Examination of Histological Methods from Frozen Gonad Samples of the Billfishes.

Akira Kurashima[1], Hirotaka Ijima[1], Yasuhiro Shibasaki[2] and Nobuhiro Mano[2]

[1]Highly Migratory Resources Division, Fisheries Stock Assessment Center, Fisheries Resources Institute (FRI), Japan Fisheries Research and Education Agency.

[2]Department of Marine Science and Resources, College of Bioresource Sciences, Nihon University.

Email: akura@affrc.go.jp

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Abstract

Histological observation needs high-quality specimens to estimate accurate maturity size. However, collecting good condition specimens for histological observation of billfish species are not easy because they are highly migration fishes and are live far sea. If we can use the frozen specimens for histological observation, the gonad sampling will be facilitating. This study observed histological changes among the paraffin sections and the cryofracture, or among raw, frozen, and defrost condition. We also considered whether we can use frozen specimens to estimate the size at first maturity of the billfishes. As a result, we frequently observed the cell shedding by cryofracture in the thin sections of frozen samples. The paraffin sections had higher morphological retention capacity than cryofracture. In the comparison among preserve condition, shedding and deteriorations is looks like morphologies of follicles. This result suggested the possibility of causing misunderstand maturity stage. Although the detailed maturity stage cannot be determined from the frozen specimens, it might be possible to determine mature or immature by referring to the cell diameter and other factors.

Introduction

Maturity size of fish has a significant impact on the population growth rate. Thus, the size at 50% maturity (L50) is considered a critical parameter for the stock assessment model (Anon 2018, 2019). To understand the accurate maturity level, histological observation using high-quality gonads are necessary. However, there are some difficulties with Japanese billfishes port samples. For example, Collecting the gonads in the Japanese port is not easy because almost billfishes have been landed without internal organs. Some frozen samples from Offshore and Distant water longline fisheries are available. If we can use frozen specimens for histological observation, facilitate sampling is to be possible.

The cryofracture technique is one of the tissue diagnosis methods that has been used for rapid tissue diagnosis. The cryofracture technique is a method that slices frozen tissues. Hence, it is possible to reduce the damage by freezing to use this method. In this study, we observed histological changes among the paraffin sections and the cryofracture and considered whether we could use frozen specimens to estimate the size at first maturity in the billfishes.

Materials and Methods

Sample Collection

The gonads of striped marlin and swordfish were collected using long-line by R/V 37 Den-maru and 188 Hannei-maru (Table 1). A fish from R/V 37 Den-maru was dissected on the deck, a part of gonad was fixed by 10% buffered formalin as soon as possible. The other fishes from R/V 188 Hannei-maru were preserved on ice until carry to our laboratory. These preserved terms were 4–11 days. These fishes were dissected in our laboratory, a part of gonad of them were fixed by 10% buffered formalin. The other gonads were preserved in -30°C. Frozen specimens were collected by disposable biopsy punch (φ4 mm), were fixed by cold 10% buffered formalin or preserved in -30°C. A part of frozen tissues was defrizzed at room temperature, fixed by cold 10% buffered formalin or preserved in -30°C. Further, a part of un-fixed frozen and defrizzed tissues were preserved in -30 °C.

Histological methods

Fixed specimen series (i.e., raw, frozen, and defrost samples fixed by 10% buffered formalin) were used for paraffin section method. These tissues were dehydrated in alcohol sires and embedded in paraffin. They were sectioned transversely to 5 μm by microtome. For cryofracture technique, the fixed specimen series and the un-fixed specimens (frozen and defrizzed samples) were embedded in O. C. T. compound, were freeze in 70% alcohol at -80°C. These specimens

were sectioned transversely to 5 μm by cryostatl in -25°C. All thin-sections were fixed by 10% buffered formalin PBS, were stained with Hematoxylin-Eosin stain method. These specimens were examined under the microscope.

Results and Discussion

Maturation stage

In this study, we observed the thin sections of the raw specimens to understand the maturity stages. Maturity stages were decided based on the most advanced cell. The specimen of female striped marlin (BF4557) had the early vitellogenic oocyte and, we categorized it as the maturing stage (Fig.1). The specimen (BF4554) had the previtellogenic oocyte (Fig.2) Thus, the specimen was categorized developing stage. These fishes did not observe the atretic oocytes. In terms of male striped marlin (BF4552), spermatozoa were observed and categorized maturing stage (Fig.3).

