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RESEARCH ARTICLE

ARTIFACTS IN MICROSCOPIC SLIDES AND TEM IMAGES OF AQUATIC ANIMALS AND INSECTS: A 10-YEAR RETROSPECTIVE OBSERVATION

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Abstract. Since histopathology is used to assess health status and pathological changes, the proper preparation of histological slides is a routine procedure under the standard criteria, laboratory equipment and histo-technicians. It is accepted that artifacts are commonly found in prepared slides and can lead to errors in assessment and diagnostic problems for histotechnologists and pathologists. In this 10-year retrospective study, data collected during observations in our laboratory was examined to identify artifacts in aquatic animal/insect histological sections and associated errors. Information of ultrastructural artifacts was also included throughout the study. Results from this research revealed that several artifacts of tissue specimen including fixation, over-decalcification, dehydration and paraffin infiltration, sectioning, flotation, staining, and mounting artifacts. Interestingly, artifacts from the aquatic animal histological slides were most introduced during fixation, tissue processing, sectioning, and mounting procedures. Consequently, these inclusions had an incomplete influence on the histological process and hardly affected the histopathological interpretation of tissue sections. Additionally, artifacts could occur in transmission electron micrographs of pre-fixative and thin sections. This article is the first to describe the most common errors and artifacts, serving as a guide to help reduce the occurrence of artifacts in animal histological slides and thin sections. However, they recognized a challenge for histological slide preparation.

Keywords: Artifacts, histological slides, aquatic animals, tissue automated processing.

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1. INTRODUCTION

The use of quantitative histopathology is strongly recommended as a suitable method of assessing the health of fish under stressful conditions and environmental threats. The accurate diagnosis of health is based on observation of histological sections prepared following standard guidelines. However, several reports note the presence of artifacts in prepared slides [1-4] that could be mistakenly associated with causative changes in normal histology and changes in histological tissue features. These mistakes could lead to diagnostic inaccuracies. Diagnostic accuracy is therefore reliant on the skill of laboratory personnel and their ability to produce high-quality sections [3-4].

An artifact can be defined as an unrelated and extraneous feature observed during slide examination [1-2]. Some artifacts are easily distinguishable as minor features, in contrast to others, which are difficult to characterize and constitute major features [1-4]. Various types of artifacts introduced during staining have been identified, for example fixation artifacts, and artifacts related to the flotation and mounting of stained artifacts [1-4]. Taqi et al. [5] reported in a review that artifacts are not usually seen in sections and can occur at different stages of slide preparation, from surgical removal to final mounting. Their study confirmed that artifacts were the primary source of diagnostic problems and impacted histologists and pathologists [3]. An artificial structure or an altered microscopic slide is one of the most important causes of diagnostic errors. Artifacts can also be problematic in transmission electron microscope (TEM) images [6-7]. Michen et al. [6] reported that artifacts in TEM images occurred particularly when laboratory personnel were trying to avoid artifacts from drying. The damage can cause significant problems when interpreting TEM images [8-9]. Artifacts commonly found in TEM images, including nuclear graphite components and epoxy embedding components, derived from specimen preparation errors [7-9].

Currently, investigations of artifacts in histological slides and associated errors are focused on samples from higher vertebrates and humans [10-13]. However, the commonly encountered artifacts and the data concerning histological slides and TEM sections of aquatic animals have not been discussed in the literature. Therefore, this study focuses on the problems of artifacts in histological slides of aquatic animals and insects. We hope this information will be helpful in histological/ultrastructural descriptions and comparisons of clinical/common diagnoses.

2. MATERIALS AND METHODS

During the period 2012–2022, histological/histochemical slides from marine organisms were accumulated at the Division of Biological Science, Faculty of Science, Prince of Songkla University. The species represented in this work include the molluscs *Amphibalanus amphitrite* and *Polinices mammilla*; the arthropods *Alpheus* sp., *Thalamita crenata*, and *Matuta victor*; the fish *Leiognathus decorus*, *L. splendens*, *Secutor insidiator*, *Ambassis kopsii*, *A. nalua*, *Allenbatrachus grunniens*, *Scomberoides tol*, *Hippocampus trimaculatus* and *H. spinosissimus*; and the insects *Hypothenemus hampei*, *Halobates hayanus*, *Aspidimorpha sanctaecrucis*, *Oligotoma saundersii*, *Asclepios annandalei*, *Epicauta waterhousei* and *Mitragyna speciose*. The slides were processed using common histological methods. All chemical reagents used in slide preparation are shown in Table 1.

All samples were fixed in 10 % neutral buffer formalin, dehydrated in a series of reagent ethanol solutions, embedded in paraffin, and then manually sectioned with a microtome set at 4–5 μm thickness [14-16]. The paraffin sections were stained using Harris's hematoxylin and eosin (H&E), periodic acid–Schiff–hematoxylin (PAS-H), or Masson's trichrome [14-15]. All slides were scanned and photographed using a 3DHISTECH panoramic Viewer (3DHISTECH, Hungary). Ultrastructural figures of *Episesarma singaporense* and *A. grunniens* (n = 50 individual figures) were also obtained from representative specimens.

