 7 days old which were still very young, the presence of pancreatic tissue was not detected inside the liver, only one of the fishes presented intrahepatic pancreocytes but in a very peripheral position. However, it was common to observe pancreatic tissue in the vicinity of the liver, particularly at the ventral 3 and dorsal 4 positions of entry point of afferent veins, sometimes accompanying the vessel to the site of entry but remaining outside the liver. However, the presence of pancreatic tissue inside the liver was already visible in both 17-day and 28-day fish. In both ages, the penetration of pancreocyts was observed accompanying the blood afferent veins. This finding allows us to infer the independent formation of these two tissues and even that the penetration of pancreocytes inside the liver happens as the development of the fish occurs until reaching the adult state. However, the origin of the pancreas that accompanies the efferent veins as observed in adult fish, has not yet been clarified (Sousa et al., 2018). The possibilities of an internal origin from metaplastic transformation of biliary epithelium into pancreatic tissue (De Wolf et al., 1992) or a heterotopic nature (Lawrence et al., 2015) have not yet been confirmed or not. Because the very young age of these fish the blood vessels in the stroma hepatic present reduced diameter making it difficult to follow them along the hepatic tissue.

As in adult fish, several entry points of blood vessels in the liver (afferent vessels) were observed, at most four locals of small venous vessels entry were identified. This observation is only possible from serial cuts that allow to follow the course of these vessel from the starting point (intestine wall) to the interior of the liver tissue. This record confirms that in this species of fish the venous blood supply to the liver is made through several points appearing as the dominant venous circulation. It has been found in all fish groups that there are several entry points of blood vessels in the liver from the gut that pierce the liver in different places. This register is in disagrees with the idea that the portal vein branches before reaching the "hilum-liver border" as was reported in Nile tilapia (Figueiredo-Fernandes et al., 2007). This fact highlights the inter-specific differences observed in fish liver and the importance of individual study in each species.

As in adult fish the presence of isolated arterioles within the hepatic tissue was not observed. However, arterioles were observed at all fish ages, in the outer zone of the liver near the main hilar zone. This is in agreement with the assumption of a reduced arterial circulation in this organ already verified in adult fish.

It was not registered in any of the guppy fish, inside the hepatic parenchyma the presence of normally aggregated of phagocytic cells designate as melanomacrophage centers or macrophage aggregates (MAs), because their cells contain a variety of pigments, namely melanins, lipofuscin, ceroid, and hemosiderin, conferring colors from light yellow to deep black (Agius and Roberts, 2003). However, their presence was common in adult fish. This lack of observation is in accordance to the idea that the presence of MAs is not always necessary as a criterion for the identification of afferent vessels, as was verified in the adult guppy in which these groups of cells appeared indifferently associated to afferent and efferent vessels (Sousa et al., 2018). Previous study with brown trout (Rocha et al., 1995), had already questioned the exclusive use of this criterion for the identification of afferent vessels in all species. On the other hand, the absence of MAs in fish as young as those of this study, is in agreement of the information that this type of cells of defense against infections caused by resistant bacteria that can cause chronic infections, increase in size and frequency with the fish age (Schwindt et al., 2006). The fact that the larvae were kept in their short life in environmentally clean and healthy conditions, that is, kept isolated in glass aquarium with dechlorinated and continuously filtered and aerated tap water, is also in agreement with the classification of this type of cells as biomarkers of water quality because they increased due environmental stress caused by oxygen-poor water and chemical pollution (Agius and Roberts, 2003; Passantino et al., 2014).

A peculiar observation, not illustrated due to poor image quality but worth noting was the presence of Rodlet Cells (RdC) in the periphery of blood vessels and also near to the pancreatic tissue. This registration is not common in all fish and although the origin is unknown, these cells will have an endogenous origin, however the non-observation of this type of cells in the adult fish may indicate that their presence is not constant throughout the life of the fish. Generally, these cells are associated with inflammatory responses, however once the larvae have been kept in environmentally clean and disinfected conditions larvae so infections by exogenous agents are not expected to occur, which is in some way in agreement with the fact that this type of cells also appear in places where there are no pathologies.

Lastly it became clear that the exocrine pancreas that accompanies the venous blood vessels into the liver comes from the intestine wall along with the blood vessels.

