**Universidade de Aveiro** Departamento de Biologia
**Ano 2012**

**Daniela Pio Quinto Castelo**

# Constrangimentos fisiológicos na produção de som no xarroco

# Physiological constraints on sound production in Lusitanian toadfish

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Professor Doutor Paulo Jorge Quintais Cancela da Fonseca, do Centro de Biologia Ambiental e da Faculdade Ciências da Universidade de Lisboa, com a coorientação científica da Professora Doutora Maria Gabriela Gomes de Figueiredo Rodrigues, do Centro de Biologia Ambiental e da Faculdade Ciências da Universidade de Lisboa, e do Professor Doutor Mário Guilherme Garcês Pacheco, do Departamento de Biologia da Universidade de Aveiro.

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**o júri**

| | |
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**palavras-chave**

Produção de som, xarroco, *Halobatrachus didactylus*, fadiga músculo sónico, comunicação acústica, variação sazonal.

**resumo**

O Sucesso reprodutivo dos machos territoriais do xarroco dependem da sua capacidade vocal. Para a produção de vocalizações são utilizados músculos sónicos intrínsecos à parede da bexiga-natatória, cuja frequência de contração pode atingir valores superiores a 100 Hz e são por isso conhecidos como os músculos mais rápidos entre todos os vertebrados. Com este estudo pretende-se averiguar se a capacidade fisiológica de produção de som é diferente no inverno e na época reprodutiva, assim como entre juvenis e adultos. Para este efeito estimulámos o nervo sónico de indivíduos jovens e adultos, no inverno e na época reprodutiva, com uma sequência de sirenes artificiais simulando a frequência, duração e taxa de vocalizações naturais. O movimento de contração do músculo sónico foi registado recorrendo a um transdutor de força e, simultaneamente foi registado o som produzido. Esperávamos que machos adultos de verão produzissem sons de maior amplitude e fossem mais resistentes à fadiga do que machos adultos de Inverno. Não esperávamos encontrar estas diferenças sazonais em juvenis pré-reprodutivos. No entanto, esperávamos que machos juvenis no geral produzissem sons de menor amplitude e fossem menos resistentes à fadiga. Em paralelo à estimulação do nervo sónico para produção de som, foi realizada a caracterização histológica e histoquímica das fibras do músculo sónico para cada um destes grupos de modo a procurar eventuais diferenças estruturais que justificassem as diferenças esperadas. Machos de verão, tanto adultos como juvenis demonstraram ter uma melhor performance vocal em termos amplitude de som. A fadiga muscular parece não variar com a estação do ano mas é, no entanto, mais acentuada em juvenis. Os resultados referentes ao movimento de contração do músculo sónico mostram que, para além da contração rápida correspondente à frequência de estimulação, este músculo apresenta uma contração lenta e sustida não descrita para outras espécies deste género. Os cortes histológicos apresentam uma distribuição heterogénea das fibras. Machos de verão apresentam mais sarcoplasma na época reprodutiva que os indivíduos de inverno, fêmeas e juvenis. Machos de inverno e verão apresentam fibras de maior diâmetro que juvenis. As fibras do músculo sónico têm uma forma poligonal e um centro de sarcoplasma rodeado de miofibrilhas. A presença de fibras em remodelação e possível divisão em xarrocos adultos nunca tinha sido descrita nesta espécie. Machos adultos de inverno, assim como machos adultos de verão que não apresentam uma alta taxa de vocalizações naturais, aparentam ter fibras mais lentas que machos adultos de verão com grande performance vocal. Não foi possível determinar o mecanismo responsável pela contração lenta e sustida do músculo sónico. No entanto, postulamos que este fenómeno terá um papel importante na ampliação e radiação do som produzido.

**keywords**

Sound production, lusitanian toadfish, *Halobatrachus didactylus*, sonic muscle fatigue, acoustical communication, seasonal variation.

**abstract**

Male territorial Lusitanian toadfish depend on their vocal capability for reproductive success. Sound is produced by a pair of sonic muscles intrinsic to the swimbladder walls, which contract as fast as 100Hz. and are therefore considerate to be among the fastest muscles in vertebrates. In this study we aimed to investigate if the physiological ability for sound production is different in the winter and in the breeding season, as well as in juveniles and adults. In that vein we have stimulated the sonic nerve of both adults and juveniles, during the winter and breading season, with sequences of artificial boatwhistles simulating the frequency, duration and rate of natural calls. The sonic muscle contraction movement was recorded using a force transducer. Simultaneously, we have recorded the produced sound. We expected that the breading adult males would be able to produce sound of higher amplitude and to be more resistant to fatigue then the non reproductive winter adult males, however we didn't expect to find seasonal differences in pre-reproductive juveniles males. However, it was expected for juvenile males to produce sounds of lower amplitude and to be less resistant to fatigue than adult males in general. We have also examined the histology and histochemistry of sonic muscle fibers to search for eventual morphological differences between these groups in order to justify the expected differences in physiological ability for muscle contractions. Summer males, both adults and juveniles, showed a better performance in terms of a higher sound amplitude. The muscle fatigue didn't seem to change between seasons but is more pronounced in juveniles than adults. The contraction movement of the sonic muscle results shows the expected fast contractions that follow the stimulation frequency and also a slow and sustained contraction that hasn't been described in any other toadfish specie. Histological sections of the sonic muscle show fibers that are arranged in several orientations. Summer males sonic muscle fibers have higher sarcoplasm area than winter individuals, females and juveniles. Winter and summer males showed a larger sonic muscle fibers diameter than juveniles. The fibers were found to have a polygonal shape and a central core of sarcoplasm surrounded by myofibrils. The presence of remodeling and possible division fibers in sonic muscle in adult males has never been described in this species. The sonic muscle of both winter and summer adult males that did not vocalize at high rates in a natural environment presented slower fibers than summer adult males that were previously found to be strongly vocal. It was not possible to determinate the mechanism responsible for the slow and sustained contraction of the sonic muscle but we postulate that this phenomenon has an important role in sound amplitude and radiation.

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% RF - Remodeling Fibers percentage per fascicule

AB – Artificial Boatwhistle

BM – Body Mass

EMG – Electromyogram

EW – Eviscerated Weight

GSI – Gonadosomatic Index

GW – Gonads Weight

MFI – Movement Fatigue Index

MSR – Myofibrils area / Sarcoplasm area Ratio

SAF - Summer Adult Female

SAM - Summer Adult Male

SBI - Swimbladder Index

SDH – Succinate Dehydrogenase

SFI – Sound Fatigue index

SBM - Swimbladder movement

SJM – Summer Juvenile Male

SL – Standard Length

SM - Sonic Muscle

SN – Sonic Nerve

SSAM - Summer Silent Adult Male

SVAM - Summer Vocal Adult Male

SW– Swimbladder Weight

TL – Total Length

TW– Total Weight

WAF – Winter Adult Female

WAM – Winter Adult Male

WJM – Winter Juvenile Male

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# INTRODUCTION

# 1. Introduction

## 1.1. Acoustic communication

Communication plays a central role in animal behavior (Kumar, 2003). Receivers engaging in acoustic communication must be able to detect and discriminate differences among signals (Wiley & Richards, 1978) that may encode diverse information such as species identity, motivation, individual condition, etc. Vocal signals may play an important role in mate choice if females can use acoustic cues to select mates associated with increased holding potential, better parental care, good territory or genetic quality (Andersson, 1994). These cues can be encoded in different acoustic signal parameters such as repetition rate or intensity (Bradbury & Vehrencamp 2011).

Just like in other taxa, sound production appears to be widespread in fish. Fish sounds are typically low-frequency, pulsed signals that mainly differ in duration, number of pulses and repetition rate (Winn, 1964; Myrberg *et al.,* 1978). There are mainly three different mechanisms used to produce sound in sonic fishes: 1) the contraction of sonic muscles (SM), that may be intrinsic or extrinsic to the swimbladder (SB); 2) stridulation by rubbing together certain structures, and 3) hydrodynamic sounds that are produced by quickly changing speed and direction while swimming (Tavolga, 1971; Demsky *et al*., 1973; Fine *et al*., 1977; Kasumyan, 2008).

Several fish species produce sounds for social communication in contexts such as courtship (Lugli *et al*., 1997), agonistic interactions (Ladich, 1997), warning alarms (Matos, 1997), distress (Vasconcelos & Ladich 2008) and competitive feeding (Amorim & Hawkins, 2000; Ladich & Fine, 2006), and vocalizations can be related to particular activities (Amorim 2006). A variety of behaviors associated with vocalizations was observed in a few species. For example, in the Mozambique tilapia (*Oreochromis mossambicus*, Peters, 1852*)* vocalizations can be used to advertise an individual's presence and are produced by a male to inform the female about its readiness for reproduction and the release of gametes (Amorim *et al*., 2003). Also, territorial fish of several

species use vocalizations in territorial defense (Ladich *et al*., 1992) and mate attraction (Myrberg *et al*., 1986). Despite the importance of acoustic communication in fish daily activities, information about the auditory capabilities is still scarce as only a few species have been investigated (Vasconcelos *et al.,* 2010a).

Batrachoidids, such as the Lusitanian toadfish (*Halobatrachus didactylus,* Bloch & Schneider, 1801), gulf toadfish (*Opsanus beta,* Goode & Bean, 1880), midshipman fish (*Porichthys spp.)* and oyster toadfish (*Opsanus tau*, Linnaeus, 1766) produce sound by fast contraction of a pair of sonic muscles embedded on the swimbladder walls. Each contraction pushes the swimbladder wall inwards, leading to a rise of the inside pressure, followed by relaxation and consequent pressure decrease. These oscillations of the swimbladder wall and following pressure variations are transmitted to the surrounding water in the form of sound waves (Skoglund, 1961; Fine *et al.*, 2001).

According to Tavolga (1964) the SB behaves like an air bubble and its natural vibration frequency is around the contraction frequency of the SM which contributes to the efficiency of the system.

The sounds produced by this family include boatwhistles, grunts, moans, growls boops, trains, croaks, double croaks, long grunt trains and a mixed grunt–croak call (Amorim, 2006). This entire set of sounds is not produced by all batrachoidids and the same sound type can differ between species. For example the boatwhistle lasts several hundred milliseconds in the *O. tau* and presents only one note, while in *O. beta* it has several notes (Fine, 1978; Thorson & Fine, 2002).

The *H. didactylus* boatwhistle is similar to the one emitted by *O. tau*. This species can produce several other sound types like grunts, croaks and double croaks (Amorim *et al*., 2008). The courtship sound of *O. beta* is very complex: it may initiate with one up to three grunts that are followed by one (long tonal) to three (short) boops and lasts more than a second (Thorson & Fine, 2002). Scarecrow toadfish (*O. phobetron*) is another toadfish that produces single boatwhistles comparable to the ones of *O. tau,* but longer (Fine *et al*., 1977).

Also, in the plainfin midshipman (*Porichthys notatus,* Girard, 1854), territorial males make short (50–200 ms) broadband agonistic grunts that are produced singly or in long trains at rates of 1–2 Hz (Brantley & Bass, 1994). During courtship, these males communicate through hums (a long duration sound that can last from a few second to over an hour) (Ibara *et al*., 1983; Brantley & Bass, 1994).

## 1.2. The Lusitanian toadfish, *Halobatrachus didactylus*

The Lusitanian toadfish, *Halobatrachus didactylus*, is a benthic, solitary and relatively sedentary batrachoidid specie (Costa, 2004). Like most batrachoidids, *H. didactylus* has a very robust body, dorsoventrally flattened at the anterior region and compressed laterally in the posterior zone (Albuquerque, 1954). The head is very large, with a big slightly protractile mouth (Bertin & Arambourg, 1958). The body is usually covered by mucus (Bauchot & Pras, 1980; Costa, 2004), which has antimicrobial properties to protect the fish from fungus and other infections (Knouft *et al.,* 2003). This specie can be up to 50 cm long, although most of the specimens do not exceed 35 cm (Roux, 1986; Bauchot, 1987). Average and maximum sizes are population dependent. In Portugal, the Tagus estuary population has the largest individuals (Costa, 2004).

It is a voracious predator, feeding from a wide range of prey (Cárdenas, 1977; Sobral, 1981; Costa *et al.,* 2000), and occupying the top position in estuarine and coastal lagoons trophic webs, where it plays an important role in the structure and balance of the existing biological communities (Costa, 2004).

### 1.2.1. Distribution

*H. didactylus* occurs in the continental shelf from the Gulf of Guinea to the Gulf of Biscay, including the western Mediterranean (Roux, 1981, 1986; Bauchot, 1987), and in The Atlantic Islands of Madeira (dos Santos *et al*., 2000), Canaries (Fowler, 1936) and Cape Verde islands (Reiner, 1996). Considerable

populations are only found between the Liberian coast and the south of Portugal (Costa, 2004). It is a marine species adapted to brackish environments. In the Portuguese coast it occurs in estuaries (Tagus, Sado, Mira, Arade and Guadiana) south of Cape Carvoeiro, in lagoons and in coastal environments (Ria de Alvor and Ria Formosa) (Costa, 1993; Sobral & Gomes, 1997; Costa & Costa, 2002; Costa *et al*., 2003).

These fish are usually associated with soft sand, muddy substrates, but can also be found in hard substrates, under stones or sheltered in rocky crevices (Roux, 1986; Bauchot, 1987; dos Santos *et al*., 2000).

### 1.2.2. Reproduction

The reproductive season of *H. didactylus*, in the Tagus estuary, occurs from February to June (Pereira, 2006). This reproduction window depends on climate conditions and may differ from year to year. Fish reproductive behavior and nests with eggs have been found in the Tagus estuary between late April and July (Amorim, personal communication, 2012). This species is gonocoric (Costa, 2004) with external fertilization.

The males may belong to one of two morphotypes. Type I males are territorial and use boatwhistles to attract the females into their nests. Upon female spawning the eggs attach to the nests ceiling and are then fertilized by the male. The females leave the parental care exclusively to males until the offspring become free-swimmers (Roux, 1986; dos Santos *et al.,* 2000).

Another male morphotype exists, presenting different morphometric and endocrine characteristics adjusted to their alternative mating strategy (type II males or sneakers). Type II males are satellite males that approach type I males' nests and sneak spawn stealing fertilizations from territorial males (Brantley & Bass, 1994; Bass, 1996, Bass & McKibben 2003). Type II males present large testes, produce large quantities of sperm and try to reproduce as many times as possible during the reproductive season (Modesto & Canário, 2003a). On the other hand, type I males have larger accessory glands. These structures have an important role on the production of substances that reduce

sperm dispersion, thus increasing sperm active life span in the nest (Barni *et al*., 2001). Sneakers also have smaller sonic muscle volume than type I males (Modesto & Canário, 2003a).

The abundance and proportion of morphotypes of *H. didactylus* males has been studied in the Mira (Costa, 2004) and Tagus (Pereira, 2006) estuaries. Results showed a significant predominance of type II males in the Mira estuary and of type I males in the Tagus estuary. According to Pereira (2006) the fact that the Tagus estuary ecosystem offers a high number of habitats and nesting places, leads to a decrease in competition for those places. This condition makes it less favorable to the development of alternative morphotypes.

