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Research article

Procedure for performing a fixed microscopic specimen of the gonads of fish

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Abstract

This study aims to provide a procedure for performing microscopy in fish gonads. Specimens used in this study were the ovaries and testes of fish collected in the Mekong Delta from April to July 2022. Organs at five different stages for each sex (ovary diameter from 1.16 cm in stage I to 4.04 cm in stage V; testis diameter from 0.42 in stage I to 2.39 in stage V) were studied in this procedure. The results showed that both ovaries and testes needed to be fixed at 4% formol for 24 h and under running water for eight hours. Ovaries and testes were then immersed in 500 ethanol for 24 h to initiate dehydration. Results showed that the time for dehydration and paraffin infiltration was longer in the ovary (26 to 55 h across stages) than in the testis (25 to 26 h across stages). The mean staining time of the ovary was 1.5 min (stage I) to 3.5 min (stage V) in Hematoxylin and 50 s (stage I) to 140 s (stage V) in Eosin. Whereas the staining time of testis was shorter, 1 min (stage I) to 3 min (stage V) in Hematoxylin and 30 s (stage I) to 90 s (stage V) in Eosin. This study provides a reference for further studies on the histological structure of fish gonads. These results could be applied to fish species inside and outside the Mekong Delta.

Keywords: Hematoxylin & Eosin, Histology, Ovary, Testis

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INTRODUCTION

Vietnam is located in Southeast Asia and has a tropical monsoon climate. Besides the narrow terrain, Vietnam has a long coastline and rich marine resources, especially a developed fishing industry (Le et al., 2006). Compared to other regions, the Mekong River Delta (VMD) is a region with strengths in fishing and aquaculture with many favorable conditions. This is the downstream area of the Mekong river system combined with a dense network of rivers, so many freshwater fish species exist. On the other hand, VMD has a long coastline of about 700 km and is bordered on three sides by the sea (Le et al., 2006). This shows that in addition to the resources of freshwater fish, this area also contains saltwater and thus brackish water fish species are also very developed. According to research by Tran et al. (2013), VMD has more than 300 species belonging to 77 families. Among them, nearly 80 species of fish are recorded with economic value (Thai, 2015). However, in recent years, the number of fish species in VMD has decreased due to climate change and human exploitation (Thai et al., 2012). With abundant aquatic resources and economic value, there have been many studies on fish biology in this area to conserve and effectively exploit these fish species.

Many studies at VMD focus on studying reproductive characteristics such as spawning season, reproductive pattern, and reproductive capacity in some fish species such as *Stigmatogobius pleurostigma* (Dinh and Tran, 2018), *Glossogobius sparsipapillus* (Nguyen et al., 2019), *Butis koilomatodon* (Dinh et al., 2021a), *Mystus mysticetus* (Vo et al., 2022), etc. The histological characteristics of the gonads play an essential role in the study of fish reproductive biology. According to Pham and Tran (2004), information on maturation cycles and the help of the education system will be more understanding and predictable about changes and the development of a natural garment. In addition, structural goniometry provides information on reproductive profiles in fish (Dinh and Le, 2017). If the fish can reproduce many times, the histological structure will appear in many different stages of sex cells. In contrast, if a fish reproduce only once, histology shows only one type of sex cell (Miller, 1984; Dinh and Le, 2017).

Currently, many studies perform gonad-fixed microscopy to study the reproductive biology of fish. This method is intended to produce thin slices with contrasting colors between the nucleus and cytoplasm (Vu et al., 1976). Thereby, it is possible to observe the structure inside the sex cell. However, the publications on the implementation of this method are minimal. Carleton et al. (1980) present a procedure to perform histological specimen with eight stages, including extraction, fixation, specimen inclusion, slicing, section staining with Hematoxylin and Eosin Y, loading of the sample onto the lamella, and stting up of the lamella. However, some of the chemicals used in this process are no longer used due to safety concerns in the laboratory. Therefore, this study aims to provide a system for performing gonadal histology of specimens from fish species. This study's results provide a procedure for performing histological specimens in certain fish species and many other species with different gonadal sizes. This provides a suitable implementation method for further studies on fish reproductive biology.

