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Seasonal changes in gonad maturity, proximate and fatty acid composition of Limbaugh's damselfish, *Chromis limbaughi* Greenfield & Woods, 1980 (Pisces: Pomacentridae)

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Abstract: Limbaugh's damselfish, *Chromis limbaughi* Greenfield & Woods, 1980, is endemic to the Gulf of California, and one of the five most exploited species for the aquarium trade in this region. *C. limbaughi* is a gonochoristic, gregarious and territorial species without sexual dimorphism that inhabits rocky, sheltered areas. Development of captive breeding techniques for this species would not only ensure a continued supply of fish for the commercial trade, but perhaps more importantly, it would also alleviate fishing pressure and support stock enhancement. Thus, as a first step towards achieving these goals, in this work, we investigated some aspects of the reproductive biology of *C. limbaughi*. Seasonal fish samplings, with a total of eighty-nine fish caught in one year, were carried out at San Esteban Island, Gulf of California, Mexico. The reproductive season of *C. limbaughi* extends, at least, from May to September. A new maximum standard length of 10.5 cm is reported for this species. The estimated size at first sexual maturity was 7.90 cm for males and 7.59 cm for females. For both male and female gonads, the major constituent fatty acids were palmitic acid, stearic acid, oleic acid, docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid. The water-quality conditions under which maturation of *C. limbaughi* took place were measured, and should prove useful for the management of broodstock in captivity.

Keywords: *Chromis limbaughi*; gonad maturity, fatty acid profile; developmental stages; proximate composition

INTRODUCTION

For many years the trade of marine ornamentals has been a profitable activity worldwide [1,2]. However, this market is not without controversy, as most of the traded marine organisms are captured from the wild [3]. This is also the case of Limbaugh's damselfish, *Chromis limbaughi*, one of the five most heavily exploited fish species in the Gulf of California, Mexico, for the aquarium trade [4]. *C. limbaughi*, a member of the family Pomacentridae, is a beloved species for fish hobbyists for several reasons. It is an eye-catching damselfish, beautifully colored in blue and yellow, especially in the juvenile stages. It is a relatively rare species, endemic to the Gulf of California. And last but not least, unlike many other damselfishes, which typically are highly territorial and aggressive, *C. limbaughi* is reasonably mild-tempered, compatible with many other fishes in the community aquarium. Although *C. limbaughi* is in the list of ornamental fishes with special protection status in the Mexican legislation for wildlife conservation NOM-059-SEMARNAT-2001 [5], the Mexican government has issued fishing permits for *C. limbaughi* and other species for small-scale fishing cooperatives in the Gulf of California. Regrettably, the actual data on fish catches are unknown because fisheries record-keeping and reporting are either nonexistent or not enforced [4]. Thus, there has long been strong suspicion of overexploitation and/or illegal fishing [6,7].

In order to not only lessen fishing pressure, but to enhance stock, the reproduction of threatened fish species in captivity is highly desirable [3,8]. However, knowledge of various aspects of fish reproductive biology is essential for developing successful, captive breeding programs, e.g., identifying and monitoring gonad developmental stages is crucial for the timing of spawning [9]. On the other hand, the allocation of nutrients for gonad development is a well-known process in fish. For example, lipids are important components

of vitellogenin [10], which is incorporated into developing oocytes [11,12]. Among lipids, fatty acids play key roles in broodstock nutrition. While saturated and monounsaturated fatty acids are usually catabolized for energy, long-chain polyunsaturated fatty acids (LC-PUFA) accumulate in sufficient amounts in oocytes to guarantee adequate hatching and early larval growth and survival [13]. Specifically, docosahexaenoic acid (22:6n-3, DHA) is an important constituent of the central nervous system tissues [14]. Eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA) act as prostaglandin precursors, which stimulate steroidogenesis in the ovaries and testicles [15]. In addition, ARA is believed to play a role in the development of the embryonic immune system, hatching and growth during early larval stages in fish [16].

To advance our knowledge of aspects of its reproductive biology and gonad biochemical composition, this study evaluated seasonal changes in gonad maturity, proximate and fatty acid composition of Limbaugh's damselfish *C. limbaughi*.

