

## 1. Introduction

Sexual reproduction with its recombination of genes and genetic variability makes far more new phenotypes available for natural selection than does mutation or any other process, thus gaining an enormous importance in speciation and evolution (Mayr 2001).

Ethological barriers are among the most important premating mechanisms of reproductive isolation to prevent interspecific crosses, and in this sense prevent also the waste of resources involved in gametes production, courtship and copulation (Mayr 1963, Alexander 1967).

Sound production and reception for recognizing and localizing a mate are among the ethological mechanisms of reproductive isolation, and they are used with this purpose (though not exclusively) in mammals, birds, fishes, anurans (frogs and toads), and insects (Mayr 1963).

So far, tympanal hearing organs have been found in seven orders of insects, including species in the most speciose and widespread orders. In some cases, a sense of hearing may have evolved independently more than once within a given order (Hoy 1998). However, this does not mean that tympanal hearing organs are used always and exclusively in sound communication.

When a signal is produced and there is a reduction of uncertainty about the signaler's identity or subsequent behavior or a change in the behavior of the receiver, this is called acoustic communication (Gerhardt and Huber 2002). The advantage of sound communication over chemical, visual and tactile signals is the possibility of rapid transmission of information over long distances with an intrinsic high information content for localization (loudness = approximate distance, position = direction) and identification (species-specific signals). Long distance communication by sound is uncommon among insects other than orthopterans and cicadas due to physical, morphological and physiological constraints related to the small body size (Greenfield 2002).

Suborders Caelifera and Ensifera in Orthoptera are phylogenetically divergent with different patterns of development and morphology in their hearing organs (confirming that ears evolved at least twice in this order, Meier and Reichert 1990), and with sound production involving different body parts (Gwynne 1995, Hoy 1998).

Considering Ensifera, long distance acoustic communication is a good choice to find a mate (Rheinlaender and Römer 1986, Römer and Lewald 1992), complemented with visual cues (Helvesen and Wendler 2000) and vibratory signals (Kalmring and Kühne 1980, Kalmring 1983, Stiedl and Kalmring 1989) in the short range. In fact, many more insects produce and are sensitive to substrate-borne signals than to airborne ones, and there is strong evidence that most tympanal ears evolved from chordotonal mechanoreceptor organs (Boyan 1993, 1998, Yager 1999).

Tympanal hearing is then a subclass of mechanoreception that involves the detection by the receiver of mechanical disturbance from the source, propagated through the air (Hoy 1998). Intraspecific communication was and still is the dominant function of the production and detection of sound in Ensifera (Yager 1999), with fossil records indicating that sound-producing and hearing organs are of equal antiquity and evolved approximately 250 million years ago (Greenfield 1997). The chronologic sequence of sound production and sound detection in Ensifera is still under debate, especially because in the New Zealand wetas (Stenopelmatidae), the plesiomorphic stridulatory condition is often associated with defence behavior and lack of hearing organs (Field 1993). It is hypothesized that after the appearance of echolocating bats 65 to 85 million years ago many Ensifera added the ultrasonic hearing range to detect and evade bats (Yager 1999).

From a phylogenetic perspective the monophyletic Ensifera (Otte 1992) are divided into two clades, the tettigonioids (bushcrickets or katydids) and grylloids (crickets) (Gwynne 1995, 2001). Both of them have tegminal sound production (Elliot and Koch 1985, Heller 1990) and a foretibial hearing organ as a pressure-difference sound detector (Hoy and Robert 1996, Greenfield 1997) associated with the tracheal system (Lakes and Schikorski 1990, Stumpner and Helversen 2001). Species in both clades have a group of auditory neurons that form the auditory network including the peripheral auditory receptor cells (Lakes and Schikorski 1990), prothoracic auditive interneurons (Stumpner and Helversen 2001), brain interneurons (Boyan 1980, Schildberger 1984) and interneurons descending from the brain (Boyan and Williams 1981).