5. Conclusion

The histological study of the liver of guppy fish at early stages of life, has generally contributed to establish an evolutionary perspective of some of the histological aspects of the liver of this species of fish. This way it was confirmed the existence of several points of entry of venous vessels (minimum four) already observed in the adult and now also in the larvae of the fish. This is an important conclusion that, while not applicable to all species, highlights the intraspecific character of some of the characteristic of the fish (Jordanova et al., 2007). The presence of exocrine pancreas within the hepatic parenchyma will definitely be associated with the age of the fish as it was evident in this study. It was clear that the pancreas that accompanies the afferent veins has an origin in the intestine wall, however it was not possible to verify the origin of the pancreatic tissue inside the liver that accompanies the efferent vessels, as was observed in adult. Age will also be a decisive factor for the occurrence of associations between different types of vessels. In guppy of early age of this study only small blood vessels (small veins and capillaries) scattered in the liver stroma can be seen inside the liver; an established biliary network was not observed in the bile ducts. As in adult fish the blood circulation within the liver is predominantly venous (Sousa et al., 2018) none intrahepatic arterioles were observed. This observation may explain the tolerance of this species to polluted environments, with low oxygenated waters (Reznick et al., 2012). The absence of melanomacrophages aggregates (MAs) in young larvae as opposed to regular presence in adults may also be considered a factor that reinforces the tolerability of this species to low quality environments. There is no mention in the literature of the ontogeny of these defense cells attached to the liver; however, it may be assumed that their non-presence is due to the fact that they have not yet formed due to the very young age of these fish.

This study may contribute to the knowlwdge of the evolutionary development of the guppy liver histology, highlighting the specific characteristics of this species, which may be useful for the early detection of environmental problems as result of the presence of chemical pollutants of anthropogenic origin.

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CHAPTER IV

Liver development in the guppy fish (*Poecilia reticulata*): Investigating 7 and 17-days-old females and males using design-based stereological estimators of volume and number.

Liver development in the guppy fish (*Poecilia reticulata*): Investigating 7 and 17-days-old females and males using design-based stereological estimators of volume and number.

Abstract

Objective and consistent knowledge of the normal structure of the liver is indispensable in order to evaluate disease states that may be associated with this organ. The use of stereology for characterization of the liver began in the sixties of the twentieth century with mammals but only later, in the 80s it was used in fish.

Although the guppy fish is a widely used model organism in sience, quantiative studies on the development of its liver do not exist. Yet, the guppy is amenable to such studies, which can help to understand how the early post-embryonic growth of the liver occurs in fish. To tackle this question, we used stereology in this work and studied 7 and 17-day old guppies, which summed 13 males and 10 females. Biometry was registered. The fish were fixed in toto and processed for histology. Each fish was serially cut into 25 µm thick sections; those with liver were picked.

Stereology was executed on light microscopy images. Target parameters were liver volume, total number of hepatocytes, and their nuclear and cellular volumes. Liver volumes were estimated by the principle of Cavalieri and the cell numbers by the optical fractionator. Nuclear and cell volumes were estimated by the nucleator.

Statistical analysis did not support significant differences between sexes at each age, for all parameters. By the contrary, the analysis of variance of the cell and nuclear volumes accounted for a significant age effect ($P<0.001$), in both sexes. Both females and males of 17 days had a smaller cell volume than females and males of 7 days (for both sexes P values $P<0.001$). This was paralleled by the increase in the number of hepatocytes from 7 to 17 days; more striking in females. The combination of a higher number but of smaller cells in 17-day-old fish resulted in that the liver volume did not vary between the two ages.

With this study, it was verified that at early stages of the guppy life, despite the body mass and length of the guppy increases, the volume of the liver did not accordingly, and passes by a state of a relatively steady volume, based on the generation of more hepatocytes but of smaller (nuclear and cell) volume. This should represent a baseline mechanism of creating a large of hepatocytes that could hypertrophy later on to support liver growth. This should be explored further.

Keywords: age; gender; stereology; hepatocyte; liver.

1. Introduction

The liver is quoted as the largest gland of vertebrates, from fish to humans (Akiyoshi and Inoue, 2004; Qin and Crawford, 2018). It is a vital organ whose functions span from the paramount role in the metabolism and depot of nutrients, serving as a dynamic energy reserve to face the organism needs, to the organ's unparalleled ability to biotransform and eliminate endogenous or exogenous toxic substances. It has been frequently restated that the liver performs over 500 independent functions (e.g., Bhatia et al., 2014). Therefore, it is quite natural that the healthy and diseased liver has be so often studied. The classic organisms of interest in hepatology have been mammals, especially rodents as experimental models, and humans, because of the liver diseases affecting the latter (Blachier et al., 2013). But not only the mammalian liver sparks the interest of scientists. Lower vertebrates, and particularly fish, have been much studied in what regards the liver structure and function. On the one hand, from long ago there was a fundamental interest in understanding the phylogenetic aspects of the organ structure, and fish, with its long evolutionary history and highest species diversity among vertebrates, have been studied since then, particularly regarding its microanatomy (Shore and

Jones, 1889; Elias and Bengelsdorf, 1952). By other hand, and exactly because of the organ's specific functions, studying the fish liver gained a renewed interest due to progresses in aquaculture and need to master nutritional aspects, and concerns about the toxicological impacts of pollutants on fish populations. Ultimately, the increasing popularity of using fish in biomedical research, particularly as model organisms of human disease, makes the liver of fish increasingly studied in both innovative and wider contexts (e.g., Zang et al., 2017; Fujisawa et al., 2018).