MATERIALS AND METHODS

Fish collection

A fish collection permit for scientific purposes (Permit No. SGPA/DGVS/06436/17) was obtained from the Mexican Agency for the Environment and Natural Resources (SEMARNAT) on August 2, 2017. Four fishing samplings were conducted during the months of May (spring), September (summer), November (autumn) and February (winter), in temperatures representing each season. Fish were captured by SCUBA diving, using dip fishing nets, at San Esteban Island, Gulf of California, Mexico (latitude 28°42'2.66" N and longitude 112°34'37.42" W). Records of water temperature, salinity, pH and depth were obtained at the sampling site. Caught fish were temporarily placed in a submerged cage, then transported live in circular polyethylene tanks (250 l capacity) to the University of Sonora, Hermosillo, Sonora. Upon arrival, they were euthanized with an overdose of tricaine methanesulfonate (MS-222 at 300 mg l^{-1}), which complies with the Official Mexican Norm (NOM-062-ZOO-1999) on the Technical Specifications for the Production, Care and Use of Laboratory Animals [17]. Fish were individually weighed and measured, and their gonads and liver were removed to calculate the gonadosomatic index

$$GSI\ (\%)=(\text{gonad weight, g/body weight, g})\times 100;$$

the hepatosomatic index:

$$HSI\ (\%)=(\text{liver weight, g/body weight, g})\times 100;$$

and condition factor:

$$K=(\text{body weight, g}\times 100)/\text{total length}^3, \text{cm}.$$

Gonads were divided into two similar portions. One of them was placed in Davidson's fixative solution (acetic acid:95% ethanol:formaldehyde:H_2O at a 1:3:2:3 ratio) for 24 h for the histological determination of the gonad developmental stage. The second portion was stored at -84.0°C and used for the determination of the proximate and fatty acid composition.

Histological evaluation of gonads

Upon storage in Davidson's solution, the gonads were transferred into a 70% ethanol solution, then into ethanol solutions of increasing concentrations (80, 95, 100%) for dehydration, and then into xylene for clearing. At least three segments per gonad were sliced and embedded in paraffin. Transverse sections, approximately 4 μm thick, were cut, placed onto microscope slides and stained with hematoxylin and eosin (H&E). These preparations were examined under an inverted trinocular microscope (VWR International LLC, Model 89404-462, Radnor, PA, USA) equipped with a digital camera (VWR International LLC, Model V-10, Radnor, PA, USA) using the Motic Images Multi-focus software (Motic Instruments, Carlsbad, CA, USA) for image acquisition. A standardized nomenclature for staging gonad development [18], which includes the stages immature, developing, spawning capable, regressing and regenerating, was followed.

Proximate and fatty acid composition

The proximate composition of gonads was determined in terms of crude protein (CP), which was analyzed via combustion by the Dumas method (N factor=6.25), method 968.06 of the Association of Official Analytical Chemists [19]. The contents of moisture and ash were determined by the methods 930.15 and 942.05,

respectively [19]. Crude fat (CF) was extracted following a gravimetric method [20], obtaining a CF extract of 30 mL. CF was then quantified gravimetrically after drying a 5 mL aliquot of extract under nitrogen. The remaining 25-mL lipid extract was employed for fatty acid analysis. Fatty acids were transesterified using boron trifluoride. Fatty acid methyl esters (FAME) were then analyzed with a Varian 3800 gas chromatograph equipped with a 30 m×0.25 mm fused silica capillary column and a flame ionization detector, following a method previously described [21]. Fatty acids were identified by comparing retention times to those of known standards and expressed as percent of FAME identified.

Size at first sexual maturity

Using the Spearman-Karber equation [22], size at first sexual maturity was calculated by:

$$M \text{ (size at first sexual maturity)} = \text{anti log } m,$$

$$\text{where: } m = xk + (X/2) - (X \Sigma pi).$$

The 95% confidence limits were determined by:

$$\text{anti log10 } m \pm 1.96 \times \sqrt{(X2 \times \Sigma (pi - qi)/(ni-1))},$$

where: xk is the $\log_{10}$ of the size class middle value at which 100% of fish are mature, X is the mean difference between $\log_{10}$ of the size class middle values, $pi=ri/ni$, where ri is the number of fish with mature gonads to class i, and ni is the total number of fish to class i.

Finally, qi is 1-pi.

Statistical analysis

Possible differences between sexes in length (total length, standard length, fork length) and body weight were assessed by one-way analysis of variance (ANOVA), using a significance level of $P \leq 0.05$. ANOVA was also applied to GSI, HSI, K and gonad proximate and fatty acid composition data for males and females at different gonad developmental stages. When significant differences were detected, Tukey's HSD test was used as the mean separation procedure. Statistical analyses were performed using the Statistical Analysis System software package (SAS Institute Inc., 2013, Software Release 9.4, Cary, NC, USA).

RESULTS

Collected fish, length and weight

Thirty-five (15 male, 20 female) fish individuals, sixteen (8 male, 8 female) individuals, nineteen (9 male, 10 female) individuals and nineteen (11 male, 8 female) fish were caught during the spring, summer, autumn and winter surveys, respectively. The male to female ratios were 0.8:1, 1:1, 0.8:1 and 1.4:1, respectively. Recorded values of water temperature were 21.0, 27.0, 20.0 and 14.0°C, respectively; water salinity values were 36.0, 36.0, 37.0 and 36.5‰, respectively; pH 8.1, 7.3, 7.8 and 7.7, respectively. Fish were caught at a depth of 15 m throughout the four seasons, except for the winter survey, in which depth of capture was 12 m. Male and female length and body weight data from all four seasons were combined in order to evaluate size differences between sexes. Statistical differences for these parameters were not detected (Table 1).

