NOAA Technical Memorandum NMFS-SEFSC-649

AN EVALUATION OF SEXUAL MACROSCOPIC STAGING APPLIED TO GULF OF MEXICO FISHES

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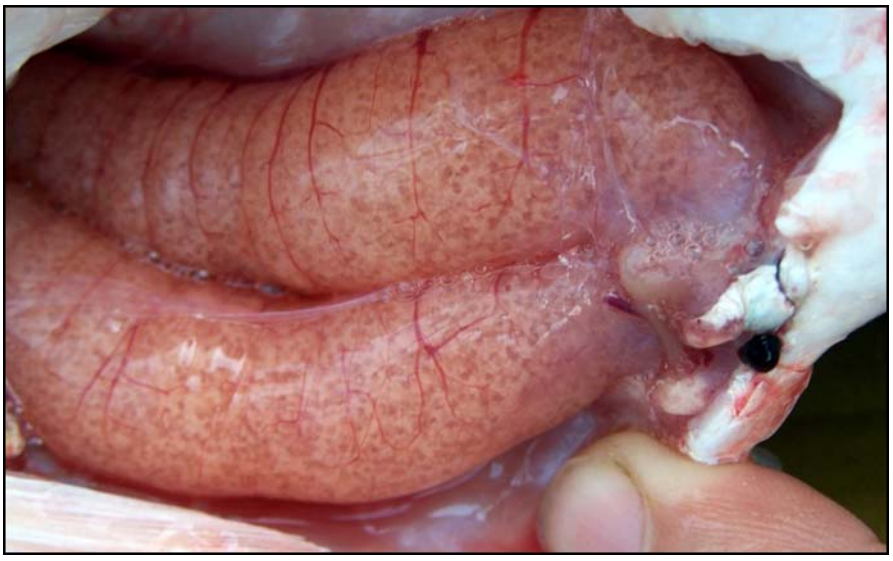

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Abstract

During 2011, a congressionally supplemented fisheries survey was conducted in the northern Gulf of Mexico (using 6 vessels) from April to October. All fish captured on hook-and-line gear were macroscopically sexed and staged for reproductive condition. Gonads were dissected and histologically examined for a subset of randomly selected fish, as well as all female red snapper (*Lutjanus campechanus*). To obtain further detail on a hermaphroditic species, macroscopic and histological data on red grouper (*Epinephelus morio*) were extracted from NOAA archives. During the survey, most gonochorists (9 species) were sexed correctly (97%) in contrast to hermaphrodites (7 species, 68% correct). The red grouper data set, which afforded a larger sample size from more experienced readers also indicated some error in assigning 'field' sex to a hermaphrodite in that 81% (n= 2,153) were sexed correctly. Almost all errors were due to misidentifying males as females. Rarely were histological females misidentified. This result may occur because testes of protogynous fish retain the ovarian form. Macroscopic classification of reproductive stage (males and females) ranged from 55-68% correct in gonochorists and 53-55% for protogynous hermaphrodites. Although spawning females were often classified correctly more errors were associated with inactive, spent and maturing stages. These findings may reflect the difficulty in discerning early development and atresia of oocytes with the naked eye. Spawning males, especially hermaphrodites, were often misclassified as maturing which may indicate that the histological readiness to spawn may not always equate with 'running ripe' condition (extruded milt) in the field. Additional training may help reduce error in macroscopic staging. However, we believe these results indicate a need for routine collection and fixation of reproductive tissues by on-board observers which will enable histological assessment of spawning condition (fraction etc.) and sex ratio of our most economically important stocks.

Introduction

Histology is the standard method to determine sex and reproductive condition of commercially and recreationally regulated fish, but it is costly and time consuming (DeMartini and Lau 1998, ICES 2007). Multiple methods of determining spawning condition without histology have been tested, such as gonadosomatic index (GSI), hepatosomatic index (HSI), oocyte diameter, ovary volume, and a mixture of all of the above (West 1990, DeMartini and Lau 1998, Korta et al. 2010). In addition, visual macroscopic staging has been used to determine sex ratio and reproductive condition, such as maturity and spawning fraction (Tomkiewicz et al. 2003, Vitale et al. 2006).

While considered low-cost and effective in some applications (e.g., Scott and Pankhurst 1992), there are limitations to macroscopic staging. Several examples of disagreements between histological and macroscopic staging are due to features of the gonad that are not readily recognized by the naked eye (West 1990, Garcia-Diaz et al. 1997, Adams et al. 2000, Tomkiewicz et al. 2003, Vitale et al. 2006, Costa 2009). More specifically, there have been problems macroscopically identifying immature individuals and recognizing atresia leading to overestimation of maturity and misclassification of spent fish (Garcia-Diaz et al. 1997, Tomkiewicz et al. 2003,Gerritsen and McGrath 2006, Vitale et al. 2006, Costa 2009).

