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Evaluation of staining techniques for the observation of growth bands in tropical elasmobranch vertebrae

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Supplementary material

Table S1. – Age and growth publications in tropical elasmobranchs (Spreadsheet in MS Excel format available at: http://scimar.icm.csic.es/scimar/supplm/sm05045TableS1.xlsx).

Table S2. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Narcine leoparda*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	21.7	76.7	27.4	38.8	0.39	65.4	**86.7**	8.6	12.1	0.25	**91.3**	76.7	**0.7**	**1.6**	0.09
Immersion oil	38.5	70.0	19.3	41.7	0.19	57.7	**86.7**	4.5	6.4	0.14	37.5	76.7	8.3	13.9	0.28
Distilled water	27.8	**86.7**	23.4	33.1	0.27	68.0	83.3	7.8	11.1	0.16	39.1	76.7	5.4	7.6	0.33
Methylene blue 1′	**75.0**	80.0	8.1	11.4	0.41	50.0	80.0	5.8	8.2	0.10	73.9	76.7	1.6	2.3	0.37
Methylene blue 2′	65.2	76.7	6.8	9.6	0.81	60.9	76.7	4.3	8.1	0.50	62.5	80.0	2.4	4.7	0.16
Methylene blue 3′	63.6	73.3	8.3	14.3	0.54	68.2	73.3	3.8	11.0	0.50	70.4	**90.0**	2.6	5.3	0.22
Crystal violet 1′	69.6	76.7	5.5	7.8	0.11	58.3	80.0	5.2	7.3	0.33	64.0	83.3	2.7	3.8	0.32
Crystal violet 3′	62.5	80.0	10.3	14.6	0.12	44.4	60.0	4.9	7.0	0.52	58.3	80.0	2.8	3.9	0.17
Crystal violet 7′	65.2	76.7	6.7	9.4	0.16	57.1	70.0	3.9	5.5	0.12	65.2	76.7	2.4	3.4	0.09
Basic fuchsin 3′	47.8	76.7	16.9	23.9	0.12	64.0	83.3	4.3	6.1	0.42	63.0	**90.0**	2.8	4.0	0.16
Basic fuchsin 5′	56.5	76.7	14.8	20.9	0.08	60.0	83.3	4.6	6.5	0.29	46.2	86.7	4.5	6.4	0.19
Basic fuchsin 7′	50.0	73.3	12.0	16.9	0.34	**73.1**	**86.7**	3.4	4.8	0.42	54.2	80.0	4.8	6.7	0.09
Alizarin red 12′	52.0	83.3	13.1	18.5	0.21	52.0	83.3	8.2	11.6	0.43	56.0	83.3	4.2	5.9	0.10
Alizarin red 14′	65.4	**86.7**	8.8	17.9	0.04	61.5	**86.7**	2.4	5.4	0.39	43.5	76.7	6.1	8.6	0.51
Alizarin red 16 ‘	73.1	**86.7**	**4.9**	**7.0**	0.14	64.0	83.3	**3.3**	**4.6**	0.22	68.2	73.3	3.2	4.5	0.41