Histological evaluation of gonads

Nineteen out of twenty females caught in spring had gonads at the developing stage, while one was at the spawning capable stage. In the summer, 37.5% were at the spawning capable stage, 37.5% at the regressing stage and 25% were immature. In autumn and winter, the vast majority of females were at the regenerating

Table 1. Comparison of length and body weight between sexes of *Chromis limbaughi* caught at San Esteban Island, Gulf of California, Mexico. Values are overall minimum, maximum and mean (± SEM), obtained by combining data from the four seasons.

	TL (cm)			SL (cm)			FL (cm)			BW (g)		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Males	4.2	13.8	9.3±0.4	3.0	10.5	7.0 ± 0.3	3.6	11.9	8.7±0.3	1.0	48.8	19.0±1.8
Females	3.8	12.7	9.6±0.3	2.8	9.8	7.3 ± 0.2	3.3	11.7	8.4±0.3	0.9	45.8	20.3±1.6
	ANOVA ($P < F$)		0.5071	ANOVA ($P < F$)		0.4204	ANOVA ($P < F$)		0.4872	ANOVA ($P < F$)		0.5876

TL – total length; SL – standard length; FL – fork length; BW – body weight.

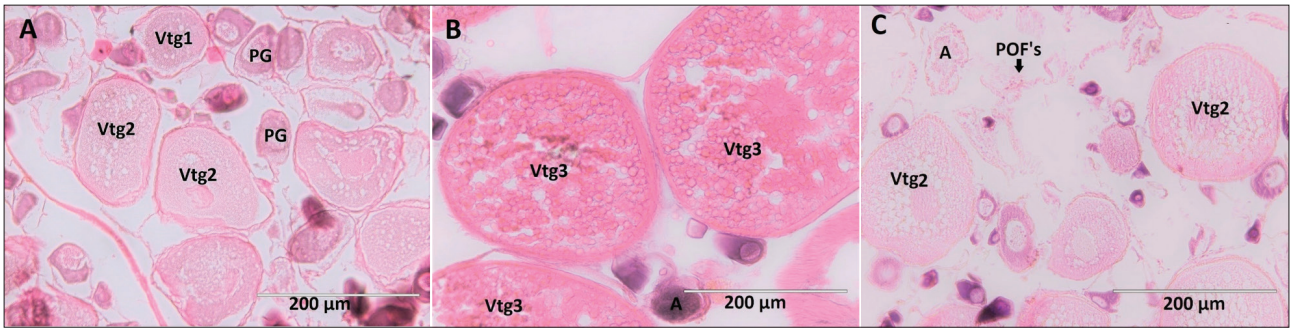

Fig. 1. Histological sections of female *Chromis limbaughi* gonads at the stages of (**A**) developing (20X), (**B**) spawning capable (20X) and (**C**) regressing (20X), following the nomenclature previously described [18]. PG – oocytes in primary growth; Vtg1 – oocytes in primary vitellogenesis; Vtg2 – oocytes in secondary vitellogenesis; A – atresia; Vtg3 – oocytes in tertiary vitellogenesis; POF – postovulatory follicles.

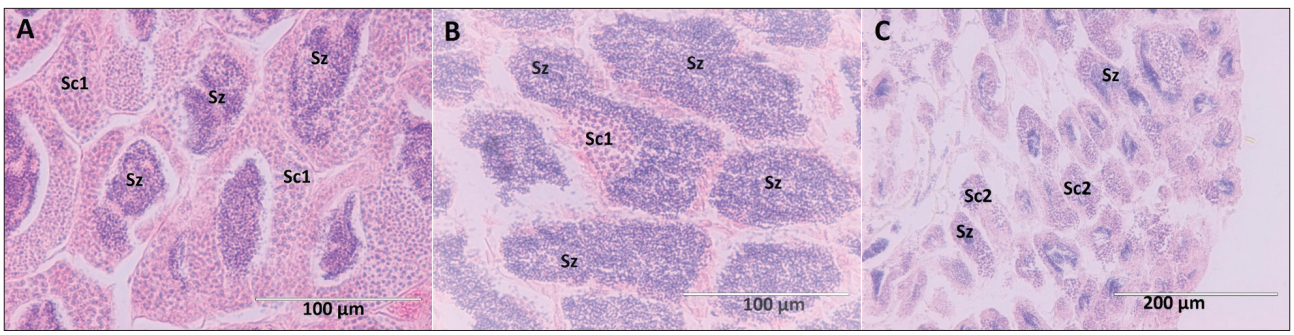

Fig. 2. Histological sections of male *Chromis limbaughi* gonads at the stages of (**A**) developing (40X), (**B**) spawning capable (40X) and (**C**) regressing (20X), following a nomenclature previously described [18]. Sc1 – primary spermatocytes; Sz – spermatozoa; Sc2 – secondary spermatocytes.

