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Testing staining techniques to determine age and growth of *Dasyatis pastinaca* (Linnaeus, 1758) captured in Iskenderun Bay, northeastern Mediterranean

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Summary

This study tested the suitability of several staining methods to determine the age of common stingray (*Dasyatis pastinaca*) from Iskenderun Bay, Turkey. A total of 384 specimens (16.6 cm–69.3 cm disc width) were obtained by trawling between September 2010 and December 2011. Sex ratio of the samples was 53% males and 47% females. Appropriate age determination was firstly demonstrated using *Safranin-O* staining. Age readings were made by two independent readers and the index of average percent error (IAPE) determined as 6.3% for *Safranin-O*, 6.8% for *Crystal Violet*, 7.9% for *Alcian Blue*, and 9.3% for *Silver Nitrate*. *Safranin-O* and *Crystal Violet* staining methods provided the best results. Verification of temporal growth ring formation was by marginal increment analysis. Disc width–weight relationships were determined by $W = 0.0272*DW^{3.06}$ for females and $W = 0.0247*DW^{3.08}$ for males. Estimates of the von Bertalanffy growth parameters indicated a larger asymptotic disc width ($DW_\infty = 127.06$ cm) for females than for males ($DW\infty = 114.54$ cm); growth parameters were $k = 0.058$ year^{-1}, $t_o = -1.508$ and $k = 0.041$ year^{-1}, $t_o = -3.632$ for females and males, respectively.

Introduction

Seven species of Dasyatidae occur in the Mediterranean (Başusta and Erdem, 2000; Golani et al., 2006). In addition to the Mediterranean and Black seas, *Dasyatis pastinaca* (Linnaeus, 1758) is common in the northeastern Atlantic Ocean, plus the South African coasts (Serena, 2005). Locally, the species is generally caught as by-catch by trawl fisheries in Iskenderun Bay (Yeldan et al., 2009). Maximum disc width (DW) is 140 cm and maximum total length (TL) is 250 cm (Fischer et al., 1987; Notarbartolo di Sciara and Bianchi, 1998). Size at maturity for females ranges from 28 to 38 cm DW, and for males from 26 to 32 cm (Ismen, 2003) and 62.5 cm total length (Yigin and Ismen, 2012). Parturition is reported to occur in the Mediterranean in early July (Ismen, 2003). Common stingray is assessed globally as data deficient (DD) by the International Union for Conservation of Nature (IUCN), due to the lack of basic biological data on catches and population trends (Abdul Malak et al., 2011), including the Mediterranean Sea. Unfortunately, quantifying life history data are lacking for most cartilaginous species within the eastern Mediterranean (Çek et al., 2009; Başusta and Sulikowski, 2012; Başusta et al., 2012; Duman and Başusta, 2013).

Age information forms the basis for calculating growth rate, mortality and productivity (Campana, 2001). Staining plays an important role in the success of precisely determining the age rings (bands). In this study, the use of four staining techniques to further enhance the clarity of the bands on stingray *Dasyatis pastinaca* vertebrae were investigated: *Crystal Violet*, Silver Nitrate, *Safranin-O* and *Alcian Blue*.

Materials and methods

Sampling

Common stingrays were collected in Iskenderun Bay between September 2010 and December 2011 as by-catch from a commercial bottom trawler (F/V Coskun Reis; depth 20–50 m). Mesh sizes were a 44 mm stretched mesh in the cod-end. Trawling lasted for 2 h with a trawling speed of 2.5 knots. Total length (TL, cm) of *D. pastinaca* was measured in a straight line from the tip of the rostrum to the end of the tail; disc width (DW, cm) was measured in a straight line between the tips of the widest portion of the pectoral fins. The total wet weight (g) was also recorded.

Preparation of vertebral samples

In the laboratory, a block of 12 vertebral centra was taken from each specimen above the abdominal cavity. A total of 384 *Dasyatis pastinaca* (209 females, 175 males) were labelled and stored frozen (Turkmen et al., 2005). Soft tissue was removed from the frozen vertebral segments using a scalpel and fine forceps. The individual vertebrae were then cut apart from each other and soaked in warm distilled water. Hypochlorite (6%) was used to remove the remaining connective tissue. However, hypochlorite can decalcify cartilage when overused, thus soak times were limited to approx. 10 min. The vertebrae were then air-dried for a minimum of 48 h.