MATERIALS AND METHODS

The specimens used in this study were the gonads of gobies collected in VMD, such as *Periophthalmodon schlosseri*, *Glossogobius aureus*, *Glossogobius giuris*, *Glossogobius sparsipapillus*, *Butis humeralis*, *Butis koilomatodon*, Butis butis, *Mystus mysticetus*, and *Periophthalmus chrysospilos*. As we removed the gonads from dead fish from fishers from July 2021 to March 2022, no animal ethics approval was required. We bought the dead fish from logcal fishers who used trawl nets/traps to collect these fish specimens with a mesh size of 2a=1.5 cm in the cod-end along Hau river (Cai Rang - Can Tho and Long Phu - Soc Trang) and VMD coastal provinces (Duyen Hai - Tra Vinh, Tran De - Soc Trang, Hoa Binh - Bac Lieu, Dong Hai - Bac Lieu and Dam Doi - Ca Mau) (Figure 1). The identification of fish samples was determined through morphological characteristics described by Tran et al. (2013). After classification, the fish samples were subjected to gonadal dissection for use in histological procedure studies. In addition, the gonadal samples were also sized to determine the time for the next steps. The average ovary diameter through stages I, II, III, IV, and V were 1.16; 1.46; 1.87; 2.70, and 4.04 cm, respectively. Meanwhile, the average diameters of the testes lines used in this study were 0.42 (stage I); 0.88 (stage II); 1.19 (stage III); 1.55 (stage IV); 2.39 (stage V), respectively. Stages of histology are performed based on the description of Carleton et al. (1980) and Vu et al. (1976).

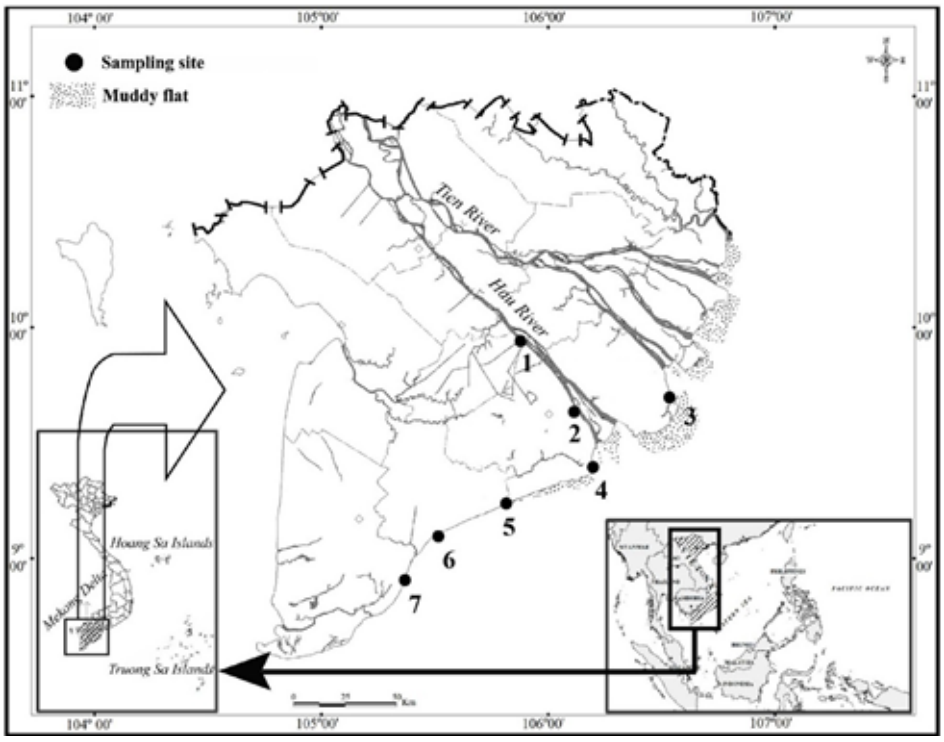

Figure 1 Map of sampling area for some fish species in VMD (1: Cai Rang - Can Tho; 2: Long Phu - Soc Trang; 3: Duyen Hai - Tra Vinh, 4: Tran De - Soc Trang, 5: Hoa Binh - Bac Lieu, 6: Dong Hai - Bac Lieu and 7: Dam Doi - Ca Mau (Dinh et al., 2018))

The equipment used in this study included: technical balance (0.01 g), analytical balance (0.01 mg), microscope (Olympus, Japan) connected to a digital camera, Motic microscope with a built-in camera, Motic magnifying glass with a built-in camera, Microtome cutter (Sakura, Japan), Nikon D5100 camera, kettle, refrigerator, oven.

