



Reproductive Biology and Embryonic Diapause as a Survival Strategy for the East Asian Endemic Eagle Ray *Aetobatus narutobiei*

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Batoids comprise five of the seven most threatened families of sharks and rays. The East Asian endemic Naru eagle ray *Aetobatus narutobiei* is a large bodied ray whose estuarine habitat overlaps with an economically valuable bivalve fishery. In response to decreased bivalve yields, the government initiated a predator control program and as a result, Naru eagle rays have faced intense and targeted fishing pressure during the last two decades. The long-term impacts of the predator control program on the population of rays and bivalves and their balance in the ecosystem are unknown because the life history of the Naru eagle ray has not been characterized. To begin to fill these critical knowledge gaps, the reproductive life history of the Naru eagle was described. Females mature at a larger size than males and require nearly twice as many years to reach maturity (DW50, 952.0 mm vs. 764.2 mm; Age50, 6.0 years vs. 3.5 years). Both males and females reproduce annually and their reproductive cycles are synchronized and seasonal. Females have a single ovary and paired uteri, are viviparous, and reproduce via matrotrophic histotrophy. Mating occurs in August and September and gestation lasts approximately 12 months including a 9.5-month diapause that begins soon after mating and ends in June of the following year, leaving 2.5 months for embryos to complete development. Fecundity ranged from 1 to 7 embryos per brood ($n = 158$, mean \pm SD = 3.36 ± 1.26) and was positively correlated with female disc width (linear regression; $F = 105.73$, $d.f. = 151$, $P < 0.05$). Naru eagle rays are vulnerable to overfishing because of their low fecundity, long reproductive cycle and long time to reach sexual maturity. Obligate embryonic diapause during overwintering and seasonal migrations is a survival strategy that benefits the adults and neonates. This research is a valuable resource to help guide science-based management, conservation and protection of the endemic Asian *A. narutobiei* and its nursery areas.

Keywords: elasmobranch, Myliobatidae, viviparous, trophonemata, fecundity, nursery area, uterus, estuary ecosystem

INTRODUCTION

Climate change and anthropogenic disturbances can have significant and deleterious effects on coastal ecosystems and regional biodiversity. Elasmobranchs are especially susceptible to environmental change (Crear et al., 2020; Nowicki et al., 2021) and increased pressure from fisheries (Camhi et al., 1998; Musick, 1999) due to their generally slow maturity, low fecundity and low population growth rates (Hoenig and Gruber, 1990; Camhi et al., 1998). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, 33.7% (387 of 1174) of sharks and rays are threatened with extinction (Dulvy et al., 2021). Additionally, the global abundance of oceanic sharks and rays has declined by 71% owing to increase fishing pressure in the past 50 years (Pacoureau et al., 2021). Among elasmobranchs, large-bodied and shallow-water species are at the greatest risk of extinction, and five out of the seven most threatened families are batoids (i.e., rays, skates, guitarfish, and sawfish) (Dulvy et al., 2014). Batoids are more threatened than sharks and chimeras (Dulvy et al., 2021) and they play an important role as mesopredators and energetic links in ecosystems, however, they have received comparatively little attention from researchers and policy makers compared to their more charismatic shark counterparts. As a result, establishing science-based management of batoids is stymied by the lack of comprehensive data and information on their reproductive biology, early life history and nursery area requirements (Martins et al., 2018). For endemic elasmobranchs with a narrow range, identification and preservation of critical habitat is an urgent conservation need.

The East Asian Naru eagle ray *Aetobatus narutobiei* is one of five species of pelagic eagle rays and are endemic to East Asia. *Aetobatus narutobiei* is very similar in appearance to the long-headed eagle ray *A. flagellum*, a tropical species distributed throughout the Indo-West Pacific, with which it was formerly considered conspecific (Yamada and Miya, 1989; McEachran and Seret, 1990; Yamaguchi et al., 2005; White et al., 2013; White and Moore, 2013). The Naru eagle ray range is narrow, and their main habitat is Ariake Bay where large aggregations of rays are observed from spring to autumn. The rays are not targeted commercially for consumption. Ariake Bay, located in the southwestern of Japan, is a unique ecosystem with a muddy substrate encompassing the largest estuary in Japan. Its estuarine and tidal flats serve as conduits for nutrients to pass into productive coastal waters. Ariake Bay also provides essential spawning and nursery grounds for many species of bivalves, teleost fishes, and elasmobranchs such as the red stingray *Hemirhamphysa akajei* (Furumitsu et al., 2019) and scalloped hammerhead shark *Sphyrna lewini* (Yamaguchi and Kume, 2011). Historically, the bay is referred to as “the sea of treasures,” with an extensive estuary, nicknamed the “uterus.” Ariake Bay is renowned for its highly productive bivalve fisheries, especially pen-shells *Atrina* spp. and short neck clams *Ruditapes philippinarum*.

By 1984, the bivalve fishery was experiencing significantly reduced yields compared to the previous decade (Kyushu Regional Agricultural Administration Office, 1964–2013) and by

the 1990s, when global warming impacts first were observed in western coastal areas of Japan (Ogino et al., 2019), an increase in the population size of Naru eagle rays in Ariake Bay was reported (Kawahara et al., 2004; Kimura et al., 2018). Warmer waters may have extended the time Naru eagle rays stay within Ariake bay and/or increased overwintering survivorship. Despite the timeline, with bivalve yields declining before Naru eagle ray populations increased, because the Naru eagle ray diet is dominated by mollusks (Yamaguchi et al., 2005), rays were assumed to be competing with the bivalve fishery causing significant reductions in yield. The national and local government responded to the decline in bivalve fishery with a government-supported and sanctioned predator control program with the goal of reducing Naru eagle ray populations as a mechanism to restore bivalve yields. As a result, the rays in Ariake Bay have been under intense fishing pressure for the last two decades. Since 2001, systematic surveys of rays culled as part of the predator control program in Japan were performed to describe the age, growth, feeding habits, and seasonal migration patterns of the Naru eagle ray (Yamaguchi et al., 2005). The largest catches of Naru eagle rays from the predator control program, including pregnant females and neonates, were from Ariake Bay, suggesting this area also might serve as a nursery ground for the species (Yamaguchi et al., 2005). Periodic assessment of bivalve and ray populations in Ariake Bay historically have not been completed and accordingly, the efficacy of the predator control program as a mechanism to recover bivalve yield is unknown. Despite the lack of data and understanding of ray predator-prey interactions that might affect bivalve population dynamics and ecosystem functions (Flowers et al., 2021), the government's predator control program remains broadly adopted as an effective control measure.

Myliobatiformes is the largest order of rays with 218 species (Last et al., 2016). All Myliobatiformes rays are viviparous and reproduce via lipid histotrophy. Despite this commonality, myliobatid reproductive features vary widely, and comprehensive studies of their reproductive biology and embryonic development, especially for large pelagic species from the family Aetobatidae and Mobulidae are incomplete for all species. Basic anatomical information including the number of functional ovaries and uteri is lacking for many species even though it is essential to determine fecundity, a critical parameter for population management. Myliobatiform rays are a diverse group in terms of their morphology, habitat, and ecology; therefore, understanding their reproductive biology and embryonic development is of interest evolutionarily as well as from a biodiversity standpoint.

Select elasmobranchs include embryonic diapause, a temporary slowing or suspension of embryonic development that extends the time required to complete a reproductive cycle, as a life history trait. Diapause lasting from 4 to 10 months has been reported previously for 16 elasmobranch species (2 sharks and 14 rays), including *A. narutobiei* as summarized by Wyffels (2009) and Waltrick et al. (2012). In addition, an abbreviated diapause, less than 2 months in duration, is suspected for the red stingray (Furumitsu et al., 2019). Embryonic diapause in elasmobranchs has independently evolved among species with a range of

reproductive strategies, suggesting that it may be regulated via several different mechanisms (Waltrick et al., 2012). Diapause is assumed to benefit neonates thereby increasing reproductive fitness of adults (Simpfendorfer, 1992; White et al., 2002), but this hypothesis has not been rigorously tested. It is essential to consider the reproductive biology and life-history parameters of a species holistically in order to understand the potential survival strategies and advantages afforded by embryonic diapause.

Recently, the Ministry of the Environment, Japan, categorized the Naru eagle ray as “Near Threatened (NT)” (Ministry of the Environment, 2017; Kimura et al., 2018). A subsequent assessment of Naru eagle ray by the IUCN Red List of Threatened Species listed Naru eagle rays as “Vulnerable,” a higher risk of extinction than what previously was assessed by the Ministry of the Environment, Japan (Rigby et al., 2021). To reduce the extinction risk of the Naru eagle ray and establish a science-based management policy for population conservation, it is essential and timely to fill biological and ecological knowledge gaps for this species. Understanding the eagle ray life history will guide future research and management measures that enable measures that reduce conflicts with bivalve fishery and guarantee the conservation of this threatened species.

The study aims to characterize the reproductive biology, including embryonic diapause and development of the Naru eagle ray in a great estuarine ecosystem and provide comment on the urgent need for conservation. Knowledge of the reproductive life history and strategies for survival for this pelagic ray will further our understanding of the reproductive systems of elasmobranchs in general, and the evolution of viviparous rays worldwide.

MATERIALS AND METHODS

Study Location and Survey Techniques

Specimens examined ($n = 1189$) were collected by commercial vessels as part of the predator control program using gill nets (20 cm mesh size, 400 m length) at depths of 8–20 m in the northern part of Ariake Bay between August 2001 and November 2019 (Figure 1). Despite continuing efforts by fishers, few Naru eagle ray were caught during December and none were caught from January to March in the first few years of the predator control program (Yamaguchi et al., 2005). As a result, the predator control program operates annually from May to November.

Morphometrics and Reproductive Data

Immediately after collection, disc width (DW, nearest 0.5 mm), body mass (BM, nearest 0.001 g for embryos or 1 g for field specimens), and sex were recorded for each Naru eagle ray. For females, uterus mass (UM, nearest 0.1 g) was measured. For males, inner clasper length (CL, nearest 0.01 mm), distance from the anteriormost limit of the cloaca to the tip of the clasper, was measured and the presence or absence of semen in the seminal vesicles was noted. Male maturity stages were determined based on the degree of development of the testes and claspers as described previously (Furumitsu et al., 2019): (1) immature—testes and claspers undeveloped; (2) premature

or sub-adult—testes becoming lobular, vas deferens beginning to thicken and coil, clasper elongated and flexible, not calcified; and (3) mature—testes and seminal vesicles fully developed, vas deferens fully developed and coiled, claspers calcified. Female maturity stages were determined by the degree of development of the ovaries and uterus: (1) immature—ovary comprising white, undifferentiated tissues, uterus thin; (2) premature or sub-adult—ovary containing numerous immature follicles pale yellow or whitish in color and translucent, uterus thickening but without contents; and (3) mature—ovary containing opaque yellow vitellogenic follicles, uterus thick and may contain eggs or embryos (Furumitsu et al., 2019). Clasper length and UM relative to disc width were used as indicators of maturity (Yamaguchi and Kume, 2009). Analysis of covariance (ANCOVA) was used to test for differences between sexes in the log-transformed linear DW–BM relationship (KyPlot 6.0, Kyenslab, Tokyo, Japan) with a critical probability threshold of 0.05 for statistical significance.

