Chapter 2
Comparative Neurobiology of Sound Production in Fishes

Andrew H. Bass, Boris P. Chagnaud and Ni Y. Feng

Abstract While studies of sonic mechanisms among vertebrates date back more than 100 years, the past 25 years have witnessed a burgeoning of interest in the bioacoustics and behavioral ecology of sound production among fishes, including in its neural basis. Here, we review the body of comparative literature on the neuroanatomical, neurophysiological, and neuroendocrine mechanisms of sound production/vocalization among fishes. Most studies have focused on teleosts, the most species-rich group of living vertebrates. The past decade alone has witnessed the demonstration of (1) an extensively connected vocal-acoustic network at forebrain, midbrain, and hindbrain levels, (2) distinct neural populations comprising a central pattern generator (CPG) that directly determines the physical attributes of social context-dependent sounds including pulse repetition rate (PRR), fundamental frequency, duration, and amplitude, (3) a highly conserved pattern for the evolutionary developmental origin of vocal CPGs between fishes and tetrapods, (4) shared origins between vocal CPGs that are dedicated to sound production and pectoral appendage motor systems that function in both acoustic signaling and locomotion, and (5) forebrain, midbrain, and hindbrain targets in the vocal-acoustic network for the modulatory actions of steroid hormones and neuropeptides. In sum, the relative simplicity of acoustic signaling in fishes and of the underlying neural circuitry offer both insights into the evolutionary history of sound producing mechanisms among all vertebrates, and practical advantages for investigating adaptations at the cellular and network levels of neural organization that sculpt behavioral phenotypes.

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Abbreviations in Text

11-KT	11-ketotestosterone
AH	Anterior hypothalamus
AR	Androgen receptor
AT	Anterior tuberal hypothalamus
AVT	Arginine vasotocin
CPG	Central pattern generator
ER	Estrogen receptor
Ir	Immunoreactivity
IT	Isotocin
PAG	Midbrain periaqueductal gray
PL	Midbrain paralemniscal tegmentum
POA	Preoptic area
POA-AH	POA Anterior hypothalamus
PRR	Pulse repetition rate
qPCR	Quantitative PCR
VIP	Vasoactive intestinal polypeptide
VMN	Vocal motor nucleus
VPN	Vocal pacemaker nucleus
VPP	Vocal pre-pacemaker nucleus

2.1 Introduction

Acoustic communication is a prominent feature of vertebrate social behavior. Fish and Mowbray's (1970) remarkable volume, *Sounds of Western North Atlantic Fishes*, revealed an astonishingly widespread occurrence of sound production among fishes, and especially among teleost fishes that are the largest group of living vertebrates (Nelson 2006). By way of both spectrograms and oscillograms, Fish and Mowbray documented broad species diversity in spectral and temporal characters, including duration, pulse repetition rate (PRR) and harmonic structure. At about the same time, neurophysiologists were beginning to record from motoneurons in the central nervous system and peripheral nerve roots that activate sound producing (sonic, vocal) muscles (Packard 1960; Skoglund 1961; Pappas and Bennett 1966; Cohen and Winn 1967), while brain stimulation studies began to reveal vocal initiation sites distributed in forebrain, midbrain, and hindbrain regions (Demski and Gerald 1972). Over 40 years later, studies of fishes now draw attention to shared attributes and evolutionary developmental origins of vocal-acoustic behaviors and neural networks across all of the major lineages of vocal vertebrates (Bass and McKibben 2003; Bass et al. 2008; Bass and Chagnaud 2012). Why study the neural basis of sound production in fish? We hope to answer this question in this review, while providing an overview of the current status of research on the neuroanatomy and neurophysiology of central vocal networks in fish.

2.2 Why Study Fish?

Fish provide ideal model systems for investigating the fundamental neural mechanisms of sound production among vertebrates for several reasons. (1) The simplicity of their pulsatile vocalizations that vary within and between species makes them especially amenable to deciphering the neural codes that determine their physical attributes (Fig. 2.1). (2) Sound production typically depends on the actions of a single pair of vocal/sonic muscles whose activity is controlled by a single hindbrain central pattern generator (CPG) (Fig. 2.2). (3) Discrete CPG populations control equally discrete acoustic characters—duration, PRR, and amplitude (Fig. 2.2). (4) The vocal CPG of fishes shares evolutionary developmental origins with the hindbrain vocal CPGs of tetrapods. In sum, the simplicity of vocal mechanisms in fishes leads to a simple translation between neural, mechanical, and behavioral events that inform our understanding of fundamental mechanisms leading to the more complex vocal CPGs and behaviors of tetrapods.

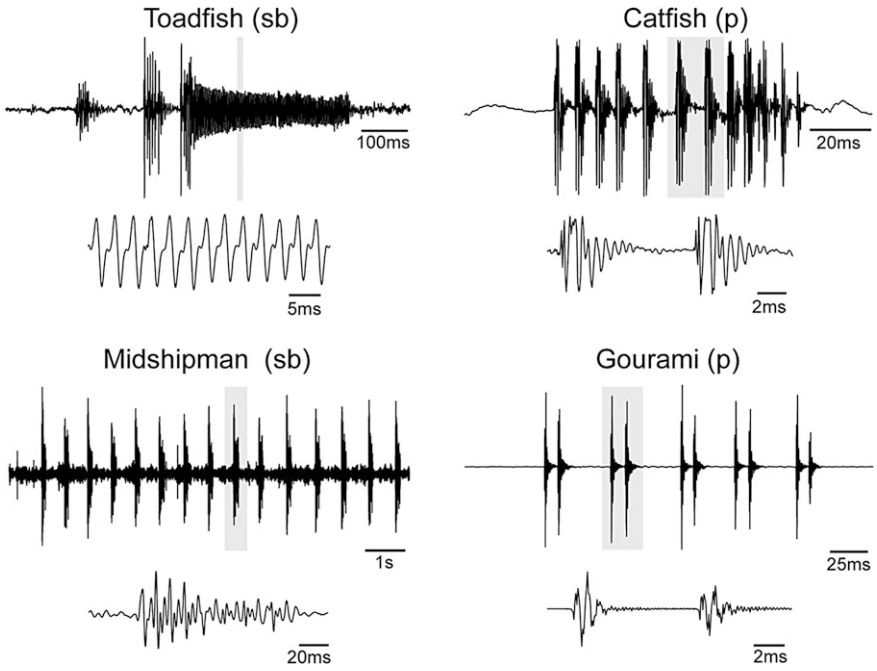


Fig. 2.1 Vocal-acoustic signals in teleost fish. Shown here are representative examples of the pulsatile-like sounds generated by fishes using either vocal swim bladder (*sb*) or pectoral (*p*)-dependent mechanisms. Signals are shown on two time scales for the boatwhistle of the Gulf toadfish (*Opsanus beta*), grunt traingrunt train of the plainfin midshipman (*Porichthys notatus*), grunt of the Raphael catfish (*Platydoras armatulus*) and croak of the croaking gourami (*Trichopsis vittata*). Catfish and gourami records courtesy of F. Ladich

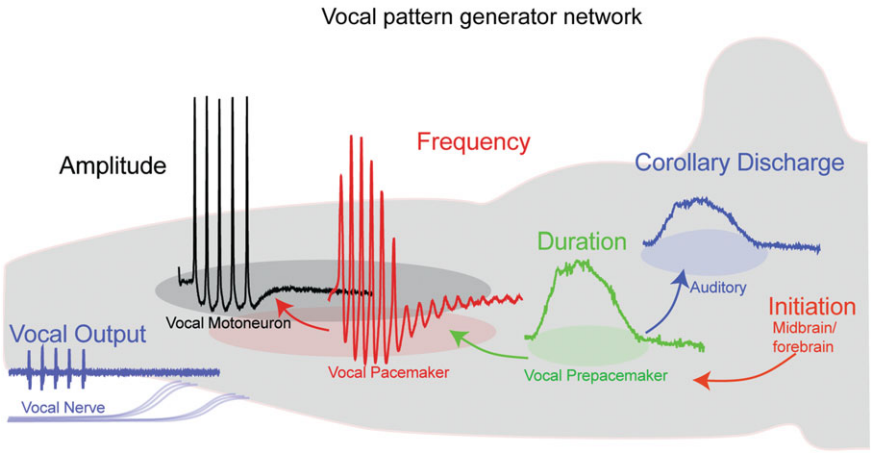


Fig. 2.2 Connectivity and temporal code of hindbrain vocal central pattern generator (CPG). The vocal CPG, depicted here for plainfin midshipman fish (*Porichthys notatus*), is activated by descending input from vocal midbrain regions. The CPG comprises three populations, superimposed here on a background sagittal image of the caudal hindbrain: vocal pre-pacemaker, vocal pacemaker, and vocal motor neurons. Representative intracellular records are shown for each population. The final output of the vocal CPG is carried by vocal nerve activity. The vocal pre-pacemaker nucleus also projects to central auditory nuclei, providing a corollary discharge that informs the auditory system about the duration of natural vocalizations. Modified after (Chagnaud et al. 2011)

The acoustic repertoire of fishes has long attracted the attention of neurobiologists. More than 50 years ago, Packard (1960) demonstrated that individual sound pulses are matched 1:1 with individual nerve spikes that reflect synchronous motoneuron activity (e.g., Chagnaud et al. 2012). Skoglund (1961) and Cohen and Winn (1967) showed for the oyster toadfish *Opsanus tau* and the plainfin Midshipman *Porichthys notatus*, respectively, that simultaneous contraction of a pair of muscles attached to the walls of the Swim bladder leads to the production of one sound pulse, with repetitive muscle contractions directly determining the sound's PRR and total Duration. Cohen and Winn (1967) also drew attention to the hearing abilities of midshipman by providing an audiogram and showing that the saccule was the main organ of hearing. These studies, along with Pappas and Bennett's (1966) description of "swim bladder motoneurons" in the oyster toadfish, set the stage for using fishes as model systems for investigations into the neural mechanisms of vertebrate acoustic communication.

2.3 Vocal-Sonic Neuroanatomy

As part of a broader survey of gap junction-mediated electrical coupling in the nervous system, Pappas and Bennett (1966) presented both neurophysiological and ultrastructural evidence for electrical coupling at the level of motoneurons

innervating the sonic swim bladder muscles in the oyster toadfish. It was, however, not until the 1980s that the first comprehensive descriptions of the vocal premotor–motor circuit were published, in this case for the closely related plainfin midshipmanmidshipman fish (Bass 1985; Brantley and Bass 1988; Bass and Marchaterre 1989; Bass and Baker 1990). Since that time, our knowledge of patterns of organization of a vocal or sonic motor nucleus, and in some cases pre-motoneurons, has greatly expanded beyond toadfishes as discussed in the next section.

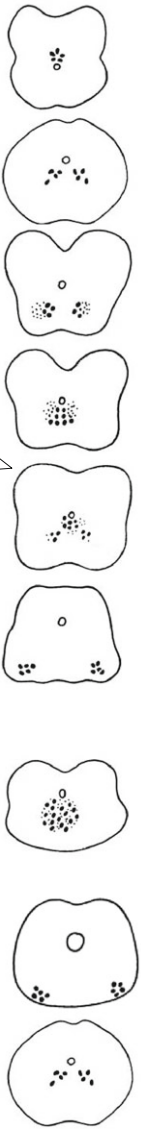
The sounds of fishes are now often referred to as vocalizations and the central network controlling sound production as a vocal circuit to draw attention to their evolutionary relationship to vocal behaviors and neural mechanisms in tetrapods. As when first proposed (Bass et al. 1994), this comparison depends on several key findings. First, the vocalizations of fishes function in social contexts comparable to those of tetrapods, namely mate attraction and aggression. Second, sonic muscles attached to the swim bladder, like sonic muscles of the syrinx and larynx, are dedicated to one behavioral function, sound production. Third, sonic muscles attached to the swim bladder, larynx, and syrinx share developmental origins from occipital somites (Tracy 1959, 1961; Huang et al. 1999; Noden and Francis-West 2006). Fourth, in a range of species, the sonic muscles are innervated by occipital nerve roots, considered homologs of hypoglossal nerve roots that innervate the avian syrinx and parts of the tongue that direct movements for speech in humans (Tavolga 1962; Vicario and Nottebohm 1988; Lieberman 2002; Bass et al. 2008; Ma et al. 2010; Laine and Bailey 2011). As reviewed below, the essential developmental evidence across vertebrate lineages to support comparisons of a hindbrain CPG for patterning the activity of sonic muscles (larynx, syrinx, swim bladder) first became available only during the past few years (Bass et al. 2008). This led to the renaming of the sonic motor nucleus that innervates swim bladder muscles as the vocal motor nucleus, or VMN (Bass et al. 2008), a nomenclature that is followed here and has recently been adopted by studies of the amphibian laryngeal system (Hall et al. 2013).