Table 1: Chemical solutions used in histological procedures and transmission electron observations (Modified from Kongthong et al. [16] and our laboratory)

No	Agents	Company
1	35-40 % Formaldehyde	RCI Labscan Limited
2	Glutaraldehyde	Electron Microscopy Sciences
3	Sodium di-hydrogen phosphate	KemAus
4	Di-sodium hydrogen phosphate dibasic anhydrous	KemAus
5	Weigert's iron hematoxylin	Tokyo chemical industry Co., Ltd
6	Eosin solution	C.V. Laboratories Co., Ltd
7	Absolute ethanol	RCI Labscan Limited
8	Biebrich scarlet	HiMedia Laboratories Pvt. Ltd. (India)
9	Acid fuchsin	HiMedia Laboratories Pvt. Ltd. (India)
10	Light green SF yellowish	Sigma-Aldrich (Merck, Germany)
11	Periodic acid	HiMedia Laboratories Pvt. Ltd. (India)
12	Schiff's reagent	Sigma-Aldrich (Merck, Germany)
13	Xylene	RCI Labscan Limited
14	Ferric chloride anhydrous	Loba Chemie Pvt. Ltd
15	Hydrochloric acid 37 %	RCI Labscan Limited
16	Acetic Acid Glacial	RCI Labscan Limited
17	Phosphomolybdic acid	HiMedia Laboratories Pvt. Ltd. (India)
18	Phosphotungstic acid	HiMedia Laboratories Pvt. Ltd. (India)
19	Surgipath Decalcifier II	LeicaBiosystems
20	Permount	Fisher Scientific
21	Osmium tetroxide	Electron Microscopy Sciences
22	Araldite 502 resin	Electron Microscopy Sciences
23	Uranyl acetate	Electron Microscopy Sciences

3. RESULTS AND DISCUSSION

Our examination of 2,000 histological slides and 100 ultrastructural figures from aquatic organisms enabled us to describe different types of errors and artifacts for the first time. The errors and artifacts were easily distinguishable from normal tissue or histopathological components and the most frequently encountered are described below. The distinction of artifacts from normal or pathological tissue in aquatic animals is a crucial step toward ensuring the accuracy of histopathological and ultrastructural analyses. The types of artifacts and errors observed greatly depended on the specimen type, slide preparation technique, and staining method used throughout the diagnostic process.

3.1 Fixation Artifacts

Fixation artifacts are alterations or distortions in tissue structure due to the fixation process. The fixation process is meant to preserve tissue in a state that resembles living tissue as closely as possible by halting enzymatic breakdown and autolysis. Tissue fixation is the first step in tissue preparation. It should be performed as soon as possible after sample collection. The aim of fixation is to prevent tissue autolysis and to stabilize the cell proteins with the fixative [14]. The appropriate fixative penetrates tissue quickly to reduce the occurrence of artifacts. If the fixative does not act quickly, autolysis artifacts, such as unidentifiable cells or tissues and loose tissue, can occur (Figures 1A-1F). The type of fixative artifact can vary depending on the specific fixative used and the preserved tissue type. Tissue contraction due to fixation can distort the architecture of the tissue and cellular components, resulting in shrinkage (Figures 1E-1G).