The female of swordfish (BF4551) was observed early vitellogenic oocyte and this fish was categorized maturing stage (Fig.4). The male swordfish (BF4575) were observed spermatozoa, and categorized maturing stage.

Paraffin section vs. Cryofracture

Compering among the thin sections by the paraffin section method and the cryofracture technique, it is increasing the cell shedding in the cryofracture technique. Especially, the cell shedding was increasing in all cases using 10% buffered formalin, it is difficult to use histological observation (Fig.1-5). At least, it is better to slice without fixation in the case of using the cryofracture technique.

The paraffin sections had higher morphological retention capacity than cryofracture. In cryofracture, the deformed oocytes were observed (Fig. 6, 7). The deformed oocytes were morphologically similar to the atretic oocytes. These oocytes are considered deformed oocytes because the atretic oocytes were not observed in all paraffin sections. This tendency affects the measurements of cell diameter. It may cause misidentification of the maturity stage.

Comparison among raw, frozen and defrost specimens in paraffin section

The deterioration of the cell was observed in frozen and defrost specimens. Especially, the damage of defrosting was greater than it of freezing. At early vitellogenic oocyte, the nuclear is unclear in frozen specimens (Fig. 6). Further, several hollows by deterioration were observed in

defrost specimens (Fig. 6). This hollow is morphologically similar to follicle, it may cause misidentification of the maturity stage. At the perinucleolus stage oocyte, the nuclear is broken in the frozen and defrost specimens (Fig. 7). It is difficult to identify whether the perinucleolus stage oocyte or oogonia. It seems necessary to collect raw samples for a detailed determination of maturity stage. However, it may be possible to estimate mature or immature from the oocyte diameter even in frozen samples.

Conclusion

We will require the raw specimens fixed by 10% formalin fixation to evaluate the detailed maturation stage. However, even frozen specimens may be possible to estimate the maturity size. Unfortunately, it is difficult to use defrosted samples for maturity determination.

Reference

Anonymous. (2019). Stock Assessment Report for Striped Marlin (*Kajikia audax*) in the Western and Central North Pacific Ocean through 2017. *19th Meeting of the International Scientific Committee for Tuna and Tuna-Like Species in the North Pacific Ocean Taipei, Taiwan July 11-15, 2019*.

Anonymous. (2018). Stock Assessment for Swordfish (*Xiphias gladius*) in the Western and Central North Pacific Ocean through 2016. *18th Meeting of the International Scientific Committee for Tuna and Tuna-Like Species in the North Pacific Ocean, Yeosu, Republic of Korea July 11-16, 2018*.