2.3.1 Vocal Nerves and Vocal Motor Nucleus

Cranial (occipital) and/or rostral spinal nerve roots innervating sound producing muscle dedicated to this one function have been identified in 12 orders, 20 families and 30 species of teleost fish (reviewed in Ladich and Bass 2005; Onuki and Somiya 2007; Boyle et al. 2013). Innervation varies from occipital only or spinal only to a combination of both. A VMN dedicated to sonic functions has been experimentally demonstrated in 22 species following the labeling of one or more vocal nerve roots with either horseradish peroxidase or a biotin derivative (neurobiotin, biocytin, dextran-biotin) (Table 2.1). Intracellular recording and staining further verifies the position of VMN in three species of Batrachoidiformes (plainfin midshipman; oyster toadfish; and Gulf toadfish, *O. beta*) and one species of Perciformes (northern sea robin, *Prionotus carolinus*) (Pappas and Bennett 1966; Bass and Baker 1990, 1991; Chagnaud et al. 2012; Chagnaud and Bass 2014).

Table 2.1 Vocal motor systems among teleost fishes having swim bladder mechanism

	Sonic organ; innervation	Motoneuron rostro-caudal extent	Motoneuron transverse location
Cohort Osteoglossomorpha			
Order Osteoglossiformes			
Family Mormyridae			
Brienomyrus sp.[a]	Ex; NI	cM	dors mid
Cohort Otomorpha			
Order Characiformes			
Family Characidae			
Pygocentrus nattereri[b,c]	Ex; S3-5	rSC	vent cc
Serrasalmus rhombeus[b]	Ex; S3, 4	rSC	vent cc
order Siluriformes			
family Ariidae			
Ariopsis felis[d]	Ex; Occ	cM/rSC	vent 4th/cc
Bagre marinus[d]	Ex; Occ	cM/rSC	vent 4th/cc
family Doradidae			
Platydoras armatulus[d]	E ; Occ	cM/rSC	vent mid
family Mochokidae			
Synodontis nigriventris[e]	Ex; Occ, S1	cM/rSC	vent mid
Synodontis nigromaculatus[e]	Ex; Occ, S1	cM/rSC	vent mid
family Pimelodidae			
Pimelodus blochii[d,f]	Ex; Occ, S1, 2	cM/rSC	vent mid/4th/cc
Pimelodus pictus[f]	Ex; Occ, S1, 2	cM/rSC	vent mid/4th/cc
cohort Euteleosteomorpha			
order Holocentriformes			
family Holocentridae			
Holocentrus rufus[g]	Ex; Occ	cM/rSC	vent lat
Sargocentron seychellense[g]	Ex; Occ	cM/rSC	vent lat
Sargocentron xantherythrum[g]	Ex; Occ	cM/rSC	vent lat
order Beryciformes			
family Monocentridae			
Monocentris japonica[h]	Ex; Occ	cM	vent lat
order Batrachoidiformes			
family Batrachoididae			
Opsanus tau[i-l]	In; Occ	cM/rSC	vent mid
Opsanus beta[m]	In; Occ	cM/rSC	vent mid
Porichthys notatus[a,k n]	In; Occ	cM/rSC	vent mid
order Perciformes			
family Cottidae			
Leptocottus armatus[a]	PV, Occ*	cM/rSC	vent lat
Myoxocephalus scorpius	PV, Occ*	cM/rSC	vent lat
family Scorpaenidae			
Sebastiscus marmoratus[o]	Ex; Occ	cM/rSC	vent lat
family Triglidae			
Prionotus carolinus[a,k, also p]	In; Occ	cM/rSC	vent lat
Series Percomorpharia			
family Chaetodontidae			
Hemitaurichthys polylepis[q]	Ex; S1-3	rSC	vent cc



The *far right column* is line drawings in transverse plane showing location of motoneurons that innervate sonic swim bladder muscles. Symbols: *Filled circles* represent the somata of motoneurons. *Open circle* represents fourth ventricle and/or spinal canal. Abbreviations: *cM* caudal medulla; *dors mid* dorsal to fourth ventricle on midline; *Ex* extrinsic swim bladder muscles;

In intrinsic swim bladder muscles; *NI* not identified; *Occ* occipital nerves; *PV* pectoral girdle vibration; *rSC* rostral spinal cord; *S* spinal nerves; *vent cc* ventral to central canal (cc), dorsal and/or lateral to medial longitudinal fasciculus (mlf); *vent 4th/cc* ventral to fourth (4th) ventricle and cc, and lateral to mlf; *vent lat* ventrolateral to mlf within ventral motor column; *vent mid* immediately ventral to 4th ventricle, on midline and dorsal to mlf. References corresponding to superscript alphabets are found below (also see for references on sonic organ). Tables 2.1 and 2.2 adapted from Boyle et al. (2013) and Bass and Ladich (2008). Although Cottidae lack a swim bladder and vibrate the pectoral girdle, they are included in Table 2.1 for developmental and functional reasons (see Sect. 2.3.2 of the text for explanation). See Wiley and Johnson (2010), Betancur-R et al. (2013) and Broughton et al. (2013) for teleost classification in Tables 2.1 and 2.2
[a] Bass (1985); [b] Ladich and Bass (2005); [c] Onuki et al. (2006); [d] Ladich and Bass (1998); [e] Ladich and Bass (1996); [f] Ladich and Fine (1994); [g] Carlson and Bass (2000); [h] Onuki et al. (2010); [i] Bennett and Pappas (1965); [j] Fine et al. (1982); [k] Bass and Baker (1991); [l] Fine and Mosca (1989); [m] Bass and Baker (1997); [n] Bass and Baker (1990); [o] Yoshimoto et al. (1999); [p] Finger and Kalil (1985); [q] Boyle et al. (2013)

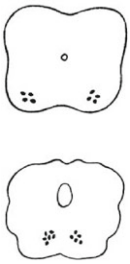
As listed in Table 2.1, VMN is positioned along the rostral-caudal axis in the caudal hindbrain medulla and/or the rostral spinal cord in all species so far investigated. VMN location varies within the transverse plane in six discernible patterns (see Table 2.1 for references). (1) A subset of motoneurons at the level of the vagal motor nucleus in the caudal medulla, positioned dorsal to the fourth ventricle and near the midline (dors mid). This pattern has only been identified in weakly electric mormyrid fish. A caveat to this study is that, while it identified neurons connected to musculature of the swim bladder, it was not performed in a highly vocal mormyrid species, namely *Pollimyrus isidori* or *Pollimyrus adspersus* (Crawford et al. 1997). More comprehensive studies should be completed in highly vocal mormyrids. (2) Rostral spinal cord only with motoneurons diffusely spread dorsal and/or lateral to medial longitudinal fasciculus and ventral to central canal (vent cc). (3) Caudal medulla and rostral spinal cord with the motor nucleus positioned ventral to the fourth ventricle and central canal, near the medial longitudinal fasciculus (vent 4th/cc). (4) Caudal medulla and rostral spinal cord with the motor nucleus positioned ventral to the fourth ventricle on the midline and dorsal to the medial longitudinal fasciculus (vent mid). (5) Combination of patterns 2–4 as described above (vent mid/4th/cc). (6) Caudal medulla and rostral spinal cord with the motor nucleus ventrolateral to the medial longitudinal fasciculus, along the ventral surface of the brain and spinal cord (vent lat). Patterns 4 and 6 are the most prevalent.

2.3.2 Sonic-Pectoral Signaling

Swim bladder dependent mechanisms of sound production are the most prevalent so far documented among teleosts (Ladich and Fine 2006; also see Chap. 3). Sonic-pectoral signaling, i.e., using some part of the pectoral girdle or appendage (fin, forelimb, wing) to generate sound, is also well known among fishes (Ladich and

Table 2.2 Vocal motor systems among teleost fishes having pectoral fin mechanism

	Sonic organ; innervation	Motoneuron rostro-caudal extent	Motoneuron transverse location
Cohort Otomorpha			
Order Siluriformes			
Family Mochokidae			
Synodontis nigromaculatus[a]	PS; Occ	cM/rSC	vent lat
Family Pimelodidae			
Pimelodus blochii[b,c]	PS; Occ, S1, 2	cM/rSC	vent lat
Pimelodus pictus[c]	PS; Occ, S1, 2	cM/rSC	vent lat
cohort Euteleosteomorpha			
order Anabantiformes			
family Osphronemidae	PT; Occ	cM/rSC	vent lat
Trichopsis vittata[d]			



The *far right column* includes a single line drawing in transverse plane showing location of motoneurons that innervate muscles linked to pectoral spine stridulation (PS) or pectoral tendon plucking (PT) for all species listed. Symbols: *Filled circles* represent the somata of motoneurons. *Open circle* represents fourth ventricle and/or spinal canal. Abbreviations: *cM* caudal medulla; *Occ* occipital nerves; *rSC* rostral spinal cord; *S* spinal nerve; *vent lat* ventrolateral to medial longitudinal fasciculus within ventral motor column. References corresponding to superscript alphabets are found below (also see for references on sonic organ)
[a] Ladich and Bass (1996); [b] Ladich and Bass (1998); [c] Ladich and Fine (1994); [d] Ladich and Fine (1992)

Fine 2006), as it is among birds (Prum 1998; Hingee and Magrath 2009; Miller and Baker 2009; Bostwick et al. 2010; Barske et al. 2011) and mammals (Reynolds 1965; Remedios et al. 2009). As noted in Sect. 2.3, the use of the term vocal for fishes was adopted in reference to systems dedicated to sound production, akin to syringeal and laryngeal systems among tetrapods (Bass et al. 1994). Although sculpin, members of the family Cottidae, lack a swim bladder and depend upon vibration of the pectoral girdle for sound production, their motor system is included in Table 2.1 (vent lat, see pattern 6 described at end of last section) as a VMN for two reasons. First, like the VMN that activates swim bladder muscles, the sonic muscle of sculpin appears to be dedicated to sound production (Barber and Mowbray 1956). Second, the sonic muscle of sculpin shares the same developmental history as the sonic muscles driving swim bladder vibration in the closely related scorpaenids (Hallacher 1974).

Several families of catfishes include species that use both swim bladder and pectoral spine sonic mechanisms (e.g., Mochokidae and Pimelodidae in Table 2.1) (also see Kaatz et al. 2010). Unlike sculpin, pectoral mechanisms in catfishes, and in the croaking gourami (*Trichopsis vittata*) that snap a pair of hypertrophied pectoral tendons during sound production, are multi-functional because of their

added involvement in posture, locomotion and defense mechanisms (see Ladich 1989; Ladich and Fine 1992). Catfishes exhibit patterns 3–5 for VMN (Table 2.1) and pattern 6 for pectoral motoneurons (Table 2.2). Gouramis also show pattern 6. We place these pectoral fin systems into a separate Table 2.2 to distinguish them from the dedicated sonic systems listed in Table 2.1. How multi-functional pectoral motoneurons switch between sonic and non-sonic functions remains a fascinating neural CPG puzzle to solve. The central control of other sonic mechanisms that are also likely multi-functional, such as those engaging pharyngeal muscles (e.g., Rice and Lobel 2002; Parmentier et al. 2007), remains to be investigated.

The charting of sonic motoneuron populations in widely divergent groups of teleost fishes that utilize dedicated vocal and/or multi-functional pectoral-sonic provides both a more complete mapping of acoustic signaling systems among vertebrates in general, as well as essential information for guiding future comparative electrophysiological investigations.

2.3.3 Brainstem Vocal Network

Besides identifying VMN location as summarized above, transneuronal transport of anatomical tracers has further recognized vocal premotoneurons in several families of fish. In 1994, Bass and colleagues showed that labeling the vocal nerve of midshipman fish at the level of the swim bladder with either biocytin or neurobiotin led to transneuronal transport and bilateral labeling of the paired VMN as well as an extensive premotor network in the caudal hindbrain. The premotor network included two expansive columns of neurons alongside each VMN that matched the location of neurons identified by Bass and Baker (1990) as pacemaker neurons because their oscillatory-like firing properties appeared to determine VMN's firing rate. Bass et al. (1994) then identified a second premotor nucleus immediately rostral to the VPN–VMN circuit that was designated the ventral medullary nucleus because of its location. Bass et al. (2008) later renamed this nucleus the vocal prepacemaker nucleus or VPP, because it directly innervates the VPN–VMN circuit (also see Goodson and Bass 2002; Chagnaud et al. 2011). A subsequent study further identified intra- and intersexual dimorphisms in the size of neurons in the VPP–VPN–VMN network (Bass et al. 1996) that expanded upon earlier observations of differences in VPN and VMN neuron size by Bass and Baker (1990) who also reported intra- and intersexual differences in the firing rate of the VPN–VMN circuit. A third transneuronal study provided an initial mapping of the early development of the VPP–VPN–VMN circuit in larval midshipman fish (Knapp et al. 1999a).