Time of sampling in relation to spawning season may be an important consideration for accuracy of staging (ICES 2007). According to previous studies, the most accurate macroscopic observations were completed at the beginning of the spawning season (Tomkiewicz et al. 2003, Vitale et al. 2006). When fishes are near spawning condition the appearance of their gonads may be less ambiguous with less error in classification, but the importance of timing may be related to the latitude of investigation (Gerritsen and McGrath 2006, ICES 2007). For instance, macroscopic staging has been found to have utility in boreal fisheries where seasonal changes in reproductive condition tend to be profound and occur over a relatively short time period, i.e. weeks to a few months (Burchard et al. 2013). In contrast, reproduction in tropical to sub-tropical waters may occur over much of the year. More hermaphroditic species and more diverse reproductive strategies are also encountered in lower latitudes (Shepherd et al. 2013). Thus the macroscopic approach may be subject to greater error.

To define reproductive potential of multiple fish stocks within a large region such as the Gulf of Mexico, spatial and temporal coverage of reproductive sampling must be generally broad. Thus onboard scientific observers have been endorsed as having the greatest potential to deliver such samples on an annual or continuing basis (ICES 2007). Subsequently, scientific observers may be a good source of low cost macroscopic information but there is evidence that a lack of experience can increase assessor variability (Gerritsen and McGrath 2006, ICES 2007). Therefore, our overall purpose was to determine whether macroscopic staging can be used to

accurately assess fish sex and reproductive stage, particularly in a sub-tropical region, and we wished to draw some inference about how experience can affect the accuracy of staging.

As part of a 2011 congressional supplemental sampling program to augment assessment of Gulf of Mexico (GOM) fisheries stocks, NOAA Fisheries contracted and trained field biologists to serve as scientific crew aboard contracted commercial fishing vessels. Among their tasks, they were asked to macroscopically sex and identify maturity/spawning stage of each fish collected. Histological samples were randomly taken from all species without regard to visual assessment of sex. In addition, all red snapper (*Lutjanus campechanus*) females (macroscopically sexed) were also sampled histologically if they were not subject to random selection. Based upon histological samples drawn from these two protocols, our objective was to determine the accuracy of the macroscopic approach for 1) sex, 2) maturity, and 3) spawning state and comment on the adequacy of the approach for these key reproductive traits.

Methods

From April through October 2011 various offshore fishes were caught in the Gulf of Mexico using two bandit reel and four bottom longline boats. The bandit reel boats deployed three electric reels with ten hooks on each reel spaced 61cm apart and baited with Atlantic mackerel (*Scomber scombrus*). Three different hook sizes (8/0, 12/0, 15/0) were rotated by reel after each set and were randomly assigned at the beginning of each fishing day. Longline boats set 1.6 km of line with 100 size 15/0 hooks and baited with Atlantic mackerel. Each boat was randomly assigned to fish from noon to midnight or midnight to noon for each cruise to allow for equal chance of catching fish at night or during daylight hours. Most cruises consisted of two surveys (10 days/survey).

Fish gonads were macroscopically and histologically sexed and staged using a classification system developed by NOAA Fisheries (Tables 1&2). Biologists were trained to find, remove, and class a gonad macroscopically, with no previous experience assumed. Reproductive seasonality information was not provided to the biologists to aid in gonad classification, and sampling only encompassed spawning season of gonochorists (Table 3). A systematic random approach was used to select every n^{th} fish caught. Gonads were removed, and subsamples were placed in 10% neutral buffered formalin for histological analysis. Slides from each sample were stained with hematoxylin and eosin-Y by Mass Histology Service Inc. For the first two months every 20^{th} fish was sampled, but protocol changed to every 5^{th} fish for the remainder of the survey. Each vessel was instructed to limit their randomly selected fish to 50 total specimens for the duration of the supplemental sampling.

For those red snapper females not selected during random sampling, gonads were also removed and frozen for storage at sea. Subsequent histological sampling as indicated above was conducted later in the laboratory (Table 1&2). This added focus on red snapper females was an effort to maximize sample sizes needed to estimate spawning fraction in the GOM stock (see

Fitzhugh et al. 2012). Thus histological sampling intensity was higher for red snapper females than for red snapper males and other species.

