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Preliminary insight into the reproductive traits of the flapper skate *Dipturus intermedius* using in-field ultrasonography and circulating hormone concentrations

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ABSTRACT: Due to global population declines, there is a pressing need for data on the life history traits of many elasmobranch species to support the development of species-specific management plans. A lack of information on the reproductive cycle of the Critically Endangered flapper skate *Dipturus intermedius* was recently identified as a hindrance to its conservation. To address this data gap, we combined non-lethal ultrasound and hormone analysis to investigate the size at maturity and reproductive cycle of the flapper skate in the Loch Sunart to the Sound of Jura Marine Protected Area off the west coast of Scotland. In-field ultrasound imagery revealed encapsulated eggs *in utero* and was used to determine the presence and size of ovarian follicles. Combining these images with levels of plasma testosterone, progesterone and oestradiol provided valuable insights into the timing of the reproductive cycle and maturity state of the flapper skate. This preliminary study suggests that male skate start to mature at 165 cm and females at 203 cm total length. Oestradiol appears to be the primary hormone controlling the female reproductive cycle and, along with ultrasound images, indicates that females lay pairs of eggs throughout a winter egg-laying season. Our study further highlights how non-lethal methods can be used to investigate the life history of oviparous elasmobranchs in the field. This information will support the identification of important life history groups and their associated habitats and contribute to the development of management strategies for these species.

KEY WORDS: Conservation · Management · Critical habitats · Non-lethal · Maturity · Reproductive cycle · Rajidae

1. INTRODUCTION

Over-exploitation of elasmobranchs has resulted in the decline of many species (Dulvy et al. 2021), prompting the implementation of conservation and management actions (Carlson et al. 2019). A key challenge is developing conservation measures that account for the elasmobranchs' strongly *K*-selective life history characteristics, a feature that can hinder their recovery (Musick et al. 2000, Barker & Schluessel 2005). Furthermore, understanding the life history of a species can help determine how it will respond to future population pressures (Cailliet 2015).

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Large, slow-growing skate species are particularly susceptible to over-exploitation (Dulvy & Reynolds 2002). This vulnerability emphasises the need to gather data to support management for this group. Garbett et al. (2021) highlighted how a lack of knowledge of reproduction in the flapper skate *Dipturus intermedius* is hampering the conservation of the species throughout its range. Current knowledge on the reproductive biology of this species suggests that females lay eggs over the summer months (Whitehead et al. 1986) and have an estimated fecundity of 40 eggs yr^{-1} (Brander 1981). However, this data was collected before 2010, when the flapper skate and the common blue skate *D. batis* were still grouped together as 'common skate'. As these 2 species have now been shown to have markedly different life history characteristics (Iglésias et al. 2010), it is unclear whether our understanding of the flapper skate's reproductive biology was obtained from *D. intermedius* or *D. batis*. Due to the flapper skate's Critically Endangered conservation status (Ellis et al. 2021), there is an obvious need for species-specific life history data.

A commonly used method to assess the life history of elasmobranchs is to evaluate the concentrations of the circulating gonadal hormones 17β-oestradiol (E_2), testosterone (T) and progesterone (P_4) in blood samples (e.g. Koob & Callard 1999, Gelsleichter 2004, Sulikowski et al. 2007, Awruch et al. 2008a, Penfold & Wyffels 2019), as these hormones have been linked to maturation events and the reproductive cycle of oviparous elasmobranchs (e.g. Koob & Callard 1999, Gelsleichter 2004, Sulikowski et al. 2004, Awruch et al. 2008a, 2021). The concentrations of these hormones can provide information on sexual maturity (e.g. Rasmussen & Murru 1992, Rasmussen et al. 1999, Gelsleichter 2004, Sulikowski et al. 2004, Kneebone et al. 2007, Awruch et al. 2008a, 2021, Awruch 2013, Verkamp et al. 2022) and reproductive state (e.g. Rasmussen & Gruber 1993, Sulikowski et al. 2005). However, there is considerable interspecific variation in the concentration levels of these hormones in relation to specific events in the reproductive cycles of oviparous elasmobranchs (Koob & Callard 1999, Gelsleichter 2004, Sulikowski et al. 2004). This variation, alongside annual cycles of hormones in year-round egg-laying species (Sulikowski et al. 2004), suggests that incorporating additional methods to assess the physical state of the reproductive organs can provide a more detailed description of the reproductive cycle in oviparous species (Whittamore et al. 2010, Anderson et al. 2018, Awruch et al. 2021).

In female elasmobranchs, the developmental stage of the reproductive tract is often determined via post-mortem assessment (Sulikowski et al. 2005, Licandeo et al. 2007, Kyne et al. 2008, Quiroz et al. 2009). However, there is an obvious need to use and promote non-lethal methods to describe the reproductive organs (Hammerschlag & Sulikowski 2011), especially for Critically Endangered species such as the flapper skate, where individual survival is a priority. Advances in ultrasound technology have made the non-lethal assessment of the reproductive tract of female elasmobranchs in field-based situations more feasible and have been successfully used on live-bearing elasmobranch species (Carrier et al. 2003, Jirik & Lowe 2012, Daochai et al. 2016, Sulikowski et al. 2016, Anderson et al. 2018, Penfold & Wyffels 2019, Tomita et al. 2019). Using ultrasound as a diagnostic tool for oviparous elasmobranchs has received comparatively less attention but has been used to measure the size of ovarian follicles and assess the presence of encapsulated eggs (Daly et al. 2007, Whittamore et al. 2010, Awruch et al. 2021).

Species-specific life history data is highly relevant in the conservation of mobile species such as the flapper skate. Several widely used management tools are based on information such as size at maturity, timing of the reproductive cycle and information on critical habitats. For example, there are 2 Marine Protected Areas (MPAs) in Scotland designated to protect the flapper skate (Neat et al. 2015, Thorburn et al. 2021, Dodd et al. 2022). One of these focuses on protecting an egg nursery (Dodd et al. 2022), which is a critical habitat and essential for aiding population recruitment. Understanding the life history of a species helps to determine how it will respond to future population pressures (Cailliet 2015), and obtaining this data via non-lethal methods can support telemetric studies, in which the tagged individual's life history data can aid in the interpretation of subsequent movement patterns.

By making use of in-field ultrasound and levels of circulating concentrations of T, P_4 and E_2 from blood samples, this study aimed to provide valuable insight into the reproductive and maturity state of the Critically Endangered flapper skate through non-lethal methods.

2. MATERIALS AND METHODS

Flapper skate were caught as part of tagging studies, mainly in the Loch Sunart to the Sound of Jura MPA, west Scotland, from March 2018 to November 2021

(see Thorburn et al. 2021, Lavender et al. 2022 for further information). Additional individuals were caught off the southwest coast of Scotland in October 2021. Skate were caught using barbless hooks with rod and line from recreational charter vessels. Once landed, sex and total length (TL; snout tip to tail tip, in cm) were recorded. Following these measurements, blood sampling and ultrasound scanning were undertaken around the focal tagging work (see Thorburn et al. 2021, Lavender et al. 2022). All skate were returned, alive, at their point of capture. All tagging procedures complied with The Animals (Scientific Procedures) Act 1986 under a Home Office Project License (no. P05E95C50) by competent Personal License holders.