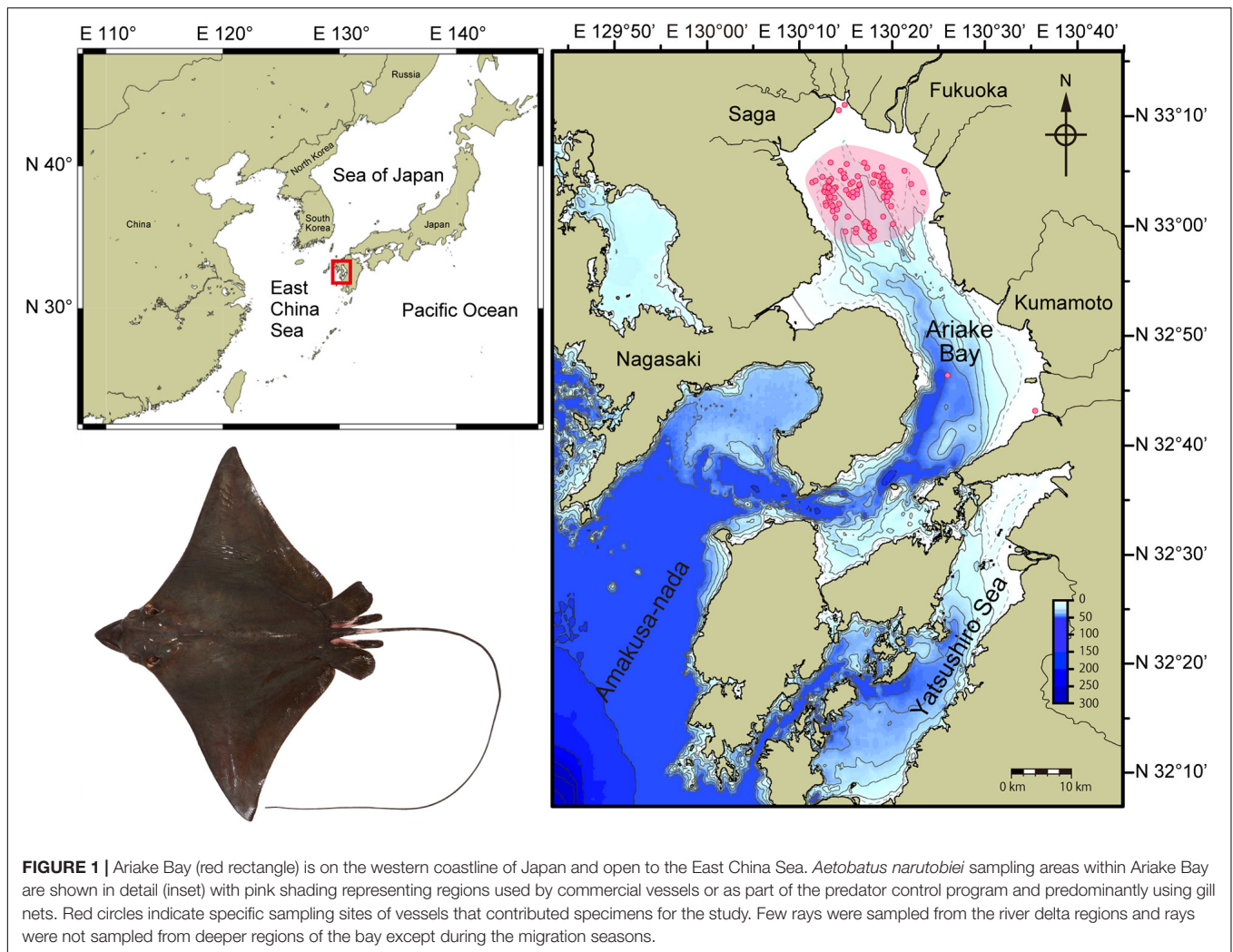
Size and Age at Maturity and Natural Mortality Rate

The percentage of mature individuals in relation to immature and premature individuals for each 10 mm DW size group was calculated and fitted to the logistic model, $Y = [1 + e(aX + b)]^{-1}$, where Y is the proportion of individuals mature at size X , and a and b are empirical parameters derived using the statistical software KyPlot 5.0 (KyensLab Inc.) (White and Dharmadi, 2007; Kume et al., 2009). Age at sexual maturity was calculated from established length-at-age relationships for the Naru eagle ray (Yamaguchi et al., 2005). Size (L50) and age (A50) at 50% maturity were calculated using the equation $L50$ or $A50 = -ba^{-1}$ (Kume et al., 2009). Using previously reported age composition data for this species (Yamaguchi et al., 2005) in combination with the age at maturity determined in this study, a natural mortality rates of adult rays was calculated by a catch-curve method (Ricker, 1975).

Male and Female Reproductive Status

Masses (nearest 0.1 g) of the liver and gonads (with associated epigonal organ) were collected for mature rays to assess reproductive status. The gonadosomatic index (I_G) was calculated using the formula: $I_G = [\text{gonad mass}/(\text{body mass} - \text{gonad mass})] \times 100$. The monthly I_G for mature males was used to approximate the mating season. Livers were removed and weighed to the nearest 0.1 g and the hepatosomatic index (I_H) was calculated using the formula: $I_H = [\text{liver mass}/(\text{body mass} - \text{liver mass})] \times 100$.

Testes removed from mature males were fixed in Bouin's solution for 48 h and transferred to 70% ethanol. They were subsequently dehydrated and embedded in paraffin wax, sectioned to a thickness of 4–5 μm , and stained with hematoxylin and eosin (HE). For the purpose of defining the developmental stages of spermatogenesis, differentiating sperm cells were classified as spermatogonia (SG), primary spermatocytes (SC1), secondary spermatocytes (SC2), spermatids (ST), immature spermatozoa (IS), and mature spermatozoa (MS), according to Conrath and Musick (2002) and Poulakis and Grier (2014). A representative cross-section was selected from the middle of



the right or left lobe and the testis were classified into one of seven stages of spermatogenesis (I–VII) based on the most developed stage of the sperm observed (Maruska et al., 1996; Furumitsu et al., 2019). Monthly proportions of the testis stages were calculated with and without the degenerate zone (VII), to characterize the proportion of newly formed spermatocytes in testis that are predominately degenerating.

For each mature female, the diameter of the largest ovarian follicles was measured (nearest 0.01 mm) with an electronic caliper and averaged. Because the Naru eagle ray was susceptible to capture-related uterine discharge, specimens that were observed to abort one or more embryos were removed from fecundity and uterine egg and embryo analyses. For mature females, 20 trophonemata were randomly selected and their length measured (nearest 0.01 mm) with an electronic caliper and averaged. The relationship between trophonemata length and stage of embryo and trophonemata length and female DW were tested using linear regression. Uterine egg and embryo mass (nearest 0.001 g), embryo DW (nearest 0.01 mm), and embryo sex (when possible) were recorded. The number of male and female embryos for all gravid rays was subjected to an χ^2 test to

determine if the sex ratio differed from parity. The relationship between the female DW and average uterine egg mass and female DW and fecundity were tested using linear regression (KyPlot 6.0, Kyenslab, Tokyo, Japan) with a critical probability threshold of 0.05 for statistical significance. Size at birth was estimated from the largest embryos and the smallest free-swimming neonates. Embryos were classified into 11 developmental cohorts based on easily recognized morphological characteristics (Table 1).

RESULTS

Size Distributions, Disc Width, Age at Sexual Maturity, and Natural Mortality Rate

Males averaged 686.6 ± 174.5 mm DW (range 298–1038 mm) and 5881.5 ± 3797.2 g (range 409.3–17350 g). Females averaged 834.2 ± 290.7 mm DW (range 297–1536 mm) and 13186.4 ± 11495.1 g (range 411.6–60000 g). The largest female was approximately 1.5 times the disc width and 3.5 times the mass

TABLE 1 | Developmental cohorts for *Aetobatus narutobiei* embryos.

Cohort	Disc width	Description
1		Uterine eggs without macroscopic embryos contained within a tertiary egg envelope. Uterine eggs include pre-gastrulation embryonic stages.
2	<2 mm	Embryos are visible on eggs contained within the egg envelope. The embryos are transparent and fragile. Dorsal, pectoral, and pelvic fins are not formed.
3	4–7 mm	Eggs and embryos are no longer contained within the egg envelope. Pectoral and pelvic fins are recognized and expanding laterally. The anterior margin of the pectoral fins does not surpass the posterior margin of the gills. The dorsal fin is formed slightly (just like raised from the base). The spiracle is obviously recognized. Claspers, dorsal, and ventral tail fold are not formed. There is no pigmentation on the body or eyes.
4	9–13 mm	The pectoral fins have grown anteriorly but the margin does not exceed the anterior margin of the gills. Gill slits exist on the lateral surface. The dorsal fin is recognized. Dorsal and ventral tail folds are formed. The eyes have slight pigmentation, brown in hue.
5	12–18 mm	The tips of the pectoral fins have a roundness and have grown anteriorly but the margin does not exceed the eyes. The pectoral fins conglutinated at the forward edge of gill slits, and the gill slits moved to the ventral side of the body. Both ventral and dorsal tail folds were enlarged. The region from which the spines later emerge is clearly defined.
6	32–43 mm	Embryos have pectoral fins fused near the mouth. The cephalic lobes are incomplete, not over the rostrum. Embryos have long external gills. Dorsal and ventral tail folds are well developed. The spine on the tail started to form. The body remains translucent and without coloration. Male and female embryos are distinguishable by the presence of claspers.
7	39–86 mm	The rostrum regresses to the body, and the cephalic lobes are formed completely. The heights of the dorsal and ventral tail folds were lower, and the tail elongated. The external gills began to shorten. The body color is translucent and without coloration at this stage. The external yolk sac began to diminish.
8	128–150 mm	The external gills of the embryos have been reabsorbed, but an external yolk sac is still present. The dorsal and ventral tail folds disappeared. The body remains translucent but with a black color (inner muscle).
9	185–206 mm	The external yolk sac was diminished, the body is turning gray–black and the tail gray. The dorsal spine begins to be covered with skin.
10	235–250 mm	The external yolk sac is completely re-absorbed. The color of the dorsal disc surface is beginning to turn gray–black. The ventral surface of the disc has already turned white. The dorsal spine is covered with skin, but not completely.
11	295–400 mm	Near-term embryos have a body shape and coloration that mimics that of adults. A slight umbilicus protrudes from the abdomen at the site of the yolk-sac attachment. The caudal spine is clearly visible but sheathed. Embryos represent full-term embryos and compare favorably with the size of free-swimming young of the year specimens.

of the largest male (**Figure 2A**). The relationship between DW and BM was significantly different ($F = 25.983$, $d.f. = 1$, $P < 0.001$) between males $BM = (4 \times 10^{-9}) DW^{3.2112}$ ($n = 540$, $r^2 = 0.986$) and females $BM = (4 \times 10^{-9}) DW^{3.1936}$ ($n = 636$, $r^2 = 0.992$). The overall sex ratio ($n = 1189$, F:M = 1:0.86) was significantly different from 1 ($\chi^2 = 6.66$, $d.f. = 1$, $P < 0.05$).

The distributions of the DW frequencies for both sexes were bimodal (**Figure 2B**). The first mode represents immature specimens, 350–400 mm DW for males and 400–450 mm DW for females, and the second mode represents mature specimens, 800–850 mm DW for males and 1000–1050 mm DW for females (**Figure 2B**).

At sexual maturity female DW was greater than males (**Figure 2C**). All specimens with DWs larger than 855 mm for males and 1096 mm for females were sexually mature. The smallest sexually mature male was 721 mm DW and smallest sexually mature female was 885 mm DW. The size at 50% sexual maturity, or DW50 ($\pm 95\%$ CI) was 764.2 (764.2–764.2) mm for males and 952.0 (951.9–952.0) mm for females (**Figure 2C**). Male specimens began maturing at age 2 and females began maturing at age 4 with first attained maturity at age 3 and 5, respectively. All specimens over age 6 for males and age 8 for females were mature. The age at 50% sexual maturity ($\pm 95\%$ CI) was 3.5 (3.5–3.5) years for males and 6.0 (6.0–6.0) for females (**Figure 2D**). Clasper length increased abruptly when DW was 700 mm (premature males, $n = 52$), and clasper growth slowed when DW was ≥ 800 mm, concomitant with the onset of sexual maturity (**Figure 2E**). Uterus mass began to increase when DW was 850 mm, concomitant with the observations of premature females ($n = 48$), and continued to increase thereafter

