Vol. 17, No. 3, Autumn, 2021, 05-13 *Original Article*
DOI: 10.22055/IVJ.2021.299877.2387
DOR: 20.1001.1.17356873.1400.17.3.1.6
Journal Homepage: http://www.ivj.ir/

Immunohistochemistry of hepatopancreas of *Litopenaeus vannamei*, in two different temperature

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Received: 14.08.2021 Accepted: 20.09.2021

Abstract

This study aimed to investigate the effect of water temperature on the shrimp hepatopancreas of *Litopenaeus vannamei* by immunohistochemical method. In this research, a total of 50 shrimp with an average length of 23 ± 1 cm and an average weight of 28 ± 0.5 gr were accidentally caught from shrimp farms of the Choebdeh Abadan from Khuzestan province in two different seasons of the year. The shrimp were sampled for the histological and structural study of the hepatopancreas. After killing the shrimp by physical method, the hepatopancreas of each shrimp was transferred to Davidson's solution. The tissue samples were processed by routine histological procedure, using ascending ethanol for dehydration, xylene for clearing, and paraffin impregnation. 4-6 µm sections were taken and stained with hematoxylin-eosin and TUNEL immunohistochemistry technique. Finally, slides were studied by a light microscope equipped with a Dino-lite lens and Dino-capture software. The results indicated that the hepatopancreas structure is a tubular organ that can be affected by water temperature. In the warm season, the diameter of the tubules was smaller than in the cold season ($p \leq 0.05$). There was a significant difference in the percentage of apoptosis between the tubule-forming cells in the warm and cold seasons.

Key words: Immunohistochemistry, Hepatopancreas, *Litopenaeus vannamei*, Temprature

Introduction

The Crustaceans are the largest class in the arthropods, with more than 42,000 species. Most are marine; some live in freshwater (Dipper, 2021). Vannamei shrimp is found native to the eastern shores of the Pacific Ocean from Sonora and Mexico in the north, throughout south and central America to Peru, and in areas where the natural water temperature reaches more than 20 ºc throughout the year (Zhao et al, 2016). Due to the great diversity of these species and the need to use high-yielding and valuable native species and opposition to the import of non-native species, and on

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the other hand, given the economic conditions, the growing tendency to breed this shrimp species has increased (Flegel, 2012). The hepatopancreas is the largest secretory-storage gland in the shrimp body and a vital organ at the Decapoda, including the shrimp, that performs the functions of the liver, pancreas, intestines, and some other organs in vertebrates. It makes up about 6 to 8 percent of body weight and is the largest organ in the middle intestine of Crustaceans (Nunes et al, 2014; Basir et al, 2010). In the middle intestinal, it occupies the most significant volume of the intestinal tract. It transports digested food to the hepatopancreas to absorb its nutrients (Vogt, 1992). The proper functioning of the hepatopancreas has a significant impact on the health and growth of shrimp and is used as an indicator of shrimp health in studies. The function of this vital organ is the synthesis and secretion of digestive enzymes, absorption of nutrients and their continuous digestion, storage of organic matter, metabolites, fats, carbohydrates, production of materials needed for periods of transformation and vitiligo, and detoxification by metal storage (Liao, 1983). The tissue structure of this organ changes in salinity and different water temperatures directly and can affect shrimp's shelf life and health of shrimp (Michiels et al, 2013). The amount of fat and glycogen reserves in this tissue indicates this animal's health, nutrition, life stage, and hunger can also cause morphological changes in this organ. Rising temperatures, especially high temperatures, increase the body's metabolism, energy intake, and on the other hand, can reduce feed intake and consequently shrimp growth rate (Panakorn, 2012). Some researchers have studied the changes in the hepatopancreas' fat, carbohydrate, and protein content during short-term and long-term starvation. But so far, comparatively, the histological and structural characteristics of this organ, with the help of immunohistochemistry techniques, the different temperature range in two seasons have not been studied yet. This study may help maintain the health of shrimp because these changes may have been physiological or sometimes pathological effects.

Materials and Methods

Shrimp sampling

A total of 50 *L. vannamei* with an average length of 23±1 cm and an average weight of 28±0.5 g sampled randomly from Choebdeh Abadan shrimp farms with longitude 29º 42' 31/11" E and latitude 37º 17' 44/23" N in two temperature range in two different seasons (Figure 1, Table 1).