2.1. Blood sampling

Blood samples were taken from the caudal vessels by phlebotomy using a 21 gauge needle attached to a 5 ml syringe, introduced at the ventral aspect of the tail, perpendicular to the long axis of the tail, approximately 10–15 cm from the base of the tail (where the anal fin joins the body; Fig. 1). A maximum volume of 2 ml of blood was sampled from each skate and immediately transferred to a lithium heparin tube which was inverted several times to mix. Tubes were stored in a cool box for a maximum of 10 h. Plasma was extracted by pipette following centrifugation for 2 min at 7000 rpm (2680 × *g*). The plasma was stored in a screw-cap vial frozen at −20°C for a maximum of 5 d, then at −80°C before analysis.

2.2. Hormone assay

The blood plasma concentrations of E_2, T and P_4 were determined via enzyme-linked immunosorbent assay (ELISA). The inter-assay coefficients of variation (CVs) were determined from the quality controls tested on each ELISA plate. See Fig. S1 in the Supplement at www.int-res.com/articles/suppl/n052p097_supp.pdf for ELISA standard curves and Fig. S2 for parallelism results. Average quality control values are shown in Table S1, including the expected range for E_2.

E_2 was assessed using the Demeditec Estradiol ELISA kit (DE2693; Demeditec Diagnostics), following the manufacturer's guidelines and analysing samples at a ×2 dilution. The limit of quantification was 0.0212 ng ml^{-1}. T was measured by an in-house-developed ELISA as described by Wilson et al. (2022). Samples were tested at a ×2 dilution in 0.1% BSA phosphate-buffered saline (PBS). In samples that were too high at this dilution, additional ×5, ×10, ×50 and ×100 dilutions were tested. The limit of quantification was 0.3 ng ml^{-1}. P_4 was measured by an in-house-developed ELISA following a protocol close to that of T. Samples were tested at a ×2 dilution in 0.1% BSA PBS for analysis. The limit of quantification was 0.2 ng ml^{-1}. The inter- and intra-assay CVs were 4.87 and 16.21% for E_2; 16.5 and 4.3% for T; and 10.0 and 4.7% for P_4, respectively. See Text S1 for details.

2.3. Changepoint analysis

A changepoint analysis using a gamma distribution was undertaken with a 95% confidence interval to identify changes in the mean and variance of hormone concentrations in relation to TL using the 'changepoint' package in R (Killick & Eckley 2014). Hormone concentrations lower than the limit of quantification were assigned their threshold value (P_4: 0.2 ng m^{-1}; T: 0.3 ng ml^{-1} E_2: 0.0212 ng m^{-1}). The changepoint analysis was run for each hormone for males and females. We aimed to use a relatively straightforward, less subjective analysis to give insight into the body size at which hormone concentrations changed. Changepoint analysis is a highly suitable analytical approach, as it detects the point at which there is a significant ($p < 0.05$) change in the statistical properties of a sequence of observations (Killick & Eckley 2014).

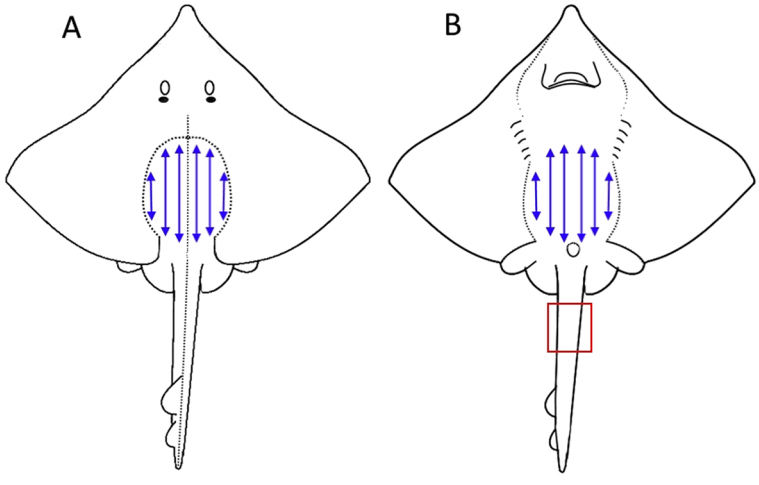

Fig. 1. Scanning protocol used for the (A) dorsal surface and (B) ventral surface (blue arrows) of flapper skate. Red square: site of blood sampling; dotted lines: body cavity limits and spine. Eyes, spiracles, and gills are also shown

2.4. Ultrasound

2.4.1. Ultrasound equipment

Two ultrasound units were used: an Easiscan 4 Digital Scanner with a micro-convex 4.0–8.5 MHz probe and a Clarius C3 with a convex 2–6MHz probe. The transducer was protected using a latex cover to prevent the denticles present on the skate's skin from damaging the probe surface. No coupling gel was used as the mucus layer on the skate's skin acted as a sufficiently conductive medium. The Easiscan had 3 image depth settings: near (6 cm), medium (8 cm) and far (12 cm). The Clarius had a maximum image depth of 40 cm. For both units, the frequency and focal zones were automatically adjusted depending on the size and depth of the scanned area.

2.4.2. Scanning procedure

Upon landing, skate were placed in a lifting sling, dorsal side up, on a soft foam support mat, and both spiracles were irrigated with seawater. The full-body cavity was scanned, initially using the far setting (Easiscan) or 30 cm penetration depth (Clarius) with the probe positioned perpendicular to the spine (Fig. 1). The skate was then turned ventral side up using the lifting sling. The entire body cavity was scanned again as described for the dorsal aspect (Fig. 1). The imagery was assessed in real-time. If features of interest were observed (e.g. ovaries or encapsulated eggs), the probe was moved through various degrees of rotation and angles, and the penetration depth was altered to obtain the best image. Multiple images were captured for each skate. On the Easiscan scanner, images were captured with an associated 1 cm reference grid. As the tagging work took precedence, it was not possible to scan every skate dorsally and ventrally, and only cursory scanning was undertaken in some cases in order to return skate to the water within a specific timeframe.

2.4.3. Image processing

Ultrasound images were assessed for the presence of encapsulated eggs and the number and size of ovarian follicles. Using either the 1 cm reference grid from the Easiscan scanner or the Clarius Cloud software, the follicles or yolk of encapsulated eggs observed in the uteri were measured across their broadest aspect and the perpendicular plane to determine the height (h) and diameter (w) measurements of the ellipse (Fig. 2), allowing the area of each feature to be estimated using the following calculation:

$$\text{Area} = \left(\frac{h}{2}\right) \cdot \left(\frac{w}{2}\right) \cdot \pi$$

3. RESULTS

Concentrations of circulating E_2, T and P_4 were analysed for 83 flapper skate: 36 females (Table 1) and 47 males (Table 2); ultrasound images were obtained for 15 of these individuals. Six additional females were imaged in September and October 2021, but no blood samples were taken. Male TL ranged from 116 to 202 cm (median: 180 cm; first and

