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ARTICLE

Marketing Program Implementation of Greek Fisheries Firms

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ABSTRACT

This study analyses the marketing program implementation in Greek fisheries firms. In this perspective, quantitative research with personal interviews to fisheries firms' executives is elaborated. Data were analyzed elaborating cluster and discriminant analysis. Findings reveal that there are two distinct groups of Greek fisheries firms regarding their decisions about the components of marketing mix. The results demonstrate that there are differences among the two groups mainly in terms of price and distribution policies. Particularly, 62.6% of sample firms seem to dispose a differential marketing mix, while 37.4% of sample firms seem to dispose a non-differential marketing mix. Notably, both clusters are not aware of quality and sustainability assurance certifications regarding seafood products. From this perspective, there is a potential for a better organized marketing program implementation aiming to respond efficiently in modern market needs.

1. Introduction

Marketing program implementation constitutes a crucial factor in marketing research field due to the fact that it has been positively related with firm performance, as well as with customer network size and knowledge [1]. Meaning that marketing mix components; product, price, promotion and distribution policies, play a significant role in the achievement of commercial success. Therefore, successful performance at firm level is strongly determined by the focus on marketing mix tactics indicating that these could have a significant impact on the ability of firms to respond efficiently to modern market needs [2]. This is particularly important for fisheries firms who are facing major challenges and threats at global level.

Specifically, fisheries firms are facing a liberazed trade regime. Analytically, rapid changes are occurred in market and consequently in consumer demand particularly in terms of quality and safety issues [3]. Undoubtedly, fisheries and aquaculture industries are of world's most globalized and interconnected industries. At the same time demand for seafood products is increasing. In a globalized economy, this situation should generate high opportunities for any seafood production activity but companies are facing key challenges, and part of the European Union (EU) fisheries sector remains at low levels of profitability. Additionally, the EU is the dominant trader of fisheries and aquaculture products at global level in terms of value, with increasing trends both in imports and in exports [4]. Particularly, Greece has a substantial production of European sea bass and gilthead sea bream at European level constituting the major supplier [5]. It is also worth noted the significance of fishing sector in economy at national level particularly in coastal areas, taking into account that Greece together with Spain, Italy and Portugal, constitute

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the 73% of employment of EU-28 in fishing sector, and Greece is representing the second highest level of employment in the sector [4].

Consequently, the present study aims to investigate and to analyze marketing program implementation of Greek fisheries firms. Since there is a limited detailed empirical research regarding stakeholders' analysis in fisheries and aquaculture sectors, and particularly in terms of marketing mix analysis at firm level, this study is critically significant, in an attempt to provide first insights in marketing program implementation. In conjunction with the fact that in marketing literature, it is continuously recommended to examine the combination of marketing mix elements rather than any single mix element on its own, indicates the necessity for an integrated approach regarding elements of the marketing mix [2]. Therefore, the objective of the study is to investigate and to further analyze marketing mix elements holistically and consequently how marketing program is implemented in Greek fisheries firms. Particularly, the paper reveals different groups of fisheries firms regarding their marketing program implementation whilst provides the potential for different dynamic relationships among firms that would not be feasible with individual observations.

2. Methodology

The study was carried out in major fish wharves in Greece, in terms of distribution volume. Field data was initiated employing personal interviews with executives from fisheries firms who are operating in the entire supply chain of fisheries and aquaculture products. The questionnaire used was simple and consisted seventeen questions separated by two sections. The first section consisted of fourteen questions covering marketing mix elements and particularly referring to product, price, promotion and distribution policies (Table 1). The second section collected general information regarding the major demographic characteristics of fisheries firms, including firm size in terms of employee number [6], firm age and type. Most of the questions in the survey tool employed a five-point Likert scale, ranging from (1) totally disagree to (5) totally agree. The advantage of providing respondents with only five choice positions contributes in avoiding responses converging on the middle response (i.e. three) and additionally, too many scale positions (e.g. seven-point scales) tend to confuse respondents [7].

Furthermore, the questionnaire was pre-tested on a sample of fifteen respondents selected by convenience to obtain face validity. Particularly, the pilot survey conducted with 15 executives from fisheries firms at the basis of personal interviews. Consequently, the necessary improvements were considered and the questionnaire was modified in accordance with the experience gained from the pilot survey. The main methodological steps followed in this study are illustrated in Figure 1.

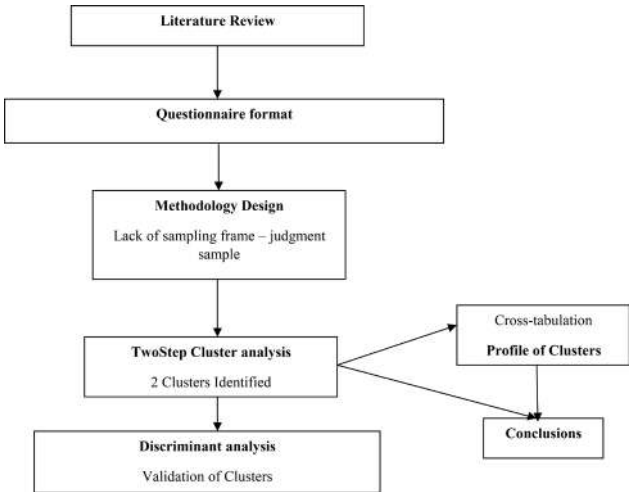

Figure 1. Flow chart of the methodology design

Table 1. Identification of variables used in cluster analysis

Code	Variables
PT	(Packaging type) wooden fish baskets =1, plastic fish boxes=2
QC	(Quality control) YES=1, NO=0
QG	(Quality gradation) YES=1, NO=0
HP	(Higher price due to lack of competition) totally agree =5 – totally disagree=1
PQ	(price policy based on quality) totally agree=5 – totally disagree=1
PMC	(price policy based on market conditions) totally agree=5 – totally disagree=1
PCP	(price policy based on competitors' price) totally agree=5 – totally disagree=1
CFP A	(Common Fisheries Policy (CFP) awareness) totally agree=5 – totally disagree=1
CFP MA	(CFP measures awareness) totally agree=5 – totally disagree=1
CFP MIBP	(CFP measures' impact on business profit) totally agree=5 – totally disagree=1
AT	(Agreement type) informal=1, formal=2
DC	(distribution channel) wholesaler=1, fish vendor=2, retailer=3, direct distribution=4
MPOs	(member in producer organizations) YES=1, NO=0
QSACA	(quality & sustainability assurance certifications' awareness) YES=1, NO=0

Due to the lack of a sampling frame based on more recent information, the snowballing procedure was chosen as the method of data collection [8]. Particularly, in this procedure, population elements are deliberately selected representing three major advantages: (1) they can meet the needs of the research, (2) they are representative of the population of interest, and (3) they can offer researchers the information they need. In all, 99 valid questionnaires

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were collected with this method. The reliability of the information source was assessed by emphasizing the identification of appropriate individuals from whom to elicit the requisite information and the willingness of these individuals to participate in the study. The respondents were considered appropriate if they were in executive positions and serving in firms as managers and/or owners and consequently if they were responsible for making decisions upon marketing program implementation. Thus, the respondents' answers applied to their field of responsibility and provided reliable and accurate information.

In order to identify similar groups of fisheries firms underlying the dimensions of Marketing Program Implementation, the method of cluster analysis was chosen. Clusters were selected aiming to obtain high internal consistency within each cluster, and high differentiation among clusters [9]. The two-cluster solution exhibited the simplest interpretation and showed the highest number of statistically significant differences among the clusters.

Additionally, in order to further validate the results of the cluster analyses [10], a discriminant analysis was applied. The latter is a method designed to derivate a linear combination of independent variables that will discriminate best between a priori defined groups [11]. However, there are limitations in applying this method, which is necessary to be considered and these are: (1) the ratio of sample size to the number of predictor variables must be at least five observations per independent variable, and (2) the sample size of each group must exceed the number of independent variables [12]. Ultimately, these limitations in the present study were considered and were in accordance with the data examined. The next step consisted of applying a cross-tabulation analysis with cluster membership.

3. Results

Table 2 presents the most important results of the methodology applied, regarding marketing program implementation of Greek fisheries firms. The two cluster solution was chosen. Particularly, the small and significant value of Wilk's Lamda represented a high level of discriminating power (Wilk's Lamda: 0.165, $\chi 2$=162,360, $\beta.\varepsilon$=14, p=0.000). Additionally, the hit-ratio (percentage correctly classified) was used, which actually provides how well the discriminant functions classified the objects. According to the hit-ratio, 99% of cases were correctly classified for clustering. Additionally, their respective cluster profiles are represented in Table 3 and are analyzed accordingly.

Cluster 1

The first cluster consisted of 62 fisheries firms, representing 62.6% of the total sample. Most of these firms (64.5%) are small-sized firms with relatively small number of employees and relative inexperienced since they have been in business for no more than 10 years. Additionally, this cluster is mostly represented by retailers. Fisheries firms in this cluster, in terms of product policy, stated that implement procedures of quality control and gradation whilst their primary packaging method regarding their seafood products involved plastic fish boxes. In terms of price policy, stated that this is strongly determined by market conditions (supply & demand) as well as prices are usually high due to lack of competition. Additionally, they use formal agreements. Furthermore, regarding promotion policy, declared that they are not aware of quality and sustainability assurance certifications regarding their seafood products and they do not belong to producers' organizations. The stakeholders also in this cluster are aware of Common Fisheries Policy (CFP) measures and considered that these measures have an impact on their business profit. Finally, in terms of distribution policy, they mainly use the direct distribution channel without intermediates. Based on these characteristics, fisheries firms in this cluster seem to dispose a "differential marketing mix".

Cluster 2

The second cluster consisted of 37 fisheries firms, representing 37.4% of the total sample. Most of these firms (40.5%) are larger firms in contrast with firms who belong to first cluster, with relatively larger number of employees and more experienced since they have been in business for more than 20 years. Additionally, this cluster is mostly represented by wholesalers (35.1%). Fisheries firms in this cluster, in terms of product policy, stated that implement procedures of quality control and gradation whilst their primary packaging method regarding their seafood products involved plastic fish boxes. In terms of price policy, stated that this is strongly determined by market conditions (supply & demand), but they declare that prices are not high due to lack of competition and their price policy is strongly determined by competitors' prices. Additionally, they use informal agreements. Furthermore, regarding promotion policy, declared that they are not aware of quality and sustainability assurance certifications regarding their seafood products and they do not belong to producers' organizations. The stakeholders also in this cluster are not aware adequately regarding CFP measures and considered that these measures may have an impact on their business profit. Finally, in terms of distribution policy, their major distribution channel is through retailers. Based on these characteristics, fisheries firms in this cluster seem to dispose a "non-differential marketing mix".

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Table 2. K-means cluster analysis results (two-cluster solution)

Variables	Codes	Cluster	Cluster
		1(N=62)	2(N=37)
Packaging Type	PT	2	2
Quality Control	QC	1	1
Quality Gradation	QD	1	1
Higher Price due to lack of competition	HP	4	2
Price policy based on Quality	PQ	5	5
Price policy based on Market Conditions	PMC	4	5
Price policy based on Competitors' Price	PCP	4	4
CFP Awareness	CFP A	4	3
CFP Measures Awareness	CFP MA	4	3
CFP Measures Impact on Business Profit	CFP MIBP	4	3
Agreement Type	AT	2	1
Distribution Channel	DC	4	3
Membership Producers Organizations'	MPOs	0	0
Quality & Sustainability Awareness Certifications' Awareness	QSACA	0	0

Note: Parentheses = % within cluster

Table 3. Cluster profiles using firm's size, experience and type

Cluster membership		Firm size	
	Small (%)[a]	Medium (%)[b]	Large (%)[c]
1st cluster	40 (64,5%)	6 (9,7%)	16 (25,8%)
2nd cluster	14 (37,8%)	8 (21,6%)	15 (40,5%)
Total	**54**	**14**	**31**
% total	**54,5%**	**14,1%**	**31,3%**
		Firm experience	
	Small (%)[d]	**Medium (%)[e]**	**Large (%)[f]**
1st cluster	25 (40,3%)	24 (38,7%)	13 (21%)
2nd cluster	8 (21,6%)	13 (35,1%)	16 (43,2%)
Total	**33**	**37**	**29**
% total	**33,3%**	**37,4%**	**29,3%**
		Firm type	
	Fishermen (%)	**Wholesalers (%)**	**Retailers (%)**
1st cluster	8 (12,9%)	16 (25,8%)	31 (50%)
2nd cluster	10 (27%)	13 (35,1%)	7 (18,9%)
Total	**18**	**29**	**38**
% total	**18,2%**	**29,3%**	**38,4%**

Note:

[a] firms with 1 - 3 employees [b] firms with 4 – 6 employees [c] firms with over 6 employees.