In addition to its multifunctionality, the liver is known for long to be quite plastic in what concerns its unusually high regenerative capacity. Indeed, the organ is capable of rapidly recovering from massive losses of mass, as very well studied using experimental surgical hepatic resections in rodents (Martins et al., 2008). It is relevant to note that loss of mass can occur after experimental or unwanted chemical liver injury (Tuñón et al., 2009), which is relevant in diverse toxicological scenarios. It is defended that the capacity of the liver to regenerate after loss of its mass apply to vertebrates from fish to humans (Michalopoulos, 2007). Restoration of the lost mass was shown to occur in days (5 to 7 in rodents and 8 to 15 days in humans) (Michalopoulos, 2007). More recently, zebrafish was found to accomplish a recover from extreme hepatocyte loss in only 5 days (He et al., 2014). Despite the liver's astonishing regenerative capacity, there is a limit. In several pathological conditions, hepatocytes are unable to regenerate themselves (He et al., 2014). Knowing fundamental mechanisms should help to circumvent those limits. Despite liver regeneration in mammals has some characteristics that markedly differ from developmental growth (Gentric et al., 2012), regeneration is a regrowth process that also uses some of the same basic underlying mechanisms the organ displays in post-natal growth (Sanders et al., 2012; Kholodenko and Konstantin, 2017). As such, studying developmental liver growth should disclose similar mechanisms as used in regeneration, as shown in zebrafish (Gao et al., 2015; Sadler et al., 2007).

The literature is rich on in depth studies of the mammalian liver embryonic, foetal and post-natal hepatogenesis and developmental growth dates a long history of findings. It was soon discovered that the postnatal proliferation of hepatocytes to sustain the necessary increase of liver mass is a more complex process than would be anticipated (Post et al., 1963; Grisham, 1969; Schultze et al., 1978). For example, in young 3-week-old rats it was soon noticed that liver cell populations were not multiplying exponentially, as initially expected, but grew with a constant generation time (Schultze et al., 1978). Nowadays, it is established that early in the postnatal period the liver enlargement is done at a slower pace, and that later on the rate increases to achieve the species normal and optimal hepatosomatic ratio (Moreno-Carranza, 2018). In contrast, the knowledge of the cellular kinetics and mechanisms underlying the liver developmental growth in fish embryos, larvae (corresponding to the postnatal period of mammals) and juvenile is comparatively meagre. Yet, not only works with diverse species show that the mechanisms of hepatogenesis are conserved in vertebrates (Lu et al., 2011), but the specific value of using fish to uncover new paradigms was recognized (Chu and Sadler, 2009).

Curiously, despite the embryonic and post-embryonic liver developmental growth implicates cell proliferation, there are very few studies that attempted to quantify under the microscope the evolution of the cell number, namely of the hepatocytes. The same applies to the cell size along the liver development. There seems to be only one study that, using classic stereological methods, reported that the rat liver volume was multiplied by 84 from 13 to 20 days of gestation, with the hepatocyte volume being multiplied by 1.5 (Vassy et al., 1988). As far as we know, there are no stereological studies made with postnatal rats. So, it is an open issue to know the relative contributions of hepatocyte hyperplasia and hypertrophy for liver growth, from the early life stages until adulthood. This seem valid for from fish to humans.

What is known about fish liver development and regeneration in fish is mostly due to studies in the oviparous zebrafish. Additionally, we know virtually nothing about such issues in the liver of fish with other reproductive strategies. The guppy (*Poecilia reticulata*) is an ovoviviparous livebearer. In addition to being popular among fish hobbyists as an easy to keep and breed species, the guppy is used in research, included in the so-called “small fish models” (Ankley and Johson, 2004).

Recently, we start exploring the guppy as a model for new hepatology insights and for building a much better normal reference to support histopathological studies. Particularly, the guppy liver has been targeted along time in various toxicological contexts (e.g., Simon et al., 1980; Antunes et al., 2017; Qualhato et al., 2018). We studied the adult liver in what respects the spatial distribution of blood vessels and biliary tree (Sousa et al., 2018). To better understand certain features seen in the adult, we started studying histologically how the vascular-biliary evolved from the early fry (unpublished data). In parallel, we asked how exactly the liver increased in mass from larvae to adults. As there are no answers in literature, we questioned to which extent hepatocyte hyperplasia/hypertrophy contributes to liver grow. Here we start answering this question, by making a stereological estimation of the liver volume, and of the hepatocyte volume and number in guppy aged 7 and 17 days.

2. Material and methods

2.1. Animals

From a production stock raised in an Israeli farm, adult pregnant female guppy (n=3) were obtained from a legally authorized reseller operating in Porto, Portugal. The fish were acclimatized in glass aquaria (5L) for 1 week to laboratory conditions, being kept thereafter in plastic breeding cages. Dechlorinated tap water was used, being continuously filtered and aerated. The temperature was kept at 24 ± 1 °C, and under a 12:12 h light-dark photoperiod. The fish were fed once a day with TetraMin Tropical Fish Flakes. Females had, on average, 4.5 cm in total length. Housing and fish handling followed the European directive of animal welfare (2010/63/EU).