Tools: plastic container, plastic tray, surgical kit, measuring table, pen, alcohol meter, measuring tube, alcohol lamp, lame, lamella, specimen tray.

Supplies: filter paper, paper towels, cotton swabs, masks, gloves, cutters, paperboard, eggs.

Chemicals: formol solution, distilled water, xylene, n - Butanol, absolute ethanol, Hematoxylin, Eosin Y, industrial paraffin, beeswax, Baune Canada, Glycerin.

RESULTS

After the testing process, this study introduced the procedure to perform gonadal microscopy in fish with specific steps as follows.

Sample preparation and fixation

The fish samples were dissected to remove the gonads. During gonadal separation, it was essential to avoid injury and damage to the membranous and internal structures of the gonad. For fresh fish samples, the gonads, after being removed, were soaked in 4% formol solution for 24 h, then continued to be washed under running water for 8 h. For fish samples stored in formalin, it was not necessary to keep them at 4% formol but they were washed under running water for 8 h. This process immobilizes the cellular components in the gonads, keeping them in the same position as when the tissue sample was alive. After fixation, the gonads need to undergo a dehydration period before being impregnated with paraffin.

Dehydration and impregnation with paraffin

This study used ethanol at different concentrations to remove water from cells to remove water of tissue samples. After dehydration, the tissue sample should be removed from ethanol because ethanol is insoluble in paraffin. Xylene needs to be used at this stage to push the ethanol out of the sample. However, the dehydration and paraffin impregnation times depend on the size of the gonads. The results are shown in detail in Tables 1 and 2.

Table 1 Dehydration and paraffin impregnation time for testes.

No.	Stage	Chemicals	Times				
			Stage I	Stage II	Stage III	Stage IV	Stage IV
1	Dehydrate	Ethanol 50^0	24 h	24 h	24 h	24 h	24 h
		Ethanol 60^0	15 s	30 s	2 m	3 m	5 m
		Ethanol 70^0	15 s	30 s	2 m	3 m	5 m
		Ethanol 80^0	15 s	30 s	2 m	3 m	5 m
		Ethanol 90^0	15 s	30 s	2 m	3 m	5 m
		Ethanol 100^0 I	15 s	30 s	2 m	3 m	5 m
		Ethanol 100^0 II	15 s	30 s	2 m	3 m	5 m
		n- Butanol I	30 s	1 m	3 m	5 m	7 m
		n- Butanol II	30 s	1 m	3 m	5 m	7 m
2	De-alcohol	Xylene I	30 s	1 m	3 m	5 m	7 m
		Xylene II	30 s	1 m	3 m	5 m	7 m
3	Sample burial	Paraffin : xylene (1:1)	10 m	15 m	20 m	40 m	50 m
		Wax : Paraffin (1:4)	20 m	30 m	40 m	60 m	70 m

Table 2 Dehydration and paraffin impregnation time for ovaries.

No.	Stage	Chemicals	Times				
			Stage I	Stage II	Stage III	Stage IV	Stage IV
1	De-hydrate	Ethanol 50^0	24 h	24 h	24 h	24 h	24 h
		Ethanol 60^0	3 m	10 m	30 m	60 m	90 m
		Ethanol 70^0	3 m	10 m	30 m	60 m	90 m
		Ethanol 80^0	3 m	10 m	30 m	60 m	90 m
		Ethanol 90^0	3 m	10 m	30 m	60 m	90 m
		Ethanol 100^0 I	3 m	10 m	30 m	60 m	90 m
		Ethanol 100^0 II	3 m	10 m	30 m	60 m	90 m
		n- Butanol I	5 m	15 m	45 m	90 m	120 m
		n- Butanol II	5 m	15 m	45 m	90 m	120 m
2	De-alcohol	Xylene I	5 m	15 m	45 m	90 m	120 m
		Xylene II	5 m	15 m	45 m	90 m	120 m
3	Sample burial	Paraffin : xylene (1:1)	30 m	40 m	90 m	240 m	360 m
		Wax : Paraffin (1:4)	50 m	60 m	120 m	360 m	480 m

Paraffin mold casting

After being soaked in wax and paraffin at the ratio of 1:4, samples were placed into a paper mold before pouring the paraffin to create volume. The shape and arrangement of the paper molds are described in Figure 2. Mold casting was carried out in two stages the preparation stage (alcohol lamp, swipe, needle, clamp, metal mold, or paper mold could be used) and the molding stage. Molding was performed according to the following steps: (1) Gently pour the molten wax: paraffin mix (1:4) into the mold; (2) Wait for about 10 s for the bottom of the mold to begin to reset, using heated tongs on the flame of the alcohol lamp, quickly grasp the sample to the center of the mold position. The operation should be done carefully to avoid forming air bubbles; (3) Use heated clamps or pliers to adjust the sample position as the desired mold can be moved to prevent sample deviation (4) The sample, after molding, was kept very well. The sample could be cut after at least 24 h.