Table S3. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Urotrygon aspidura*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	66.7	96.7	14.7	20.6	0.56	45.7	80.0	9.5	13.4	0.18	71.4	100.0	3.3	4.7	0.29
Alcohol	70.0	**100.0**	10.9	15.4	0.37	40.6	78.1	15.6	22.1	0.47	65.2	100.0	3.9	5.5	0.41
Distilled water	56.7	96.7	20.2	28.6	0.48	43.8	75.0	9.7	13.7	0.29	76.0	100.0	2.9	4.1	0.34
Methylene blue 7′	31.3	**100.0**	34.6	48.9	0.26	–	–	–	–	–	–	–	–	–	–
Methylene blue 10′	41.2	94.1	26.3	37.1	0.06	50.0	95.8	12.7	18.0	0.80	**80.0**	**100.0**	**2.2**	**3.1**	**0.39**
Methylene blue 15′	29.4	88.2	34.7	49.0	0.41	56.0	92.0	11.3	16.0	0.50	73.3	100.0	3.2	4.5	0.56
Methylene blue 20′	–	–	–	–	–	**84.2**	**89.5**	**1.2**	**1.7**	**0.32**	60.0	100.0	4.3	6.1	0.20
Crystal violet 7′	31.3	93.8	31.3	44.3	0.29	29.2	54.2	16.3	23.1	0.31	25.0	75.0	8.2	11.7	0.31
Crystal violet 10′	18.8	87.5	34.3	48.5	0.50	25.0	58.3	20.5	29.1	0.41	50.0	75.0	4.1	5.8	0.26
Crystal violet 15′	68.8	81.3	9.2	13.1	0.37	50.0	70.8	4.7	6.7	0.39	31.3	93.8	9.5	13.4	0.59
Basic fuchsin 1′	35.3	82.4	37.1	52.5	0.26	45.0	85.0	16.5	23.3	0.03	–	–	–	–	–
Basic fuchsin 5′	29.4	94.1	47.9	67.8	0.10	40.9	95.5	19.1	27.0	0.42	50.0	100.0	8.4	11.8	0.38
Basic fuchsin 10′	46.7	93.3	27.6	39.1	0.56	45.5	95.5	21.3	30.1	0.55	68.8	100.0	4.4	6.3	0.53
Basic fuchsin 20′	–	–	–	–	–	–	–	–	–	–	50.0	100.0	7.0	9.9	0.86
Alizarin red 3′	–	–	–	–	–	–	–	–	–	–	66.7	100.0	5.1	7.3	0.08
Alizarin red 7′	61.1	77.8	7.1	10.1	0.57	52.9	82.4	14.4	20.3	0.56	60.0	100.0	6.1	8.6	0.57
Alizarin red 10′	50.0	72.2	14.4	20.3	0.22	52.9	76.5	8.8	12.4	0.51	46.7	100.0	8.8	12.4	0.55
Alizarin red 15′	**70.6**	94.1	11.7	16.5	0.26	73.3	**100.0**	5.5	7.7	0.56	–	–	–	–	–
Light green 3′	56.3	81.3	9.2	13.1	0.51	81.3	93.8	3.6	5.0	0.37	73.3	100.0	5.1	7.2	0.26
Light green 5′	**64.7**	**76.5**	**5.1**	**7.3**	**0.16**	75.0	93.8	4.5	6.4	0.39	73.3	100.0	4.7	6.7	0.51
Light green 10′	53.0	70.6	6.1	8.6	0.22	62.5	87.5	6.7	9.4	0.51	66.7	100.0	4.7	6.7	0.39

Table S4. – Results of precision and bias tests between readers, for each of the treatments applied to the small, medium and large vertebrae of *Hypanus longus*. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test for each vertebrae size are highlighted in bold and the selected treatments are shaded in gray.

	Small (30)					Medium (30)					Large (30)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	86.7	100	8.9	12.6	0.37	73.3	100	3.4	4.8	0.39	80.0	100	1.6	2.3	0.57
Basic fuchsin 1′	73.3	100	8.9	12.6	0.05	66.7	100	4.4	6.2	0.29	80.0	100	1.4	1.9	0.39
Basic fuchsin 2′	80.0	100	6.7	9.4	0.08	**93.3**	**100**	**2.2**	**3.1**	**0.32**	86.7	100	0.9	1.3	0.37
Basic fuchsin 3′	73.3	100	13.3	18.9	0.14	53.9	100	7.9	11.1	0.55	86.7	100	1.2	1.7	0.16
Bismarck brown 0.5′	86.7	100	4.4	6.3	0.16	–	–	–	–	–	93.3	100	0.5	0.7	0.32
Bismarck brown 1′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	–	–	–	–	–	–	–	–	–	–
Bismarck brown 1.5′	–	–	–	–	–	–	–	–	–	–	86.7	100	1.0	1.4	0.37
Bismarck brown 2.0′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	86.7	100	1.9	2.7	1.00	–	–	–	–	–
Bismarck brown 3.0′	–	–	–	–	–	86.7	100	1.9	2.7	1.00	86.7	100	1.0	1.4	0.32
Bismarck brown 5.0′	–	–	–	–	–	80.0	100	3.6	5.1	0.61	–	–	–	–	–
Alizarin red 1′	–	–	–	–		–	–	–	–	–	86.7	100	1.0	1.5	1.00
Alizarin red 2′	–	–	–	–	–	–	–	–	–	–	93.3	100	0.5	0.7	0.32
Alizarin red 3′	–	–	–	–	–	–	–	–	–	–	86.7	100	0.8	1.2	0.37
Light green 0.5′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	–	–	–	–	–	–	–	–	–	–
Light green 1′	**93.3**	**100**	**2.2**	**3.1**	**0.32**	80.0	100	3.2	4.6	0.22	–	–	–	–	–
Light green 2′	86.7	100	8.9	12.6	0.37	80.0	100	3.2	4.6	0.22	80.0	100	1.2	1.8	0.22
Light green 3′	–	–	–	–	–	86.7	100	2.3	3.2	0.37	–	–	–	–	–
Light green 5′	–	–	–	–	–	–	–	–	–	–	86.7	100	0.8	1.2	0.37
Light green 7′	–	–	–	–	–	–	–	–	–	–	**100**	**100**	**0.0**	**0.0**	**NA**