#### 1.2.2.1. Maturation scale of the gonads of *H. didactylus*

Costa (2004) created a scale of sexual maturation for both males and females based on histological observations of fresh gonads. The Gonadosomatic index (GSI) is used to analyze the development level of the ovary and testicles.

Immature or stage I gonads characterize small and virgin individuals. Females and males in this state have a very low GSI. The second stage (II) is called resting/recovery stage and occurs in individuals that have never reproduced before and in those that are between breeding seasons. On stage III (developing stage) the gonads prepare to reproduction and start to grow. Especially in females, the oocytes occupy 1/5 of the abdominal cavity. On the next stage (IV), maturing state, GSI is higher than in the previous stages. In males the growing testicles occupy 1/5 of the abdominal cavity. After that, the mature stage (V) is when the gonads present their largest size and GSI is at its highest value in the cycle. The last state, spent (VI), occurs after spawning and the GSI values decrease again.

### 1.2.3. Vocal repertoire

*H. didactylus* has a particularly large vocal repertoire for teleost fishes. Type I males produce at least five different sounds depending on social context:

boatwhistles, grunt trains, croaks, double croaks, long grunt trains and also a mixed grunt–croak call (Amorim & Vasconcelos, 2006; Amorim *et al.,* 2008). The fundamental frequency of the sound depends on muscle contraction rate (Skoglund, 1961; Edds-Walton *et al.,* 2002).

Boatwhistles are tonal courtship sounds produced by type I males to attract females into their nests on shallow waters in the rocky intertidal zone (Skoglund, 1961; Edds-Walton *et al*., 2002; Thorson & Fine, 2002, Vasconcelos *et al.,* 2012). Boatwhistles are multi-harmonic sounds lasting *c.* 800 ms with a fundamental frequency of *c.* 60 Hz (Amorim *et al.,* 2006). Grunts are short pulsed sounds emitted in trains with fundamental frequency around 100 Hz. Grunts are produced throughout the year and are generally associated to distress or to agonistic contexts (Amorim, 2006).

Croaks are low frequency pulsed sounds emitted in isolation (Amorim *et al.,* 2006). Double croaks are composed by two croak-like elements that present both amplitude and frequency modulation (dos Santos *et al.,* 2000; Amorim *et al.,* 2006).

### 1.3. Muscle structure and contraction

The skeletal muscle produces movements of the limbs or jaws and is the most abundant tissue in the vertebrate body (Keeton & Gould, 1993). This type of muscle is formed by bundles of cylindrical and elongated cells, known as muscle fibers or myofibers which are multinucleated (with peripheral nucleus in some vertebrates) and exhibit transverse striations. It is responsible for a vigorous and fast contraction subjected to voluntary control (Junqueira, 2010).

The muscular tissue contains a big amount of cytoplasmic filaments composed of contractile proteins. These proteins are responsible for the forces necessary to generate muscle contraction, using ATP molecules for fuel (Junqueira, 2010). The myofibrils present four important proteins: actin, myosin, tropomyosin and troponin. The thick filaments are constituted by myosin and the thin filaments are composed by the three other proteins (Junqueira, 2010).

### 1.3.1. Types of muscle fibers

Muscular tissue growth can happen by two different mechanisms protein retention (hypertrophy – characterized by the increase in muscle mass and size or the cross-sectional area of the myofibers) and cell proliferation (hyperplasia). These mechanisms can have different contributions to muscle growth in teleost fish of different species (Veggetti *at al.,* 1993; Lin *et al.,* 2011). In fish, the precursor cells (or blast cells) can be responsible for the proliferation and synthesis of new fibers that continue to play a key role in muscle growth after the juvenile states (Johnston, 2001).

The skeletal muscle fibers do not all have the same structure or function. They differ in color, myoglobin levels, vascularization, contraction speed (depends on their ability for ATP usage), fatigue resistance, localization and metabolic processes (oxidative or glycolytic) (Kelly & Rubinstein, 1994; Purves *et al.,* (2001).

According to Kelly & Rubinstein (1994), Chen *at al*. (1998), Johnston (2001) and Junqueira (2010), depending on the structure and molecular composition, there are slow fibers and fast fibers known has type I and type II fibers respectively.

Type I fibers are dark red, have higher sarcoplasm content, myoglobin (which is responsible for oxygen storage in the muscle) and mitochondria. They are enriched with blood vessels, its contraction rate is slow and present minor fatigue on long-term activities. Fatty acids are the primary source of energy. In terms of metabolic processes, the oxidative capacity is higher than in type II fibers (Chen *at al*., 1998; Johnston, 2001; Junqueira, 2010).

Type II fibers are specialized on fast and discontinuous contractions with consequent rapid fatigue in a short period of time, have less myoglobin and therefore they are pink or white. These cells use anaerobic breakdown of glycogen for energy and have a fast contraction (Kelly & Rubinstein, 1994; Junqueira, 2010).

These fibers are subdivided in at least 5 types (IIA, IIB, IIC, IIAC, and IIAB) according to functional and biochemical features, but we will only focus on IIA

and IIB fibers type (Kelly & Rubinstein, 1994; Chen *at al*., 1998; Scott *et al.,* 2001).

The first group, IIA, has faster fibers than type I but have equal mitochondria content as well as oxidative capacity (Johnston, 2001).

Type IIB fibers are the fastest, they are white and depend on glycolysis to obtain energy but are the ones that fatigue faster. These fibers are the ones with less mitochondria, vascularization (due to this fact they are called white) and oxidative capacity. The glycolytic capacity is the highest among all fibers (Johnston, 2001; Junqueira, 2010).

The different types of fibers can be identified by histochemical methods, such as ATPase activity of the myosin (higher in type II fibers) and Succinate Dehydrogenase (SDH) (higher in type I fibers). SDH is a very important enzyme in the Kreb's cycle indicating aerobic metabolism. This enzyme is used to distinguish between oxidative and nonoxidative (in fact, "less" oxidative) fibers. High oxidative capacity fibers generate ATP using oxidative phosphorylation in the mitochondria, so cells that contain more mitochondria have a higher oxidative capacity (Chen *et al*., 1998; Holmes *et al*., 2007; Lieber, 2010).

### 1.3.2. Sonic muscle

Not much is known about the SM fibers in *H. didactylus*. Following the thought of Tavolga (1964) that compared several sonic muscles of different fishes (squirrelfish, toadfish, sea robin red hind) and based on dissections, stimulation experiments and serial cross sections, we can conclude that all these muscles must be homologous structures, despite the differences in appearance and location. We therefore assume some similarity between the SM structure among the toadfish family.

At very fast contraction frequencies, independent contractions are not possible to produce, leading to fused contractions, with no relaxation period between them (*e.g.* tetanization of the muscle). But in some batrachoidids there is no

tetanization until 300 Hz at 25ºC and each contraction is seen despite the high contraction rate. For this reason, the sonic muscles in this family (Batrachoididae) are known as the faster muscle in vertebrates (Rome *et al.,* 1996).

In *O. tau* sonic muscles are very fast and tetanize at 340 Hz (Fine *et al*., 2001) corresponding to a contraction period of only about 3 ms. This is exceptionally fast when compared to the contraction frequency of the swimming muscles that in this family is around 1-5 Hz (Rome, 2006) with a twitch lasting 500 ms (Rome *et al.,* 1996). To achieve this exceptional performance, the fibers of the sonic muscle have several morphological and biochemical adaptations, such as a very abundant sarcoplasmic reticulum that represents one third of the fiber volume (Appelt *et al*., 1991; Franzini-Armstrong *et al*., 1983) with numerous $Ca^{2+}$ pumps (Appelt *et al*., 1991) that allow the faster calcium transportation ever recorded in vertebrates (Rome *et al*., 1999).

Several batrachoidids show sexual dimorphism and hypertrophy of the sonic muscles during the mating season (Fine *et al*., 1990; Brantley *et al*., 1993; Connaughton *et al*., 2000; Modesto & Canário 2003b). Furthermore during the breeding season type I males present a considerable increase in the SM mass compared to females and type II males that do not show changes throughout the year (Modesto & Canário, 2003a).

In *P. notatus* the SM is made of a group of specialized muscle fibers controlled by the central nervous system (Bass 1989; Bass & Baker, 1990). These fibers have an interesting radial morphology exhibiting a polygonal shape with a sarcoplasm center surrounded by a contractile tube of alternating streams of sarcoplasmic reticulum and myofibrils (Fine *et al.,* 1993; Nahirney *et al*., 2006). Fine *et al*. (1990) postulate that this arrangement results from the need for fast muscle contraction. The cylinders' organization allows for the minimization of the distance, and therefore time, for calcium transportation between the sarcoplasmic reticulum and the myofibrils. At the periphery of these cells there are multiple nucleus and below the sarcolemma glycogen granules and mitochondria are present (Fine *et al*., 1990).

In *P. notatus* type I males have abundant mitochondria surrounding the tubes that can occupy approximately 53% of the myofiber volume (Lewis *et al*., 2003). During the breeding season, besides the higher development of the SM previously described, territorial males have a higher storage of glycogen (Mitchell *et al*., 2008) and more mitochondria (Appelt *et al*., 1991) due the a supplementary demand for vocal activity. Another interesting feature in the *Porichthys* genus is that the SM fibers of type I males have the myofibrils' largest Z band, which can reach a width of 1.2 μm (Bass & Marchaterre, 1989; Lewis *et al*., 2003), 20 times wider than comparable Z bands of type II males and females, or of typical vertebrate skeletal muscle (Bass & Marchaterre, 1989).

## 1.4. Objectives

In the present work we have evoked muscle contractions by electrical stimulation of the sonic nerve and studied the movement changes induced at the swimbladder wall and the resulting sound produced by toadfish collected both during and outside the reproductive season. We expected to find seasonal differences in the sonic muscles fatigue resistance, force upon contraction and sound production amplitude in adult type I males but not in juvenile pre-reproductive males.

Furthermore we have examined sonic muscle histology and histochemistry and have searched for possible morphological differences between winter and summer individuals that might explain differences in physiological ability for muscle contractions. We also inspected the muscle fibers for possible differences associated with fast and slow muscle contraction types.

# MATERIALS AND METHODS

# 2. Materials and methods

## 2.1. Fish collection and maintenance

*H. didactylus* type I males and females were caught in the Tagus estuary by fisherman, with nets or beam trawl, or collected from artificial nests between January and July of 2012. Fish obtained at the artificial nests in the summer had their vocal activity previously monitored for a period of about two weeks for another project. Vocal activity in the field was recorded as in Jordão *et al*. (2012). The fish might be temporarily kept in the field in tanks (3 m in diameter and 0.5 m deep) but were always transported in the same day to sea water stock tanks (80 l) kept at room temperature until recordings. The tanks were provided with aeration and filters (for suspended particles and protein). All fish were used within 15 days of capture.

## 2.2. Electrical stimulation of the sonic nerve

In a preliminary study the electrical stimulation was delivered, via a stimulus insulating unit (Grass, Model SIU-V; West Warwick, U.S.A.), to the double hook electrodes positioned at the sonic nerve and electrically insulated from the fish tissues with a mixture of vaseline (DAB 9; Hagen, Germany) and mineral oil.

The electrical stimuli were generated by a Phipps and Bird Isolated Square Wave Stimulator (Model 7092-611; Richmond, U.S.A.). The stimuli consisted of a 0.5 ms square wave, with amplitude large enough to elicit clear muscle contractions delivered at increasing frequencies, starting at 1 Hz and manually adjusted up to about 120 Hz, a rate corresponding to the maximum contraction frequencies measured in grunt sounds of vocalizing fish. In the subsequent experiments an electrical stimulus was prepared to mimic the contraction frequency and duration observed on an average natural boatwhistle. Each “artificial boatwhistle” (AB) stimulus, created by a Digital Stimulator (Cygnus Technology Inc., model PG4000; Pennsylvania, U.S.A.), consisted of 0,5 ms square wave pulses of 9 V delivered at 50 Hz for 700 ms (*i.e*. 35 pulses per AB)

and were repeated every 3 seconds for a total 5 min. This protocol resulted in a 5 min stimulation program at 20 boatwhistles/min, a rate observed in natural vocalizations of highly motivated fish (Amorim *et al*., 2010), producing a total of 100 AB.

### 2.3. Anesthesia and surgical procedure

The subjects were anesthetized in a saltwater bath with benzocaine (Sigma-Aldrich) (300 mg/litre) for *c.* 15 minutes. Subsequently they were moved from the anaesthetizing container to a foam holder, and positioned with the ventral side upwards.

The subjects were kept breeding but anaesthetized during the entire experiment by perfusing the gills with water containing the anesthetics through a T-shaped tube positioned in the fish mouth. The tube was continuously fed by a small pump (Hailea, HX-800; Raoping County) in a water closed circuit (Fig.1).

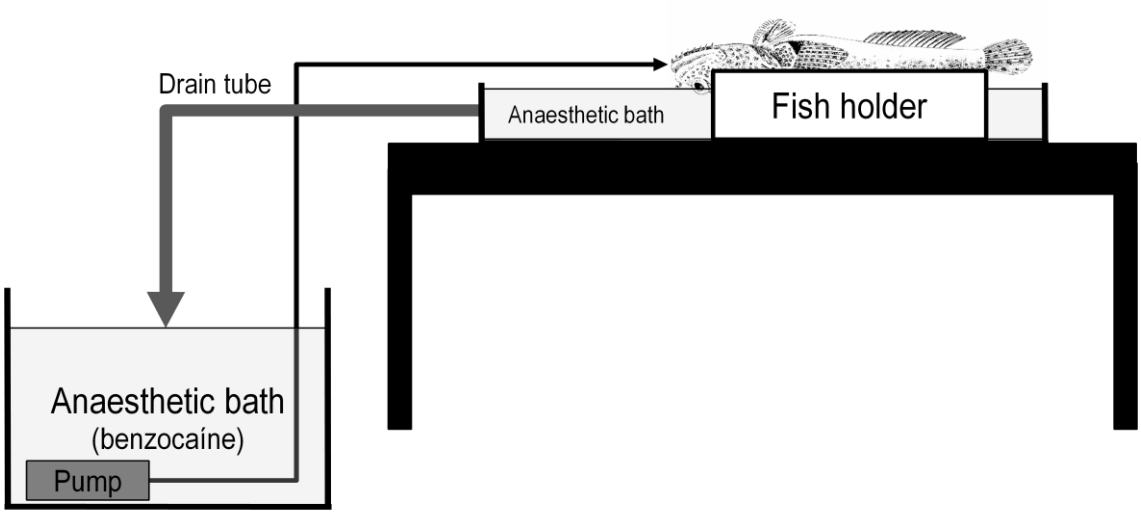


Figure 1 - Diagram of the water system used during the surgical procedure and Sonic Nerve (SN) stimulation.

An incision was made on the ventral surface of the fish to expose the swimbladder and the sonic muscles. The abdominal wall and the intestines were pushed aside. One of the sonic nerves was exposed with a needle, involved using a double hook silver electrode and isolated with vaseline (DAB 9; Hagen, Germany). The sonic nerve (SN) was then stimulated with the AB

stimulus described in the previous section. The experiments were performed at room temperature (approx. 22ºC).

## 2.4. Recordings

Sonic muscle electrical activity was recorded by inserting one stainless steel electrode, insulated for its extension but the tip, in the sonic muscle, while a silver reference electrode was positioned into the abdominal cavity. This allowed for electromyogram (EMG) recording of the SM during the contractions elicited by electrical stimulation of the SN.