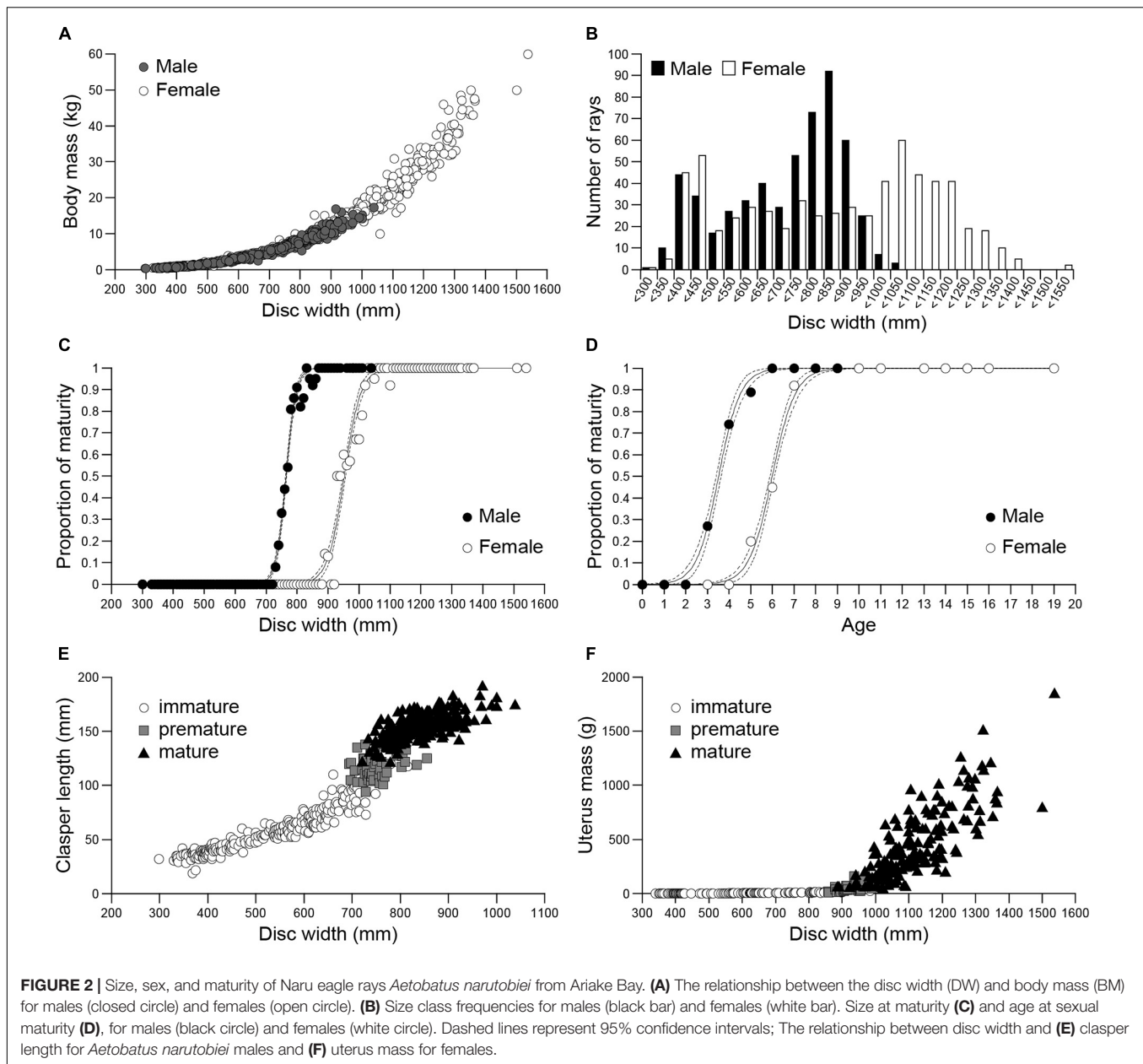
(**Figure 2F**). Natural mortality rate for mature females (more than 6 years old) is 0.23 and that for mature males (more than 4 years old) is 0.75.

Temporal Periodicity of I_G and I_H

Monthly I_G for males (**Figure 3A**) showed clear seasonal variations. Male I_G increased from May to June, reached its maximum in July, and decreased precipitously from August to September. In contrast, there were no seasonal trends in I_G for females during the same timeframe (**Figure 3B**). Monthly I_H showed a similar trend between the sexes, although there were different seasonal patterns for I_H vs. I_G for both sexes. The I_H was lowest from April to July for males and April to August for females, and for both sexes increased thereafter until November (**Figures 3C,D**). In November, just before the overwintering period, male and female I_H was approximately two-fold higher than the lowest values measured in early spring.

Testis Morphology, Spermatogenesis, and Temporal Periodicity

The testes of Naru eagle rays are paired, dorsoventrally flattened organs on the dorsal surface of the epigonal organ located in the anterior peritoneal cavity and suspended from the dorsal body wall by mesorchia (**Figure 4A**). Both testes are well developed and functional. The germinal zone of each testis lies in the central (middle) portion of each lobe, not visible externally (**Figures 4B,C**). Spermatogenesis proceeds radially from the central germinal zone, toward the outer margin of the lobe where spermatocysts connect to efferent ductules. Spermatocysts



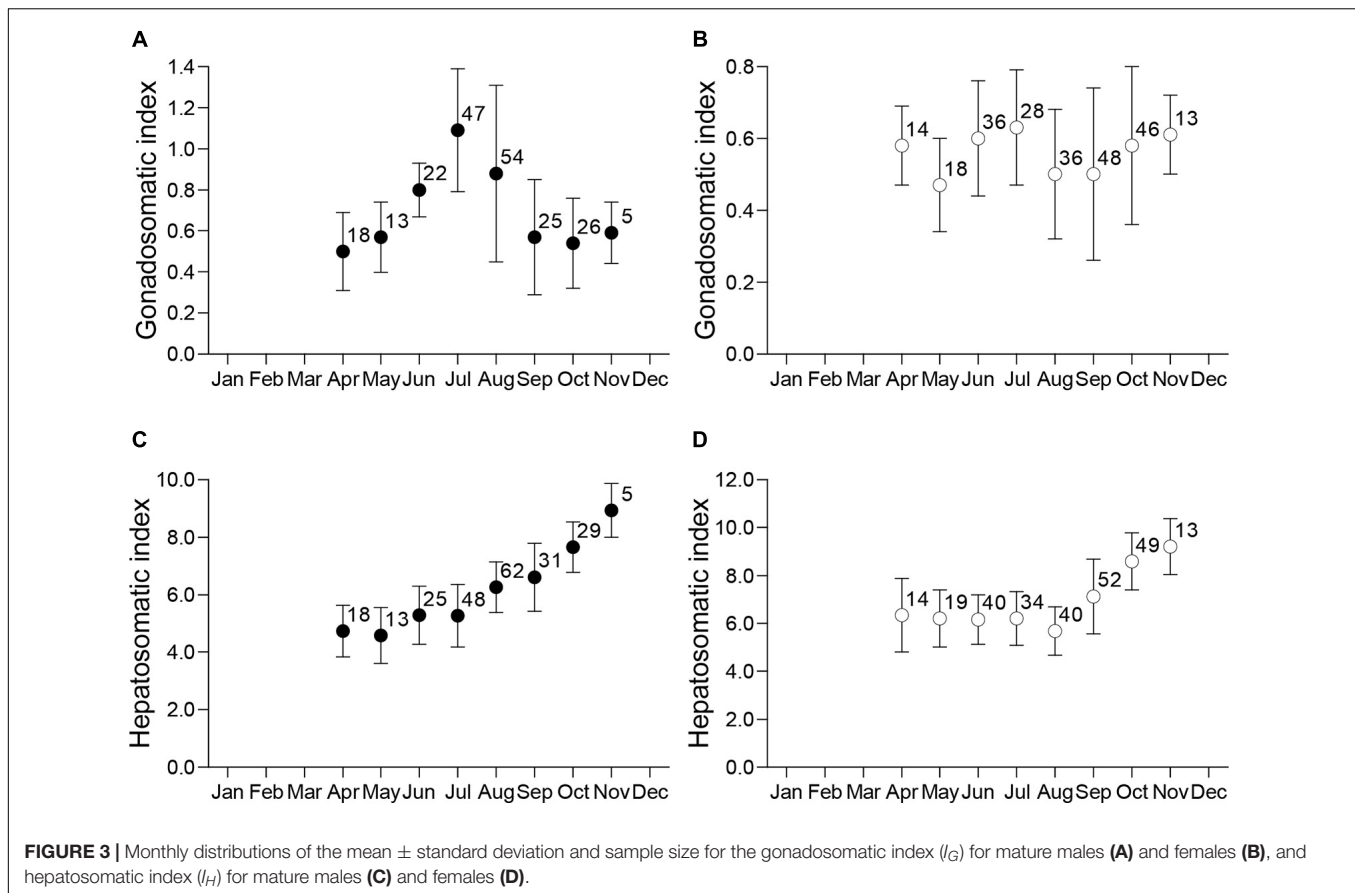
in the final stages of spermatogenesis are visible around the circumferential perimeter of each lobe (Figures 4B,C).

Stage I (Figure 4D) was observed in all months. Stage II spermatocysts (Figure 4E) occurred in April–June, October, and November, peaking in April and May (100%), and reached stage III (Figures 4F,G) in June, July, and September to November (Figure 5B). Stage IV spermatocysts (Figure 4H) were observed in July and September, and stage V (Figure 4I) in July and August (Figure 5B). Stage VI spermatocysts (Figure 4J) were observed from August until October (Aug 96.6%, Sep 86.7%, Oct 8.3%). Stage VII spermatocysts (Figures 4J,K) were observed in April and September–November (Figure 5A). In October, approximately 67% of males had degenerate zones in their testes. Sperm production was confirmed by direct observation of semen

in the seminal vesicles of rays from August through October (Aug 91.7%, Sep 100%, Oct 75.0%).

Reproductive Cycle of Females

Similar to testes, the ovary of Naru eagle rays is observed on the dorsal surface of the epigonal organ in the anterior peritoneal cavity and is suspended from the dorsal body wall by mesorchia. Unlike the paired testes, only the left ovary was functional (Figures 6A,B). Maximum ovarian follicle diameter showed clear seasonal variations. Diameters were smallest during April and July, 10.11 ± 2.51 mm (range 5.40–18.50 mm), and abruptly increased in August, 25.20 ± 5.32 mm (range 13.50–32.00 mm) (Figure 6C). Pre-ovulatory ova were observed in females collected from August to September. The largest ovarian



follicle diameter (32.20 mm) was observed in September. Mature females were post-ovulatory with encapsulated uterine eggs in early September with the exception of two females that cycled in August (7th and 30th). These observations indicate that ovulation and fertilization were synchronous and occurred in September.

Gestation and Embryonic Development

Aetobatus narutobiei had paired functional uteri and pregnant females had multiple eggs or embryos in both, apart from ten females that had a single egg or embryo in either the right or left uterus. The total number of eggs or embryos examined from the right (133 eggs and 73 embryos) and left (143 eggs and 61 embryos) uterus of pregnant females ($n = 158$) was not significantly different (right: left = 1.01:1, $\chi^2 = 0.01$, $d.f. = 1$, $P > 0.05$).

Uterine eggs were enclosed in a fragile, soft, smooth textured, light brown translucent tertiary egg envelope (Figure 7A). Mean uterine egg mass varied widely (3.145–8.019 g) among females with larger females producing eggs with greater mean mass (linear regression; $F = 4.03$, $d.f. = 63$, $P < 0.05$) (Figure 7B). There was no relationship between the number of uterine eggs per female and mean egg mass (linear regression; $F = 1.07$, $d.f. = 63$, $P > 0.05$) (Figure 7C).

Uterine eggs without macroscopic embryos (stage 1, $n = 86$) were observed from April–June and September–November with the exception of two females with stage 1 embryos observed in

August. No data is available from December to March because Naru eagle rays overwinter outside of the bay and specimen collection proved difficult (Figure 8). The ratio of pregnant females with stage 1 embryos was 100% in April and May, dropped to 18.2% in June and 0% in July before increasing to 8.7% in August, 80.6% in September and reaching 100% in October and November.

Uterine eggs with macroscopic embryos were observed first in mid to late June and embryonic growth was rapid (Figure 8). Parturition occurred in late August with the exception of four pregnant females examined in the first 3 days of September, approximately 2.5 months after the smallest macroscopic embryos first were observed. All females examined after September 3 had encapsulated uterine eggs in stage 1 of development. Embryos ($n = 224$) were sexed when possible ($n = 132$). Embryos were assigned to one of 11 developmental cohorts (Table 1): Stage 1 ($n = 86$); Stage 2 ($n = 2$); Stage 3 ($n = 7$); Stage 4 ($n = 4$); Stage 5 ($n = 5$); Stage 6 ($n = 3$); Stage 7 ($n = 4$); Stage 8 ($n = 6$); Stage 9 ($n = 11$); Stage 10 ($n = 4$); and Stage 11 ($n = 13$), based on easily recognized external morphological characteristics (Figure 9). Embryo sex ratio (F:M 1:1.03) was not different from 1:1 ($\chi^2 = 0.03$, $d.f. = 1$, $P > 0.05$). At the onset of stage 2, embryonic development was rapid and embryos reached stage 10 after only 4–5 weeks (Figure 8). Easily observed morphological changes in body form were complete by stage 10 with large increases in embryo size during stages 10 and 11,