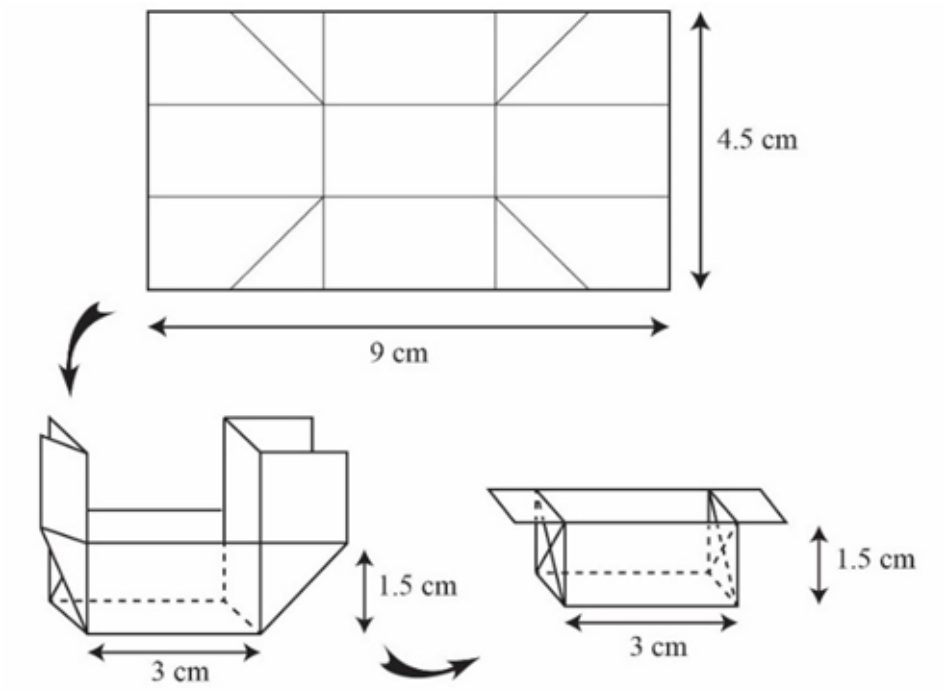

Figure 2 How to fold paper molds.

Sample cutting

After 24 h, the sample and paraffin formed a uniform mass. Before making thin slices, it was necessary to cut the sample block into a suitable shape to facilitate the cutting process. Usually, the sample block was cut into a truncated cube. The sample block cutting stage was performed in the following sequence of steps: (1) Use a knife to cut the paraffin block containing the specimen into a cylinder whose two bottoms are isosceles trapezoids (this was also the cutting section); (2) Attach the trimmed sample block to the wood block by heating the block and then attach them so that the block cools, and finally attach the block to the cutter (Figure 3).

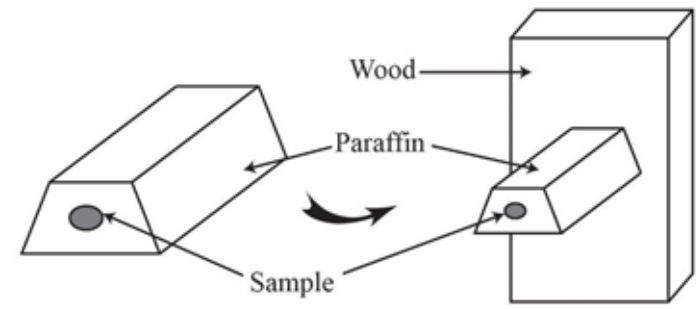

Figure 3 Paraffin block shape.