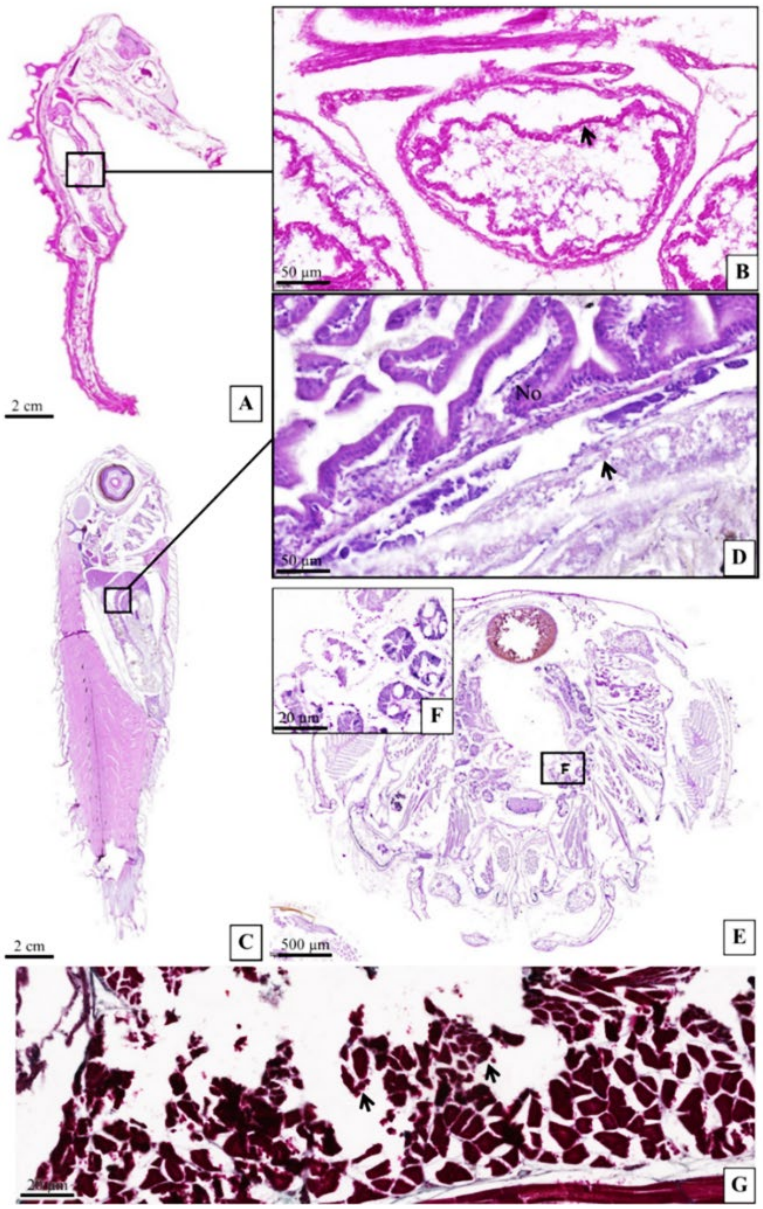

Figure 1: Artifacts from the degradation of fixative are visible in slides of fish (A-D, G) and crab (E-F). Autolysis artifacts, unidentified cell/tissue (B, D, arrows and F), and shrinkage in muscle tissue (G, arrow) can be identified.
Abbreviation: No = normal structure

The degree of shrinkage can be influenced by the type of fixative used, the fixative concentration, and the duration of fixation. Over fixation can occur when tissues are left in fixative solutions for too long, resulting in tough tissues that may be difficult to cut and process (Figure 1F). Overstaining can also be a problem, resulting in dark, dense tissues that are difficult to interpret. Under fixation, on the other hand, occurs when the tissue is not fixed for long enough, resulting in poor preservation of cell detail and possible autolysis. However, prolonged fixation should not be disregarded as it affects microtome sectioning and the commonly found bleaching artifacts [12]. Some fixatives can react with substances in the tissue or other chemicals in the processing sequence, resulting in precipitations, which appear as small, often granular, deposits that can be confused with, or mistaken for, histological features.

Fixatives can also cause color changes in tissues, making it difficult to identify certain structures correctly. It has been noted that thick tissues/samples are a major problem in animal histological sections, along with inadequate quantities of fixative [13,17-19] The maximum thickness of sections should not exceed 6 mm to ensure a good image and the efficacy of fixatives. Identifying and avoiding fixative artifacts is critical to the accuracy of histopathological analysis. Using standardized fixation protocols, appropriate fixative types and concentrations, and suitable fixation times can help minimize the occurrence of fixative artifacts. Advances in digital image analysis and machine learning can also assist in identifying these artifacts, thereby increasing diagnostic accuracy.

3.2 Over-decalcification Artifacts

Hard tissues and bones (or skeletal structures), especially in fish, have to be decalcified to soften the hard tissues so that they may be cut into thin sections for microscopic examination. Failure to do so may result in a poor section or damage to the microtome blade (Figures 2A-2B).

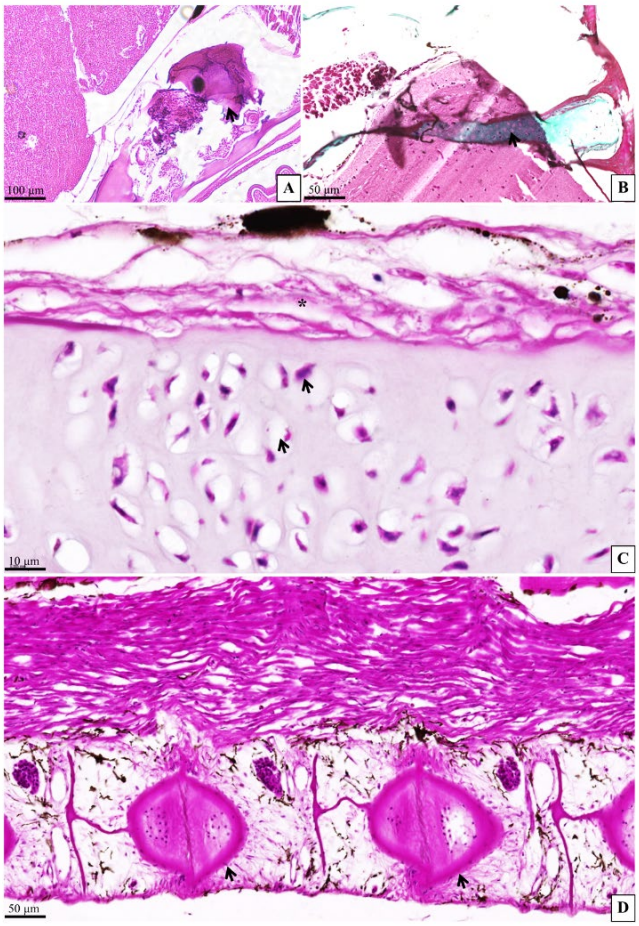

Figure 2: Slides of fish show artifacts from over-decalcification with a poor section component. Impacts on cartilage and bone are visible (A-B, arrows) and loss of collagen structure can be seen (C, arrows). Nuclei appear darkly stained under the microscope. The loss of organic components of bone is found (arrow, D).