Smaller centra were sanded with a Dremell TM tool to replicate a sagittal cut. Processed vertebrae were mounted horizontally on glass microscope slides and ground successively with a finer-grit (400 then 600) with wet or dry sandpaper. Each vertebra was then remounted and one side was ground to produce a thin (0.4–0.5 mm) sample (Başusta et al., 2008). Large centra more than 5 mm in diameter were sectioned using a gem saw (Ray Tech) with two diamond

blades separated by a 0.6 mm spacer (Başusta and Sulikowski, 2012).

Staining methods

Four staining techniques to enhance the clarity of the bands were tested on a subsample of the common stingray: *Crystal Violet, Silver Nitrate, Safranin-O* and *Alcian Blue*.

Safranin-O. This staining method was modified by Kahveci et al. (2000) and Tran et al. (2000). The cartilage staining varied from orange to red in colour.

Weigert's Iron Hematoxylin solution was used as stock solution A that contained: 1 g hematoxylin, and 100 ml alcohol (95%). Stock solution B contained: 4 ml ferric chloride in water (29%), 95 ml distilled water, and 1 ml of hydrochloric acid (concentrated). The working solution requires mixing equal parts of stock solutions A and B. The Fast Green (FCF) solution (0.001%) contained: 0.01 g fast green, FCF, C.I. 42053, and 1000 ml distilled water. Acetic Acid Solution (1%), 1 ml acetic acid, glacial, and 99 ml of distilled water. The *Safranin-O* solution (0.1%) contained: 0.1 g *Safranin-O*, C.I. 50240, and 100 ml distilled water. The slides were than hydrated with distilled water and stained with Weigert's Iron Hematoxylin working solution for 10 min. Thereafter the slides were washed under running tap water for 10 min, stained with FCF) solution for 5 min and then rinsed quickly with acetic acid solution for no more than 6 s. Staining in *Safranin-O* solution lasted for 5 min. Thereafter, samples were dehydrated and cleaned first with ethyl alcohol (95%) then transferred to absolute ethyl alcohol, using two changes of 2 min each.

Table 1
Precision of four different staining techniques on *Dasyatis pastinaca* vertebrae. The calculated precision indexes are: average percent error (APE), coefficient of variation (CV), and precision index (D). Percentage of readings that did not allow estimation of a valid age (ND) also given

			Precision indices			
Species	N	Technique	APE	CV	D	% ND
Dasyatis pastinaca	384	Crystal violet	6.8	9.1	5.2	5.6
		Silver nitrate	9.3	12.7	7.5	13.9
		Safranin O	6.3	7.5	4.5	1.3
		Alcian blue	7.9	10.2	6.1	12.9

Table 2
Average total length and weight at age, *Dasyatis pastinaca*, Iskenderun Bay, 2010–2011 fishing season

Age Groups	N	Average total length (cm)	Size range (cm)	Average total weight (g)	Weight range (g)
1	9	17.52	16.60–19.90	159.89	94–242
2	64	23.48	20–26.7	421.78	264–614
3	74	28.73	26–31.90	767.69	518–982
4	128	34.22	29–40.1	1376.10	976–1940
5	42	41.10	37.40–45.50	2450.38	1922–3142
6	10	47.22	45.20–49.30	3486.40	3018–4000
7	16	49.31	45.50–52.50	4434.53	3596–5814
8	23	51.65	50–56	4836.78	4006–5870
9	8	54.41	53–55.80	5833.63	5290–6650
10	7	57.04	55–58.10	6420.57	5000–8660
11	1	64.20	64.20	8396	8396
12	1	67.30	67.30	9480	9480
13	1	69.30	69.30	10564	10564