[d] firms with 1 - 10 years of experience [e] firms with 11 – 20 years of experience [f] firms with over 20 years of experience.

4. Discussion

In this study, a cluster analysis was implemented in an effort to identify possible distinct groups among fisheries firms regarding their actual choices and perceptions based upon marketing program implementation. Results revealed two distinct clusters.

Analytically, regarding cluster 1, fisheries firms in this cluster stated that prices are usually high due to lack of competition, while fisheries firms in cluster 2 stated that prices are not high due to lack of competition and their price policy is strongly determined by competitors' prices. Additionally, fisheries firms belonging to cluster 1 declare that their type of agreements is formal while in cluster 2, fisheries firms declare that they use informal agreements. This finding is in accordance with previous research results [13], where it has been found that most sales agreements are informal.

However, it is worth mentioned that both clusters declare that they do not belong producers' organizations. This is particularly important finding, since it has been in accordance with previous studies [13], indicating the lack of information services among the whole of supply chain in the examined sectors and suggesting the necessity for the development of an information network. Further studies have revealed [14] that especially in fisheries sector, where producers' organizations have been established, they have represented a modern and sustainable approach to fishery covering all the steps along the value chain, and consequently contributing in balance between supply with market demands and developing added value. Therefore, producers' organizations could contribute in a more stable supply and demand, in a better product quality, and even in better environmental management procedures particularly regarding sustainable aquatic resources.

Furthermore, another important finding is that that both clusters are not aware of quality and sustainability certifications concerning fisheries and aquaculture products. Failure to ensure sustainable fisheries and aquaculture may have consequences for firms' performance as well as the consumers. The results may be deteriorating taking into account possible ineffective policy measures. Although, criticisms to relevant certifications like Marine Stewardship Council (MSC) are growing due to several relevant issues that compromise the future of marine ecosystems [15], several studies have stressed the issues that purchasing is influenced by both context and attribute variables, such as environmental preferences [16] [17] as well as food safety standards [13] [18]. Additionally, in agrofood businesses pursue sustainability as an opportunity to offer new value propositions to customers and improve their competitive

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advantage [19]. Furthermore, particularly in the sectors of fisheries and aquaculture, sustainability has become an important issue, indicating that both for fisheries as well as for aquaculture sector, the conservation of biodiversity and consequently the sustainable management of aquatic resources are main issues [20]. This is also in accordance with the fact that it has been observed that particularly organic seafood is a major concern at consumer level, and consequently it has been found that consumers show a relatively positive attitude towards it, indicating potentially a general trend for organic seafood demand [20,21].

Ultimately, due to the snowballing procedure used in this study, the collection of data with the questionnaire depended on the single-informant approach. Therefore, the validity of research findings is affected by the original choice of quantitative methodology [9].

However, this study may provide an opportunity for further research regarding marketing mix elements analysis at firm level. Possible research avenues may pertain to a more detailed investigation of the factors affecting policy reforms, such as Common Fisheries Policy of E.U and considering changes in consumers' demand at global level for the sectors examined.

5. Conclusions

This study potentially is the first that brings new insights regarding marketing program implementation particularly in fisheries and aquaculture sectors at firm level. However, the research has limitations. The first is the relatively small size of the sample, which limit the external validity of the results. Second, this study was limited at national level. Potentially, field survey data derived by different sample prefectures and countries could additionally reveal major differentiation in marketing program implementation of fisheries firms. Therefore, generalizations of these findings to markedly different contexts should be made cautiously, taking into account the competitive and market structure differences that most likely exist between different prefectures and countries. Finally, this study focused on actual perceptions and choices regarding marketing mix elements, rather than stated preferences of. Future studies could integrate an analysis of perceptions, stated preferences and actual choices.

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ARTICLE

Life Stage, Gender and Movement of Blue Crabs (*Callinectes sapidus*) in Lake Mattamuskeet and Connecting Canals

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ABSTRACT

In their ranges on east and south coasts of the Americas as well as their established invasions in the Adriatic and Baltic, blue crabs, *Callinectes sapidus*, inhabit estuaries, sounds and coastal oceans and are commercially and ecologically important. How crabs move in response to physical variables is important to management. We monitored life stages at canal control structures, assessed gender ratios with recreational crabbing, learned from crabbers, and studied movements of tagged crabs in a canal connecting Lake Mattamuskeet to the Pamlico sound. Juveniles enter the lake through two of 4 canals connecting to the sounds. Females migrate out through one canal. The lake standing population is about 70% male. Movements of 240 crabs in August 2012 and 102 crabs in October 2014 were quantified using RFID tags with co-located meteorological and oceanographic devices. Non-spawning females and males are nomadic. Crabs released in the canal move in response to changes in water depth and go with the flow, toward the Pamlico Sound (summer 76% and fall 78%). What crabbers describe as a fall migration appears to be concentration of crabs in warmer deeper canals and then southern movement with flow generated by strong north winds. To be effective, management strategies like migratory corridors require understanding of crab movements.

1. Introduction

Blue crabs, *Callinectes sapidus*, range naturally from Argentina to Cape Cod [1] where they occupy aquatic habitats from coastal oceans to fresh to hypersaline sounds and estuaries. Invasive populations are established from Italy to Turkey and in the Baltic [2-5]. The blue crab life cycle is dominated by movements. Understanding movements is a key to effective active

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management.

In lunar tidal systems, early-stage larvae, settlement-stage megalopa, dispersing juveniles, and spawning females migrate using tidal stream transport. Early stage larvae move out of estuaries into the coastal ocean [1-6,7]. Zoea ride falling tides at night out of estuaries [6]. Planktonic, settlement stage larvae change vertical migration patterns in response to estuarine odors [8-11] migrating up-estuary on flood tides [12].

Females migrate to high salinity to spawn in 22 PSU or higher. Migrations are often over 5 km per day [13,14]. The spawning migration is punctuated by foraging [15,16] as migrating females gather energy and mature additional clutches of eggs [17-19]. Spawning female crabs move first to high salinity water and then into the coastal ocean [13,14, 20-22].

Cues that stimulate movements are known for some blue crab life stages. Megalopa respond to salinity, turbulence and light [7,23-25]. Juveniles respond to day vs night and to flow[12]. Spawning females respond to rates of change in salinity and water depth and walk and swim with falling tides [13,22,,26,27]. In strongly tidal systems, spawning females move with the flow on falling tides and walk against the flow with rising tides [13]. In the tidal Chesapeake Bay, movements of spawning females reported by Aguilar et al., [14] were 10 fold higher than movements of spawning females in the tributaries of the non-tidal Neuse River in north Carolina [28]. Spawning females in the South Atlantic Bight are found at least 13 km offshore [29]. In the weakly tidal Gulf of Mexico spawning female crabs are found over 70 km offshore (unpublished GOMEX cruise data). Spawning females have sequential clutches and are likely to complete their life cycle in the coastal ocean.

How non-spawning adult and late stage juvenile blue crabs behave is less well understood. In an embayment in North Carolina [19] and sub-estuary of the Chesapeake, late stage juveniles were found in shallower water than adult males. Both meandered with punctuated times of faster movement. Juveniles meander about 12 m/hour and adults meander about 24 m/hour. Adult males are nomadic. Juveniles and adults overwinter in deeper water [30]. Vertical swimming in juvenile crabs enables rapid planktonic dispersal over tens of kilometers. Thus, the little that is known suggests movements of juveniles and males are a combination of directed and non-directed movements.

Environmental cues that stimulate movement, decision rules, should be universal and work in the vast range of environments crabs occupy. Cues common in all habitats are changes in water height, turbulence, flow and night vs day.

Our goal is understanding the roles of cues in triggering activity, determining movement direction and rates of movement in wind driven systems. This knowledge has potential to inform management decisions for blue crab fisheries and conservation. Whooping cranes mainly eat blue crabs while wintering in Texas. Part of the impetus for doing this study is management maximizing crab populations in and around the Aransas National Wildlife Refuge. For fisheries man is the major crab predator. This information could inform management of large commercial blue crab fisheries in predominantly wind-driven sounds and estuaries such as in the Albermarle-Pamlico Estuarine System, NC and in large tidal fisheries like the Chesapeake.

The massive size of most wind driven estuarine systems and their complexity make experimental approaches intractable. We chose to study movements of crabs in Lake Mattamuskeet National Wildlife Refuge. Lake Mattamuskeet is a large shallow wind driven lake with a robust crab population, an extensive canal system and water control structures. The lake has only a spatially restricted and bag limited recreational fishery. Though still a large and complex body of water the system has features that make experimentation tractable.

Major questions

Five major questions guided this study: (1) do crabs show directioned movements analogous to STST? (2) do male and female crabs behave similarly? (3) does behavior change with season? (4) which environmental variables cue movement behavior? (5) is there a discrete fall migration? We also gathered life stage movement information which could be used in active management of crabs in the refuge.

2. Materials and Methods

2.1 Lake Mattamuskeet

Lake Mattamuskeet is a ~15,400 ha coastal bay lake modified first by settlers of Dutch origin with slave labor and becoming a wildlife refuge during the US great depression [31-33]. Though the mission of the refuge is to manage the lake and wetlands for wild fowl, the lake level is managed to support surrounding agriculture and lake water is used for irrigation. Moorman et al. (2017) [34], provide a succinct history of the lake. In the 19th century, the Lake was connected to the Pamlico sound with a network of canals that enabled draining of swamps for harvest of cypress and conversion to farm land. Today, there are 4 canal systems: 1 to the east, 2 to south and 1 to the west that connect the Lake to the rest

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of the APES (Figure 1A). Each canal is approximately 20 m wide and has a water control structure controlled presently by pressure-activated gates. When the water level in the Lake surpasses that of the estuaries the gates open; conversely, if the estuary water levels exceed that of the Lake, the gates shut [35]. Until 2013, water levels in the canals averaged between 0.61–0.88 m [35]. In 2013 and 2014, the control structures were rebuilt and canals dredged to a depth of 1.2 m. Lake Mattamuskeet averages 46 cm deep. At the beginning of the study, the side of the lake west of highway 94 was phytoplankton dominated and the east side was submerged aquatic vegetation dominated. By the end of the study the lake was virtually entirely phytoplankton dominated [34]. The lake experiences strong wind-driven circulation with very strong wind driven flows though the culverts on Highway 94 as well as into the canals.

Lake Mattamuskeet's status as a National Wildlife Refuge prohibits commercial fisheries. The lake supports a vibrant but spatially restricted recreational crab fishery with catch limits of 12 per person per day. Crabbing is primarily at the control structures on the 4 canals, at five 10 m wide culverts under the Route 94 causeway, which crosses the lake north to south and divides the lake into 1/3 to the west and 2/3 to the east, and at 2 bridges near the refuge headquarters. The lake is famous for crabs of consistently large size. A creel survey funded by the federal government and conducted by NC Fish and Wildlife personnel [36] estimated that 220,000 adult crabs were harvested recreationally that year. We chose a 1-km stretch of the Central Canal for detailed study of tagged crab movements in relation to environmental variables. Tagging studies were conducted in one summer interval and in one fall interval.

2.2 Initial Observational Data

In 2012 from June to August, we spent time learning about the lake. Targets were control structures, north and south culverts on US 94 and two bridges over canals near the refuge office frequented by crabbers. During June and July 2012 crabs were hand captured daily with a net and baited crab lines, photographed and sexed determined at the 7 locations. Flow conditions were noted. This effort was used to determine adult sex ratios and gain experience with where crabs could be most easily and consistently captured for tagging studies. While crabbing we made an effort to meet the resident crabbers and learn from them. Based upon their input, we verified some anecdotal comments with observations.