The EMG signal was monitored with an oscilloscope (EZ Digital, OS-5020; Long Branch, New Jersey, U.S.A.), amplified (single ended; 1000 times; homemade amplifier MPIV Nr.20905476), digitized (50 kHz) by an Axon Instruments A/D converter board (Digital data 1200; Union City, California, U.S.A) and recorded to a PC running Axoscope 9.0 (Axon Instruments Inc.; Union city, California, U.S.A.).

Upon contraction of the sonic muscles the pressure increased inside the swimbladder and this change pulled out the blade of a force transducer (UFI, 9 mV/g, 0-15g, UFI 1030; California, U.S.A.) previously made to contact with the ventral surface of the relaxed swimbladder. Thus, the movement/force exerted on the sensor by the swimbladder wall (SBM) and due to the SM contraction was recorded. The sound generated by SM contraction was captured by a condenser microphone (Beyerdynamic, CK 703 7200, frequency response 20Hz - 20kHz, +/-3 dB; Farmingdale, New York, U.S.A.) positioned 5 cm from the swimbladder. The microphone signal was conditioned by an audio pre-amplifier (Edirol UA 25 EX; Roland, Los Angeles, U.S.A.) and simultaneously digitized and recorded through the same Axon Instruments device (see above).

After the procedure, the subjects were euthanized with an excess dose of anesthesia. They were measured (total length, TL and standard length, SL) and weighed (total weight, TW and eviscerated weight, EW). The swimbladder was removed, measured (volume, length and width) and weighed (SW). The gonads

were weighed (GW) and their state of maturity was determined (see table I). The GSI and the swimbladder index (SBI) were calculated as 100 x GW/WE (Modesto & Canário 2003a) and 100 x SW x (EW) $^{-1}$, respectively. Eighteen swimbladders were fixed in formaldehyde for further histological work.

## 2.5. Histology of the sonic muscle

For histological work 17 toadfish were used. From those, two were winter adults males (WAM), three were summer vocal adult males (SVAM) and three were silent summer adult males (SSAM) (see data analysis). Regarding juveniles, two were winter males (WJM) and 3 were summer males (SJM). We have also used one winter adult female (WAF) and three summer adult female (SAF).

SM were isolated and fixed in formaldehyde (10%) for a period of 30 days. After a month in fixative, the SM were prepared for stereological analysis by a method adapted from Emerson *et al*. (1990).

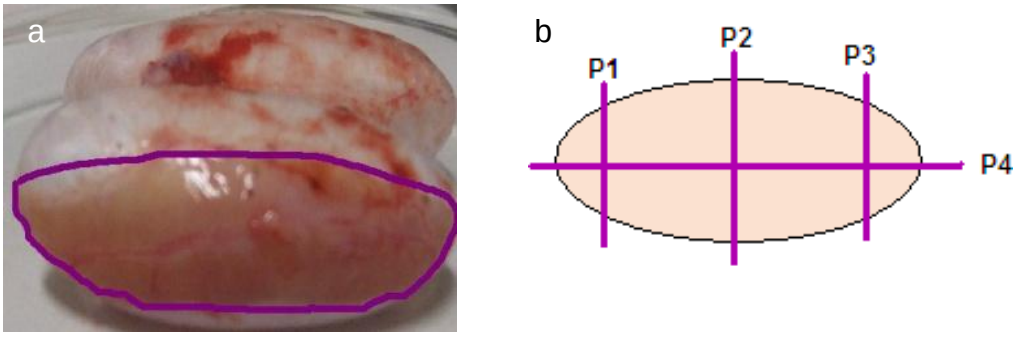


Figure 2 - (a) Lateral view of the SB of the *H. didactylus*. The purple line depicts a SM.; (b) Transversal (P1, P2, P3) and longitudinal (P4) section of the sonic muscle.

After SM from adults fishes were removed from the fixative, four slices with 1 cm$^2$ were dissected, each one corresponding to the P1, P2, P3 and P4 sections, as depicted in Figure 2. In the case of juveniles it was only possible to take P2 and P4 due to sonic muscle size. The small fragments were placed in biopsy cassettes and transferred to 70% ethyl alcohol and dehydrated until 95% ethanol. The muscles were embedded in 2-hydroxyethyl-methacrylate (GMA) resin (Heraeus Kulzer Products, Technovit 7100; South Bend, Indiana, U.S.A.)

to produce the blocks. Using a microtome (Leica Biosystems, RM 2155) three sections with 3 µm were made from each block. The sections were stained with toluidine blue, after which the preparations were cleaned with Neo-Clear (Merck; Darmstadt, Germany), and mounted in Neo-Mount (Merck; Darmstadt, Germany).

Six histological sections with transversal fibers from P1, P2, P3 and/or P4 (see table I) of each fish were photographed in an Olympus BX60 light microscope (Japan) equipped with a with an Olympys DP50 camera (Japan).

Table I – Number of sections and division used on each fish.

| Fish | No of sections | Divisions used | Fish | No of sections | Divisions used |
|---|---|---|---|---|---|
| WAM 1 | 3 | P2, P3, P4 | WJM 2 | 1 | P4 |
| WAM 2 | 3 | P2, P3, P4 | SJM 1 | 1 | P4 |
| SVAM 1 | 2 | P1, P3 | SJM 2 | 1 | P4 |
| SVAM 2 | 1 | P4 | SJM 3 | 1 | P4 |
| SVAM 3 | 1 | P4 | WAF 1 | 2 | P3, P4 |
| SSAM 1 | 1 | P4 | SAF 1 | 1 | P1 |
| SSAM 2 | 1 | P1 | SAF 2 | 1 | P4 |
| SSAM 3 | 1 | P4 | SAF 3 | 1 | P4 |
| WJM 1 | 1 | P4 | | | |

## 2.6. Histochemistry

Some small fragments from SM from one Winter Adult Male (WAM), Summer Vocal Adult Male (SVAM) and Summer Silent Adult male (SSAM) were collected, covered with free bubble air Tissue-Tek (Bioplus OCT Compound; Korea), frozen in isopentane (VWR; Radnor, Pennsylvania, U.S.A.) over dry ice and stored at -80ºC.

Eight micrometers sections were made from each muscle pieces in the cryostat (Bright, Model OTF Cryostat; Huntingdon, England) dried at room temperature

and stored again at -80ºC. The sections were washed with distilled water and incubated at 37ºC with an incubation solution (0.2 M phosphate buffer with a 7.4 pH, 0.2 M sodium Succinate (Sigma Aldrich) and 1 mg/ml Nitro Blue Tetrazolium (Sigma Aldrich) for 1:15 hour. A new wash of the sections was made and the preparations were mounted in glycerol. The results were photographed in an Olympus BX60 light microscope equipped with a with an Olympys DP50 camera.

### 2.7. Data analysis

The test subjects were divided in summer (caught in the breading season) and winter (caught out of the breading season) fish according to date of capture and gonads maturity stage (see table II). Summer adult males were also divided as vocal and silent according to the amount of monitored vocal activity during the previous 15 days. Fish that produced a significant vocal activity were classified as vocal. Silent toadfish were fish that either did not produce any sound or only vocalized for very short periods. Sound and swimbladder movement data from singing and silent summer adult fish were pooled together to increase sampling size for analysis. It was not possible to determine the morphotype of juvenile individuals.

In the case of artificial boatwhistle sounds we have measured the pulse amplitude of the first 4 pulses of all identifiable sounds. We have then calculated the maximum amplitude and the mean amplitude of each boatwhistle. After a variable amount of time, depending on the subject, no sounds were produced in spite of the continuing stimulation of the sonic nerve. We have counted, for each subject, the number of artificial boatwhistles with 4 identifiable pulses and used this as a sound fatigue index (SFI) (Fig. 3).

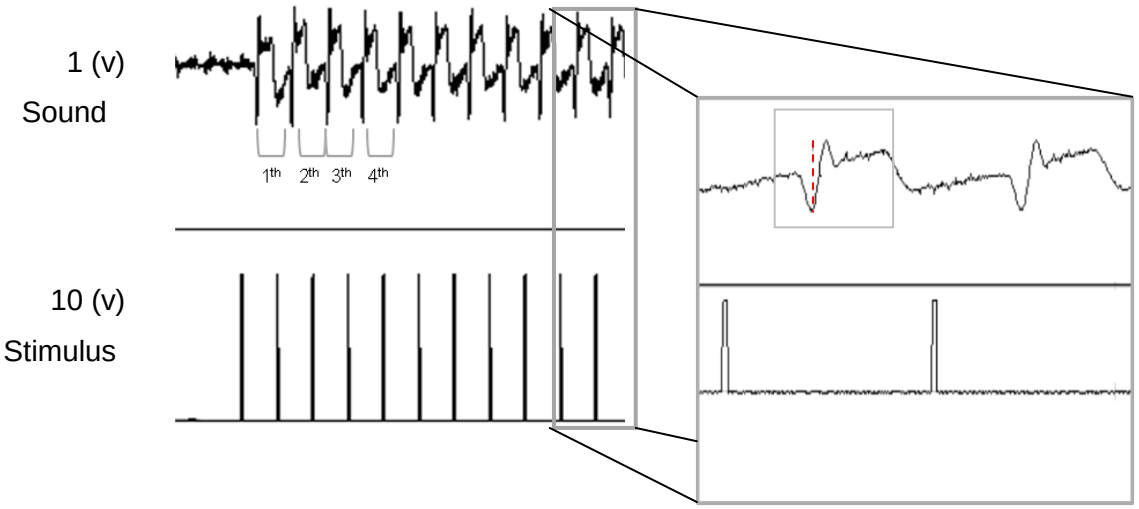


Figure 3 – Sound waveform (top) created by the stimuli (bottom). The dotted red line represents the sound amplitude.

Regarding the swimbladder movement, we have applied two different filters (created using Adobe Audition 3.0 - Adobe Systems, San Jose, California, U.S.A.) to the SBM recorded signal. The low pass filter had a 20 Hz frequency of cut, Fast Fourier Transform base with 2048 point and a Blackman Window. The other filter differs only in the cut off frequency, 14 Hz.

The first one was a high pass filter were the mean amplitude and the time since the stimuli until the maximum amplitude of the first 4 and last 4 pulses of the $1^{th}$, $2^{th}$, $3^{th}$, $28^{th}$,$29^{th}$ and $30^{th}$ artificial boatwhistles were recorded.

After the SBM signal was filtered with the low pass we noticed that there were different patterns of movement (see table III).

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Table II – Morphometric measurements of the individuals used on each procedure.

| Category | | Gonads Maturation State | Sound | | | SBM | | | Histology | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | n | Standard Length (cm) | Body mass (g) | n | Standard Length (cm) | Body mass (g) | n | Standard Length (cm) | Body mass (g) |
| Winter Adult Male (WAM) | | II | 5 | 28.4-43.8 | 604-2485 | 9 | 24.7-43.8 | 408-2485 | 2 | 28.3 | 704 |
| Winter Adult Female (WAF) | | II | 0 | n.a. | n.a. | 0 | n.a. | n.a. | 1 | 25.4 | 505 |
| Winter Juvenile Male (WJM) | | I | 3 | 9.3-10.5 | 16 -28 | 3 | 9-10 | 16-28 | 2 | 9.7-10.5 | 16-23 |
| Summer Adult Male (SAM) | Vocal (SVAM) | IV;V;VI | 4 | 29.5-37 | 624-1495 | 4 | 29.5-37.0 | 624-1495 | 3 | 29.5-39.1 | 624-1147 |
| | Silent (SSAM) | IV;V;VI | 4 | 35.4-39.1 | 1102-1390 | 5 | 32.0-39.1 | 814-1390 | 3 | 32.6-37.2 | 804-1495 |
| Summer Adult Female (SAF) | | V | 0 | n.a. | n.a. | 0 | n.a. | n.a. | 3 | 21.4-25.6 | 334-425 |
| Summer Juvenile Male (SJM) | | I | 5 | 8.0-10.7 | 14-20 | 6 | 8-10 | 14 -36 | 3 | 6.8-14.1 | 13 -17 |

Table III – SBM patterns (a, b, c and d) in toadfish in the 1th, 30th and 100th artificial boatwhistles.

| | Boatwhistle no. 1 | Boatwhistle no. 30 | Boatwhistle no. 100 |
|---|---|---|---|
| a |  |  |  |
| b |  |  |  |
| c |  |  |  |
| d |  |  |  |

Due the diverse force/movement patterns exhibited by the AB along time we integrated (custom made program by Paulo Fonseca) the area under each recording of an AB (which can be regarded as a proxy for the total work developed by the SM during the time of one AB) for all 100 ABs produced during an experiment. From this integration data we measured the maximum shown by an AB recording within the 100 repetitions and the mean per fish. As a measure of fatigue we determined movement fatigue index (MFI) where the AB in an experimental sequence corresponded to 50% of the maximum.

Regarding the histological cuts, we have quantified the number of normal and remodeling fibers percentage per fascicule (%RF), as well as the diameters of 10 fibers in each SM fascicle using a random grid from Image J Software (National Institutes of Health, U.S.A). This grid had two different sizes: 500 µm for adults and 200 µm for juvenile's fascicules. The density of the fibers (number of fibers/fascicle area) was also estimated.

To determine the myofibrils and sarcoplasm areas we used Image J. This software measures the area based on color intensity but some histological structures such as blood vessels and erythrocytes had similar intensities comparing to the myofibrils area. In order to exclude those structures, we assessed manually case by case their diameters range and their morphology. Only elements between 45 µm - 4500 µm were analyzed. The myofibrils area/sarcoplasm area ratio (MSR) was then calculated.

### 2.7.1. Statistical analyses

Statistical analyses were conducted with Statistica 10 (Statsoft Inc.; Tulsa, U.S.A.) and all data was transformed when necessary to meet assumptions of the used parametric tests (Zar, 2009).

#### 2.7.1.1. Sound

To test the effect of season (summer *vs* winter) and ontogenetic stage (adult *vs* juvenile) on both the mean and maximum amplitude of AB we used factorial ANOVAs. Similarly, we tested the effect of the above factors on the SFI with a factorial ANOVA.

#### 2.7.1.2. Swimbladder movement

To test the effect of season (summer *vs* winter) and ontogenetic stage (adult *vs* juvenile) on the high-pass filtered mean amplitude and mean time (of 100 AB of al fish within each category) of the SBM we used ANCOVAs using male SL, GSI, and SBI as covariates. As none of the covariates has a significant effect we removed them from the analysis and carried out a factorial ANOVA instead.

Regarding the low pass filtered amplitude of the SBM we have tested the effect of season (summer *vs* winter) and ontogenetic stage (adult *vs* juvenile) both maximum and mean amplitude and MFI with a factorial ANOVA. Both amplitude data were log-transformed.

2.7.1.3. Histology

We tested the effect of the factor 'vocal activity' (two levels 0 = silent, 1= vocal) on each the following dependent variables: the myofibrils/sarcoplasm ratio (MSR), the percentage of remodeled fibers of each fascicle (% RF) and fibers density, with ANCOVAs using male SL, GSI, and SBI as covariates. On the fibers test only SL was included in the analyses, because it was the sole significant covariate.

We also tested the effect of the factor “gender” (two levels M = male, F = female) on MSR, Remodeling fibers percentage (% RF) and the fibers density with an ANCOVA using SL and GSI as covariables.