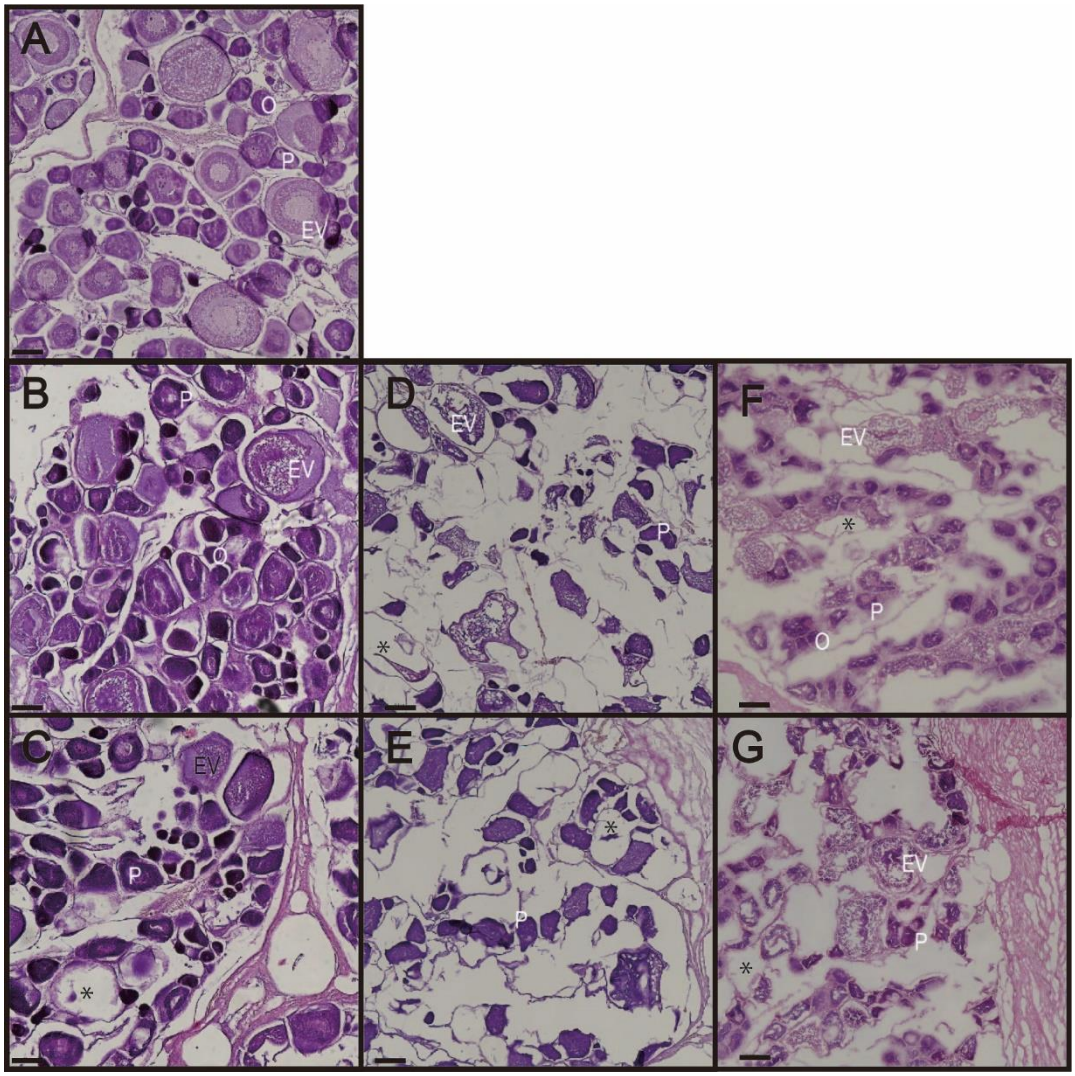

Fig. 1 Female of striped marine, EFL=192cm（BF4554）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-G: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Frozen specimen non fixation. G: Defrost specimen non fixation. O, oogonia, P, perinucleolus stage oocyte, EV, early vitellogenic oocyte, Asterisks, shedding. Scale bars = 100μm.