Place the blade in the microtome cutter and secure the knife tightly. Adjust the offset of the edge for the sample block accordingly (usually, the deviation is from 10 – 15°). Adjust the slice thickness from 4 to 6 µm depending on the sample size. In this study, the appropriate thickness for oocytes in stages I (1.16 cm in diameter), II (1.46 cm in diameter), and III (1.82 cm in diameter) oocytes was 5 µm; The stage IV (diameter 2.70 cm) and V (4.04 cm diameter) oocytes are 6 µm. Testes samples with diameters from 0.42 (stage I) to 2.39 cm (stage V) were cut at 4 µm. Rotate the cutter at a moderate and even speed. The cut sample meets the requirements when the slices are consecutive in series. Inspiring sample cut with white cardboard. After cutting, the sample should be stored in a container with a lid. A good slice was a slice that was arranged in a row, was not folded, torn, or overlapped, and has the shape shown in Figure 4. During the use of the microtome cutter, the room temperature should be between 18 and 23°C. With a higher room temperature, the cutting piece was easily broken or stuck to the machine.

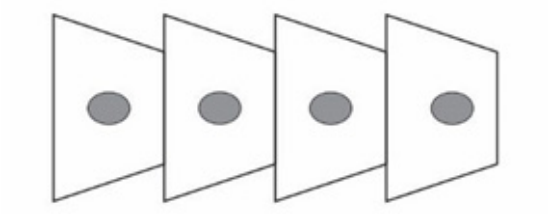

Figure 4 The shape of the array of slices.

The cut piece was loaded onto the lame in preparation for the dyeing process. Steps to apply the cut piece to the lamella include: (1) use a lamella to dip a little albumin-glycerin solution, spread a skinny layer on the clean, dry lamella surface, and let this edge of the lamella come in contact with the clean lame at an angle of 45°, pull from to the end of the lame; (2) Then use a paper towel to wipe the laminated lame in a specific direction gently; (3) Place the albumin-lined slide on the tray and place it in the oven at 70°C for 5 m; (4) Spread the cut pieces into a basin of warm water (60°C); (5) Remove the lame from the oven and use the lame to remove the cut pieces from the basin of warm water; (6) Wipe off excess water with a paper towel and place the sample-affixed slide in the oven for 7 m.

Dyeing samples

During the study, there was a time adjustment in the steps to match the ovaries and testes. Due to the smaller size of the testes glands, the dyeing time of Eosin and Hematoxylin dyes was significantly shorter than that of the ovary gland. The results of staining time according to the size of the testes and ovaries at each stage are shown in Tables 3 and 4.

Table 3 Testis staining process.

No.	Stages	Chemicals	Times				
			Stage I	Stage II	Stage III	Stage IV	Stage V
1	De-paraffin	Xylene I	1 m	1 m	1 m	1 m	1 m
		Xylene II	1 m	1 m	1 m	1 m	1 m
		Ethanol I	30 s	30 s	30 s	30 s	30 s
		Ethanol 90^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 80^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 70^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 60^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 50^0 I	30 s	30 s	30 s	30 s	30 s
		Distilled water I	1 m	1 m	1 m	1 m	1 m
2	Hematocylin staining	Hematocylin	1 m	1.5 m	2 m	2.5 m	3 m
		Machine water	1 m	1 m	1 m	1 m	1 m
		Distilled water II	4 m	4 m	4 m	4 m	4 m
		Ethanol 50^0 II	30 s	30 s	30 s	30 s	30 s
3	Eosin Y staining	Ethanol 60^0 II	30 s	30 s	30 s	30 s	30 s
		Ethanol 70^0 I	30 s	30 s	30 s	30 s	30 s
		Eosin Y	30 s	40 s	50 s	70 s	90 s
		Ethanol 80^0	30 s	30 s	30 s	30 s	30 s
		Ethanol 90^0 II	30 s	30 s	30 s	30 s	30 s
4	Dehydrate	Ethanol 100^0II	30 s	30 s	30 s	30 s	30 s
		n-Butanol I	1 m	1 m	1 m	1 m	1 m
		n-Butanol II	2 m	2 m	2 m	2 m	2 m
5	Make clarity samples	Xylene I	2 m	2 m	2 m	2 m	2 m
		Xylene II	Until clarity	Until clarity	Until clarity	Until clarity	Until clarity

Table 4 Ovary staining process.