To test the “season” factor (two levels W = winter, S = summer) effect on MSR, % RF and the fibers density we tried to use an ANCOVA with two factors but there was no homogeneity of variances. We had to test each factor using separate T-tests. To correct for multiple testing we used Bonferroni corrections for probability levels.

To analyze the effect of the “Ontogenic state” (two levels A = adult and J = juvenile) on “fibers diameter” we used T-tests individually for winter and summer data. Since the homogeneity of variances was not achieved with data transformations we did not use ANOVA. To correct for multiple testing we used Bonferroni corrections for probability levels.

## 2.8. Ethical note

All efforts were made to maximize animal welfare. The performed procedures respect all the current Portuguese animal welfare laws, guidelines and policies.

# RESULTS

# 3. Results

## 3.1. Sound

50 Hz stimulation of the sonic nerve generated boatwhistles (Fig. 4) with an initial vigorous response that decreased over the stimulation period. At the end of stimuli bout only a single weak pulse was generated.

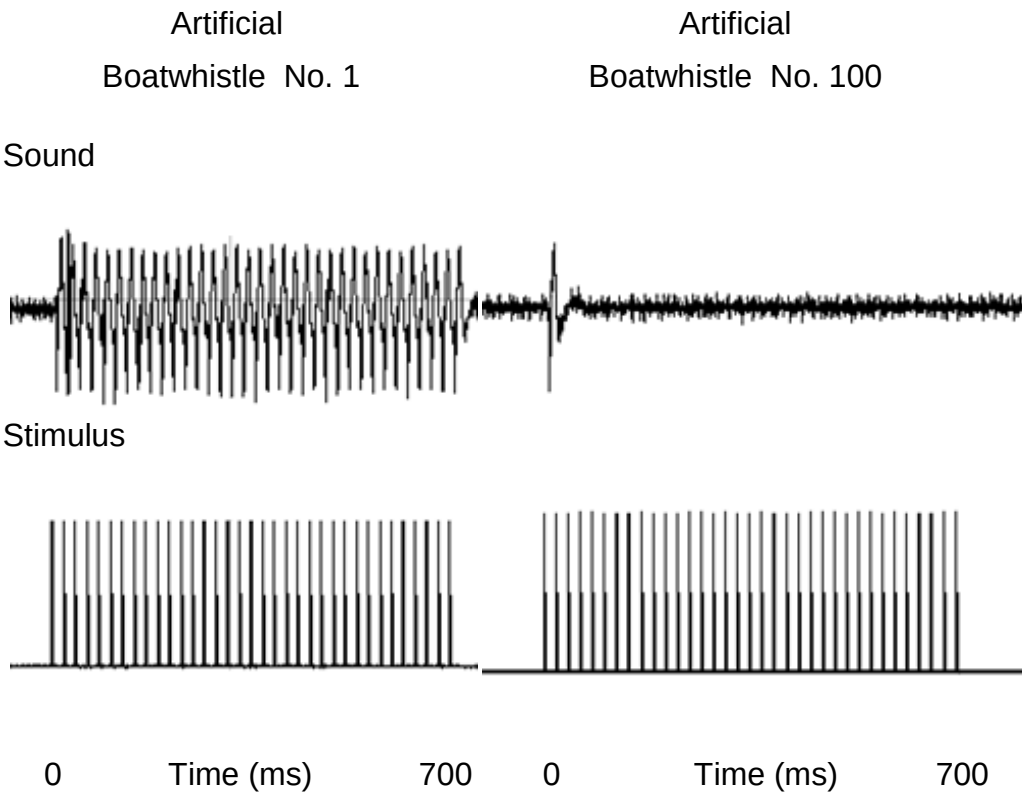



Figure 4 – sound waveform (top) and Stimuli (bottom) evoked by 700 ms trains of stimuli (50Hz) applied to the sonic nerve.

Artificial boatwhistles with maximum amplitudes were produced in the beginning of the stimuli (Fig. 5). Summer males (both adults and juveniles) produce significantly higher amplitude boatwhistles compared to winter individuals (Table IV, Fig. 6b).

AB mean amplitudes were higher in summer specimen than in winter ones but there was no difference between adult and juvenile fish (Table IV, Fig. 6a). At

the 30th artificial boatwhistle all toadfish presented little sound amplitude, except for the summer adults (Fig. 5).

Adult fish fatigues significantly less as the first four pulses could be measured until a higher order of artificial boatwhistles (Table IV; Fig. 7).

Table IV - Results of Factorial ANOVA showing the effects of season and ontogenic state on sound amplitude and fatigue on sound. All data was log-transformed and square root transformed when necessary to meet the ANOVA assumptions.

**Variables**

| **Log Maximum Amplitude** | **F1,17** | ***p*** |
|---|---|---|
| Season | 9.9 | ** |
| Ontogenic stage | 1.1 | 0.3 |
| Season x Ontogenic stage | 0.8 | 0.4 |
| Interception | 54.4 | *** |
| **Log Mean Amplitude** | **F1,17** | **p** |
| Season | 6.3 | ** |
| Ontogenic state | 2.6 | 0.1 |
| Season x Ontogenic stage | 17.3 | *** |
| Interception | 54.4 | *** |
| **Log Sound Fatigue Index** | **$F_{1,17}$** | ***p*** |
| Season | 0.0 | 0.9 |
| Ontogenic state | 6.0 | ** |
| Season x Ontogenic state | 1.5 | 0.3 |
| Interception | 1210.9 | *** |

*** p<0.001; ** p<0.01; * p<0.05.

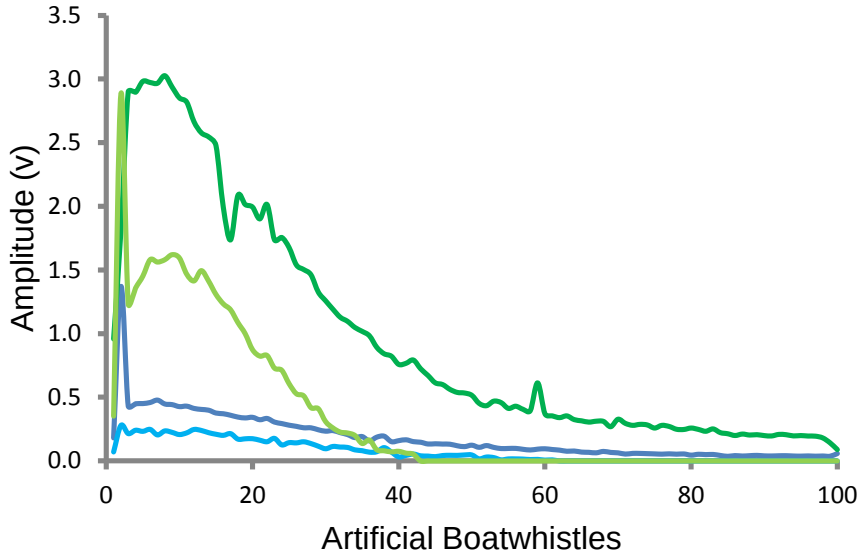



Figure 5 – Sound amplitude mean in winter adult males (dark blue), winter juvenile males (light blue), summer adult males (dark green) and summer juvenile males (light green).

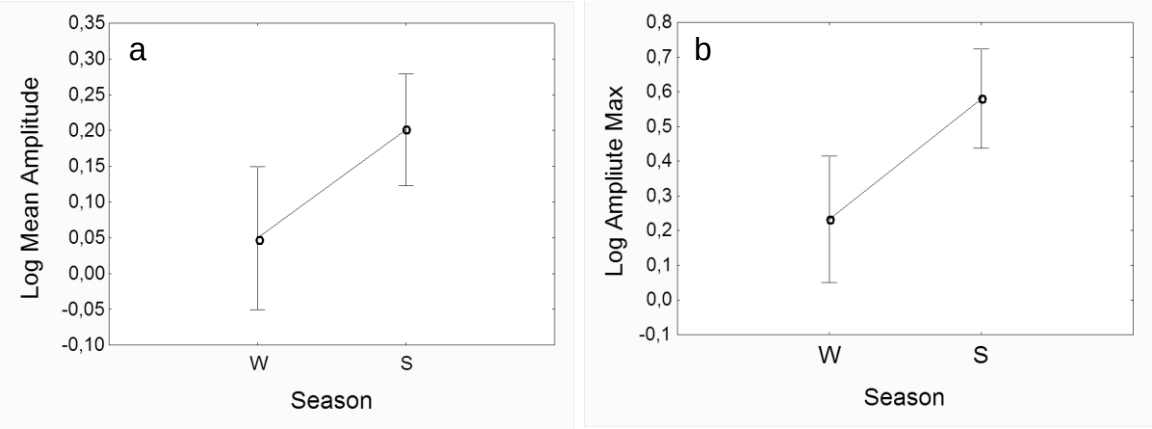



Figure 6 – Comparison of the mean (a) and maximum (b) sound amplitude between winter (W) and summer (S) males. All the differences are significant. Dots and error bars are means and 95% confidence intervals, respectively. All data was log-transformed to the ANOVA assumptions.

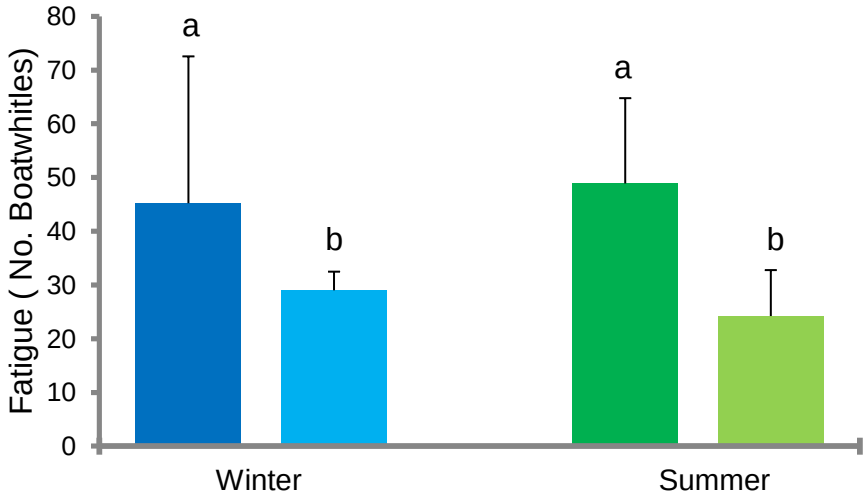



Figure 7 – Comparison of sonic muscle fatigue resistance in winter adult males (dark blue), winter juvenile males (light blue), summer adult males (dark green) and summer juvenile males (light green). Different letters indicate statistically significant differences (factorial ANOVA) ($p<0.01$). Vertical bars represent standard deviation.

### 3.2. Swimbladder Movement

Stimulation at 1Hz (Fig. 8a) and 50 Hz (Fig. 8b) allowed us to see that the swimbladder movement has two different components. A fast twitch contraction follows the stimulus pattern. The other component is a sustained slow contraction of the sonic muscle during the entire stimulation lingering until after the stimulus end until muscle relaxation. At 1 Hz and 50 Hz there is a latent period of 6 ms.

Figure 8a shows the contraction and relaxation of the two components. The black full arrow indicates the contraction of the fast component whereas the dotted arrow shows the relaxation. The full grey line illustrates the contraction of the slow component and the relaxation is marked with the dotted grey line.

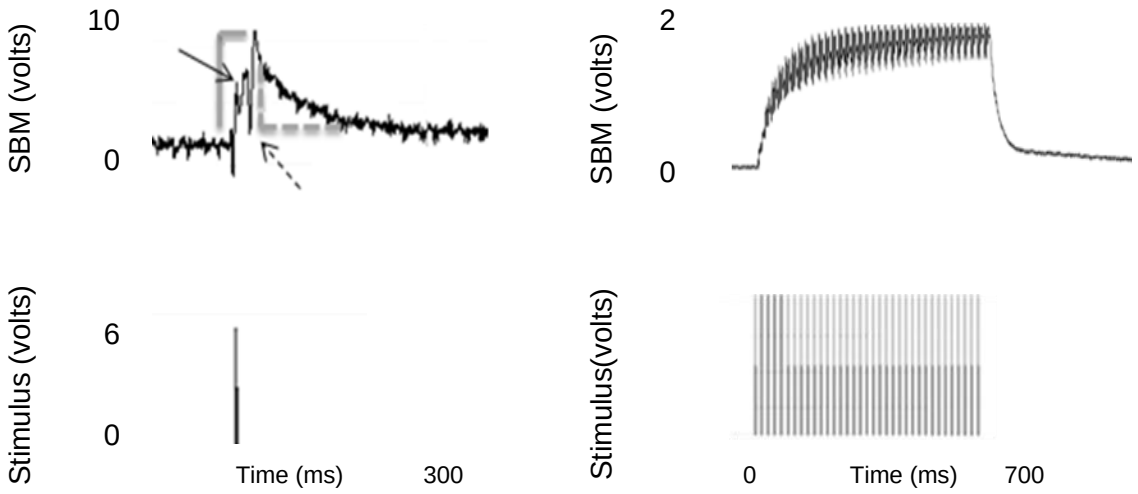
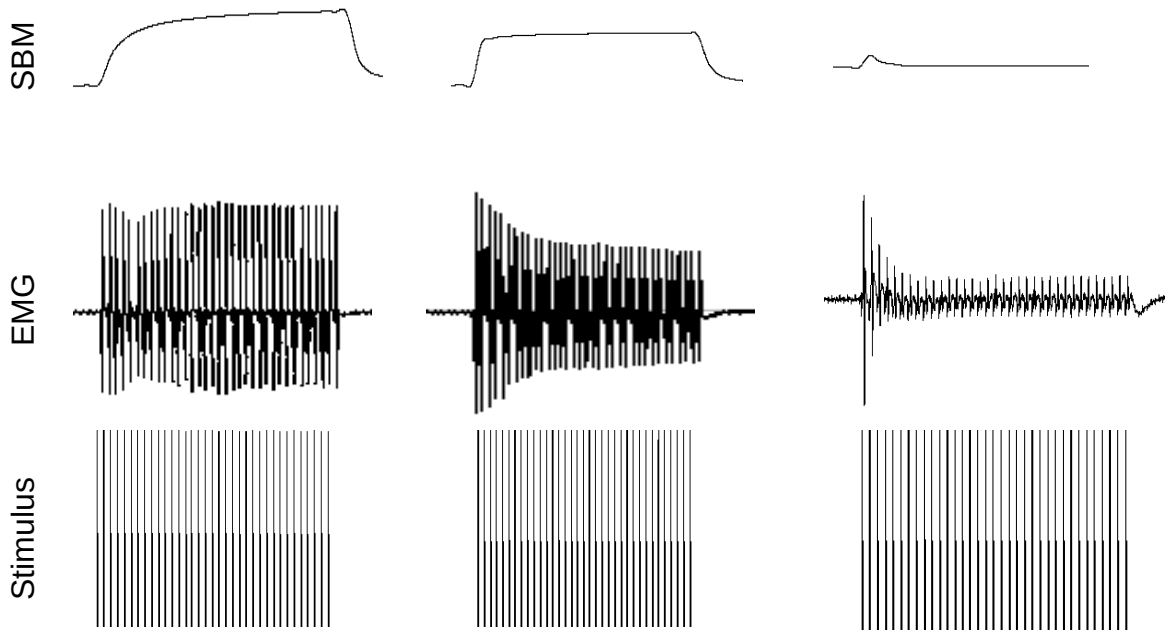



Figure 8 – SBM (top) at the beginning of stimulation (1st stimulus – bottom) with 1 Hz (a) 50 Hz (b) frequency with 12.5 ms and 700 ms trains respectively of electrical stimuli.

