

## Vocal Sac of *Rana ridibunda*: A Morphological and Immunohistochemical Study

Sengezer-Inceli M. \*, Sancar-Baş S. Kaptan E.

Istanbul University, Faculty of Science, Department of Biology, Section of Zoology, Vezneciler 34134 Istanbul

### Abstract

The goal of this study is to investigate the structure of the vocal sac in males of *Rana ridibunda* and to detect the presence of prolactin receptor immunohistochemically under light microscopy in its components. The vocal sac of *Rana ridibunda* were bilaterally localized on each side of the mouth and contained two different layers, namely an internal and an external sheath. External and internal sheaths were formed with multilayered squamous keratinized epithelium and single layered columnar epithelium with rarely ciliated cells, respectively. The connective tissue under the epithelium in the internal sheath possessed predominantly skeletal muscle fibers. Furthermore, the prolactin receptor immunoreactivity was observed in the uppermost cell layer of the epidermis in the external sheath. This paper is one of the first studies for the purpose of displaying the morphological aspects of the bilaterally located vocal sac and its hormonal interaction. In our opinion, this study also contributes to the field of amphibian reproductive biology.

**Key words:** *Rana ridibunda*, vocal sac, prolactin receptor

**\*Corresponding author:** Meliha Sengezer-Inceli (E-mail: melis@istanbul.edu.tr)

### Introduction

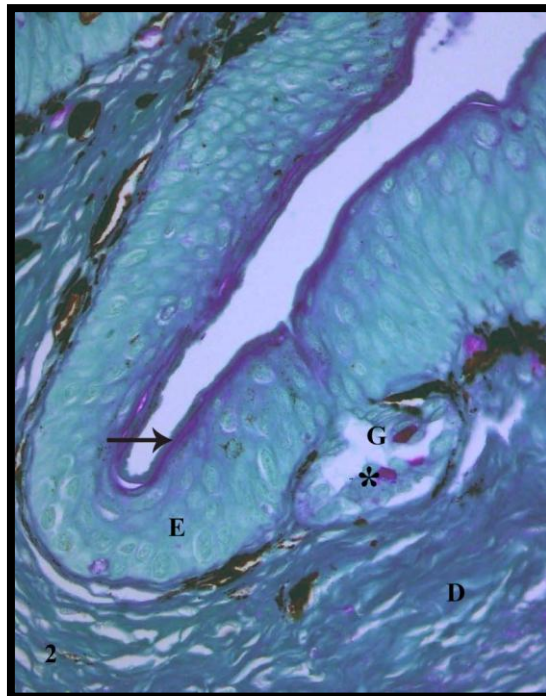
Sound production by animals is primarily a method of advertising the presence of one individual to others of the same species. Among vertebrates, vocalization is highly developed in anurans and birds (Demirsoy 1993). In many terrestrial species, a chorus of simultaneously calling males attracts females to a breeding area; reproductive females then choose and locate one male, using distinctive acoustic cues. Anuran acoustic signaling systems are thus subject to the strong pressures of sexual selection (Kelley, 2004).

Especially male individuals of amphibians breathing with the lung have the capacity of sound production. Anuran calling is produced by their laryngeal apparatus. However, the vocal sac mediates the calling process as an expandable pouch that performs as a sound resonator and radiator. Generally, terrestrial anurans have a supralaryngeal type vocal sac

but aquatic anurans have a bilateral type vocal sac (Demirsoy 1993). The position of the single vocal sac in *Physalaemus pustulosus* is median and subgular. The vocal sac is external and exhibits extensive dermal modification. A pair of elongated vocal slits connects the vocal sac and buccal cavity. Histological analysis of the vocal sac and body wall in the leptodactylid frog *Physalaemus pustulosus* suggests that both muscle and elastic fibers are important in call production (Jaramillo et al., 1997). The morphological and physiological aspects of the supralaryngeal type vocal sac has been described in several species (Duellman and Trueb, 1986) but have not been completely elucidated. Nevertheless, no detailed information is available on the acoustic and morphological properties of the bilateral type of vocal sac.