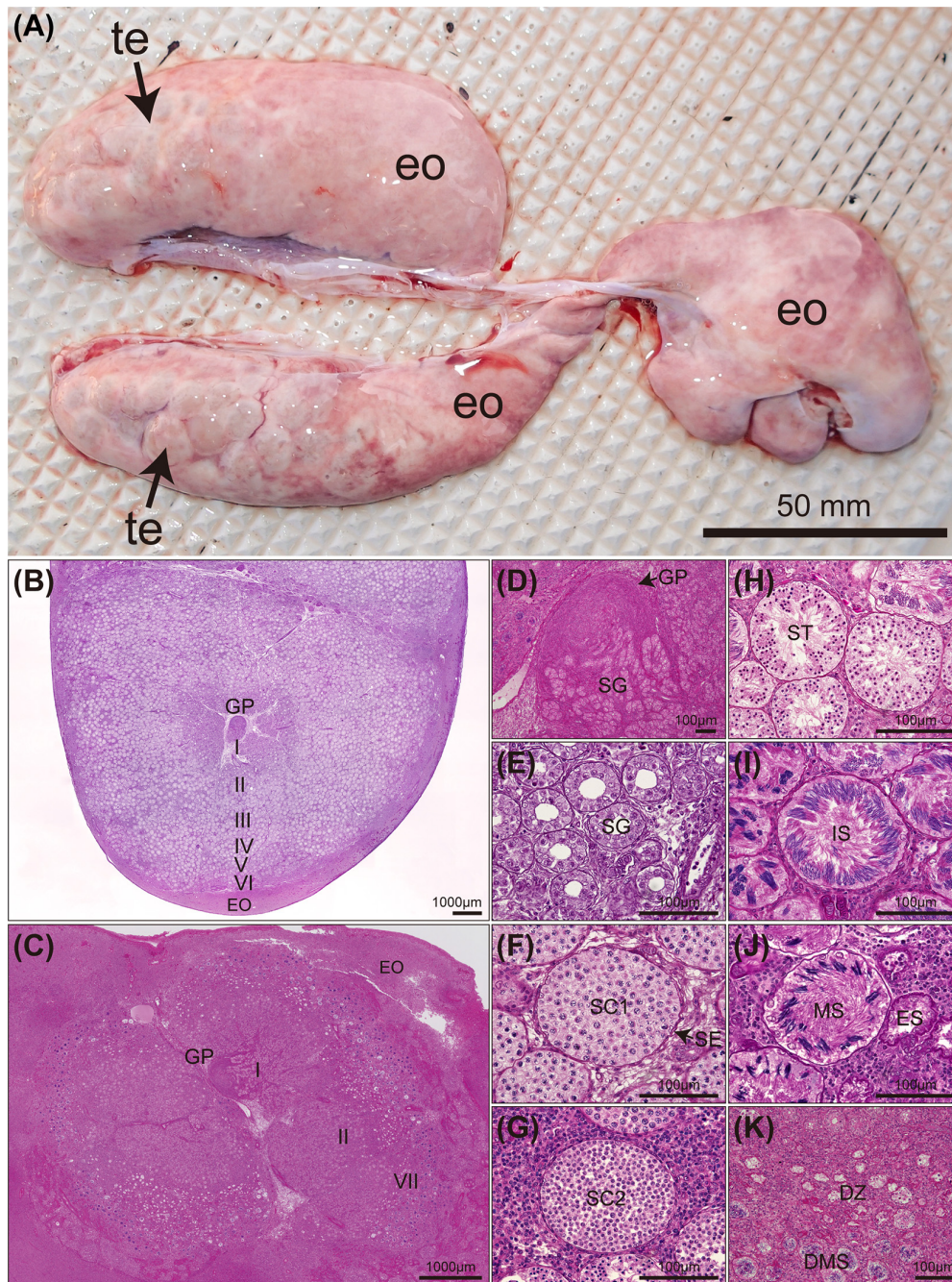
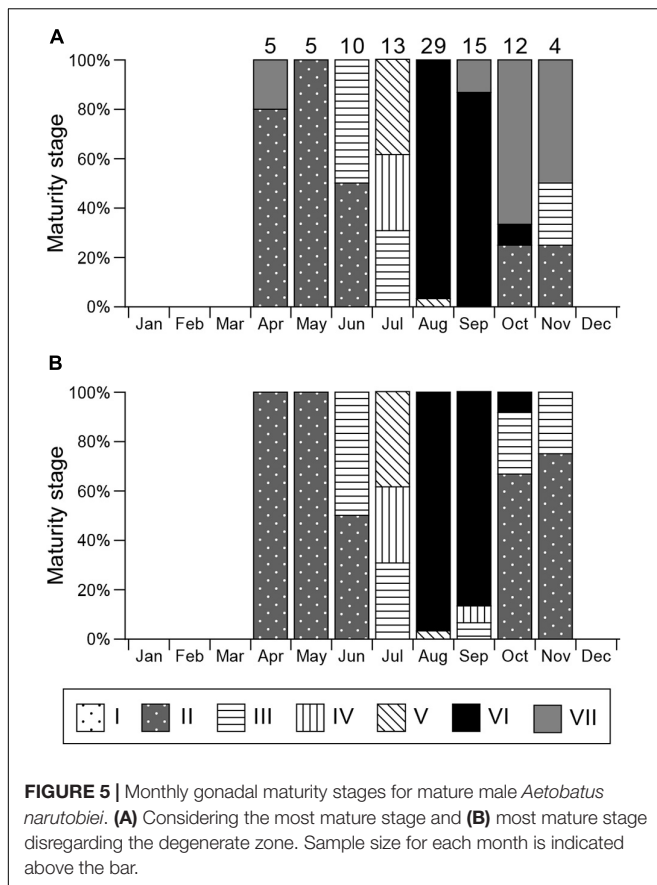


FIGURE 4 | Mature *Aetobatus narutobiei* testes anatomy and stages of spermatogenesis **(A)** (te) testis lobe, (eo) epigonal organ and histology **(B–K)**. **(B)** Cross section through a single testicular lobe with germinal papilla (GP) and epigonal organ (EO) (August). **(C)** Cross section through a single testicular lobe (October). **(D)** Stage I primary spermatogonia (SG). **(E)** Stage II early spermatocysts with spermatogonial cells (SG). **(F)** Stage III primary spermatocytes (SC1) with Sertoli cells (SE). **(G)** Stage III secondary spermatocytes (SC2). **(H)** Stage IV spermatids (ST). **(I)** Stage V immature sperm (IS). **(J)** Stage VI mature sperm (MS) and an empty spermatocyst (ES). **(K)** Stage VII degenerate zone (DZ) and degenerate mature sperm (DMS).

the months preceding parturition (**Figure 8**). Monthly embryo DW (average \pm SEM) increased from 14.25 ± 2.98 mm in June ($n = 23$) to 328.23 ± 24.22 mm in September ($n = 4$).

As embryo DW increased, mass of the external yolk sac decreased (**Figure 10**). External yolk sac mass ranged

2.47–5.73 g during embryonic stages 2–6, and was mostly absorbed (<0.015 g) by stage 10. Embryo body mass increased exponentially once embryos reached stage 5 and by stage 11 embryo mass was (average \pm SEM) 624.4 ± 53.6 g. The heaviest embryo was 1089 g, representing an increase in wet weight of

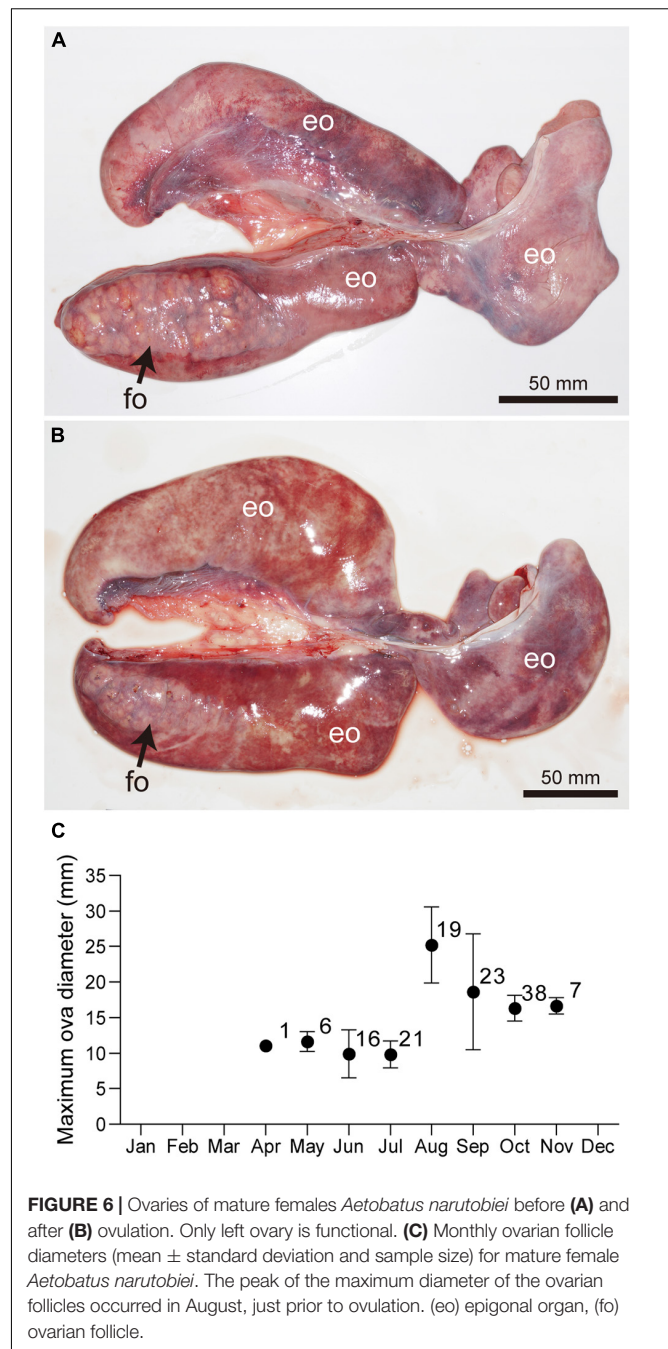


12,874–34,626% compared to the lightest (3.145 g) and heaviest (8.459 g) egg mass measured from stage 1 eggs or 19,179% compared to the average egg mass (5.678 g) observed for females of similar DW.

Parturition occurred during late August through to early-September, and the DW at birth ranged from 300 to 400 mm. The earliest free-swimming specimen (329 mm DW and 540 g) was observed on August 22nd and the smallest free-swimming specimen (297 mm DW and 420 g) was observed on August 30th. Both the largest and the latest embryos in stage 11 (390 mm DW and 1089 g) were observed on September 2nd. Gestation was almost 1 year including a 9.5 months embryonic diapause phase and 2.5 months embryonic growth phase.

Trophonemata and Embryonic Growth

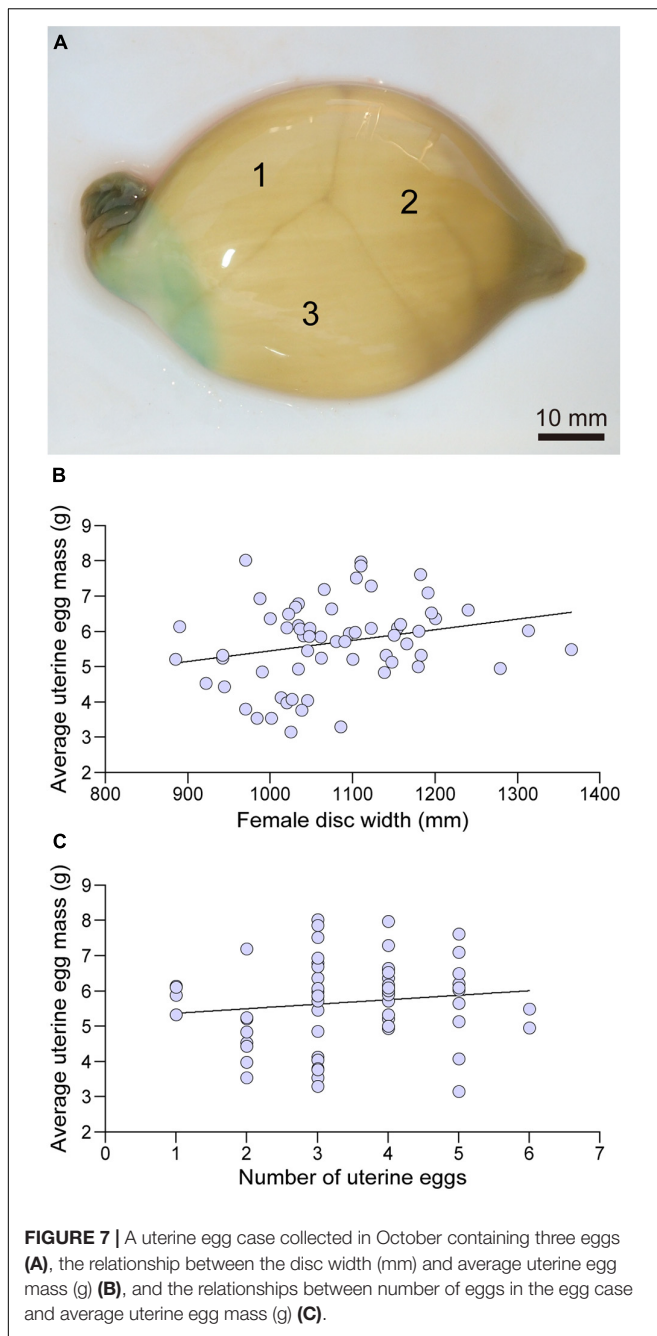
Embryo body mass increased rapidly after external yolk was consumed (Figure 10). In conjunction with yolk depletion, embryonic nutrition transitioned to histotroph. Trophonemata were conspicuous as villous and highly vascular projections of the uterine mucosa (Figure 11A). There was no relationship between the length of the trophonemata and the yolk mass or embryo DW during the embryonic growth phase (Figure 11B). However, there was an increasing trend for trophonemata length from stages 1 to 4 and a decreasing trend from stages 10–11 (Figure 11C). Trophonemata length increased with disc width of pregnant females (linear regression; $F = 56.37$, $d.f. = 50$, $P < 0.05$)



(Figure 11D). Histotroph was not observed for females with stage 9 embryos (Figure 11E) but it was present in uteri of females with stage 11 embryos (Figure 11F). When or at what embryonic stage (10 or 11) histotroph is available to embryos was not determined.