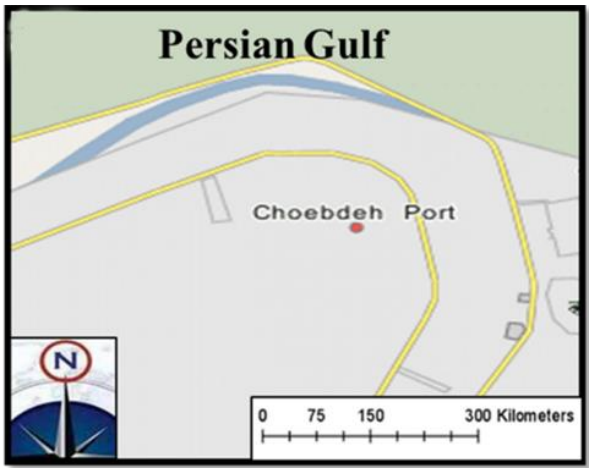

Figure 1. Map of sampling area in Choebdeh Abadan in southern Iran

Table 1. Physicochemical characteristics of shrimp ponds in the sampling times

Parameter	Temperature (°C)	Salinity (ppt)	pH	Dissolved oxygen (mlL^{-1})	Transparency (cm)	Water height (cm)
Warm season	30-35	22-25	7-8	6-12	85	110
Cold season	13-17	15-23	7-8	7-12	85	110

Tissue sampling

After killing the shrimp by physical method, the hepatopancreas of each shrimp was transferred to Davidson's solution. Before dissection of all shrimps, they were injected with Davidson solution to confirm intra-tissue fixation (Berry et al, 2019; Chen et al, 2019).

Tissue processing and staining

The usual histological method on the samples was performed with a tissue processor device, dehydration with ethanol ascending degrees, clearing with xylene, and paraffin impregnation (Devi et al, 2015). Then from the samples were cut 4-6 µ slices and stained with Hematoxylin-Eosin (H&E) dye (Ghasemi et al, 2014).

For the immunohistochemical technique and TUNEL test, tissue paraffinic sections with a thickness of 4 µ were prepared from the samples. The sections were de-paraffined by xylol and dehydrated by ethanol descending concentrations. Then these were washed with PBS, and 20µl of protease K working solution (0.19 g of protease K was added to 10 ml of 10 mM tris solution) was added to each section and heated for 30 min at 37 ºC were incubated and finally the sections washed three times with PBS. To each section, 50µl of TUNEL working solution (Roche, Germany) was added and incubated at 37 ºC for 60 min. It should be noted that prepared this solution immediately before use and according to the instructions of the German company Roche. Then washed the sections with PBS and with three repetitions. At this stage, 50 µl of POD solution were added to each section and incubated for 30 min at 37 ºC. The sections were re-washed three times with PBS. Finally, 100 µl of DAB working solution (according to the instructions of Roche Germany, 125 µl of DAB substrate solution add 1.125 ml of peroxidase) were added to each section and kept at room temperature for 15 min away from light. The sections were re-washed three times with PBS (Glick et al, 2010; Qiu et al, 2017; Moradkhani et al, 2020). Then, the percentage of apoptotic is equal to the number of apoptotic cells in total cells multiple 100. Finally, the study of the slides produced by the light microscope of the Olympus model (OLYMPUS, BH-2) equipped with a Dino-lite lens and a computer system equipped with a Dino capture 2.0 software and 200-magnification was performed (Gholami et al, 2018).

Statistical analysis

All statistical analyses were performed using the Graph Pad Prism (V.5.04.San Diego, CA, USA). Data are expressed as mean± SEM, and the results were statistically evaluated using a one-way ANOVA test. In all cases, it was considered significant between individual groups ($P \leq 0.05$)