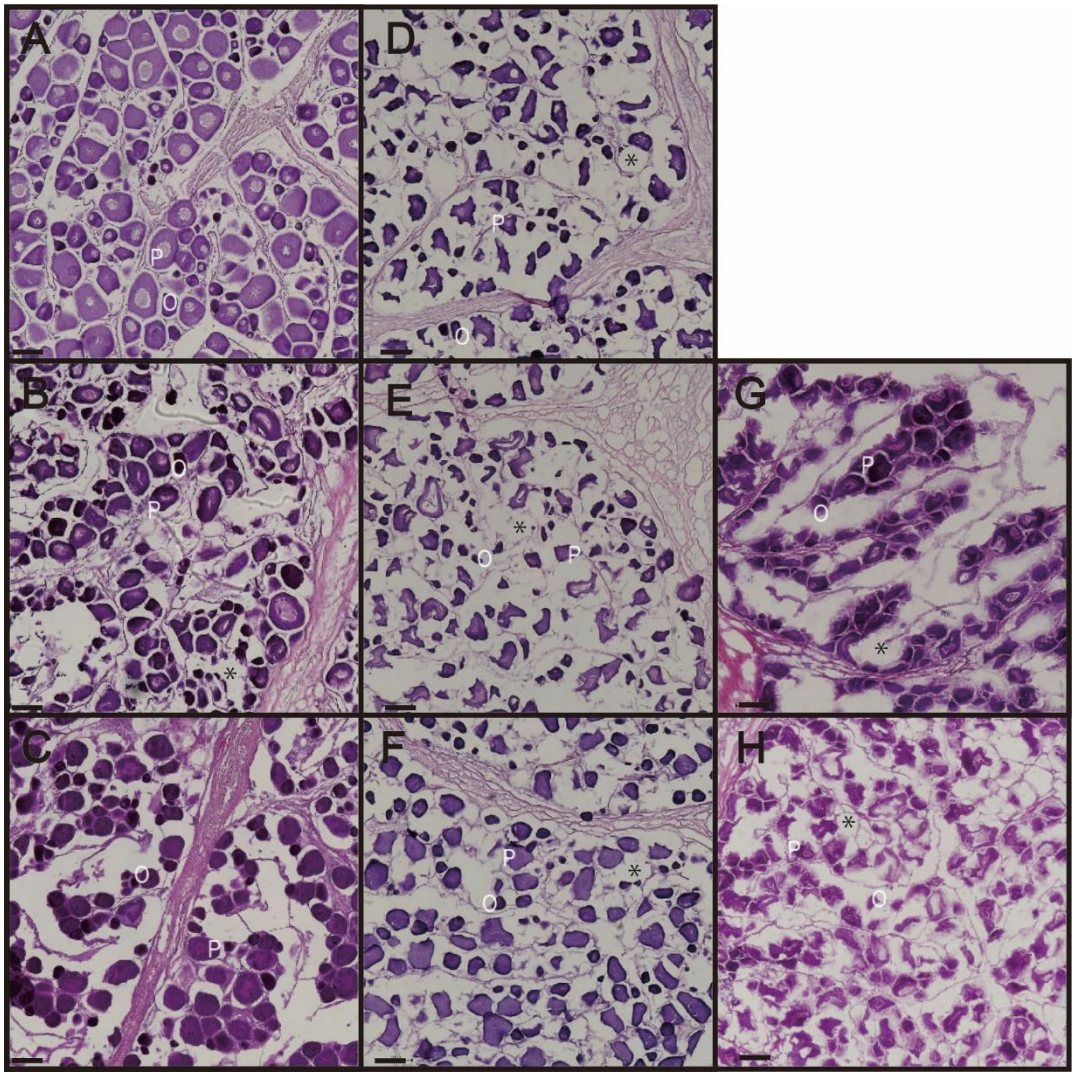

Fig. 2 Female of striped marine, EFL=154cm（BF4557）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-H: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Raw specimen non fixation. G: Frozen specimen non fixation. H: Defrost specimen non fixation. O, oogonia, P, perinucleolus stage oocyte, Asterisks, shedding. Scale bars = 100μm.