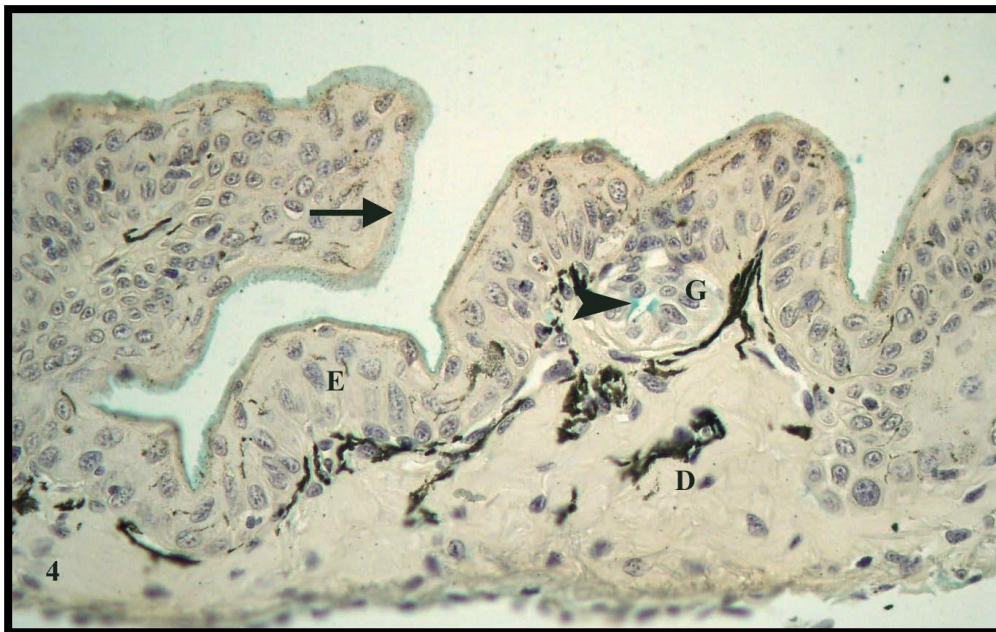
**Figure 1:** External sheath of vocal sac. HE X 400. E: Epidermis, D: Dermis, G: Gland, →Pigment Cells.



**Figure 2:** External sheath of vocal sac. PAS-fast green X 400. E: Epidermis, D: Dermis, G: Gland, \*PAS- positive material in the gland, → PAS-positive reaction in the keratin layer.



**Figure 3:** External sheath of vocal sac. Aldehyde fuchsin (AF) X 400. E: Epidermis, D: Dermis, G: Gland, → AF – positive reaction in the keratin, ▶ AF-positive reaction in the elastic fibers.



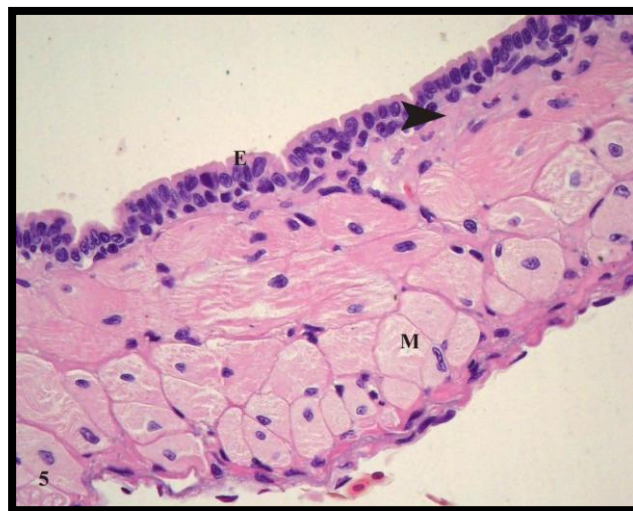
**Figure 4:** External sheath of vocal sac. Alcian blue (AB) X 400. E: Epidermis, D: Dermis, G: Gland, → Weakly AB- positive reaction in the keratin, ▶ AB-positive reaction in the gland.