However, if the process is not carefully controlled, various artifacts can be introduced that alter the appearance of tissue and make accurate histological evaluation difficult. Over-decalcification is a common artifact encountered in the histological processing of mineralized tissues such as bone. Excessive decalcification can damage the organic components of cartilage and bone (Figures 2C-2D), causing these components to appear distorted or out of focus under the microscope. While the main goal of decalcification is to remove the mineral content of the bone to facilitate sectioning, over-

decalcification can remove too many minerals, distorting the typical architecture of the bone and potentially causing tissue shrinkage. The decalcification solution used can cause distortion and even cell loss, which can alter the appearance of bone marrow and complicate the accurate identification of different cell types. Sections that have been decalcified too much show intense eosin staining with a significant shrinkage of basophilic nuclei and reduced nuclear and cytoplasmic detail. Cell nuclei that have shrunk due to over-decalcification can appear darkly stained under a microscope (Figure 2C).

3.3 Dehydration and Paraffin Infiltration Artifacts

Dehydration is the process of removing the water from tissue samples by exposing the tissue to increasing concentrations of alcohol, usually ethanol. This procedure is performed before the tissue is cleaned and infiltrated with paraffin. Artifacts can result from improper or rushed dehydration. If dehydration occurs too quickly, tissues can harden leading to difficulty in sectioning and potential damage to tissue architecture (Figure 3A). Insufficient or uneven dehydration can lead to tissue shrinkage, which distorts tissue architecture and cellular structures (Figure 3B). Over-dehydration can also lead to shrinkage but makes tissues brittle. If the dehydration process is faulty, infiltration with paraffin and embedding in paraffin will be affected [20]. These preparatory mistakes can cause samples to be inadequately infiltrated by paraffin. Air bubbles can become trapped in the tissue during infiltration and appear as free spaces in the final sections (Figure 3A). Insufficient paraffin infiltration leads to wrinkles that can run in any direction and produce paraffin-embedded tissues that are difficult to cut [21]. Tears or holes can appear in tissue samples during sectioning.

If the paraffin is too hot during embedding, heat artifacts (Figures 3C-3D) such as tissue charring can occur. Automated tissue processors can help obtain consistent results by ensuring that each step is performed at the right time. Regular equipment maintenance and appropriate quality control procedures can also help prevent the introduction of artifacts into samples. Choosing the proper processing protocol for the type of tissue being processed is important as different tissues can have different sensitivities to these processing steps.

3.4 Sectioning Artifacts

Sectioning artifacts can occur while cutting thin slices of tissue embedded in a paraffin block, typically using a microtome. Such artifacts can be caused by the physical properties of the tissue, the type and sharpness of the microtome blade, the embedding medium, and the techniques employed by the histotechnologist. It is accepted that sectioning artifacts are more common than other types of artifacts (Figures 3E-3F). Sectioning artifacts are typically a pattern of ridges or waves on the tissue section that occurs when the microtome blade is not secure, the block is too hard, or the cutting speed is too high for a dull blade. Sectioning artifacts usually manifest as smudging or wrapping of the tissue, which occurs when the blade is dull, the cutting angle is inappropriate or the cut is made too rapidly, or when the tissue is complicated or poorly infiltrated. If, during floating on the water bath, a section is not adequately flattened before being picked up on the slide, tears can occur that distort the architecture of the tissue. Vibrations in the microtome blade, and loose blades, screws or block holders can produce sections of uneven thicknesses (Figures 3G-3H), as similarly reported [22].

Hard particles, especially calcifications, and incomplete impregnation were causes of sectioning artifacts. Therefore, a sharp blade and trimmed blocks should be used to prevent these artifacts. Sometimes, the paraffin block should be cooled with ice to improve sectioning but if tissue blocks are not correctly stored and become frozen, ice crystals can form, which leads to empty spaces or gaps in the tissue section. To prevent sectioning artifacts, the best histological practices should be followed, such as ensuring proper fixation, dehydration, and paraffin infiltration prior to sectioning. Additionally, using a sharp blade, maintaining the correct cutting angle and speed, and adequately floating the sections in the water bath can all help produce high-quality, artifact-free sections. Finally, well-trained and experienced histotechnologists are essential to ensuring artifact-free sections.