After birth, the fry were separated from their mothers and left to grow in 5 L plastic tanks under the same conditions used to keep the parents. The fry were fed crushed flakes of the above mentioned brand. When the fish had the targeted ages, either 7 or 17 days, they were sacrificed by an overdose of anaesthetic; 2-phenoxyethanol, (Merck, Germany), used at a concentration of 1 μL/mL.Twenty five fish aged 7 days and 14 fish aged 21 days were collected for histology. Before fixation, each animal was weighed (in mg) and photographed with a digital camera (Olympus, DP21) coupled to a stereomicroscope (Olympus, SZ61). Later, the fish total and standard lengths were measured on images imported to ImageJ software (Ver. 1.52).

2.2. Histological processing

Each fish was fixed in toto in Bouin's solution, for 24 h, at room temperature (ca. 20 ºC). The fixed animals were washed in 70 % ethanol and then dehydrated with an ascending series of alcohol (70, 95, and 100 %), followed by clearing by xylene. Then, samples were put in embedding cassettes, dehydrated and impregnated by high quality paraffin (Paraplast Plus, Sigma-Aldrich). The processing was made using an automatic tissue processor (Leica TP1020, Germany). The final embedding was made in a tissue embedding centre (Leica EG1150, Germany). Fish were orientated to generate sagittal planes. To avoid losing sections containing the liver, each block was totally serially sectioned with a fully automatic rotary microtome (Leica RM2155, Germany). Section thickness was set at 25 µm. Sections were glued to slides coated with aminopropyl triethoxysilane (Sigma A3648, Germany). On average, 7 days old animals produced ~56 sections and fish of 17 days old raised ~68 sections. Sections were stained with hematoxylin-eosin (HE) and mounted with DPX (Sigma-Aldrich).

As the fish sex could only be identified with a histological analysis, we sectioned randomly picked animals with 7 days of age until we obtained a sample of 5 males and 5 females. The same approach was done for animals aged of 17 days. For the latter case, from the initially fixed fish we could sampled 5 females but just 3 males.

2.3. Stereological analysis

The serial sections from each fish were first qualitatively evaluated to select those that had liver. The liver sections were then subjected to a systematic sampling (with a random start), so that, on average, 10 sections would be selected for stereology.

The procedures were done semi-automatically, using a stereological workstation, run with the software CAST-Grid (version 1.5, Olympus Denmark), and integrating a light microscope (Olympus BX50, Japan), equipped with a microcator (Heidenhain MT-12, Germany), a motorized stage with a 1 μm X-Y movement accuracy (Prior, USA), and a CCD video camera (Sony) displaying live image in a 17'' CRT monitor (Sony Trinitron, Japan). The primary stereological parameters were liver volume (V), total number hepatocytes per liver, volume of the hepatocyte nucleus [$\bar{v}_V$ (nucleus)] and volume of the hepatocytic cell [$\bar{v}_V$ (hepatocyte)]. Secondary parameters were derived from the primary estimations, namely one hepatic-somatic index (HSI) and nucleus-to-cell volume ratio $\hat{V}_V\ (nucleus, cell)$. As this is a comparative study of fish treated equally as to histotechnology, the volumes were not corrected for shrinkage.

The Cavalieri's principle was used to estimate the V. Accordingly, in every section sampled for stereology, a point-counting grid was superimposed on images of the liver (captured with the ×4 objective lens). Knowing the grid and the points that laid on the liver, the V was estimated with the formula (Gundersen and Jensen, 1987):

$$V(liver) = \bar{t} \cdot \frac{1}{ssf} \cdot \frac{a}{p} \cdot \sum_{i=1}^{m} P_i$$

where $\bar{t}$ is the average section thickness (in μm) in a series of sections from one fish, sampled for stereological purposes, ssf is the section sampling fraction (i.e., the ratio of sections sampled for stereology facing the total number of liver sections in a fish (a/p) is the area per point (in μm, corrected for the magnification used), and P_i is the number of point over the liver in a section, summed over all the sampled sections (from i to m). The $\bar{t}$ derived from measurements made in every disector (see below).