*Rana ridibunda* is an aquatic anuran species. Male individuals of this species have a bilaterally located vocal sac opening at the corner of mouth. Generally in aquatic species of anuran, the presence of a thumb pad, found on

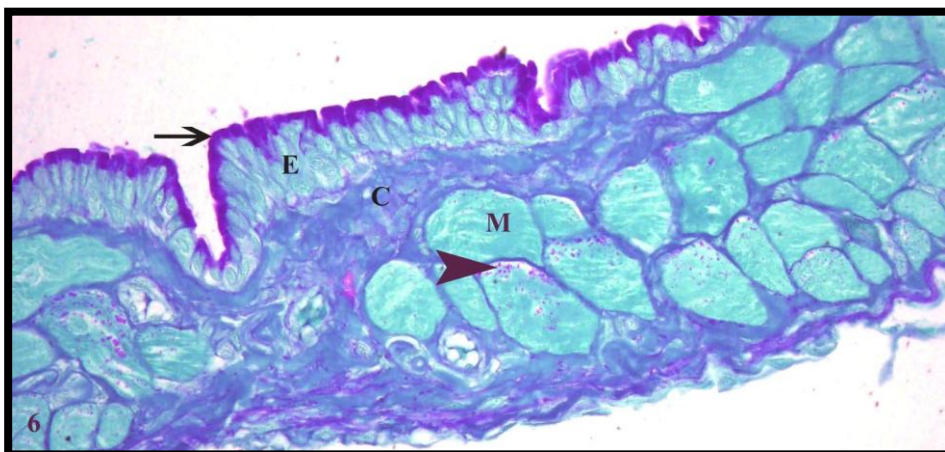
first finger and a coarse muscular forearm is also one of the characteristic properties of the male individuals (Demirsoy 1996). Vocal sac, thumb pad, and skin are described as secondary sex characteristics (D'Istria et al.; 1982, Kao et

al., 1994). These structures exhibit morphological and physiological changes during an annual reproductive cycle. The cyclic changes correlate with plasma androgen levels (Lofts et al., 1972; Kao et al., 1994; Emerson et al., 1999). Researchers have indicated that prolactin is one of the hormones affecting secondary sex characteristics of urodel amphibians such as cloacal glands and caudal fin (D'Istria et al., 1984; Kikuyama et al., 1986). Several studies showed that there is a synergy between prolactin and androgen hormones on the secondary sex characteristics

(Norris et al., 1989; Iwata et al., 2000a; Matsumoto et al. 1997). It has been shown that the development of the vocal sac is dependent on plasma androgen levels in the male Chinese bullfrog, *Rana rugulosa* Wiegmann (Kao et al., 1994). Prolactin participates in the processes of regulating growth, metamorphosis, osmoregulation and reproduction in amphibians (Iwata et al., 2000b; Mosconi et al., 2002). However, it has not been established whether there is such a synergistic effect of prolactin in the anuran species.



**Figure 5:** Internal sheath of vocal sac. HE X 400. E: Epithelium, ▶ Connective tissue, M: Muscle fibers.



**Figure 6:** Internal sheath of vocal sac. PAS- fast green X 400. E: Epithelium, C: Connective tissue, M: Muscle fibers. → Strongly PAS-positive reaction in the apical cytoplasm of epithelial cells, ▶ Glycogen granules.

Numerous studies carried out on various amphibian species have been published to show the relationship between secondary sex characteristics and hormones. However, this relationship has not been completely evaluated in *Rana ridibunda*. Up to now, no reports have been found on the histological features of the vocal sac related to this species. In our study, the histological structure of the vocal sac was described in detail at the light microscopic level and the functional relationship between vocal sac and prolactin were investigated.

### Materials and methods

In this study, five adult male frogs (*Rana ridibunda*) (Amphibia: Anura: Ranidae) were used. Animals were collected in February from Thrace. The internal and external sheaths of vocal sacs were taken and fixed in Bouin's fluid for 24h for morphological studies. Vocal sacs were also fixed overnight in 10% neutral buffered formalin for immunohistochemical staining. After dehydration, samples were embedded in paraffin and 4 µm thick sections were prepared. Thereafter, the sections were stained with haematoxylin-eosin, periodic acid-Schiff (PAS), aldehyde fuchsin and alcian blue (pH 2.5).