Red grouper (*Epinephelus morio*) data from a NOAA Fisheries archive (Panama City Laboratory) was used to develop a larger sample size for hermaphroditic fish. Historical macroscopic and histological classification records dating back to 1992 were compared for macroscopic staging validation of red grouper.

Macroscopic assignments were compared to histological (independent) classifications via contingency table analysis. The results were analyzed separately for hermaphroditic and gonochoristic fish species. Due to the increased effort for red snapper females and red grouper, monthly results were also tabulated (% correct) and compared to the Gonadosomatic index (GSI) trend. GSI was calculated using the equation:

$$GSI = \left(\frac{GW}{GFBW}\right) * 100$$

with GW representing gonad weight and GFBW representing gonad-free body weight.

Results

The bottom longline gear captured 149 species of which 28 teleost species, key to fisheries management, comprised 2,647 of the total fish caught. The bandit reel boats collected a total of 67 species which was comprised of 25 teleost species and 2,528 teleost fish (see Campbell et al. 2011). Of these, sex and spawning class were determined for 16 species of fish from April through August. Nine species were gonochorists and 7 were hermaphrodites (but see Lombardi-Carlson 2012 for re-consideration of *Lopholatilus chamaeleonticeps*). There was only one individual captured for almaco jack (*Seriola rivoliana*), blackfin tuna (*Thunnus atlanticus*), dolphin (*Coryphaena hippurus*), speckled hind (*Epinephelus drummondhayi*), and wahoo (*Acanthocybium solandri*), but other species were collected in higher numbers (Table 3). Red snapper (*Lutjanus campechanus*) and red grouper (*Epinephalus morio*) were caught most often, with 80 and 70 individuals taken respectively via random sampling.

Random samples: Of the 143 gonochorists examined 97% were sexed correctly and 55% were staged correctly within sex. Among females, contingency table results indicate that no inactive fish were macroscopically identified whereas n= 21 histologically classified inactive females were detected (Table 4). In addition, 11 of the 38 maturing females were misclassified as ‘running ripe’. Among males, the running ripe class was the only macroscopic spawning class where a majority of male gonochorists were correctly classified. Eight of the 12 inactive males

were mis-classified as maturing and 4 of the 6 maturing males were mis-classified as running ripe.

Of the 92 hermaphrodites examined 68% were sexed correctly and 21% were staged correctly within sex. It was readily apparent that protogynous hermaphrodites were more difficult to sex and more difficult to classify within sex. Despite the overall misclassification, there were trends in staging. Inactive hermaphrodite females were sometimes misclassified as male (9 of 58) and when sexed correctly they were often misclassified as maturing (25 of 58). Maturing hermaphrodite females were macroscopically classified in many different ways, but only 4 of 13 were done so correctly. Among male hermaphrodites, histological analysis indicated all were actively spawning (spermatozoa evident and filling ducts) however none were macroscopically classified as running ripe. There were only five transitional individuals sampled: four were macroscopically classed as inactive or spent and one was classified as a running ripe female. None of the transitional individuals were labeled macroscopically as undetermined; all were sexed as male or female. Seven fish were noted to be "undetermined" sex in the field and histology results indicated all were inactive females (Table 5).

Red snapper females: Distinct from random selection, a total of 992 female red snapper were caught from April through October with a macroscopic and histological stage assigned. Given the larger sample size and collections made throughout the April-October survey duration, immature females were observed and more "spent" females were noted. The majority of immature, maturing and running ripe females were correctly classified, overall 68%. For example, 6 of the 8 (75%) immature females were classified correctly and over 70% of maturing and running ripe females were classified correctly. However, similar to the trend for random gonochorists many inactive females were misclassified, mainly being mis-identified as maturing (47%) in the field. Every histologically assessed "spent" female was mis-identified as maturing (Table 6).

The gonadosomatic index (GSI) trend was similar to previous findings about reproductive seasonality. Ovary weight and spawning activity peaked in July, declined though August and September and signaled cessation of spawning in October (Figure 1A). Macroscopic classification by month also indicated that a peak in running ripe females occurred in July and that by September a large percentage of inactive females were present, suggesting reduced spawning (Figure 1B). However, there was no obvious relationship between monthly GSI (reflecting reproductive seasonality) and the overall ability to classify ovaries. Percent correct classifications were relatively low at the beginning and end of the season (Apr, Oct) as well as mid season (July) (Figure 1A), albeit for different reasons. The high degree of misclassification in July (54% correct) was due to mistaking maturing ovaries as running ripe. By contrast, misclassification of April females (46% correct) and October females (53% correct) was due to mistaking inactive ovaries as maturing.