Figure 1A. Lake Mattamuskeet National Wildlife Refuge [37]

Note: Lake Mattamuskeet is about 0 .5 m deep 10 km wide and 20 km long. Major recreational crabbing is at 5 culverts on NC-94 which crosses the lake, 4 control structures controlling water entry and exit from the lake, and two bridges near the refuge. The area of detailed study by RFID tagging is in the box labled study area.

Figure 1B. Satellite view of the study area (Google Maps)

Note: The northern end of the Central Canal is the study area. The canal runs due North to due South. The refuge office is to the south west of the southern end of the location of water instruments under New Bridge. The weather station was located on a treeless area about 1 km east of the canal.

2.3 Study Site and Physical Data Instrumentation

We chose our study site (Figure1B) to maximize security and simplify data collection and interpretation. Major considerations were:

(1) The north/south heading of the canal and consistent canal width and conditions. These features simplified RFID antenna placement in north and south flow patterns.

(2) Approximately 8 miles of lateral canals draining

wetlands immediately south of the study area. These canals and wetlands acted as a buffer enabling wind forced flows in the canal for extended periods even when the control structure was closed.

(3) Security considerations included: proximity to the Refuge Headquarters and staff housing; Restricted access at New Bridge, enabling deployment of expensive and sensitive equipment under New Bridge.

(4) Ease of access by roads on both sides of the canal. A restricted access road west of the canal enabled secure placement of antenna controllers and facilitated battery exchanges.

Physical data were collected with a meteorological station and oceanographic instruments. The meteorological station (Campbell Scientific, WXT-520) was deployed at the end of a restricted access road in a treeless area on a blunt point (Figure 1B) 0.8 km east of the Central Canal. This station measured barometric pressure, air temperature, relative humidity, rainfall, wind speed and wind direction. The sensors were mounted on a tower 5 m above ground and recorded to a self-contained data logger (Campbell Scientific, CR-800) at 10 min intervals.

In concert with the meteorological station, the oceanographic instruments continuously collected data on water velocity, dissolved oxygen, water temperature, water pressure and salinity in the Central Canal. Water velocity was measured with a bottom-mounted 2 MHz acoustic Doppler current profiler (ADP, Nortek AquaDopp), that measured velocity profiles at 1 Hz and recorded at 1 min intervals. In 2012 and 2014 this device was deployed under New Bridge. New Bridge is part of the main access road over the central canal immediately north of the pump house. Water temperature, salinity, and pressure were measured with a bottom-mounted pumped CTD (Seabird SBE-16plus). Dissolved oxygen (DO) was measured with a Seabird SBE-43 cabled into the CTD. All CTD/DO data were recorded at 10 min intervals. In 2014 second instrument was mounted under a no-wake buoy, at the west side of the crab release site. Water velocity, water quality, and meteorological data were post-processed with a 1-hr low pass filter, and then interpolated onto a common time base with 15-min intervals. Weather and oceanographic sensor data products were then used as candidate environmental parameters to test whether they had an effect on crab movement behavior based upon synchronized RFID tag data.

2.4 RFID Antennas

Arrays of radio frequency antennae were deployed across the Outfall Canal. Antennae were constructed of 43 meters of 8 gage insulated copper wire in a loop separated by 1.2 m PVC pipe spacers. The antennae were tuned, activated, and data recorded using multiple antenna readers and control boxes from Oregon RFID, Inc. (Portland, OR). In 2012 two sets of two antennae were deployed (Figure 2). The pair of antennae between the release point and the lake was 112 m from the release point. The pair of antenna south of the release point was 612 meters away from the release point hidden under the New Bridge. Each antenna was comprised of 20 meters of 8 gage coated copper wire in a loop with 1.2m PVC pipe spacers. In 2014 the design was modified to loops with a width of 35 cm to improve excitation and detection and because the larger arrays became rapidly fouled in the fall with detached aquatic vegetation.

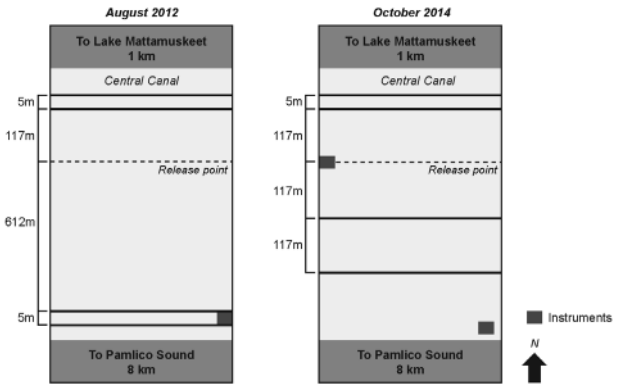

Figure 2. Study area in 2012 and 2014

Note: Sketch of study area, instruments and RFID antenna arrays in August 2012 and October 2014 in the Outfall Canal (not to scale). In 2012 each pair of antenna was approximately 5 meters apart and separated by 624 m. In 2014 the arrays were modified to the pattern depicted due to a combination of our improved understanding and practical equipment limitations. Crabs were released individually in the 2012 experiment and in groups in the 2014 experiment.

In October 2014, based upon what we learned from previous year, we altered the RFID array design (Figure 4). The antennae pair under New Bridge was also separated, with one antenna placed 117 meters south of the release point and one 234 Meters south of the release point. The antenna were 378 and 495 m away from crabbing activity at New Bridge.

2.5 Crab Collection and Tagging

In 2012 we employed a commercial fisher to soak 20 crab pots in the Central Canal from the mouth at the lake to the water control structure. Potting was after sunset when the refuge was closed and small diurnal turtles were inactive. Ten pots were set to the north from about 50 meters above the North antenna array to the lake and south from 50 meters above the release point to the water control structure for the Central Canal. After deployment, pots were

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soaked for 1 hour, emptied and redeployed. Crabs were delivered to the boat ramp where each crab was tagged across the back with a unique 32mm HDX+ PIT tag. The tags were inserted in silicone tag sleeves with a string with a loop at each end. The string was fitted around the large lateral spines across the back and loops were secured on the crab with a cable tie. After tagging a crab was photographed and released immediately. We tagged 202 crabs with the commercial crabber. An additional 38 crabs were captured in the day by net using weighted hand lines baited with chicken. All crabs were photographed with their RFID number displayed on an Agrident AWR 100 tag reader (Figure 3). In October 2014, crabs feeding on chicken were pulled to the surface, netted, and then fitted with across-the-back radio frequency identification (RFID) tags. Tag number and sex were determined from photographs. In 2014, 102 crabs tagged as they were captured were photographed and stored in coolers separated by damp clothes for up to 2 hours until release of a group within 5 minutes.

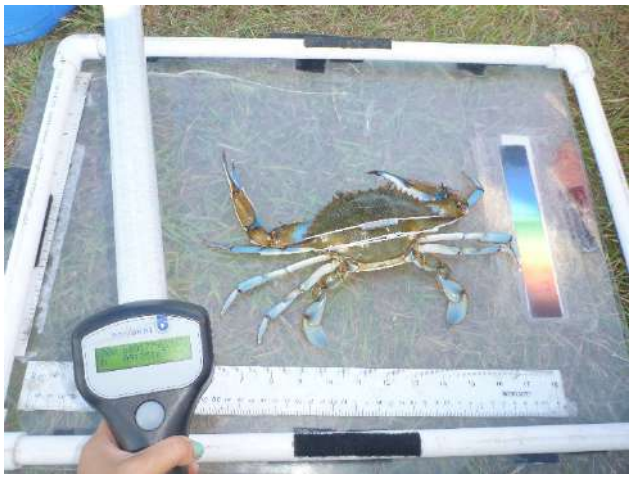

Figure 3. Photograph of a male crab with an RFID tag. All photographs are available on Fickr: Callinectes sapidus

2.6 Analysis

Crab movement and environmental variable data were synthesized using Microsoft Access and Excel. Direction of movement was based on the first detection. Direction of flow was determined for that time. To determine movement velocity we used crabs with a second detection. Time and distance between detections were used to calculate direction as well as movement velocity. We had simultaneous information on flow direction which enabled us to determine crab velocities in relation to water flow speed and direction.

Salinity, water temperature, water velocity, and dissolved oxygen were analyzed for patterns that could explain crab movements between the north (Lake Mattamuskeet) and the south (Pamlico Sound). Frequency analysis, chi square or contingency, was used to accept or reject specific hypotheses described in the text. Two-sided t-tests were performed in order to determine whether there was a significant relationship between direction of movement and each respective environmental variable. Statistical analyses were conducted in R Studio and through web-based statistical packages.

3. Results

3.1 Confirmation of Recreational Crabber Information

Most recreational crabbers were friendly and made every effort to help us. Over the three years that this study was conducted we were able to confirm many of their observations. Although we visited all control structures, most small juvenile crabs were encountered predominantly at Lake Landing and Waupoppin. We hand captured and sampled 100, 4 mm cw crabs with a pool leaf skimmer net in 20 minutes at Lake Landing in late May. As an aside we also found dozens of eel elvers in in the net. Later in the summers, July and August, larger, 30 to 60 mm cc, juvenile crabs were observed on the sound sides of the outfall, Lake Landing, and Waupoppin control structures. Rather than in the hundreds, the number of crabs was 1 to 10 on any particular day. We experienced the "fall migration" at Lake Landing all three years. Finally, we documented the reports of crabbers that most females could be captured at the outfall canal.

3.2 Sex Ratios of Crabs Captured Summer 2012

Seven locations were fished with chicken baited hand lines daily from June until August 2012. If there was flow, crabs were routinely captured at 6 of the 7 locations. No crabs were captured at the Rose Bay control structure. In total, 876 crabs were captured with 70% male and 30% female. Depending on location, crab gender ranged from over 85% male to 60% female (Figure 4). Female crabs were most common at the outfall canal water control structure and next most common at new bridge between the lake and the extensive southern canal system. Male crabs were most common at the North Culvert on highway 94. The largest female crabs, average 170 mm cw, were captured at the north culvert. The largest males crabs, average 178 mm cw. Were captured at the south culvert (data not shown). Episodically, and consistent with local crabber conversations, large numbers (10s to 100s) of small crabs (approximately 4mm to 30 mm cw) were observed

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swimming on the sound side and attached to floating vegetation at the control structures at Waupoppin and Lake Landing when the water control gates were closed.

Figure 4. Sex ratios of crabs captured by hand line during the summer of 2012

Note: Seven locations were fished with chicken baited hand lines every week from June until August 2012. Crabs were routinely captured at 6 of the 7 locations. No crabs were captured at the Rose bay control structure. n is the number of crabs caught at the site. Pink is female crabs. Blue is male crabs. Note high proportion of female crabs captured at the Outfall Canal control structure which is beyond the extensive canal system south of the lake. Highest proportions of males were captured at the culverts under the road crossing the lake. Image google earth.

3.3 Environmental Variables

The August 2012 RFID tagging interval was a typical of late summer weather in Coastal NC with prevailing winds from the southwest, gradual changes in air pressure which ranged from 1009 hPa to 1024 hPa, and air temperatures that fluctuated, usually diurnally, between 21°C and 32°C. The canal experienced daily heating and consistently high water temperatures between 26°C and 29°C. In contrast, in the fall (October) variations in pressure ranged between 1001 and 10024 hPa and were related to the passage of fronts. With warm fronts, winds were from the south and west and with cold fronts winds were from the north. Air temperatures were more variable in fall than summer and ranged from to 6°C to 28°C. Cold temperatures were associated with high pressure cold fronts and strong nighttime cooling with clear skies. Water temperatures ranged from about 9°C to 24°C. Fronts with cooling events were exemplified by cold fronts in the 10/07 and 10/ 20 intervals (Figure 5.) The canal experienced daily heating with larger changes than observed in summer. On 10/07 air temperatures dropped from the high 20s to below 10°C , a 19°C change). In the 10/20 interval air temperatures dropped from the Mid 25°C to about 6°C, a 19°C drop. On 10/23 water temperature dropped briefly to a level below 15°C where crabs are reported to be inactive. Maximum wind speeds were about 3 times higher in the fall than in the summer. Thus, August weather and October weather were very different and these differences are summarized in Table 1 and displayed in Figure 5.