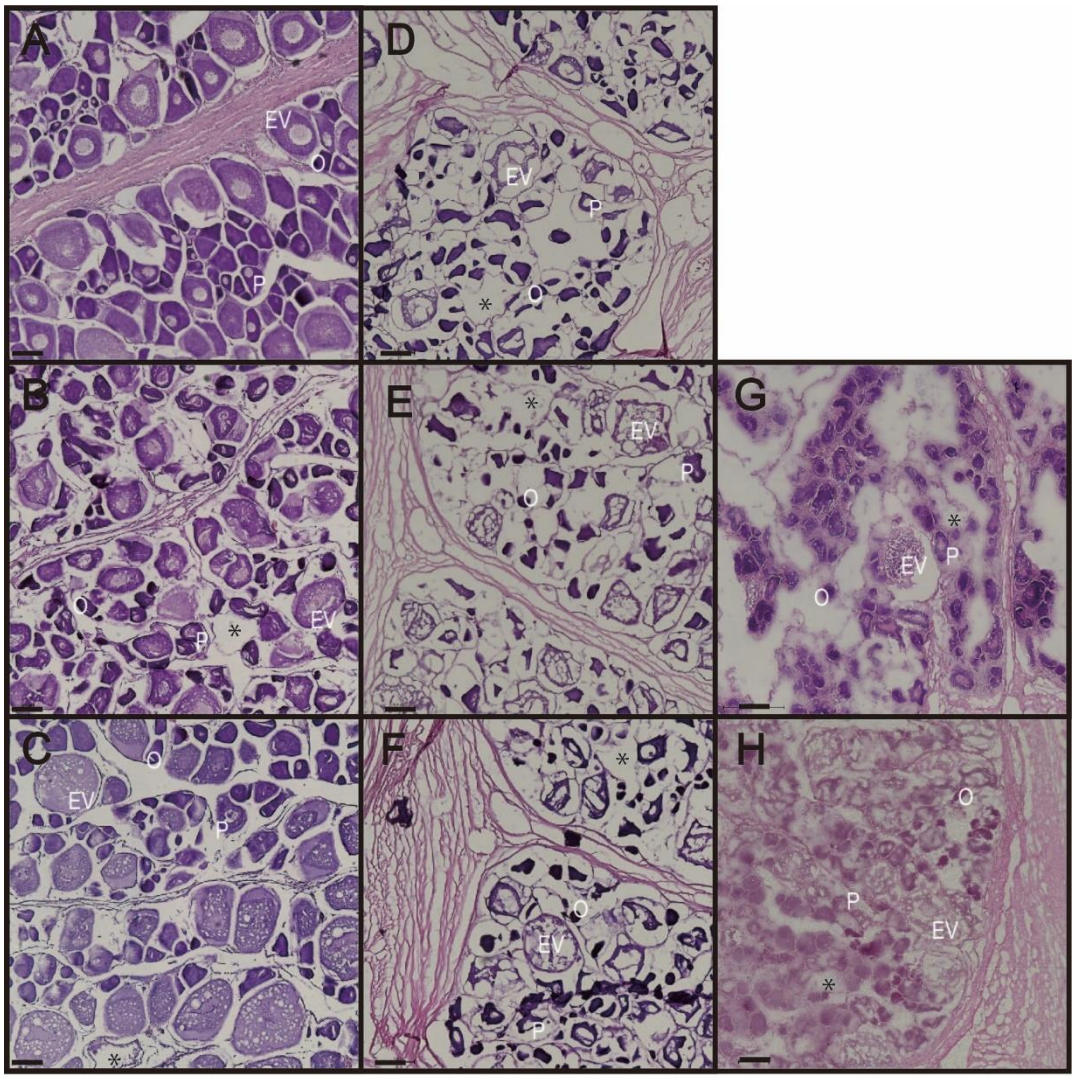

Fig. 3 Female of swordfish, EFL=143cm（BF4551）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-H: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Raw specimen non fixation. G: Frozen specimen non fixation. H: Defrost specimen non fixation. O, oogonia, P, perinucleolus stage oocyte, EV, early vitellogenic oocyte, Asterisks, shedding. Scale bars = 100μm.