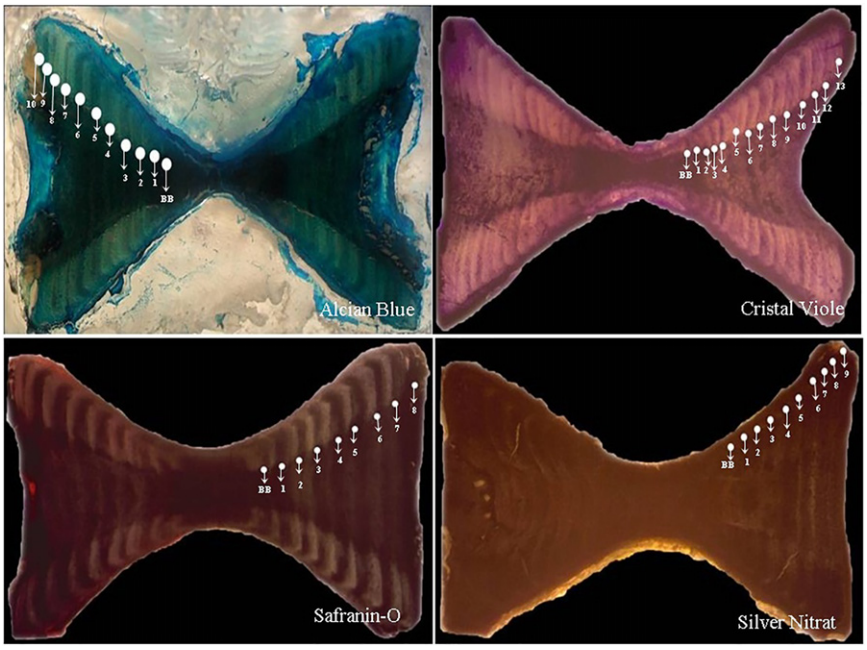

Fig. 1. Longitudinal cross-sections of different vertebral centra from same specimen of *Dasyatis pastinaca* (69.3 cm, DW) showing opaque and translucent seasonal growth bands according to four different staining methods (BB: Birth Band).

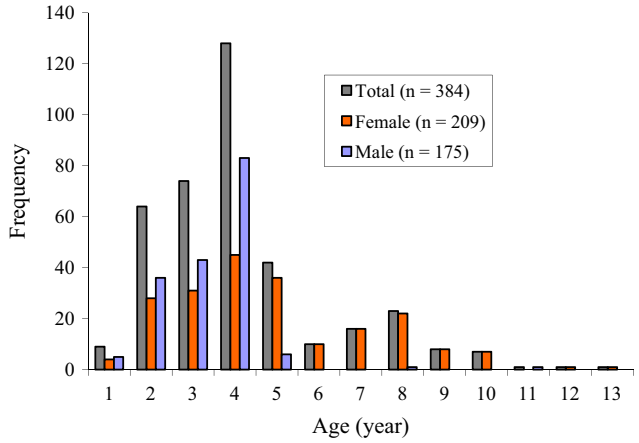

Fig. 2. Frequency distribution of *Dasyatis pastinaca,* Iskenderun Bay, 2010–2011 fishing season.

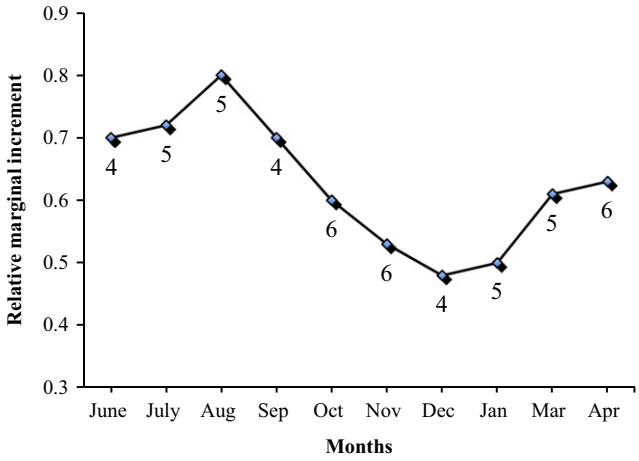

Fig. 3. Monthly variation values of Marginal Increment Ratio in *Dasyatis pastinaca*, Iskenderun Bay, 2010–2011 fishing season.

Alcian Blue. In this method, the *Alcian Blue* dye technique was used to enhance visibility of the vertebrae band. The section was soaked in *Alcian Blue* solution (16 ml 100% ethanol, 2 mg *Alcian Blue* and 4 ml glacial acetic acid in 0.8 ml distilled water) for 12 h (Başusta et al., 2008).

Crystal Violet. After each vertebra was cleaned of excess tissue, all samples were soaked in a 0.01% solution of *Crystal Violet*. Schwartz (1983) used a soak-time of 10–15 min for 12 different elasmobranch species (10 min for sharks <70 cm FL, 15 min for sharks >100 cm FL).