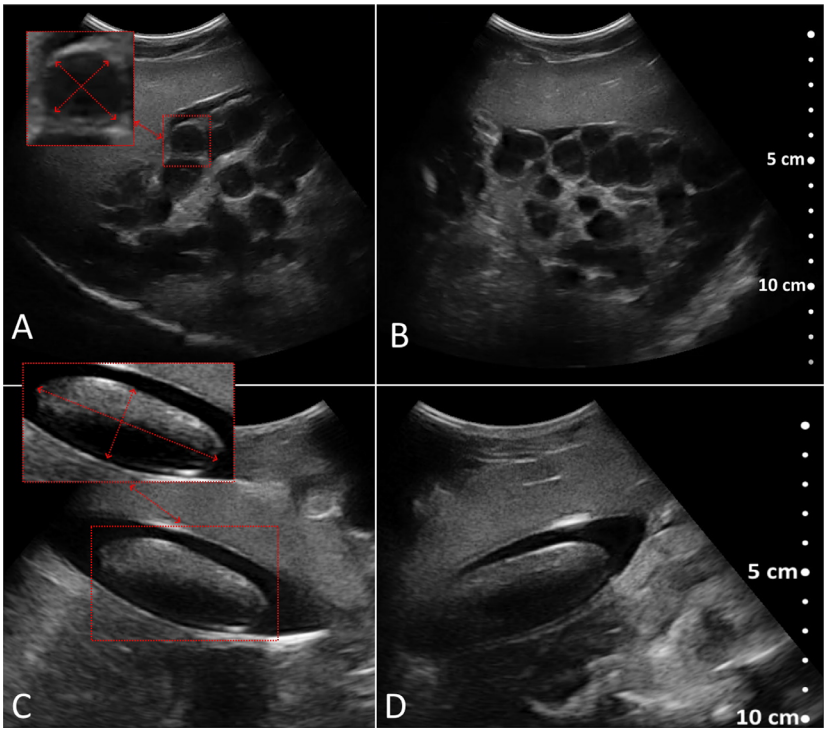

Fig. 2. Ultrasound images from 2 female flapper skate. (A,B) Skate 2216; both ovaries appear to be active. (C,D) Skate 3329; egg case present in each uterus. All images were taken with a Clarius C3 convex probe scanner

third quartile: 164 and 191 cm), and female TL ranged from 112 to 229 cm (median: 203 cm; first and third quartile: 153.5 and 216.5 cm) (Table 3).

3.1. Plasma hormones

For females, changepoint analysis revealed significant ($p < 0.05$) changes in all 3 hormones (Fig. 3). The changepoints occurred at 203 cm TL for E_2, 208 cm TL for T and 229 cm TL for P_4. Although the changepoint for P_4 occurred at a larger TL than that of E_2 and T, there were some concentrations of P_4 above mean levels in individuals of 210 cm TL. For males, changepoint analysis only identified a change in T, which occurred at 165 cm TL. While no changepoint was detected for other hormones ($p > 0.05$), there were elevated concentrations of P_4

Table 1. Female flapper skate sampled as part of this study, showing month and year of sampling, size (total length), sex, concentrations of each hormone, whether the skate was imaged using an ultrasound, and if an encapsualted egg was observed *in utero*. Under Skate ID, '_1' indicates the first capture event and '_2' indicates the second. Further information on associated follicle sizes can be found in Table S2

Skate ID	Month and year	Total length (cm)	Sex	Oestradiol (ng ml^{-1})	Testosterone (ng ml^{-1})	Progesterone (ng ml^{-1})	Imaged	Encapsulated egg observed
2092	Aug-2018	218	F	0.06319	1.58	<0.2	N	–
2902	Aug-2018	218	F	0.06357	2.59	0.3	Y	No
3338	Aug-2018	221	F	0.24146	4.11	0.2	Y	No
2972	Aug-2018	224	F	0.25264	3.12	<0.2	N	–
2935	Aug-2018	226	F	0.17665	4.12	0.29	Y	No
3576	Apr-2019	137	F	<0.0212	<0.3	<0.2	N	–
3330	Apr-2019	183	F	0.04043	0.43	0.33	N	–
1486	Apr-2019	211	F	1.37707	19.58	0.57	N	–
1467_1	Apr-2019	229	F	2.03683	25.91	1.83	Y	No
1491	Aug-2019	118	F	0.02926	<0.3	<0.2	N	–
2910	Aug-2019	124	F	0.02266	1.17	<0.2	N	–
2920	Aug-2019	140	F	0.03388	<0.3	<0.2	N	–
1524	Aug-2019	152	F	0.04992	0.36	<0.2	N	–
1363_1	Aug-2019	152	F	0.02296	<0.3	<0.2	N	–
2877	Aug-2019	155	F	<0.0212	1.49	<0.2	N	–
2433	Aug-2019	155	F	0.05537	<0.3	<0.2	N	–
2316	Aug-2019	203	F	0.37958	0.95	<0.2	Y	No
3977	Aug-2019	208	F	0.26702	4.3	<0.2	N	–
1473	Aug-2019	212	F	0.92744	1.02	0.28	Y	No
2980	Aug-2019	216	F	3.34036	2.44	<0.2	Y	No
3367	Aug-2019	220	F	1.54334	1.06	<0.2	Y	No
1334	Nov-2019	217	F	3.81202	4.96	<0.2	Y	No
9492	Mar-2020	112	F	<0.0212	<0.3	<0.2	N	–
9293	Mar-2020	130	F	0.02227	2.75	<0.2	N	–
3370	Mar-2020	132	F	0.03729	<0.3	<0.2	N	–
2215	Mar-2020	172	F	0.02455	1.04	<0.2	Y	No
4522_1	Mar-2020	179	F	0.05884	<0.3	<0.2	Y	No
3185	Mar-2020	213	F	2.54538	25.2	<0.2	Y	Yes
9256	Mar-2020	213	F	0.86852	78.42	0.53	Y	No
1513	Mar-2020	222	F	1.30618	19.75	<0.2	Y	Yes
1467_2	Mar-2020	229	F	1.96573	20.99	0.39	N	–
9172	Jun-2021	150	F	0.02632	0.45	<0.2	N	–
1363_2	Jul-2021	170	F	0.02986	1.26	<0.2	N	–
9205	Jul-2021	180	F	0.07915	<0.3	<0.2	N	–
4522_2	Jul-2021	185	F	0.07625	1.76	<0.2	N	–
3033	Jul-2021	211	F	0.15344	6.25	<0.2	Y	No
22160	Sep-2021	196	F	–	–	–	Y	No
31780	Sep-2021	200	F	–	–	–	Y	No
21580	Sep-2021	210	F	–	–	–	Y	Yes
22090	Sep-2021	212	F	–	–	–	Y	No
22150	Sep-2021	220	F	–	–	–	Y	No
3329	Oct-2021	214	F	–	–	–	Y	Yes

Table 2. Male flapper skate sampled as part of this study, showing month and year of sampling, size (total length), sex, and concentrations of each hormone. Under Skate ID, '_1' indicates the first capture event and '_2' indicates the second