Table S5. – Results of precision and bias tests between readers for each of the treatments applied to *Potamotrygon magdalenae, Alopias pelagicus* and *Carcharhinus falciformis* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

	Potamotrygon magdalenae (24)					*Alopias pelagicus* (27)					*Carcharhinus falciformis* (21)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	87.5	80.0	1.2	2.5	0.16	41.7	52.2	3.6	5.1	0.54	0.0	54.6	12.6	17.8	0.42
Distilled water	53.3	80.0	2.6	3.7	0.61	41.7	52.2	4.9	6.5	0.54	60.0	45.5	4.3	5.0	0.39
Immersion oil	81.3	75.0	6.6	9.4	0.14	60.0	65.2	4.6	6.9	0.42	57.1	63.6	3.5	6.1	0.37
Methylene blue 15′	–	–	–	–	–	0.0	25.0	20.8	29.4	0.39	–	–	–	–	–
Methylene blue 20′	58.8	85.0	8.5	12.0	0.29	25.0	33.3	4.3	6.1	0.39	66.7	54.6	2.2	3.1	0.37
Methylene blue 25′	–	–	–	–	–	16.7	50.0	9.4	13.3	0.42	16.7	54.6	9.3	13.1	0.42
Methylene blue 30′	57.9	**95.0**	6.3	8.9	0.09	–	–	–	–	–	83.3	54.6	2.8	3.9	0.32
Methylene blue 35′	–	–	–	–	–	–	–	–	–	–	57.1	63.6	6.9	9.7	0.39
Methylene blue 40′	47.4	**95.0**	10.6	14.9	0.43	–	–	–	–	–	–	–	–	–	–
Crystal violet 15′	–	–	–	–	–	20.0	45.5	2.3	3.3	0.37	–	–	–	–	–
Crystal violet 20′	–	–	–	–	–	28.6	58.3	6.5	9.2	0.29	57.1	63.6	5.7	8.0	0.32
Crystal violet 25′	–	–	–	–	–	22.2	81.8	9.1	12.8	0.43	55.6	81.8	3.5	5.0	0.41
Crystal violet 30′	–	–	–	–	–	–	–	–	–	–	55.6	81.8	2.9	4.1	0.41
Crystal violet 35′	–	–	–	–	–	–	–	–	–	–	85.7	63.6	**0.5**	0.7	0.42
Crystal violet 40′	66.7	90.0	3.8	5.4	0.20	–	–	–	–	–	–	–	–	–	–
Crystal violet 50′	88.2	85.0	1.3	1.8	0.37	–	–	–	–	–	–	–	–	–	–
Crystal violet 60′	**100.0**	75.0	**0.0**	**0.0**	NA	–	–	–	–	–	–	–	–	–	–
Acid fuchsin 40′	–	–	–	–	–	–	–	–	–	–	75.0	72.7	1.4	2.0	0.37
Acid fuchsin 45′	–	–	–	–	–	–	–	–	–	–	75.0	40.0	2.8	3.2	0.32
Acid fuchsin 50′	–	–	–	–	–	–	–	–	–	–	80.0	45.5	0.7	1.0	0.37
Basic fuchsin 35′	–	–	–	–	–	33.3	75.0	4.4	6.2	0.54	–	–	–	–	–
Basic fuchsin 40′	–	–	–	–	–	22.2	75.0	6.8	9.6	0.43	20.0	45.5	6.5	9.1	0.39
Basic fuchsin 45′	–	–	–	–	–	36.4	**91.7**	4.8	6.8	0.43	66.7	54.6	2.6	3.6	0.32
Basic fuchsin 50′	–	–	–	–	–	–	–	–	–	–	75.0	36.4	1.6	2.2	0.32