Red grouper: Of the 2,153 red grouper used for the sex comparison 81% were sexed correctly (Table 7). This suggests that more experienced readers can judge sex more accurately compared to the result for randomly selected hermaphrodites (68% correct). While females were correctly sexed in 97% of macroscopic observations, males and transitionals were only correctly sexed 48% and 10% respectively; suggesting a much lower ability to recognize a testis as opposed to an ovary (Table 7). Within sex, spawning classifications were 59% correct for females (n=1470) and 28% correct for males (n=642). It was apparent that some classes, by sex, were more difficult to distinguish than others. Every running ripe female red grouper was classified correctly and 81% of inactive and 76% of maturing ovaries were classified correctly. However, only 2 of the 366 (0.5%) histologically assessed spent red grouper females were recognized, which diminished the overall accuracy of female macroscopic staging. Of the specific classifications for males, 45% of the maturing individuals were recognized and only 28% of running ripe individuals were correctly classified (Table 7).

As with red snapper females, red grouper females showed no apparent relationship with the seasonality of gonad weight (GSI) and the ability to discern spawning classes (Figure 2A). However there was a decline in correct classifications as the reproductive season progressed and concluded (February – June, Figure 2A). This decline largely coincided with misclassification of inactive females and the increasing prevalence of spent females by May to July (Figure 2B). Distinct from female red snapper and red grouper, male red grouper exhibited a trend of increased gonad weight in April which appeared to be readily distinguished as an increased proportion of running ripe males (Figure 3).

Discussion

Macroscopic staging is sometimes considered a cost-effective means to determine sex, maturity, and spawning state. But in large part, most of the macroscopic classifications that are conducted for fisheries assessment are done so for gonochoristic species in temperate to boreal waters (e.g., Scott and Pankhurst 1993, Burchard et al. 2013). Extended seasonality and more rapid physiological changes typical of warmer latitudes may affect the ability to visually stage fish reproductive condition. As well, more hermaphrodites are found at lower latitudes. In the sub-tropical Gulf of Mexico, 43% of teleost species under a Fisheries Management Plan are hermaphrodites (see list; Gulf of Mexico Fisheries Management Council 2012). Sex-based information for managing hermaphrodites is vitally important (Shepherd et al. 2013), but the sexual transitioning of hermaphrodites may create a challenge for macroscopic sexual classification.

Our findings clearly revealed that gonochorists could be sexed more accurately than hermaphrodites. During the 2011 congressionally supplemented survey 97% of the gonochorists could be accurately identified to sex as opposed to 68% of hermaphrodites. The fact that every hermaphrodite in the transitional phase was misidentified and six of the 13 male gonads were

incorrectly sexed, mostly as female, indicates that external morphological changes may not be readily evident. Based upon the larger archived data specific to red grouper sampled throughout the year, males and transitional fish were also more likely to be identified as females. When a protogynous hermaphrodite transitions from female to male, morphological changes can be barely noticeable (Ozen and Balci 2012), likely the principal factor leading to macroscopic errors.

Several previous studies indicated an advantage in conducting macroscopic staging during the period of reproductive development and spawning (Tomkiewicz et al. 2003, Vitale et al. 2006, Gerritsen and McGrath 2006, ICES 2007). However, we found no clear tendency of overall more accurate macroscopic observations during reproductive periods. The gonochorists captured in this survey tended to be summer spawners while the hermaphrodites tended to be winter to spring spawners (cf. red snapper *Lutjanus campechanus* and scamp *Mycteroperca phenax* respectively, Table 1). The error we detected may in part be due to experience level of the observers and in part due to the previously mentioned challenge of staging hermaphrodites. Many large maturing red snapper ovaries were misclassified as running ripe in July, which is the peak period of spawning for that species. Clearly, more training and experience would ameliorate this type of error. In contrast, female red grouper tended to be misclassified in months, with a larger proportion of spent females, and red grouper males were misclassified in all months except for the peak period in April when a large proportion were running ripe. In these instances, barely discernible changes in gonad morphology are likely resulting in inaccurate classification. As with previous studies (Garcia-Diaz et al. 1997, Tomkiewicz et al. 2003, Vitale et al. 2006, Costa 2009), we found errors associated with inactive and spent stages. These findings may reflect the difficulty in discerning early development and atresia of oocytes with the naked eye. Additional training and experience may not adequately reduce these types of errors.

While the histological results are not shown, we noted that in every month at least some red grouper males were histologically assessed as actively spawning. However the actual secretion of milt from a cut in the testes confirms spermatozoa in the lumen and this condition was much more prevalent during peak spawning (April) based upon the macroscopic results. This raises a question of whether our histological criteria used to confirm spawning readiness in males (spermatozoa merely evident and present in ducts) sufficiently captures the seasonal dynamics of red grouper reproduction. Some measure of abundance of spermatozoa from histological assessment maybe a more appropriate indicator of running ripe condition as opposed to mere presence in the sperm ducts.