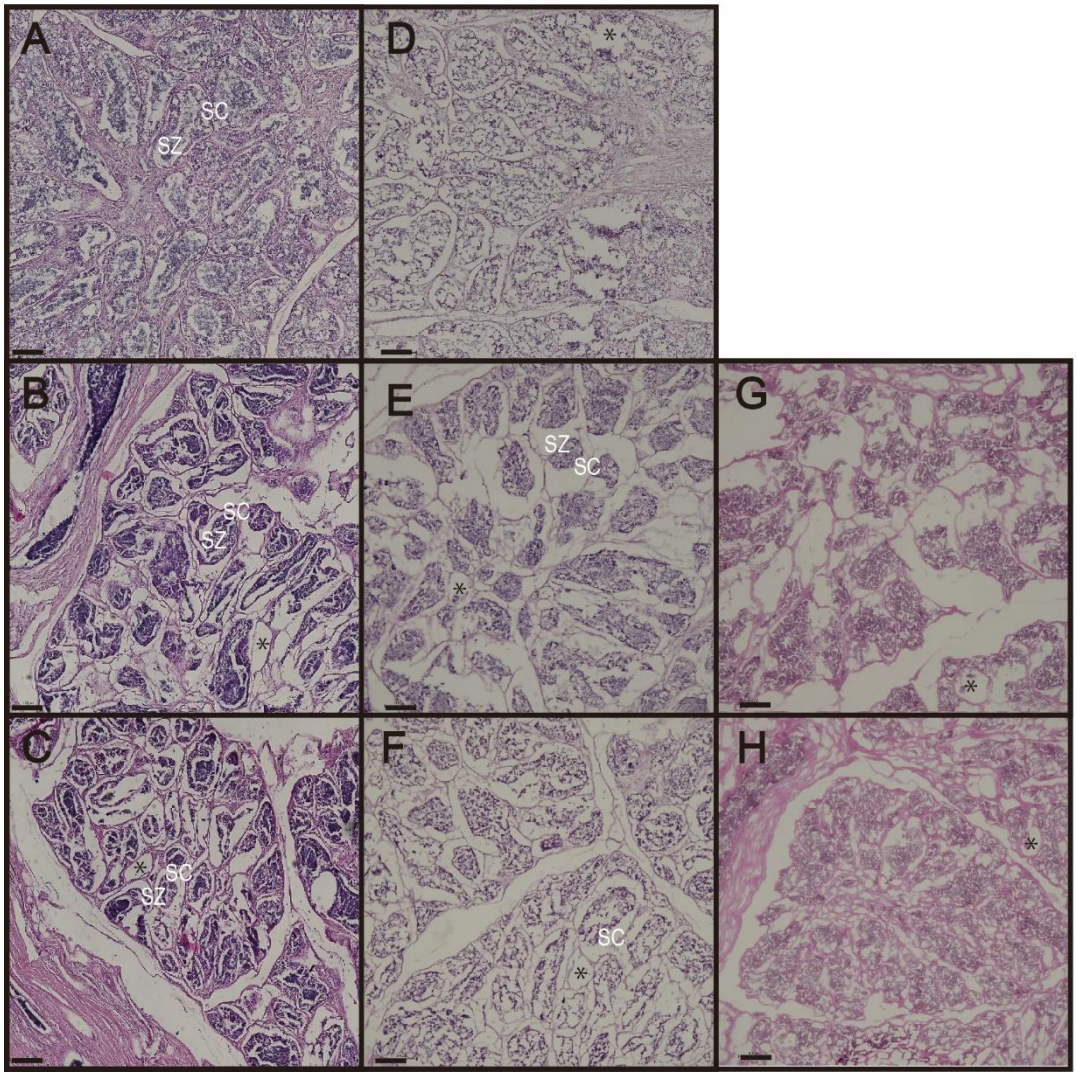

Fig. 4 Male of striped marlin, EFL=164cm（BF4552）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-H: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Raw specimen non fixation. G: Frozen specimen non fixation. H: Defrost specimen non fixation. SC, spermatocytes, SZ, spermatozoa, Asterisks, shedding. Scale bars = 100μm.