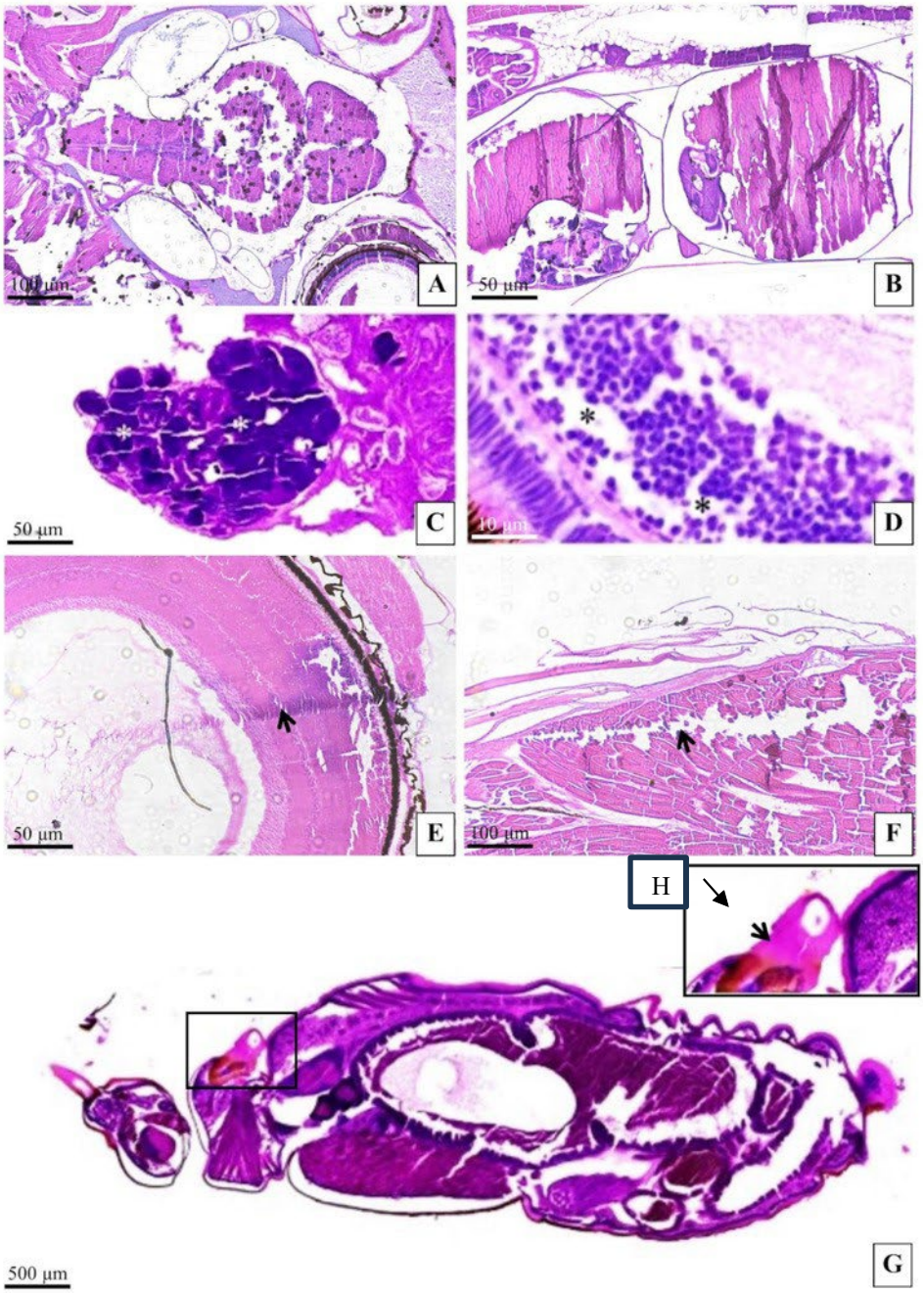

Figure 3: Slides A-D show artifacts of dehydration. Slides E-H show artifacts of sectioning. Slides of fish (A-B) show a hardened section due to rapid dehydration from heat. Slides of a crab (C) and a fish (D) show shattering and cracking. Slides of a fish (E-F) show possible heat artifacts and damage to tissue architecture from a microtome blade (arrows). Slides of an insect show areas of uneven thickness (G-H, arrow). Abbreviation: asterisks = tissue detachment.

3.5 Flotation Artifacts

Flotation artifacts can occur when tissue sections are floated on a water bath before being placed onto slides**.** A poor flotation procedure commonly results in poor tissue adherence to the slide **(**Figures 4A**-**4D**).** The primary purposes of floating sections in a water bath is to remove wrinkles and allow the section to expand to its proper size and shape after sectioning**.** However, various artifacts can occur that are associated with an improper procedure during processing or cutting and an inappropriate water bath temperature**.** Too high a temperature can cause section loss**.** If the tissue section is not adequately

flattened during the floating process, it can form wrinkles or folds that can obscure other tissue areas and interfere with the microscopic examination.