Vocal premotoneurons directly within, adjacent to, or rostral to VMN have also been identified by transneuronal biotin transport in catfish (Mochokidae, Table 2.1) (Ladich and Bass 1996), squirrelfish (Holocentridae, Table 2.1) (Carlson and Bass 2000) and rockfish (Scorpaenidae, Table 2.1) (Yoshimoto et al. 1999). For these groups of fish, as in midshipman and toadfish (Bass et al. 1994, 2008; Chagnaud

and Bass 2014), biocytin or neurobiotin labeling also showed connections between the vocal system and the central auditory system, including the octavolateralis efferent nucleus (OEN) that directly innervates the inner ear and lateral line organs. The activity of putative OEN axons projecting to the inner ear and lateral line system was correlated to call duration and frequency (Weeg et al. 2005), as later verified via intracellular OEN recordings (Chagnaud and Bass 2013). VPP neurons were shown to be the predominant source of the vocal-auditory coupling using intracellular recording and single cell staining in midshipman (Chagnaud et al. 2011; Chagnaud and Bass 2013). The identification of a VPP in other species, along with its connection to the OEN, however, awaits comparable studies.

2.4 Evolutionarily Conserved Hindbrain Compartments

2.4.1 Vocal Hindbrain Compartment

Experimental investigations of the early development of the vocal pattern generator network in midshipman and toadfish larvae soon after hatching show that it maps onto the same region of the developing hindbrain as the hindbrain-spinal vocal circuitry in tetrapods (Bass et al. 2008). This mapping was accomplished by using confocal microscopy in combination with labeling of the developing sonic swim bladder muscle and either the spinal cord or other musculature with different wavelength fluorescent dextran-amines and Alexa-biocytin. In this way, vocal neurons could be positioned within the context of well-documented hindbrain landmarks (e.g., reticular formation neurons projecting to the spinal cord, and other cranial and spinal motor neuron populations). The results showed that VMN forms in a segment-like compartment in the caudal hindbrain that extends into the rostral spinal cord (Fig. 2.3a). Transneuronal transport of neurobiotin in progressively older larvae further showed that the premotor VPN and VPP populations formed in the same neural compartment (Fig. 2.3b–d). Given the conserved location of vocal circuitry in the caudal hindbrain and rostral spinal cord among diverse groups of teleosts (Sects. 2.3.1 and 2.3.2, Table 2.1), the developmental phenotype in midshipman and toadfish (batrachoidids) likely extends to these other groups. A comparison to the early development of vocal systems in birds and frogs, along with the available evidence on motoneuron development in mammals and organization of the vocal circuitry in adult mammals, revealed that the VPP–VPN–VMN network in fish developed in the same neural compartment as in tetrapods (Fig. 2.3e) (Bass et al. 2008).

Teleosts, including sonic species, belong to a larger clade of bony vertebrates known as Actinopterygii or ray-finned fishes (Fig. 2.4a, b). So far, all of the neurobiological studies of sound communication in fishes have focused on teleosts. Sound production is, however, also reported for more basal groups of actinopterygians, namely Polypteriformes (bichir; Ladich and Tadler 1988), Acipenseriformes (sturgeon; Johnston and Phillips 2003) and Amiiformes (bowfins; Fülleborn 1894).

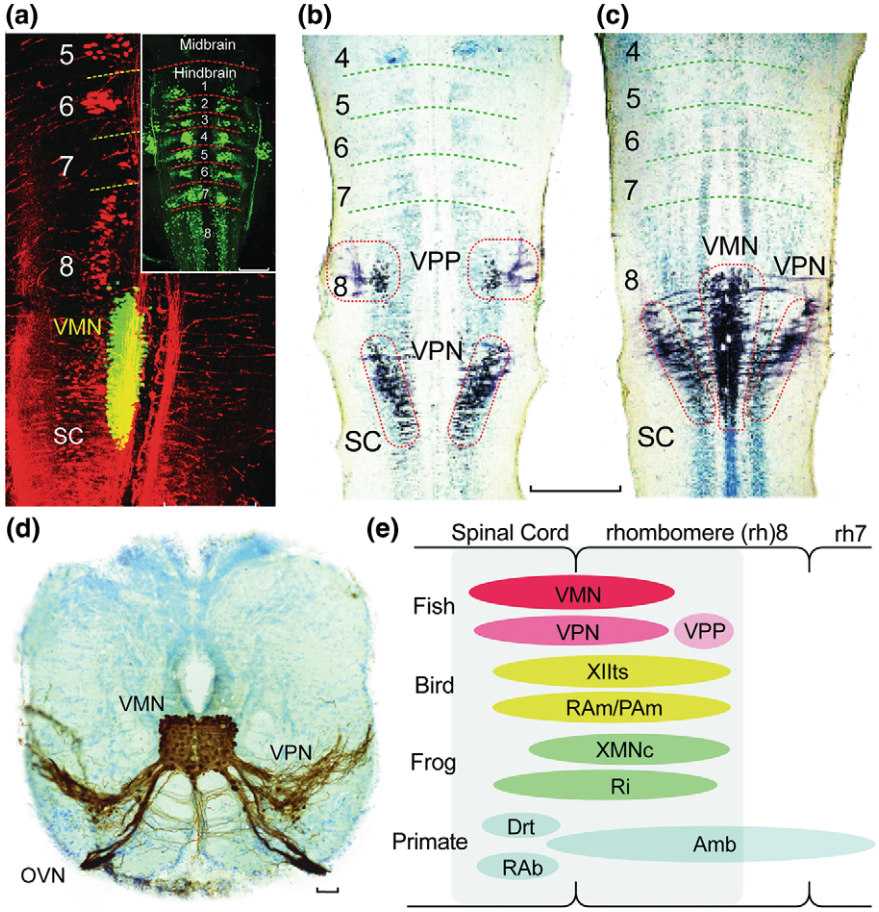


Fig. 2.3 Map of developing hindbrain vocal central pattern generator. The location of the developing vocal motor nucleus (VMN) was mapped onto the embryonic segments of the developing hindbrain known as rhombomeres (rh) and the spinal cord (SC) in larval plainfin midshipman fish (*Porichthys notatus*). **a** Fluorescently labeled neurons are visualized with laser scanning confocal microscopy (*horizontal plane*). Simultaneous visualization of clusters of reticular formation neurons labeled in rh 5–8 via retrograde transport from the spinal cord (Alexa 546 dextran-amine, *red*), and VMN labeled via retrograde transport from the developing vocal muscle (Alexa 488 dextran-amine, *green*). *Yellow* is composite overlap and does not indicate double labeling. *Inset* clusters of reticulo-spinal neurons (Alexa biocytin 488, *green*) in each rh, from 1 to 8. (*Scale bar* 0.2 mm.) **b**, **c** Mapping in *horizontal plane* of vocal pre-pacemaker (VPP), vocal pacemaker (VPN), and VMN neurons (*black*) in Gulf toadfish (*Opsanus beta*) larvae; labeling via transneuronal transport of neurobiotin from developing vocal muscle. Cresyl violet counterstain reveals reticulospinal clusters in rh segments. (*Scale bar* 0.2 mm.) **d** Transverse section in caudal hindbrain of juvenile Gulf toadfish showing extensive transneuronal bilateral labeling with neurobiotin (*brown*) of dendritic and axonal branching of VMN and VPN. VMN axons exit brain via occipital vocal nerve root (OVN; cresyl violet counterstain). (*Scale bar* 100 μm.) **e** Schematic in sagittal plane summarizing relative positions of hindbrain vocal premotor–motor networks in rh8-spinal compartment of several lineages of vocal vertebrates (see Bass et al. 2008 for details). Abbreviations: *Amb*, nucleus ambiguus; *Drt* dorsal reticular nucleus; *PAm* nucleus parambigualis; *RAb* nucleus retroambiguus; *RAm* nucleus retroambigualis; *Ri* inferior reticular formation; *XIIts* tracheosyringeal division of hypoglossal motor nucleus; *XMNc* caudal XMN. From Bass and Chagnaud (2012)

There are also reports of sound production in Dipnoi (lungfish; Thompson 1968) belonging to the Sarcopterygii or lobe-finned fishes, a group basal to tetrapods in the sister clade to Actinopterygii (Fig. 2.4b). While only comprising a small percentage of fishes in general, it remains important to investigate both the biomechanical and neural bases for sound production in these non-teleost lineages of fishes. If the adult and early developmental vocal phenotypes observed so far in teleosts do *not* extend to more basal actinopterygians, we will conclude that the teleost phenotype evolved

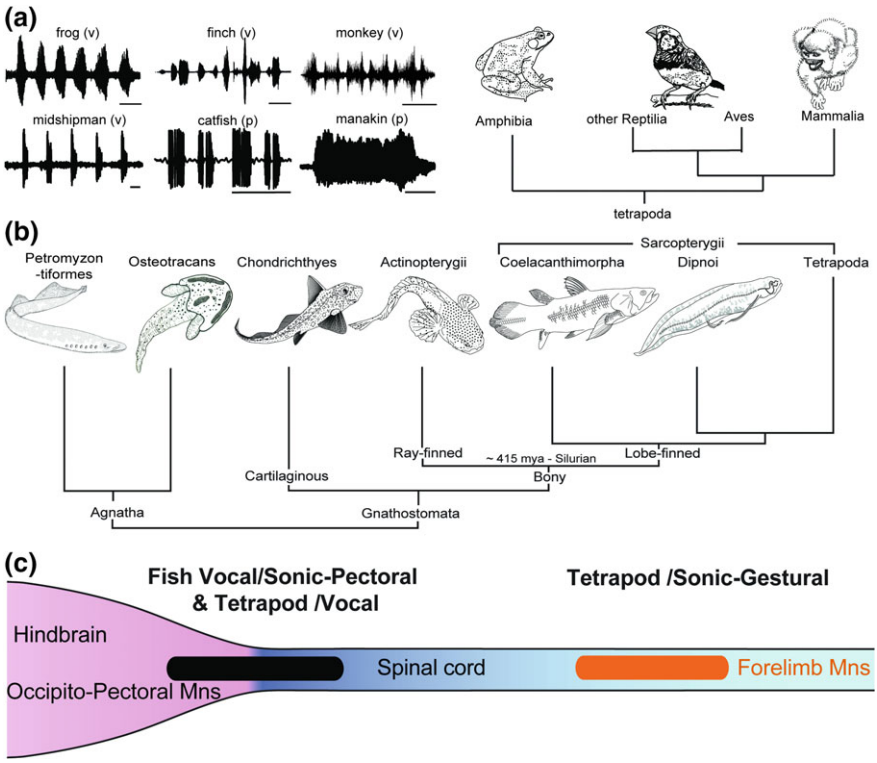


Fig. 2.4 Evolution of vocal–pectoral motor systems in fishes and tetrapods. **a** Waveforms of vocalizations of bullfrog (*Lithobates catesbeiana*, time base 1 s), zebra finch (*Taeniopygia guttata* 250 ms), squirrel monkey (*Saimiri sciureus*, 200 ms), midshipman fish (*Porichthys notatus*, 500 ms), catfish (*Platydoras armatulus*, 250 ms), and club-winged manakin (*Machaeropterus deliciosus*, 100 ms). Vocal (v) and pectoral (p) basis for sound production is shown. **b** Vertebrate cladogram depicting both jawless (agnathan) and jawed (gnathostome) radiations (Osteostracans represent an extinct agnathan group with pectoral fins). **c** Schematic summary in sagittal plane showing location of vocal and sonic-pectoral motoneurons. The occipito-pectoral motor column (*black*) in fishes gives rise to motoneurons innervating muscles of vocal organs dedicated to sound production (e.g., swim bladder) and pectoral fins that can also serve a sonic function. The occipito-pectoral column also gives rise to vocal motoneurons in tetrapods. Forelimb motoneurons (*orange*) that function in both sonic and gestural signaling in tetrapods are located in the spinal cord. (**a**, **b**) From Bass and Chagnaud (2012). (**c**) Modified from Ma et al. (2010)

independently among actinopterygians. If the teleost pattern applies broadly to actinopterygians, but not to lungfish, then the pattern observed in teleosts and/or actinopterygians, in general, versus that observed in tetrapods (Fig. 2.3e) is a case of convergent evolution.

2.4.2 *Vocal and Sonic-Pectoral Motor Systems Share Developmental Origins*

Bass et al.'s (2008) study of the early development of the vocal system in fish also shows that motoneurons innervating pectoral musculature are located in the same compartment as the developing vocal (swim bladder muscle) circuitry. This finding led to a more comprehensive, phylogenetic analysis of pectoral motoneuron development by Ma et al. (2010). Using similar methods to identify the developing pectoral system in the embryos and larvae of three orders of teleosts—Batrachoidiformes (midshipman), Cypriniformes (zebrafish and goldfish), and Salmoniformes (salmon and trout), Ma et al. (2010) also included a more basal group of fishes, Acipenseriformes (paddlefish), in their investigation. Using a more complete set of developmental landmarks such as molecular marking of the hindbrain-spinal boundary and the location of myotomes, Ma et al. (2010) precisely mapped a pectoral column extending between the caudal hindbrain and rostral spinal cord in all species investigated. Labeling of occipital myotomes at the same stages of development in several species, including midshipman, also delineated the entire extent of the occipital motor column (Fig. 2.5). The results showed overlapping caudal occipital and rostral pectoral columns, including the region where vocal circuitry is located in larval and adult toadfishes (Bass et al. 2008) (Fig. 2.5). This finding led to the conclusion that sonic systems, both vocal and pectoral, share developmental origins in fishes (Bass and Chagnaud 2012) (Fig. 2.4c).