Silver Nitrate. All connective tissue must be removed from the centrum to ensure chemical substitution by the silver nitrate. Centrae can then be placed in a 1% silver nitrate solution for 1–3 min and simultaneously illuminated with an ultraviolet light source for anywhere between 2 and 4 min, depending on the species and size of the centrum (Stevens, 1975; Schwartz, 1983). The timing used by Cailliet et al.

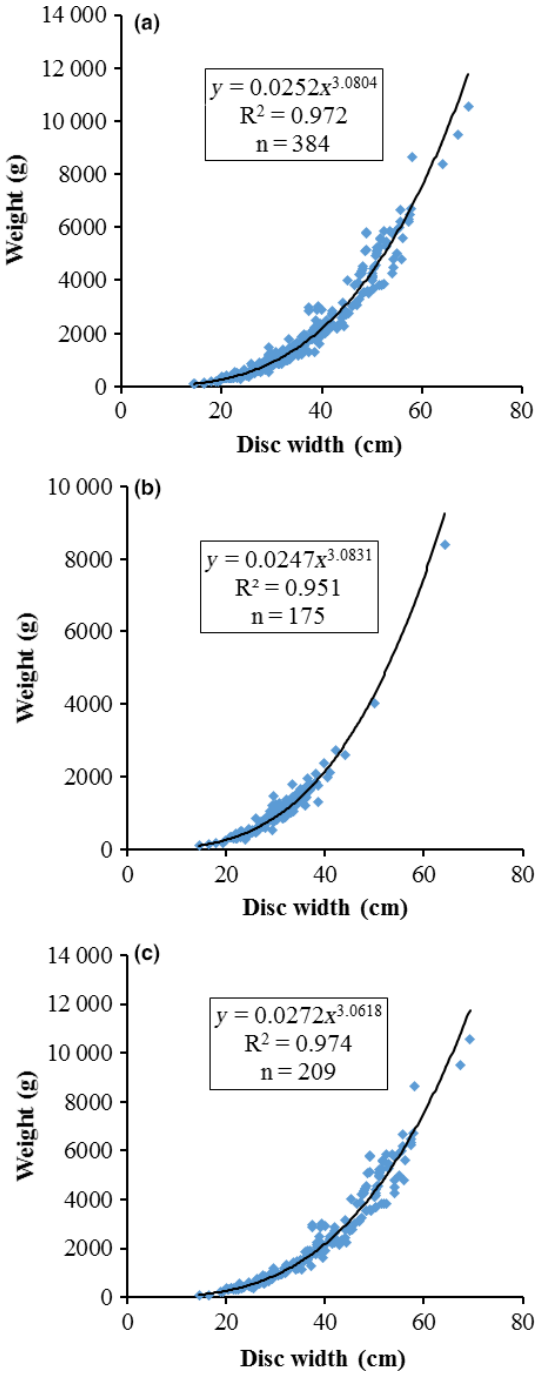

Fig. 4. Disc width–weight relationships of *Dasyatis pastinaca*, Iskenderun Bay. (a) sexes combined; (b) males; (c) females.

Table 3
von Bertalanffy growth parameters for *Dasyatis pastinaca*, Iskenderun Bay

Sex	L_∞ (cm)	k (year^{-1})	t_o(year)
Males	114.54	0.041	−3.63
Females	127.06	0.058	−1.51
Combined	104.43	0.075	−1.43