When stimulated at 50Hz (Table V) the muscle response in adults is robust until the 30th AB, just like in sound production, but the movement amplitude decreases towards the end of the stimulus (100th AB), where the SM does not contract in spite of the action potentials measured by the EMG following the stimuli pattern.

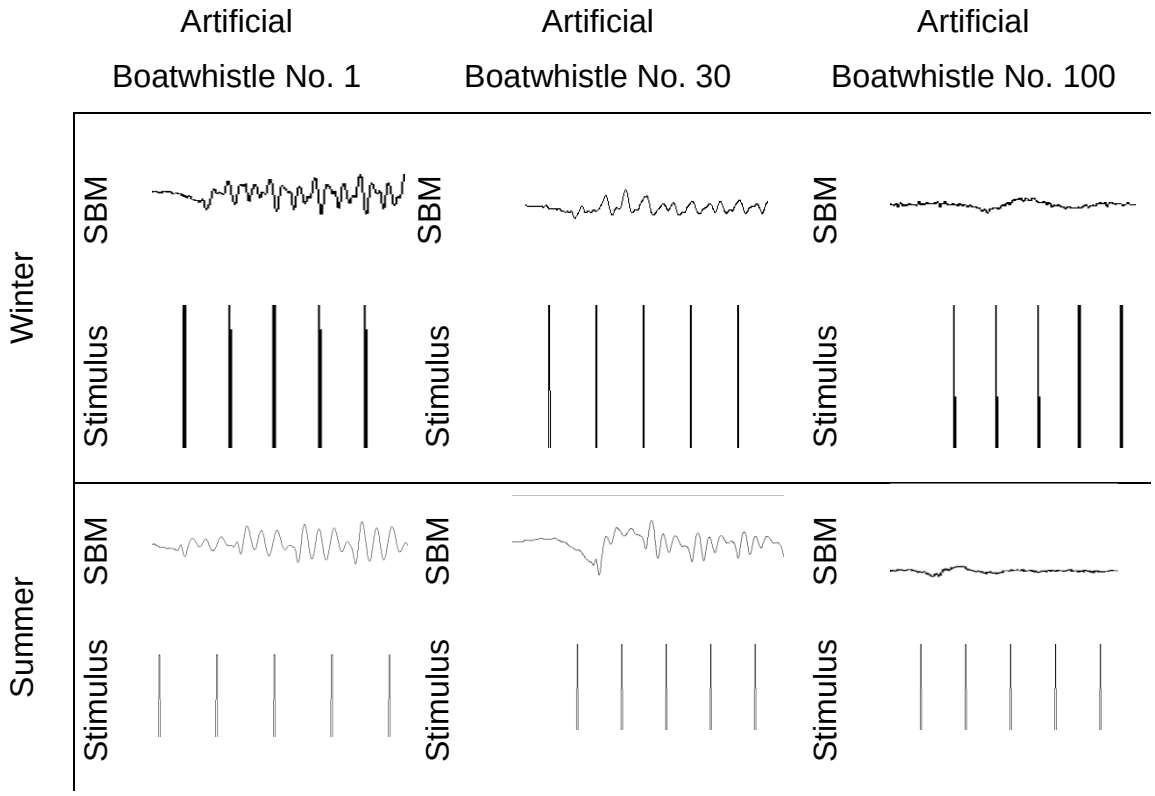
Table V – Slow component swimbladder movement on the first, thirtieth and hundredth artificial boatwhistles in summer adult males.

| | Artificial Boatwhistle No. 1 | Artificial Boatwhistle No. 30 | Artificial Boatwhistle No. 100 |
|---|---|---|---|




The SBM at 100$^{th}$ AB is low or inexistent compared to the first or 30$^{th}$ AB (Table VI). The high pass filter analysis was made using the 1$^{th}$, 2$^{th}$, 3$^{th}$, 28$^{th}$, 29$^{th}$ and 30$^{th}$ AB. The amplitude of each AB results from the mean of 1$^{th}$, 2$^{th}$, 3$^{th}$, 33$^{th}$, 34$^{th}$, 35$^{th}$ pulses.

Table VI – Observed swimbladder movement (Low pass filter) on the first, thirty and hundredth artificial boatwhistle in winter and summer males.




The mean amplitude with high pass filter is higher in the summer (Table VII, Fig. 9a), but doesn't differ between ontogenic state (Table VII). The time of contraction is smaller in summer adult individuals (Table VII, Fig. 9b). There are no differences between seasons (Table VII).

Table VII - Effects of season and ontogenic state (factorial ANOVA) on mean amplitude SBM, and duration of swimbladder movement (700 ms) in the fast component of the muscle movement.

| **Variables** | **Fast contraction** | |
|---|---|---|
| **Sqrt Mean Amplitude** | **$F_{1,16}$** | ***p*** |
| Season | 7.8 | * |
| Ontogenic stage | 0.8 | 0.4 |
| Season x Ontogenic stage | 5.1 | * |
| Interception | 41.6 | 0 |
| | | |
| **Log Mean Time** | **F1,16** | ***p*** |
| Season | 0.4 | 0.5 |
| Ontogenic stage | 5.1 | * |
| Season x Ontogenic stage | 0.2 | 0.6 |
| Interception | 323.7 | 0 |

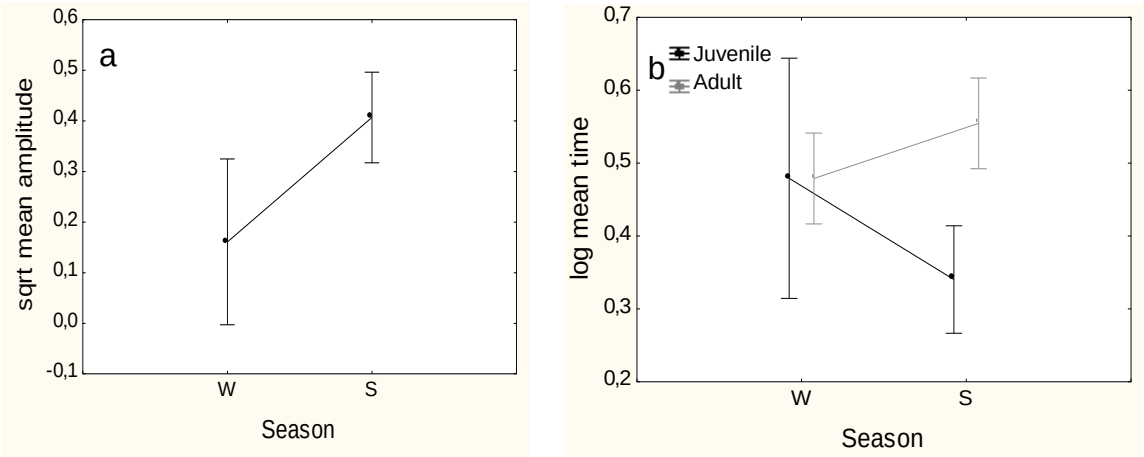



Figure 9 – Comparison of the mean amplitude (a) of fast contraction SBM in males from winter (W). Comparison of the mean time (b) of fast contraction SBM in adult and juvenile males from winter (W) and summer (S). All differences are significant. Dots and error bars are means and 95% confidence intervals, respectively. Data was log or square root -transformed when necessary.

The maximum area as well as the mean area of the integration is higher during the summer (Table VIII, Fig. 10a, 10b and 11), there are no differences in the ontogenic state for both of this variables. The fatigue index doesn't show differences between seasons or ontogenic stages.

Table VIII - Effects of season and ontogenic state (factorial ANOVA) on maximum and mean amplitude SBM and fatigue in the slow component of the muscle movement.

| **Variables** | **Slow contraction** | |
|---|---|---|
| **Log Maximum Amplitude** | **$F_{1,19}$** | ***p*** |
| Season | 6.23 | * |
| Ontogenic stage | 0.002 | 0.97 |
| Season x Ontogenic stage | 1.72 | 0.2 |
| Interception | 277.3 | 0.0 |
| | | |
| **Sqrt Mean Amplitude** | **$F_{1,19}$** | ***p*** |
| Season | 7.2 | * |
| Ontogenic stage | 0.6 | 0.5 |
| Season x Ontogenic stage | 0.3 | 0.6 |
| Interception | 93.0 | *** |
| | | |
| **Movement Fatigue Index** | F1,19 | p |
| Season | 0.14 | 0.72 |
| Ontogenic stage | 0.01 | 0.92 |
| Season x Ontogenic stage | 0.8 | 0.4 |
| Interception | 88.9 | *** |

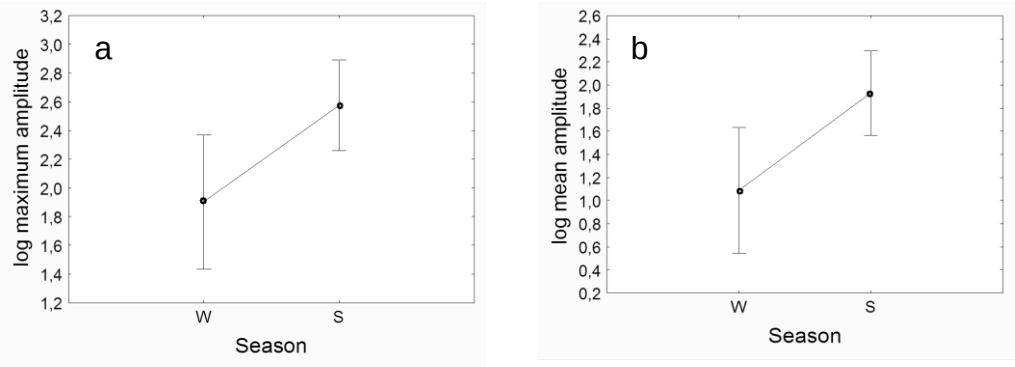



Figure 10 - Comparison of the maximum and mean amplitude of the slow contractions movement from winter (W) and summer (S). Dots and error bars are means and 95% confidence intervals, respectively. All data was log-transformed to meet ANOVA assumptions.

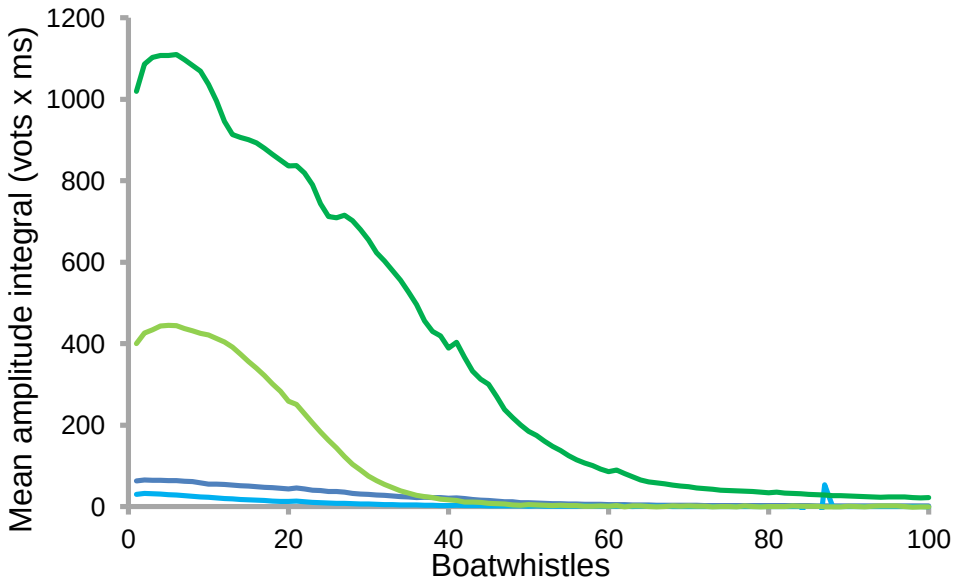



Figure 11 – Integrated mean amplitude displacement observed in winter adult (dark blue) and juvenile (light blue) males as well as in summer adult (dark green) and juvenile (light green) males.

When the last peak of sound occurs the slow component of the SBM starts to relax (dotted grey line in Figure 12).

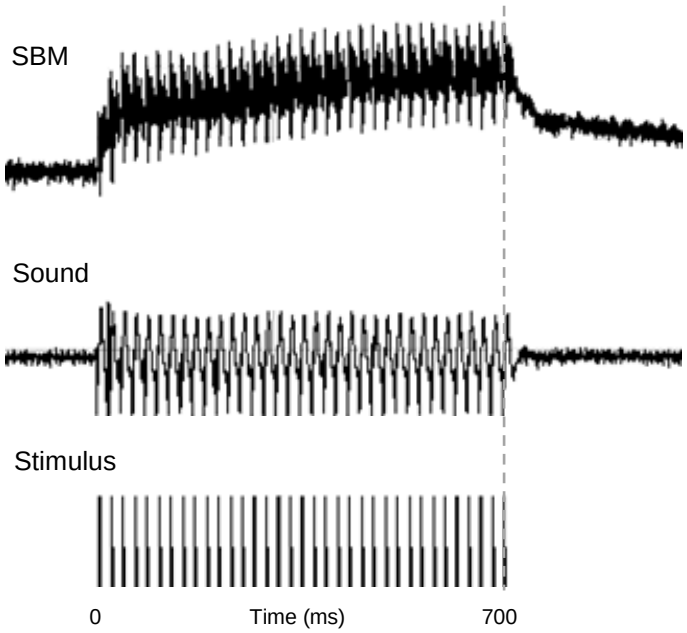



Figure 12 – Stimulus, sound waveform and SBM (movement if the swimbladder) evoked by 700 ms trains of stimuli (50Hz) applied to the sonic nerve.

### 3.3. Histology

The mapping of fibers orientation in the sonic muscle is presented in figure 13. Using four sections (Fig. 2b), transversal (P1, P2, P3) and longitudinal (P4), it is possible to observe that there are fibers in three different directions on the transversal sections (P1, P2 and P3): some are longitudinal, others transversal or oblique. In contrast, the fibers presented on the P4 cuts are all transversely arranged.

Based on these results the samples used on the following histological analysis (Fig.13) were collected from the middle (P2 and P4) or edge (P1 and P4) of the SM as being representative of the entire muscle.