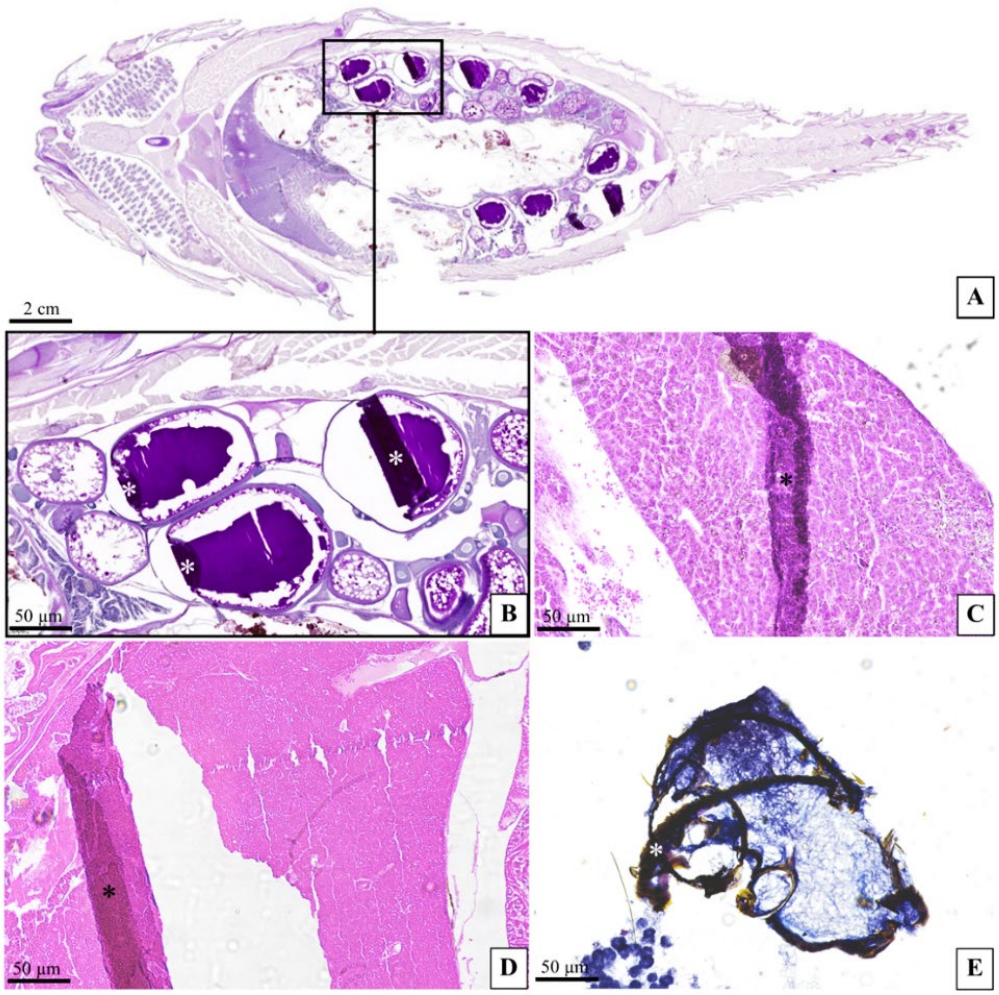

Figure 4: The representative slides of various fish (A-D) and an insect (E) show flotation artifact with an incomplete section and wrinkled or folded sections (asterisks).

Thin paraffin sections can stretch around tissue structures with different consistencies; wrinkles and folds may occur, resulting in darker-stained strands. These artifacts can be removed by gently stretching and tapping the section with forceps while the section is in the water bath. The water bath can sometimes be a source of contamination if it is not cleaned regularly. Debris or residues from other tissues can adhere to sections and introduce foreign material (Figure 4E) that can be confused with actual tissue structures. If the water bath is too warm or the tissue section stays too long in the water bath, sections may expand too much, distorting tissue architecture and complicating histological interpretation. Artifacts can also occur when the water in the bath is disturbed, creating waves that distort the section. These artifacts can be prevented by carefully monitoring the temperature of the water bath (usually set at 37 °C to 45 °C for paraffin sections) and the duration of flotation, and by ensuring that the water bath is cleaned regularly to avoid contamination. Gently agitating the water can also help spread the section flat. However, care must be taken to avoid creating waves or turbulence that can distort the sections. Furthermore, when transferring sections onto slides a gentle and careful technique can help prevent wrinkles or creases (Figures 4B-4D).

3.6 Staining Artifacts

The problem of uneven H and E staining is well-known and commonly reported [21]. This problem affects overall diagnostic accuracy. H and E staining allows various structures in tissue samples to be differentiated based on their chemical properties. Staining artifacts can occur due to several factors, including improper handling, poor technique, or issues with staining reagents. Most of the time, over-staining and under-staining were the artifacts we observed (Figures 5A-5F).