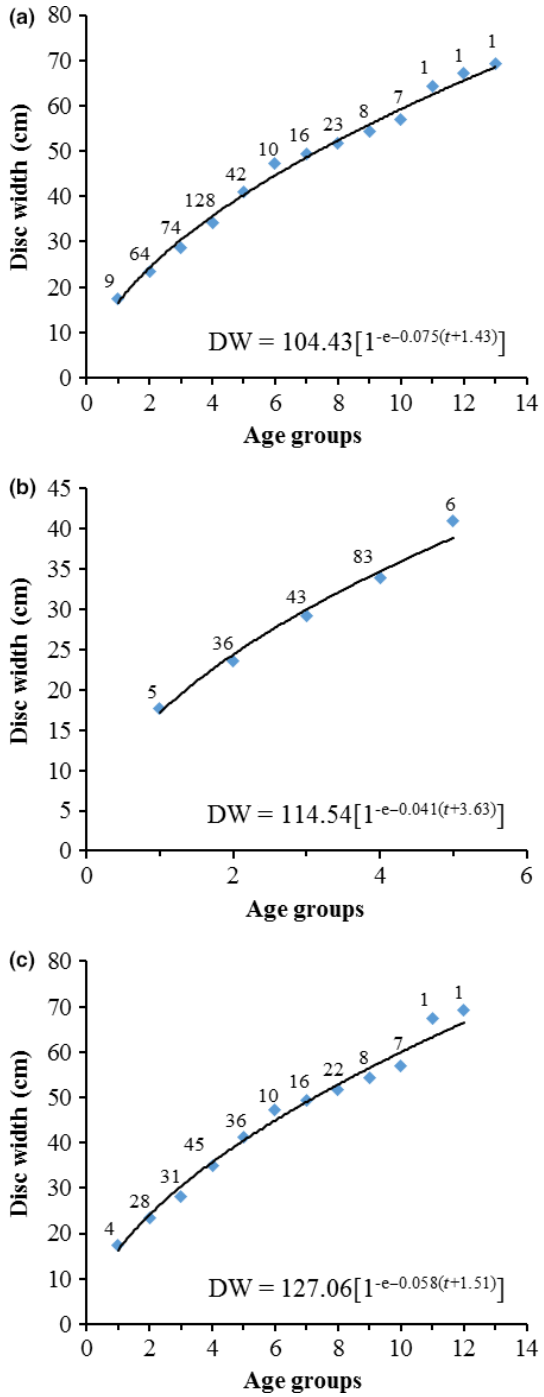

Fig. 5. von Bertalanffy growth curve of *Dasyatis pastinaca*, Iskenderun Bay. (a) sexes combined; (b) males; (c) females. Mean data, sample size (n) above data point.

(1983), however, ranged from 3 to 15 min, which is longer than what was employed here.

Counts of annuli

Vertebral sections were examined under a compound microscope using reflected light (25–40 × magnification). One growth band was defined as an opaque and translucent band