Table 1. A summary of environmental conditions in the August and October study intervals

	August 2012		October 2014	
	Minimum	*Maximum*	*Minimum*	*Maximum*
Air Temperature (Celsius)	21°	32°	6°	28°
Barometric Pressure (hPa)	1009	1024	1001	1024
Wind Velocity (min/sec)	-5.0	4.9	-4.0	12.2
Water Temperature (Celsius)	26°	29°	9.0°	24.0°
Salinity (PSU)	–	–	0.74	0.75
Dissolved Oxygen (mg/L)	–	–	6.3	9.3
Water Velocity (min/sec)	-0.228	-.004	-0.330	0.040

Note: Negative numbers indicate winds from the south and water flow in the outfall canal to the south toward the sound. Positive numbers indicate winds from the north and water flows to the north toward the Lake.

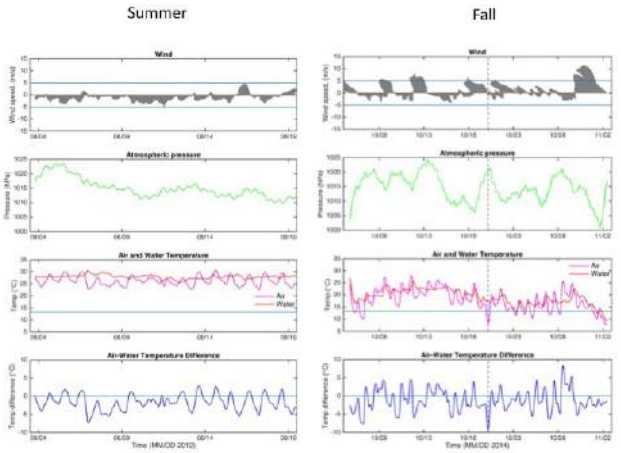

Figure 5. Wind, atmospheric pressure, air and water temperature, and the air-water temperature difference during the two tagging intervals

Note: The line at 13.3°C on the temperature figure is the reported low temperature cutoff for crabs to pot.

3.4 RFID Tagging, Detection, Gender and Direction

Of the 240 crabs caught haphazardly and tagged in 2012, 179 individuals (75%) were detected at least once. Forty-five crabs were female and 134 were male. In 2014, 89 (88%) of the 102 haphazardly caught, tagged and released crabs were detected at least once. For these, of the 60 crabs of known gender 15 were female and 45

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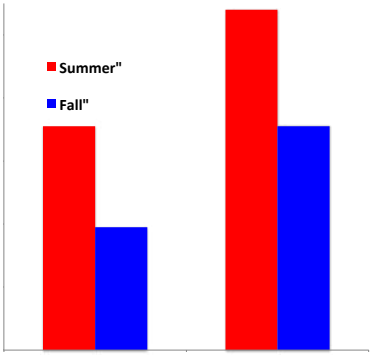
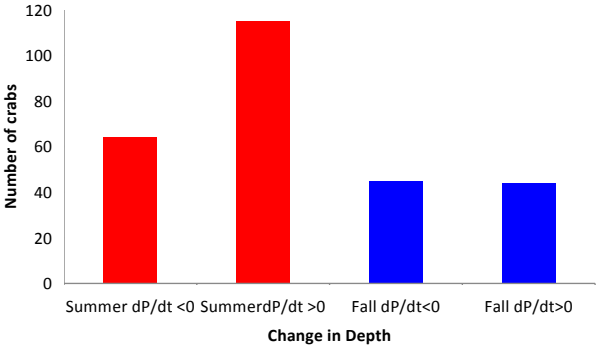
were male. We detected significantly more of the tagged crabs in 2014, rejecting the hypothesis that initial detection was equally likely (chi sq= 6.678, p =0.009 1 d.f.). In both tagging intervals sex ratios of captured and released crabs were similar, approximately 3:1 males to females(2012-2014 comparison chi sq=0.0005 p=0.981 df ns). The null hypothesis that sex ratios were equal was rejected for each year (2012 chi sq = 17.65, p<0.001 1 df; 2014 chi sq = 7.29 p= 0.006, 1 df). Sex ratios were skewed towards males and were similar in both seasons. In general, movements were not randomly oriented, but were primarily toward the sound. The vast majority of adult crabs (76% in summer and 78% in fall) moved toward the sound. In the 2012 and 2014 crab release intervals water flow was predominantly south toward the Pamlico Sound. Most crabs moved with the flow but at a slower pace than flow.

3.5 Rate of Change in Environmental Variables

Previous studies show crabs respond to rates of change in environmental variables [8,25,27].We compared frequencies of summer and fall movement and direction of rate of change in water depth, temperature, salinity, and dissolved oxygen. Using all of the movement data, we tested the hypothesis that overall direction of change made no difference. This hypothesis was accepted for temperature, salinity and dissolved oxygen, fall water depth (chi square = 0.006, 1 d.f., p = ns) but rejected for summer 2012 water depth (chi square = 5.4, 1 d.f., p = 0.02) Figure 6A. Next, we combined directional movements and compared directional responses of crabs in the summer and fall in response to rates of change less than 0 and greater than 0 (Figure 6B). The null hypothesis that there was no difference in total movement responses between summer and fall was accepted for temperature, salinity and dissolved oxygen and rejected for response to water depth (chi sq= 5.41, 1 df, p=0.02; Figure 5). Almost twice as many crabs moved in response to increased water depth than to decreased water depth in the summer (Figure 6B).



A.



B.

Figure 6. Movement of Crabs and rates of change in water depth in relation to movement in summer (red bars) and fall (blue bars)

Note: In both seasons more crabs moved south than north (6A). Crabs moved more frequently to increases in water depth in summer (6B). In fall the responses were approximately equal (6B).

The clearest example of movement in relation to the direction of flow is from October data before, and then after high pressure cold fronts moved through the region (Figure 7). This set of fronts resulted in flows predominantly to the north as the front approached and then strongly to the south after the front had passed. When flow was to the north, crabs move to the north. When flow was to the south crabs moved to the south. The null hypothesis that there was no effect of flow on movement direction was rejected (chi sq 1 df =19 p<<0.05. Crabs go with the flow.

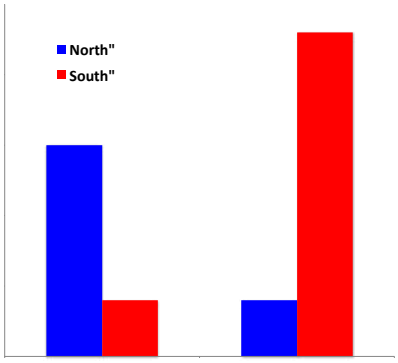


Figure 7. Movements of Crabs with flow predominantly to the north (<October 20) and then with flow predominantly to the south (>October 23)

Note: Blue bars are crabs detected moving north toward lake. Red bars are crabs detected moving south toward sound.

3.6 Crab Movements During the Fall

We were particularly interested in the movements of crabs during what is anecdotally called the fall migration. This

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is an interval in October where recreational crabbing picks up and a crabber can often catch his limit of 12 crabs in less than an hour. In 2014 the "migration" started when the water temperature dropped below 17°C (Figure 8). Inspection of the figure shows crab movements are usually clustered by direction. Crabs did not move across antennae when the water temperature was below 15. 5°C.

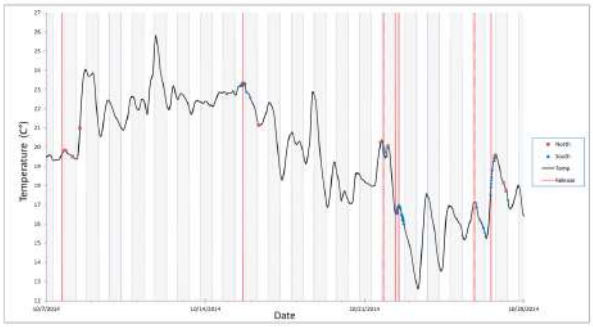

Figure 8. Crab Movement Direction and Temperature

Note: This figure was generated to show that movements are clustered in one direction or the other and that crabs stop moving as the water temperature approaches 15 C and then resume activity when the water warms above 15 C. The lowest temperature a crab was detected was 15.6 C.

3.7 Mean Crab Velocity

Mean crab velocity was computed for 47 crabs detected at more than one antennae for the 2014 data. Velocities were more variable for crabs moving towards the lake than for crabs moving towards the sound (Figure 9.). The October 25th 20:15 release was particularly striking. In that released crabs moving towards the lake moved at a significantly higher velocity than those moving towards the sound.

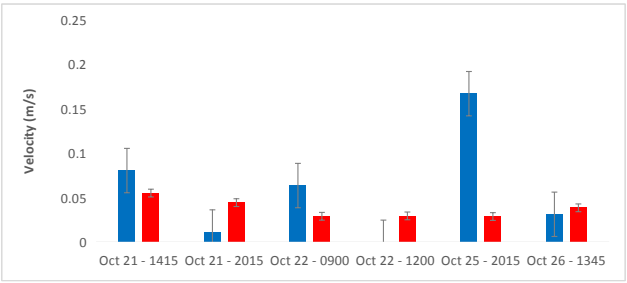

Figure 9. Crab velocities for crabs moving between antennae

Note: Velocities computed for crab detected at two antennae during the 2014 experiment. Velocities were calculated for crabs that were detected at two different antennae after release. Blue is North toward lake, Red is south toward sound. The numbers after the date are the 24 h time of crab releases.

3.8 Individual Crab Velocity

Individual crab velocity computed for the 47 individual crabs used to generate Figure 10. Crab velocity and flow velocity were converted to meters per minute and crab velocity plotted against flow velocity. All but two of the individual crab velocities ranged between a little over 0 m/m to about 4.5 m/min. Crab velocity was low as water flow approached 0 meters per minute and neared 4.5 meters per minute with flows approaching 20 meters per minute. Two individual crabs had movement velocities approximately double the highest velocities observed for the other crabs. These two crab velocities were approximately double the water flow velocity.

3.9 Flow and Crab Movement

Using the absolute value of flow, we binned flow into 15 minute bins and grouped the bins into four categories from slow, less than 0.09 m/s to fast, >0.3 m/s flows. We then grouped crab detections in the same time intervals. We tested the hypothesis that crab detection was equally likely at all flows. We found that significantly fewer crabs were detected at low flows than expected ($G > 7.8$, $p < 0.05$)and significantly more crabs were detected at high flows ($G > 11.34$, $p < 0.01$) than expected (Figure 10).

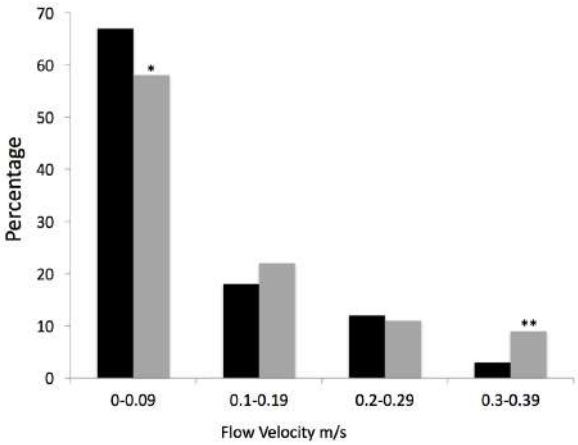

Figure 10. Histogram of percentages of crabs moving at ranges of flow velocities

Note: Statistics were based upon contingency analysis of frequencies. The null hypothesis that crabs were equally likely to move at all flows was rejected. Significantly fewer crabs were detected at low flows than expected and significantly more crabs were detected at high flows than expected. * $p<0.05$; ** $p<0.01$.

4. Discussion

Our primary interest was to determine how adult crabs move in response to environmental variables in the absence signals associated with lunar tides. In the shallow lake system that we chose for our study, flows were due to wind forcing and geometry. We simplified the system by working in a man-made canal oriented in a north-south

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direction with dominant flows to the south about ¾ of the time. Our goal was to compare crab movements in the summer foraging season with movements during the recreational crabber reported "fall migration" in which crabs were reported to move in high numbers from the lake to canals and then to the sounds.

4.1 We Asked 5 Questions:

(1) Do crabs move with the flow analogous to selective tidal stream transport (STST)? We found crabs move with the flow, but movement is not analogous to STST because crabs move with the flow independent of the direction of the flow.

(2) Do male and female crabs behave similarly? Yes, male and female crabs behave similarly. However, based on gender ratios from our early crab capture data, some females, presumably those with mature ovaries appear to actively migrate out of the outfall canal toward the sound. The cues for this movement are unknown.