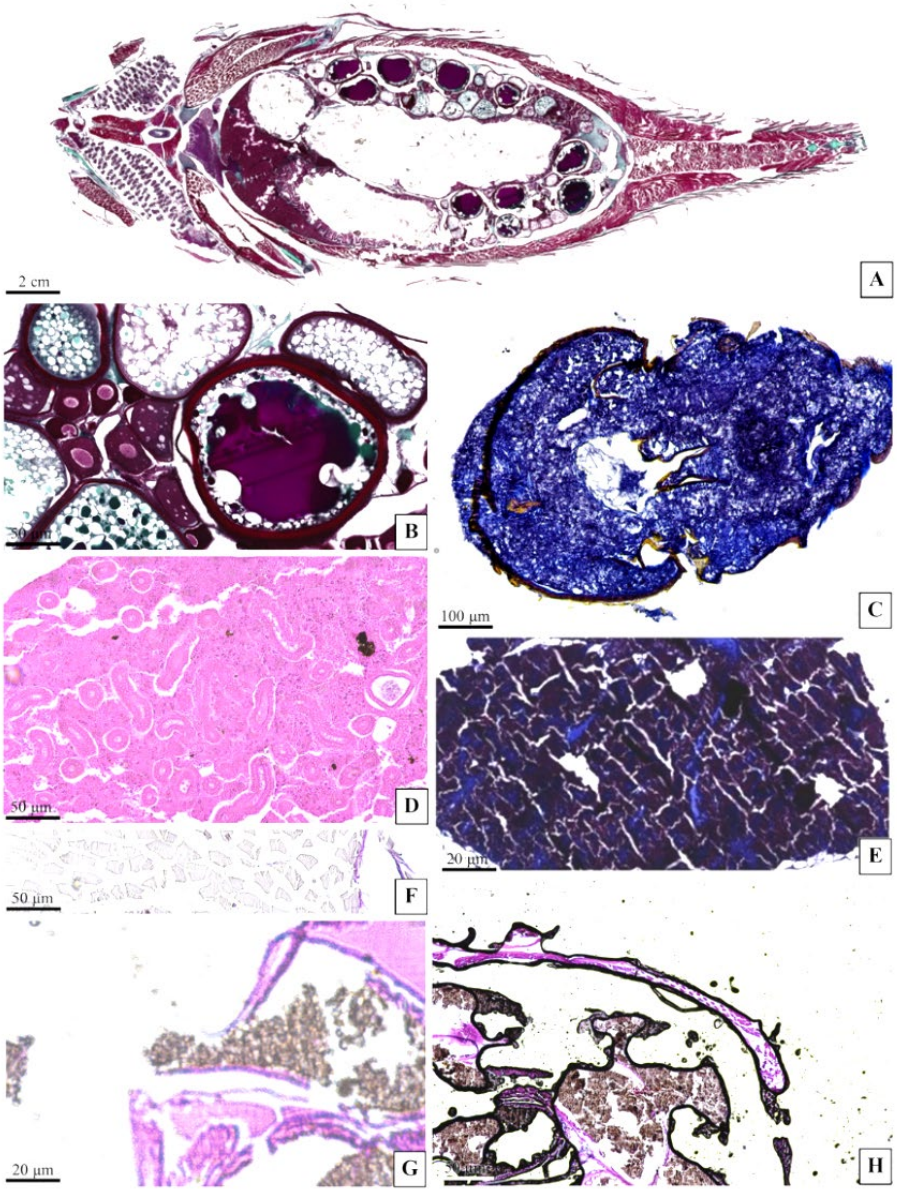

Figure 5: Representative slides show the overstaining of sections of various fish (A-B, D), of an insect (C) and a gastropod (E); and weak staining of a section of a fish (F). Slides of sections of fish (G-H) show dry areas due to thick mounting medium (G) and thin mounting medium.

Overstaining happens when the tissue is left in the staining solution for too long or when the staining solution is too concentrated. The obtained stains are darker than usual and the fine details of the tissue structure can be obscured or tissue components can be unclear (Figure 5E). Shattered sections are associated with various causes, such as prolonged fixation and drying, inadequate fixation, and thick sections. Drying can be a problem if the tissue section dries out before staining or if the section is not

fully submerged in the stain, causing uneven coloration across the tissue. This can also happen if the staining time is too short or the stain is too dilute.

Under staining can make it difficult to see certain vital structures. If the staining solution is not adequately filtered or is old, dye particles may be precipitated that adhere to the tissue section and may be mistaken for pathological structures. Foreign material (like dust or oil) on the slide or in the staining solution can introduce artifacts during staining. These contaminants can often be misinterpreted as part of the tissue. Sometimes, artifacts such as air bubbles or knife lines from sectioning, that were introduced in earlier stages of slide preparation can be stained along with the tissue, confusing diagnostic interpretation.

Troubleshooting any of the above influential quality factors should be analyzed before proceeding with further observation. To avoid staining artifacts, it is important to use freshly prepared and correctly diluted stains, to filter them to remove any precipitates, to ensure slides are fully submerged in the staining solution, and to adhere to the correct staining time. Additionally, it is essential to handle slides carefully to prevent drying out, and to clean staining equipment regularly to avoid contamination (Figures 5G-5H).