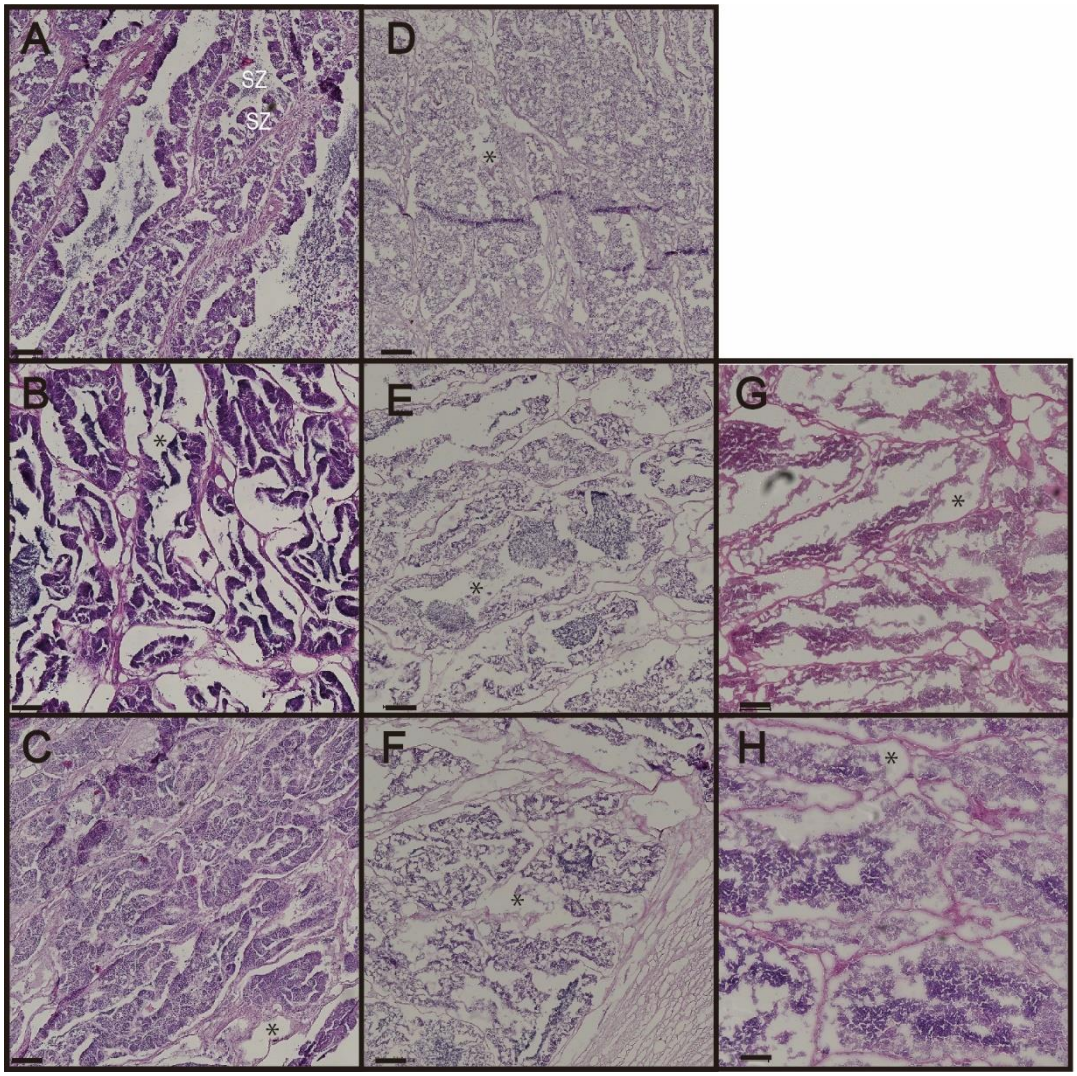

Fig. 5 Male of swordfish, EFL=153cm（BF4575）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-H: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Raw specimen non fixation. G: Frozen specimen non fixation. H: Defrost specimen non fixation. SC, spermatocytes, SZ, spermatozoa, Asterisks, shedding. Scale bars = 100μm.

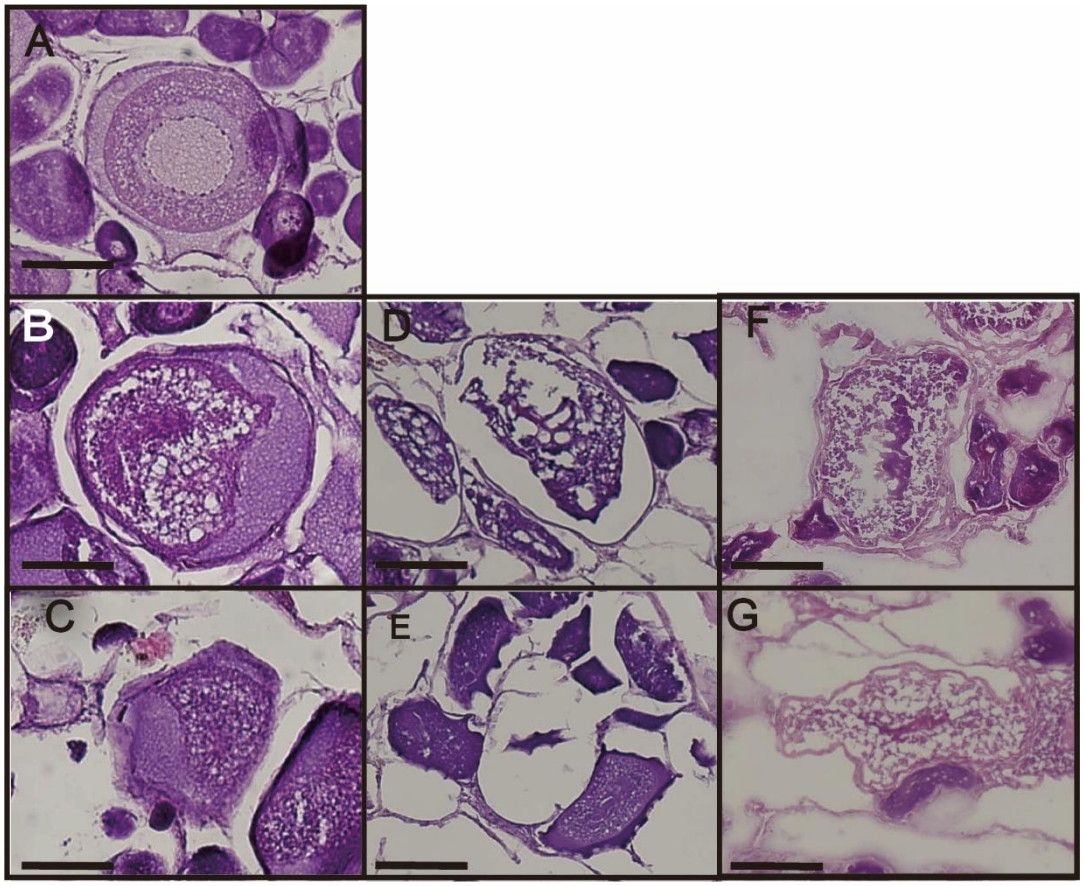

Fig. 6 Early vitellogenic oocyte（BF4554）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-G: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Frozen specimen non fixation. G: Defrost specimen non fixation. Scale bars = 100μm.