The liver volumes were used to compute the hepatic-somatic index (HSI) per fish:

$$HSI = Liver\ Volume\ (mm^3) \div Body\ Mass\ (mg)$$

The optical disector-fractionator technique (Gundersen, 1986; West et al., 1996) was to estimate the total number (N) of hepatocytes, using the next general formula:

$$N(hepatocytes) = Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{hsf}$$

where Q is the total number of hepatocytes counted in all the optical disectors; hsf is the height sampling fraction, i.e., the ratio of section thickness that was screened in z-axis for counting; asf is the area sampling fraction, i.e., the ratio between the area of the counting frame and the area covered by each *x* and *y* movements of the motorized stage; ssf is the section sampling fraction, i.e., the ratio of total sections that was sampled for stereological purposes. As for the Cavalieri, an average of 10 sections were sampled per liver. From 215 up to 953 hepatocytes were counted per liver. Counting was done in live images displayed on the monitor, captured with ×100 (NA = 1.35) oil immersion lens in systematically sampled fields. The x-y moving step between fields varied from 100-100 µm to 120-120 µm, according to liver size (the bigger the liver the smaller the step). The real section thickness (*t*) was measured in every sampled field. Because it was quite consistent, revealing no section deformation, the averaged *t* for each fish was used to compute $hsf = h/t$ (Dorph-Petersen et al., 2001). The overall average (all fish) of *t* was 23.4 µm and the pre-settled disector height *h* was 15 µm. A minimal top guard zone of 5 µm was set as there was no heterogeneous distribution of hepatocytes in the z-axis (Bartheld, 2002). To avoid biases by edge effects, the digital counting frame had inclusion and exclusion lines (Gundersen, 1977). Hepatocytes seen within optical disectors and that had one visible nucleolus in sharp focus were counted – and sampled too for volume estimation (see below) – if the nucleolus was inside the area of the sampling frame or if it touched the inclusion lines, but it could not touch the exclusion lines.

The $\bar{v}_V$ (nucleus) and $\bar{v}_V$ (hepatocyte) were estimated in the same cells sampled for counting N. The nucleator was used, based on the formula (Gundersen, 1988):

$$\bar{v}_N(nucleus\ or\ cell) = \frac{4\pi}{3} \cdot \bar{l}_n^3$$

were l is the length measured from the centre of nucleoli to the point where the line intercept the nucleus/cell boundary. We used not the original nucleator but its more efficient variant, the "four-way nucleator" (Tandrup, 1993), where measurements of l are made not in two but in four directions, and therefore in the cited formula n = 4.

At last, the volume density of the nucleus in relation to the cell, $\hat{V}_V\ (nucleus, cell)$, in %, was estimated with the formula:

$$\overline{V}_V(nucleus, cell) = 100\ \cdot\ \bar{v}_N(nucleus) \div\ \bar{v}_N(cell)$$

2.4. Statistical analysis

Data are presented as mean, standard deviation (SD), and coefficient of variation (CV), with animals being grouped in accordance with the sex and the age. Inferential statistics was made with the open source software Past 3.21 (Hammer et al., 2001). After confirming the assumptions of normality and homogeneity of variances of the data sets, using the Shapiro-Wilk's and the Levene's tests, respectively, the effect of age and sex were investigated using two-way ANOVA. To grant the validity of the procedures, a log or a rank transformation was implemented whenever a data set did not meet any of the assumptions. Post-hoc analyses to compare specific groups were done using the Tukey's test. The significance level was set at the usual 5%. Data are given as group means, standard deviations and coefficients of variation.

3. Results

3.1. Body morphometry

The primary gross morphometric parameters are accessible in **Table 1.**

The 2-way ANOVA revealed a significant result for the factor age (p = 0.015), with 17 days-old fish being heavier. However, such increase was fundamentally due to the females (41% difference of older vs younger fish). Indeed, in males the mean body mass in the older animals was only 9% greater (which turned out to be a non-significant difference under the post-hoc Tukey's test). The effect of sex and of the interaction between age and sex were both not-significant.

As to the total and to the standard body length, in both cases the 2-way ANOVA revealed non-significant effects for the factors sex and interaction of sex and age. Yet, there were marked statistically significant results for the factor age ($p < 0.001$), with the 17 days-old fish being lengthier than the younger. Anyway, females had more pronounced differences ($p < 0.001$, with the older fish being ca. 50% lengthier) when compared with the males ($p = 0.017$ for total length and $p = 0.018$ for standard length, being the older fish ca. 35% lengthier).

Overall, body mass tended to have higher interindividual variability (greater CVs).

Table 1 – Body mass, total length and standard length of female and male guppy aged of 7 and 17 days after birth*.

Age (days)	**Body Mass** (mg)		**Total Body Length** (mm)		**Standard Body Length** (mm)	
7	Females	Males	Females	Males	Females	Males
Mean	6.9	8.2	7.1	7.0	5.3	5.3
SD	1.4	1.1	0.7	0.2	0.5	0.2
CV	21%	14%	9%	2%	10%	3%
17	Females	Males	Females	Males	Females	Males
Mean	9.7	8.9	10.7	9.4	8.1	7.2
SD	1.0	2.4	0.4	1.8	0.2	1.5
CV	10%	26%	3%	19%	2%	20%

* 7 Days: n = 5 fish / sex. 17 days: n = 3 for males and n = 5 for females. Data sets are displayed as mean, standard deviation (SD) and coefficient of variation (CV).

3.2. Stereologically derived parameters

The parameters (directly or indirectly) estimated with the use of stereology are given in **Table 2** and **Table 3**.