3.7 Mounting Artifacts and Air Bubbles

Mounting is the final step in preparing histological slides. The mounted tissue section is protected with a coverslip that also enhances image quality when viewed under the microscope. Mounting artifacts can occur if this step is not carried out correctly. Several problems were found that involved both thick and thin mounting media (Figure 5G). If not enough mounting medium is used, air pockets can form when the coverslip is placed on the slide. If the mounting medium is thick, cell or tissue components may not be identified because light from the microscope does not fully illuminate the section (Figure 5G). Whether the medium is thick or thin, small and large air bubbles may still form if the mounting medium begins to dry before the coverslip is fully attached, and more air can be drawn under the edges of the coverslip as the medium dries further (Figure 5H). If the coverslip is placed flat on the section and not at an angle, air can become trapped underneath, causing bubbles to form and producing poor-quality sections. Sometimes, microorganisms can enter the slide.

The mounting medium must be sufficiently thick and any air bubbles must be removed from the slide. To prevent air bubbles from forming during mounting, it is important to use the proper technique when applying the coverslip, to ensure an adequate amount of mounting medium is used, and to work quickly to prevent the medium from drying. If air bubbles occur, they can sometimes be removed by applying gentle pressure to the coverslip to push the bubbles to the edge of the slide. However, care must be taken not to shift the coverslip or damage the tissue section. If the bubbles cannot be removed, it may be necessary to remove the coverslip, clean off the mounting medium, and remount the section. Other mounting artifacts include those caused by using a mounting medium that is incompatible with the staining technique, which may result in colors fading or scattering. Therefore, it is important to use a mounting medium that is appropriate for the specific staining method.

3.8 TEM Artifacts

Some problems in the TEM figures of aquatic animals and insects are related to poor ultrathin sections. In our experience, when poor or delayed fixation was indicated (Figure 6A), it showed a loss of fine structure and heterogeneous electron density (Figures 6A-6B). We recommend basing the fixation protocol on the tissue size, the fixative, and the temperature, as poor results will follow the wrong protocol. One of the artifacts most encountered in the TEM study was poor thin sections, which occurred due to incorrect fixation times, unsuitable ultramicrotomy skills, and poor tissue embedding (Figure 6B). Hence, the preparation of TEM slides is a methodologically demanding technique that not only requires experienced laboratory staff, but it is also a highly practice-oriented equipment.

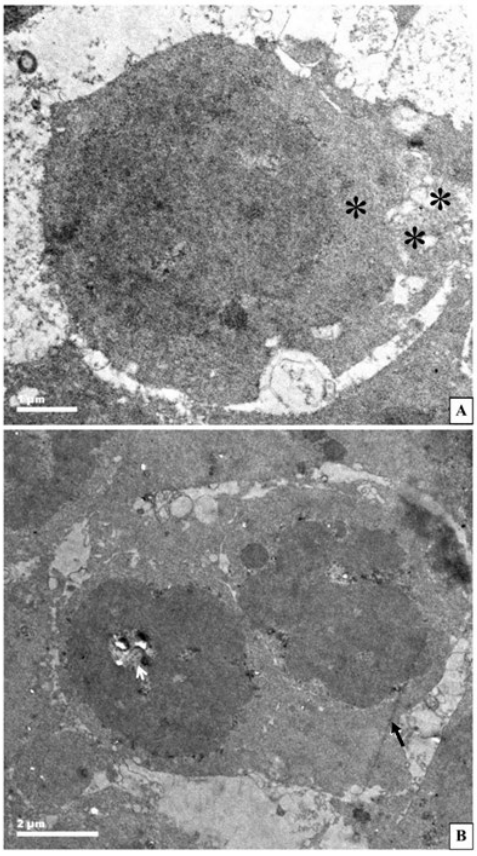

Figure 6: The TEM images of sections from a crab show artifact that include the loss of fine structure (asterisks and white arrow) and scratch lines in the thin section (black arrows).

4. CONCLUSIONS

There has been an increase in histological and histopathological observations of aquatic organisms. We have explained the efficacy and advantages of following the correct histological techniques for tissue slide preparation and described the common tissue artifacts observed in slides of marine organisms and insects. We have associated different types of artifacts with certain steps in specimen processing and their impact on diagnostic accuracy. We hope that our observations will support us in further projects and give suggestions for solving and avoiding problems.

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Author Contributions

All authors contributed toward data analysis, editing the manuscript and agreed to be accountable for all aspects of the work. Piyamat Kongtueng wrote the original manuscript, supporting

facility and equipment, data collection and analyzed the data. Natthawut Charoenphon and Sinlapachai Senarat wrote and edited manuscript throughout the data analysis. Narit Thaochan provided the main conceptual ideas, performed analyses, wrote the manuscript, planned and supervised the work.

Disclosure of Conflict of Interest

The authors declare no potential conflict of interest in the publication of this work.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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