2.5 Vocal Neurophysiology

While sound production among vertebrates has received considerable attention in terms of biomechanics and behavioral context (Bradbury and Vehrencamp 2011), and the location of sonic motoneurons has been mapped in all of the major lineages of vocal vertebrates (Bass et al. 2008), there is an astonishing lack of knowledge of how vocal muscles are patterned by the central nervous system. For tetrapods, this is due, in part, to several factors: (1) The strong coupling of CPGs for respiration and vocalization in birds and mammals makes functional characterization technically difficult due to simultaneous activity of CPGs (exhalation during breathing and vibration of vocal chords; Riede and Goller 2010). (2) The surgical accessibility of the vocal hindbrain in songbirds, the most studied vertebrate lineage in terms of

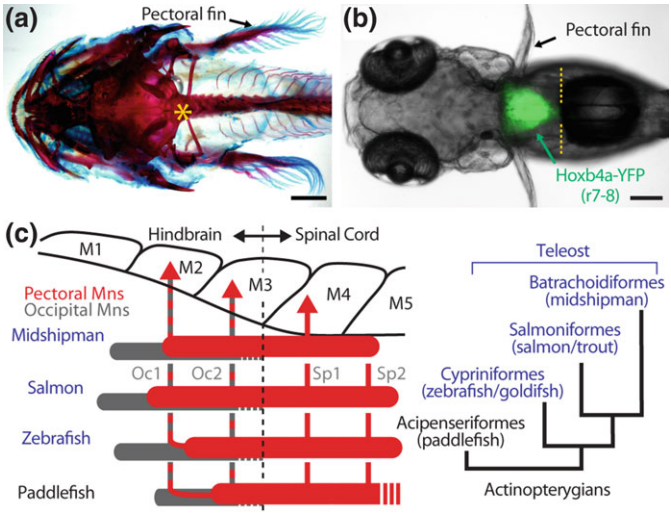


Fig. 2.5 Map of developing pectoral motor column in actinopterygian fish. **a** Dorsal view of juvenile plainfin midshipman fish (*Porichthys notatus*) cleared and stained with *alcian blue* and *alizarin red*; *asterisk* indicates location of craniovertebral junction. **b** Dorsal view showing the boundary between hindbrain rhombomere (r)8 and spinal cord (*yellow* hatching) defined by hoxb4a expression in zebrafish (*Danio rerio*) hoxb4a enhancer trap line. **c** Alignment in sagittal plane of columns of pectoral (*red*) and occipital (*gray*) motoneurons, segmental myotomes ("M"), and occipital (Oc1, Oc2) and spinal (Sp1, Sp2) nerves. Phylogeny of study species is shown to the *right*. Modified from Ma et al. (2010)

vocal production, is limited, thus discouraging neurophysiological recording from hindbrain premotor and motoneuron populations in an intact, in vivo preparation (e.g., Sturdy et al. 2003; Kubke et al. 2005). (3) The complexity of the vocal apparatus in birds and mammals poses significant technical challenges in delineating the neuromuscular patterning of individual syllables and entire vocalizations. The avian syrinx, for example, has several muscles that are controlled independently, thus potentiating the complexity of sequencing muscle contraction patterns (Vicario 1991; During et al. 2013).

Vocal fish lack all of the above complications and thus offer an optimal model to investigate CPG(s) underlying the neuromuscular patterning of vocalization, especially at the hindbrain level. In addition, due to the evolutionary conserved organization of the vocal hindbrain (Bass et al. 2008), functional comparisons to other vocal lineages can be drawn from the organization of vocal fish neuronal patterning. Clearly, the simplicity of vocal structure in fishes (Fig. 2.1) presents limitations in providing explanations for the more complex repertoires of birds and mammals, including answers to the question of fully innate versus partially learned vocalizations. Given the technical challenges of single unit recordings in a freely behaving animal in the aquatic medium (Palmer et al. 2003), this aspect of vocal behavior has remained largely unexplored in vocal fishes, unlike the case for

songbirds and primates (e.g., Hage and Jürgens 2006; Goldberg et al. 2012). While the central mechanisms underlying the switching between call types, e.g., advertisement versus agonistic signals, and the patterning of vocalizations that include both amplitude and frequency modulated components such as toadfish boatwhistles (Mosharo and Lobel 2012) (Fig. 2.1) and midshipman growls (Bass et al. 1999) remains to be demonstrated for any vertebrate, fish have already offered significant contributions to our understanding of vocal patterning as reviewed below.

The mechanical decoupling of the vocal and respiratory systems makes vocal fishes an optimal model to study vocal CPGs. This is not to say that the vocal system of fish neither influences, nor is influenced by, other motor systems, including respiration. For instance, Bass and Chagnaud (2012) provide behavioral evidence in midshipman for interactions between vocalization, respiration and pectoral appendage movement (Fig. 2.6). Midshipman fish also inflate their swim bladder during advertisement humming (Fig. 2.7), likely to increase call amplitude

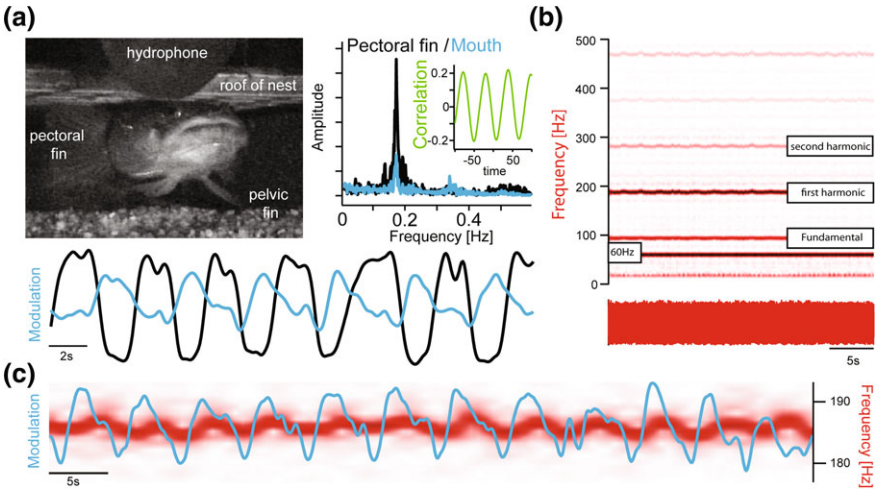


Fig. 2.6 Spatiotemporal patterning of vocal-respiratory-pectoral mechanisms in fish. **a** *Upper left* Photograph from video sequence at night of a type I male plainfin midshipman fish (*Porichthys notatus*) that is producing his advertisement "hum" call while in an artificial nest constructed of elevated ceramic tile in an aquarium (video courtesy of A. Bass and M. Marchaterre, Cornell University). *Lower* Waveforms of modulation rhythm of pectoral fin (*black*) and mouth (*blue*) movement during humming are superimposed to indicate temporal relationship (analyzed by video tracking). *Upper right* Corresponding fast Fourier transforms of pectoral fin and mouth waveforms shows matched modulation frequencies; correlation (*inset*) shows high temporal matching of both rhythms. **b** Sonogram (*upper*) and waveform (*lower*) of hydrophone recording of type I male midshipman hum (dense *bottom trace*) recorded simultaneously with the video in (**a**). The fundamental frequency, first two upper harmonics and 60-Hz band from nearby power line are indicated. **c** Overlay of waveform of mouth waveform (*blue*, approximate breathing rhythm) and modulation hum frequency (*red*, first harmonic) of simultaneous recorded midshipman hum in (**b**). Despite call generation in fishes not requiring airflow over a membrane as in most terrestrial vertebrates, the respiratory and vocal modulations are in phase. From Bass and Chagnaud (2012)

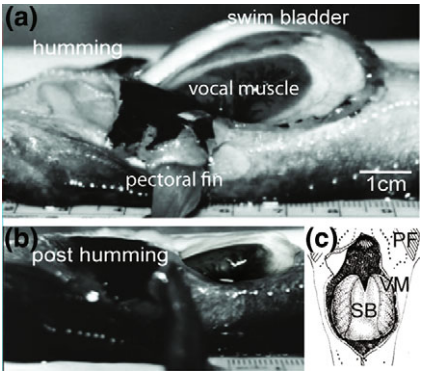


Fig. 2.7 Swim bladder inflation during hum advertisement calling in type I male midshipman (*Porichthys notatus*). *Photomicrographs* showing swim bladder of type I males that were recently humming (**a**) and several hours after humming (**b**). See Genova et al. (2012) for experimental details (**c**). Also shown is *line* drawing of ventral view of type I male swim bladder showing paired vocal muscles (*VM*) attached to the lateral walls of the swim bladder (*SB*); the position of the pectoral fin (*PF*) is also indicated

(Bradbury and Vehrencamp 2011) and perhaps also resonance frequency. Some species of weakly electric fish that employ their electric organ discharge (EOD) for social communication (Hopkins and Bass 1981) are also vocal, using a sonic swim bladder mechanism (Crawford et al. 1986; Crawford and Huang 1999). Pectoral-sonic mechanisms are coupled to swimming/hovering and intraspecific signaling movements when fish such as sculpins, gouramis, and catfish spread their pectoral fins (Ladich 1989; Ladich and Fine 2006). The neurophysiological basis for the coupling between sonic and other motor functions remains to be investigated.

2.5.1 Peripheral Vocal Physiology

Tower (1908) provided a historical overview of physiological observations and theories of how fish produce sound up to the time of his report. In a landmark series of experiments, Tower also provided the first demonstration of the relationship between *sonificus* muscle contraction and sound production in the Squeteague, *Cynoscion regalis*. He also evoked "a perfectly normal grunt" in the northern sea robin, *P. carolinus*, after directly stimulating either the swim bladder nerves and/or muscles in vivo and in the isolated swim bladder. Tavolga (1962) carried out similar experiments in two species of ariid catfishes, the gaff-topsail catfish, *Bagre marinus* and the sea catfish, *Ariopsis* (formerly *Galeichthys*) *felis* as well as Schneider (1964) for the tigerfish, *Therapon jarbua*. Together, these studies showed the close relationship between muscle contraction and sound production.

Barber and Mowbray (1956) showed in the longhorn sculpin, *Myoxocephalus octodecimspinosus*, a 1:1 relationship between individual muscle contractions and

sound pulses. The same correlations with the pattern of nerve activity were reported by Packard (1960) and Skoglund (1961) for the pectoral muscles of the southern pigfish, *Congiopodus leucopaecilus* and the swim bladder muscles of the oyster toadfish, respectively. Nerve firing patterns have since been identified in several other distantly related species. Figure 2.8 shows drawings outlining the position of the VMN and bilateral recordings from occipital nerves in representative Batrachoidiformes (plainfin midshipman and oyster toadfish) and Perciformes (the shorthorn sculpin, *Myoxocephalus scorpius* and the northern sea robin). Nerve firing patterns highlight four important behavioral and neural attributes of vocal motor systems in fishes. First, each of the nerve's spike-like compound action potentials directly translates into individual sound pulses (also see beginning of this section). Second, each nerve spike reflects the synchronous firing of VMN neurons (Pappas and Bennett 1966; Chagnaud et al. 2012). Third, the nerves on either side of the midline fire either in (e.g., midshipman, toadfish, sculpin; Fig. 2.8a–c) or out of (e.g., searobin, Fig. 2.8d) phase. Fourth, nerve activity directly determines the PRR and duration of natural sounds (Packard 1960; Skoglund 1961; Cohen and Winn 1967; Connaughton 2004). Fifth, the pattern of nerve activity does not reflect pattern of VMN organization. For example, sea robin and sculpin both display a ventrolateral VMN at the same hindbrain-spinal level (Fig. 2.8) (also see Bass and Baker 1991; Ladich and Bass 1998). However, sculpin, like midshipman and toadfish, display bilateral nerve firing that is in phase, while it is out of phase in sea robins (Fig. 2.8) (see Bass and Baker 1991 for comprehensive study). These firing patterns are consistent with patterns of sonic muscle contraction (Skoglund 1961; Cohen and Winn 1967; Connaughton 2004). The mismatch between a ventrolateral VMN and neurophysiological mechanisms (bilateral synchrony in sculpin, but not in sea robins) extends to peripheral sonic mechanics—pectoral girdle vibration in sculpin which lack a swim bladder (Barber and Mowbray 1956) and vibration of sonic muscles directly attached to the swim bladder in sea robins (Tower 1908; Bass and Andersen 1991) (also see Table 2.1). These findings show at a gross level that underlying neurophysiological patterning cannot be easily predicted from either central anatomy or peripheral mechanics.