Fecundity

Fecundity for 158 pregnant females with uterine eggs ($n = 86$) and embryos ($n = 72$) ranged from 1 to 7 embryos per brood ($n = 158$, mean \pm SD = 3.36 ± 1.26) and was positively correlated with female disc width (linear regression; $F = 105.73$, $d.f. = 151$, $P < 0.05$) (Figure 12).



Fetal Mortality and Abnormal Embryo

Two dead embryos and four undeveloped uterine eggs were observed among 224 embryos from six of 72 females examined from late June to early September (Figures 13A–C). Assuming the undeveloped uterine eggs were fertilized, the fetal mortality rate for Naru eagle ray, in Ariake Bay was 2.68%. Females with fetal mortality ($n = 6$) had a combination of live embryos with one or more dead embryos or undeveloped eggs. Fetal mortality rates were 50% ($n = 2$), 33.3% ($n = 3$) and 16.7% ($n = 1$) by brood. The first female (1010 mm DW) with fetal mortality was collected August 2003. She had one embryo and one unfertilized

or undeveloped egg, i.e., 50.0% fetal mortality. The remaining five females (with DWs of 1142 mm, September 2003; 1275 mm, June 2010; 1047 and 1081 mm, July 2018; 1026 mm, July 2019) had undeveloped eggs, or dead embryos in their uteri, resulting in individual fetal mortality rates of 33.3%, 16.7%, 50.0%, 33.3%, and 33.3%, by brood, respectively.

One morphologically abnormal but live embryo, alongside two healthy embryos was observed in a single gravid female (DW of 1058 mm) collected on 2 September 2009. All other females ($n = 65$) presented normal embryos. The abnormal embryo was at embryonic stage 11 and its deformities were associated with fusion of the cranial margin of the pectoral fins or disc (Figure 13D). The percentage of morphologically abnormal Naru eagle ray embryos observed in Ariake Bay was 0.45% ($n = 1$).

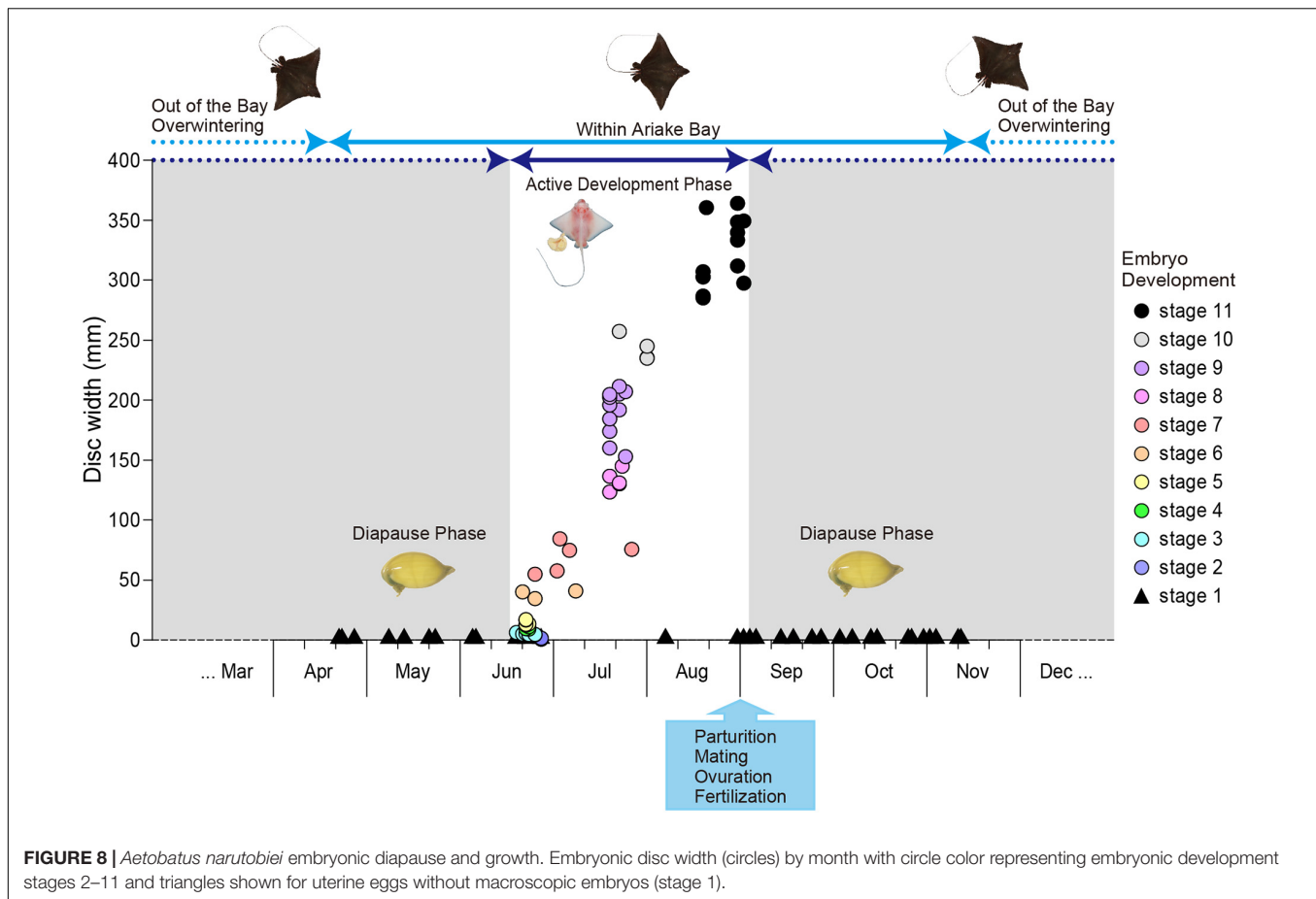
DISCUSSION

This research represents the first comprehensive reproductive life history study for an Aetobatidae ray and provides data fundamental for the development of future science-based management strategies of *A. narutobiei*. Naru eagle rays are considered specialists that feed mainly on bivalves (Yamaguchi et al., 2005) whose foraging activities are assumed to negatively impact populations of commercial bivalves, for both fisheries and aquaculture. Consequently, developing programs to reduce their population rather than protecting the rays as an important component of the ecosystems has been the focus of previous research (Fukuda and Zenitani, 2010; Yokoyama et al., 2014). This research identifies Ariake Bay as the site of mating, gestation and parturition and a nursery ground for Naru eagle rays (Heupel et al., 2007). Therefore, coastal areas such as Ariake Bay that are important bivalve fishery grounds also serve as critical habitat for all life stages of Naru eagle rays.

Naru eagle rays reproduce synchronously and annually. They are especially vulnerable to overfishing because of their low fecundity and long reproductive cycle. Their reproductive strategy includes rapid embryonic development, parturition, and mating in Ariake Bay during late summer, and a long period of embryonic diapause in relation to seasonal migrations outside of Ariake Bay.

Reproduction and Size at Sexual Maturity

Size and age at 50% sexual maturity for Naru eagle ray females was 20% and 42% greater than that of males, respectively. In the family Aetobatidae, size and age at sexual maturity were known previously for only male spotted eagle ray *A. narinari* (ocellated eagle ray *A. ocellatus* at present) from Australia (DW at 50% sexual maturity of 130.6 cm) (Schluessel et al., 2010). Size and age disparity between sexes has been reported for other stingray species such as the longtail butterfly ray *Gymnura poecilura* (Menon et al., 2020), whiptail stingray *Dasyatis chrysonota* (Ebert and Cowley, 2009), and bat ray *Myliobatis californica* (Martin and Cailliet, 1988b) might be typical of Myliobatiforms and related to their reproductive mode (Martin and Cailliet, 1988b; Capapé, 1993; Furumitsu et al., 2010, 2019). For viviparous



species larger females are advantageous, as the increased internal space for developing embryos enables larger young and/or higher fecundity at parturition (Furumitsu et al., 2019). However, the long time needed for females to reach maturity makes *A. narutobiei* especially vulnerable to anthropic pressure, particularly targeted fishing efforts that remove gestating females such as the Ariake Bay predator control program.

The bimodal size distribution for male and female *A. narutobiei* in Ariake Bay was similar to the size distributions of red stingrays in Ariake Bay (Furumitsu et al., 2019) and Atlantic stingray *Hypanus sabinus* from Florida (Snelson et al., 1988). This bimodal pattern may be the result of differing habitat preferences for smaller (the immature or premature stage) and larger size classes. Naru eagle rays were usually sampled from size-segregated schools of immature or premature rays (including both sexes), and mature (males and females, separately) life stages. The observed bimodal distribution in sizes in Ariake Bay also may be related to fishing pressure with larger rays and bigger aggregations of eagle rays targeted by fisher, presumably to maximize profit from the predator control program.

Reproductive Seasonality

In general, sampling Naru eagle rays proved to be extremely challenging because of their large size and mass. Moreover, the testis and ovaries were particularly susceptible to post-mortem

deterioration. Pregnant females were prone to spontaneous abortion when caught and brought onto the vessel as has been reported for many elasmobranchs, especially rays (Adams et al., 2018). This may be responsible for the lack of reproductive life history information for Naru eagle rays, especially fecundity and gestation time, despite its importance for conservation and management.

In common with many elasmobranch species that reproduce seasonally, the I_G for males decreased in advance of the mating season (Yamaguchi et al., 1997, 2000; Kyne and Bennett, 2002). Similarly, *A. narutobiei* I_G peaked 1 month prior to the observation of semen in seminal vesicles and mating activity. The decrease in I_G represents seasonal testicular atrophy, where active spermatogenesis has ceased and sperm are stored in the seminal vesicles. The presence of mature sperm in the testes and seminal vesicles coincided with parturition and observation of pre-ovulatory ova for females.

Naru eagle rays undertake annual and seasonal migrations between the inner and outer bay areas (Yamaguchi et al., 2005). Male and female body condition deteriorated during winter and early spring while outside Ariake Bay. Seasonal patterns for I_G were different between males and females and may be a consequence of the energetic demands of reproduction. However, monthly variation in I_H was not different between males and females and may be attributed to common seasonal migration