In **Table 2** data are for HIS, liver volume and number of hepatocytes. The 2-way ANOVA for the HSI and liver volume did not reveal significant effects of sex, age or interaction. By the contrary, for the number of hepatocytes there was a significant effect of age ($p = 0.012$), with older fish having more cells. However, considering the post-hoc test used, the pair difference was significant for females only ($p = 0.04$). In females the cell number increased 80% while in males increasing trend was 32%.

Table 2 – Hepatic-somatic index (HSI), liver volume and number of hepatocytes per liver of female and male guppy aged of 7 and 17 days after birth*.

Age (days)	**HSI** (mm^3/mg)		**Liver Volume** (mm^3)		**Hepatocyte Number** (per liver)	
7	Females	Males	Females	Males	Females	Males
Mean	0.017	0.016	0.11	0.13	102,630	121,577
SD	0.009	0.006	0.04	0.03	34,167	29,189
CV	50%	37%	35%	24%	33%	24%
17	Females	Males	Females	Males	Females	Males
Mean	0.014	0.016	0.14	0.14	185,121	160,094
SD	0.002	0.003	0.01	0.03	98,768	18,078
CV	12%	20%	9%	21%	53%	11%

* 7 Days: n = 5 fish / sex. 17 days: n = 3 for males and n = 5 for females. Data sets are displayed as mean, standard deviation (SD) and coefficient of variation (CV).

In **Table 3** data are the cell-size parameters: $\bar{v}_V$ (nucleus), $\bar{v}_V$ (hepatocyte), and $\overline{V}_V$ (nucleus, cell).

As to the $\bar{v}_V$ (nucleus), according to the 2-way ANOVA there was no effect of sex and of the interaction between sex and age. Yet, the effect of age was significant, ($p < 0.001$), with older fish hepatocytes having smaller nuclei. The post-hoc analysis revealed significant pair differences in females ($p = 0.022$, with a decrease of 24% in volume from 7 to 17 days) and males ($p = 0.001$, with decrease of "only" 17%).

As to the $\bar{v}_V$ (hepatocyte), the 2-way ANOVA also exposed no effects of sex and of the interaction between sex and age. Again, there was a marked effect of age ($p < 0.001$), with older fish having less voluminous cells. Post-hoc testing showed significant pair differences in females ($p < 0.001$, with a decrease of 57% in volume from 7 to 17 days) and males ($p < 0.001$, with a comparable size decrease of 60%).

Finally, as to the $\overline{V}_V$ (nucleus, cell), the 2-way ANOVA did not unveil significant effects for the factor sex for the interaction between those parameters, but exposed an overall significant effect for the factor age, with an increasing trend in older fish ($p = 0.017$). However, the post-hoc analyses did not identify a single significant difference between pairs. As such, only when male and female were combined there was enough power to highlight an overall 15% increasing of the parameter with age.

Table 3 – Nuclear volume [$\bar{v}_V$ (nucleus)], cellular volume [$\bar{v}_V$ (hepatocyte)], and volume density of the nucleus in relation to the cell body [$\bar{V}_V$ (nucleus, cell)] of the hepatocytes of female and male guppy aged of 7 and 17 days after birth*.

Age (days)	**$\bar{v}_V$ (nucleus)** (μm³)		**$\bar{v}_V$ (hepatocyte)** (μm³)		**$\hat{V}_V$ (nucleus, cell)** (%)	
7	Females	Males	Females	Males	Females	Males
Mean	25	24	485	468	5%	6%
SD	2	2	48	99	1%	1%
CV	9%	8%	10%	21%	14%	20%
17	Females	Males	Females	Males	Females	Males
Mean	19	20	209	188	7%	8%
SD	3	1	52	21	2%	2%
CV	13%	3%	25%	11%	32%	30%

* 7 Days: n = 5 fish / sex. 17 days: n = 3 for males and n = 5 for females. Data sets are displayed as mean, standard deviation (SD) and coefficient of variation (CV).

3.3. Correlation analysis

Linear correlation analisis investigated the degree of linear association between some of variables. It was found that the liver volume is positively correlated with the number of cells; the volume of the nucleus somewhat related to cell volume and cell volume is negatively correlated with the racio N:C. The number of cells is negatively correlated with the liver mass, ie, the lower the number of cells the greater the mass of the liver. This negative correlation will be related to a first phase of increase of the number of cells due to an intense cellular multiplication without increase of the volume of the liver, since the cells are more numerous, but of smaller dimensions in this phase of the liver's development and later there will be a phase of cell growth without increasing the number of cells which will lead to an increase in liver volume.

In the two ages, the number of cells is positively correlated with the liver volume, this positive correlation is more influenced by the 7-day age group, which presents a lower number of cells, but with a higher cell volume.