Skate ID	Month and year	Total length (cm)	Sex	Oestradiol (ng ml^{-1})	Testosterone (ng ml^{-1})	Progesterone (ng ml^{-1})
2885	Aug-2018	124	M	<0.0212	7.39	0.24
3028	Aug-2018	151	M	<0.0212	4.17	<0.2
3359	Aug-2018	163	M	<0.0212	4.7	0.26
2883	Aug-2018	168	M	0.0222	29.19	0.3
4606	Aug-2018	168	M	<0.0212	29.64	<0.2
2921	Aug-2018	188	M	<0.0212	38.54	0.42
3231	Aug-2018	191	M	<0.0212	250.25	0.33
1061	Aug-2018	199	M	0.03041	35.37	0.37
3194	Aug-2018	202	M	0.04984	31.85	0.59
1556	Aug-2019	119	M	0.03599	6.16	0.24
1483	Aug-2019	133	M	<0.0212	7.29	<0.2
995	Aug-2019	142	M	0.05551	1.16	<0.2
3357	Aug-2019	157	M	0.02795	2.96	<0.2
3226	Aug-2019	163	M	<0.0212	7.26	<0.2
1510	Aug-2019	179	M	0.0258	6.04	<0.2
3005	Aug-2019	180	M	0.03564	8.15	<0.2
3118	Aug-2019	180	M	<0.0212	30.61	<0.2
1545	Aug-2019	180	M	<0.0212	12.03	0.23
420	Aug-2019	185	M	<0.0212	10.57	<0.2
8867_1	Aug-2019	188	M	0.02582	39.4	0.24
2986	Aug-2019	193	M	0.02273	26.29	0.69
2939_1	Aug-2019	197	M	0.06032	25.9	0.57
1562	Mar-2020	116	M	0.02318	0.5	<0.2
2889_1	Mar-2020	163	M	0.02364	2.67	<0.2
1470	Mar-2020	183	M	0.04559	22.03	<0.2
2918_1	Mar-2020	189	M	0.12548	28.58	<0.2
9309	Mar-2020	191	M	0.02139	231.35	0.28
1480	Mar-2020	192	M	<0.0212	28.01	<0.2
289	Mar-2020	198	M	0.04329	26.88	<0.2
2268	Mar-2020	198	M	0.09016	28.31	0.26
1530	Jun-2021	142	M	0.03214	3.81	<0.2
1515	Jun-2021	163	M	0.02344	3.47	<0.2
1549	Jun-2021	173	M	0.05095	39.81	<0.2
2889_2	Jun-2021	175	M	0.02999	13.23	<0.2
1337	Jun-2021	176	M	<0.0212	25.16	<0.2
9214	Jun-2021	185	M	0.10599	8.56	<0.2
658	Jun-2021	191	M	0.04574	30.94	<0.2
9284	Jun-2021	196	M	0.04843	108.9	<0.2
9031	Jul-2021	132	M	0.08493	11.01	<0.2
9321	Jul-2021	165	M	0.02677	20.74	<0.2
9435	Jul-2021	170	M	0.02749	27.61	<0.2
1325	Jul-2021	173	M	0.11002	13.51	<0.2
1963	Jul-2021	178	M	0.056	178.5	<0.2
7904	Jul-2021	185	M	0.03004	85.9	<0.2
2918_2	Jul-2021	190	M	0.24877	20.85	<0.2
8867_2	Jul-2021	193	M	0.0672	22.61	<0.2
2939_2	Jul-2021	196	M	0.05342	163.1	<0.2

above mean levels in individuals larger than 168 cm TL. E_2 showed more variation across all sizes, with concentrations above mean levels at 132 cm TL. Higher concentrations of E_2 (>0.1 ng ml^{-1}) were observed from 173 cm TL. Using the smallest TL identified by the changepoint analysis (F = 203 cm; M = 165 cm), our sample consisted of 31 immature skate (M = 13, F = 18) and 53 mature skate (M = 34, F = 18).

In females, the highest E_2, P_4 and T values were observed in different individuals. For E_2, the highest value (3.812 ng ml^{-1}) was observed in one female in November, but concentrations above 2 ng ml^{-1} were also observed in March, April and August; concentrations were lowest in July (Table 1, Fig. 4). The highest concentration of T was observed in one female in March (78.42 ng ml^{-1}). This concentration was considerably higher than in other females, with concentrations above 20 ng ml^{-1} observed in April. Concentrations were all less than 10 ng ml^{-1} in July, August and November (Table 1, Fig. 4). There was a high occurrence of P_4 concentrations below the limit of quantification in individuals (n = 28) reported in March, July, November and August. The highest P_4 concentration was observed in one female in April (1.83 ng ml^{-1}); this was substantially higher than other high P_4 concentrations in March (>0.5 ng ml^{-1}; Table 1, Fig. 4).

For males, E_2 concentrations were similar in March and July, but the highest value was recorded in one male in July (0.249 ng ml^{-1}); concentrations were all below the quantification threshold in August (Table 2, Fig. 4). T concentrations were mainly below 50 ng ml^{-1}, although concentrations above 75 ng ml^{-1} were observed, predominantly in July but also in March and August. As with females, numerous samples had P_4 concentration levels below the quantifiable threshold (n = 33). P_4 concentrations above the threshold limits were only observed in August (0.23–0.69 ng ml^{-1}; n = 13) and March (0.26–0.28 ng ml^{-1}; n = 2) across different years (Table 2, Fig. 4). There were 3 recaptures of females and 4 recaptures of males, all at least 339 d apart (Table 4). Of note, for skate 1467, E_2 concentrations were high in both samples (1.966 and 2.037 ng ml^{-1}). See Table 1 for further information.

Table 3. Hormone concentration ranges, medians and first and third quartiles (ng ml^{-1}) for all male and female flapper skate (n = 83). The number of individuals that are below quantifiable threshold concentrations is shown for each sex. Median and quartile values are all based on individuals over threshold limits

	Males					Females				
	No. below threshold	Min.–max.	Median	First quartile	Third quartile	No. below threshold	Min.–max.	Median	First quartile	Third quartile
Oestradiol	13	0.021–0.249	0.039	0.026	0.057	3	0.022–3.812	0.079	0.0356	1.117
Testosterone	0	0.5–250.25	22.61	7.29	30.94	9	0.36–78.42	2.59	1.06	6.25
Progesterone	33	0.23–0.69	0.29	0.24	0.457	27	0.2–1.83	0.33	0.285	0.55

3.2. Ultrasound

The 21 scanned female flapper skate ranged in size from 172 to 229 cm TL. Ovarian follicles were observed in 18 females (Fig. 2) that ranged in length from 185 cm (max. follicle area: 0.98 cm^2) to 229 cm TL (max. follicle area 1.9 cm^2). Ovarian follicle area ranged from 0.027–5.36 cm^2 (median: 0.66 cm^2; first and third quartiles: 0.33 and 1.38 cm^2) with seasonal variation, although there were insufficient data to