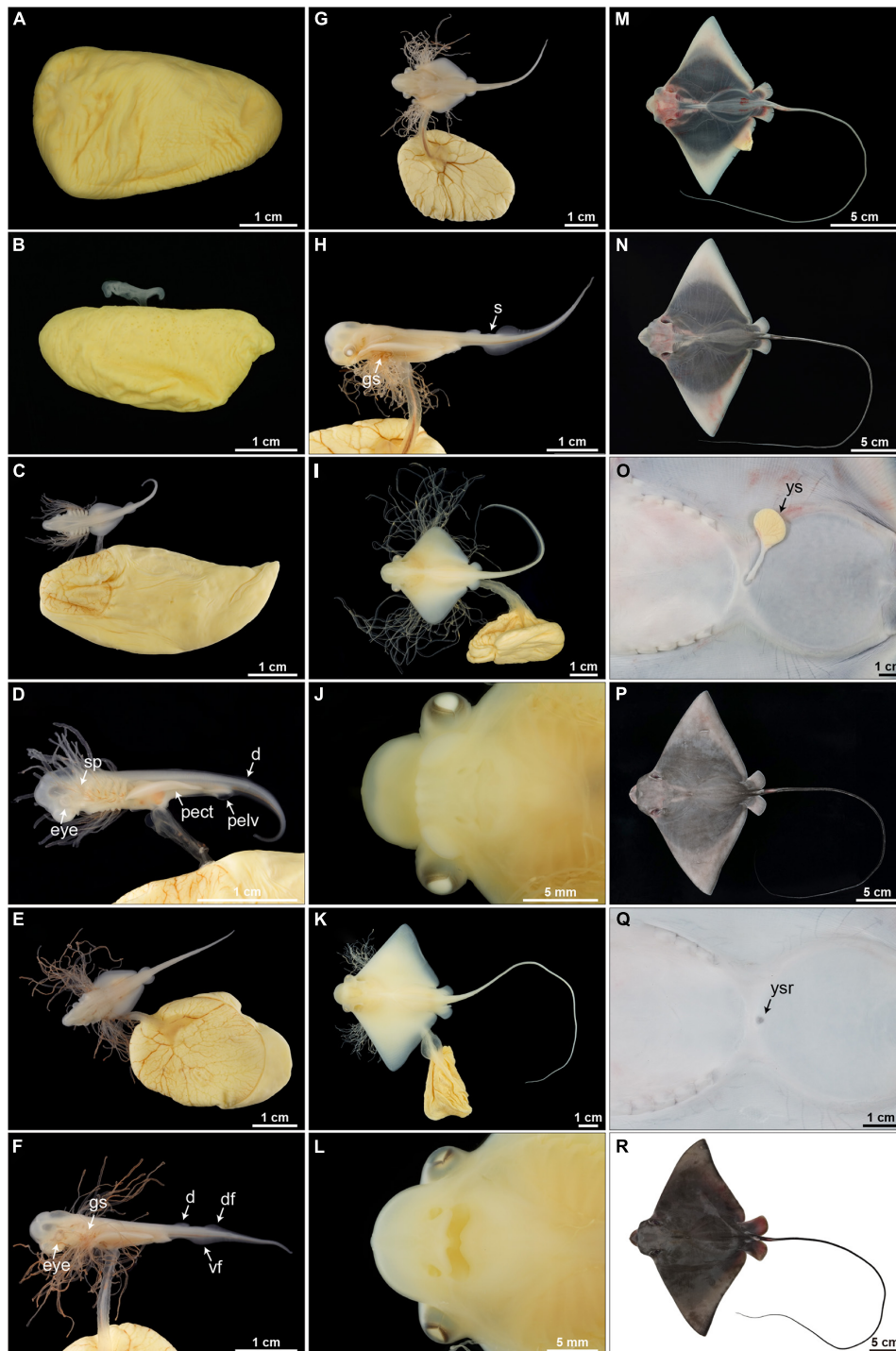
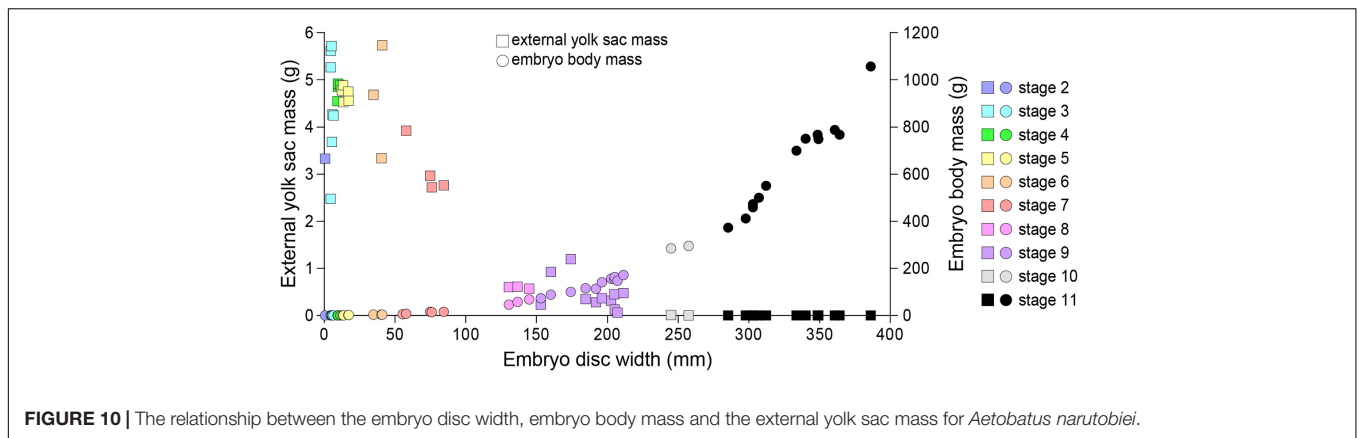


FIGURE 9 | Embryonic development (stages 1–11) of *Aetobatus narutobiei*. **(A)** Stage 1: The egg is within an egg case and without a macroscopic embryo. **(B)** Stage 2: Macroscopic embryos on uterine eggs within an egg case (11.72 mm TL). **(C,D)** Stage 3: The pectoral fins within the posterior margins of the gills (6.56 mm DW). **(E,F)** Stage 4: The pectoral fins beyond the posterior margin of the gills but within the anterior margins of the gills (10.79 mm DW). **(G,H)** Stage 5: The pectoral fins beyond the gills but within the posterior margin of the eyes (16.10 mm DW). **(I,J)** Stage 6: The pectoral fins are fused forming the disc, the cephalic lobes are developing (36.09 mm DW). **(K,L)** Stage 7: The cephalic lobes are formed completely, the yolk sac and gill filaments persist (75.04 mm DW). **(M)** Stage 8: Gill filaments are resorbed but the yolk sac remains, muscle tissue in the center of the disc appears black but the disc margins remain translucent (132.80 mm DW). **(N,O)** Stage 9: The yolk sac is diminished and the body is free of pigmentation (116.35 mm DW). **(P,Q)** Stage 10: The yolk sac is resorbed and the dorsal surface is begins to turn gray–black (241.00 mm DW). **(R)** Stage 11: Body pigmentation is present (312.00 mm DW). d, dorsal fin; df, dorsal fold; gs, gill slit; pect, pectoral fin; pelv, pelvic fin; s, spine; vf, ventral fold; ys, external yolk sac; ysr, yolk sac remnant.



and feeding habits. In April, when rays return to Ariake Bay, male and female I_H was at its nadir. Before leaving the bay in November, I_H had doubled. Peak I_H corresponded with completion of spermatogenesis for males and the end of gestation and parturition for females. Hepatic energy stored from feeding activity in Ariake Bay during late summer and fall is used as an energy source during the overwintering period and for spring and early summer reproductive activity.

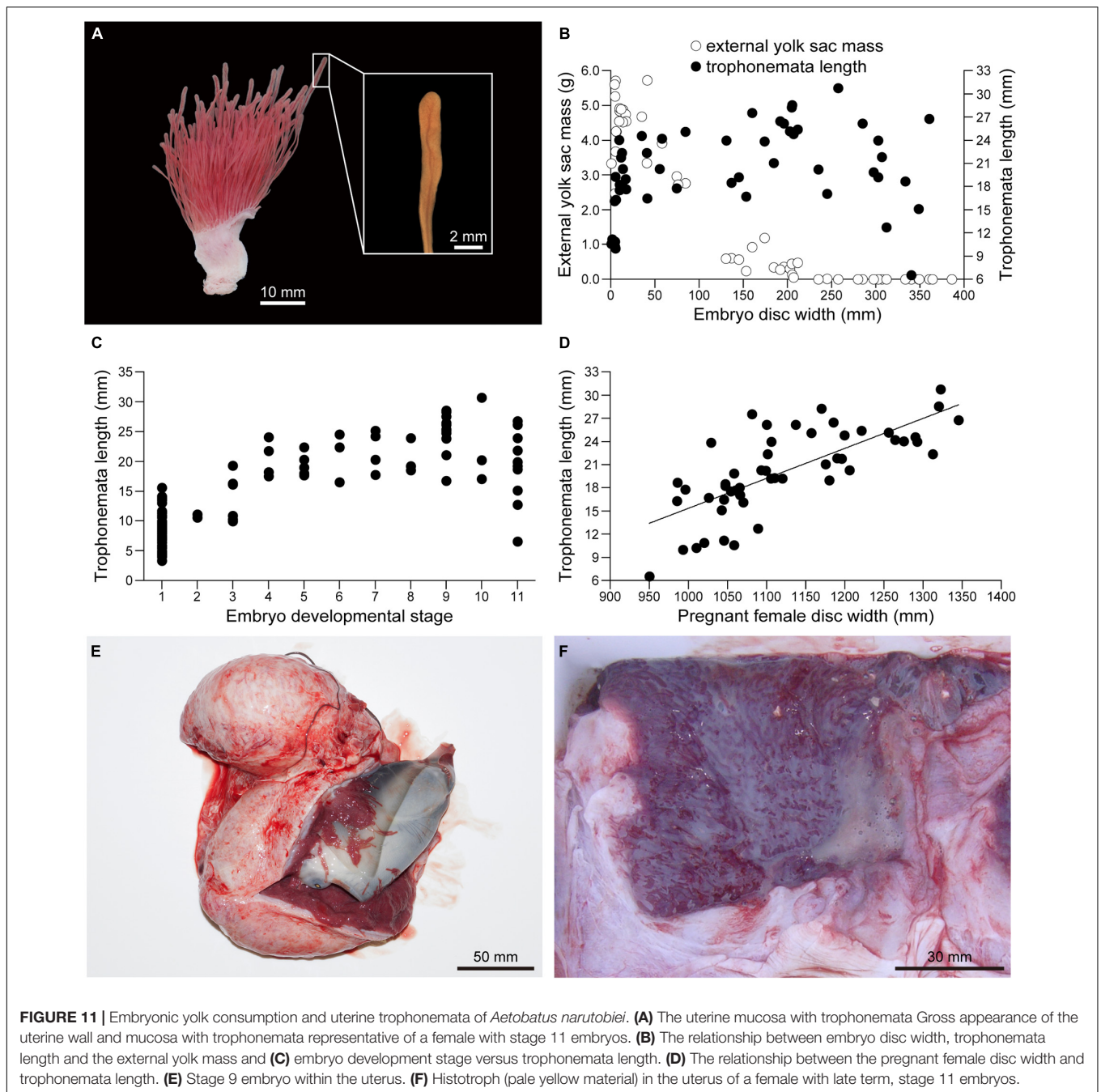
Male Reproductive Anatomy

Shark and ray testes are classified into three major organizational types: radial, diametric, and compound (Pratt, 1988). However, the testis organization type for some batoids does not conform to one of these types and has been described as lobate with radial or compound organization (Pratt, 1988). This research is, to the best of our knowledge, the first to describe testis structure and spermatogenesis for rays in the Aetobatidae family. *Aetobatus narutobiei* testes had internal lobes composed of spermatocysts that developed radially from a central germinal zone. Lobate testes with radial development were observed also for a Potamotrygon ray (Pratt, 1988). Other rays where testis organization has been described [Atlantic stingray (Maruska et al., 1996), red stingray (Furumitsu et al., 2019), bat ray (Pratt, 1988), smooth butterfly ray *Gymnura micrura* (Pratt, 1988), cownose ray *Rhinoptera bonasus* (Poulakis, 2013) and *Fluvitrygon signifer* (Chatchavalvanich et al., 2005)] have lobate testes with compound development of spermatocysts across each lobe. Future studies on the testicular organization for other batoids may reveal additional diversity in reproductive anatomy as well as provide insight into the implications of these alternative organizational patterns on reproduction.

Female Reproductive Anatomy and Fecundity

Elasmobranchs utilize a plethora of reproductive modes and their reproductive anatomy and physiology is equally diverse (Wourms, 1977; Hamlett and Koob, 1999; Lutten et al., 2005). All myliobatid rays are viviparous and histotrophic, with female reproductive anatomy, species specific with respect to the symmetry and functionality of ovaries and uteri. This

is important because the number of functional ovaries and uteri may affect fecundity and ultimately fitness for viviparous species. Previously, little was known about fecundity for family Aetobatidae. Fecundity for spotted eagle ray based on 60 pregnancies for rays held at six aquaria worldwide (Underwater World Singapore, SeaWorld Antonio, Discovery Cove, Georgia Aquarium, Burger's Zoo, Disney's The Seas, Okinawa Expo Aquarium and Ripley's Aquarium of the Smokies) ranged from 1 to 4 young with a mean of 1.8 ± 0.6 young per litter, and neonate DW 44.7 ± 5.1 cm at birth (Swider et al., 2017). The left ovary and uterus were functional and the right ovary and uterus vestigial based on examination of a single post-partum spotted eagle ray (Swider et al., 2017). However, at least one of the included institutions (Okinawa Expo Aquarium) houses ocellated eagle ray that were previously identified as a spotted eagle rays. Uchida et al. (1990) reported fecundity was 1 or 2 with DW at birth 50–59 cm for the ocellated eagle rays. *In situ* spotted eagle rays sampled from Venezuela have one functional uterus and their fecundity ranges from 1 to 5 (mean 3.09) (Tagliafico et al., 2012). Both ovaries and uteri are functional for Naru eagle rays and their fecundity (max 7) is the highest of all Aetobatids. Although fecundity of *in situ* spotted eagle rays from Venezuela is similar to *in situ* Naru eagle rays (this study), the maximum DW (2260 mm) of the largest Venezuelan female was almost 1.5 times that of the largest female Naru eagle ray from Ariake Bay (Tagliafico et al., 2012). This suggests that the reproductive life history strategy of Naru eagle rays maximizes the number of young over their size at birth. This contrasts with the majority of myliobatids, whose reproductive strategy includes producing a small number of large neonates. Fecundity of 23 eagle ray species ranges from 1 to 12 with 14 species less than 2 (Figure 14A), putting the relatively high fecundity of Naru eagle rays into phylogenetic perspective. From an anatomical perspective, fecundity for Myliobatidae and Aetobatidae species with paired uteri was higher compared with Rinopterae and Mobulidae species with one functional uterus and usually, only one offspring (Bigelow and Schroeder, 1953; Notarbartolo-di-Sciara and Serena, 1988; White et al., 2006; Mendonça et al., 2012). The Naru eagle ray is the only Aetobatid species with paired uteri and females had higher fecundity and smaller neonates at birth than similarly sized congener females (Figure 14B). Overall, embryo size at birth is smaller among



myliobatids with two uteri, regardless of female size. However, even though Naru eagle ray fecundity is high among myliobatid species, it is lower than that reported for 21 dasyatid species with only a single uterus (Furumitsu et al., 2019), and therefore the call for conservation of Naru eagle rays remains an urgent necessity. Functionally there was no relationship between maximum female size and fecundity among eagle ray species (Figure 14A), but larger species generally bore larger young, similar to dasyatid species (Figure 14B).