While we can't argue that more training and more (esp. seasonal) reference information may improve macroscopic classifications, we generally found that macroscopic results were reliable for providing sex of gonorchoristic species (97% correct) with newly trained observers. During the spawning season, identification of mature females (maturing and spawning females)

was correct 77% of the time which may have utility for inter-annual or regional (stock-based) contrasts. More work is needed to judge the reliability of sexing hermaphrodites. Certainly our samples reveal that macroscopically sexing hermaphrodites is fraught with error. Thus we recommend that efforts must be extended to obtain routine collection and fixation of reproductive tissues by on-board observers which will enable histological assessment of spawning condition (fraction, etc.) and sex ratio of our most economically important harvested stocks.

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Table 1. Terminology used to determine female macroscopic and histological spawning class with description and characteristics for each classification.

Class	Description	Female Macro Characteristics	Female Histo Characteristics
IM	**Immature**	Ovaries are small or ribbon-like, opaque and jellied grayish color. No oocytes visible to naked eye.	Primary growth oocytes only with no evidence of prior spawning
MA	**Maturing**	Class includes gonads with small opaque oocytes, barely seen with naked eye and large, granulated gonads with oocytes easily seen with naked eye	Cortical alveolar or vitellogenic oocytes present (for the purposes of this comparison post-ovulatory follicles and oocyte maturation can be present)
RR	**Running Ripe (hydrated)**	Some clear, hydrated oocytes seen through the tunic.	Late Hydrated oocytes present
ST	**Spent**	Ovaries are slack and flaccid, often reddish or bloody in color. Gonads may contain residual oocytes.	Greater than half of yolked oocytes undergoing atresia
IA	**Inactive (Regressed)**	Macroscopic distinctions between inactive (regressed) and immature ovaries can be imprecise, but the regressed ovary is likely more opaque and jellied with a more reddish-grey cast than the immature ovary. Class also includes skipped spawners.	A large majority of primary growth oocytes present and a small amount of cortical alveolar oocytes can be present. Must be some indication of prior spawning or maturity.

Table 2. Terminology used to determine male macroscopic and histological spawning class with description and characteristics for each classification

Class	Description	Male Macro Characteristics	Male Histo Characteristics
IM	**Immature**	Testis is small, thin, string-like, often translucent or pink in color. No spermatozoa are evident.	Only spermatogonia present, no residual spermatozoa
MA	**Maturing**	Testis is larger, firm, white and often triangular shaped. Spermatozoa not released when testis is cut.	All stages of spermatogenesis with little to no spermatozoa present in the lumen
RR	**Running Ripe (hydrated)**	Testis is often thick and white. Spermatozoa (milt) are observed in the lumen or ducts when testis is cut. Spermatozoa are often released when pressure applied to abdomen.	All stages of spermatogenesis present with spermatozoa evident in lumen and filling sperm ducts
ST	**Spent**	Testis is elongated, flaccid, and may be reddish or blood stained. No spermatozoa released when cut.	Spermatogenesis is ceasing and spermatozoa is present in shrinking lobules. Spermatogonia proliferation common
IA	**Inactive (Regressed)**	Testis ribbon-like but usually larger than the testis of an immature fish. No spermatozoa are present.	Spermatogonia dominate in testes, no active spermatogenesis. Lobules and ducts primarily empty with some residual spermatozoa present

Table 3. The number of each species randomly selected for comparison of histological and macroscopic spawning class with max and min length (mm), spawning season (if known), and whether the species is a gonochorist or hermaphrodite.

Species	Males (n)	Females (n)	Transitional (n)	Fork Length (max-min, mm)	Spawning Season	Sexual Strategy
Centropristis striata	1	2	0		December-April	Hermaphrodite
Epinephelus drummonhayi	1	0	0	954	–	Hermaphrodite
Epinephelus flavolimbatus	4	10	0	800-490	January-October	Hermaphrodite
Epinephelus morio	10	60	5	690-373	March-May	Hermaphrodite
Epinephelus niveatus	0	1	0		April-September	Hermaphrodite
Mycteroperca phenax	0	2	0	536-400	February-July	Hermaphrodite
Lopholatilus chamaeleonticeps	26	13	–	1045-436	May-August	Gonochorist*
Coryphaena hippurus	1	0	–	606	January-July	Gonochorist
Seriloa dumerili	1	1	–	708-650	January-June	Gonochorist
Seriloa rivoliana	0	1	–	868	–	Gonochorist
Lutjanus campechanus	43	37	–	870-232	May-October	Gonochorist
Lutjanus synagris	1	1	–	337-168	March-August	Gonochorist
Romboplites aurorubens	7	12	–	525-199	April-October	Gonochorist
Pagrus pagrus	1	3	0	522-337	December-February	Hermaphrodite
Acanthocybium solandri	0	1	–	985	May-August	Gonochorist
Thunnus atlanticus	0	1	0	675	–	Gonochorist