(3) Does behavior change with season? This is a qualified yes. Basic crab behavior is the same. However, as water cools in the fall crabs concentrate in the deeper warmer canals. Then when the wind blows and water moves concentrated crabs move in the canals and can move though the control structures or back toward the lake.

(4) Which environmental variables cue movement behavior? Change in water depth is the only variable we found that cued movement. We couldn't test salinity because there is only a weak salinity signal in the lake.

(5) Is there a discrete fall migration? No, there is no evidence of a discrete fall migration. Crab activity increases with increased flows driven by stronger winds associated with the passage of weather fronts. Although the crabs still move with the flow, this is nomadic movement because crabs move with the flow independent of flow direction. Crabbers do not see the crabs moving toward the lake at control structures as we did with Tags in canals with antennae because gates block flow toward the lake.

Crabs move in response to wind forcing when relative water depth changes. Crabs move with the flow. This is consistent with lunar tidal systems in which crabs move in and out of habitats with the tides [19]. In the lake, foraging male and female crabs move similarly. Males and adult females in the first ≈ 2-3 months post molt (the time it takes to mature ovaries and seminal vesicles) would forage and move nomadically, while females physiologically ready to begin spawning would show more directed down-stream movements and reproductive males would periodically spend time on station for breeding.

There are two potential explanations for the observed three to one sex ratio between male and female crabs. One explanation is fewer female crabs migrate to very low salinity water than males. The other explanation is males stay and female crabs begin their spawning migration and actively move down stream after their ovaries mature. In North Carolina, female crabs molt to maturity and mate from March to November.The higher percentage of females at the outfall canal supports he interpretation that small numbers of the females change behavior and actively migrate to high salinity water[39]. This is consistent with our gender data. Our RFID tag data sets are too small to detect the females starting this migration. Pulses of spawning migrations observed in other estuaries[14,22] are probably due to spring and fall synchronization of physiological responses by temperature and other environmental variables.

Based upon the proportions of males and females captured, the Lake Mattamuskeet population is unusual when compared to commercial fishery populations in that mating is not male limited as predicted for US East Coast fisheries populations[17,40-43]. Condition of females in Lake Mattamuskeet would be an interesting comparison with females from male limited populations like the Chesapeake with reduced reproductive output through sperm limitation[17].

Movement velocities of individual crabs provides a sense of how crabs move in flow. Our interpretation is at this life stage, crabs go with the flow and by and large are walking. The slow walking in lower flows suggest crabs are casting laterally as they walk down current. This behavior is consistent with what we observe with crabs foraging in shallow water. We think the slow walking in low flow velocities (near 0 flow) reflects a lack of clear direction and activity and the fast walking in high flow reflects directed movement. The velocities we observed are similar to movement velocities of crabs walking seaward during the spawning migration[13]. They are considerably faster than average 0.3m/min speeds observed in a flume walking up current in response of chemical stimulation [44].

We hypothesize the two very high individual crab movement velocities in Figure 10 are crabs swimming in flow. Although swimming is routine for blue crabs undergoing STST during migrations up or down estuary, it is observed in male and female crabs occasionally in Lake Mattamuskeet. On days when one crab is seen swimming usually others are also seen swimming (Rittschof P.O.). It may be that in the lake and canals nonmigrating crabs swim over a very narrow range of flow velocities (Figure 10). It is likely that swimming can be stimulated by several environmental variables including turbulence[25] and potentially low oxygen at depth.

Clark et al. (1999) [45] followed male crabs by ultra-

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sonic telemetry in a tidal creak in the Chesapeake Bay and provided elegant information on a small number of male crabs spend their days. Hooper (1999) [46] in a sea grant study of recently molted male crabs showed newly molted crabs generally stayed in the area where they were captured until their exoskeletons were calcified. Our data expand these findings to include the observation that males are nomads responding by foraging with the flow at movement rates comparable to those observed for females [19].

Consistent with the extensive literature reporting environmental control of movement behavior, we hypothesized that crabs would move in response to currents in the larger context of physiological state and biological rhythms. Our data support this hypothesis. Rates of change in water depth predict movement of adult male and female crabs in the summer and in the fall. Supporting the hypothesis that crabs at this life stage are nomadic is the result that crabs move with rates of change in water depth independent of direction of the change and crabs go with the flow. Thus crabs are not performing the equivalent of STST by moving in one direction, using STST observed in megalopae and spawning females. However, since the net flow in the lake is out of the lake and into the sound, crabs in canals that move with the flow would eventually end up in the sound.

In the fall, especially after cold shocks, crabs moved in groups. However, our interpretation of the data suggests that rather than a true migration, individual crabs are responding to physical factors that result in their aggregation and then cause them to move in the same direction. During these intervals crab movements are with the direction of the water flow. Thus the reported "fall migration" is a response of individual crabs to environmental conditions that result in the appearance of a coordinated fall migration. These movements are similar to what has been reported for aggregations of mole crabs on ocean beaches [47].

There is a clear seasonality to crab capture in Lake Mattamuskeet [36]. It is easiest to catch crabs in the spring and in the fall when the wind blows the strongest impacting water height and flow. The most difficult times to catch crabs are when the wind blows the least. In our intervals of catching crabs by baited lines, when there was low to no flow one was lucky to catch one crab. In contrast, if there was flow one caught crabs.

In addition to setting the lower limit for crab foraging and movement, temperature may play other important roles in crab activity. In summer, there is less wind forcing, and water temperatures in all portions of the lake support crab activity. Hypothetically crabs are dispersed maximally and less likely to be in canals in the summer. In the spring and the fall, crabs, if they behave as they do in tidal systems tend to move into deeper warmer water as nighttime cooling impacts the shallow water. The deepest water in Lake Mattamuskeet is the canals. Relic canals from when the south side of the lake was a vegetable farm may serve as crab collectors as the lake cools. Thus, when the wind blows and if temperature is high enough to support crab activity, crabs go with the flow and are funneled to specific canals.

There is extensive overlap between the nomadic behavior of crabs and what has been termed Phase I migration[21]. As noted by Aguilar et al. [8], and seen as well in the APES, mature females are routinely the majority of the pot fishery catch during the fall. These mature females are recently molted, nomadic and foraging. In North Carolina these crabs have little value in the basket markets and are sold to the crab meat picking industry.

4.2 Management Implications

In applying our findings to place-based management practices, our most important findings for Lake Mattamuskeet crab management are: (1) juvenile crabs enter the lake through Lake Landing and Waupoppin canal control structures and entry is episodic; (2) female crabs leave the lake though the Outfall Canal; (3) crabs that grow up in the lake are nomadic with movements triggered by changes in relative water depth and movement is in the direction of flow; (4) approximately 70% of the crabs in the lake are male rendering the lake female limited. Active management of the control structures when juvenile crabs are migrating in would maximize the lake population. The lake is a potential source of very large male and female crabs for improving depleted fisheries. However, as managed, the long canals are choke points and filled with commercial crab pots. It is unlikely any crabs survive that gauntlet. Only one canal, the outfall canal appears important to female egress from the lake. Female crabs could be captured by weir at the outfall canal control structure and transported to sanctuaries or there could be a ban on commercial capture of female crabs on some schedule in the outfall canal and sound.

5. Conclusions

The simplicity of the logic of water height change and flow determining movement direction is an attractive decision rule because it applies to lunar tidal as well as wind driven tidal systems. It predicts that if one has a real time model of the physical forcing of flow in an estuary and if one knows where the crabs are that one can predict where and how fast crabs will go. As reported by Carr et al. [13]

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and Darnell et al. [28], and seen here, crab movement rates are a fraction of the actual flow rates. It is likely that expert commercial crabbers know the relation between flow and crab movements and that knowledge explains why some crabbers are consistently more effective at harvesting crabs than others.

That the lake population has a high proportion of males and is not male limited has implications for the reproductive quality of the female crabs. In the lake proper, male and female crabs average approximately 170 mm CW which is routinely larger than all but the very largest crabs in the fishery and in two fecundity studies [18,19] . If Lake Mattamuskeet crabs could travel to high salinity water and spawn, these crabs could help counteract fisheries pressure for smaller more rapidly reproducing males [40] and females [49] .

Management of the life stages of crabs such as spawning females using migration corridors requires sophisticated understanding of crab behavior. For example, migrating crabs do not simply move down current and remain in deep channels. Rather crabs migrate using STST and then, if the water is warm, routinely move into shallow water to forage [13,15,48]. Thus establishment of migration corridors as a management tool should also include shallow foraging habitat. Migration corridors would not be an effective management option for the majority of crabs in the fishery as these crabs are nomadic rather than migratory.

The water control structures which function to keep farm fields from flooding are choke points for blue crab movements in and out of the lake. As sea level continues to rise and the land in Eastern NC continues to subside, the water control structures will increasingly impact crab entry and exit as the gates will be closed increasing periods of time. This issue needs to be addressed with an active management plan if the lake is to continue to be a vibrant recreational crab fishery and continue to contribute a large proportion of the anadromous fish spawning habitat in North Carolina and Virginia.

Blue crabs are the most valuable and largest fishery in North Carolina. However this position is threatened by a severely declining stock. Populations have declined steadily since the mid-1990s, with reasons for that decline rooted in anthropogenic [50] and environmental [51] stressors. By more thoroughly understanding the biology of crabs in APES extensive wind-driven estuarine systems, state fishery and wildlife managers can better adaptively manage crab resources—such as crabs in Lake Mattamuskeet National Wildlife Refuge-potentially to enhance the current commercial stock. If science-driven management is an option, then the blue crab fishery in Lake Mattamuskeet could be successfully managed. The success of the blue crab fishery in North Carolina and the sustainability of the state seafood economy are intimately coupled. Potentially, lessons from Lake Mattamuskeet could be applied to the larger fishery.

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ARTICLE

Growth Pattern and Morphological Variation of *Labeo calbasu* Found in Indus River, Sindh-Pakistan

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ABSTRACT

The present work reports the length–weight relationships (LWR) and condition factor relationships for Labeo calbasu collected from Upstream (Matyari) Kotri barrage at, River Indus, Pakistan, because stock assessment helps the fisheries managers to conserve the commercially important fish. Morphological characters of fish as well as Length-weight relationship are an important tool for fishery management. The results of LWR (W= aLb), for L calbasu. Representing negative allometric growth pattern. LWRs and condition factor relationships were found significantly correlated. A total of 200 and 190 specimens from upstream and downstream were collected, respectively. The assessed values of length-weight correlation and condition factor were calculated as Kn=39.663 (LeCren), and K=11.915 (Fulton) for upstream and Kn=44.066 and K=13.872 for downstream. Length-weight was found with a strong correlation of n= 2.892, a=0.0235 with r2=0.934 for upstream population then the downstream population. The results of this work would be beneficial for sustainable management as well as fishery managers.

1. Introduction

Fisheries are one of the very imperative bases of income and socio-economic industry of our country and serves as an important food sector in human nutrition [1]. Labeo calbasu is a freshwater fish species belonging to the family Cyprinidae under the order Cypriniformes. It is a popular best food fish having delicious flavor. Less intramuscular bones and high protein contents is significance of this species. This is known as sport fish and having delicious taste, recently introduced as ornamental fish in the market of Pakistan and abroad [2]. In last few years, the wild inhabitants of this fish species have extremely dropped due to over fishing and other anthropological causes [3,4,5,6]. In Pakistan it has been recounted as Lower Risk near endangered and in Bangladesh as rare species [4,7]. Labeo calbasu supports an important commercial fishery in the Rivers Ganga [8], Yamuna [9], Ghaghra [10] and middle stretch of Ken [11] and Indus River Pakistan. In our country, Labeo calbasu is a one of great commercial important species resembling three other Indian Major Carps such as Catla, Rohu and Mrigal. The Rivers in Pa-

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kistan are challenging several problems intended for severe water pollution, over extraction, intrusion, dams and barrages which cut off the connectivity of the River with its associated ecosystems, climate change, deforestation in catchment areas, etc. In fisheries science, relationships of length weight provide measurements, which are keystone in research and management and are main gears for accurate assessment of biomass and calculation of length frequency samples to total catch [12].