Regardless of reproductive mode, reproductive output of viviparous species is confined by internal space. If the total

area available to developing embryos is similar for females with a single uterus versus two uteri, then overall reproductive output might be similar from a total mass standpoint but not necessarily from a reproductive output standpoint. Females maximize the number of young or the size of young as part of their reproductive strategy. The potential maximum size of young at birth for viviparous species is ultimately bound by the size of the uterus. *Aetobatus* is an important species to study in order to improve our understanding of the trade-offs between number of functional uteri and the size and number of young produced during each reproductive cycle and their influence on other life

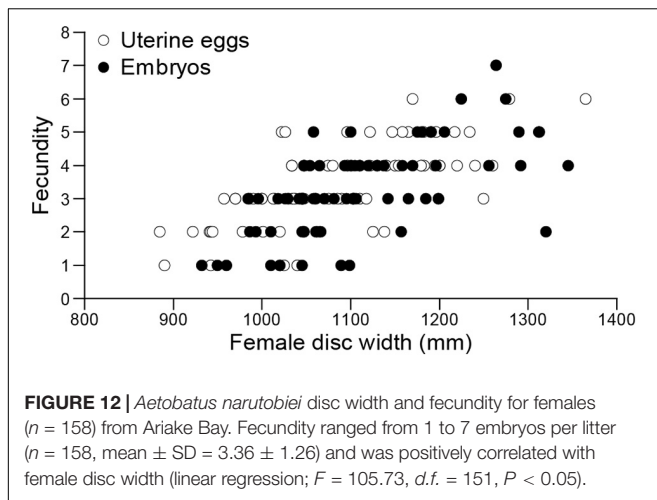


FIGURE 12 | *Aetobatus narutobiei* disc width and fecundity for females ($n = 158$) from Ariake Bay. Fecundity ranged from 1 to 7 embryos per litter ($n = 158$, mean \pm SD = 3.36 ± 1.26) and was positively correlated with female disc width (linear regression; $F = 105.73$, $d.f. = 151$, $P < 0.05$).

history characteristics. For example, manta and devil rays have a single functional uterus and produce a small number of very large young. They have acquired larger bodies and are among the largest of all batoids with expansive oceanic and pelagic ranges.

Functional Morphology of the Uterus

Elasmobranch reproduction includes lecithotrophic and matrotrophic reproductive modes (Wourms, 1977; Hamlett et al., 2005). Histotrophy is a type of matrotrophy where embryos are provided supplemental nutrition as histotroph or uterine milk that contains variable proportions of lipids, carbohydrates and proteins. All stingrays are histotrophic and the uterine mucosa is specialized with villous projections termed trophonemata (Hamlett et al., 1985, 2005; Compagno, 1990; Hamlett and Hysell, 1998). Trophonemata are involved in one or more critical physiological processes during gestation including gas exchange, nutrient provision, and osmoregulation (Babel, 1967; Hamlett et al., 1985, 1996; Smith and Merriner, 1986; Johnson and Snelson, 1996; Hamlett and Hysell, 1998).

Aetobatus narutobiei trophonemata length was proportional to female size but not embryo size. This suggests that trophonemata elongation and histotroph (uterine milk) secretion are not directly related and that gas exchange and osmoregulation may be more important. A similar finding was observed for *Myliobatis goodei* where the prominent vascularity and the thin epithelium of trophonemata aids gaseous exchanges to supply the oxygen demands of the embryos (Colonello et al., 2013). Previous studies on the uterus of the cownose ray (Hamlett et al., 1985), southern stingray *Hypanus americanus* (Hamlett et al., 1996), and manta ray *Mobula alfredi* (Tomita et al., 2012) suggest that oxygen may be supplied to the uterine fluid from the uterine mucosa by diffusion. Colonello et al. (2013) proposed that the source of the histotroph for *M. goodei* was the basal crypts observed between the trophonemata, especially later in gestation, when embryonic nutritional requirements are increased. The function(s) of Naru eagle ray uterine trophonemata may include one or more critical aspects of embryonic respiration and nutrition but further study is needed for confirmation.

The energy derived from consumption of yolk and histotroph (uterine milk) resulted in a 34,626% increase in embryo mass at birth compared to the mass of the egg yolk. Increases in embryo mass of more than 3,000% during gestation are common for histotrophic rays, such as *Bathytoshia centroura* (approximately 3,000%) (Capapé, 1993), *Aetomylaeus bovina* (3,120%) (Seck et al., 2002), *Pteroplatytrygon violacea* (1,680%) (Babel, 1967), butterfly ray (3,564%) (Yokota et al., 2012), red stingray (2,080–15,643%) (Furumitsu et al., 2019) and yellow stingray *Urobatis jamaicensis* (4,600%) (Fahy et al., 2007). For Naru eagle rays, the percent increase in embryo mass is 2–10 times higher in comparison. *Aetobatus narutobiei* embryos showed the greatest degree of matrotrophy or growth efficiency of any ray; however, based on female size we suspect Mobulidae might have an even higher degree of matrotrophy. Future work will focus on characterizing histotroph and uterine mucosa dynamics to identify the source of histotroph and its nutritional content for Naru eagle rays. This information could help to elucidate mechanisms for the extreme degree of matrotrophy observed for Naru eagle rays and the reproductive strategy of the largest eagle and manta rays.

Embryonic Diapause for Overwintering Survival

Aetobatus narutobiei gestation was nearly a year, but embryos were in diapause for 79.2% of the duration. Embryonic diapause, a temporary arrest of development, is a reproductive strategy that extends the time required to complete a reproductive cycle. For Naru eagle rays diapause was obligate, occurring each year and observed for every pregnant female. In elasmobranchs, diapause is recognized among species with seasonal and synchronous reproductive cycles, when females possess uterine eggs without macroscopic signs of embryonic development during sequential months (Wyffels, 2009). This is the first observation of embryonic diapause for any species of the family Aetobatidae, though diapause has been observed for nearly 20 elasmobranch species (Waltrick et al., 2012). Embryonic diapause is typically thought to enable young to be born when environmental conditions such as prey abundance, predator presence, and water temperature are optimal for their growth and survival (Simpfendorfer, 1992; Marshall et al., 2007). Diapause may also be due to the energetic demands of reproduction for adults and the seasonal availability of mates. It is likely that the drivers and advantage(s) of diapause are species and habitat specific, requiring a thorough understanding of the complete life history of each species under investigation (Waltrick et al., 2012).

Considering the advantages of diapause from the perspective of Naru eagle ray neonates, prey abundance, water temperature, and predator presence are all factors potentially influencing gestation time. At the end of summer when *A. narutobiei* give birth, bivalves are abundant in Ariake Bay's estuary. Adult hammerhead sharks *Sphyrna* spp. and white cheek sharks *Carcharhinus tjtjtjot* are the most likely predators of Naru eagle rays. These species also use Ariake Bay as mating and nursery grounds, but migrate into the bay in May and depart in July after breeding. Only neonate sharks remain in the bay, thereby

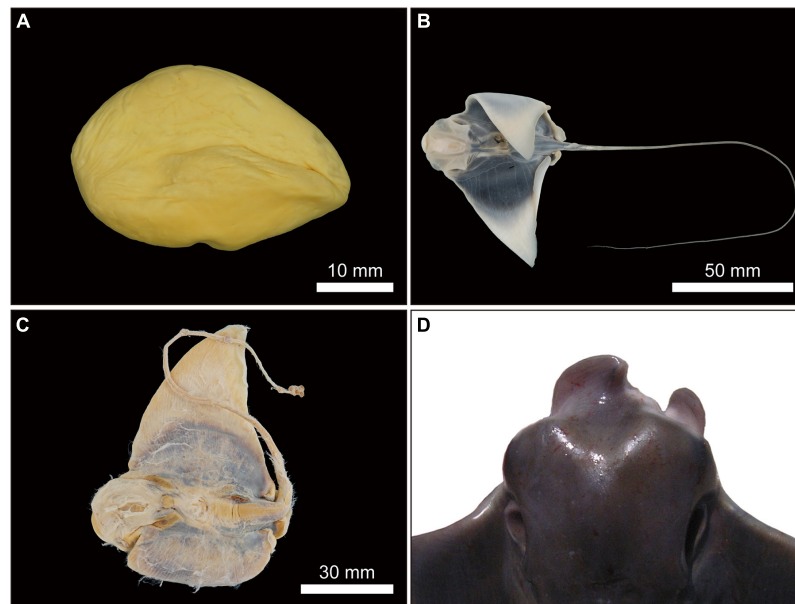


FIGURE 13 | Undeveloped or unfertilized uterine eggs or dead embryos and abnormal morphological embryos of *Aetobatus narutobiei*. **(A)** Egg without embryonic development from a female collected in June 2010; **(B,C)** dead embryos from females collected in July 2019 **(B)** and September 2003 **(C)** and **(D)** an abnormal embryo in September 2009.

minimizing the risk of predation on neonate Naru eagle rays. Summer water temperature in Ariake Bay when Naru eagle rays give birth is high, but with the onset of the autumn season water temperature declines. The water temperature of the estuary and shallow areas of Ariake Bay is below 10°C during the winter period, which can threaten survival of Naru eagle rays. Therefore, when water temperature reaches 18–19°C in late October and November, Naru eagle rays gradually move from the shallow regions to the deeper, southern habitat within Ariake Bay, before migrating to the open sea to overwinter. Our observations include a 200 km migration from the innermost area of Ariake Bay to Amakusa-nada in the East China Sea over the period of a month (Yamaguchi and Furumitsu, unpublished data). The timing of parturition for Naru eagle rays occurs when water temperatures are highest, predator presence lowest, and is optimized for young to maximize food consumption and energy stores in the 2–3 months period prior to their winter migration.

The active embryonic development phase occurs quickly, from June to August, after a protracted diapause phase where embryonic development is arrested at a very early stage. The timing of parturition gives young an optimized environment for survival, but the drivers of diapause also should be considered from the perspective of the adult Naru eagle rays. Diapause may allow females to recover body condition depleted from the demands of reproduction and migration (Waltrick et al., 2012). During summer, *A. narutobiei* females produce ova for the next reproductive cycle at the same time as secreting histotroph for actively developing embryos. The energy demands from reproduction were reflected in female body condition that did not improve until September, after parturition and ovulation. Male and female body condition was at its maximum, nearly twice as

high as in April, by the end of autumn just before migration out of Ariake Bay. Females were pregnant prior to migration and overwintering. During overwintering the embryos are arrested and as a result, the energetic demands of pregnancy may be minimal and relatively constant. The rays remained in deeper waters in the open sea where the water temperature is stable during the winter, and likely use stored energy from feeding in Ariake Bay as sustenance in the absence of prey. As the water temperature in Ariake Bay increased in the spring, *A. narutobiei* migrated from the depths of the open sea to the shallow estuaries of Ariake Bay where bivalves were prevalent from April to June allowing them to recover depleted body condition before meeting the energetic demands of histotroph production and concurrent folliculogenesis. In autumn, the size and the stock of their main food, bloody clam (ark shell) *Scapharca kagoshimensis*, increased after the end of the fishing season (Masaki and Onohara, 2003) providing energy for their winter migration.

There are few potential predators for the adult Naru eagle rays in Ariake Bay during late summer and autumn. Their migration to the open sea to avoid low water temperatures in Ariake Bay is expected to be extremely dangerous with regard to large predator encounters, long-distance movement, and acquisition of food. Males are smaller than females (Max: 1000 mm DW vs. 1500 mm DW) and their lifespans (9 years vs. 19 years) were less than half that of the females (Yamaguchi et al., 2005). The natural mortality rate of males is 3.2 times higher than for females. The smaller size of males may have left them more susceptible to predation and thus, particularly higher mortality rates for the males compared to females during migration and overwintering can be expected. Thus, mating prior to exposure to risks associated with overwintering is advantageous for adult males.