Prolactin receptor (PRLR) was detected in the sections using an immunohistochemical staining method called streptavidin-biotin complex technique. The following steps indicate the staining procedure of the sections:

1-Deparaffinization

2- Rehydration

3-Blocking of endogenous peroxidase activity; Hydrogen Peroxide Block (Diagnostic BioSystems C825) (at room temperature for 20 min.)

4-Incubation in mouse monoclonal anti-prolactin Ab-1 receptor (Neomarkers MS-1338-R7) primary antiserum (at 4 °C for 3 days)

5-Incubation in anti-mouse biotinylated secondary antiserum (Diagnostic BioSystems C723) (at 35 °C for 20 min)

6-Incubation in streptavidin-biotin peroxidase complex (Diagnostic Biosystems C728) ( at 35 °C, for 20 min)

7-Incubation in chromogen substrate solution (Zymed, 85-6143) (at room temperature for 15 min)

During this procedure, sections were washed with a phosphate buffer (0.01 M, pH: 7.2) three times after each step.

Immunoreactivity in place of primer antibody was controlled by phosphate buffer (0.01 M, pH: 7.2) and also, human breast carcinoma was used as a positive control tissue.

### Results

#### *Histological results*

The vocal sac of *Rana ridibunda* was composed of two sheaths: the internal sheath and the external sheath. The outer sheath, in contact with the environment, consisted of two layers being epidermis and dermis. The epidermis was composed of multilayered squamous keratinized epithelium being formed by 4-5 cell layers (Fig. 1). The keratin layer gave positive reactions with PAS (Fig. 2) and aldehyde fuchsin (Fig. 3), whereas Alcian blue gave a weak positive reaction (Fig. 4). The thickness of the dermis was proportional to the thickness of the epidermis. In this layer, various types of connective tissue cells, pigment cells, blood vessels, and nerves were present (Fig. 1). However, multicellular alveolar glands were rarely present. The cytoplasm of some cells of these glands gave PAS and Alcian blue positive reactions (Fig. 4). In the sections stained with aldehyde fuchsin, there was a purple band formed by elastic fibers in the outer zone of the dermis (Fig. 3).

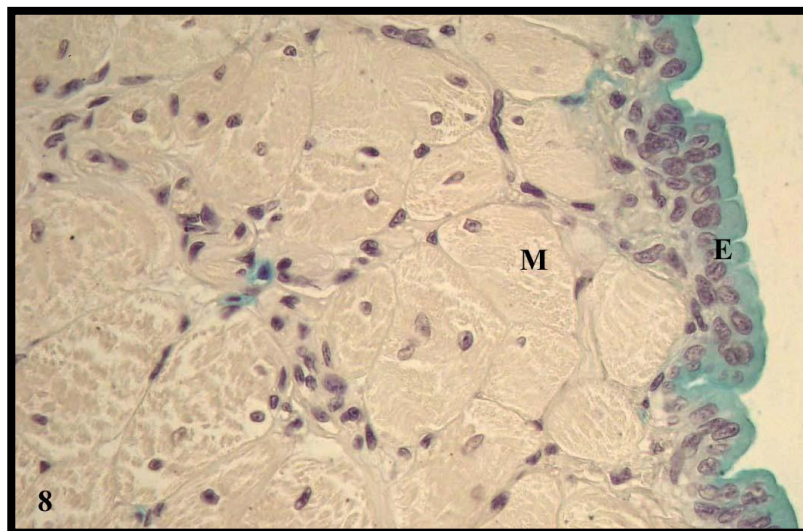
The internal sheath of the vocal sac facing the oral cavity was composed of single layered columnar epithelium with rarely ciliated cells (Fig. 5). The epithelial layer contained many folds (Figs. 6, 7). The apical cytoplasm of epithelial cells gave strong PAS-positive (Fig. 6) and weak aldehyde fuchsin (Fig. 7) and Alcian blue (Fig. 8) positive reactions. A connective tissue was present in the underside of the epithelium. The transverse and longitudinal

sections of the skeletal muscle fibers splaying in different directions were seen in the following connective tissue (Figs. 5, 6). The glycogen granules gave PAS-positive reaction