4. Discussion and Conclusion

This study allowed to evaluate the development of guppy fish liver over time through the stereological analysis of different biological parameters at two different ages of fish, 7 and 17 days. Besides the age factor, the influence of the sex factor on the histological development of the liver was also evaluated. This study made it possible to determine the total number of hepatocytes per liver, the total volume of the liver, the hepatic-somatic index, nuclear and cellular volume of the hepatocytes and nucleus to cell volume ratio (N:C) and to establish comparisons between the values found for males and females within the same age group and between the two age groups studied. These data allow an objective evaluation of the parameters under study that can thus establish comparisons between different species or between individuals of the same species and to evaluate eventual pathologies associated with variations of the volume of this organ.

Statistical analysis of the data allowed us to conclude that there are no significant differences between genders of the same age group. It was verified that the parameters determined in the two age groups of fish were more influenced by the age factor than by the sex factor. The very young age of these two group of guppy fish with reduced differentiation may be the reason to explain this similarity of results between genders. However, we can not be sure that this similarity remains in the adult state because there are no quantitative data regarding these parameters in adult fish. However, there are significant differences in some of the parameters between different ages of which emphasize the increase in the number of cells and the reduction of their volume from 7 to 17 days. This fact is important because it allows marking a phase of intense cell multiplication without a corresponding increase in liver volume occurring at a higher age.

A comparison can be made with other species in which the stereological evaluation of the liver was studied, e.g. brown trout, *Salmo trutta* f. *fario* (Rocha et al., 2009), rainbowtrout *Oncorhynchus mykiss* (Arnold et al., 1995) but also in this fish there are no significant differences between genders.

However, this studies above mencionated was not done with fishes of the same ages as the guppy fish, and a significant comparison can't therefore be made.

In summary, we can consider this study provides us with important information for the overall knowledge of guppy liver. This stereological tool makes it possible to complement the histological studies. According to the main objective of this study the estimation of the parameters studied using this technique combined with the histological studies for the same age of the animal (Chapter 3) allows us a more objective and therefore more real knowledge of the guppy fish liver.

It is not possible to establish a good correlation with studies performed at more advanced ages. However, the results obtained from this analysis reinforce the idea that the liver has characteristics intrinsic to each species and only from an individualized analysis will it be possible to obtain a more detailed knowledge of this organ.

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FINAL REMARKS AND CONCLUSIONS

Final remarks and conclusions

Fish liver was the central subjet study of this Thesis.

Fish liver is a gland of special relevance on the control of several vital functions and is therefore used as a study model to evaluate the health status of natural populations (Bernet et al., 2004; Ramírez-Duarte et al., 2008). As far as liver morphology and histology are concerned, they can be used as a sensitive bio-monitoring tools about toxicant impact assessment to indicate the effect of toxicants on fish health in polluted aquatic ecosystems (Stentiford et al., 2003; Bernet et al., 2004; Ramírez-Duarte et al., 2008). Histopathological assessment of fish tissue allows for early warning signs of disease and detection of long-term injury in cells, tissues, or organs (Peakall, 1992).

Moreover, as the liver is very important when studying fish diseases, the knowledge of normal microanatomy is essential to assess histological changes, e.g., related to environmental change or toxic pollutants.

The main objective of this Thesis is to contribute to the identification of anatomical and structural variations of this organ in different teleost species. We chose guppy fish as a model due mainly to the favorable characteristics of this species, we emphasize the high reproduction rate, females have continuous reproductive cycles, high resistance to unfavorable environmental conditions specialy to the temperature (Chung, 2001; Reeve et al., 2014).

The high tolerance to environmental factors of the *Poecilia reticulata,* Peters, 1859, makes it possible when appears in a natural environment, to be used as a bioindicator of negative environmental disturbances in the environment where it occurs, since this species has ecological adaptations which gives it a great success in colonizing in many types of environments such as high competence in inter and intraspecific competition (they use the same resources of native species) and the ability to withstand extreme variations in the environment (Reznick et al., 2012).

We studied for the first time the distribution and spatial organization of the different types of blood vessels and biliary ducts and the relationships between them are established (chapter 2). For this, each liver was totally sectioned, and the serial sections inspected in detail. The guppy liver presented intra-hepatic pancreatic tissue named hepatopancreas (Rocha et al., 1994), such as *Ictalurus punctatus* (Kendall and Hawkins, 1975; Hinton and Pool, 1976) and so reported its association with the vascular and biliary elements.