Table 2. Numbers of female and male *Chromis limbaughi* at different gonad developmental stages caught during each season at San Esteban Island, Gulf of California, Mexico.

Season	Immature	Developing	Spawning capable	Regressing	Regenerating	Total No. fish
	Females					
Spring	0	19	1	0	0	20
Summer	2	0	3	3	0	8
Autumn	2	0	0	0	8	10
Winter	3	0	0	0	5	8
	Males					
Spring	1	13	0	0	1	15
Summer	1	0	5	2	0	8
Autumn	2	0	0	3	4	9
Winter	1	0	0	0	10	11

or immature stages (Table 2). Female gonads at the developing stage were characterized by oocytes in primary and secondary vitellogenesis (Vtg1 and Vtg2, respectively), some oocytes in primary growth (PG), but no oocytes in tertiary vitellogenesis (Vtg3) (Fig. 1). In spawning-capable females, large proportions of Vtg3, greater than 240 µm in diameter, could be seen, as well as some oocytes in atresia (A) (Fig. 1). In the regressing stage, there were postovulatory follicles, oocytes in atresia and some Vtg1 and Vtg2 (Fig. 1). Similarly, the majority (87%) of males caught in spring had gonads at the developing stage, while one was immature and one in the regenerating stage. Of the males caught in the summer, 62.5% were spawning capable, 25% were at the regressing stage and 12.5% were immature. Most of the males collected in autumn had gonads at either the regressing (33.3%) or regenerating (44.5%) stages, while 22.2% were immature. All but one of the males caught in winter were in the regenerating stage (Table 2). The developing stage in male gonads was characterized by large proportions of primary spermatocytes (Sc1) and small amounts of spermatozoa (Sz) (Fig. 2), while spawning capable gonads had large proportions of spermatozoa, and small amounts of primary or secondary spermatocytes (Sc2) (Fig. 2). Secondary spermatocytes predominated in gonads at the regressing stage, with residual spermatozoa and large empty spaces in the lumen of lobules (Fig. 2).

Table 3. Body indices (means±SEM) by sampling season and by gonad developmental stage of *Chromis limbaughi* caught at San Esteban Island, Gulf of California, Mexico.

	Females			Males		
	By sampling season					
	GSI	HSI	K	GSI	HSI	K
Spring	1.93^{a}±0.26	1.61^{a}±0.09	2.08±0.05	1.42^{a}±0.16	1.49^{a}±0.31	2.11^{a}±0.04
Summer	1.84^{ab}±0.53	0.88^{b}±0.09	1.87±0.10	0.84^{ab}±0.22	0.71^{c}±0.08	1.82^{b}±0.05
Autumn	0.65^{b}±0.02	0.73^{b}±0.10	2.03±0.08	0.16^{b}±0.22	1.00^{bc}±0.16	1.83^{b}±0.05
Winter	0.75^{b}±0.07	1.02^{b}±0.07	2.03±0.10	0.25^{b}±0.18	1.25^{ba}±0.17	1.95^{ab}±0.05
ANOVA (P < F)	0.0068	<0.0001	0.3362	<0.0001	0.0008	0.0002
	By gonad developmental stage					
	GSI	HSI	K	GSI	HSI	K
Immature	0.28^{b}±0.05	1.70^{a}±0.26	1.60^{b}±0.09	0.39^{bc}±0.06	2.45^{a}±0.61	1.62^{c}±0.09
Developing	1.97^{a}±0.27	1.66^{a}±0.09	2.09^{ab}±0.07	1.47^{a}±0.23	1.51^{a}±0.08	2.10^{a}±0.04
Spawning capable	2.53^{a}±0.60	0.98^{b}±0.12	1.90^{ab}±0.09	1.11^{a}±0.14	0.65^{b}±0.09	1.87^{bc}±0.04
Regressing	1.04^{ab}±0.54	0.85^{b}±0.15	1.90^{ab}±0.06	0.16^{b}±0.01	1.14^{ab}±0.16	1.71^{c}±0.07
Regenerating	0.71^{b}±0.03	0.87^{b}±0.08	2.03^{a}±0.04	0.25^{b}±0.07	1.11^{ab}±0.13	1.95^{ab}±0.04
ANOVA (P < F)	0.0004	<0.0001	0.0009	<0.0001	<0.0001	<0.0001

Means with different superscripts within the same column are significantly different (P < 0.05). GSI – gonadosomatic index; HSI – hepatosomatic index; K – condition factor.