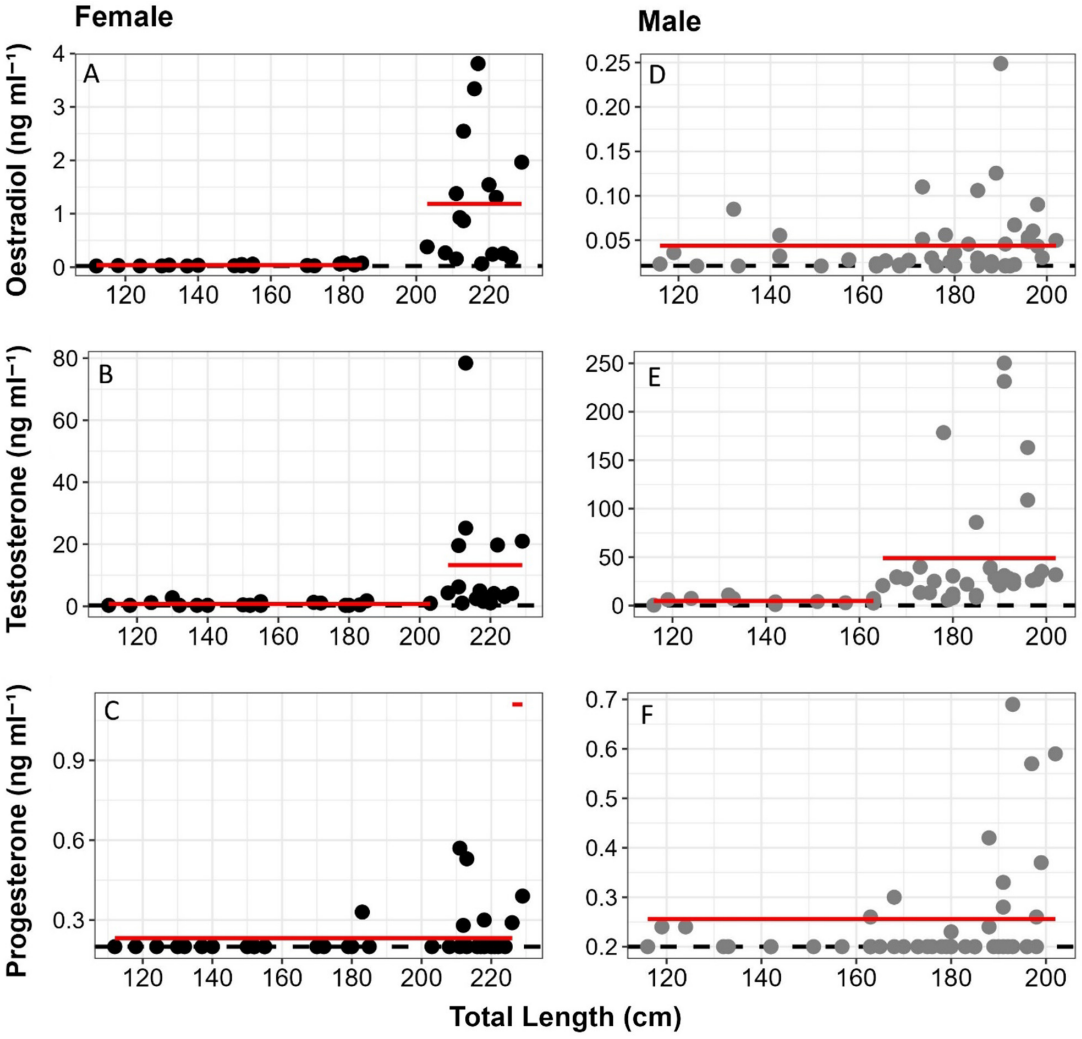

Fig. 3. Changepoint analysis of (A,D) oestradiol, (B,E) testosterone and (C,F) progesterone levels vs. body length of (A–C) female (n = 36) and (D–F) male (n = 47) flapper skate. Red line: group mean (ranges of total lengths on either side of the identified changepoint); black dashed line: threshold detection concentration for each hormone

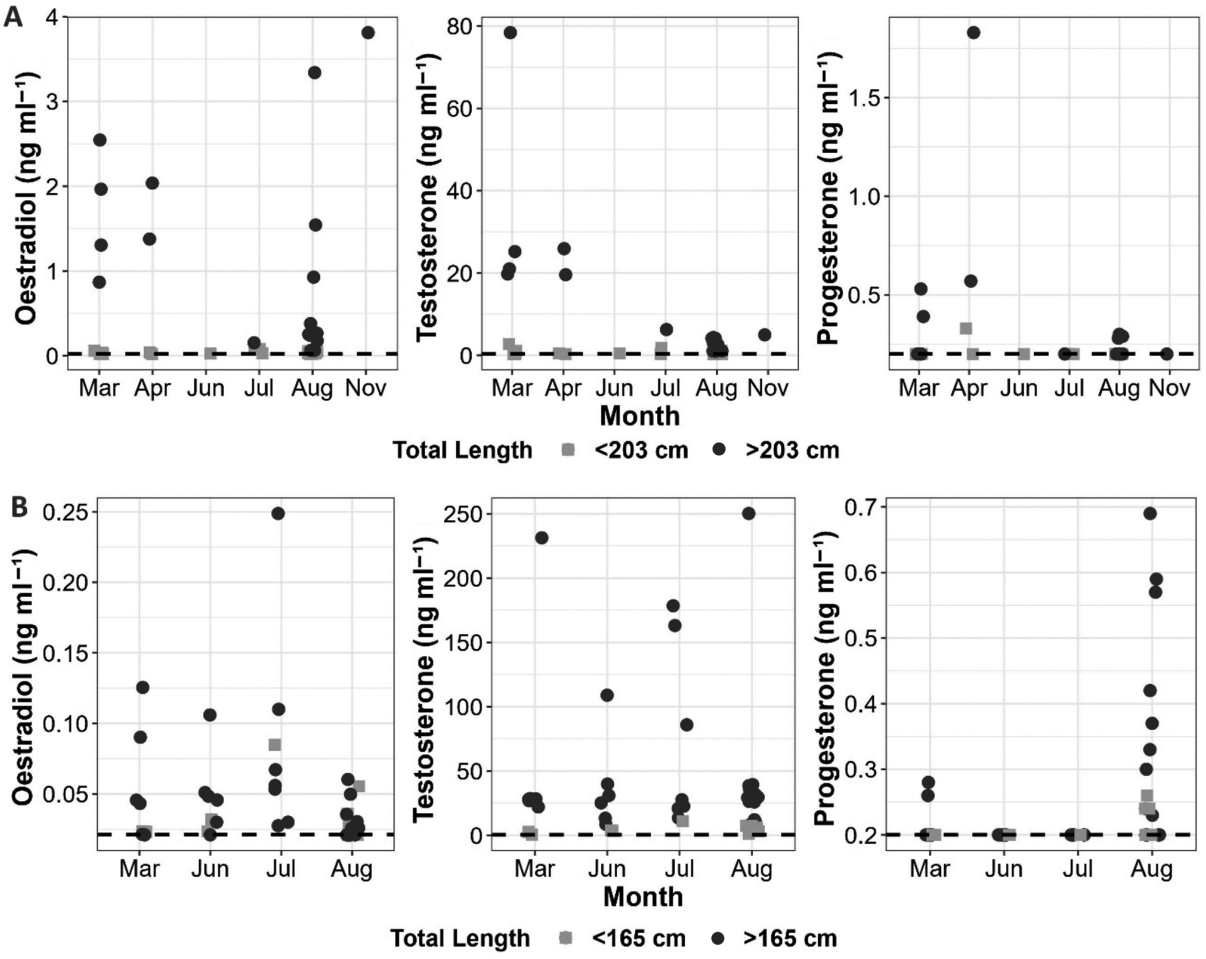

Fig. 4. Hormone concentrations from (A) female and (B) male flapper skate sampled per calendar month: March (F = 9, M = 8), April (F = 4), June (F = 1, M = 8), July (F = 4, M = 9), August (F = 17, M = 22), September (F = 5) and November (F = 1). Points are coloured and shaped based on the threshold length detected by the changepoint analysis (see Fig. 2). Black dashed line: threshold detection concentration for each hormone

Table 4. Hormone concentrations for the 7 recaptured flapper skate. Under Skate ID, '_1' indicates the first capture event and '_2' indicates the second. TL: total length. Days apart: number of days between the first and second capture events