Bismarck brown 10′	85.7	87.5	9.5	13.5	0.37	50.00	72.7	2.5	3.5	0.41	–	–	–	–	–
Bismarck brown 15′	–	–	–	–	–	**57.1**	**77.8**	1.8	2.5	0.39	–	–	–	–	–
Bismarck brown 20′	78.6	87.5	10.0	14.1	0.22	44.4	75.0	5.5	7.8	0.42	75.0	72.7	1.8	2.5	0.37
Bismarck brown 25′	–	–	–	–	–	–	–	–	–	–	57.1	70.0	2.7	3.9	0.39
Bismarck brown 30′	78.6	87.5	3.2	4.6	0.39	–	–	–	–	–	83.3	66.7	0.8	1.1	0.32
Bismarck brown 35′	–	–	–	–	–	–	–	–	–	–	66.7	60.0	1.4	2.0	0.32
Dahl staining10-10	–	–	–	–	–	–	–	–	–	–	50.0	40.0	3.4	4.8	0.37
Dahl staining 15-15	–	–	–	–	–	–	–	–	–	–	80.0	50.0	0.9	1.2	0.32
Dahl staining 5-5	–	–	–	–	–	–	–	–	–	–	50.0	60.0	5.5	7.8	0.39
Silver nitrate 1-1	–	–	–	–	–	–	–	–	–	–	60.0	35.7	1.8	2.5	0.39
Silver nitrate 2-3	–	–	–	–	–	–	–	–	–	–	**100.0**	16.7	0.0	**0.0**	NA
Alizarin red 3′	82.4	85.0	3.8	5.4	0.16	–	–	–	–	–	–	–	–	–	–
Alizarin red 5′	95.0	83.3	0.5	0.8	0.16	**100.0**	28.6	**0.0**	**0.0**	0.43	–	–	–	–	–
Alizarin red 7′	95.5	91.7	0.1	1.3	0.16	66.7	42.9	1.6	2.2	0.32	–	–	–	–	–
Alizarin red 9′	–	–	–	–	–	33.3	42.9	4.7	6.5	0.37	–	–	–	–	–
Alizarin red 10′	–	–	–	–	–	–	–	–	–	–	28.6	70.0	4.2	5.9	0.70
Alizarin red 15′	–	–	–	–	–	–	–	–	–	–	16.7	60.0	5.9	8.3	0.26
Alizarin red 20′	–	–	–	–	–	–	–	–	–	–	33.3	60.0	6.0	8.5	0.41
Light green 20′	–	–	–	–	–	42.9	77.8	4.6	6.5	0.41	33.3	33.3	2.6	3.6	0.37
Light green 25′	–	–	–	–	–	50.0	44.4	3.6	5.1	0.37	33.3	33.3	3.6	5.1	0.37
Light green 30′	81.8	64.7	2.12	3.0	0.37	66.7	66.7	2.1	2.9	0.37	22.2	22.2	5.2	7.4	0.31
Light green 35′	75.0	70.6	4.1	5.8	0.39	–	–	–	–	–	33.3	**100.0**	5.4	7.7	0.39
Light green 40′	**100.0**	52.9	**0.0**	**0.0**	NA	–	–	–	–	–	28.6	70.0	5.7	8.0	0.42

Table S6. – Results of precision and bias tests between readers for each of the treatments applied to *Sphyrna lewini*, *Sphyrna corona* and *Mustelus lunulatus* vertebrae. IAPE, index of average error percentage; CV, coefficient of variation; PA (± 0 bands), percentage of total agreement; RV, percentage of read vertebrae; P, p-values of the Bowker symmetry test. Best results of each test are highlighted in bold and the selected treatment for each species.is shaded in gray.