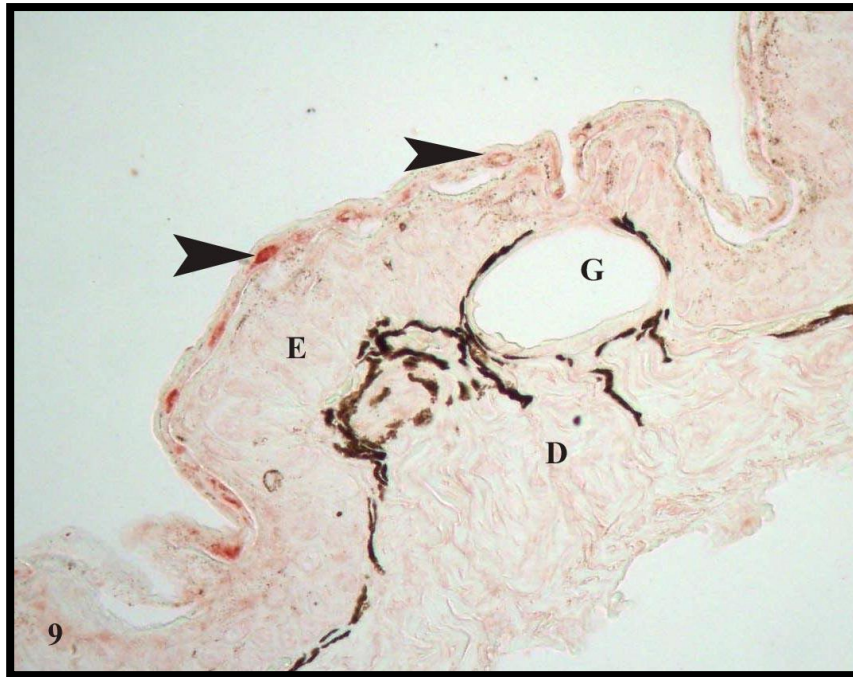
in the muscle fibers (Fig. 6). In the sections stained with aldehyde fuchsin, the elastic fibers gave a positive reaction among the muscle fibers in the connective tissue (Fig. 7).



**Figure 7:** Internal sheath of vocal sac. Aldehyde fuchsin (AF) X 400. E: Epithelium, M: Muscle fibers. ▶ AF-positive reaction in the elastic fibers, → weakly AF- positive reactions in the apical cytoplasm of epithelial cells.



**Figure 8:** Weakly AB- positive reactions in the apical cytoplasm of internal sheath epithelial cells. Alcian blue (AB) X 400. E: Epithelium, M: Muscle fibers.



**Figure 9:** PRLR positive reaction in external sheath of vocal sac ( ▶ ) X 400. E: Epidermis, D: Dermis, G: Gland.



**Figure 10:** PRLR positive reaction in external sheath of vocal sac ( ▶ ) X 1000.

### **Immunohistochemical results**

The cytoplasm of the squamous cells in the uppermost layer of the epidermis of the external sheath in the vocal sac gave PRLR positive reaction (Figs. 9, 10). The other layers of cells of the epidermis, dermis, the glands in the dermis and internal sheath were PRLR negative (Figs. 9, 10).

### **Discussion**

Vocalization is one of the important aspects of the social behavior in anurans. Most male amphibians produce species-specific mating calls and this attracts only con-specific mates (Girgenrath and Marsh, 1997). Frog vocalizations, except for species that vocalize underwater, are powered by respiratory airflow. Pulmonary air is compressed by the trunk muscles and forced from the lungs through the larynx into the oral cavity and vocal sac. As air passes through the larynx it generates sound by causing oscillation of the vocal cords, which are located upstream from the glottis. In some frogs, sound may also be produced by forcing air in the vocal sac back through the larynx into the lungs. Four pairs of laryngeal muscles control the glottal aperture as well as the position and tension of the vocal cords. These are composed of elastic tissue and lack intrinsic muscles. In ranid frogs, partial or total surgical removal of the vocal cords abolishes or substantially modifies the vocalizations (Suthers et al., 2006).