This species occurs mainly inhabits Rivers and Ponds, also in natural Lakes, reservoirs, streams and canals [13,14,15,16,17]. Its favorite habitation is the abysmal pools of Rivers, where it largely remains localized during the winter and summer months, and ascend to adjacent shallower region of the river for breeding during monsoon months [14]. It can be cultured in ponds and tanks [1316]. It can tolerate slightly brackish water also. Fish populations are natural control processes that continually modify with adjust structure, abundance and wide range of factors. Besides some factors as overfishing, species composition, population outbreak, behavior, species switching from small size to large, ecosystem degradation, seasonal fluctuations, pesticide and aquatic pollution, diseases, introduction of exotic species, destruction of breeding grounds and unlawful fishing practices [18]. The maximum reported size for this fish is 90cm [19] but during the last few decades has not been reported. Length weight relationships assist in adaptation of growth in length equations to growth in weight equations particularly in fishes, which is a useful parameter for ichthyologists and fish farmers in assessment, culture, and stocking of fish. Length weight relationships are good indicators of fitness and fish condition [20].

2. Materials and Methods

2.1 Data Collection

In the present study report, Labeo calbasu (200 from upstream and 190 from downstream) was collected from Indus River, Sindh-Pakistan. The specimens were brought to the Department of Freshwater Biology and Fisheries, University of Sindh for species documentation and evaluating growth parameters. Identification of selected fishes was done with the help of related literature, accounts and keys specified [17]. A total of 15 morphological traits (Table.1-2) and 6 meristic traits (Table.3) were measured and calculated.

Morphometric was measured on the fish measuring board while meristic traits were calculated with the help of magnifying lens. The eye diameter was measured by the caliper. The weight of fish was taken by the digital balance machine. The meristic traits were calculated with the help of magnifying lens. The data was analyzed by using SPSS (11.5) software package length weight relationship was calculated as standard [20]. All the parameters were measured for data analysis to calculate the effect at different environments of Indus River, Sindh.

2.2 Statistical Analyses

2.2.1 Length Weight Relationship and Condition Factor

Length weight relationships were calculated using the least square fitted method to Log transformed data using the function as suggested by the Le Cren [20] equation $W= aL^b$. Whereas: W is the total weight of fish in g, L was the length of fish in cm, a was constant condition factor and b was an exponent indicating isometric/allometric growth.

The parameters a and b were estimated by linear regression on transformed equation. The equation 1 could be expressed in the linear form by using logarithms, as given below:

$$\text{Log } W = \text{Log } a + b \text{ Log } L$$

The estimates of the constants c and n were obtained empirically by using the formulae, as given below:

$$Log\ = \frac{\Sigma LogW \times (\Sigma logL2) - \Sigma LogL \times \Sigma(LogL \times LogW)}{N(\Sigma LogL2) - (\Sigma(LogL)2)}$$

$$n = \frac{\Sigma LogW - NLogC}{\Sigma LogL}$$

2.2.2Condition factor 'k':

The condition factor of the adult fish was determined the Fulton's Condition Factor (K) was computed by using the formulae, as given below:

$$\text{Condition Factor } (k) = \frac{Weight\ (g)}{(\ length)3\ (cm)} \times 100$$

(K= condition factor, W= weight of the fish and L= length of fish). Condition factor (K) was determined for different length groups using length and weight data following the equation given by LeCren[20]:

$$\text{The LeCren Condition Factor } Kn = \frac{(w \times 100)}{L3}$$

3. Results

3.1 Length Frequency Distribution

The smallest length was witnessed 7.5cm and the highest

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length was noted 28cm with an average length of 20.892 cm for average of upstream and downstream population.

3.2 Length Weight Relationship

The expected b values in current study for Labeo calbasu from Indus River (Matyari) Sindh Pakistan was a= 0.0235, b= 2.892 and correlation r^2= 0.934 for upstream and a= 0.0458, b= 1.792 and correlation r^2= 0.849 for downstream population (Table 4).

4. Discussion

The achieved parameters of Length-weight relationship specify the evidence about the seasonal changes in their environment and about physical well being of the fish. It is also states the isometric or allometric growth of the fish, this evidence about the growth pattern of the fish is considered to be an essential feature to know the fish population dynamics. The statistical correlation between the length and weight is highly significant tool for the estimation of the weights of the fish of known lengths [21]. The estimated Length weight parameters in current research were compared to Length weight parameters of the other scientist's work (Table 5). The projected results of (a) 0.024 in our study for Labeo calbasu were normally smaller than previous assessed results and the estimated results (b) 2.892 in our study for Labeo calbasu were generally moderate as the previously estimated results from various parts of the world for related species. However the estimated results of (b) value 2.892 in our study for Labeo calbasu were less than 3, which indicates negative allometric rate. While small variances in results may be due to availability of food, condition of maturity and spawning, sex differences [22,23,24].

When we calculated the correlation of traits, with body weight and among other morphological traits, then we found that SnL (0.160) and AFL (0.027) show weak correlation while all other had strong correlation for upstream population (Table 1). While, only ED shows (0.098) weak correlation for downstream population (Table 2). Further, we investigated the correlation of TL with all other traits. It was found that SnL (0. 172) and AFL (0.031) had weak correlation for upstream population. While, all other traits show strong correlation with TL in downstream population, except ED (0.211) as shown in Table 6.

The Condition factor (k) reflects, through its variations, information on the physiological state of the fish in relation to its welfare. The Fultons condition factor k value recorded 11.915 and the Le Cren condition factor (kn) value of labeo calbasu was 39.663 for upstream population. While, the k and kn was recorded 13.872 and 44.066 for downstream population, respectively (Table 4). The fluctuation in the value of k if fish has been mainly assigned to dependency on many factors such as feeding, intensity, fish size and availability of fish.

5. Conclusion

The results indicate that Labeo calbasu showed an almost negative allometric pattern of growth in the present habitat and the condition factor values showed that it is in not good condition or health due to environmental factor. It reveals that present environmental situation of Indus River has great influence on the growth of L.calbasu. These findings may be is useful to the study of fishery biology; conservation biologist, successful development, production and management of fishes and ultimate conservation of this threaten species.

Acknowledgment

The present work is partial work of M.Phil research scholar.

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Appendixes

Table 1. Correlation of various morphological traits of Upstream Population L. calbasu from Indus River, Sindh-Pakistan

	w (gm)	TL (cm)	ED (mm)	SL	HL	FL	Girth	DFL	PFL	VFL	AFL	CFL	SnL	PVD	VAD
w (gm)	1														
TL (cm)	0.841	1.000													
ED (mm)	0.635	0.730	1.000												
SL	0.869	0.965	0.750	1.000											
HL	0.751	0.863	0.700	0.908	1.000										
FL	0.820	0.892	0.664	0.918	0.812	1.000									
Girth	0.767	0.890	0.742	0.915	0.877	0.862	1.000								
DFL	0.762	0.813	0.608	0.837	0.740	0.777	0.772	1.000							
PFL	0.706	0.803	0.559	0.825	0.759	0.764	0.813	0.778	1.000						
VFL	0.749	0.807	0.656	0.823	0.760	0.800	0.825	0.700	0.687	1.000					
AFL	0.027	0.031	-0.146	0.039	0.010	0.037	-0.088	0.056	-0.006	-0.099	1.000				
CFL	0.756	0.857	0.736	0.884	0.848	0.779	0.911	0.763	0.738	0.806	0.000	1.000			
SnL	0.160	0.172	0.143	0.184	0.162	0.196	0.156	0.149	0.127	0.167	-0.123	0.146	1.000		
PVD	0.813	0.910	0.766	0.920	0.835	0.873	0.903	0.789	0.762	0.793	-0.052	0.855	0.142	1.000	
VAD	0.737	0.776	0.673	0.798	0.725	0.776	0.767	0.671	0.605	0.794	-0.086	0.743	0.099	0.794	1

Note: TW=Total weight, TL =Total Length, ED=Eye diameter, SL=Standard Length, HL=Head length, FL=Fork length),Gr= Girth, DFL=Dorsal Fin base, PFL=Pectoral Fin Length, VFL=Ventral Fin Length, AFL=Anal Fin Length, CFL=Caudal Fin Length, SnL=Snout Length, PVD=Pectoral Ventral Distance and VAD=Ventral Anal Distance (1= strong correlation, 0.5= moderate correlation and 0.5<, weak correlation)

Table 2. Correlation of various morphological traits of Downstream Population L. calbasu from Indus River, Sindh-Pakistan

	w	TL (cm)	ED (mm)	SL	HL	FL	Girth	DFL	PFL	VFL	AFL	CFL	SnL	PVD	VAD
w	1														
TL (cm)	0.921	1													
ED (mm)	0.098	0.211	1												
SL	0.935	0.991	0.175	1											
HL	0.826	0.918	0.170	0.919	1										
FL	0.902	0.955	0.172	0.954	0.861	1									
Girth	0.865	0.948	0.177	0.940	0.906	0.923	1								
DFL	0.922	0.980	0.146	0.986	0.895	0.946	0.938	1							
PFL	0.879	0.919	0.004	0.929	0.859	0.893	0.919	0.933	1						
VFL	0.897	0.921	0.124	0.925	0.856	0.899	0.918	0.925	0.880	1					
AFL	0.845	0.884	0.113	0.887	0.813	0.878	0.796	0.865	0.867	0.782	1				
CFL	0.839	0.919	0.171	0.926	0.877	0.862	0.947	0.923	0.880	0.922	0.746	1			
SnL	0.842	0.855	0.090	0.857	0.801	0.887	0.827	0.857	0.787	0.900	0.800	0.802	1		
PVD	0.831	0.921	0.259	0.923	0.839	0.899	0.877	0.912	0.831	0.868	0.784	0.897	0.828	1	
VAD	0.892	0.912	0.185	0.918	0.844	0.888	0.882	0.911	0.826	0.909	0.801	0.895	0.891	0.877	1

Note: TW=Total weight, TL =Total Length, ED=Eye diameter, SL=Standard Length, HL=Head length, FL=Fork length),Gr= Girth, DFL=Dorsal Fin base, PFL=Pectoral Fin Length, VFL=Ventral Fin Length, AFL=Anal Fin Length, CFL=Caudal Fin Length, SnL=Snout Length, PVD=Pectoral Ventral Distance and VAD=Ventral Anal Distance (1= strong correlation, 0.5= moderate correlation and 0.5<, weak correlation)

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Table 3. Meristic traits of Upstream and Downstream Population L. calbasu from Indus River, Sindh-Pakistan

Meristic characters	Upstream population		Downstream population	
	MAX	MINI	MAX	MINI
DFR	17	14	17	14
PFR	19	12	18	10
VFR	11	8	10	7
AFR	9	7	23	26
CFR	26	21	24	18
L.LS	56	46	56	44

Note: In the table DFR = (Dorsal fins rays), PFR= (Pectoral fins rays), VFR= (Ventral fins rays), AFR= (Anal fins rays), CFR=(Caudal fins rays), L.LS=(Lateral line scales).