	Sphyrna lewini (52)					*Sphyrna corona* (18)					*Mustelus lunulatus* (15)				
Treatment (Stain+Time)	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P	PA	RV	IAPE	CV	P
No staining	76.0	96.2	20.8	29.5	0.41	75.0	66.7	6.1	8.7	0.39	45.5	68.8	11.0	15.6	0.31
Immersion oil	**89.8**	94.2	**6.9**	**9.8**	0.14	78.6	**77.8**	4.0	5.2	0.57	45.5	73.3	8.6	12.1	0.67
Agua destilada	72.3	90.4	16.6	23.5	0.05	66.7	66.7	4.0	5.2	0.39	27.3	73.3	11.9	16.9	0.30
Methylene blue 20′	–	–	–	–	–	–	–	–	–	–	53.3	93.8	7.6	10.7	0.41
Methylene blue 25′	68.2	84.6	19.5	27.6	0.51	81.8	61.1	2.5	3.6	0.37	–	–	–	–	–
Methylene blue 30′	79.5	84.6	12.1	17.1	0.56	60.0	55.6	6.0	8.5	0.14	60.0	93.8	5.7	8.1	0.20
Methylene blue 35′	84.4	86.5	12.2	17.2	0.26	55.6	50.0	10.8	15.2	0.14	–	–	–	–	–
Methylene blue 40′	–	–	–	–	–	–	–	–	–	–	69.2	81.3	5.0	7.1	0.40
Crystal violet 20′	61.9	89.4	34.9	49.4	–	**100.0**	55.6	**0.0**	**0.0**	0.16	71.4	87.5	3.2	4.5	0.41
Crystal violet 25′	62.7	**98.1**	27.9	39.4	0.06	80.0	55.6	1.8	2.6	0.51	–	–	–	–	–
Crystal violet 30′	64.0	96.1	29.0	40.9	0.09	55.6	50.0	5.3	7.4	0.14	62.5	**100.0**	5.7	8.1	0.32
Crystal violet 35′	–	–	–	–	0.31	–	–	–	–	–	–	–	–	–	–
Crystal violet 40′	–	–	–	–	––	–	–	–	–	–	56.3	**100.0**	6.2	8.8	0.20
Basic fuchsin 40′	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–
Basic fuchsin 45′	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–	–
Basic fuchsin 50′	–	–	–	–	0.19	–	–	–	–	–	–	–	–	–	–
Bismarck brown 20′	71.1	91.8	23.8	33.7	0.15	50.0	66.7	8.7	12.3	0.42	66.7	64.3	6.9	9.7	0.50
Bismarck brown 25′	71.1	90.0	20.2	28.6	0.02	60.0	55.6	6.5	9.2	0.41	–	–	–	–	–
Bismarck brown 30′	65.1	87.8	22.8	32.3	0.21	76.9	72.2	5.0	7.0	0.39	55.6	64.3	9.4	13.2	0.39
Bismarck brown 40′	–	–	–	–	–	–	–	–	–	–	44.4	64.3	10.4	14.7	0.41
Alizarin red 5′	70.6	89.5	27.5	18.7	–	61.5	72.2	7.6	10.8	0.42	36.4	73.3	12.4	17.5	0.41
Alizarin red 10′	78.7	88.7	13.2	32.4	0.15	53.8	72.2	7.0	9.8	0.41	53.8	76.5	7.6	10.8	0.56
Alizarin red 15′	75.0	95.7	22.9	38.8	0.26	75.0	66.7	3.7	5.2	0.39	40.0	88.2	9.0	12.7	0.55
Alizarin red 20′	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–
Light green 20′	–	–	–	–	–	–	–	–	–	–	69.2	92.9	3.6	5.1	0.55
Light green 25′	69.8	93.5	24.0	34.0	–	75.0	66.7	3.4	4.8	0.22	–	–	–	–	–
Light green 30′	73.9	90.2	21.7	30.7	0.17	64.3	77.8	6.1	8.7	0.39	91.7	85.7	1.2	1.7	0.61
Light green 35′	74.5	92.2	17.3	24.5	0.26	75.0	66.7	4.4	6.2	0.22	–	–	–	–	–
Light green 40′	–	–	–	–	0.12	–	–	–	–	–	**100.0**	78.6	**0.0**	**0.0**	0.30