The follow-up from the observation of serial cuts of the liver of the different types of vessels within the liver allowed their observation either in isolation or in different types of associations, such as venous-arteriolar tracts (VAT), and venous-biliary arteriolar tracts (VBAT). The liver of the guppy has pancreas inside and sometimes, pancreocytes appear within the liver surounding isolated veins, forming venous tract with pancreatic acini (VT-P), or dual associations with afferent vessels, forming venous–arteriolar tracts with pancreatic acini (VAT-P). Intrahepatic pancreatic ducts were tiny and rare, putting in question the functional role of the acini. No isolated arterioles were observed inside the liver or in association with bile ducts (BAT). This fact shows a reduced arterialization of this organ contrasting with an over predominance of a random distribution of the venous vascularization. We found aggregates of macrophages, namely associated with afferent and efferent venous vessels this observation is not common in other fish species. The guppy differs to some extent from other previously studied models as for exemple chum salmon, *Oncorhynchus keta* (Takahashi et al., 1977), coho salmon, *Oncorhynchus kisutch* (Leatherland, 1982), Atlantic salmon, *Salmo salar* (Robertson and Bradley, 1991, 1992) and rainbow trout, *Oncorhynchus mykiss* (Hacking et al., 1978; Schär et al., 1985; Hampton et al., 1985, 1989; Hinton et al., 1988; Chapman, 2006; Figueiredo-Fernandes et al., 2006), highlighting the importance of making this kind of study to offer specific frameworks that can explain specific physiological processes.

This experimental work contributed to the qualitative characterization of the adult fish liver. It was necessary to achieve the objectives initially proposed to characterize the liver of the Guppy in younger stages of life and thus be able to monitor and analyze the changes that this organ is suffering throughout its development.

The histological analysis was done using three groups of fish with different age: 7, 17 and 28 days old (chapter 3). In the three groups were confirmed some of the observations made in the adult: the presence of endocrine pancreas and its entrance accompanying afferent vessels; the absence of arterioles within the liver; existence of several entry points of blood vessels in the liver. No macrophage aggregates (MAs) were observed in any of the groups studied, however, in adult fish they were frequent near blood vessels and bile ducts. This is in agreement with the association between the presence of these aggregates (MAs) and the age of the fish (Agius and Roberts, 2003), increasing their frequency in environments with oxygen deficiency and polluted by different chemical agents, thus being considered as biomarkers of water quality (Agius and Roberts, 2003). There were no associations within the pancreatic liver between different vessel types as had been observed in adult, this is probably due to the age of the larvae and consequently to the poor development of various types of blood vessels and bile ducts which haven`t yet established a defined biliary network.

The observation of several entry points of afferent vessels in adult fish was reinforced by the same observation in these very young age groups. These vessels come from the intestine and some of them are accompanied by pancreatic tissue that penetrates the liver along with the blood vessel.

In adult fish, it was observed that the pancreocytes were both adjacent to afferent and efferent vessels. Thus, a fundamental question is yet to be clarified: what is the origin of the pancreas that is next to the efferent vessels.

The combination of these two studies (chapters 2 and 3) contributed to the qualitative characterization of the guppy liver. But beyond this morphological characterization it was important to make a quantitative analysis that complemented this work.

Thus, was made the first study that used unbiased stereology techniques (Gundersen and Jensen, 1987) to estimate the volume of the liver of the guppy fish in early stages of life, 7 and 17 days of age (chapter 4). Cell parameters (nucleous and cell volume) and their number per liver were quantified and liver volume determined. It was investigated the influence of two independent variables, gender and age, on the liver development of the guppy fish. Statistical analysis of the data allowed us to conclude that there are no significant differences between genders of the same age group. It was verified that the parameters determined in the two age groups of fish were more influenced by the age factor than by the sex factor. The very young age of these two group of guppy fish with reduced differentiation may be the reason to explain this similarity of results between genders. However, we can not be sure that this similarity remains in the adult state because there are no quantitative data regarding these parameters in adult fish. However, there are significant differences in some of the parameters between different ages of which emphasize the increase in the number of cells and the reduction of their volume from 7 to 17 days. This fact is important because it allows marking a phase of intense cell multiplication without a corresponding increase in liver volume occurring possibly at a higher age.

In summary, this study allowed to evaluate the development of guppy fish liver over time through the stereological analysis of different biological parameters at two different ages of fish, 7 and 17 days.

This is the first study that used unbiased stereology techniques to estimate the volume of the liver of the guppy fish in early stages of life. The determination of these biological parameters in two different ages allows to know the main alterations in this organ during the development of the fish. We may compare our study with others e.g. brown trout, *Salmo trutta f. fario* (Rocha et al., 2009), rainbowtrout *Oncorhynchus mykiss* (Arnold et al., 1995), however these were not done in young stages of life.

The histological study showed us that there are several entry points of blood vessels in the liver, and not just a single hilar zone.

The origin of the exocrine pancreas that goes along the afferent vessels into the liver has been clarified.

The increase in the number cells and the reduction of their volume from 7 to 17 days of fish age allows marking a phase of intense cell multiplication without a corresponding increase in liver volume – Guppy liver growth is not continuous.

This investigation paved the way for further studies.

It will be important to know the origin of the pancreocytes observed in the adult next to efferent blood vessels and also to investigate when the pigmented macrophages appear inside the liver.

It will be important in the future to do a similar study using a group of fish close to the adult state and also a group of adult individuals in order to know all the development stages of the guppy liver.

Finally, I think that the work developed throughout this Thesis can make an important contribution to the knowledge of the morphology, histology and ontogenic development of the liver of the guppy fish (*Poecilia reticulata*).

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