*See Lombardi-Carlson 2012

Table 4. Spawning class for each male and female gonochorists determined histologically along with the corresponding macroscopic spawning class recorded. Spawning classes included are inactive (IA), maturing (MA), running ripe (RR), and spent (ST).

Gonochorists			Macroscopic Spawning Class							
			Female				Male			
			IA	MA	RR	ST	IA	MA	RR	ST
Histological Spawning Class	Female	IA		15	3	2			1	
		MA		**27**	11				1	
		RR			**6**					
		ST								
	Male	IA					**4**	8		
		MA						**2**	4	
		RR		1	1		1	14	**39**	1
		ST						1	1	

Table 5. Spawning class for each male and female hermaphrodite determined histologically along with the corresponding macroscopic spawning class recorded. Spawning classes included are immature (IM), inactive (IA), maturing (MA), running ripe (RR), and spent (ST).

Hermaphrodites			Macroscopic Spawning Class							
			Female				Male			Undetermined
			IA	MA	RR	ST	IA	MA	RR	
Histological Spawning Class	Female	IA	**14**	25		3	2	7		7
		MA	4	**4**	2	1	1	1		
		RR								
		ST				**1**				
	Male	IA								
		MA								
		RR		3		3	4	5		
	Transitional		1		1	2	1			

Table 6. Counts of histological spawning classes present for female red snapper (*Lutjanus campechanus*) collected outside the random sampling regime along with the corresponding macroscopic spawning class. Spawning classes included are immature (IM), inactive (IA), maturing (MA), running ripe (RR), and spent (ST).

Female Red Snapper		Macroscopic Spawning Class				
		IM	IA	MA	RR	ST
Histological Spawning Class	IM	**6**	2			
	IA	18	**150**	147	1	4
	MA	6	8	**461**	96	2
	RR		1	20	**59**	
	ST			11		

Table 7. Histological sex and spawning class with corresponding macroscopically identified sex and spawning class for red grouper (*Epinephelus morio*). Spawning classes included are inactive (IA), maturing (MA), running ripe (RR), and spent (ST).

Red Grouper			Macroscopic Spawning Class											
			Female				Male			Transitional		Unknown		
			IA	MA	RR	ST	IA	MA	RR	IA	MA	IA	MA	ST
Histological Spawning Class	Female	IA	**375**	76	5	5	3	1	1	19		2	7	
		MA	54	**443**	84	2			4	2				
		RR			**16**									
		ST	185	168	11	**2**				3	2			
	Male	IA												
		MA	11	2	1		6	**5**		5		3		
		RR	137	56	2	6	38	176	**82**	91	2	4	14	1
	Transitional		23	12		2				**4**				