Table 4. Length-Weight relationship of Labeo calbasu (Upstream and Downstream population)

Groups	Average Length (cm)	Average Weight (gm)	a	b	Fulton's Condition factor (*k*)	Le Cren Condition factor (*kn*)
Upstream Population	20.892	193.57	0.0235	2.892	11.915	39.663
Downstream Population	26.758	305.4743	0.04577	1.7923	13.872	44.066

Table 5. Comparison of estimated results of length weight relationship of Labeo calbasu in present study with species from other parts of the world

Source	Species	*a*	*B*	R^2
Naeem M. *et al.*, (2017)	*L. gonius*	0.729	3.29	0.974
Das B.K et al., (2013)	*L. calbasu*	1.719	1.557	..
N.C. Ujjania *et al.*, (2012)	*L. rohita*	-2.409	3.316	0.976
Shehla et al (unpublished)[25]	*L. gonius*	0.005	2.782	0.879
Present study (Upstream Population)	*L. calbasu*	0.024	2.892	0.934
Present study (Downstream Population)	*L. calbasu*	0.046	1.792	0.849

Table 6. Correlation of morphometric traits with total length (TL) for Upstream and Downstream Population L. calbasu from Indus River, Sindh-Pakistan

S: No	Morphological traits	Correlation Upstream Population	Correlation Downstream Population
1	ED (mm)/TL	0.730	0.211
2	SL (cm)/TL	0.964	0.991
3	HL (cm)/TL	0.862	0.918
4	FL (cm)/TL	0.892	0.955
5	Girth (cm)/TL	0.889	0.948
6	DFL (cm)/TL	0.812	0.980
7	PFL (cm)/TL	0.803	0.919
8	VFL (cm)/TL	0.807	0.921
9	AFL (cm)/TL	0.031	0.884
10	CFL (cm)/TL	0.857	0.919
11	SnL (cm)/TL	0. 172	0.855
12	PVD (cm)/TL	0.910	0.921
13	VAD (cm)/TL	0.776	0.912

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ARTICLE

A Preliminary Technique for the Isolation and Culture of Brown Trout (*Salmo trutta macrostigma*, Dumeril, 1858) Spermatogonial Stem Cell

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ABSTRACT

This study was aimed to find a practical technique for isolation and culture spermatogonial stem cells from male brown trout (*Salmo trutta macrostigma*). Twelve wild juvenile male were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey). The juveniles were taken alive to the aquaria unit and stored in a 1000-liter capacity fiberglass tank. In order to identify the best size, age and testis structure of *S.t. macrostigma* for spermatogonial stem cell isolation and culture. Morphological and histological testis conditions were assessed. Fish were anesthetized with 0.04% 2-phenoxethanol. The surface of the fish was sterilized with 70% ethanol. Twelve fish were divided into two groups for enzyme digestion, and each group was divided into two replicates (three fish per replicate). Testis tissue of group one were digested by 0.25% trypsin- EDTA, and testis tissues of group two were digested by 0.05% trypsin-EDTA. At the end of the trial, first, the best age, size and weight of the male fish for spermatogonial stem cell isolation and culture were identified as 5+ month old, 12.13±1.5 cm, 19, 25±7.05 g respectively. Then, the highest spermatogonial stem cells were measured in the stage one and two of the testes. Finally, isolation and culture conditions were optimized for male *S.t. macrostigma*. Spermatogonial stem cell isolation and culture techniques were defined for fish in order to be used in surrogate reproduction technologies and gene transfer systems.

1. Introduction

Spermatogenesis defined as the process by which haploid spermatozoa develop from germ cells in the seminiferous lobules of the teleost fish testis [1,2]. This process starts with the mitotic division of the primordial germ cells (PGCs) laid close to the basement membrane of the lobules. Primordial germ cells differentiate into ovary or testis after reaching the gonadal anlage [1,3,4,5]. Histologically, the spermatogonia still resemble the PGCs with only a minimal size difference where the spermatogonia are smaller than the PGCs [1].

The isolation and culture conditions were well optimized for *O. mykiss* [3,4,6,7]. These authors concluded that, in vitro and in vivo studies on *O. mykiss* spermatogonial stem cells can differentiate into mature egg and sperm in the recipient's (*O. mykiss*) gonads [4,7]. Although, *O. mykiss* and Anatolian mountain trout *Salmo trutta macrostigma* (*S. t. macrostigma*) are belongs to the same family, Salmonidae family, its unique morphology, phenotip and immune system (sexual differentiation, maturation age, weight, size and each fish species has its own immune system) assurances to

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identify isolation and culture conditions for *S.t. macrostigma*. *S. t. macrostigma* is commercially important and is found in Turkey's natural inland water habitats. The scientific name of *S. trutta* macrostigma is still under discussion and somehow remains controversial [8,9]. Tougard and his colleagues suggested the name, Brown trout, *S. trutta* [10]. As it previously mentioned above *S.t. macrostigma* has unique phenotypic characteristics Therefore, the scientific name *S.t. macrostigma* has been used throughout this study and distribution of *S.t. macrostigma* in Turkey is given in Figure 1A[11,12,13,14].

Females reach sexual maturity at the age of four whereas males reach sexual maturity at the age of 2 years [11]. Commercial value of *S. t. macrostigma* when compared to *O. mykiss*, is four to ten-folds higher. Maximum body weight is 25Kg in *S. t. macrostigma* whereas *O. mykiss* has body weight of only 10Kg. It is difficult to spawn in captivity, is unable to stand stress, it cannot tolerate high water temperature and comparatively it takes longer time to reach sexual maturity than *O. mykiss*. Therefore, gamete production for this species is expensive in terms of time, cost, labor and space. However if, *S. t. macrostigma* spermatogonial stem cells could be transplanted into the *O. mykiss*, which is a closely related species that reaches sexual maturity only in just 1,5 years at a body weight of 1Kg, able to stand stress, high water temperature *S. t. macrostigma* gametes might be more easily and rapidly produced. In addition, the natural stock of *S. t. macrostigma* is declining and it is also an endangered species [15]. Therefore, after isolation and culture of spermatogonial stem cells it could be possible to preserve the cells by cryopreservation [16].

Although, spermatogonial germ cell isolation and culture is well established for *O. mykiss*, there is only one study using this technology for *S. t. macrostigma*. The study was performed by Lujic´and his colleagues[16]. As stated above, *S. t. macrostigma* have different phenotype, morphology and immune system. Therefore, the main objective of the present study was to isolate and culture of spermatogonial stem cells from the *S. t. macrostigma*.

2. Materials and Methods

2.1 Experimental Design

Wild mature, immature and juvenile male *S.t. macrostigma* were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey). The males were taken alive to the aquaria unit and stored in three 1000-liter capacity fiberglass tanks. Water was supplied from a tap. Mature, immature and juveniles were fed three times a day with commercial trout feeds during the experiment (IDL ALFA, 2.2mm; Inve, Aquamaks, Turkey). The water was continuously aerated with a pump. The fiberglass tanks were housed inside an experimental room with a natural photoperiod (12 h dark, 12 hour light). A static water system was used, and 80% of the water in each tank was changed weekly, before the morning feed. One day before gonadal sampling the fish starved. The average weight and length of fish recorded. The testis structure of *S.t. macrostigma* were morphologically and histologically studied in order to identify the best size, age and testis structure of *S.t. macrostigma* for spermatogonial stem cell isolation and culture.

2.2 Histological Procedures

Males were anaesthetized in 0.04%, 2-phenoxethanol (Sigma Chem. Dorset, UK). The testes were dissected from three sacrificed males for each week. The testis tissue was divided in two lobes and half of the testes lobes was fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and then sectioned at 5µm thickness and stained with hematoxylin and eosin (MERCK) for histological evaluation [17,18]. The second lobe of the testes tissue was cut into small pieces for spermatogonial cell isolation. After histological work, all slides were examined under a light microscope (CH-2 Olympus-Japan). Photomicrographs were taken to illustrate the most abundant number of spermatogonia in the testes of *S.t. macrostigma*[1]. Spermatogenesis and spermatogonia were classified by developmental stages adapted from Çek and Yılmaz [2].

2.3 Isolation of Spermatogonial Stem Cells

Before sacrificing of the male St. macrostigma, the whole fish body was sterilized with 70% isopropanol to prevent any possible contamination. *S.t. macrostigma* was dissected using a sterilized dissection set and the testes were placed in HBSS in a sterile petri dish. The used tubes, lids and dissection set were kept in a continuous burner to prevent possible contamination. The testes were chopped into very small pieces with pre-sterilized scalpel were cleared of blood vessels and peritoneum in the sterile cabinet (uv system was turned on 15 minute before the study started and burner flame was used continuously). For proper enzymatic digestion and maximum dispersion of the testes, 0.25% and 0.05% Trypsin-EDTA (ethylene diamine tetra acetic acid) added in two different groups (each containing 3 male fish). EDTA decouples cell-cell connections and allows the cells to be homogeneously dispersed. The trypsin was neutralized by 10% fetal bovine serum (FBS).

After washing the testes in streptomycin, gentamycin

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and fungizone containing HBSS, the testes in petri dish were left in the bleach solution in pH 7.4 (1/10 clorox bleach, 9/10 ultra-distilled pure water) for 2 minutes, followed immediately by 3 times washed with HBSS [19,20,21]. Minced testes tissues of each fish were transferred into one 50 mL autoclaved glass flask, which contained a stir bar. All the samples were incubated on ice for 30 min followed by 1 hr. at 20 ℃ with a magnetic stirrer to achieve higher digestion efficiency. In order to eliminate cell clumps, and to obtain single cell suspension, the cell suspension from each replicate was then filtered using a 40 µm cell strainer (nylon mesh, Falcon, BD Falcon) and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 mL HBSS. The trypan blue dye exclusion tests were used to determine the number of viable spermatogonia cells. It was based on the principle that live cells possessed intact cell membranes that excluded trypan blue, whereas dead cells did not. Therefore, a viable cell had a clear white cytoplasm whereas a non-viable cell had a dark blue cytoplasm. Five µL of cell suspension were gently mixed with 45 µL Trypan blue.

2.4 Spermatogonial Stem Cell Culture

After centrifugation, the cells were washed 3 times with 50µg / ml penicillin, 50µg/ml streptomycin, 50µg/ml l gentamycin and 50µg / ml fungizone containing HBSS. Following resuspension of cells in 2 mL HBSS, 20µl of prepared cell suspension was transferred to each culture dish and 60µl culture medium (L-15) was added (BIOCHROM AG). Stock solution of culture medium prepared as 25mM HEPES, antibiotics, 1.0µg/ml $NaHCO_3$, 0.3µg/ml L-glutamine, 10% FBS, 5% *S.t. macrostigma* serum, 50µg/ml insulin and 1ng/ml bFGF. Previously six compartments polystyrene sterile culture plates were well coated with poly-L lysine, and then the cells were seeded into these culture plates including culture medium (GREINIER BIO-ONE CELLSTAR). Culture media pH was maintained at 7.45. Cells were cultured at 19 ºC in a refrigerated incubator. Osmolality adjustment was done by using an osmometer (Automatic semi-micro osmometer, model A0300, Knauer). They were checked daily under an inverted microscope (OLYMPUS CKX41SF). Spermatogonia were removed by medium change daily in the first week and daily dilutions were made.

3. Results

3.1 Morphological Suitability of the Testes

The best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture were identified as 5+ month old (age), 12.13±1.5 cm (in length), 19,25±7.05 g (in weight) respectively. At these age, size and weight, the testes paired and attached to the dorsal lateral lining of the peritoneal cavity. They were inactive and the experience needed to separate testis from the ovaries. At 5 months old, testes were creamy white in color. Thin ribbon like translucent structure observed (Figure 2A). Morphological characteristics of the testes are given in Table 1.

3.2 Histological Suitability of the Testes

Histologically testes conditions were assessed as seen in Figure 2B and C. The highest spermatogonial stem cells were measured in the stage one and two of the testes. This stage was characterized by a large nucleus in the central position, surrounded by little cytoplasm, at five months old (Figure 2B and C). Histological characteristics of the testes are given in Table 1.

3.3 Isolation of Spermatogonial Stem Cells

The 0.25% Trypsin-EDTA enzymatic digestion indicated the best efficiency and the best amount of cell isolation (Figure 3A). Isolation conditions were optimized for *S.t. macrostigma*. Viable spermatogonial stem cells and non - viable spermatogonial stem cells were shown (Figure 3B). In the figures the germinal vesicle of the viable cells which carry the genetic material were bright and white in color whereas the germinal vesicle of the death spermatogonial cells were dark blue (Figure 3B). Trypsin-EDTA enzymatic dissociation was terminated by adding 5% sterilized *S.t. macrostigma* 's serum and culture medium.

3.4 Culture of Spermatogonial Stem Cells

After testicular germ cell isolation, culturing of these cells were performed. Cell filtration carried out through a 40 µm cell stainer, and then the cell suspension from each testis was centrifuged at 500 g for 10 min. The supernatant discarded and the pellet resuspended in culture medium. Cells seeded in attachment factor and poly-L lysine coated 6- well plates. Using poly-L lysine was successful and the cells attached to the plate during the first five days of the culture (Figure 3C). Plates were incubated at 19˚C in an incubator without 5% CO_2. Cells were sub cultured when 80% culture surface was covered by cells. After chancing the 50% of the media, cells and tissue were observed under an inverted microscope and images were taken (Figure 3C and D). In the following days, colonization of these cells was detected (Figure 3E). Culture conditions were optimized for *S.t. macrostigma*. Survival rate of spermatogonia and mitotic activities in L-15 culture media, with 5% serum of *S.t. macrostigma* (Culture media

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pH, 7.45; temperature 19ºC, Osmolality was maintained at 235±0.5 mOsm kg^{-1}) were developed.