Skate ID	Date (dd/mm/yyyy)	Oestradiol (ng ml^{-1})	Testosterone (ng ml^{-1})	Progesterone (ng ml^{-1})	Sex	TL (cm)	Days apart
1363_1	13/08/2019	0.0230	<0.3	<0.2	F	152	
1363_2	02/07/2021	0.0300	1.26	<0.2	F	170	689
1467_1	02/04/2019	2.0368	25.91	1.83	F	229	
1467_2	06/03/2020	1.9657	20.99	0.39	F	229	339
4522_1	06/03/2020	0.0588	<0.3	<0.2	F	179	
4522_2	02/07/2021	0.0763	1.76	<0.2	F	185	483
2889_1	05/03/2020	0.0236	2.67	<0.2	M	163	
2889_2	29/06/2021	0.0300	13.23	<0.2	M	175	481
2918_1	04/03/2020	0.1255	28.58	<0.2	M	189	
2918_2	02/07/2021	0.2488	20.85	<0.2	M	190	485
2939_1	12/08/2019	0.0603	25.9	0.57	M	197	
2939_2	02/07/2021	0.0534	163.1	<0.2	M	196	690
8867_1	06/08/2019	0.0258	39.4	0.24	M	188	
8867_2	01/07/2021	0.0672	22.61	<0.2	M	193	695

test for monthly differences. The largest follicles were observed in September (images from 4 individuals), with large follicles also present in March (images from 4 individuals) and August (images from 7 individuals). The follicle count in ultrasound images (1–19; median: 5; first and third quartiles: 4 and 7) was highest in September (n = 19; Fig. 5), with August also having some high counts (n = 11). The smallest follicles were observed in April (images from 1 individual) and November (images from 1 individual), which aligned with the months with the lowest follicle counts (Fig. 5). Both ovaries appeared to be active in several skate (e.g. 2216; Fig. 2). High concentrations of E_2 were associated with increasing follicle size but were also noted in ovaries with smaller follicles (1–1.5 cm^2; Fig. 6). P_4 and T concentrations were highest with smaller follicles (1–1.5 cm^2; Fig. 6). See Table S2 for details on follicle size for each skate.

Encapsulated eggs were observed in 4 females (210, 213, 214 and 222 cm TL) in March, September and October, including one female carrying 2 encapsulated eggs in October (Fig. 2). Of these 4 females, only 2 had accompanying hormone concentrations. Yolk area ranged from 4.58 to 13.27 cm^2 (median: 8.13 cm^2; first and third quartiles: 5.28 and 10.57 cm^2).

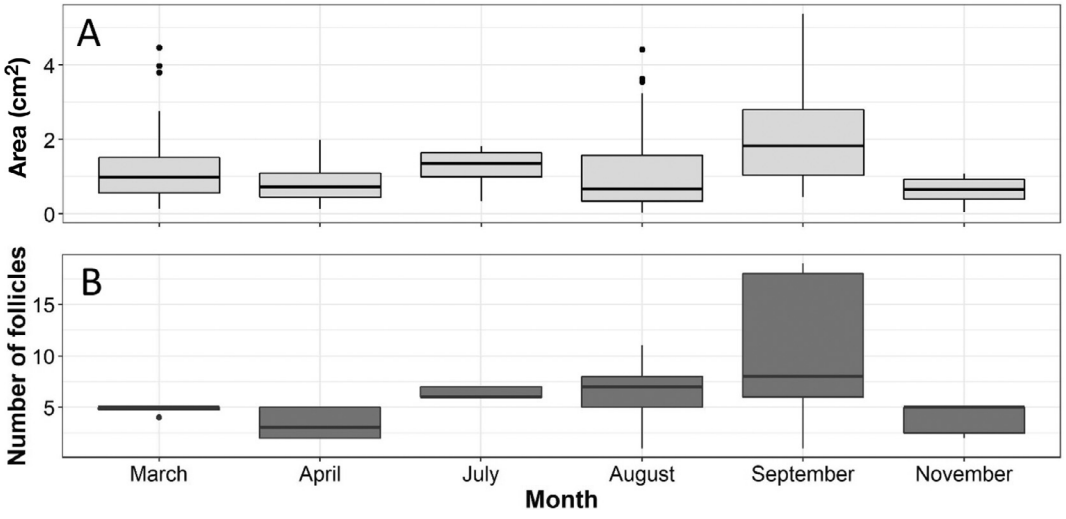

Fig. 5. (A) Areas of measured follicle sizes per month sampled and (B) number of follicles counted in each ultrasound image of flapper skate; sample sizes for each month: March: 4; April: 1; July: 1; August: 7; September: 4; and November: 1. Error bars: ±95% confidence intervals; boxes: 25th, 50th (median) and 75th percentiles. No mature skate were imaged in June. Encapsulated eggs were observed in March, September and October, but no ovaries were imaged in October

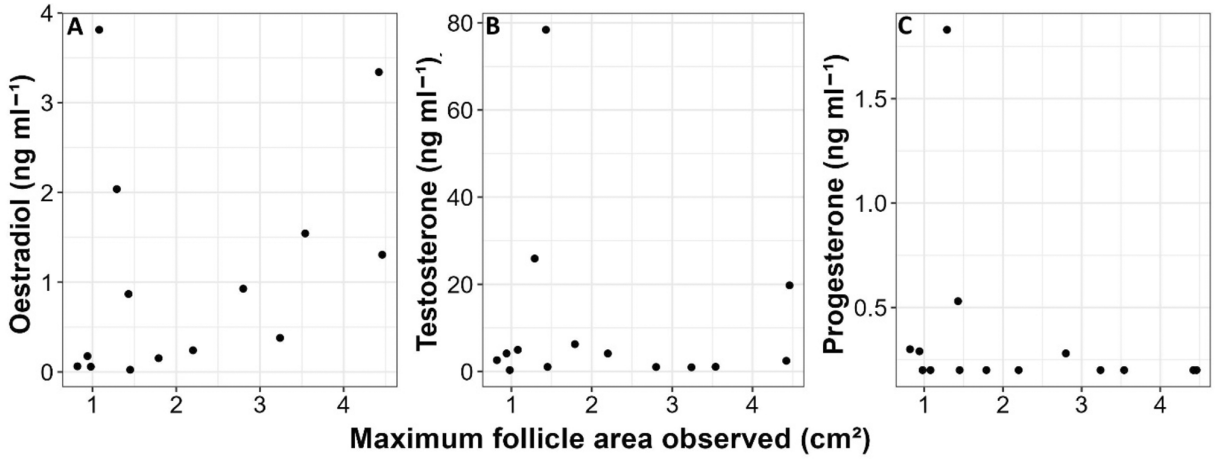

Fig. 6. Concentrations of (A) oestradiol, (B) testosterone and (C) progesterone compared to maximum follicle size observed per imaged flapper skate (n = 18), including those with concentrations below threshold values (shown as 0). Number of skate with hormone concentrations above the quantification threshold: oestradiol: n = 15; testosterone: n = 15; progesterone: n = 5