Macroscopically, spawning capable gonads in females were large, with prominent blood vessels, and orange in color. Gonads in the regressing stage were also orange, but somewhat translucent, smaller and flaccid. Regenerating gonads were whitish, small, and with reduced blood vessels. For males, spawning capable gonads were whitish and large. Gonads at all other stages, although relatively smaller, were also whitish and did not display distinctive features to tell them apart.

Body indices

The body indices GSI, HSI and K of males and females, as arranged by sampling season, are shown in Table 3. GSI was highest in spring and summer for both males and females, declining significantly in autumn and winter. HSI also varied significantly with sampling season. For females, HSI in spring was statistically higher than in the other seasons. For males, it was highest in spring and lowest in summer. With respect to K of males, it was significantly higher in spring than in other seasons. For females, no statistical differences were detected for this parameter (Table 3). When data were arranged by gonad developmental stage, greater values of GSI were observed in males and females with developing and spawning capable gonads, which decreased at later stages of gonad development. HSI was significantly higher in females with immature and developing gonads, as compared to females at other stages of gonad development. In males, the highest HSI values corresponded to males with immature gonads and developing gonads, statistically higher than fish with spawning capable gonads, but not different from fish with regressing or regenerating gonads (Table 3).

Size at first sexual maturity

Size at first sexual maturity for females was 7.59 cm, with 95% lower and upper confidence limits of 6.82 and 8.44 cm, respectively. For males, it was 7.90, with intervals of 7.05 and 8.85 cm, respectively.

Proximate and fatty acid composition of gonads

The proximate composition of gonads varied significantly with sampling season in terms of the contents of moisture, ash and CP in males, and in terms of the ash content in females. However, the data did not depict clear patterns of variation (Table 4). For males, gonad tissues collected during the winter were insufficient to perform proximate composition analysis.

In females, as arranged by stage of gonad maturity, the major constituent fatty acids were, among

Table 4. Proximate composition of male and female gonads of *Chromis limbaughi* caught at San Esteban Island, Gulf of California, Mexico, during each season.

Season	Moisture (%)	Ash (%)	Crude protein (%)	Crude fat (%)
	Females			
Spring	69.67±1.27	1.74^{b}±0.07	21.84±0.82	7.49±0.66
Summer	66.37±4.65	3.78^{a}±0.90	22.87±1.54	8.54±1.19
Autumn	69.34±0.63	1.48^{b}±0.21	21.50±0.93	8.50±1.87
Winter	69.35±0.67	2.94^{ab}±0.57	21.87±0.46	6.02±1.48
ANOVA (P < F)	0.7914	0.0112	0.8645	0.5360
	Males			
Spring	80.38^{a}±0.29	1.94^{b}±0.08	15.71^{b}±0.34	4.37±0.90
Summer	70.17^{b}±0.44	3.84^{a}±0.13	19.76^{a}±0.48	6.20±0.90
Autumn	70.05^{b}±0.53	1.49^{b}±0.16	21.63^{a}±0.67	7.40±0.90
ANOVA (P < F)	<0.0001	<0.0001	<0.0001	0.1287

Values are means (±standard error). Means with different superscripts within the same column are significantly different (P<0.05).

Table 5. Determined composition of selected fatty acids (% of FAME identified) in female gonads of *Chromis limbaughi* at different developmental stages, caught at San Esteban Island, Gulf of California, Mexico.

Fatty acid	Developing	Spawning capable	Regressing	Regenerating	ANOVA (P < F)
14:0	5.14±0.20	5.04±0.65	3.90±0.78	4.21±0.48	0.1363
14:1	2.05±0.17	1.63±0.46	1.62±0.30	1.46±0.18	0.2086
16:0	21.00±1.84	31.00±9.63	24.83±2.75	24.63±1.76	0.2552
18:0	6.04^{b}±0.16	6.37^{ab}±0.79	10.66^{ab}±1.59	11.72^{a}±2.36	0.0050
18:1n-9	10.98±0.57	9.03±1.70	12.21±2.69	7.42±1.73	0.0860
18:2n-6	0.81^{b}±0.09	1.23^{b}±0.20	2.51^{a}±1.00	1.58^{ab}±0.38	0.0077
18:3n-3	1.75^{a}±0.25	1.10^{ab}±0.37	0.95^{ab}±0.39	0.59^{b}±0.19	0.0266
20:4n-6	6.59^{a}±0.98	1.90^{ab}±1.90	0.00^{b}±0.00	3.70^{ab}±0.51	0.0344
20:5n-3	6.89^{a}±0.50	3.62^{ab}±1.94	2.18^{b}±0.51	4.41^{ab}±1.01	0.0154
22:5n-3	4.06±0.45	3.63±0.49	5.76±3.20	2.57±0.34	0.1044
22:6n-3	15.11±0.95	13.63±2.27	10.09±1.24	17.60±2.66	0.2965
Saturates	34.01±1.72	43.58±8.90	40.32±2.20	39.17±2.44	0.1890
Monounsaturates	15.40±0.58	12.17±1.86	15.37±2.04	15.32±2.21	0.5603
LC-PUFA	50.59±1.42	44.25±7.09	44.31±4.24	45.51±4.39	0.4153
Total n-3	33.46±1.29	30.30±4.74	29.87±4.28	30.64±3.55	0.7304
Total n-6	10.56±1.16	7.63±1.78	8.58±0.03	10.07±1.19	0.6558
n-3/n-6	3.43±0.34	4.27±0.53	3.48±0.51	3.06±0.15	0.3649