No.	Stages	Chemicals	Times				
			Stage I	Stage II	Stage III	Stage IV	Stage V
1	De-paraffin	Xylene I	1 m	1 m	1 m	1 m	1 m
		Xylene II	1 m	1 m	1 m	1 m	1 m
		Ethanol I	30 s	30 s	30 s	30 s	30 s
		Ethanol 90^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 80^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 70^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 60^0 I	30 s	30 s	30 s	30 s	30 s
		Ethanol 50^0 I	30 s	30 s	30 s	30 s	30 s
		Distilled water I	1 m	1 m	1 m	1 m	1 m
2	Hematocylin staining	Hematocylin	1.5 m	2 m	2.5 m	3 m	3.5 m
		Machine water	1 m	1 m	1 m	1 m	1 m
		Distilled water II	4 m	4 m	4 m	4 m	4 m
		Ethanol 50^0 II	30 s	30 s	30 s	30 s	30 s
3	Eosin Y staining	Ethanol 60^0 II	30 s	30 s	30 s	30 s	30 s
		Ethanol 70^0 I	30 s	30 s	30 s	30 s	30 s
		Eosin Y	50 s	70 s	90 s	120 s	140 s
		Ethanol 800	30 s	30 s	30 s	30 s	30 s
		Ethanol 90^0 II	30 s	30 s	30 s	30 s	30 s
4	Dehydrate	Ethanol 100^0II	30 s	30 s	30 s	30 s	30 s
		n-Butanol I	1 m	1 m	1 m	1 m	1 m
		n-Butanol II	2 m	2 m	2 m	2 m	2 m
5	Make clarity samples	Xylene I	2 m	2 m	2 m	2 m	2 m
		Xylene II	Until clarity	Until clarity	Until clarity	Until clarity	Until clarity

After transferring it to the xylene vial, it was necessary to clean the lamella because the albumin-glycerin layer had been stained with Hematoxylin, and then glue the lamellae with Baume Canada. Wipe off excess Baume Canada with xylene tissue, then label the specimen. Avoid leaving the sample in the air for a long time, which would lead to its blackening. The sample had to be in contact with Baume Canada when covering lamellae. After staning with Hematoxyline and Eosin we got the fix microslide of gonads (Figure 5-6).

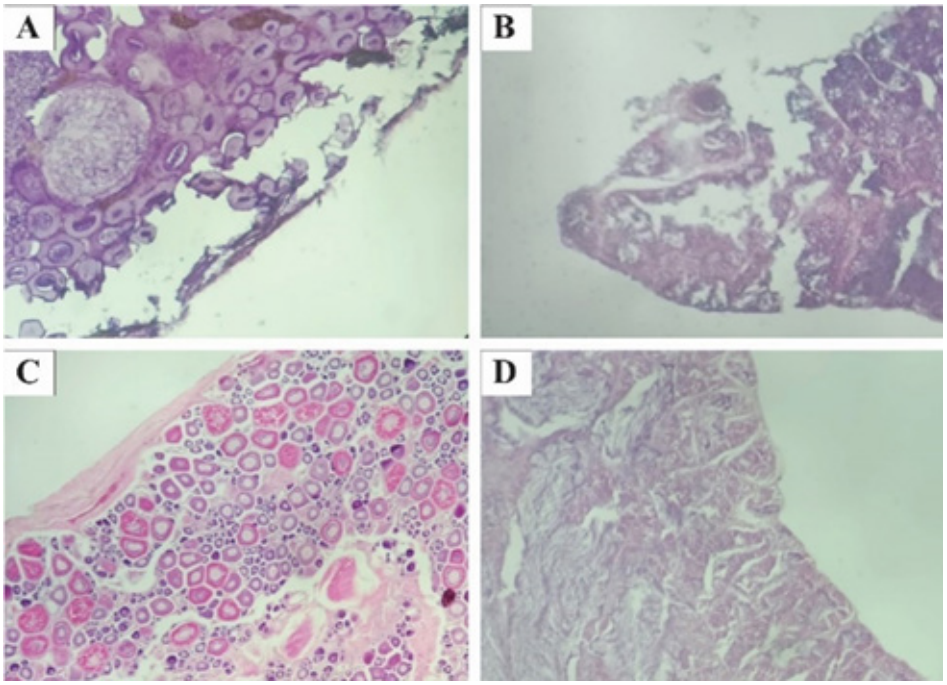

Figure 5 Dehydration time test results and paraffin soak (A: The sample of the egg gland was not soaked long enough time; B: the sample of the testis was soaked too long; C and D: samples of the ovary and testis were soaked for the adequate time).