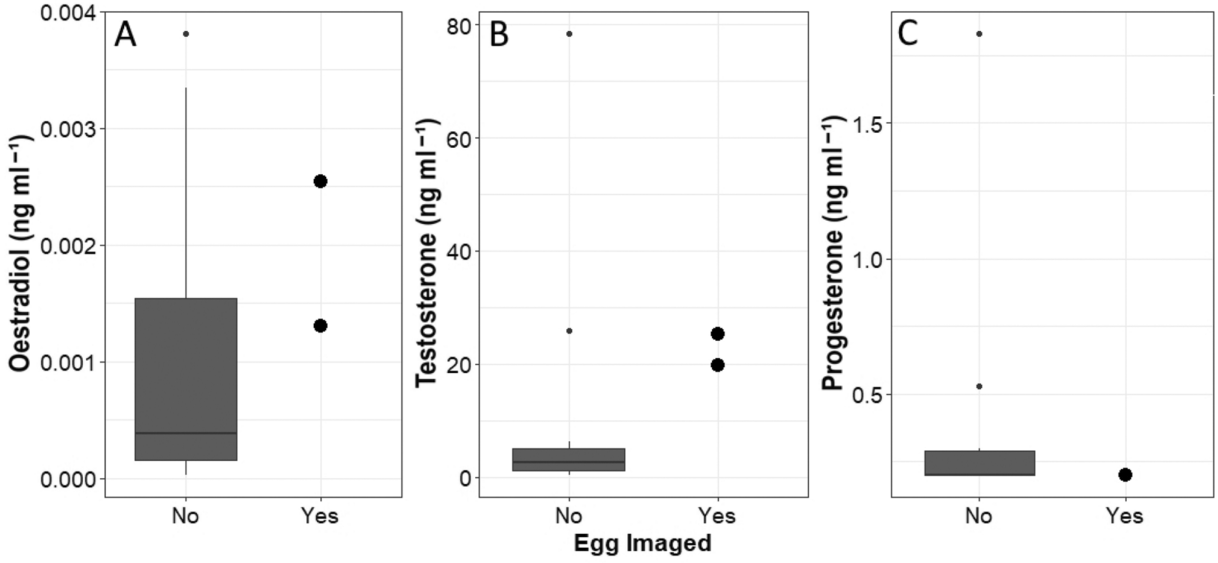

Fig. 7. Concentrations of (A) oestradiol, (B) testosterone and (C) progesterone for female flapper skate larger than 203 cm total length that were scanned and eggs were observed (n = 2; the 2 additional skate that had encapsulated eggs present had no associated hormone levels) vs. those scanned with no eggs observed (n = 11). For no encapsulated eggs observed, error bars: 95% confidence interval; boxes: 25th, 50th (median) and 75th percentiles. As there were only 2 skate with encapsulated eggs imaged and plasma hormones measured, hormone concentrations are represented by single points

Ovaries were imaged in 2 of the females carrying encapsulated eggs, and the maximum follicle sizes in these females were 4.46 and 5.3 cm^2; see Table S2 for further details. In the 2 skate with encapsulated eggs for which there were associated hormone levels, E_2 and T concentrations were comparatively high (E_2: 1.306 and 2.545 ng ml^{-1}, T: 19.75 and 25.2 ng ml^{-1}; Table 1, Fig. 7) and P_4 was below threshold values. In female skate that were larger than the changepoint threshold of 203 cm TL with no encapsulated eggs imaged (n = 16), E_2 concentrations were between 0.06 and 3.812 ng ml^{-1} (median: 0.624 ng ml^{-1}; first and third quartiles: 0.193 and 1.860 ng ml^{-1}), and T concentrations were between 0.95 and 78.42 ng ml^{-1} (median: 4.115 ng ml^{-1}; first and third quartiles: 1.795 and 16.247 ng ml^{-1}). Eight females had P_4 levels above the quantification threshold, ranging between 0.2 and 1.83 ng ml^{-1} (median: 0.345 ng ml^{-1}, first and third quartiles: 0.283 and 0.56 ng ml^{-1}) (Table 1, Fig. 7).

On the Easiscan unit, the far mode was the most suitable for full-body penetration, but the resolution did not allow for accurate visualisation of ovarian follicles. Near mode allowed for good visualisation of follicles close to the epidermis but did not penetrate far enough to visualise the whole ovary. The variable depth penetration range of the Clarius unit allowed for more detailed imaging of the ovary by allowing user selection of the most suitable penetration depth for the individual animal.

4. DISCUSSION

We provide valuable insight into the life history traits of the Critically Endangered flapper skate, including the timing of the reproductive cycle and size at maturity and the associated hormone concentrations for these life history events. Encapsulated eggs imaged in females inside the Loch Sunart to the Sound of Jura MPA suggest that egg-laying occurs within the site, which increases our understanding of the life history stages that this MPA protects.

As with other *Dipturus* species (Gedamke et al. 2005, Licandeo et al. 2007, Kyne et al. 2008, Follesa et al. 2012), both reproductive tracts of female flapper skate were observed to be active at the same time, and the simultaneous presence of encapsulated eggs in the left and right uterus suggests that flapper skate produce pairs of eggs. However, we also observed females carrying only one egg capsule. This may be due to female flapper skate having a level of plasticity in the number of eggs they lay at one time, as observed in other species of *Dipturus* (Licandeo et al. 2007). However, it could also be due to scanning occurring after the skate had laid one egg, or to encapsulated eggs moving into each uterus at different times. More females should be scanned to investigate the prevalence of pairs of encapsulated eggs. Both ovaries were imaged in an individual that was captured in September, with 18 and 19 follicles per ovary counted on one image. These findings suggest

that ovarian fecundity is at least 37 eggs yr^{-1}, similar to the fecundity estimate of 40 eggs yr^{-1} for the common skate (Brander 1981). While the observed ovarian fecundity is similar to that reported for other *Dipturus* species (*D. trachyderma*, Licandeo et al. 2007; *D. polyommata*, Kyne et al. 2008; and *D. chilensis*, Quiroz et al. 2009), the actual ovarian fecundity of the flapper skate is likely to be higher, as follicles were only counted from 2D images. In future work, video recordings of ovary scans may allow for a more precise ovarian follicle count.

The egg nurseries of the flapper skate can be used to support the conservation of the species (Phillips et al. 2021, Dodd et al. 2022); thus, their identification is important. If, as in other skate species, flapper skate hold encapsulated eggs in the uterus to harden for several days (Koob & Hamlett 1998), the presence of a fully encapsulated egg case in the uterus suggests that the female will be close to the deposition site, helping to identify these critical areas. Furthermore, the fecundity of the flapper skate suggests that egg-laying females are likely to remain in the vicinity of egg nurseries for protracted periods, as most oviparous species lay eggs over extended oviposition periods (Rasmussen et al. 1999, Luer et al. 2007). Consequently, protecting egg nurseries could safeguard both the deposited eggs and, for a period of time, reproductively active mature females, promoting recovery in strongly *K*-selective species (Frisk et al. 2001).

Encapsulated eggs were observed in March, September and October, suggesting that egg-laying occurs in these months. Additional insight into the egg-laying seasons was gained from the imaged follicles and the concentrations of circulating hormones, especially E_2. Previous studies on oviparous elasmobranchs have shown a strong association between concentrations of E_2 and the size of the ovary and ovarian follicles (Koob et al. 1986, Sulikowski et al. 2004, Awruch 2013, Nozu et al. 2018). Female flapper skate with high concentrations of E_2 were in an active egg-laying state, as indicated by the presence of encapsulated eggs or follicles larger than 2 cm^2 (Serra-Pereira et al. 2011). In 2 females, one sampled in April and one in November, high concentrations of E_2 were associated with smaller follicles. In oviparous elasmobranch species, E_2 levels rise before follicular development; therefore, elevated E_2 levels and smaller follicles may indicate a female at the beginning of the reproductive cycle (Nozu et al. 2018). The females sampled in July had low levels of E_2 and comparatively small follicle sizes, suggesting they were not reproductively active.