2.5.2 *Anatomically Distinct Brain Nuclei Code for Vocal Characters*

As reviewed in Sects. 2.3.1 and 2.3.2, several anatomical studies have identified the neuronal circuitry involved in sonic signaling among fishes. Most of these investigations are limited to the identification of the position and extent of motoneurons with few also identifying pre-motoneuron circuitry (Sect. 2.3.3). Taken together, these studies provide a comparative basis for neurophysiological investigations of vocal pattern generation.

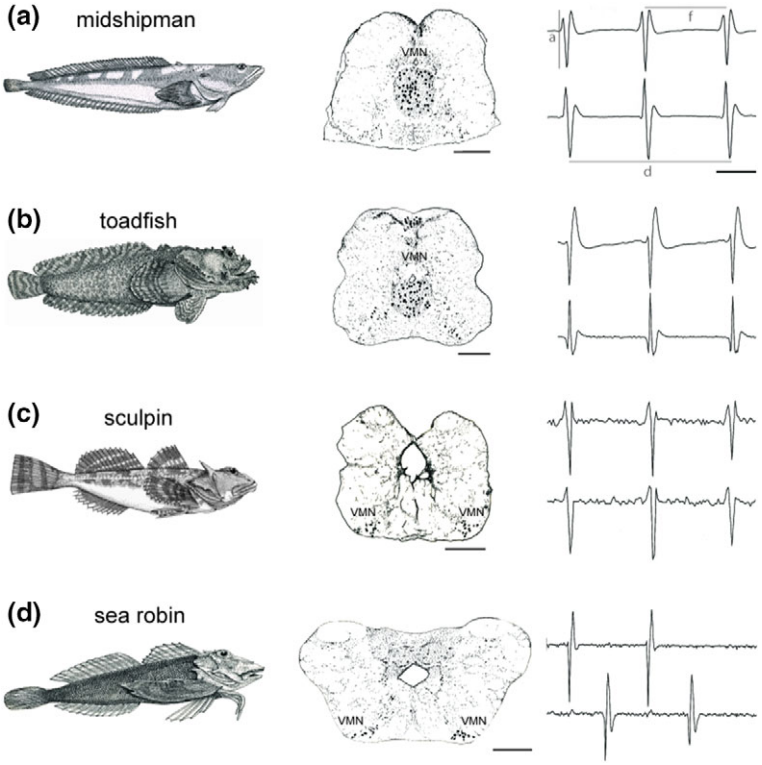


Fig. 2.8 Location of the vocal motor nucleus (VMN) does not predict phase relationship of bilateral vocal nerve firing in fishes. Portrait (*left column*) of fishes using swim bladder mechanisms (plainfin midshipman fish, *Porichthys notatus*; oyster toadfish, *Opsanus tau*; and northern sea robin, *Prionotus carolinus*) and pectoral girdle vibration (shorthorn sculpin, *Myoxocephalus scorpius*) to produce sound. *Line* drawings of transverse sections (*middle column*) of caudal hindbrain depict location of the bilateral VMN (*midline* VMNs are directly apposed to one another). *Bar scales* indicate 500 μm. Vocal nerve records from the *left* and *right* (*right column*) sides of the body demonstrate a synchronous motor volley in midshipman, toadfish, and sculpin, but an asynchronous volley in sea robins. *Bar scale* indicates 5 ms for midshipman and applies to other species. The paired vocal nerve volleys directly determine the duration (d) and PRR/fundamental frequency (f) and amplitude (a) of the vocalization as schematically indicated in the midshipman record

Most of the available cellular neurophysiological data on vocal patterning in fishes originates from studies of toadfishes that include midshipman and species generally referred to as toadfish (Nelson 2006). The success of these studies has relied, in part, on the direct translation between the neural output of the hindbrain vocal CPG, and the physical attributes of the vocal behavior. Hence, the spike-like appearance of vocalizations in fishes directly reflects distinct spike-like events comprising the activity pattern of occipital nerves (Fig. 2.8). The pattern of vocal nerve activity is referred to as a *fictive call* because it directly determines multiple

call characters and is produced in the absence of muscle activation in an in vivo neurophysiological preparation.

Demski and Gerald (1972, 1974) and Fine (1979) used electrical stimulation to identify vocally active sites in the hindbrain, midbrain and forebrain of Gulf and oyster toadfishes. These studies provided landmarks for later studies to reliably elicit vocal activity in identified vocal hotspots. Anatomical tract tracing studies in plainfin midshipman and Gulf toadfish revealed an extensive network of connections between vocally active sites from forebrain to hindbrain levels, including what came to be identified as the hindbrain vocal CPG (Bass et al. 1994; Goodson and Bass 2002). These and other (Bass et al. 2000) studies that also revealed extensive connectivity with the central auditory system continue to provide anatomical guideposts for neurophysiological investigations of central vocal mechanisms.

Kittelberger et al. (2006) showed in the plainfin midshipman that vocally active neurons in the periaqueductal gray (PAG), a highly conserved midbrain region integral to vocal function in birds and mammals (see Kingsbury et al. 2011), shared a role with mammalian vocal PAG neurons in the initiation of downstream vocal CPG activity. A PAG role in the direct patterning of either fictive call duration or PRR (equal to fundamental frequency for multi-harmonic sounds in fishes) was lacking, thus leaving hindbrain targets of descending PAG pathways (Goodson and Bass 2002) as candidates for a vocal CPG in fishes. Demski and colleagues proposed that a set of hierarchically organized nuclei determined the duration and frequency of teleost fish/toadfish vocalization (Demski et al. 1973). The model was largely verified in midshipman fish by Bass and colleagues who provided the essential cellular level experiments (Bass and Baker 1990; Goodson and Bass 2000a; Kittelberger et al. 2006). As recently shown by Chagnaud and colleagues (Chagnaud et al. 2011, 2012; Chagnaud and Bass 2014), the VPP and vocal pacemaker nuclei (VPN) discussed earlier code for call duration and fundamental frequency/PRR, respectively. VPP neurons receive PAG input (Kittelberger and Bass 2013) and project to the VPN-VMN region (Chagnaud et al. 2011), acting as a link that gates forebrain/midbrain activation of hindbrain vocal sites (Kittelberger et al. 2006). Pharmacological inactivation experiments demonstrate that decreasing or increasing the activity of VPP neurons leads to an increase or a decrease in fictive call duration, highlighting the importance of this nucleus in determining call duration (Chagnaud et al. 2011). In contrast, VPN neurons exhibit prominent subthreshold oscillations that underlie an oscillatory-like firing, not present in VPP neurons, that directly determines the fundamental frequency/PRR of calls (Fig. 2.2). Pacemaker neurons project to VMN and fire in a 1:1 relationship with motoneurons; each VPN action potential directly precedes VMN action potentials. The duration of VPN-VMN spiking activity strongly correlates with the duration of the VPP long-lasting membrane depolarization from which they receive direct input (Fig. 2.2). Taken together, this evidence is highly supportive of VPN neurons determining the frequency/PRR and VPP the duration of natural vocal activity.

Vocalizations, however, are not characterized by frequency and duration alone, but also by other characters such as amplitude, and modulations of amplitude and/or frequency. The amplitude of midshipman vocalization has been proposed to be

directly related to the recruitment pattern of VMN motoneurons (Chagnaud et al. 2012), i.e., motoneurons are recruited in accordance with the size principle, with the smallest ones activated first (Henneman and Mendell 1981). The question remains, however, as to which premotor nucleus determines the number of motoneurons to be recruited at any one time. One potential mechanism could originate from a differential activation of VPP neurons that, besides projecting to VPN, also project to VMN (Chagnaud et al. 2011). This further raises the question as to which brain region determines the differential recruitment of VPP neurons and by which means (e.g., size principle again?). The midbrain PAG would be an excellent candidate due to its role in vocal initiation, but also because of its suggested contribution to shaping vocal duration (Kittelberger et al. 2006). Direct testing of these hypotheses awaits further experimental investigation.

2.5.3 Neuronal Synchrony—An Essential Trait of Vocal Neurophysiology

Few studies have investigated the neurophysiological adaptations of single vocal motoneurons or of the activity pattern of the entire VMN population. As noted earlier, each vocal nerve spike reflects the synchronous firing of VMN neurons, and directly determines the onset of muscle contraction and in turn, the production of one sound pulse. Hence, the vocal nerve motor volley provides a direct readout of natural vocal behavior, providing information about the onset of a call, as well as its frequency, duration, and amplitude (see Fig. 2.8) (Cohen and Winn 1967; Bass and Baker 1991; Remage-Healey and Bass 2004; Remage-Healey et al. 2006; Rubow and Bass 2009; Chagnaud et al. 2012). The firing frequency of the vocal motor volley, which directly translates into natural call PRR/fundamental frequency, increases linearly with increasing temperature across multiple species irrespective of peripheral vocal mechanism, i.e., swim bladder (oyster toadfish, midshipman, sea robin) versus pectoral girdle vibration (sculpin) (Bass and Baker 1991). Together, the available evidence suggests that stable and precise temporal patterning of a high degree of neuronal synchrony is a shared motoneuron character across distantly related groups of vocal fishes (Bass and Baker 1991).

Sonic swim bladder muscles qualify as superfast muscles known to be adapted to generate speed rather than force (Rome et al. 1996; Rome 2006). To do so, these muscles require simultaneous activation of muscle fibers within a brief period of time in order to reach a high PRR. In Gulf toadfish, for instance, full muscle contraction and full muscle relaxation has to occur within a period of 5 ms in order to reach PRRs of 200 Hz at ambient temperatures of 25–30 °C (Demski et al. 1973; also see Rome 2006 for midshipman muscle and Bass and Baker 1991 for temperature dependency). The presence of superfast muscles is, however, not limited to toadfishes. They have also been found in other sonic systems such as in the rattlesnake tail shaker (Rome et al. 1996), the avian syrinx (Elemans et al. 2004, 2008) and the bat larynx (Elemans et al. 2011).

The first neurophysiological studies investigating the properties of a superfast motor system were in the oyster toadfish by Bennett and colleagues (Pappas and Bennett 1966; Bennett et al. 1985) who provided brief descriptions of several key characters contributing to vocal motoneuron synchrony, including gap junctional (electrotonic) coupling, low motoneuronal excitability, and inhibitory input. Pappas and Bennett (1966) showed electrotonic synapses onto swim bladder motoneurons (see Bass and Marchaterre 1989 for midshipman). Antidromic activation of motoneurons and presynaptic afferents via stimulation of the "swim bladder nerves" provided neurophysiological evidence of gap junctional coupling, later supported by anatomical studies showing extensive transneuronal transport of biotin compounds throughout the VPN–VMN circuit (Bass et al. 1994). In-depth studies of these neurophysiological characters were recently completed in midshipman fish by Chagnaud et al. (2012) who also demonstrated the importance of oscillatory excitatory input from vocal pacemaker neurons and a hyperpolarizing conductance that is activated upon network activity in generating an extreme level of synchrony. While each mechanism alone can establish synchronous activity (see Chagnaud et al. 2012 for details), the entire suite of characters contributes to the extreme level of neuronal synchrony controlling superfast muscle vocal systems, comparable to levels observed for electric fish (Bennett 1971). Interestingly, both systems share key neuronal components, including pacemaker circuitry in the same hindbrain compartment (Bass and Baker 1997; Bass and Zakon 2005). We propose similar vocal CPG adaptations in any sonic system where either nerve or EMG recordings, or sound characters themselves, indicate modulation rates of >60 Hz. This would include pectoral-dependent mechanisms in sculpin (Barber and Mowbray 1956; Bass and Baker 1991) and croaking gouramis (Ladich et al. 1992).

While midshipman and other toadfish species provide insights into the cellular mechanisms of vocal CPGs, there is an urgent need for more in-depth comparative studies of other toadfishes and other groups of sound producing fishes to test how widespread the cellular mechanisms so far identified mainly for midshipman can be applied to other species and lineages. Is extreme neuronal synchrony found among other fishes using other sonic mechanisms, e.g., sonic muscles not directly attached to the swim bladder, unlike toadfishes (see Ladich and Fine 2006)? While the dependency of superfast sonic muscle activation on synchronous neuronal activity is perhaps obvious, the question remains whether this synchrony will also be found in sound producing systems that do not apparently utilize superfast muscles given their low PRRs (e.g., damselfish; Lobel and Mann 1995) compared to toadfishes or other superfast motor systems (see above).