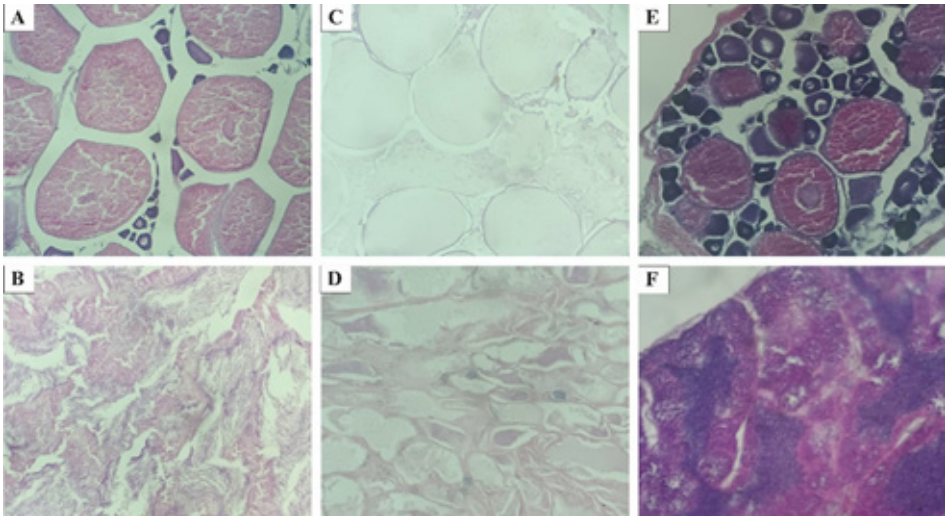

Figure 6 Comparison of the results of different dyeing times (A and B: ovary and testis successfully stained; C and D: ovary and testis did not catch the colors: E and F: ovary and testis too strongly colored).

DISCUSSION

The immobilization solution used in this study differed from that of the survey by Carleton et al. (1980) who used Bouin fluid to fix the sample. Compared with the Bouin fluid, formol has a lower toxicity and is cheaper while giving similar results. Besides, the process of mixing Bouin fluid made it flammable and explosive, and this chemical was challenging to find on the market. The sample fixation period was of the same duration for all sizes of gonad.

The time of dehydration and paraffin infiltration plays a vital role in performing fish gonadal microscopy. If the sample soaking time is too long or too fast, it leads to paraffin not fully penetrating the gonadal sample (Figure 5). Due to the large size of the ovary, the time to dehydrate and absorb paraffin was often lacking. On the contrary, in small size and manipulation, it was easy to have spare time to drain and soak paraffin. When dehydration and paraffin infusion over time or lack of time caused cells in the sample to shrink or burst. The operation should be done quickly and accurately during the dyeing process because the dye picks up colors quickly, which can affect the final result. The staining was successful when the cytoplasm was stained pink with Eosin, and the nucleus was stained blue-violet with Hematoxylin (Figure 6A, B). If the staining time was insufficient, the sample did not have the desired colors, the sample was blurred, and the components were not distinguishable (Figure 6C, D). If the operation was slow, leading to excess staining time, the sample became dark, making it challenging to observe the components inside the cell (Figure 6E, F). The staining process was successful when the cytoplasm was stained pink with Eosin, and the nucleus was purple-blue with Hematoxylin.

This procedure has been successfully applied to many species in VMD like *Periophthalmodon schlosseri* (Tran et al., 2019), *Glossogobius aureus* (Dinh et al., 2021b), *Glossogobius sparsipapillus* (Nguyen et al., 2019; Nguyen et al., 2021), *Butis koilomatodon* (Dinh et al., 2021a), *Mystus mysticetus* (Vo et al., 2022), *Periophthalmus chrysospilos* (Dinh et al., 2022a), and *Glossogobius giuris* (Dinh et al., 2022b; Dinh et al., 2022c).

CONCLUSIONS

This study provided the procedure for performing gonadal microscopy in fish. This process used less toxic chemicals and was easier to find on the market than previous processes. In addition, this procedure also gave suitable timings for ovaries with diameters from 1.16 to 4.04 cm and testes with diameters from 0.42 to 2.39 cm. The results from this study suggested a uniform procedure for implementing fish specimens. This was the basis and material for further studies on implementing samples in fish species from the VMD and surrounding regions.

AUTHOR CONTRIBUTIONS

Quang Minh Dinh; Conceptualization and design the experiment, investigation, supervision, editing and finalization
Ton Huu Duc Nguyen; Investigation, methodology, formal analysis, manuscript preparation

CONFLICT OF INTEREST

We have no conflict of interest.

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