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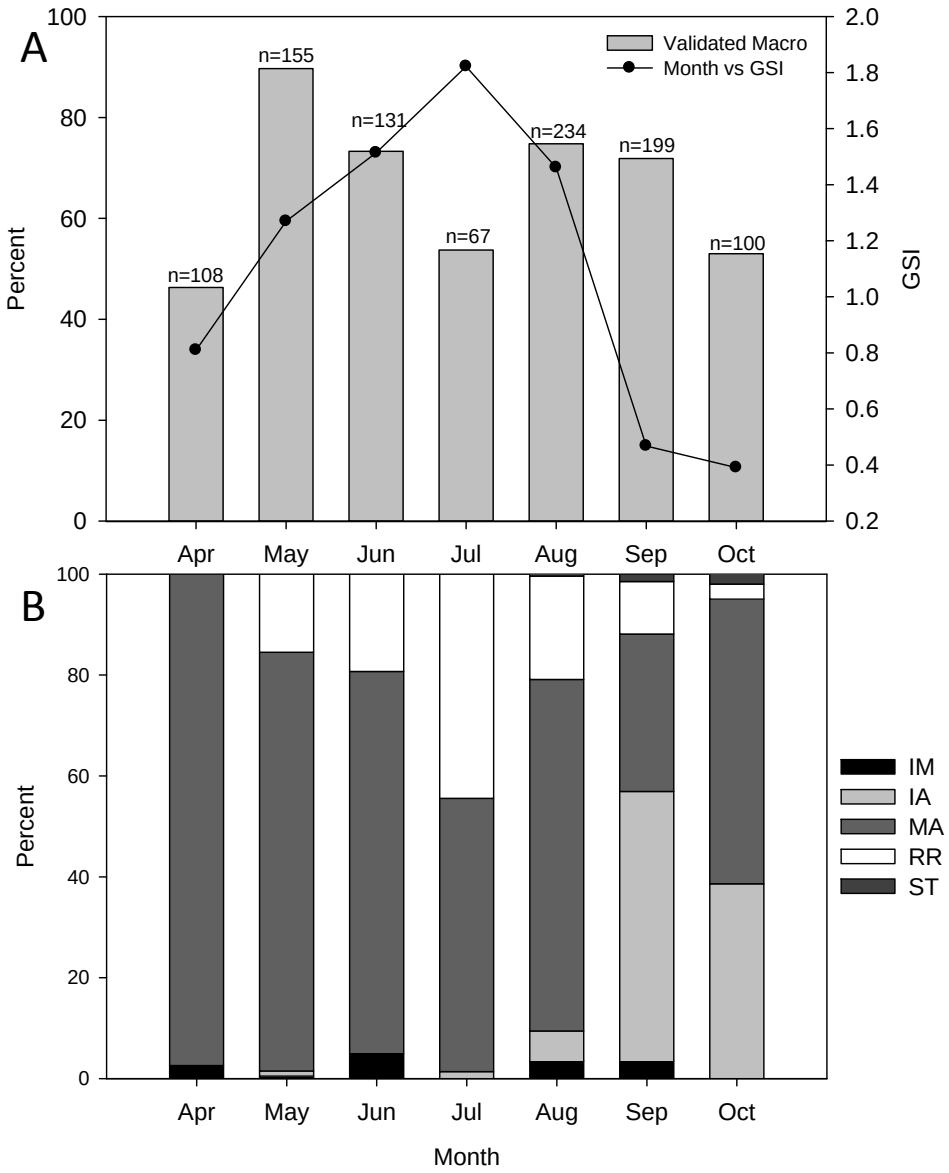
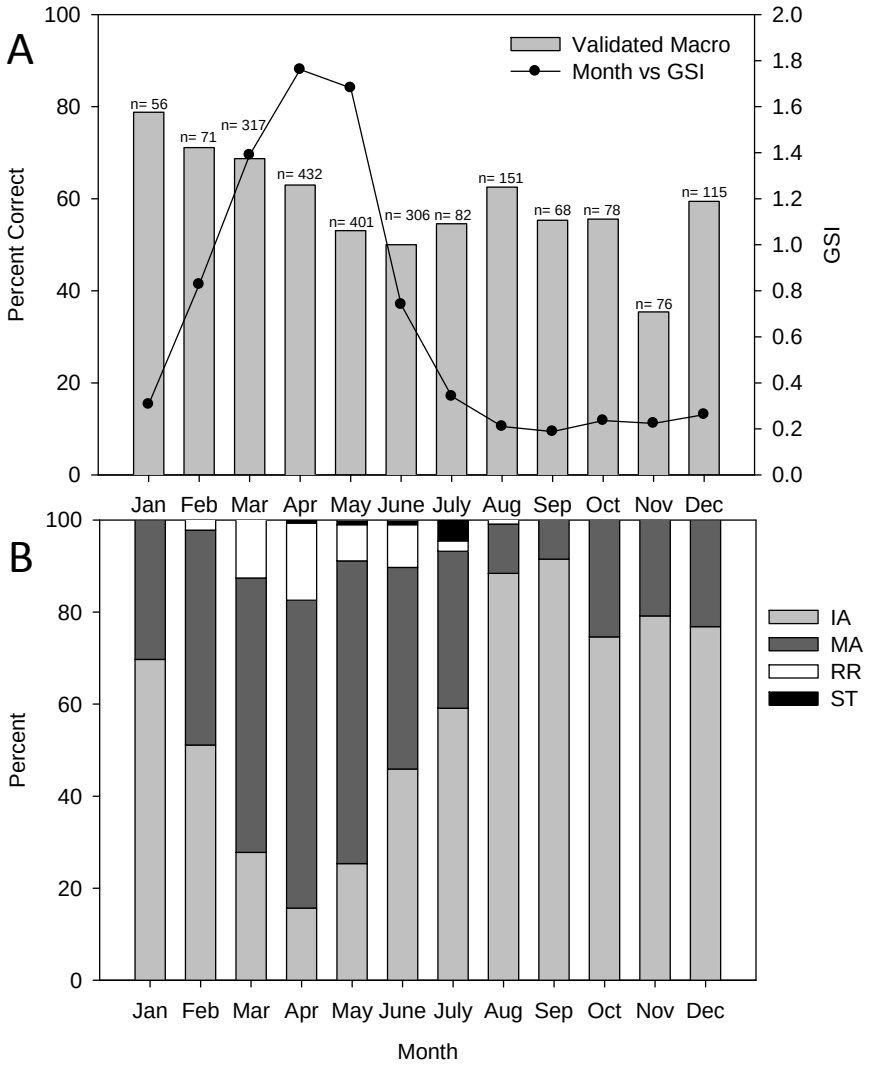