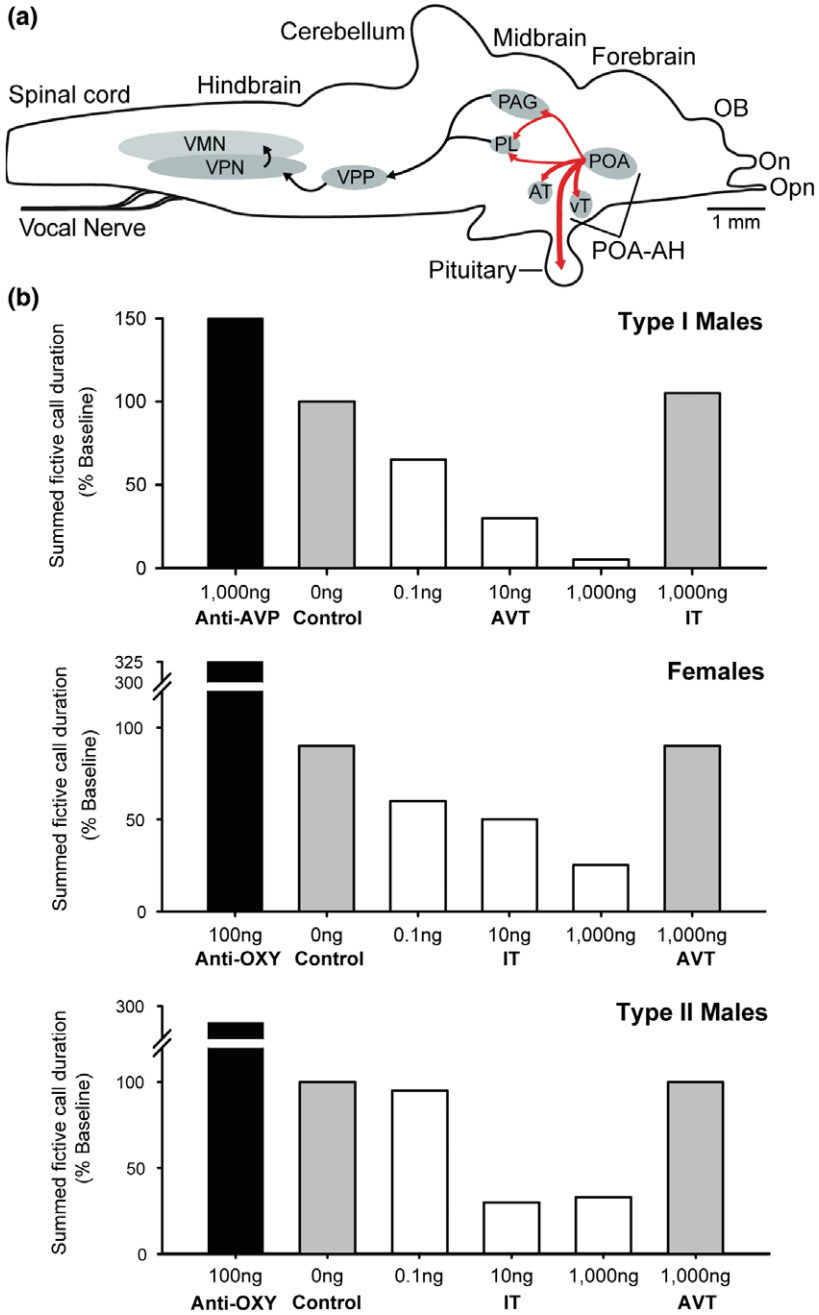
Many aspects of the neural coding of vocal behavior in fishes and among vertebrates in general remain elusive. Besides those noted at the end of the last section, other unanswered questions include: Will a single premotor nucleus be the critical node for switching between call types? What role might hormones, neuromodulators, and inhibitory and excitatory neurotransmitters play in such network level decisions and in the sculpting of call parameters in general? We next consider how hormones might contribute to the diversity of vocal signaling.

2.6 Hormonal Modulation of Vocalization and Central Sites of Action

In many vertebrates, including sonic fish, vocalizations are inextricably tied to the expression of social and reproductive states, and as such are under the influence of hormones and neuropeptides that regulate a host of related behaviors and physiology (Remage-Healey and Bass 2006; Bass and Remage-Healey 2008; Goodson 2008). Much of this is likely related to the seasonal and, in some cases daily, cycles in reproductive related acoustic communication. Among the hormones shown to modulate vocal mechanisms in fish are steroids such as androgens, estrogens, and glucocorticoids, as well as neuropeptides such as vasoactive intestinal polypeptide (VIP), arginine-vasotocin (AVT; the non-mammalian homolog to arginine-vasopressin (AVP)), and isotocin (IT; the non-mammalian homolog to oxytocin) (Goodson and Bass 2000a, b, c; Remage-Healey and Bass 2004; Remage-Healey et al. 2006). AVT is the ancestral nonapeptide in vertebrates that gave rise to closely related nonapeptides such as IT, and is found in all non-mammalian vertebrates examined to date (Bentley 1998). Several studies in midshipman fish have found divergent hormone profiles and sensitivity to specific hormone types between the sexes as well as between two male morphs exhibiting alternative reproductive strategies and vocal repertoires (Brantley et al. 1993a; Goodson and Bass 2000a; Sisneros et al. 2004a; Remage-Healey and Bass 2007; Arterbery et al. 2010a). These alternative male morphs include type I males that defend nests and acoustically court females, and type II males that sneak fertilization from type I males by aligning with females in morphology and behavior (Brantley and Bass 1994). Here, we review the effects of AVT-like peptides and steroids on the vocal behavior and neurophysiology of toadfishes, their neuroanatomical sites of action, and differences found between and within the sexes therein.

2.6.1 Neuropeptide Regulation of Vocal Motor Output

Modulation of vocal output by nonapeptides of the AVT family is widespread among vertebrates such as fish (Goodson and Bass 2000a, c), amphibians (Diakow 1978; Marler et al. 1995; Tito et al. 1999; Trainor et al. 2003), and birds (Voorhuis et al. 1991; Maney et al. 1997; Goodson 2008). The two main nonapeptides produced by the teleost preoptic area (POA) are AVT and IT (Bentley 1998; Goodson 2008). While all vertebrates produce vasotocin-like peptides in the POA-anterior hypothalamus (AH), the teleost brain produces AVT and IT almost exclusively within the parvocellular and magnocellular nuclei of the POA-AH, making vocal teleosts like toadfishes an ideal model for understanding vasotocin-like peptidergic regulation of vocal behavior in a fundamental and well-conserved neural circuit (Fig. 2.9a) (Goodson and Bass 2002; Goodson 2008).


(a)
Cerebellum
Midbrain
Forebrain
Spinal cord
Hindbrain
PAG
OB
VMN
VPN
VPP
PL
POA
On
Opn
AT
vT
1 mm
Vocal Nerve
POA-AH
Pituitary
(b)
Type I Males
Summed fictive call duration (% Baseline)
150
100
50
0
1,000ng Anti-AVP
0ng Control
0.1ng
10ng AVT
1,000ng
1,000ng IT
Females
325
300
100
50
0
100ng Anti-OXY
0ng Control
0.1ng
10ng IT
1,000ng
1,000ng AVT
Type II Males
300
100
50
0
100ng Anti-OXY
0ng Control
0.1ng
10ng IT
1,000ng
1,000ng AVT

◀**Fig. 2.9** Arginine vasotocin neural expression and regulation of vocal central pattern generator output. **a** Simplified representation of AVT pathways (*red arrows*) mapped onto a sagittal view of the plainfin midshipman (*Porichthys notatus*) vocal network (*black arrows*) based on immunocytochemistry (Goodson and Bass 2000c). *Arrow thickness* denotes projection density. AVT-immunoreactive (AVT-ir) pathways originate almost exclusively from the preoptic area (POA) and innervate vocally active brain sites in the POA, anterior tuberal nucleus (AT), ventral tuberal hypothalamus (vT), paralemniscal midbrain tegmentum (PL), and midbrain periaqueductal gray (PAG). Other abbreviations: *OB* olfactory bulb; *On* olfactory nerve; *Opn* optic nerve; *POA-AH* pre-optic area-anterior hypothalamus; *VMN* vocal motor nucleus; *VPN* vocal pacemaker nucleus; *VPP* vocal pre-pacemaker. **b** Vocal motor output of the midshipman in responses to POA-anterior hypothalamus stimulation 10 min after delivery of AVT, isotocin (IT), or their respective mammalian antagonists (Anti-AVP; Anti-OXY). *Top panel*, type I males; *middle panel*, females; *bottom panel*, type II males. Total fictive call duration of type I males is inhibited in a dose-dependent manner by AVT, disinhibited by anti-AVP, and not affected by IT. By contrast, fictive calls of females and type II males are not affected by AVT, but inhibited by IT and disinhibited by anti-OXY. Adapted from Goodson and Bass (2000a)

In the midshipman vocal network, the in vivo fictive call preparation in combination with central or peripheral delivery of hormones and/or their receptor antagonists has been an effective and essential tool for investigating hormone action. Delivery of AVT into, and subsequent electrical stimulation of, the POA-AH of midshipman type I males induced a dose-dependent decrease in fictive call duration and number of elicited calls, while delivery of an AVP V_{1a} receptor antagonist significantly increased call duration but not the number of calls elicited (Fig. 2.9b) (Goodson and Bass 2000a). When AVT is delivered to vocally active sites in the paralemniscal midbrain tegmentum (PL), the number of fictive calls evoked was significantly decreased and the latency of fictive call onset after stimulation was increased, while fictive call duration was not significantly affected (Goodson and Bass 2000c). Thus, in type I males, AVT inhibits vocal network excitability at both forebrain and midbrain levels, but seems to act in the forebrain to inhibit both call duration and initiation and in the midbrain to inhibit only call initiation.

Despite the conservation in amino acid sequence (AVT and AVP differ in only one amino acid) and expression within the POA-AH, the vasotocin family of nonapeptides are evolutionarily labile in that their effects are often sex-, morph-, and species-dependent (Goodson 2008; Bass and Grober 2009). A good example can be found in their differential modulation of vocal output between and within the sexes of midshipman (Fig. 2.9b). As discussed above, AVT is effective in decreasing vocal output in the nest-guarding and highly vocal type I male morph. However, AVT has negligible effects on vocal output in females or type II males (Fig. 2.9b), the alternative male morph that steals spawning from type I males and shows female-typical behaviors and morphology (Brantley and Bass 1994). Instead, IT injection in the POA-AH of females and type II males exerts dose-dependent inhibition of vocal output, while an oxytocin antagonist facilitated vocal output (Fig. 2.9b) (Goodson and Bass 2000a). These results suggest that in species with alternative reproductive tactics such as the midshipman, vasotocin-like peptides are dissociated from gonadal sex and align instead with phenotypic sex.

Placing neurophysiological results within a neuroanatomical framework (Fig. 2.9a) provides a more comprehensive view of nonapeptide action within vocal neural networks, and is made possible by immunocytochemistry studies in midshipman (Goodson and Bass 2000c; Goodson et al. 2003). Based on AVT injection studies reviewed above, projections from AVT-immunoreactive (AVT-ir) neurons in the POA to anterior (AT) hypothalamic areas (Fig. 2.9a) could be important for modulating call duration, while the AVT-ir pathway from the POA to the PAG and nearby PL (Fig. 2.9a) (Goodson and Bass 2000c) likely plays a crucial role in gating vocal initiation, since AVT injections into PL only affect call initiation and not duration (Goodson and Bass 2000c). The importance of the PAG in gating vocal output was highlighted by single unit recordings in the PAG that correlated PAG neuronal firing with call initiation. Furthermore, no fictive calls could be evoked from the POA-AH when the PAG was pharmacologically inactivated (Kittelberger et al. 2006). Thus, AVT terminals originating from the POA could be influencing call initiation via the PAG and adjacent midbrain regions.

Although females and type II males diverged from type I males in nonapeptide sensitivity (see above), no qualitative sex or morph differences in AVT or IT neuroanatomical distribution were observed (Goodson et al. 2003). Like AVT, IT-ir somata were found in the POA and projected to vocal regions in the hypothalamus and midbrain (see above). At hindbrain levels, both AVT-ir and IT-ir terminals could be found within the vicinity of the vocal CPG, especially within the vocal pacemaker region (Goodson and Bass 2000c; Goodson et al. 2003). Since no sex or morph differences were observed in AVT or IT distributions, the differential effects of these peptides on vocal motor output could be mediated by sex- or morph-specific expression of AVT or IT receptors within individual vocal nuclei, which remain to be characterized in midshipman.

2.6.2 *Sex Steroid Hormone Regulation of Vocal Behavior and Neurophysiology*

To our knowledge, the first report of hormone effects on sound production in fish were qualitative observations for the satinfin shiner, *Cyprinella analostana* (formerly *Notropis analostanus*), where testosterone injections led to increased sound production over a period of 5–10 days (Winn and Stout 1960). Since then, more comprehensive studies have shown that steroids exert rapid and robust effects on vocal behavior and the output of the underlying vocal network (Remage-Healey and Bass 2004, 2005, 2007; Remage-Healey et al. 2006).