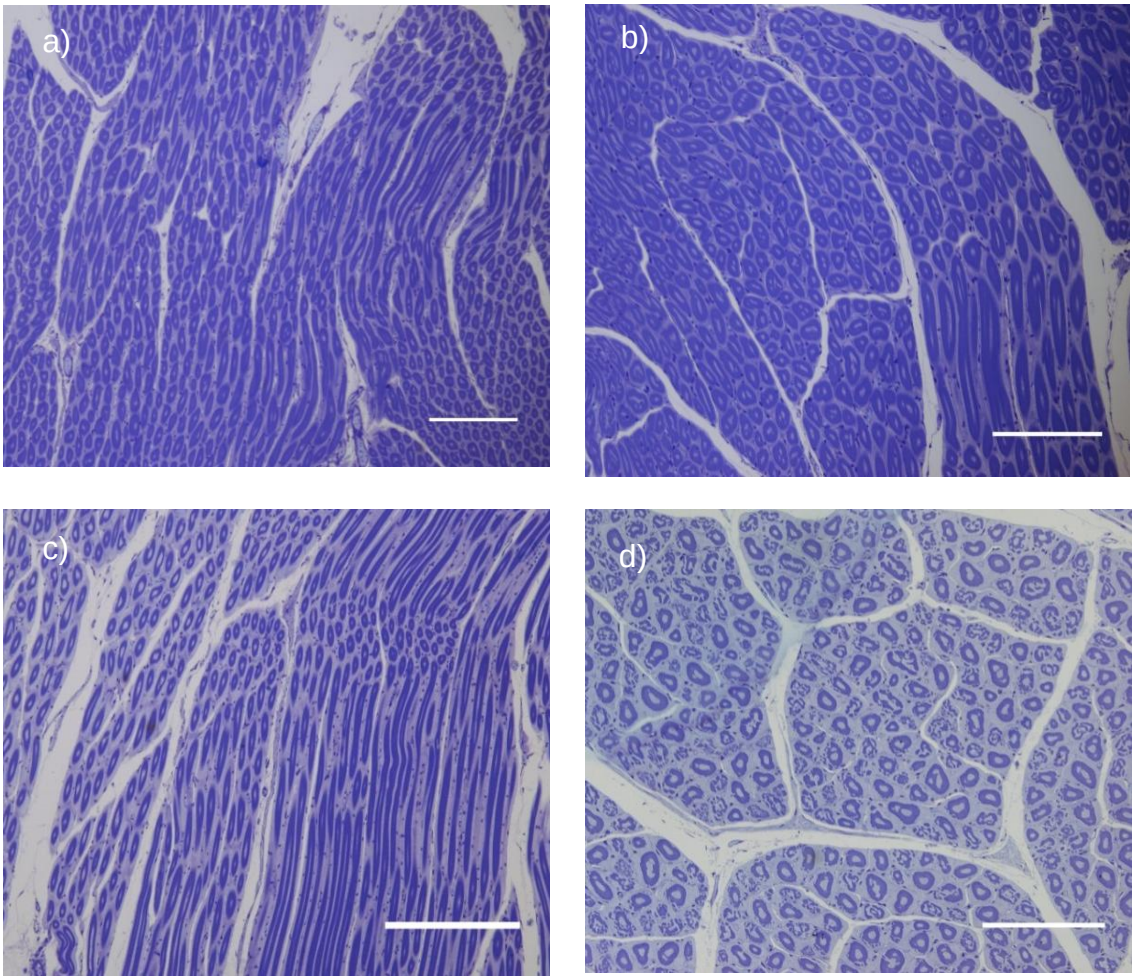



Figure 13 – SM sections of *H. didactylus* dyed with blue toluidine: (a) P1 section with transversal and longitudinal fibers. (b) P2 section with transversal and longitudinal fibers. (c) P3 section with transversal and longitudinal fibers. (d) Transversal fibers in the P4 section. Scale bar: (a)100 µm, (b) 200 µm, (c) 150 µm, (d) 200 µm.

The sonic nerve is surrounded by connective tissue and it has the same orientations of the fibers around (Fig. 14).

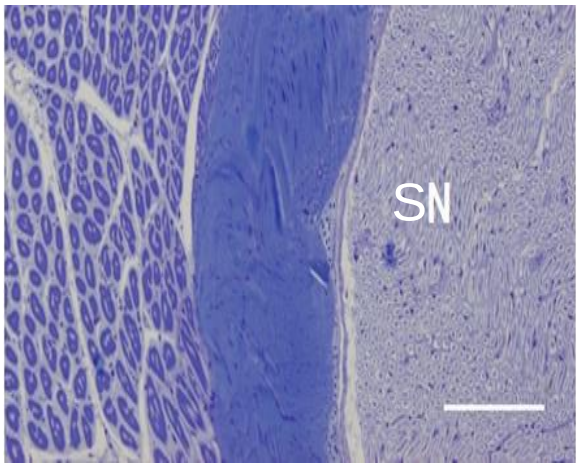



Figure 14 - The sonic nerve is surrounded by connective tissue with the same orientations of the fibers around it.

The fibers have a sarcoplasm core around and in the middle of the myofibrils. The same fascicle can have fibers with a single and multiple sarcoplasmic cores of sarcoplasm as is demonstrated in Figure15a. Adult males in the breading season appear to present promptly small fibers (Fig. 15).

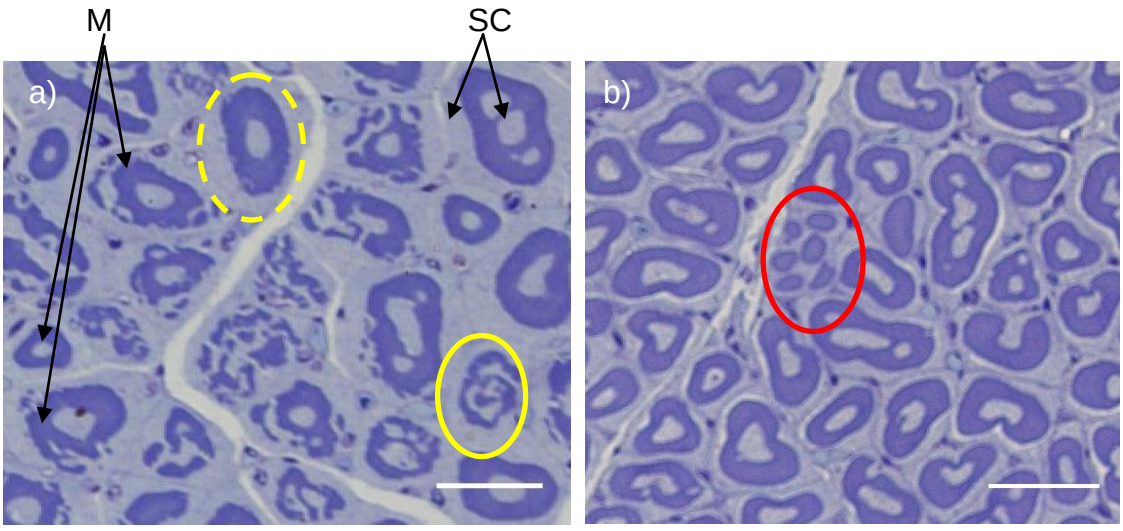



Figure 15 – SM sections of a Summer Adult Male *H. didactylus* SM stained with blue toluidine. a) The sarcoplasmic core (SC) that's light blue and myofibrils (M) that are dark blue in the middle and in the periphery of the SM myofibrils. Normal fiber (inside the yellow broken line) and remodel fiber with multiple core of sarcoplasm (inside the full yellow line). b) Smaller fibers near remodeling fibers (inside the red line). Scale Bar a) 40 μm; b) 50 μm.

The sarcoplasmic central core of juveniles is nonexistent or very small (Fig. 16e, 16f and 16g).

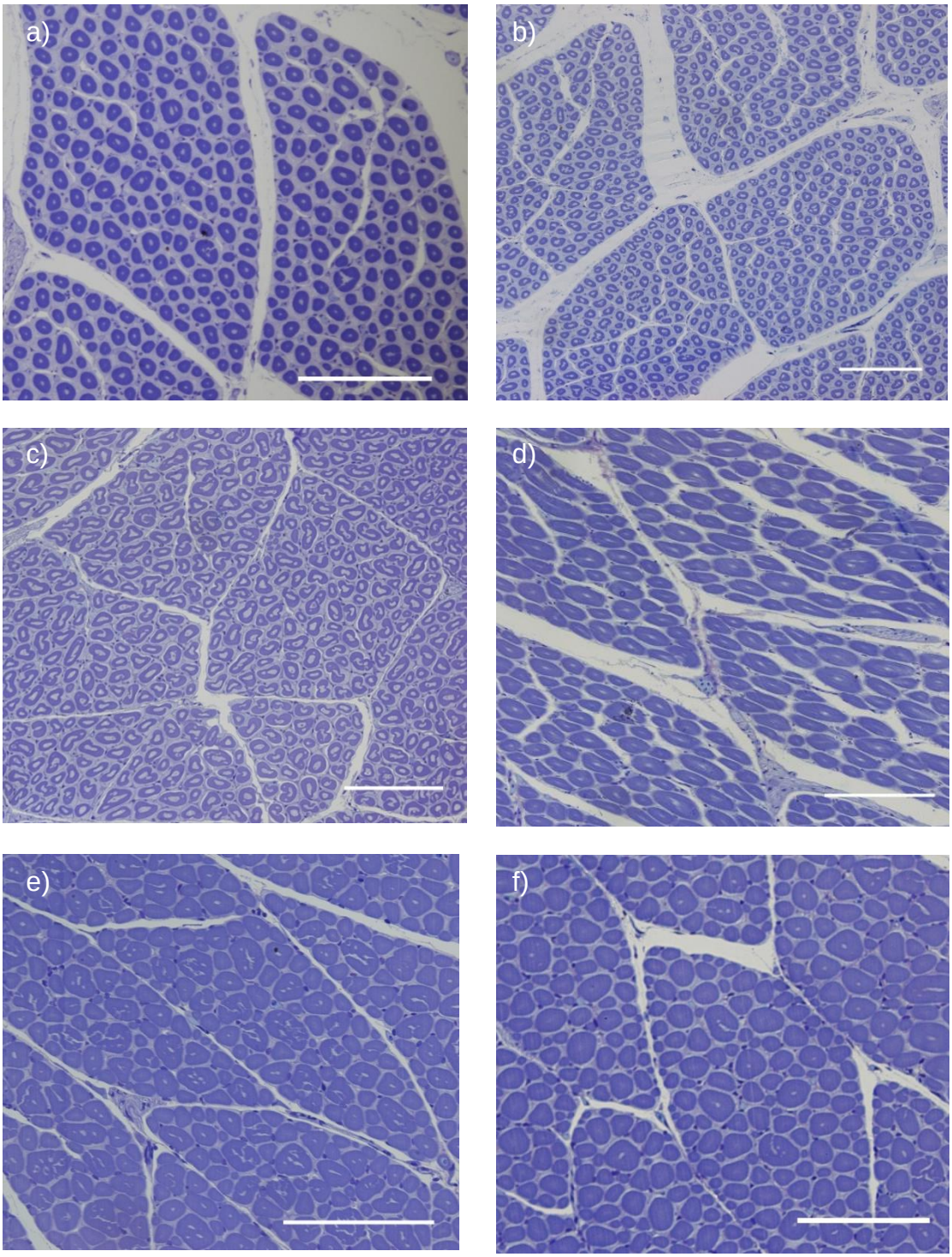



Figure 16 – Sonic muscle sections of *H. didactylus* dyed with blue toluidine: a) Winter adı male; b) Summer vocal adult male; c) Summer silent adult male; d) Summer adult female; e) Winter juvenile male; f) Summer juvenile male. Scale bar a) 200 µm; b) 200 µm; c) 200 µm; d) 200 µm; e) 250 µm; f) 120 µm.

Vocal males had marginally non-significant larger MSR than silent males (Table IX, Fig. 17a) and also presented higher fiber density (Table IX, Fig. 17b). However the % RF did not differ between vocal and silent males (Table IX).

Females have higher MSR than males during all year round, and don't show seasonal changes (Table IX, Fig.16d and 18a). Males MSR decrease in the summer (Table IX, Fig. 16a, 15b, 16c and 18a). The remodeling fiber percentage is higher in females (Table IX, Fig.18b). In the winter there are more remodeling fibers (Table IX, Fig.19c). Females have higher fiber density than males all year round and show an increase in the summer (Table IX, Fig.18d). On the contrary, males have lower fiber density during the breading season (Table IX, Fig.18d).

Table IX - Effects of season and gender on histological measurements (MSR - myofibrils area/sarcoplasm area ratio, % RF – Remodeling fibers percentage and fibers density). Standard length (SL), gonadosomatic index (GSI) and swimbladder index (SBI) were used as covariates The gender data ware log-transformed and square root transformed when necessary to meet the ANCOVA assumptions.

| | **MSR** | | **% RF** | | **Fibers density** | |
|---|---|---|---|---|---|---|
| | **$F_{1,32}$** | ***p*** | **$F_{1,31}$** | ***p*** | **$F_{1,33}$** | ***p*** |
| **Vocal activity** | 4.1 | 0.052 | 0.4 | 0.055 | 7.1 | * |
| SL | - | - | 6.6 | * | 19.9 | *** |
| GSI | 17.1 | *** | 9.2 | ** | - | - |
| SBI | 8.9 | ** | 12.9 | ** | - | - |
| Intercept | 11.6 | * | 1.0 | 0.33 | 3.9 | 0.06 |
| | **$F_{1,68}$** | ***p*** | **$F_{1,60}$** | ***p*** | **$F_{1,60}$** | ***p*** |
| **Gender** | 224.3 | *** | 22.0 | *** | 15.0 | *** |
| Season | 7.9 | ** | 15.8 | ** | 7.2 | ** |
| Gender x Season | 7.1 | ** | 0.2 | 0.66 | 15.6 | *** |
| SL | - | - | 28.4 | *** | 4.4 | * |
| GSI | - | - | - | - | 11.2 | ** |
| SBI | - | - | 5.0 | * | - | - |
| Intercept | 76.1 | 0 | 12.9 | *** | 15.5 | *** |

*** p<0.001; ** p<0.01; * p<0.05

Winter toadfish had higher MSR than summer individuals (Table X, Fig. 15a, 15b, 15c and 19a), but no differences were found in % RF and fiber density (Table X).

When we compared the MSR between juveniles and adults, juveniles have a higher ratio in the summer (Table XI, Fig.19a). No differences were found in MSR during the winter (Table XI) although, during the summer, adults have more remodeling fibers than juveniles (Table XI, Fig.19c). Adults have higher fiber density then juveniles during the summer (Table XI, Fig.19b).

The diameter of the fibers does not change from winter to summer in males (Table XII). In the summer juveniles have tight fibers (Table XII, Fig. 20a). Adult males have larger fibers than juveniles especially in the summer (Table XII, Fig. 20b and 20c). Males show fibers with larger diameter than females (Table XII, Fig. 20d).