There is almost a perfect association between the presence of a vocal sac and vocalization. This suggests that the vocal sac is an important, if not essential, structure for normal anuran vocalization. However, Duellman and Trueb (1986) state: “a few male frogs that vocalize do not have vocal sacs” (Hedges, 1987).

The vocal sacs which are special resonance organs are functional to strengthen the produced vibrations. These structures are sac-shaped and blown up like a balloon when they are filled with air. Otherwise, they shrink in their slits. Both sheaths of the vocal sacs of *Rana ridibunda* include elements contributing to the

expansion and shrinkage movements. The external sheath of the vocal sac contacts with the environment and is accepted as an extension of the skin. A layer formed by elastic fibers is present in the dermis of this sheath. The internal sheath being an extension of the oral mucosa is composed of epithelial and muscle layers. According to our histochemical results, the apical cytoplasm of epithelial cells contains mainly neutral glycoconjugates and little sulphated sialomucins (Bancroft and Stevens, 1982). The elastic fibers are present among the muscle bundle in this layer. The muscle and elastic fibres present in both layers are the supporting structures of the changes in the shape which occur dependent on the function of the vocal sac.

Small mucous glands that are also found in the skin (Sengezer-Inceli et al., 2004) were observed rarely in the external sheath. Only, some cells of these glands gave PAS and Alcian blue-positive reactions. The large mucous glands were present in the skin and all cells of these glands gave PAS-positive reaction. However, these glands were not present in the external sheath of the vocal sac. According to our histochemical staining results, secretion of the small mucous glands includes neutral and acidic glycoconjugate (Bancroft and Stevens, 1982). Because of their contact with the environment, secretion of these glands in the dermis of vocal sacs may have a moisturizing function even when they are located in the slits.

We observed the PRLR-positive reaction only in the uppermost cells of the epidermis in the external sheath. This sheath is an extension of skin. PRLR was detected in amphibian skin by several researchers (Goldenberg and Warburg, 1983; Brown, 1988; Takada, 2005) and was reported to be effective on osmoregulation. PRL in urodeles induces changes in skin texture and skin secretion. Thyroid hormone also helps in these changes induced by prolactin to some extent (Dent et al., 1973). In another study, it was reported that in the red spotted newt, *Notophthalmus viridescens*, PRL increased levels of sialic acid and n-acetylglucosamine in the stratum



corneum. In contrast, glucoconjugates containing fucose, galactose, n-acetyl-galactosamine and galactose-(1,3)-n-acetyl-galactosamine were decreased by PRL within both the glands and epidermis of the skin (Singhas and Ward, 1993). It has been reported that testosterone and prolactin have synergistic effects on secondary sex characteristics such as urodel cloacal glands, tail fin and nuptial pads (Kikuyama et al., 1975; Singhas and Dent, 1975) The larynx of *Xenopus laevis* is a sexually differentiated vocal organ. Androgen-induced changes in the contractile properties of laryngeal muscle are inhibited in prolactin-deprived animals. This prevents the rapid rates of muscle contraction required for males to produce courtship songs (Edwards et al., 1999). In the study of d'Istia et al (1984), the interactions between thyroid hormones and receptors for steroid hormones and prolactin in dorsal skin and caudal fin of *Triturus cristatus carnifex* were investigated. The results indicate that in *Triturus cristatus carnifex* the thyroid induces an increase of androgen receptors and a decrease, that is removed by thyroidectomy, of prolactin receptors.

The mentioned studies show the relations between secondary sex characteristics and hormones such as PRL, androgens and thyroxin. The same functions may be acceptable for the vocal sac. But further studies are needed on this topic. This paper is one of the first studies for the purpose of displaying morphological aspects of bilaterally located vocal sac and its hormonal interaction. In our opinion, this study also contributes to the field of amphibian reproductive biology.

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