Results

The microscopic studies showed that the hepatopancreas in shrimp was an organized glandular-tubular structure consisting of several tubes that connect to the midgut through the main duct in the gastrointestinal tract. The gland is covered on the outer surface by a capsule of connective tissue and two layers of muscle. From this capsule, threads go inwards and divide into lobules and form the lobules' stroma. The structure of all lobules was similar. The gland is a type of compound tube gland in which tubular secretory units radiate around the gland and pour their secretions into the central ducts of the gland. There are tubules of different diameters in each lobule. The tube duct had a star-shaped appearance lined with a row of epithelial cells. Around the tubes is a network of myoepithelial cells with large, stretched, and distinct nuclei attached to the basement membrane, which was responsible for contracting and pressing on the secretory cells. Hepatopancreatic tubules consisted of several types of cells; each different cell type was seen in each part of the tubule. Some lesser-diameter tubules were found

inside and around the lobules, and their walls were covered with short columnar, basophilic cells with an oval nucleus. These cells are called basal cells. Adjacent to these tubules were larger diameter tubules with oval-shaped and highly basophilic columnar cells in the wall. Their cytoplasm above the nucleus has transverse lines seen in an optical microscope; these cells are called resorbtive cells. In the middle of the lobules, there were tubules with large diameters that covered the swollen and bright cells with a spherical, coarse-grained nucleus, and these cells swelled toward the inner of the gland. Thus the internal shape of the star tube has formed, and these cells are called blister-like cells. Another type of cell, In smaller numbers, is short cuboidal cells with microvilli that placed on the surface of the tubules (Figure 2, 3). In histometrical studies and counting the number of cells that make up the tubules in the samples related to each season, five microscopic fields of each slide with a magnification of 40 were determined that the number and type of cells that make up the wall in different studied temperature are different. In the warm season, most types of cells were related to basal cells and myoepithelial cells. In contrast, in the cold season, hepatopancreas was normal, bister-like, and fibrillar cells were the most common type of cells ($p \leq 0.05$) (Figure 5). In the warm season, the diameter of the tubules was smaller than in the cold season ($p \leq 0.05$) (Table 2). There was a significant difference in the percentage of apoptosis between the two warm seasons and the cold season ($p \leq 0.05$) (Figure 4, 6).

Table 2. Comparison of the diameter of the tubules in the hepatopancreas of *L. vannamei* in the warm and cold seasons (mean ± SEM).

Parameter	warm season	cold season
Diameter (µm)	2.8±39.7	4.7±109.8

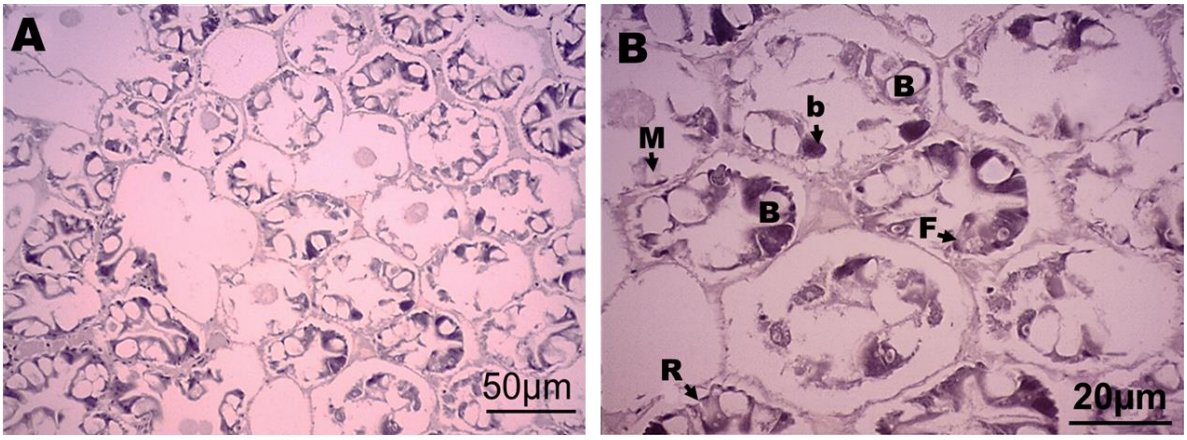

Figure 2. Microscopic view of the hepatopancreas morphology of *L. vannamei* in the warm season (H&E). Fibrillar cells (F), Blister-like cells (B), basal cells (b), Resorbtive cells (R), Myoepithelial cells (M). Scale bars indicate 50 µm for A and 20 µm for B.