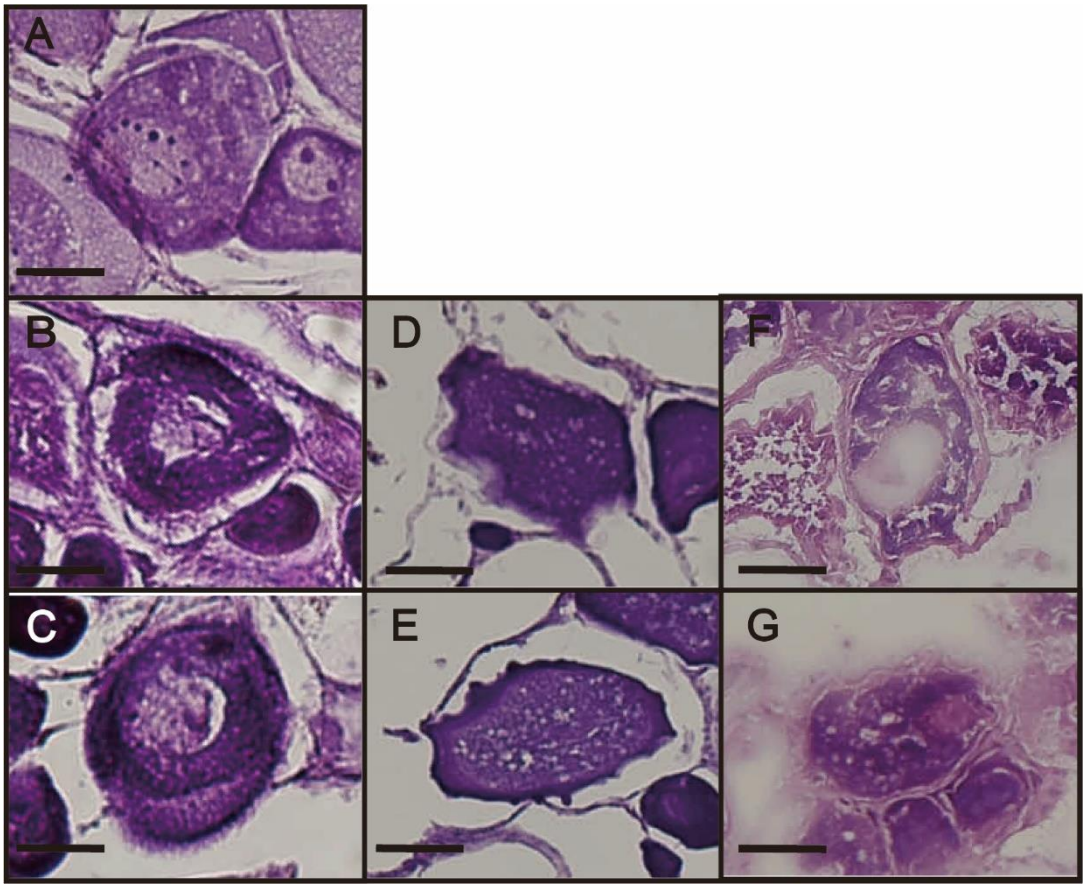

Fig. 7 Perinucleolus stage oocyte（BF4554）. **A-C: Paraffin section method.** A: Raw specimen fixed by 10% buffered formalin. B: Frozen specimen fixed by 10% buffered formalin. C: Defrost specimen fixed by 10% buffered formalin. **D-G: Cryofracture technique.** D: Frozen specimen fixed by 10% buffered formalin. E: Defrost specimen fixed by 10% buffered formalin. F: Frozen specimen non fixation. G: Defrost specimen non fixation. Scale bars = 50μm.

Table 1 Specimens information.

Species	ID	Sex	EFL(cm)	Weight(kg)	Catch Day	Preserved Days	Vessel
Striped marlin	BF4552	Male	164.6	51.66	2020.5.14	11	188 Hanei-maru
Striped marlin	BF4554	Female	192.8	87.54	2020.5.17	5	188 Hanei-maru
Striped marlin	BF4557	Female	154	35.28	2020.5.21	4	188 Hanei-maru
Swordfish	BF4575	Male	153	82	2020.5.21	0	37 Den-maru
Swordfish	BF4551	Female	143.8	60.92	2020.5.14	11	188 Hanei-maru