Values represent means (±standard error). Means with different superscripts within the same column are significantly different (P<0.05). FAME – fatty acid methyl esters; LC-PUFA – long-chain polyunsaturated fatty acids.
Saturates: 14:0, 16:0, 18:0, 20:0.
Monounsaturates: 14:1, 16:1, 18:1n-9, 20:1, 22:1, 24:1.
LC-PUFA: 16:2, 18:2n-6, 18:3n-6, 18:3n-3, 18:4n-3, 20:3n-6, 20:4n-6, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3, 22:6n-3, 24:4n-6, 24:5n-6, 24:5n-3, 24:6n-3.
Total n-3: 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3, 24:5n-3, 24:6n-3.
Total n-6: 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6, 24:4n-6, 24:5n-6.

saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0); among monounsaturated fatty acids, oleic acid (18:1n-9); among fatty acids from the n-3 family, DHA and EPA; and among fatty acids from the n-6 family, ARA. Of these major constituents, EPA, ARA and stearic acid varied significantly with stage of maturity, but without showing clear patterns. Significant differences also were detected for linoleic acid (18:2n-6) and linolenic acid (18:3n-3), but these were minor components. No significant differences were detected for fatty acid groups or the n-3/n-6 ratio (Table 5).

For males, because of insufficient gonad tissue, fatty acid analysis is presented only for the developing,

Table 6. Determined composition of selected fatty acids (% of FAME identified) in male gonads of *Chromis limbaughi* at different developmental stages, caught at San Esteban Island, Gulf of California, Mexico.

Fatty acid	Developing	Spawning capable	Regressing	Regenerating	ANOVA ($P < F$)
14:0	5.49^{a}±0.09	1.45^{c}±0.22	5.26^{a}±1.36	3.38^{b}±0.06	<0.0001
14:1	2.18^{ab}±0.08	0.89^{c}±0.05	2.71^{a}±0.78	1.49^{bc}±0.42	0.0004
16:0	17.46^{c}±0.48	21.93^{a}±0.86	20.97^{ab}±1.62	18.68^{bc}±1.57	0.0017
18:0	7.58^{b}±0.34	9.53^{ab}±0.32	10.25^{a}±1.35	9.70^{a}±0.85	0.0070
18:1n-9	9.78^{a}±0.42	9.36^{a}±1.00	2.08^{b}±0.25	2.17^{b}±0.13	<0.0001
18:2n-6	1.04^{a}±0.12	1.15^{a}±0.12	0.32^{b}±0.11	0.42^{b}±0.00	0.0072
18:3n-3	2.40^{a}±0.21	0.33^{b}±0.15	0.09^{b}±0.09	0.19^{b}±0.19	<0.0001
20:4n-6	9.69^{a}±1.19	0.00^{b}±0.00	0.00^{b}±0.00	0.00^{b}±0.0	<0.0001
20:5n-3	7.22^{a}±0.41	2.04^{b}±0.41	0.87^{b}±0.29	1.64^{b}±0.68	<0.0001
22:5n-3	6.05±0.53	4.93±1.31	2.50±0.29	3.32±0.78	0.0529
22:6n-3	11.89^{b}±0.89	14.05^{b}±1.14	14.88^{b}±1.99	24.24^{a}±1.98	0.0005
Saturates	32.68±0.59	34.31±0.91	36.86±3.33	31.91±0.72	0.1092
Monounsaturates	14.18±0.57	10.91±1.15	13.22±2.05	11.58±0.43	0.0772
LC-PUFA	53.14±1.11	54.78±0.77	49.92±5.36	56.53±0.29	0.3567
Total n-3	32.69^{b}±0.89	38.77^{a}±0.72	27.34^{c}±3.87	40.63^{a}±0.25	0.0003
Total n-6	14.47±1.15	12.92±0.50	16.63±4.84	11.25±0.28	0.4973
n-3/n-6	2.48±0.28	3.03±0.17	1.86±0.43	3.62±0.07	0.1100