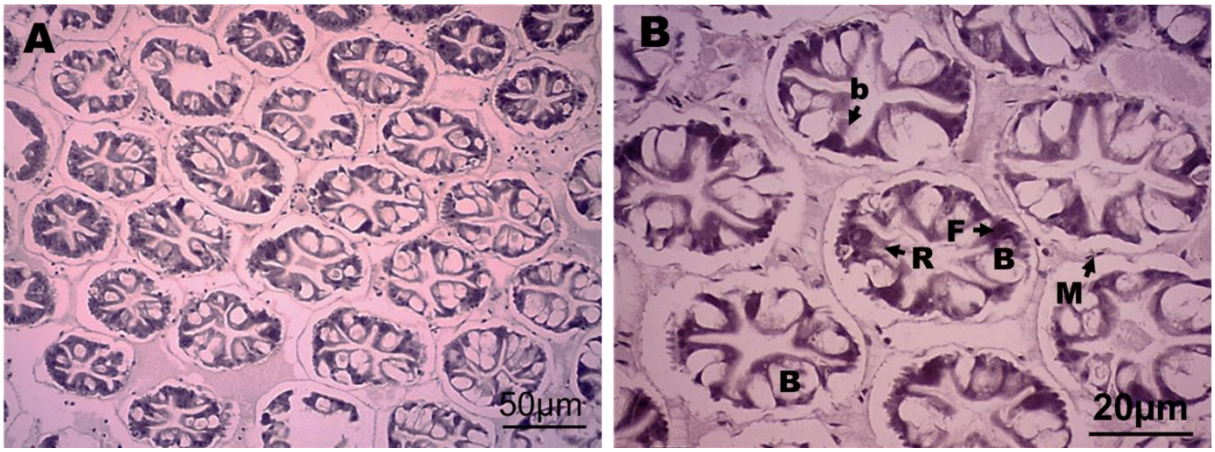

Figure 3. Microscopic view of the hepatopancreas morphology of *L. vannamei* in the cold season (H&E). Fibrillar cells (F), Blister-like cells (B), basal cells (b), Resorbtive cells (R), myoepithelial cells (M). Scale bars indicate 50 µm for A and 20 µm for B.

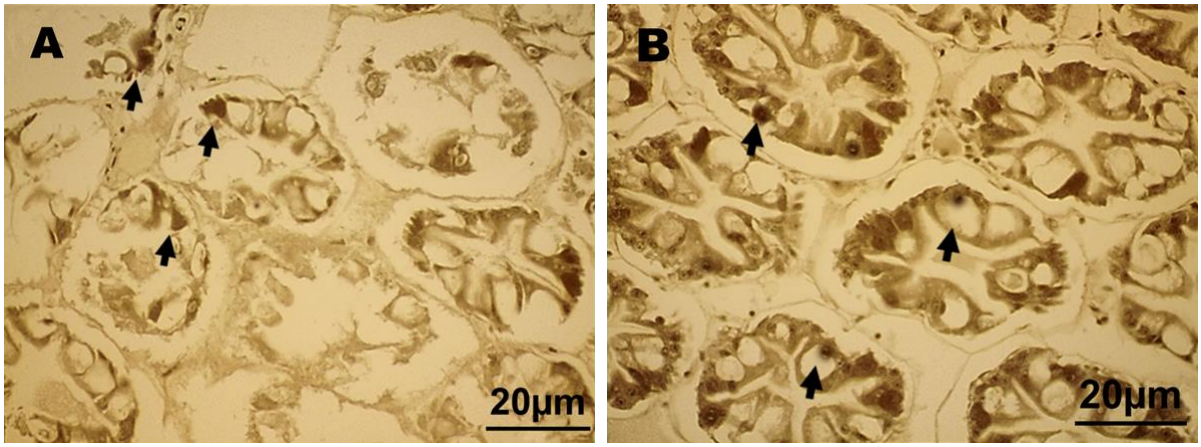

Figure 4. Microscopic view from the morphological of the hepatopancreas of *L. vannamei* in the warm season (A) and cold season (B) (TUNEL, x200); arrow indicates apoptotic cell. Scale bars indicate 20 µm.