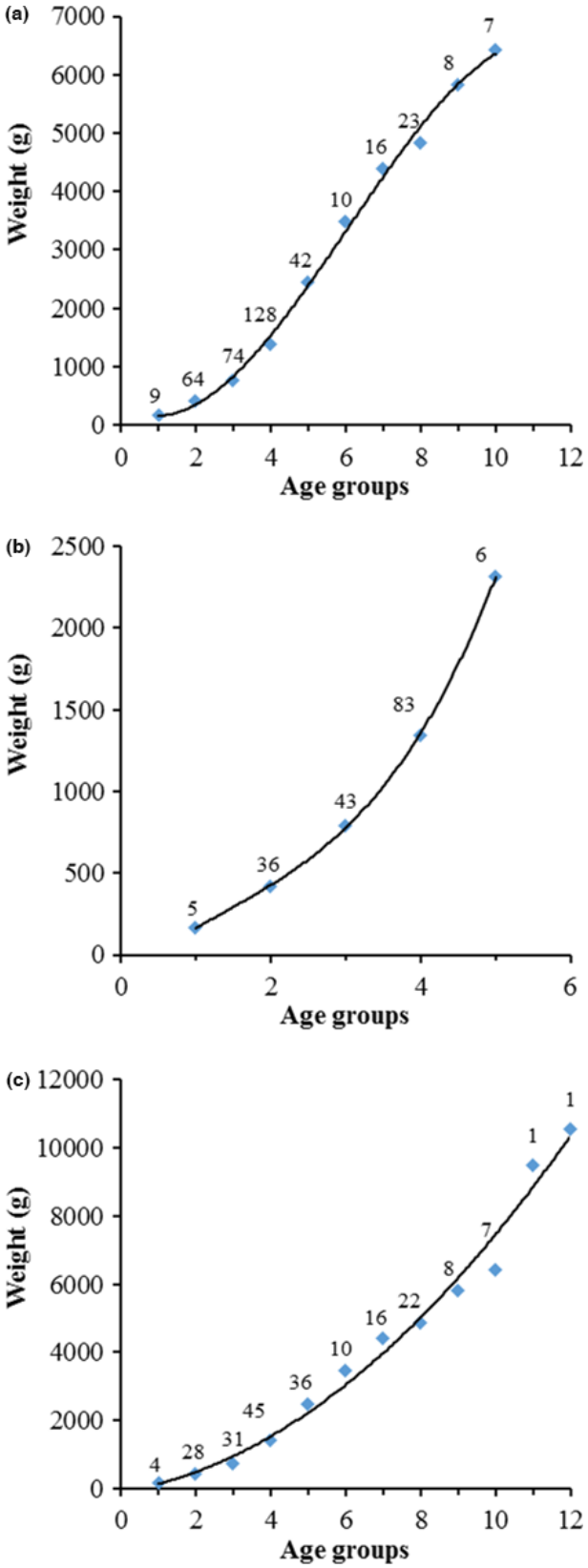

Fig. 6. Age–weight relationships of *Dasyatis pastinaca*, Iskenderun Bay. (a) sexes combined; (b) males; (c) females. Mean data, sample size (n) above data point

Table 4
Average condition values of *D. Pastinaca*, Iskenderun Bay

Age Groups	Condition factors
1	2.88
2	3.25
3	3.23
4	3.42
5	3.57
6	3.33
7	3.71
8	3.52
9	3.62
10	3.44
11	3.17
12	3.11
13	3.17
Average	3.34 ± 0.234

pair that traversed the intermedialia and clearly extended into the corpus calcareum.

The index of the average percentage error (IAPE) was calculated to assess the precision of age determinations between two independent readers. The equation (Beamish and Fournier, 1981) is expressed as:

$$\mathrm{IAPE_j} = \frac{1}{N}\sum_{j=1}^{N}\left(\sum_{j=1}^{R}\frac{x_{ij}-x_j}{x_j}\right) * 100\%$$

where N is the number of fish aged, R is the number of times each fish was aged, x_{ij} is the *ith* age determination of the *jth* fish, and x_j is the mean age calculated for the *jth* fish and the coefficient of variation (CV) and precision index (D) by Chang (1982) and Coelho and Erzini (2002). Equations used for CV and D were:

$$\mathrm{CV} = 100\% \times \frac{\sqrt{\sum_{i=1}^{R}(X_{ij}-X_j)^2/(R-1)}}{X_j}$$

where CV is the age precision estimate for the *jth* fish; *Xij* is the age determination of the *jth* fish by the *ith* reader; *Xj* is the mean age of the *jth* fish and R is the number of readings.

Index of precision (D) was calculated using $\mathrm{D} = \mathrm{CV}/\sqrt{\mathrm{Z}}$ where Z = the number of readers.

A von Bertalanffy growth function (VBGF) was fitted to the data with the von Bertalanffy (1938) equation:

$$DW_t = DW_\infty\left[1 - e^{-k(t-t_0)}\right]$$

where as, DW_t is the expected disc width at age t years. DW is the asymptotic average maximum disc width, k is the growth coefficient, and t_0 is the theoretical age at zero length.

Individual values of condition factor were obtained using the formula $K = (W/DW^b) \times 100$, where W is total weight and DW is disc width; b is the coefficient of allometry (Bagenal and Tesch, 1978).

Marginal increment analysis

The periodicity of band pair formation was investigated using the 'Marginal Increment Ratio' (MIR) (e.g. Sulikowski et al., 2003). A sub-sample of 50 vertebrae was randomly selected, which comprised both juvenile and adult specimens collected each month. The MIR was calculated as the ratio of the distance between the last and penultimate opaque bands as measured with an optical micrometer. MIR was calculated using the equation (Natanson et al., 1995):

$$\mathrm{MIR} = (VR - Rn)/(Rn - Rn - 1)$$

where R is the vertebral radius, and *Rn* and *Rn-1* are the last and penultimate opaque bands, respectively. The MIR average was plotted by the month of capture to identify trends in band formation (Cailliet, 1990; Simpfendorfer et al., 2000).

Results

Images for each individual thin vertebral section are presented in Fig. 1. These were stained with *Crystal Violet*, *Silver Nitrate*, *Safranin-O* or *Alcian Blue* to enhance the appearance of the growth rings. The application of ageing statistical measures (IAPE, CV and D) on the assessments made by readers (Table 1) showed that *Safranin-O* and *Crystal Violet* staining tended to yield lower values than *Silver Nitrate* and *Alcian Blue* and also provided the best optical resolutions of *Dasyatis pastinaca* growth rings.