Table X - Effect (T-test) of ontogenic stage on histological measurements (MSR - myofibrils area/sarcoplasm area ratio, % RF – Remodeling fibers percentage and fibers density).

| **Ontogenic state** | **MSR** | | | **% RF** | | | **Fibers density** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **df** | **T** | ***p*** | **df** | **T** | ***p*** | **df** | **T** | ***p*** |
| Winter | 12.2 | -2.5 | 0.02 | 22 | 2.1 | 0.05 | 16.4 | -4.3 | * |
| Summer | 18.4 | -8.8 | *** | 47.7 | 4.9 | *** | 18.6 | -11.6 | *** |

usual levels of significance after Bonferroni correction *** p<0.0002; ** p<0.002; * p<0.008

Table XI - Effect of season on histological measurements (MSR - myofibrils area/sarcoplasm area ratio, % RF – percentage of remodeling fibers and fibers density). Tested with T-tests.

| | **MSR** | | | **% RF** | | | **Fibers density** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **df** | **T** | ***p*** | **df** | **T** | ***p*** | **df** | **T** | ***p*** |
| **Season** | 46 | 5.67 | *** | 32 | -1.45 | 0.16 | 13 | -1.83 | 0.09 |

usual levels of significance after Bonferroni correction *** p<0.0003; ** p<0.003; * p<0.017

Table XII - Effect of season, ontogenic state and gender on fibers diameter. All data was log-transformed and square root transformed when necessary to meet the T-test assumptions.

| **Fibers diameter** | Adult Male | | | Juvenile Male | | |
|---|---|---|---|---|---|---|
| | **df** | **T** | ***p*** | **df** | **T** | ***p*** |
| Season | 478 | 0.12 | 0.90 | 123.5 | 12.02 | *** |
| | Winter | | | Summer | | |
| Ontogenic state | 232.2 | 7.7 | *** | 410.3 | 41.5 | *** |
| Gender | - | - | - | 460.0 | 34.6 | *** |

usual levels of significance after Bonferroni correction *** p<0.0003; ** p<0.003; * p<0.013

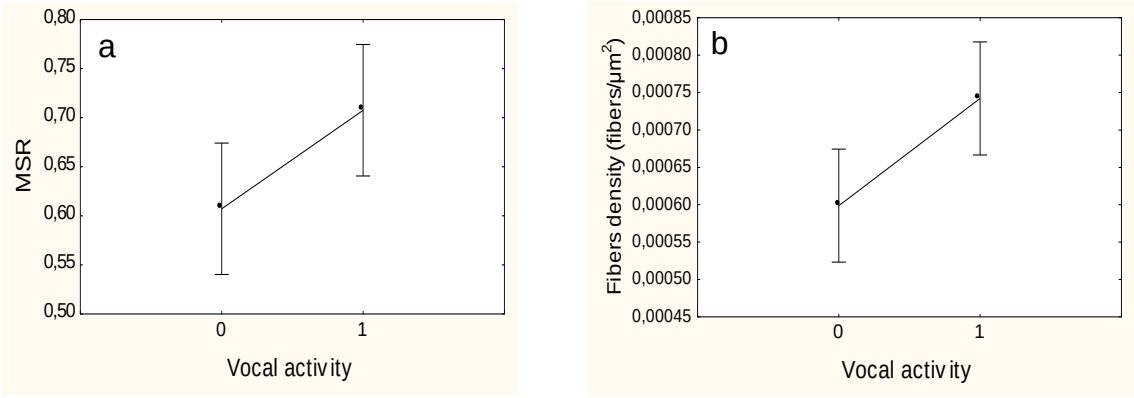



Figure 17 – Comparison of the MSR (myofibrils area/sarcoplasm area ratio) (a) and fibers density (b) depending on the vocal activity (0 = silent and 1 = vocal) on summer males. Dots and error bars are means computed for the covariates GSI (gonadosomatic index), SBI (swimbladder index) and SL (standard length) means and 95% confidence intervals, respectively.

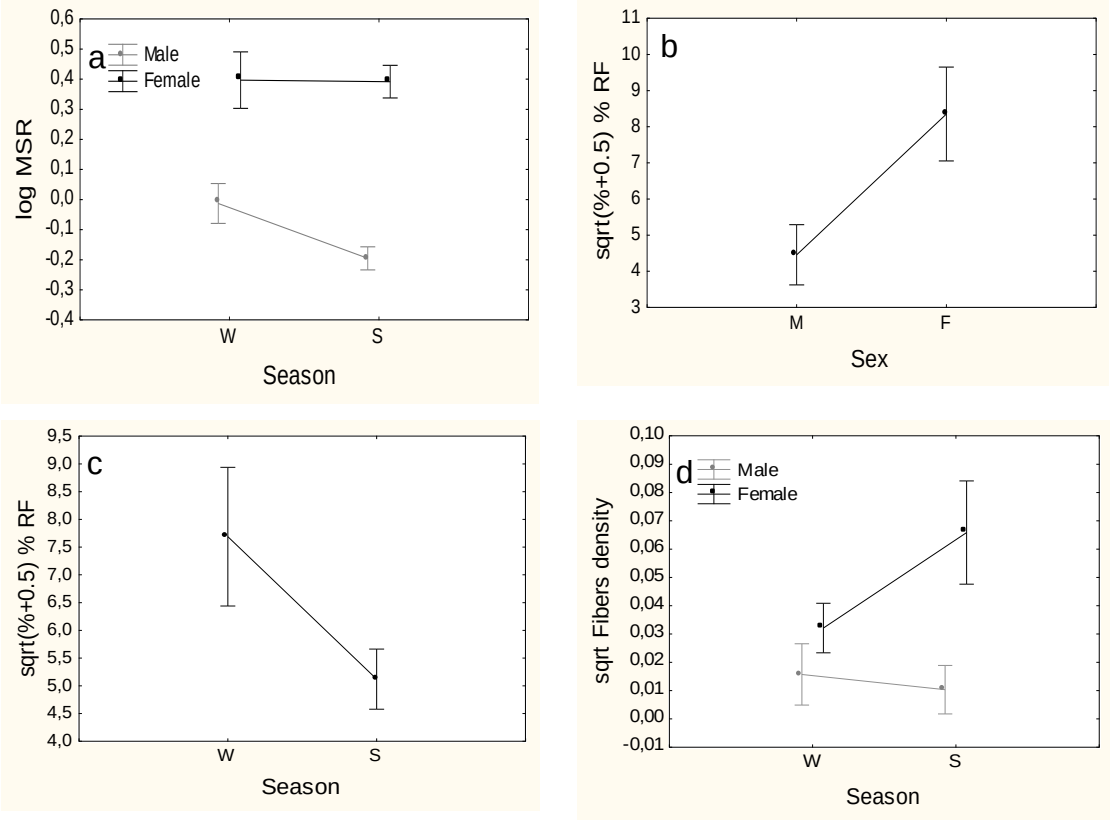



Figure 18 – Comparison of the MSR (myofibrils area/sarcoplasm area ratio) (a) and fibers density (d) in males and females during in winter (W) and summer (S). Comparison of % RF (Remodeling fibers percentage) between males and females (b) during in winter (W) and summer (S) (c). Dots and error bars are means computed for the covariates GSI (gonadosomatic index), SBI (swimbladder index) and SL (standard length) means and 95% confidence intervals, respectively. All data was log or square root -transformed to meet ANCOVA assumptions.

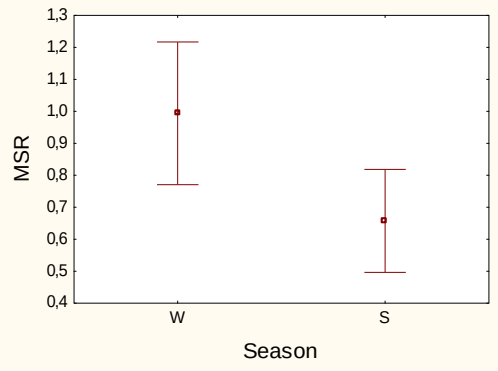



Figure 19 – Comparison of the MSR (myofibrils area/sarcoplasm area ratio) on winter (W) and summer (S) males. Dots and error bars are means and 95% confidence intervals, respectively. The differences are significant.

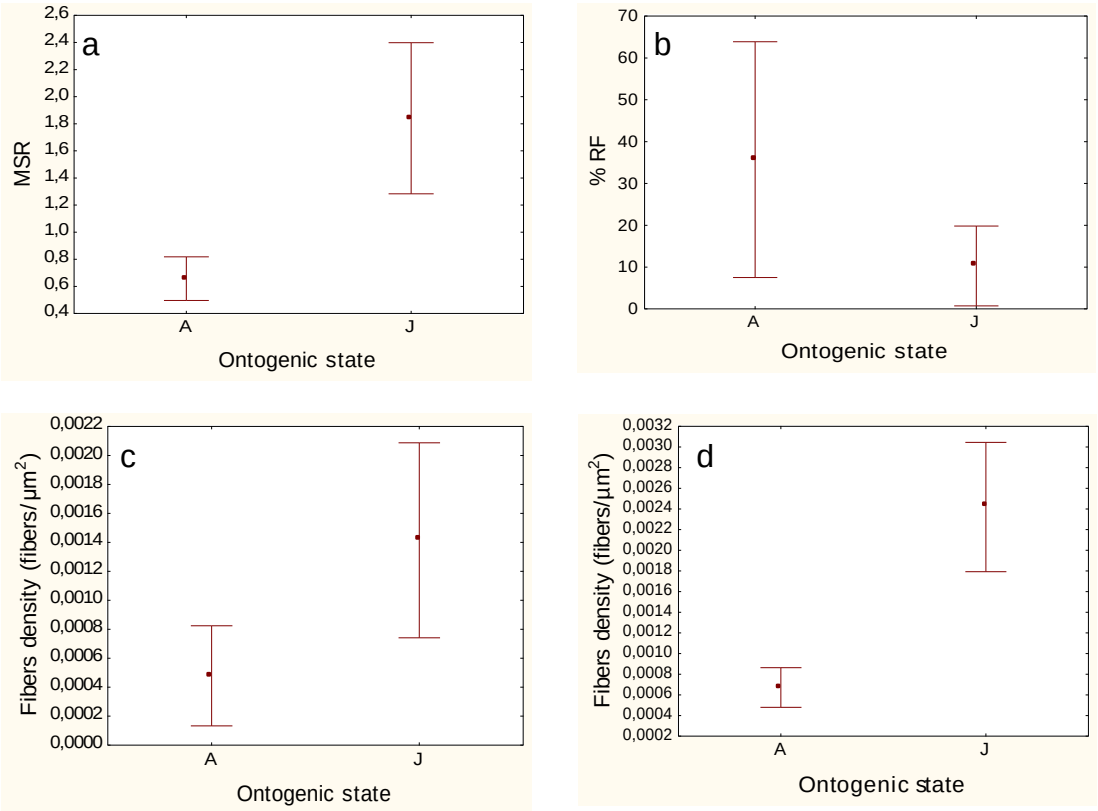



Figure 20 – Comparison of the MSR (myofibrils area/sarcoplasm area ratio) of adults (A) and juveniles (J) in summer (a). Evaluation of %RF (Remodeling fibers percentage) on the adult males (A) and juveniles (J) during summer (b). Comparison of fibers density during winter (c) and summer (d), between adults and juveniles. All differences are significant. Dots and error bars are means and 95% confidence intervals, respectively.

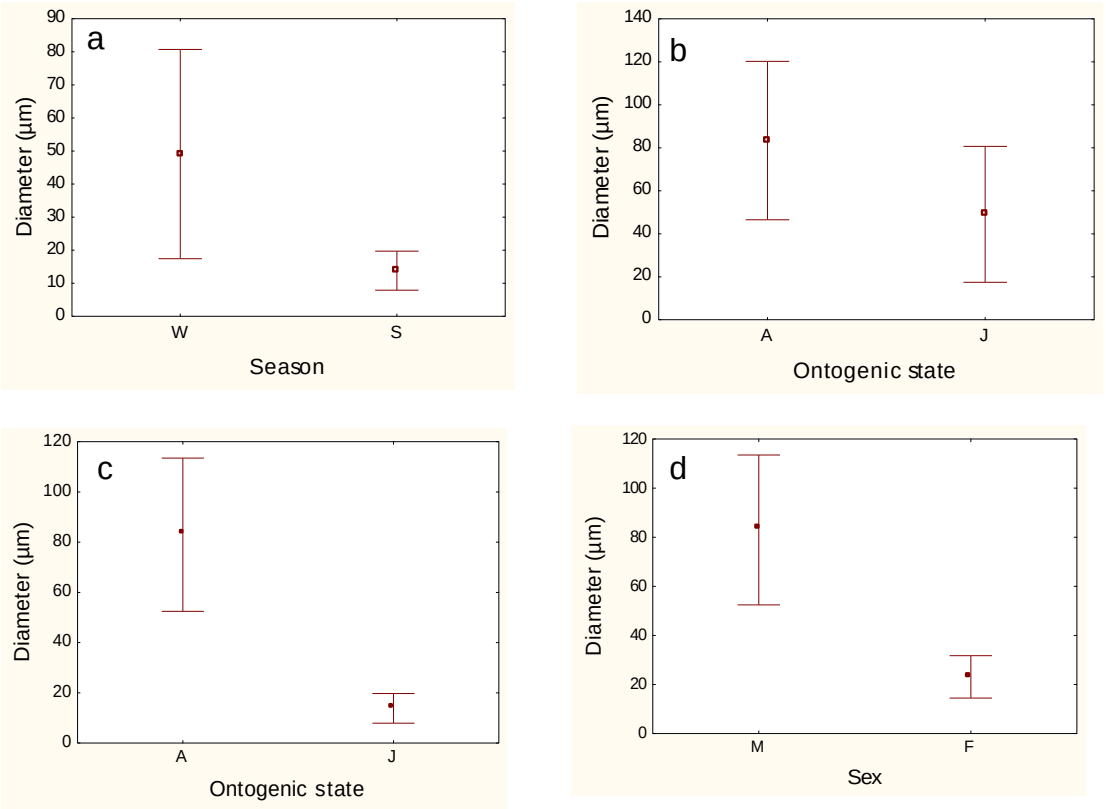



Figure 21 – Comparison of diameter in juveniles during winter (W) and summer (S) (a). Comparison of diameter in adult and juveniles during Winter (b) and summer (c). Comparison of fibers diameter between males and females (d). All differences are significant. Dots and error bars are means and 95% confidence intervals, respectively.

Summer vocal adult males (Fig. 22a) show a less accentuated SDH activity compared to both winter's adult males (Fig. 22b) and summer's silent males (Fig. 22c). Some individuals show different SDH activity in the same section of the muscle (Fig. 22a and 22d).

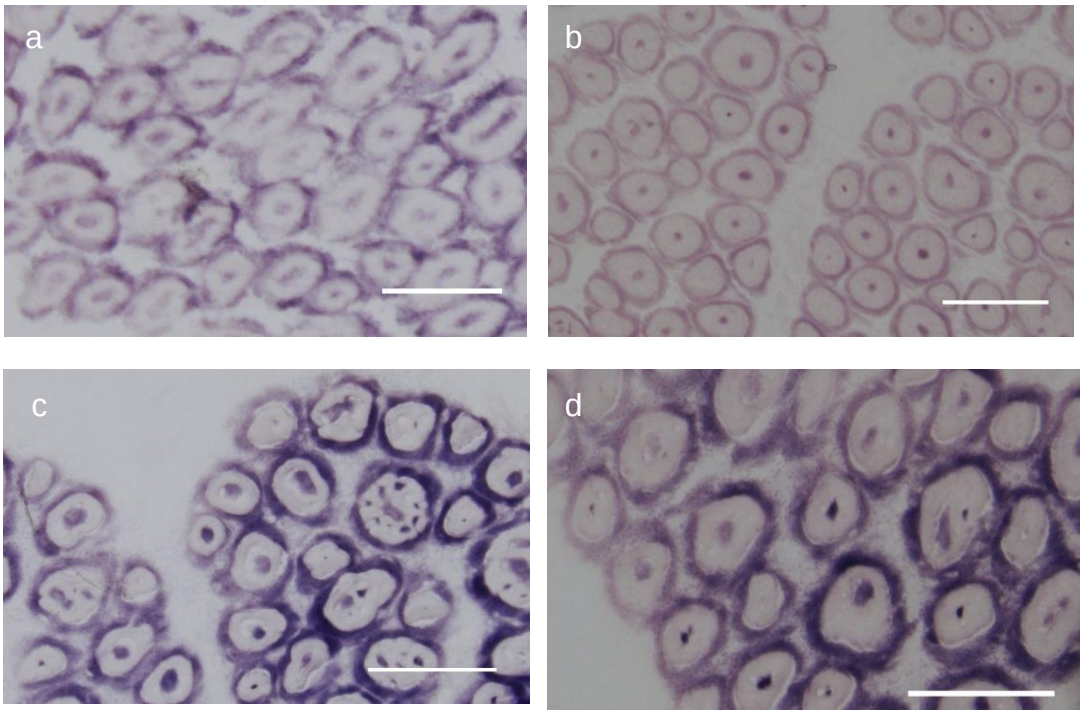



Figure 22 – SDH activity in SM sections of *H. didactylus*: a) Winter Adult male; b) Summer vocal adult male; c) Summer silent adult male; d) Fibers with different SDH activity. Scale bar: a) 60 µm; b) 100 µm; c) 160 µm; d) 100 µm.