The classic model of steroid action is the regulation of gene transcription by binding to intracellular cytosolic receptors, forming a steroid-receptor complex that translocates into the nucleus and binds to steroid-specific DNA response elements (Lösel and Wehling 2003). The resulting genomic response could take from longer than 30 min to days (e.g., as suggested for satinfin shiner), whereas rapid,

nongenomic steroid effects occur on the order of seconds–minutes and may involve activation of non-classical membrane receptors in addition to classical intracellular receptors that lead to changes in secondary messenger pathways (Lösel and Wehling 2003). From the field to the laboratory, investigations in vocal teleosts have cemented the role of steroid hormones in regulating vertebrate vocal-acoustic communication over dynamic time scales, from seasonal changes in reproductive states to rapid changes in context- and stimulus-driven vocalization (see Table 2.3 for summary).

11-ketotestosterone (11-KT) is the main circulating androgen found in many male teleosts (Bentley 1998), including in type I males of the plainfin midshipman (*P. notatus*) and the Lusitanian toadfish (*Halobatrachus didactylus*), as well as in male oyster toadfish (Brantley et al. 1993b; Genova et al. 2012; Knapp et al. 1999b; Modesto and Canário 2003; Fine et al. 2004; Sisneros et al. 2004b; Maruska et al. 2009). By contrast, testosterone, which is the main circulating androgen in female teleosts, is predominant in both type II male and female midshipman and Lusitanian toadfish (Brantley et al. 1993b; Modesto and Canário 2003; Sisneros et al. 2004a). Circulating 11-KT levels peak during the pre-nesting and nesting season in the summer, coinciding with peak levels of acoustic courtship and spawning activity (Modesto and Canário 2003; Sisneros et al. 2004b). Several field studies in the plainfin midshipman strongly implicated androgens in facilitating vocal behavior in individually identified fish by showing significant correlations between plasma 11-KT levels and advertisement calling activity (Genova et al. 2012), including over rapid time-scales (within 5 min) in response to acoustic playback challenge (Remage-Healey and Bass 2005). A causal relationship linking androgens to increased vocalization was established by field studies in the Gulf toadfish, where exogenous food delivery of 11-KT rapidly increased the rate of natural calling behavior within 10–20 min (Remage-Healey et al. 2006). Complementing field behavioral studies, in vivo neurophysiological investigations in type I male midshipman and male Gulf toadfish (this species exhibits no evidence of alternative male morphs) showed that systemic injections of 11-KT, but not testosterone, rapidly elevated the duration of the vocal pattern generator output within 5 min, an effect that lasted for at least 2 h (Remage-Healey and Bass 2004; Remage-Healey et al. 2006). Thus, 11-KT is the main androgenic steroid that rapidly facilitates male vocal behavior, likely by exerting non-genomic actions via classical intracellular receptors, which was supported by receptor-specific antagonist treatments during fictive calling (Remage-Healey and Bass 2004). Steroid actions are presumably taking place within specific vocal nuclei that express androgen receptors (ARs) (see Fig. 2.10a) (Forlano et al. 2010), though effects of focal steroid injections directly into vocal nuclei remain to be investigated.

In the Gulf toadfish, field experiments showed that cortisol levels were elevated in experimental males who began vocalizing after two days of being placed among a natural population of calling males (Remage-Healey and Bass 2005). Unlike 11-KT, cortisol levels did not rapidly elevate in response to acoustic playbacks of tones resembling natural toadfish boatwhistle calls (Remage-Healey and Bass 2005). Food delivery of cortisol to naturally vocalizing toadfish also did not rapidly

Table 2.3 Summary of steroid hormone profiles and effects of hormone injection on vocal behavior and central pattern generator output (measured in fictive call duration)

Ref	Species	Measured parameter	11-KT	T	E2	Cort
1	*Cyprinella analostana,* formerly *Notropis analostanus*	Number of sounds	N.M.	↑ In ♂ 5–10 days post-injection	N.M.	N.M.
2	*Porichthys notatus*	Plasma levels	**Higher** in hummers than non-hummers	**Equal** between hummers and non-hummers	N.M.	N.M.
3	*Opsanus beta*	Plasma levels	↑ In calling population ↑ After playback	N.M.	N.M.	↑ In calling population ✖ After playback
4	*Opsanus beta*	Natural call rate and fictive call duration	↑ Natural call rate and ↑ fictive call duration in ♂	✖ Fictive call duration in ♂ and ♀	✖ Fictive call duration in ♂ and ♀	↑ Fictive call duration in ♂ and ♀
5	*Porichthys notatus*	Fictive call duration	↑ In I♂	✖ In I♂	↑ In I♂	↑ In I♂
6	*Porichthys notatus*	Fictive call duration	✖ In II♂ and ♀	↑ In II♂ and ♀	↑ In II♂ and ♀	↓ In II♂ and ♀

I♂, II♂, ♀ respectively represent type I males, type II males, and female midshipman (*Porichthys notatus*)
↑, ✖, ↓ respectively denote an observed increase, no change, and decrease in measured parameter
Abbreviations: *11-KT* 11-Ketotestosterone; *T* testosterone; *E2* 17β-estradiol; *Cort* cortisol; *N.M.* not measured
Ref references; (1) Winn and Stout (1960); (2) Genova et al. (2012); (3) Remage-Healey and Bass (2005); (4) Remage-Healey and Bass (2006); (5) Remage-Healey and Bass (2004); (6) Remage-Healey and Bass (2007)

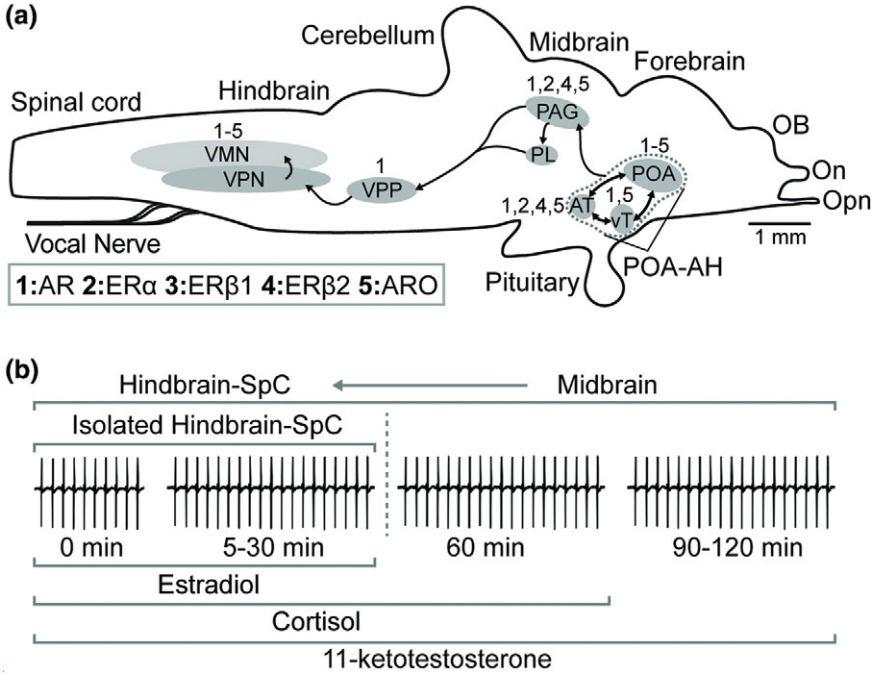


Fig. 2.10 Steroid sensitivity of vocal pattern generator. **a** Steroid receptors and aromatase (ARO) localization mapped onto the descending vocal network based on studies in plainfin midshipman (*Porichthys notatus*). Abbreviations: *AR* androgen receptor; *AT* anterior tuberal nucleus; *ER* estrogen receptor; *OB* olfactory bulb; *On* olfactory nerve; *Opn* optic nerve; *PAG* periaqueductal gray; *POA-AH* preoptic area-anterior hypothalamus; *VMN* vocal motor nucleus; *VPN* vocal pacemaker nucleus; *VPP* vocal pre-pacemaker; *vT* ventral tuberal hypothalamus. Numbers within or apposing each nucleus indicate localization of corresponding receptors or aromatase. Note that "1–5" indicate the presence of all receptors and aromatase, whereas *1*, *5* indicate the presence of only ARβ and aromatase. *Dotted lines* enclose the reciprocally connected vocal nuclei within the POA-AH. **b** Summary for type I male midshipman fish of rapid steroid hormone action on the spike-like vocal nerve motor volley, or fictive call, that is the output of vocal central pattern generator activity; adapted from (Remage-Healey and Bass 2006). The isolated hindbrain-spinal cord (SpC) is sufficient for responding to rapid stimulatory actions of steroids only up to 30 min. Intact descending midbrain input is necessary for sustained effects of steroids lasting up to 2 h. Depicted on the bottom is temporal specificity of estradiol-, cortisol-, and 11-ketotestosterone-induced increases in fictive call duration that directly determines natural call duration; each nerve spike corresponds to a single sound pulse

increase call rate, unlike what was observed for 11-KT (Remage-Healey et al. 2006). Playback experiments using foraging sounds of Atlantic bottlenose dolphins, major predators of soniferous fish including toadfish, led to decreases in call rate within 5 min by toadfish housed in enclosures in their natural habitat, while increasing their plasma cortisol levels (Remage-Healey et al. 2006). Although this study suggested a negative correlation between cortisol and natural call rate, exogenous cortisol delivered to Gulf toadfish did not change their call rate

(Remage-Healey et al. 2006). These field results would lead to predictions that either cortisol has no direct effect on natural vocal behavior or that it inhibits calling specifically in the presence of sonic predators.

Results from in vivo neurophysiology experiments, however, strongly support a regulatory role for cortisol at the vocal neural circuit level. Peripheral intramuscular cortisol injections induced a rapid increase in fictive call duration within 5 min that lasted at least 2 h in both male and female Gulf toadfish (Remage-Healey et al. 2006). Similarly, neurophysiology experiments in type I male midshipman showed that intramuscular injections of cortisol rapidly increased fictive call duration within 5 min, which lasted for 60 min, an effect blocked by the glucocorticoid receptor antagonist mifepistone (Remage-Healey and Bass 2004). In summary, although strong evidence supports cortisol action within vocal neural circuitry, a direct demonstration of consequences for vocal behavior is still lacking.

Similar to nonapeptides, steroid action on the vocal neural network in midshipman is sex- and male morph-specific (Remage-Healey and Bass 2007). In contrast to the stimulatory action of cortisol on the type I male vocal circuit, intramuscular injections of cortisol in type II male and female midshipman rapidly inhibited fictive call duration, which also lasted from 5 to 60 min (Remage-Healey and Bass 2007). Male morph-specific patterns in the abundance of corticosteroid receptors and steroid synthetic enzymes in different brain regions could mediate observed differences in neurophysiology, although such receptors have yet to be localized to specific parts of the central vocal network (Arterbery et al. 2010a, b).

As mentioned earlier, 11-KT, but not its androgenic precursor, testosterone, stimulated the output of the vocal circuitry in type I male midshipman (Remage-Healey and Bass 2004). In type II males and females, the opposite is true, where testosterone rapidly increased vocal motor output and 11-KT had no effects (Remage-Healey and Bass 2007). Testosterone's rapid action in type II males and females only lasted for 45 and 30 min, respectively, consistent with testosterone being 10 times less potent than 11-KT in activating teleost ARs (Bentley 1998), though other factors could have accounted for sex and morph differences in androgen sensitivity. Although type II male neuroendocrine sensitivity profiles align mostly with females instead of type I males, sex-specific divergence between type II males and females was discovered by the use of cyproterone acetate, an AR blocker, and fadrozole, an inhibitor for aromatase, the enzyme that converts testosterone into estradiol (Remage-Healey and Bass 2007). In these experiments, pre-injection of cyproterone acetate blocked the ability of testosterone to increase fictive call duration in type II males but not in females. By contrast, pre-injections of fadrozole blocked testosterone's ability to increase call duration in females but not in type II males. These results suggest that in type II males, testosterone acts through ARs to increase vocal output, whereas in females, testosterone is converted into estradiol, which presumably acts through estrogen receptors (ER) (Fig. 2.10a) to increase vocal output.

Estradiol injections rapidly increased fictive call duration in both sexes and male morphs of midshipman (Remage-Healey and Bass 2004, 2007), but were not effective in either male or female Gulf toadfish (Remage-Healey et al. 2006).

Furthermore, while testosterone induced rapid increases in fictive call duration in type II male and female midshipman (Remage-Healey and Bass 2007), it was ineffective in female Gulf toadfish (Remage-Healey et al. 2006). In fact, the only steroid tested that exerted rapid effects on female Gulf toadfish fictive vocalization was cortisol (Remage-Healey et al. 2006). These differences reveal striking species divergence in steroid hormone regulation of vocal neural networks.