SPECIES COMMENTS

Narcine leoparda

For this species a detailed analysis of vertebrae size effect on the growth bands visualization was performed. This is an approach rarely used, since most studies applying staining techniques do not discriminate them by size. Results showed that large vertebrae were easily read without staining, while medium and small vertebrae needed alizarin red for 16 min too enhance growth bands visualization. Thus, IAPE analysis of the assessed treatments showed an effect of the dyes on the increase of growth bands readings accuracy. There are no comparison studies for the species, however, alizarin red has shown good performance in other batoid species, even in families with significant differences in size, distribution and phylogenetic affinity, as *Raja undulata* (Coelho and Erzini 2002).

Urotrygon aspidura

Section thickness can affect the appropriate visualization of growth bands, near the focus (when defining the birthmark) and in those vertebrae with the greatest number of bands towards the edges (due to their accumulation), making difficult to count them. In the present study, appropriate thickness was chosen at 0.4 mm for best visualization of growth bands in *U. aspidura* since 0.3 mm allowed too much light to pass through the vertebra section and at 0.5 mm it was to dark, affecting the clarity of the growth bands. This result is in the range (0.3 to 0.5 mm) of age and growth studies in similar sizes species of the families Urotrygonidae, Uroplophidae and Narcinidae (White et al. 2001, Hale and Lowe 2008, Pérez-Rojas 2013, Mejía-Falla et al. 2014, Guzmán-Castellanos 2015, Santander-Neto 2015), as well as in numerous elasmobranch species (Goldman et al. 2012).

For *U. aspidura*, treatments without dyes showed a lower performance than those with it. Methylene blue for 10 min and 20 min were the most suitable for large and medium vertebrae, respectively; while light green for 5 min was the most accurate technique for small vertebrae. Mejía-Falla et al. (2014) identified that *U. rogersi* vertebrae without staining showed better results than those stained with alizarin red, crystal violet and methylene blue. Studies in other species of the family (*Urobatis halleri, Urotrygon microphthalmum, Urotrygon chilensis*) have not assessed any technique to enhance the growth bands visualization, always working on unstained vertebrae sections (Hale and Lowe 2008, Guzmán-Castellanos 2015, Santander-Neto 2015).

Hypanus longus

Sections of 0.3mm did not allow an adequate visualization of growth bands, since it increased presence of sub and pre-bands, which is related to: 1) amount of light that passed through the sample was greater, which directly affects the ability of the reader to detect the presence of the bands; 2) sections obtained with this thickness were more fragile than the other samples, which causes these structures to fracture easily and therefore lose samples, increasing the number of individuals needed for an age and growth study.

Sections of 0.5 and 0.6 mm did not facilitate the reading of growth bands in the vertebrae and, as with thin samples, light was an important factor. In these sections, the amount of light that passed through was not sufficient and, consequently, contrast between calcium deposits was hard to identify, underestimating the growth bands counting. Results of qualitative evaluation of the thickness in the present research were similar to those made in other studies with rays, where these thicknesses (0.5 and 0.6mm) were also not suitable for growth band count in *Narcine leoparda* and *Urotrygon aspidura* (Pérez-Rojas 2013, Torres-Palacios et al. 2019).

Best results were obtained using sections of 0.4 mm since light that passed through the sample was enough and allowed an adequate contrast between the calcium deposits. Additionally, vertebrae sections were not so fragile compared to 0.3 mm, reducing the loss of samples. This result is also reported for other related species (*Squalus acanthias* and *Urotrygon aspidura*), where 0.4 mm was reported as the optimum thickness for growth bands readings (e.g. Bubley et al. 2012, Torres-Palacios et al. 2019).

It is important to note that there are also contrasting results to those found in the present study, since larger or smaller section thicknesses have been proposed as the most suitable for counting growth bands in other species such as *Mustelus canis, Mustelus asterias, Prionace glauca* and *Pristis pectinata* (e.g. Conrath et al. 2002, Parra et al. 2008, Farrell et al. 2010, Scharer et al. 2012). This implies that adequate visualization of bands with a certain section thickness is not an attribute shared in all chondrichthyan species, nor even in species of the same genus, as was the case of *Dasyatis centroura* and *Dasyatis pastinaca* (Ba usta and Sulikowski 2012, Yigin and Ismen 2012), where the section thickness chosen by the authors was 0.6 mm and 0.5 mm, respectively, differing from the optimum in the present study.