A total of 384 *Dasyatis pastinaca* were examined (209 females, 175 males), ranging in disc widths from 16.6 to 69.3 cm to weights from 94 to 10564 g (Table 2). Age of the sampled population ranged from 1 to 13 years. The results showed that most individuals were in age group 4 (Fig. 2).

The band observed in the age readings represents the first year. In researching cartilaginous fish, it was found that the formation process does not equal 1, and therefore a marginal increment ratio is used (Natanson et al., 1995). Monthly variations of MIR values in *D. pastinaca* are given in Fig. 3. According to existing data, MIR began to increase from February onward and reached its highest level during summer, then began to decrease thereafter. Based on these observations, band formations tend to occur in autumn and winter.

Disc width–weight relationships for *D. pastinaca* are presented for both sexes in Fig. 4 (a-c) following an exponential relationship: $W = 0.0252*DW^{3.0804}$, $R^2 = 0.971$ for both sexes, $W = 0.0272*DW^{3.0618}$, $R^2 = 0.973$ for females and $W = 0.0247*DW^{3.0831}$, $R^2 = 0.951$ for males.

The von Bertalanffy growth equation was used to calculate growth parameters for each sex and sexes combined (Table 3). Results of the multivariate analysis show that males and females grow differently. The calculated student t-test is significant ($P < 0.05$). Females attain a larger asymptotic disc width ($DW_\infty = 127.06$ cm) than males ($DW_\infty = 114.54$ cm) and grow more slowly ($k = 0.058$ year^{-1}, $t_o = -1.508$ females and $k = 0.041$ year^{-1}, $t_o = -$-3.632 for males). Age-disc width relationships for *D. pastinaca* for both sexes are given in Fig. 5a-c. Age-weight relationship for *D. pastinaca* for both sexes, females, and males are presented in Fig. 6a-c.

Condition factors calculated for all age groups are presented in Table 4. Average condition factor value of the pop-

ulation was calculated as 3.34, with the highest condition factor value of 3.71 in age group 7.

Discussion

The precision indices (IAPE, CV and D) demonstrated that age readings stained with *Safranin-O* and *Crystal Violet* were more reproducible than the others. Although both *Safranin-O* and *Crystal Violet* staining techniques are the most suitable techniques, we recommend the *Safranin-o* technique. In addition, when the sectioning of vertebrae is made accurately, it is possible to see age rings clearly without using any chemicals for staining. However, this depends on the choice of vertebrae.

Basusta et al. (2013) identified the best choice of vertebrae for age determination in *D. pastinaca* as between the 22nd and 32nd vertebrae in the abdominal cavity. The differences in our values from the others might be by using various vertebrae from non-predefined positions.

Ismen (2003) used the *Crystal Violet* staining technique to determine the age of *D. pastinaca* in Iskenderun Bay and found a maximum age for this species of 10 years. When our data on length groups belonging to this age was compared with those of Ismen (2003), there was a similarity on lengths at early ages, but not at later ages. The reason for this difference could be the use of different techniques for determining the age of the vertebrae. We stained vertebrae after taking a cross-section, a technique that made age rings clearer.

In the present study, the weights of *D. pastinaca* individuals ranged from 159.9 to 10,560 g, with a maximum disc width of 69.3 cm. Maximum disc widths for the same species were lower in the Iskenderun Bay [51 cm in Ismen (2003) and 63.5 cm in Yeldan (2005)].

L_∞ values for *D. pastinaca* were calculated as 121.5 cm by Ismen (2003) and 294.9 cm by Yeldan (2005). At 104.87 cm the L_∞ value was smaller in our study; the reason may be the use of the disc width (fin width) to calculate L_∞, while the other studies used total length. The differences in the L_∞ values of the two cited studies may be due to differences in the total lengths obtained. This species has a tail like a whip, and tail breakage is common in this species, which affects measurements. Therefore, using the disk width (fin width) instead of total length is the most reliable method. Using the disc width is strongly recommended rather than using the total length when calculating growth values in *D. pastinaca* and other rays.

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