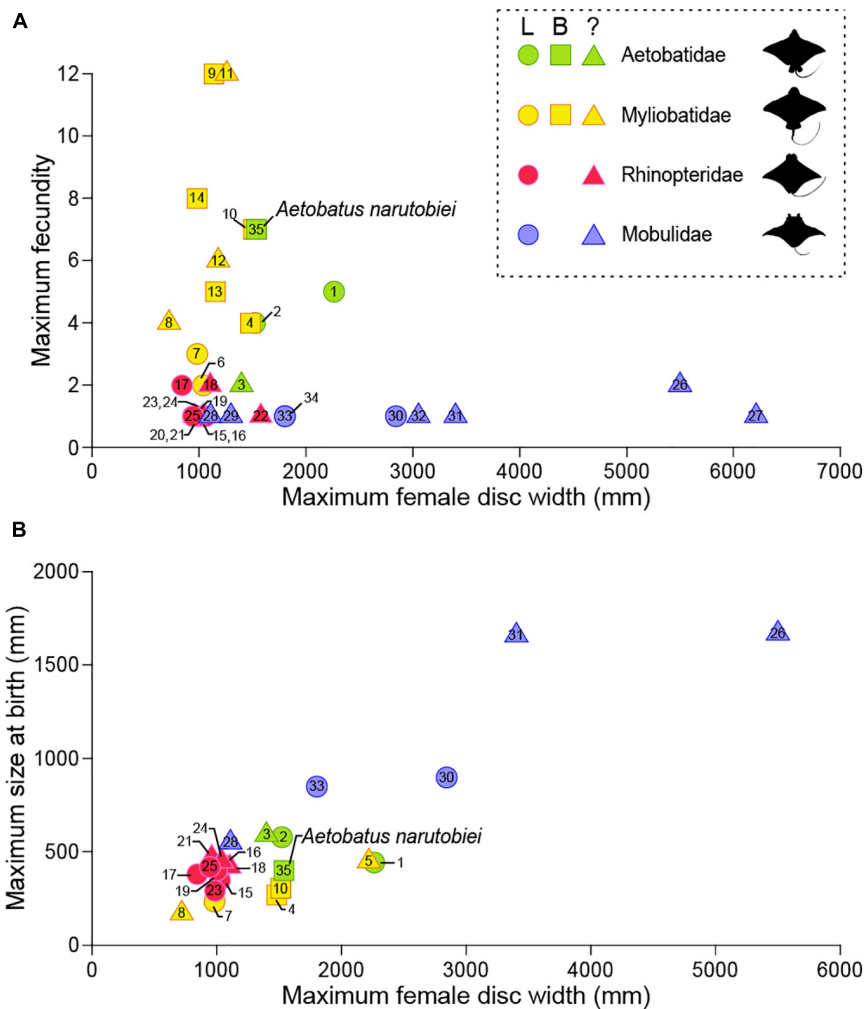


FIGURE 14 | Variability in the number of functional uteri compared to **(A)** fecundity and **(B)** maximum size at birth for subfamily Myliobatinae. Rays with two functional uteri are represented by squares, circles are shown for rays that have a single (left) functional uterus, and triangles are shown for species that have not been described. *Aetobatus narinari*: 1 (Tagliafico et al., 2012) and 2 (Swider et al., 2017); *Aetobatus ocellatus*: 3 (Uchida et al., 1990); *Aetomylaeus bovinus*: 4 (Seck et al., 2002) and 5 (Dulčić et al., 2008); *Aetomylaeus maculatus*: 6 (Devadoss, 1982); *Aetomylaeus nichofii*: 7 (Devadoss, 1998) and 8 (Last and Stevens, 2009); *Myliobatis aquila*: 9 (Capapé et al., 2007); *Myliobatis californicus*: 10 (Martin and Cailliet, 1988a) and 11 (Villavicencio Garayzar, 1996); *Myliobatis freminvillei*: 12 (Tagliafico et al., 2016); *Myliobatis goodei*: 13 (Araújo et al., 2016); *Myliobatis ridens*: 14 (Araújo et al., 2016); *Rhinoptera bonasus*: 15 (Neer and Thompson, 2005), 16 (Pérez-Jiménez, 2011), 17 (Poulakis, 2013), 18 (Fisher, 2010) and 19 (Smith and Merriner, 1986); *Rhinoptera brasiliensis*: 20 (Domingues et al., 2009); *Rhinoptera javanica*: 21 (Uchida et al., 1990) and 22 (James, 1962, 1971); *Rhinoptera marginata*: 23 (Tıraşın and Başusta, 2018); *Rhinoptera steindachneri*: 24 (Bizzarro et al., 2007) and 25 (Burgos-Vázquez et al., 2019); *Mobula alfredi*: 26 (Marshall and Bennett, 2010); *Mobula birostris*: 27 (Cabanillas-Torpoco et al., 2019); *Mobula hypostoma*: 28 (Bigelow and Schroeder, 1953); *Mobula kuhlii* cf. *eregoodootenkee*: 29 (Broadhurst et al., 2018); *Mobula mobular*: 30 (White et al., 2006) and 31 (Notarbartolo-di-Sciara and Serena, 1988); *Mobula tarapacana*: 32 (Notarbartolo-di-Sciara, 1988); *Mobula thurstoni*: 33 (Notarbartolo-di-Sciara, 1988) and 34 (Mendonça et al., 2012); *Aetobatus narutobiei*: 35 this study.

Neonates are born when food is abundant, water temperature is highest, and predator presence is lowest. Adult females take advantage of diapause to allow them to recover body condition lost during overwintering before the active phase of embryonic development begins and they need to simultaneously produce histotroph and undergo folliculogenesis, both energy intensive physiological processes. Parturition is timed to allow females several months to improve body condition before winter migration. Similar to females, adult males have several months after returning to Ariake Bay after overwintering to recover body

condition before the mating season and have several months after mating to gain condition before migration. Therefore, embryonic diapause benefits both *A. narutobiei* adults and neonates.

The reproductive life history of up to nine batoid species in Ariake Bay may include embryonic diapause (Furumitsu, 2009; Kume et al., 2009; Yamaguchi and Kume, 2009; Furumitsu et al., 2019; Yamaguchi, unpublished data). Embryonic development periods are 2.5–3 months for most species; however, the timing of parturition is different for each species, from July to September (e. g., *Hemitrygon laevigata* in July; *H. akajei*, *Rhinobatis*

hynnicephalus, and *Platyrrhina tangi* in July and August; and *Myliobatis tobijei* and *A. narutobiei* in August and September). We hypothesize another strategy where species utilize the Ariake Bay estuaries in turn by controlling the length of the diapause phase of gestation based on environmental conditions (water temperature, predator presence, competition, and food supply). Ariake Bay is remarkable with a high concentration of species that have adopted diapause as a reproductive life history strategy. Further research including the identification of endogenous and exogenous factors that initiate diapause and resumption of embryonic development are needed.

Fetal Mortality and Morphologically Abnormal Embryos

The fetal mortality rate for wild-caught viviparous rays including observations of dead embryos and unfertilized or undeveloped uterine eggs has been reported previously (1.25%) for only one species, red stingray (Furumitsu et al., 2019). In comparison, the fetal mortality rate for *A. narutobiei* was 2.68%, or twice as high. For both Ariake Bay species, the observation of mummified embryos was rare compared to undeveloped or unfertilized eggs. No estimates of fetal mortality rate are available for viviparous sharks, however, similar observations of mummified embryos and unfertilized eggs have been reported (Clark and von Schmidt, 1965; Randall, 1977; Rosa-Molinar et al., 1983; Sandoval-Castillo and Villavicencio-Garayzar, 2008; Baremore and Hale, 2012). Unfertilized eggs waste both the energy required for ova formation during folliculogenesis and uterine space during gestation. In the absence of a fitness advantage, females would be expected to minimize unfertilized eggs in a reproductive cycle. Multiple mating is one strategy females may use to ensure fertilization of all ova and unfertilized uterine eggs could be attributed to reduced mating or insufficient sperm. Of the two Ariake Bay species, only *A. narutobiei* is a target of the predator control program. The higher rate of fetal mortality for *A. narutobiei*, particularly unfertilized eggs, may be a result of lower numbers of male available for mating due to the predator control program and natural mortality. This would be especially important if *A. narutobiei* include multiple paternity as part of their reproductive life history (Portnoy et al., 2007).

The frequency of morphologically abnormal embryonic specimens for *A. narutobiei* in Ariake Bay was relatively low (0.45%), and similar to the red stingray (0.72%) from Ariake Bay (Furumitsu et al., 2019). All abnormalities observed for *A. narutobiei* in Ariake Bay were associated with fusion of the pectoral fins. This physical deformity is common and has been reported among embryos of other batoids (Honma and Sugihara, 1971; Lamilla et al., 1995; Yamaguchi, 2004; Escobar-Sánchez et al., 2009; Blanco-Parra and Niño-Torres, 2011; Ramírez-Amaro et al., 2013). Most reports of morphological abnormalities describe embryos close to their birth size, an indication that the deformity does not affect ray survival *in utero*. Indeed, adult specimens with abnormal morphologies are observed rarely in batoid species, recently summarized by Furumitsu et al. (2019). The rate of developmental abnormalities described for Naru eagle rays and red stingrays are similar and may represent the

natural incidence of birth defects for rays in a healthy ecosystem. As anthropogenic influences and climate change increasingly affect critical habitats like Ariake Bay, monitoring the rate of mummified embryos, unfertilized and undeveloped eggs and developmental abnormalities might be a useful to ensure the incidence does not increase over time.

Predator Control Programs and Science-Based Ecosystem Management

A government sponsored predator control program was implemented to increase productivity of the bivalve fishery by reducing predation on bivalves from Naru eagle rays based on the marine trophic cascade hypothesis (Myers et al., 2007). According to the hypothesis, a decline in large shark species and subsequent increase in mesopredator rays led to a decline in bivalve yield in the Chesapeake Bay. This trophic cascade imbalance was used to justify the development of targeted cownose ray fisheries in the northwest Atlantic (Myers et al., 2007). Subsequent analyses using expanded regional data and temporal analyses for shark, ray and bivalve abundance found little support for the shark-mediated trophic cascade or vilification of cownose rays in the fishery collapse (Grubbs et al., 2016). Similarly, for Naru eagle rays and bivalves in Ariake Bay, bivalve catches were in decline already since the mid-1980s (Yamaguchi et al., 2005). Therefore, increased eagle ray populations observed in the late-1990s do not overlap with long-term declines of bivalve catches. Therefore, it is essential to test the hypothesized trophic cascade using predator-prey linkages in this estuarine ecosystem and the impacts of long-term fishing pressures on the Naru eagle ray.

Estuaries serve as nursery grounds for many coastal fishes (Takita and Yamaguchi, 2009). This study identified the Ariake Bay estuary as critical habitat for reproduction and as a nursery for *A. narutobiei*. Previous studies have identified coastal areas as nurseries for elasmobranchs (Simpfendorfer and Milward, 1993; Yokota and Lessa, 2006; Rangel et al., 2018) but there are few estuarine habitats that function as nurseries for elasmobranchs (Heupel and Simpfendorfer, 2011; Francis, 2013). In Ariake Bay, many elasmobranchs, including scalloped hammerhead sharks and white cheek sharks, use the estuary for parturition and breeding during early summer and then depart the bay. Their neonates remain in the bay until autumn and are frequently caught by commercial fisheries using set nets and gill nets. Stingrays such as the red stingray also use the estuary as a nursery (Furumitsu et al., 2019). Thus, abundant neonates for several elasmobranch species during summer and autumn are dependent on the estuarine areas of Ariake Bay. The use of the estuarine areas may be a successful ecological strategy to improve survival rates, because of reduced predation (Heupel and Simpfendorfer, 2011).

Naru eagle rays have specific environmental requirements for food, reproduction, and overwintering, and Ariake Bay, an expansive estuary ecosystem, is extremely important and one of the only habitats known to support their requirements for life. The rays leave the bay in winter, when water temperatures are coldest, and are dependent on the estuary to replenish depleted

energy stores from overwintering and migration and to complete gestation, parturition and mating in rapid succession during the short summer season. The same estuary also serves as a nursery area. Adults leverage diapause to minimize the energetic demands of reproduction during migration and overwintering outside of the estuary and diapause benefits neonates by delaying parturition until summer, the optimum season for their survival. The combination of long lifespan, slow maturity, low fecundity, and long gestation along with increased and targeted fishing pressure during periods when rays are aggregated for reproduction has resulted in rapid population decline. These same life-history traits will prevent a rapid recovery. Science-based management is essential to ensure survival of this threatened and endemic ray. This study details consequences of targeted removal of an apex/meso predator from a complex ecosystem and serves as a model to assess the effects of natural and anthropogenic marine environmental change.

DATA AVAILABILITY STATEMENT

Datasets collected and analyzed as part of this research are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for this animal study in accordance with institutional requirements. Animal samples used in this study were collected from operators involved in a predator control program authorized by various national and prefectural governments.

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AUTHOR CONTRIBUTIONS

AY conceived the study and obtained funding. AY and KF completed field surveys and JW joined when in country. KF curated and analyzed the data. AY, KF, and JW interpreted the data and wrote and reviewed the manuscript. All authors approved the final manuscript.

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