Use of dyes in calcified structures has been widely used in several studies of elasmobranchs (Cailliet and Goldman 2004), where they assessed the effectiveness of stains in different species. Although for *H. longus* some treatments (methylene blue, crystal violet, acid fuchsine, immersion oil) were not adequate, it is important to highlight that these dyes allowed correct visualization in other species of rays, as *U. aspidura*, where the methylene blue treatment yielded better results for large and medium vertebrae (Torres-Palacios et al. 2019), and *Myliobatis californica* where crystal violet allowed a better visualization of growth bands (Aguirre-García 2009).

Data obtained in the present study suggest that observation and counting of growth bands in *H. longus* should be carried out using dyes treatments, since control vertebrae (without staining), belonging to the large and medium sizes, presented very low values of percentage of agreement (PA). This situation differs from other sharks and rays' studies (*Prionace glauca* and *Rhinoptera steindachneri*), which have shown high counting effectiveness of growth bands without using any dyes (e.g. Lessa et al. 2004, Pabón-Aldana 2016).

According to the results here obtained, it is recommend that age and growth studies of *H. longus* consider using light green for seven minutes in larger vertebrae ($\approx \geq 8$ 7.57 - 12.34mm), basic fuchsin (0.01%) for two minutes in medium vertebrae ($\approx$ 4.40 5 - 8.37 mm) and Bismark brown (0.05%) for one or two minutes, or light green (0.05%) for 0.5-1 min in small vertebrae ($\approx \leq$ 3.08 - 5.15 mm).

However, these treatments should not be generalized to other species of the same genus, since it has been shown that, in *Hypanus dipterurus*, the light green dye is the least suitable for growth bands observation (Carmona-Sánchez 2017). This author found that the best treatment for large vertebrae was Bismarck brown for seven minutes; for medium samples it was not necessary to apply stains, since an adequate visualization of the growth bands without staining was obtained; and finally for small vertebrae, Bismarck brown three minutes was selected as the best treatment (Carmona-Sánchez 2017).

This procedure, in spite of appearing simple and at the same time requiring an additional time, will allow that in future studies of age and growth, readings can be obtained with greater precision and less bias, and therefore estimates of growth parameters with less uncertainty. Additionally, it will reduce the waste of samples, which implies a smaller number of individuals to be slaughtered to carry out the respective studies of age and growth.

Potamotrygon magdalenae

Results obtained here showed that alizarin red for seven minutes was the technique that generated the greatest accuracy in estimating age of the species, without showing systematic bias. This treatment was followed by crystal violet. Species phylogenetically close to the Potamotrygonidae family, such as *D. parsnip*, have shown more accurate age estimates with the use of safranin (Girgin and Basusta 2016) and crystal violet (Yigin and Ismen 2012), while in *P. leopoldi* Charvet et al. (2018) used no stained treatments to assess age and growth using vertebrae sections. Also, the results of this study in *U. aspidura* identified methylene blue and light green as the best enhancement techniques, indicating that dyes and treatments can vary according to their affinity to calcium and phosphorus concentrations in each species, and therefore ratified the importance of making this type of techniques assessment for each species, before carrying out an age and growth study.

Alopias pelagicus

This species presented a high degree of difficulty for the growth bands observation and therefore the pattern definition. This influenced the final decision on the technique that was considered as the most appropriate. Alizarin red (with five and seven minutes) showed the best precision results, but a very low %R, while Bismarck brown 15 min showed an acceptable performance in precision and reading, being defined as the most suitable treatment for the species. According to this, it is highly recommended to perform different precision tests, assessing them and choosing according to what the indices show. Thus, a treatment can present high precision and very low bias, but it will not be useful if the %R is low. This situation can occur in species with poorly defined band patterns, which is generated by the low calcification of the same, as has been proposed for *Alopias superciliosus* (Fernández-Carvalho et al. 2011), who found that crystal violet stains generated the lowest values of CV and IAPE.