Values represent means (±standard error). Means with different superscripts within the same column are significantly different (P<0.05). FAME – fatty acid methyl esters; LC-PUFA – long-chain polyunsaturated fatty acids.
Saturates: 14:0, 16:0, 18:0, 20:0.
Monounsaturates: 14:1, 16:1, 18:1n-9, 20:1, 22:1, 24:1.
LC-PUFA: 16:2, 18:2n-6, 18:3n-6, 18:3n-3, 18:4n-3, 20:3n-6, 20:4n-6, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3, 22:6n-3, 24:4n-6, 24:5n-6, 24:5n-3, 24:6n-3.
Total n-3: 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3, 24:5n-3, 24:6n-3.
Total n-6: 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6, 24:4n-6, 24:5n-6.

spawning capable, and regressing stages. Similarly to what was previously observed in females, the major constituent fatty acids were 16:0, 18:0, 18:1n-9, DHA, EPA, and ARA (Table 6). Statistical differences were detected for all but docosapentaenoic acid (22:5n-3). However, the relative abundance of individual fatty acids varied from one developmental stage to the next without following definite patterns. Among fatty acid groups, significant differences were observed only for fatty acids of the n-3 family (Table 6).

DISCUSSION

One prominent aspect of the reproductive biology of *C. limbaughi* that can be inferred from the results of the present study is its sexual system. Either male or female gametes, but never both types, were present in the same individual. The population structure consisted of males and females equally represented in all sizes, as shown by the lack of differences in size and weight between sexes, and with a male to female ratio close to 1:1. This growing body of evidence clearly indicates that *C. limbaughi* is a gonochoristic species, which is consistent with gonochorism found in other species of the genus *Chromis* [23], as well as with findings of male to female ratios close to 1:1, without differences in size between sexes, reported for the also gonochoristic species *Chromis notata*, *Chromis chromis*, and *Chromis hypsilepis* [24-26]. As opposed to gonochoristic species, in damselfishes known to be protandrous hermaphrodites, such as *Dascyllus*, males are notably larger than females [23,27], while the opposite is true for protogynous hermaphrodites, which, among Pomacentrids, is only known to occur for the genera *Amphiprion* and *Premnas* [23].

Mating systems in Pomacentridae include monogamy and polygamy. In turn, polygamy can be subdivided into polygyny and promiscuity. Polygynic fishes are known for aggregating into harems where one or two males monopolize mating with several females [23]. In promiscuity, well known in the genus *Chromis* (e.g., in *C. caerulea*, *C. chromis* and *C. pembae*), females spawn

with several males [28]. Although the actual spawning act was not observed in the present study, it is likely that *C. limbaughi* also conforms to the promiscuity mating system, taking into consideration that during scuba diving it was observed that *C. limbaughi* does not form harems, and that, unlike haremic systems, with notably less males than females, the male to female ratio was close to 1:1 for this species.

The influence of season on gonad maturity of *C. limbaughi* was clear. Both male and female fish with gonads in forthright preparation for reproduction predominated in spring. Although scarce, the incidence of females with spawning capable gonads in spring is indicative of the onset of reproduction for this species; however, summer is undoubtedly the most reproductively active season. No evidence of mature gonads was found in autumn or winter, from which it is inferred, based on sampling dates, that the spawning season for *C. limbaughi* extends, at least, from May to September. Undoubtedly, increasing water temperature and daylight hours, among other factors, play key roles on this pattern, which corresponds well with those of other pomacentrids of the same geographical area, such as *Stegastes rectifraenum*, with a spawning season from March to October, and *Microspathodon dorsalis* and *Abudefduf troschelii*, both with spawning seasons lasting from April to October [29]. In addition, *C. limbaughi* seems to be a partial spawner because spawning capable females had mature oocytes, as well as oocytes at other stages of development, i.e., asynchronous development, a feature described also for the partial spawners *S. rectifraenum*, *M. dorsalis* and *A. troschelii* [29].

With regard to water temperature, during the summer season it was evident that *C. limbaughi* avoids excessively warm waters by occupying niches at relatively greater depths (15 m), where the mean temperature was 27°C, while that at the surface was 31°C. Actual water quality conditions under which the maturation of *C. limbaughi* takes place were measured in this study. The changes in these conditions can be mimicked for the management of *C. limbaughi* broodstock in captivity.

A new maximum standard length of 10.5 cm was recorded in the present study for *C. limbaughi*, which exceeds the maximum standard length of 10.0 cm reported previously for this species [30]. On the other hand, the range of length measurements recorded here position *C. limbaughi*, among other species of the same genus, as a medium-sized damselfish. For example, *Chromis fieldi* is smaller, with SL ranging from 1.5 to 5.7 cm [31], while *Chromis crusma* and *Chromis punctipinnis* are large species, reaching up to 24.5 and 33.0 cm, respectively [32,33].

As stated earlier, because of their more vivid coloration, juvenile, and thus, immature *C. limbaughi*, are targeted for capture and marketing. Since the estimated size at first sexual maturity was 7.90 cm for males and 7.59 cm for females, this implies that the rate of recruitment of fish to reproductive age is being directly impacted. The extent to which this harmful extraction practice may be affecting natural populations is difficult to assess, since there is no certainty about actual numbers of fish catches [4]. However, it brings to light the need for promoting the development of captive breeding programs to supply the ornamental fish trade, as is the case for other pomacentrids, such as some clownfish, which are currently successfully reared in captivity [34], and at the same time, for halting fishing or enforcing regulations for the extraction of wild organisms.