4. Discussion

The best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture in *S.t. macrostigma* has not been comprehensively investigated. Previously, Kise and her colleagues [22] and Sato and his team[23] studied in a closely related species, Rainbow trout, Oncorhynchus mykiss at 10ºC and recommended 11-12 months old fish for spermatogonoal isolation and culture. In their study, the best total length and weight were 13.9±1.4 cm and 42.4±11.5 g, respectively. Hayashi and his colleagues [24] used 10-15 months old transgenic *O. mykiss*. In the present study, the best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture were identified as 5+ month old (age), 12.13±1.5 cm (in length), 19,25±7.05 g (in weight) respectively. When compared those studies with the age and weight of *S.t. macrostigma* in the present study is much smaller. Although *S.t. macrostigma* and *O. mykiss* are belong to the same genus, both are quite different phenotypically. In previous studies, the temperature was 10ºC whereas the temperature in our study was 19ºC. The differences between current and other studies may be explained by the differences in phenotype and temperature.

In the present study, the highest spermatogonial stem cells were measured in the stage one and two of the testes.

Therefore, the stage one and two of testes development was suggested to be used for spermatogonial stem cell isolation and culture. This founding was similar to that of Lujić and his colleagues [16,25].

In fish, the first isolation of spermatogonial stem cells was in *O. mykiss*. Okutsu and his team [3] cultured *O. mykiss*. Testes of immature male *O. mykiss* were incubated in PBS (pH, 8.2) with 0.5% trypsin and spermatogonia were isolated [3]. In catfish, to determine the enzymatic efficiency in testicular tissue trypsinization, Shang [19,20,21] compared two different concentration of trypsin. First concentration was 0.05% trypsin-EDTA and the second one was 0.25% trypsin-EDTA. Based on their studies the 0.25% trypsin-EDTA showed higher efficiency and a higher amount of cell isolation than 0.05% trypsin-EDTA enzymatic digestion. Trypsin has been successfully used for dissociation of spermatogonial stem cells in *O. mykiss* [3,6], blue catfish Ictalurus furcatus [19,20,21], goldfish, Carassius auratus [26], and Neotropical catfish, Rhamdia quelen [27]. However, Lujić and her colleagues [27], investigated the efficiency of different concentration of collagenase on gonadal dissociation of *S.t. macrostigma*. The highest total yield was recorded in two groups without trypsin (2 and 6 mg/ml collagenase). The protocol using 6 mg/ml collagenase displayed the lowest number of viable cells. Therefore, Lujić and her colleagues [27], suggested the use of 2mg/ml collagenase for the dissociation of *S.t. macrostigma* spermatogonial stem cells. In the present study, the protocol described by shang [19] was modified. In the present study, the 0.25% trypsin-EDTA enzymatic digestion was found to be more efficiency then the 0.05% trypsin-EDTA and amount of cell isolation was satisfactory (pH was maintained at 7.45 and temperature at 19˚C).

Spermatogonial stem cell culture had been studied in *O. mykiss* [27] and blue catfish Ictalurus furcatus [19,20,21], testicular cell suspensions were prepared from six to 9 month old pvasa-Gfp transgenic *O. mykiss*. Testes were minced and incubated with 1ml of L-15 containing 2 mg/ml collagenase and 500IU/ml dispase for 7-9 hours at 10 ˚C. The resultant cell suspension was filtered through a 20-mm pore-size nylon screen to eliminate cell clumps and blood vessels. Testicular germ cells isolated from two-year-old juvenile blue catfish. The cells were easily cultured in L-15 medium at 27 ˚C in air and had the same morphological characteristics as channel catfish testes cell line. The cell suspension was filtered using a 42 μm cell strainer[19,20,21]. The authors stated that spermatogonial stem cell culture was more difficult than oogonial stem cell culture.

In the present study, the cells were cultured in L-15 culture media, with 5% serum of *S.t. macrostigma* (Culture media pH, 7.45; temperature 19ºC, Osmolality was maintained at 245±2 mOsm kg^{-1}). The main difference between present study and the studies done by Okutsu and his colleagues[3], Hayashi and his colleagues [24] and Lujić [16] was the temperature. In the present study, the temperature of the incubator was 19ºC, whereas the temperature of the incubator in those studies was 10 ºC. In the current study sterilized 5% serum of *S.t. macrostigma* were used very often therefore, the media was quite rich. In conclusion, The current study suggest that the best age, size and weight of *S.t. macrostigma* for spermatogonial stem cell isolation and culture should be 5+ month old, 12.13±1.5 cm, 19,25±7.05 g respectively. The testes should be at the stage one and two. The environment in the optimum culture media should maintained at pH, 7.45; temperature 19ºC, Osmolality at 245±2 mOsm kg^{-1}(with 5% sterilized serum of *S.t. macrostigma*). The spermatogonial stem cell isolation and culture technique was developed for *S.t. macrostigma* in order to be used in surrogate reproduction technologies and gene transfer systems.

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Statement of Conflict of Interest

The authors declare that they have no conflict of interest.

Statement on the Welfare of Animals

Fish experiments were approved by the Mustafa Kemal University in Turkey and were conducted in agreement with the guidelines of Republic of Turkey University of Mustafa Kemal Laboratory Animal Ethics Committee.

Appendixes

Tables

Table 1. Description of Testes Stages. Classification was based on the histological criteria adapted from Cek et al. 2001

Stages	Morphological Description	Histological Description
Stg1 (Spermatogonia)	Testes were colorless, thread like structure, thin and were like ovaries. Elongated and close to the vertebrate column. No morphological differences between the testes and ovaries were visible.	Spermatogonia was visible and, was characterized by a large nucleus in the central position which surrounded by little cytoplasm. Somatic cells were also abounding.
Stg2 (Primary spermatocytes)	Testes were slightly larger and distinctively longer than the previous stage. They were white and smooth	Few primary spermatocytes and at the periphery, many spermatogonia were detectable. Somatic cells were greater in number. Primary spermatocytes were smaller than the spermatogonia.
Stg3 (Secondary spermatocytes)	Testes were larger and elongated, more distinctive in color, which was white. Two lobes begin to conjugate.	Secondary spermatocytes were detected at 4 months of age. Nucleolus was not clear. Secondary spermatocytes were morphologically similar to Primary spermatocytes; however, the size was somehow smaller than the Primary spermatocytes.
Stg4 (Spermatids)	Red spots were visible on both sides of the testes. They were very white. Milt was not observed.	At this stage, secondary spermatocytes divided meiotic ally and turned into spermatids. The cells were irregular in shape and extremely basophilic.
Stg5 (Spermatozoa)	Red spots were still visible. Drops of milt were observed under pressure.	At this stage, cell division was not observed. Just before maturation stage, all stages of spermatogenesis were detectable.

Figure Legends

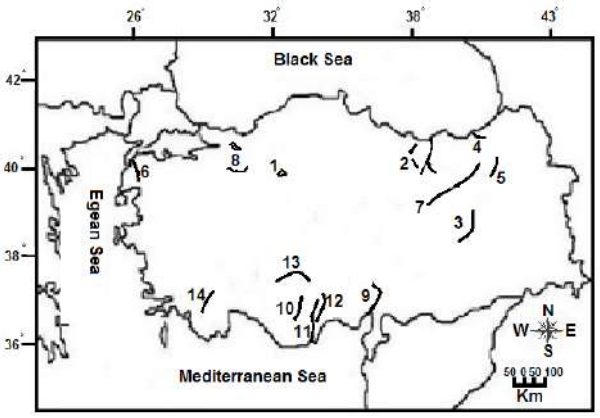

Figure 1. Map showing distribution of *Salmo trutta macrostigma* in Turkey

Note: Numbers indicates the location of the species. 1. Bolu (Yedi Göller National Park); 2. Trabzon (Uzun Göl Supplies, Uçarsu-Çatak, Arpalı-Sultanmurat); 3. Tunceli (Munzur Stream); 4. Rize (Ovit Mountain); 5. Erzurum (Tortum Stream); 6. Çanakkale (Kaz Mountains); 7. Gümüşhane (Siran Stream) [11]; 8. Sapanca (Sapanca Lake, Mahmudiye Stream[12]; 9. Ceyhan (Ceyhan Stream) [13]; 10. Antalya (Alakır Stream); 11. Antalya (Köprüçay Stream); 12. Antalya (Alara Stream); 13. Karaman (Ermenek Stream);14. Fethiye (Eşen Stream) [14].

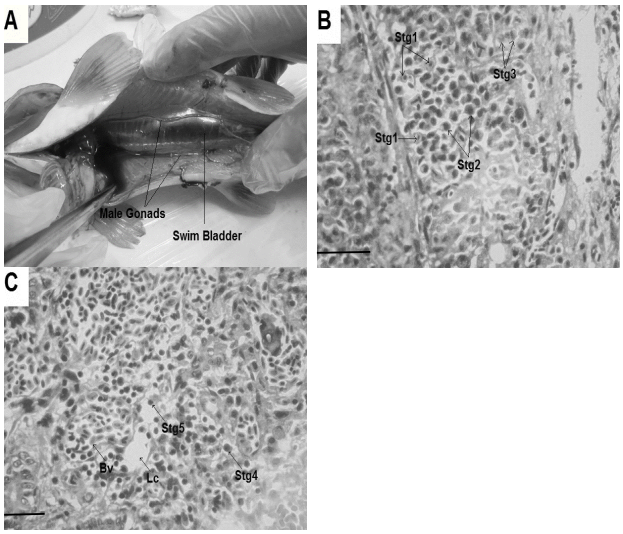

Figure 2. Morphological and histological suitability of the testis for spermatogonial stem cells isolation and culture

Note: (A) Morphology of the testis; (B) Histology of the testis; (C) Matured testes and blood vessels, which is less suitable for spermatogonial cell isolation and culture. Stg1, Spermatogonia; Stg2, Primer spermatocytes; Stg3, Seconder spermatocytes; Stg4, Spermatids; Stg5, Spermatozoa; Bv, blood vessels; Lc, Luminal cavity. Scale bar = 100 μm (B, C), Stained with Hematoxylin & Eosin.

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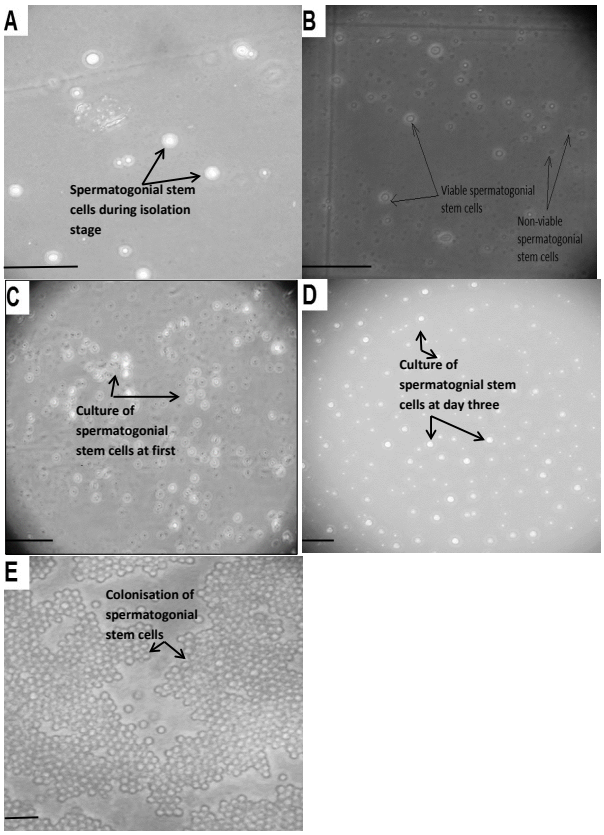

Figure 3. Isolation and culture of spermatogonial stem cells

Note: (A) Viable spermatogonial stem cells are shown at the isolation stage. (B) Counting of viable and non viable spermatogonial stem cells by hemocytometer. (C) Culture of spermatogonial stem cells, first day. (D) Culture of stem cells, Day 3. (E) Culture of spermatogonial stem cells day seven. Colonization of cells is shown. Scale bar= 125μm (A, B, C, D and E).

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