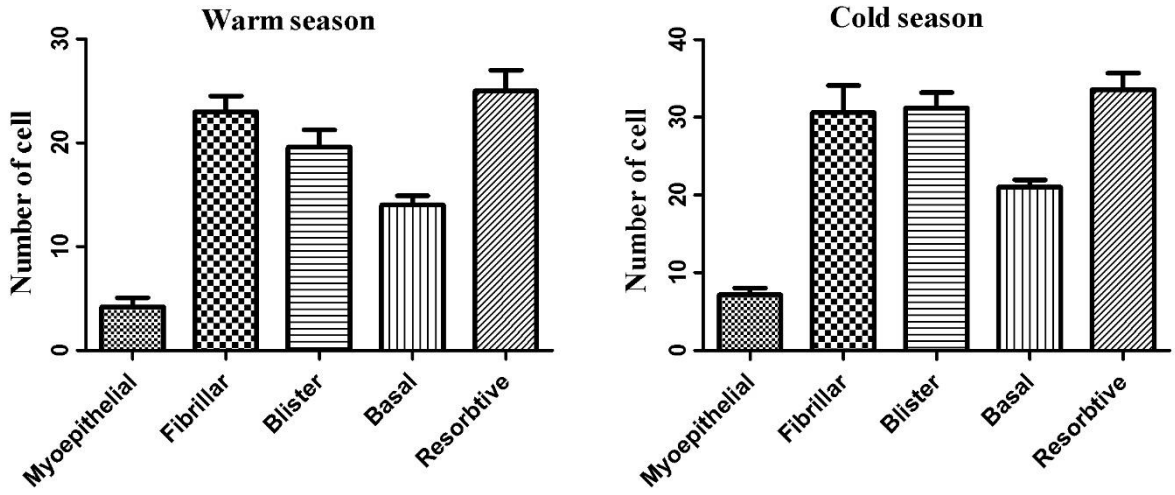

Figure 5. The number (mean ± SEM) of different cells of the hepatopancreas in samples related to warm and cold seasons ($p \leq 0.05$).

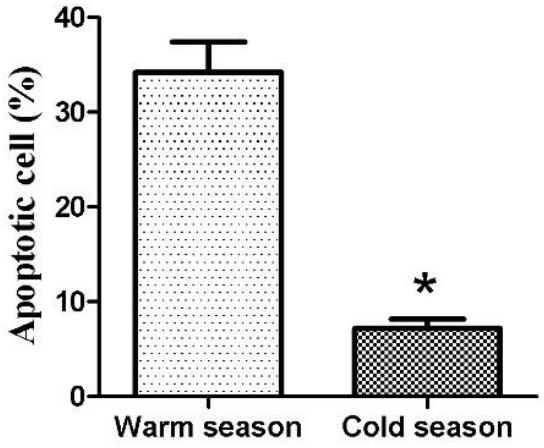

Figure 6. The percentage of apoptotic cells of the hepatopancreas in samples related to warm and cold seasons (p≤ 0.05).

Discussion

Based on the researchers' studies on the vannami species, it was found that due to the advantages of this species compared to other breeding species, including growth speed, high feed conversion ratio (Medina-Beltran et al, 2012), tolerance of the wide range of temperature and salinity changes, the survival rate and high production efficiency in larval stages and breeding period with a lower protein diet (Cavalli et al, 2001), the possibility of resistance to certain diseases, reduced production costs, and high consumer market have shown themselves to be good alternatives to low-yield shrimp (Cuzon et al, 2010). Shrimp's digestive system includes a long digestive tract that extends from the mouth to the animal's anus. The oral cavity, esophagus, and stomach are in the front of this tube to pick up and crush food. The middle section, studied in the recent study, is responsible for chemical digestion and absorption of nutrients. In the middle part of the digestive tract, various enzymes are released that help digest food (Panakorn, 2012). Researchers believe that hepatopancreas have a variety of functions, such as releasing certain digestive enzymes, storing fats, and some elements, such as calcium, due to their structural and physiological connection to the gastrointestinal tract and having certain types of cells. Studies by researchers have shown that the decrease in the number of cells may be due to the cessation of mitotic divisions due to the animal's living conditions, especially at high temperatures (Qiu et al, 2017; Panakorn, 2012). A study by Rezaian al. (2002) reported that the epithelium covering the secretory units has four types of cells. This finding is based on the present research on the vannami species in the hot season. Hepatopancreatic size, shape, and cell types appear to be affected by environmental factors such as temperature, salinity (Masson et al, 2012; Pinoni et al, 2013), and internal or physiological factors (Pinoni et al, 2013). Based on the report of Zilli et al. (2007), in the pre-peeling period, along with the calcification of the cuticle, the number of resorbtive-storage cells increases compared to other cells in the hepatopancreas (Zilli et al, 2007). These changes are probably due to increased calcium absorption by these cells, which means that more absorption-storage cells are produced due to the need for more calcium. A study by Sanchez et al. (2007) reported that short-term starvation also reduced the weight, surface, and size of the hepatopancreas relative to the weight and size of the shrimp (Sanchez-paz et al, 2007). As shown in the studies of