Concentration levels of T and P_4 did not provide additional data to support or define the egg-laying season in more detail. However, there were some indications that these hormones could provide further insight into the female flapper skate reproductive cycle if more samples were collected. High levels of T were only observed in March and April, including in the 2 females carrying encapsulated eggs for which we had recorded hormone levels. This suggests that T may be related to oviposition or egg encapsulation, as in other oviparous species (Rasmussen et al. 1999, Sulikowski et al. 2004, Awruch et al. 2008a,b, Nozu et al. 2018). It should be noted, however, that this observation was limited to 2 females and may not apply to those carrying eggs in September and October. T did not appear to increase with follicle size, and the highest concentration observed was related to comparatively small follicles, suggesting that this hormone is not related to follicle development as suggested for other species (Koob et al. 1986). Despite being linked to relatively small follicles, the high levels of P_4 observed in March and April suggest that these females were reproductively active, as P_4 regulates encapsulation and oviposition in some oviparous species (Awruch 2013). The low levels of P_4 observed in the flapper skate carrying encapsulated eggs are in keeping with other oviparous species that experience a drop in P_4 levels after ovulation (Koob et al. 1986, Sulikowski et al. 2004, Awruch et al. 2008b, Awruch 2013). P_4 concentrations often demonstrate short peaks in relation to specific events, resulting in high levels of fluctuation in P_4 (Nozu et al. 2018) and making it challenging to link these levels to specific events in the reproductive cycle. For the flapper skate, further research into combining P_4 levels with ultrasound imaging is needed to identify specific reproductive activities related to increased P_4 levels, such as encapsulation (Koob et al. 1986) and oviposition (Rasmussen et al. 1999). In viviparous species, T and P_4 have been linked to sperm storage, with concentrations increasing towards the end of the storage term (Gonzalez De Acevedo & Gelsleichter 2021). While further work on the relationship between these hormones and sperm storage in oviparous species is required, increased levels of both hormones observed in females in March and April may indicate the cessation of the need for sperm storage linked to the end of the egg-laying season.

With the caveat of a small sample size, the hormone and ultrasound evidence support a seasonal, overwinter reproductive cycle, similar to the timing observed in other *Dipturus* species (Gedamke et al. 2005, Licandeo et al. 2007, Kyne et al. 2008). An over-

winter egg-laying season would align with female flapper skate spending large amounts of time in waters shallower than 50 m (Thorburn et al. 2021), depths that are associated with egg-laying habitats in Scottish waters (Phillips et al. 2021, Dodd et al. 2022). However, an overwinter egg-laying season for the flapper skate differs significantly from the spring–summer egg-laying season previously suggested for the common skate (Whitehead et al. 1986), although this information may be based on observations of *D. batis* rather than *D. intermedius*. It is also worth noting that not all females of mature size had increased levels of hormones over the winter. This may indicate that not all females were reproductively active and may have differing reproductive seasons, do not reproduce yearly, or were incapable of breeding. Large females with small, inactive ovaries, comparable to maturing females, have been observed in several skates, including other *Dipturus* species (Licandeo et al. 2007, Kyne et al. 2008). This apparent quiescence of the ovaries has been attributed to reproductive senescence (Ebert 2005), and exploring whether this occurs in the flapper skate would be of interest as it suggests that the largest females, i.e. those that are >40 yr old (Régnier et al. 2021), may not contribute to population recruitment.

The changepoint threshold size for females was 203 cm TL based on E_2 and 208 cm TL based on T. As E_2 and T are strongly linked to processes in mature female elasmobranchs, such as breeding behaviour (Rasmussen & Gruber 1993, Awruch 2013) and ovarian follicle development (Koob & Callard 1999, Gelsleichter, 2004, Awruch et al. 2008b), high concentrations of these hormones are likely to indicate maturity in female flapper skate. P_4, conversely, does not appear to be helpful for determining the maturity of female flapper skate. The ultrasound images of the ovaries provided further insight into maturation based on their developmental state. Specifically, larger follicles were only imaged in individuals that were >196 cm TL, indicating that some female skate had reached maturity at this length (Licandeo et al. 2007, Kyne et al. 2008, Quiroz et al. 2009). These results suggest that mature females range in size from 196 to 208 cm TL, comparable to the L50% maturity of 197.5 cm estimated by Iglésias et al. (2010). The broader inference is that ultrasound imaging combined with circulating hormone concentrations, notably E_2 and T, are valuable tools for assessing the maturity status of female flapper skate.

While the use of hormone levels for determining the maturation of male elasmobranchs is not necessary, as it can easily be determined based on clasper development (Carrier et al. 2004), the changepoint analysis detected a change in T for males at 165 cm TL, suggesting the onset of maturity (Rasmussen & Murru 1992, Rasmussen & Gruber 1993, Tricas et al. 2000, Gelsleichter 2004, Awruch et al. 2008b). While no changepoints were detected for P_4 or E_2, the high concentrations of P_4 observed in male skate in August above the changepoint threshold of 165 cm TL detected for T may indicate an important reproductive event (Tricas et al. 2000, Awruch 2013, Verkamp et al. 2022), and further investigation is required to determine if these elevated levels are associated with spermiation (Gelsleichter 2004) or mating behaviour (Henningsen et al. 2008). To further investigate this question, it would be helpful to document physical evidence of mating in both males and females, such as mating scars, male milting and female cloaca redness. However, the evidence from this study suggests that T is the only hormone that requires assessment to distinguish juveniles from adults in male flapper skate, consistent with similar studies on oviparous elasmobranchs (Awruch et al. 2008a). Conducting clasper assessments in future studies will allow further insights into male maturation (Carrier et al. 2004). As changepoint analysis can detect multiple changepoints, a more extensive data set of hormone concentrations might allow for differentiation between maturing and mature female skate. Changepoint analysis could also be used on variables collected by ultrasound imaging, such as follicle size, which was not possible in this study. By utilising changepoint analysis, we can gain valuable information regarding the maturation size of elasmobranchs, and this method has the potential to offer valuable insights into life history events and complement more comprehensive techniques.

A significant challenge in using hormone concentrations in the wild involves relating concentration ranges to specific reproductive events. In captivity, this is achieved by multiple sampling of the same individual (e.g. Rasmussen & Murru 1992). The high level of site fidelity that flapper skate display in the Loch Sunart to the Sound of Jura MPA has resulted in a high recapture rate (Neat et al. 2015, Lavender et al. 2022), with 37 individuals recaptured 10 times or more (Skatespotter 2021). This recapture rate suggests that the flapper skate presents a rare opportunity to sample wild elasmobranchs over multiple occasions, allowing for greater insight into seasonal changes in hormones and the reproductive tract. This opportunity also highlights the benefits of using nonlethal methods to assess the development state of the reproductive organs. The value of this approach is

shown in skate 1467, sampled over consecutive years. The comparatively high E_2, T and P_4 concentrations observed in both samples suggest that this skate was reproductively active in both years. The 2 other females recaptured were <185 cm TL and therefore were likely immature in both capture incidences. Repeated sampling of these 2 skate (and others) offers the chance to identify maturation events by comparing individual hormone concentrations over time. For males, tracing how concentrations of T change over time in the same individual through maturation and into adulthood would give insight into how the concentrations of this hormone change and may allow for specific events, such as mating, to be identified. For example, skate 2889 was initially caught at 163 cm TL, below the changepoint size for T, but was 175 cm TL when recaptured, which is over the changepoint size. An increase in T was observed between these 2 captures, indicating that this male had either begun to mature or had matured in the 15 mo between captures. Additional data on clasper development would support these investigations.

A lack of data has hindered the development of species management plans for large skates worldwide (Licandeo et al. 2007). The rapid increase in the commercial exploitation of elasmobranchs (Dulvy et al. 2021) increases the importance of collecting life history information to support the management of remaining populations (Cailliet 2015, Carlson et al. 2019). Within this context, the data and tools presented in this study will contribute to the management of the flapper skate and, more widely, add to the body of work promoting minimally invasive research into the maturity and reproductive state of oviparous elasmobranchs in the field (e.g. Awruch et al. 2021).

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