Figure 1 A.) Percent of macroscopic staging that was correctly identified with gonadosomatic index (GSI) for female red snapper (*Lutjanus campechanus*) across months and B.) Percent of each macroscopic stage present per month for red snapper females.



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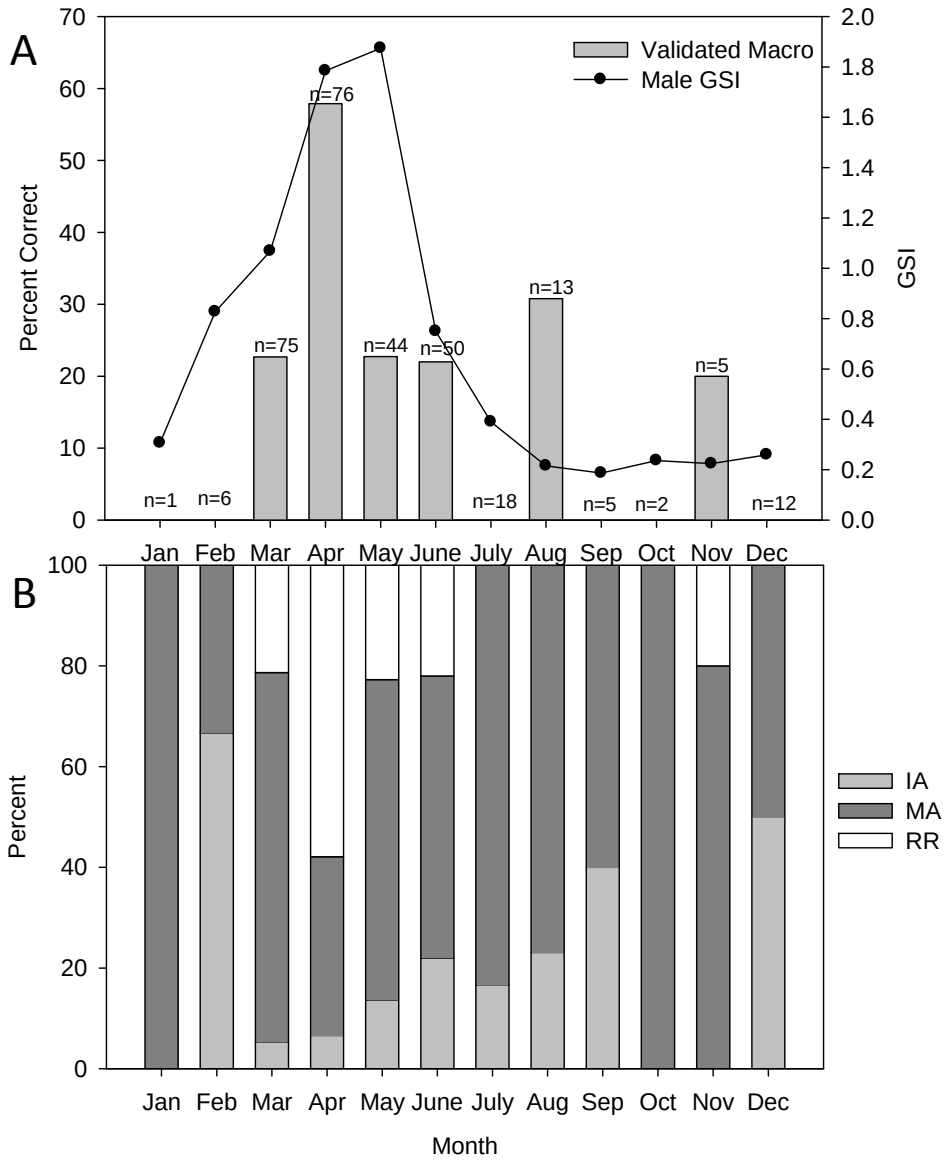

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