Drew et al. (2015) solved the problem of the vertebrae porosity of *A. pelagicus* by imbibing them in resin for later cutting. Of course, that procedure eliminated the possibility of staining. Therefore, to combine these two procedures and enhance the results, we suggest dyeing the entire vertebrae and then proceed with the cut in resin.

Carcharhinus falciformis

Age and growth studies of this species, have used undyed vertebrae (Sánchez-de Ita et al. 2011), vertebrae dye with alizarin red for 30 min (Oshitani et al. 2003) and vertebrae subjected to X-rays (Joung et al. 2008); however, none of these studies have precision tests to evaluate age estimation and therefore the results of this study compared with studies conducted for other species of the same genus. Carlson et al. (2003) estimated a higher accuracy for *Carcharhinus isodon* with vertebrae stained with 0.01% crystal violet. On the other hand, Cruz-Martínez et al. (2005) concluded that alizarin red was the best technique for improving the visualization of growth bands of *Carcharhinus leucas*. In this study, crystal for 35 min was defined as the best treatment, obtaining values of IAPE and PA (± 0 bands) similar to those obtained in the mentioned works. The low performance obtained with Dahl method, basic fuchsin and alizarin red, suggest that they can be discarded in future studies for this species.

Sphyrna lewini

Vertebrae treated with violet crystal for 25 min and immersion oil did not show significant differences between the precision indexes and the %R. Based on this, it is more efficient to use immersion oil since time investment is significantly lower. About other dyes, Zarate-Rustrián (2010) found a better bands visualization using unstained vertebrae sections, compared to vertebrae stained with alizarin red, being this similar to the present research. However, Zarate-Rustrián (2010) did not perform an analysis of precision between techniques to compare their effectiveness. In this species it has also been recorded that vertebrae stained with 0.01% crystal violet generate acceptable reading results (Anislado-Tolentino and Robinson-Mendoza 2001, Anisaldo-Tolentino et al. 2008, Piercy et al. 2007). This information shows that different techniques can generate acceptable results in the same species, and hence the need to evaluate several treatments.

Sphyrna corona

Crystal violet for 20 min showed the best visualization results for this species. Given that this is a rare and endemic species of the Tropical Eastern Pacific, there is no comparable age and growth research on it. However, close species like *S. tiburo* has estimates of age and growth (Carlson and Parsons 1993, Frazier et al. 2014), but these studies did not include techniques assessment to improve the visualization of growth bands, and the authors made age estimation using unstaining vertebrae, founding good PA and IAPE values for Florida and the western Atlantic.

Given that crystal violet was the best dye for *S. corona* and the second best for *S. lewini*, it is suggested that this dye may be a promising option for age studies in tropical species of this genus. However, and given that the use of immersion oil and unstained vertebrae have been successful in *S. lewini* and *S. corona*, it is also possible that natural configuration of the vertebrae is good enough to develop the studies without the need for stains. This, however, should be evaluated by each researcher at the time of developing his research.

Mustelus lunulatus

Assessment of precision and bias highlighted the fact that all treatments presented a %R higher than 60%, being light green for 40 min the best treatment in this species assessment. As in many other tropical species, there are no previous studies of age and growth for *M. lunulatus*. At genus level, Conrath et al. (2002) and Farrell et al. (2010) estimated age and growth of *M. canis* and *M. asterias* in the Northwest Atlantic and England, respectively, using unstained vertebrae, and defining a PA = 84% and 93%. In this study, all dyes treatments showed PA values (± 0 bands) higher than Conrath et al. (2002). On the other hand, Méndez (2008) founded a PA = 84%, IAPE = 8.5% and CV = 0.12 in vertebrae stained with violet crystal at 0.001%, for *M. henlei* in the Gulf of California, Mexico. These results show lower PA values and higher CV and IAPE in comparison with the results for *M. lunulatus* presented

in this research. However, the author did not compare the accuracy of the age estimation with other methods. Finally, Yudin and Cailliet (1990) tested different techniques in *M. californicus* and *M. henlei*, finding that, X-ray radiographs were the most successful technique to enhance visualization. However, as already mentioned, these techniques require greater logistic and economic investment.

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