Interestingly, when the descending midbrain input to the hindbrain vocal pattern generator was severed in type I male midshipman, the effects of 11-KT and cortisol on fictive call duration did not last more than 30 min, compared to 2 h (11-KT) and 45 min (cortisol) in intact preparations (Fig. 2.10b) (Remage-Healey and Bass 2004). In male Gulf toadfish, a similar reduction in the duration of call potentiation resulting from 11-KT and cortisol was observed when descending midbrain input into the hindbrain vocal pattern generator was severed (Remage-Healey et al. 2006). These results suggest that steroid action within forebrain and midbrain vocal acoustic centers mediate sustained stimulation of vocal behavior, while steroid action within the hindbrain alone is sufficient to elicit rapid increases in vocalization (Remage-Healey and Bass 2004).

In summary, complementary field and neurophysiological studies in toadfishes demonstrate steroid regulation of dynamic social behaviors that fluctuate on the minute-by-minute time scale, and neurophysiological studies have uncovered both sex- and male morph-specific steroid sensitivities of the midshipman vocal network (Table 2.3) (Remage-Healey et al. 2006). The ability to monitor changes in natural vocal behavior in the field together with the highly accessible and predictive readout of the vocal neural network makes toadfishes an ideal model for studying hormone regulation of vocal behavior at various levels of analyses.

2.6.3 Central Sites of Steroidogenesis and Hormone Action

2.6.3.1 Aromatase

All steroids are derived from cholesterol and converted by steroidogenic enzymes to bioactive or inactive metabolites (Bentley 1998; Adkins-Regan 2005). The brain itself contains all the necessary enzymes to locally synthesize steroids in addition to being capable of responding to and processing circulating steroids derived from the gonad or the adrenal gland (Baulieu and Robel 1990; Forlano et al. 2006; Saldahna and Schlinger 2008). One of the steroidogenic enzymes that has received the most attention with respect to regulating reproductive and courtship behavior is aromatase, which converts aromatizeable androgens such as testosterone into estrogens (Adkins-Regan 2005). Since estrogens cannot be converted back to androgens, aromatase levels in the brain regulate the local ratio of testosterone and estrogens to modulate steroid-dependent behaviors (Adkins-Regan 2005; Remage-Healey et al. 2008). It is important though to note that the more potent androgens, such as 11-KT in teleosts and 5α-dihydrotestosterone in other tetrapods, cannot be aromatized and

thus cannot be shunted from exerting androgen action (Bentley 1998; Adkins-Regan 2005). Neuroanatomical studies in midshipman have localized steroid receptor messenger RNA (mRNA) and protein expression as well as the estrogen synthetic enzyme aromatase within forebrain, midbrain, and hindbrain regions controlling vocal behavior. Figure 2.10a summarizes steroid receptor expression within the midshipman vocal network.

The teleost brain has a remarkably high level of aromatase activity when compared to other taxa (Callard et al. 1990; Forlano et al. 2001, 2006). In plainfin midshipman, studies that quantified enzymatic conversion of radioactive ligand found that the forebrain had higher aromatase activity compared to midbrain and hindbrain (Schlinger et al. 1999). Furthermore, aromatase activity in the hindbrain region containing the vocal pattern generator of females and type II males was significantly higher than in type I males (Schlinger et al. 1999). Type II male hindbrain had higher aromatase activity than type I males even after castration, indicating that the male morph difference in aromatase activity was not dependent on circulating gonadal testosterone (Schlinger et al. 1999). These sex and morph differences were later confirmed by quantitative PCR (qPCR) of midshipman aromatase in isolated hindbrain tissues with the VMN removed (Fig. 2.11) (Fergus and Bass 2013). These results suggest that differences in constitutive hindbrain aromatase activity could prevent testosterone from masculinizing the vocal pattern generator in females and type II males.

Neuroanatomical distribution patterns within the midshipman vocal network were extensively characterized by in situ hybridization using midshipman-specific aromatase mRNA probes, and immunocytochemistry using midshipman-specific aromatase antibodies (Forlano et al. 2001). Overall aromatase immunoreactivity (ir) confirmed brain region differences in aromatase activity summarized above. Aromatase containing cells were identified as glial and not neuronal (Forlano et al. 2001).

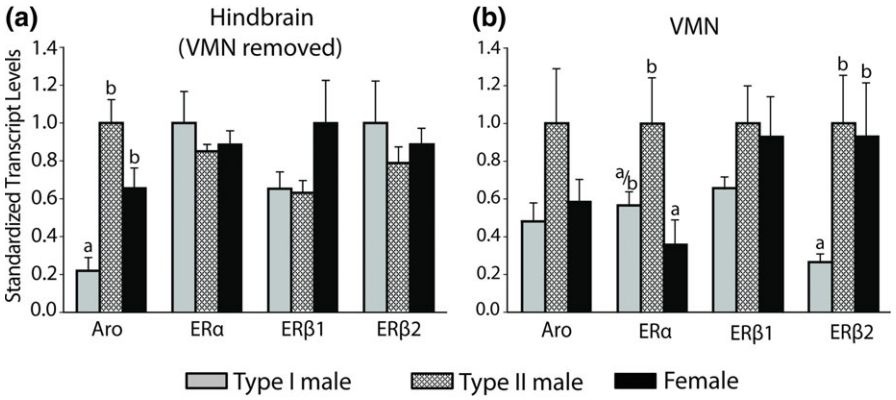


Fig. 2.11 Sex and morph differences in hindbrain estrogen sensitivity. Sex- and male morph-specific mRNA expression profiles of aromatase (*Aro*) and estrogen receptor (*ER*) subtypes, quantified by real-time PCR, for vocal motor nucleus (*VMN*) dissected in toto and for surrounding hindbrain region without VMN. Taken from Fergus and Bass (2013)

Aromatase-ir and mRNA expression were abundant throughout the vocal network, including the forebrain POA, midbrain PAG and hindbrain VMN (Forlano et al. 2001). Dense aromatase-ir containing glial cells around the dorsolateral margin of VMN extended their processes throughout the nucleus in a radial-like pattern. Thus, aromatase is found within every major node of the midshipman vocal control network, likely providing sources of locally converted estradiol, which was shown to rapidly increase fictive call duration (Table 2.3; Fig. 2.10b) (Remage-Healey and Bass 2004, 2007).

2.6.3.2 Androgen and Estrogen Receptors

Two isoforms of AR (ARα and ARβ) have been characterized in teleost fish that are sensitive to both 11-KT and testosterone (Sperry and Thomas 1999, 2000; Ogino et al. 2009). Compared to ARα, teleost ARβ shows higher sequence identity to tetrapod ARs and therefore likely resembles the ancestral AR in function (Sperry and Thomas 1999, 2000; Ogino et al. 2009; reviewed in Forlano et al. 2010). Midshipman ARβ mRNA was highly expressed in the POA, AT and ventral tuberal nuclei, sites that evoke fictive calls when electrically stimulated (Goodson and Bass 2000a, c; Kittelberger et al. 2006; Forlano et al. 2010). ARβ mRNA was also found in midbrain vocal-acoustic regions, such as the PAG, as well as in hindbrain vocal pattern generator, including the VPP and VMN (Forlano et al. 2010). An early report in the oyster toadfish using steroid autoradiography reported a sparse distribution of ARs in the medulla at the level of VMN, including a region that likely corresponds to VPP (Fine et al. 1996). It is unclear if ARα and ARβ receptor subtypes exhibit divergent localization or expression patterns in the midshipman brain, as have been shown in other teleosts (Harbott et al. 2007). Furthermore, what accounts for the differential sensitivity of the vocal network of male morphs and sexes to 11-KT versus testosterone is also not clearly understood. We know from studies in the peripheral vocal muscle that ARα mRNA expression is elevated in type I males who were actively humming at the time of tissue collection compared to males who were not, whereas ARβ was higher in non-humming type I males (Genova et al. 2012). Perhaps then, divergent sex- or morph-specific AR subtype profiles in vocal nuclei could account for differences in neural androgen sensitivity.

Several ER, including ERα, ERβ1, and ERβ2 have been sequenced and localized to vocal control regions in the midshipman brain (Fig. 2.10a) (Forlano et al. 2005; Fergus and Bass 2013). Strikingly, all three ERs are strongly expressed in the POA, previously shown to express aromatase (see above; Forlano et al. 2001, 2005; Fergus and Bass 2013). In the hypothalamus, ERβ2 showed robust expression in vocally active AT (Goodson and Bass 2000a; Fergus and Bass 2013). At midbrain levels, only ERβ2 was distributed within the PAG. In the hindbrain vocal pattern generator, ERα, ERβ1, and ERβ2 were all strongly expressed within VMN neurons (Forlano et al. 2005; Fergus and Bass 2013). Interestingly, females and type II males showed stronger staining for ERβ2 in VMN neurons compared with type I males, which was verified by qPCR using the isolated VMN (Fig. 2.11) (Fergus and

Bass 2013). These qPCR studies also revealed a significant elevation of ERα within type II male VMN compared to female VMN (Fig. 2.11). These sex and morph differences in ER expression were specific to the VMN, as qPCR of ER expression in surrounding hindbrain tissue did not show any significant differences (Fig. 2.11).

Taken together, the brain of toadfishes can be characterized as an exquisitely sensitive endocrine organ that directs the expression of adaptive vocal behavior under a variety of social and environmental contexts, including territorial defense, mate attraction, and predator avoidance.

2.6.4 *Hormone Regulation of Daily Rhythms in Vocal Behavior*

Midshipman courtship vocalization follows dramatic daily and seasonal rhythms, occurring at night during the summer breeding season (Brantley and Bass 1994; McIver et al. 2014). The daily nocturnal peak in vocal activity is similar to that observed for other toadfish species and sonic fish (Locascio and Mann 2008; Rice and Bass 2009). In vivo physiological studies show that the duration of type I male fictive calls increases and threshold levels decrease in midshipman tested during the summer at night (Rubow and Bass 2009). This nocturnal-dependent increase in vocal excitability is abolished in fish held under 5 days of constant light, and is substantially increased in fish held under constant darkness (Rubow and Bass 2009). Although we have accumulated strong evidence for the rapid modulation of fish vocal behavior by steroids and neuropeptides, the hormone control of rhythmic vocal behavior over the daily cycle has not been well studied.

Since the vertebrate time-keeping hormone melatonin shows elevated levels at night that is abolished under constant light (Falcón et al. 2010), we recently tested whether changes in vocal excitability under constant photo-regimes are melatonin-dependent in type I male midshipman (Feng and Bass 2014). Melatonin implants in fish held under 5 days of constant light decreased fictive call threshold and increased fictive call duration, effectively rescuing the decreased vocal excitability seen in control animals. However, pilot studies did not show a rapid effect of melatonin on the order of minutes–hours, suggesting that melatonin's actions on increasing vocal network excitability may depend on its interaction with other effectors, such as the steroid hormones and/or neuropeptides discussed above. It remains to be shown that melatonin receptors are expressed in vocal brain regions as seen in songbirds (Gahr and Kosar 1996; Bentley and Ball 2000; Jansen et al. 2005) and whether receptor expression levels change across the day. Future work also needs to test whether fish vocalizations are under endogenous circadian control by recording vocal behavior under constant photo-regimes, which has recently been demonstrated in a songbird (Wang et al. 2012). Thus, although we have some evidence for a melatonin-sensitive vocal network, the cascade of cellular events leading to daily and seasonally rhythmic vocal behaviors remain to be defined.

2.7 Future Directions

Fishes have become a prime model system to understand not only the neuronal patterning of vocalizations, but also the modulation of vocal behavior. However, most of these studies have so far been performed in only one group of fishes, toadfishes. There is an urgent need for comparative neurophysiological data at the level of vocal nerve output and of premotor and motor circuitry. We need to know whether the mechanisms of vocal pattern generation found in toadfishes hold true for other fish species using the same or a different vocal apparatus. While we now know how simple sounds such as grunts are patterned, we are still lacking any data demonstrating how CPGs pattern calling sequences like grunt trains or repetitive boatwhistles. We also need to better understand how vocal CPGs interact with other CPGs, especially given the proposal that vocal-pectoral appendage and vocal-respiratory coupling in fishes may be the evolutionary antecedent of vocal-gestural and vocal-respiratory coupling exploited among tetrapods during the course of vertebrate evolution (Bass and Chagnaud 2012). To best elucidate these and related questions, novel recording systems to acquire physiological data in freely behaving animals, like those being pioneered in birds and mammals, are essential.

Although we have strong evidence for steroid and neuropeptide regulation of fish vocal behavior over rapid timescales, how hormones regulate daily rhythms in vocal behavior is still unknown. Specifically, are fish vocalizations under endogenous circadian control and is the time-keeping hormone melatonin involved? Finally, investigations of other neuromodulators, such as dopamine and serotonin, are needed to paint a more complete picture of hormone regulation of vocal behavior and underlying vocal motor networks.

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