# DISCUSSION

# 4. Discussion

The sonic muscle fibers orientation is arranged in several directions. Of all the different sections examined only the P4 (longitudinal) sections sowed mostly transversal fibbers. Despite differences in organization all fibers have a similar general morphology. The fibers were found to have a polygonal shape and a central core of sarcoplasm surrounded by myofibrils which is in accordance with the descriptions from Fawcett & Revel (1961), Fine *et al*. (1993), Loesser *et al.* (1997) and Nahirney *et al.* (2006) for other toadfish species. Unlike Modesto & Canário (2003b) we found a sarcoplasmic central core on the SM fibers in *H. didactylus*. We did use a different histological dye but both were general dyes. As Modesto & Canário (2003b), we used fibers from different locations in the muscle. However, Modesto & Canário (2003b) used a different histological methodology and this might be the one explanation for the differences morphological. Modesto & Canário (2003b) used a different fixative and the muscles were extended before sectioned which may alter the myofibrils' structure. However, in an ongoing study using different histological methods we have extended the sonic muscle of some of our subjects resulting in a morphological alteration of the sonic muscle myofibrils structure but different from the observations of Modesto & Canário (2003b). Our sections of extended sonic muscles showed only longitudinal fibers, but no transversal fibers as those found by Modesto & Canário (2003b). We also found that the central core was present in most fibers of all fascicules on juveniles but it was smaller than in adults. These results are slightly different from the ones described by Fine *et al.* (1990), which only found the central core in a few fibers.

Our stimulation experiment results show that, besides the individual contractions following the stimuli frequency (50 Hz), there is also a sustained contraction of the SM during sound production. This may be due to muscle tone (or tonus) where a different group of fibers maintain the muscle in a partially contracted state (Keeton & Gould, 1993). So we postulated that there may exist two different types of fibers, some responsible for the fast muscle contraction and others responsible for the slow and sustained muscle contraction. This has

been described in carapid fish (Parmentier *et al*., 2003) and verified once in *O. tau* by Appelt *et al.,* (1991) that found a histological sample from one swimbladder which contained two types of fibers. These authors refer the presence of a few slow fibers that could be tonic and that were probably related to sound modulation.

Rome *et al.,* 1996 stimulated the sonic muscle of a different but not specified toadfish specie at a rate of 200 Hz, which they claim to be the frequency of the boatwhistle call in that specie, as well as red slow muscle at a much slower rate of 3.5 Hz. Like us, they also measured the force/movement of both these muscles with a force transducer. They did find a sustained muscle contraction in the slow red muscle while the results from the swimbladder muscle only show fast contractions (not only at 200 Hz but also at 67 Hz, a similar frequency to ours). Although they did not explore the idea of this sustained contraction in the slow red muscle they conclude that toadfish do not need sonic muscle fibers to produce movement that is both fast and sustained at the same time due the fact that sonic muscles contract synchronously during sound production enhancing sound amplitude by this mechanism. However, since no antagonist muscular mechanism exists, the swimbladder wall must regain its initial position by only elastic forces upon muscle relaxation. It is conceivable that an increased internal pressure caused by a sustained contraction of putative slow fibers would reduce the time needed for the swimbladder wall to resume its position, therefore allowing an increased efficiency at high SM muscle contraction rates.

To confirm the presence of two fiber types, fast and slow, we tried a histochemical SDH approach. We found some fibers with different color intensity in these assays that may indicate the presence of fibers with different contraction speeds as found by Appelt *et al.,* (1991). However, we did not find structural differences between those fibers, such as shape, distribution or myofibrils length like in the study with *O. tau* (Appelt *et al.*, 1991). Summer males apparently showed less SDH activity (thus indicating the

presence of faster fibers). However, despite the difference in color between summer and winter individuals, the SM fibers of all males (winter and summer) appear to be mostly like Type IIa fibers (fast oxidative glicolytic) which is in accordance with the results of Fine *et al*. (1986) with *O. tau*. Determination of glycogen content would confirm this. Fine & Pennypacker (1988) described that energy depletion in stimulated sonic muscle is expected since all the fibers in this muscle are Type IIa, *i.e.* fibers that use glycogen as a major energy substrate (Hoyle, 1983). Whatever the mechanism responsible for this slow sustained contraction, its probable role is enhancing muscle recovery upon relaxation and possibly sound amplitude.

Mitchell *et al.* (2008) stimulated *O. tau*'s sonic muscle with 100 ms trains at 200 Hz every 4 s for 5 min. They obtained a robust sound during 2 minutes but with some decrease in amplitude towards the end of each stimulus train (100 ms). The authors refer that, after 3 minutes, sound amplitude was markedly reduced even though the action potentials were still vigorous in the EMG's. This same event happened in our experiments. Therefore, it is not surprising that the maximum sound amplitude is reached during the early boatwhistles after which the muscle starts to fatigue and consequently the sound amplitude reduces. In our study, at the $30^{th}$ evoked boatwhistle (approximately after 1.5 minutes) all toadfish sounds exhibited a very small amplitude, except for the breading (summer) adults that showed a decrease in sound amplitudes a bit later, starting at the $40^{th}$ evoked boatwhistle (approximately 2 minutes into the stimulation train). Summer males also presented higher mean and maximum sound amplitudes compared to winter males. This increased ability in sound production shown by summer males (both in sound amplitude and fatigue resistance) is in accordance with our expectations since, during the breading season males have additional vocal tasks such as the attraction of females (Vasconcelos *et al*., 2012) and defending their nests from other males (Vasconcelos *et al*., 2010b).

We must take into consideration that to access the sonic nerves the fish had to be surgically opened and the swimbladder exposed to air instead of the more

dense water (Fine *et al.*, 2001). Although this certainly affects the sound produced due to acoustic impedance changes, the comparison of sound amplitudes among fishes subjected to the same procedures shall still be valid However, one factor that we could not control for was possible gas pressure differences in the swimbladder among individuals and this might have affected the sound amplitude (Skoglung, 1961).

Modesto & Canário (2003b) showed that summer males have larger sonic muscles, resulting from larger, and possibly also increased numbers of fibers. In accordance top this, our data shows that winter males have a higher MSR than summer individuals indicating a larger sarcoplasm area in summer fish, since the myofibrils area do not present seasonal changes (data not showed). This explains why the sound production fatigue is lower in summer adults. The sarcoplasm area (area surrounding the fiber and its central core) contains mitochondria, responsible for making energy available to the muscle cells. More sarcoplasm area is a proxy for a higher number of mitochondria and, consequently, more energy availability and a higher fatigue resistance (Fine *et al*., 1977; Connaughton *et al.*, 1997). Strangely, we did not find seasonal differences in fiber density (hyperplasia, or increase in fibers quantity during the breading season) described by Loesser *et al.* (1997) in *O. tau*.

The fast component of the SBM has higher amplitude in summer and the time of contraction is smaller in adults, indicating that sonic muscle fibers contract with greater force and shorter duration. A similar observation was described in the Weakfish (*Cynoscion regalis*) sonic muscle fibers by Connaughton *et al.* (1997). We found that adult summer males present a higher rate of remodeling fibers than juveniles. Fragmented multiple cores appear to result from larger postmitotic sonic fibers fragmentation (possible division) into smaller units energetically more efficient (Fine *et al*., 1993; Loesser *et al.* 1997). This should indicate a larger amount of multiple cores in summer males probably related to vocal activity, since this is a very energy demanding action in these animals (Prestwich, 1994).

The small fibers in the summer adult male, found only once by us, appear to be related to Fine *et al.* (1993) findings. Those authors postulate that the hyperplasia recorded during the breading season is not only originated from satellite cells proliferation and differentiation (Kelly & Rubinstein, 1994). Fine *et al*. (1993) claim that sonic fibers divide and that this division is essential because mitochondria only occur on the fiber's cylinder. Furthermore, these authors advocate that fiber fragmentation, possible division, and the presence of these smaller fibers with minor diameter contractile cylinders in males are seen as adaptations for repeated rapid contraction and fatigue. That idea is also claimed by Connaughton *et al*. (1997). This fibers division hypothesis has been described by Calve *et al*., (2010) on fragmentation of myofibers that contribute to the regeneration blastema (cells capable of growth and regeneration into organs or body parts) in adult red spotted newts.

Fine *et al.* (1990) found that toadfish SM fibers increase in diameter during life. In accordance to this, our data shows that adult males have larger fibers than juveniles. Although SM fiber sizes increased with fish size, small fibers were still present in adults. The same authors showed that the toadfish in *O. tau* presents an increased number of SM fibers during the breading season which shall assist the large increase in vocal activity. We have also found higher density of fibers in Lusitanian toadfish vocal males compared to silent ones. This may explain the superior vocal activity recorded in the natural environment.

The SDH processing on SM fiber sections of both winter and silent summer adults show higher enzyme activity when compared to vocal summer adult males. These differences seem to indicate that winter and silent summer males may have slower SM fibers. However, we couldn't link this observation with any physiological results (sound amplitude, fatigue or SBM) due to the low number of vocal and silent summer fishes. Besides the small histological differences observed in vocal fish (compared to silent and winter males), these individuals seem to be more motivated to perform (at least in a natural environment). This suggests that hormonal variations may play a significant role in sound production ability.

According to the Von Bertalanffy growth model for the reproductive specimens of *H. didactylus* in the Tagus estuary created by Pereira *et al.* (2011), the juveniles that we used were one to two years old and their gonads were at stage I, corresponding to an immature condition based on Costa (2004) maturation scale. In other words, those fish were not sexually active. However, they showed similar mean amplitude sound to adult males, both in the winter and in the summer. This suggests that juvenile summer males had better sound amplitude performance that winter adult males. We did not expect such result because the juveniles have sonic muscles much smaller them the adults and, like winter males, non-reproductive juveniles do not vocalize as to attract females, although they interact acoustically with other toadfish. Ours results suggest that juveniles may undergo through some early morphological or hormonal seasonal adaptation that allows them to vocalize at high rates, as much as adults during the reproductive season. The slow component of the SBM shows similar results to de sound amplitude values. Likewise, we did not obtain any ontogenic differences which corroborate Fine *et al.* (2009) previous assumptions about lack of differences on sound amplitude between different sized fish.

On the other hand, the histological results point to another direction. Juveniles did not have the morphological features that supposedly allow for an extraordinary vocal performance. The MSR was higher in juveniles than adults which indicate less sarcoplasm in the fiber than those of adults. This suggests that morphological and structural changes are not enough to explain seasonal differences in vocal performance and that some hormonal alterations may be at play both in adults and juveniles during this period. Juveniles might have some seasonal changes that allow them have similar performance to adults, however they don’t show changes in others structures like gonads due to energy costs associated.

We have used some females as control in histological assays. During breading season only males must increase vocal activity. We have hypothesized that females would show sonic muscle morphology similar to winter males. We have found that adult males have larger fibers than females, an observation also

made by Loesser *et al.* (1997). In contrast, Fine *et al.* (1990) found that in *O. tau* female sonic muscles are composed by larger fibers. We only observed one female and so our observations need to be extended.

Modesto & Canário (2003a) found that females did not change the SM mass along the year. Our results corroborate this statement. Indeed we did not find seasonal variation in the myofibrils/sarcoplasm ratio and we assume that this is possibly related to no changes in the females vocal activity during the year (Brantley & Bass, 1994). It is therefore normal that females exhibit, during the breading season, higher MSR than males, since females do not experience SM hypertrophy during the breeding season (Modesto & Canário, 2003b).

# CONCLUSION

## 5. Conclusion

Summer males present better sound production performance than winter individuals, exhibiting increased sound amplitude, muscle contraction velocity (as evaluated from EMGs) and amplitude of swimbladder displacement. This is likely due to the need of increasing vocal activity in this period.

Using electrical stimulation to induce SM muscle contractions that produced artificial boatwhistles we demonstrated that duration of vocalizations activity is limited by fatigue. Juvenile males have shown higher vocal fatigue that adults, but exhibited similar swimbladder movement compared to adults from the same season. We found that during the production of boatwhistles the sonic muscle contraction of *H. didactylus* presents two components: a slow sustained component that likely increases the overall gas pressure in the swimbladder, and fast contractions that generate the sound pulses. We suggest that these different contraction modes can be related to different muscle fiber types. At least rapid contraction and fatigue resistant fibers of type IIa seem to be present. It remains to determine if another slow contraction muscle fiber type exists in the sonic muscles of this species. Therefore this issue deserves further investigation and we have now refined the tools to tackle this question.

Histological sections of the sonic muscles showed fibers arranged in several orientations. Breeding males have bigger fibers and more sarcoplasm than winter individuals, females and juveniles, allowing increased vocal activity during the breeding season. The presence of fibers in a remodeling state and possible fiber division (hyperplasia) in adults is reported for the first time in this specie. The SDH assays proved to be a useful tool to determine the oxidative status of muscle fibers but this approach needs to be complemented with other histochemical analyses.

# FUTURE PERSPECTIVES

## 6. Future Perspectives

Our experiments should be extended to increase the sample size and thus allowing for more robust results. We should also add to the analysis breeding toadfish males studied immediately after capture, since we only used animals that were confined in a nest during two weeks for monitoring acoustic activity.

We should also extend the SDH assays to more sections of the same sonic muscle in order to understand the distribution of fiber types within the muscle (Rome *et al*., 1996).

Further research may allow detection of different types of sonic muscle fibers responsible for the slow and fast components observed in this muscle contraction. To confirm this we will have to expand the histochemical methods beyond the succinate dehydrogenase (SDH) assays. Other methods can include the detection of ATPases and enzymes catalyzing oxidative reactions (*e.g.* nicotinamide adenine dinucleotide dehydrogenase - NADH and lactate dehydrogenase - LDH) (Chen *et al*., 1998). In addition, since faster fibers are described as having a fast myosin and a greater concentration of parvalbumin when compared to slow twitch fibers, the quantification of this protein could be a suitable method to identify the two fiber types (Rome *et al*., 1996). Other approaches such as the use of specific antibodies can be helpful to answer this question.

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