The seasonal changes in GSI corresponded well with the stages of gonad maturity, reaching the greatest GSI values, by sampling season (Table 3), in spring and summer. When arranged by gonad developmental stage, GSI values were greatest in fish with gonads at the developing and spawning capable stages, reflecting active vitellogenesis and spermatogenesis mainly in spring, and spawning in summer. Then, GSI decreased notably in autumn and winter, when fish were reproductively inactive. A similar seasonal pattern in GSI was observed in the damselfish *A. troschelii* [35]. The GSI values found in this study (0.25-2.53) are comparable to those reported previously for *Chromis pelloura*, ranging from approximately 0.2 to 1.4, but for *C. notata*, very high GSI values of up to 8.5 have been reported [36].

The changes observed in HSI illustrate the active role of the liver in the process of gametogenesis, reaching high values in spring during active vitellogenesis and spermatogenesis for gonad growth, then dropping significantly in the summer, showing not only a reduction in these processes, but also a decline of nu-

tritional reserves. Furthermore, for males, the drastic change observed is probably also a reflection of egg guarding, a task normally reserved for this gender in species of the family Pomacentridae [23], in which feeding activity may be reduced by up to 85%, for example, for *Chromis hypsilepis* [37,38]. The relative increase of HSI observed in autumn and winter is not just a consequence of suppression of reproduction, but is likely the result of abundant food supply caused by upwelling during the cold months in this region, which favors primary productivity [39] and allows *C. limbaughi* to recover hepatic reserves.

The condition factor for some fish species, such as *Scorpaena mystes*, increases significantly during the spawning season and, thus, can be used as an index of gonad maturity [40]. However, in this study, even if K was significantly influenced by sampling season and gonad developmental stage in males and females, the data did not depict clear trends. Thus, K does not appear to be a good estimator of gonad maturity for this species. The K values observed in this study (1.62 to 2.11) were higher than those reported for *Chromis pelloura*, which ranged from ca. 0.3 to 0.8 [41].

The proximate composition of gonads showed that the ash content for both males and females was significantly higher in summer, which can be explained by high mobilization of nutrients, including minerals, to this tissue for maturation, as previously reported for *Epinephelus diacanthus* [42]. The moisture content of female gonads did not vary significantly with season, agreeing with the lack of differences in moisture content of immature, developing and spawning capable ovaries of *E. diacanthus* over two years of sampling [42]. Because they are important components of egg yolk, CP and CF have been shown to increase in maturing ovaries [42]. However, in the present study, statistical differences in CP and CF were not observed in females. For males, significant differences were observed only in CP, but the highest value corresponded not to that of summer, but of autumn.

The recurrent presence among LC-PUFA, of DHA, EPA and ARA as main constituent fatty acids in gonads of *C. limbaughi*, agrees with the fatty acid profile observed in broodstock gonads of *Holacanthus passer*, another marine fish species present in the same geographical area [43]. The elevated levels observed are most likely a reflection of the availability of these fatty acids through the food chains in the area, and they could possibly also be the result of selective retention in specific tissues, as suggested for some species [44]. The fatty acid profile found also endorses the relevance of these LC-PUFA for key reproductive processes such as gametogenesis, embryogenesis, hatching, as well as successful early larval growth and survival [13-15].

CONCLUSIONS

It was observed that *C. limbaughi* is a gonochoristic species without sexual dimorphism, with a polygamous, promiscuous mating system. The reproductive season of *C. limbaughi* extends, at least, from May to September. A new maximum standard length of 10.5 cm was recorded for this species. The estimated size at first sexual maturity was 7.90 cm for males and 7.59 cm for females. The gonadosomatic index reached the greatest values in spring and summer in fish with gonads at the developing and spawning capable stages, decreasing notably in autumn and winter. The changes observed in the hepatosomatic index illustrate the active role of the liver in the process of gametogenesis, reaching high values in spring during active vitellogenesis and spermatogenesis for gonad growth, then decreasing significantly in the summer, showing not only a reduction in these processes, but also a decline in nutritional reserves. Even if the condition factor was significantly influenced by sampling season and gonad developmental stage in males, and in females by gonad developmental stage, it was not a good estimator of gonad maturity for this species. For both male and female gonads, the major constituent fatty acids were palmitic acid, stearic acid, oleic acid, DHA, EPA and ARA. All in all, the present findings bring to light the urgency for promoting the development of captive breeding programs to supply the ornamental fish trade, and at the same time, for halting fishing or, at the very least, properly enforcing regulations for the extraction of wild organisms.

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