immunohistochemistry of hepatopancreatic shrimp during the hot and cold seasons in the present study, the highest apoptosis and cell death in the cellular structure of this organ occurred in the warm season and at high water temperatures. Researchers also believe that whenever shrimp are exposed to high temperatures, they will defend themselves against these adverse conditions (Danya et al, 2014). In cases where organisms are exposed to conditions such as temperature or starvation, they choose methods to prevent injuries such as optional cell death, cyst formation, hibernation, and energy storage (Norfaadila et al, 2013). Because in the process of apoptosis or programmed cell death as a protected method, it is under the control of a gene used to remove unwanted or unnecessary cells in living organisms. Therefore, apoptosis provides an opportunity to remove cells without adverse damage to adjacent tissues (Cottin et al, 2010). In a study, the hepatopancreas structure of green tiger prawn changed during the summer (Basir and Salari Aliabadi, 2020). In a recent study on hepatopancreas microscopic observations, especially in the warm season, reduced the number of hepatopancreatic cells compared to the cold season, which is consistent with other scientists' research because researchers believe that the early processes of apoptosis are cellular and pyknosis contraction (Niu et al, 2013). During cell contraction, the cells become denser cytoplasm in size and the masses become compact (Peng et al, 2016). Apoptotic bodies include cell cytoplasm with extremely dark organelles. Finally, based on the findings of the histomorphometric and immunohistochemistry of shrimp hepatopancreas, in two different seasons, the best time for higher yields in the cultivation of vannamei species is in seasons with moderate downward temperatures because in this condition has the lowest changes in hepatopancreas as the most shrimp body important gland.

Acknowledgements

This work was supported by the Honorable Vice Chancellor for Research and Technology of the Shahid Chamran University of Ahvaz.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was funded by the research number (SCU.VB99.103)

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Received: 14.08.2021
Accepted: 20.09.2021

DOI: DOI: 10.22055/IVJ.2021.299877.2387
DOR: 20.1001.1.17356873.1400.17.3.1.6

ایمونوهیستوشیمی هپاتوپانکراس میگو، *Litopenaeus vannamei*، در دو دمای متفاوت

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دریافت: ۱۴۰۰/۵/۲۳ پذیرش: ۱۴۰۰/۶/۲۹

چکیده

هدف از این مطالعه بررسی مقایسه‌ای سلول‌های هپاتوپانکراس میگوی وانامی در دو فصل سرد و گرم به روش ایمونوهیستوشیمی است. در این تحقیق، در مجموع ۵۰ قطعه میگوی وانامی با متوسط طول ۱±۲۳ و وزن متوسط ۰/۵± ۲۸ گرم به طور تصادفی از مزارع پرورش میگو چوئبده آبادان در استان خوزستان در دو فصل سرد و گرم گرفته شدند. برای بررسی بافت‌شناسی و ساختاری هپاتوپانکراس از تمامی میگوها نمونه‌برداری شد. برای تثبیت، با تزریق داخل دهانی و غوطه‌وری توسط محلول دیویدسون منتقل شدند. سپس نمونه‌های بافتی با روش معمول بافت‌شناسی و با استفاده از اتانول صعودی آبگیری، گزیلول برای شفاف‌سازی و آغشته‌سازی با پارافین انجام شد. برش‌های بافتی ۶-۴ میکرونی زده شد و توسط رنگ هماتوکسیلین- ائوزین و ایمونوهیستوشیمی با تکنیک تانل رنگ‌آمیزی شدند. سرانجام مورد بررسی قرار گرفتند. Dino-capture و نرم افزار Dino-Lite میکروسکوپ نوری مجهز به لنز اسلایدها توسط نتایج نشان داد که هپاتوپانکراس اندامی لوله‌ای شکل بوده است. در فصل گرما، قطر لوله‌ها از فصل سرما کوچک‌تر بود. در درصد آپوپتوز بین سلول‌های تشکیل دهنده توبول فصل گرم و فصل سرد تفاوت معنی‌داری وجود داشت.

کلمات کلیدی: ایمونوهیستوشیمی، هپاتوپانکراس، *Litopenaeus vannamei*، دما

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