The first level of central auditory processing occurs in the prothoracic auditive interneurons after receiving ipsilateral stimulation from the auditory receptor cells

(Stumpner and Helversen 2001), making the prothoracic auditory network extremely important for behaviors associated with acoustic signals.

Auditory neurons can also be considered as models for sensory networks in general. Their investigation offers the following advantages:

- a) Sounds can be recorded, synthesized, and played back with high fidelity (Gerhardt and Huber 2002).
- b) All of the biologically relevant acoustic information is coded and transmitted by a handful of groups of interneurons that can be recorded, stained and individually identified according to their physiological and morphological responses (Casaday and Hoy 1977, Popov et al. 1978, 1982, Wohlers and Huber 1978, 1982, Boyan and Williams 1982, Atkins and Pollack 1987).
- c) Physiology and activity in interneurons can be related to important behavioral responses (Nolen and Hoy 1984, Harrison et al. 1988, Libersat and Hoy 1991, Stumpner 1997, 1999a, Faure and Hoy 2000a, b).
- d) Manipulation of single interneurons may have drastic effects on behavioral responses (Atkins et al. 1984, 1992, Schildberger and Hörner, 1988).
- e) Unlike in many birds and mammals, the development of species-specific signals and their recognition does not require learning from other conspecific individuals, thus ensuring that much of the diversity within and between members of the species is not attributable to individual differences in acoustic experience. The lack of learning and the short breeding periods also create selective pressure for mating and other decisions to be made without extensive assessments and to be based on relatively simple acoustic criteria (Gerhardt and Huber 2002).
- f) Some possible connections between the interneurons have been suggested by neurophysiological and ultrastructural data (e.g. in crickets: Harrison et al. 1988, Hennig 1988, Horseman and Huber 1994a, b, Hirtz and Wiese 1997).
- g) Neurotransmitters involved in the synaptic connections have been detected in the prothoracic ganglion (Skiebe et al. 1990, Hardt and Agricola 1991, Hardt and Watson 1994, 1999, Hörner et al. 1995, 1996).
- h) Inhibitions in some interneurons have been removed after pharmacological treatments (Harrison et al. 1988, Stumpner 1998, 2002, Faulkes and Pollack 2001).

- i) Chemical and physical methods are available to eliminate interneurons (Mountcastle and Jay 1993).
- j) Comparisons between related species at the level of single identified neurons can be done to understand potentially underlying evolutionary trends (Stumpner 2002), and,
- k) Animals with acoustic communication face a set of common problems, with remarkably convergent solutions in insects and vertebrates (Gerhardt and Huber 2002, Stumpner 2003). Some studies indicate that there are parallels between vertebrate and insect auditory 'perception' in psychoacoustic domains traditionally considered as typical for vertebrates, such as habituation (May and Hoy 1991), precedence effect (Wyttenbach and Hoy 1993), and categorical perception (Wyttenbach et al. 1996).

Elements involved in the auditory network in *Ensifera* include:

- a) Auditory receptor cells with tonotopic organization at the level of the crista acustica (Oldfield 1982, 1988, Oldfield et al. 1986, Stumpner 1996a, Stölting and Stumpner 1998) and in the terminations in the ipsilateral prothoracic auditory neuropile (Oldfield 1983, Römer 1983, Römer et al. 1988, Ebendt et al. 1994, Stölting and Stumpner 1998), except for crickets (Esch et al. 1980, Pollack and Imaizumi 1999), auditory receptor cells give (phasic-) tonic responses and copy the incoming signals, although adaptation may influence signal representation (Stumpner and Helversen 2001).
- b) Local auditory interneurons, especially well-known is a pair of Omega neurons 1 or "ON1" (Casaday and Hoy 1977, Popov et al. 1978, Wohlers and Huber 1982, Schul 1997, Stumpner 2002), which seems to be involved in processing directional information (Selverston et al. 1985), but also some other interneurons that could mediate inhibition of local and intersegmental interneurons (Stumpner 1995, Stumpner and Helversen 2001).
- c) Intersegmental interneurons that include ascending interneurons (AN) with axons projecting and sending information to the brain (Wohlers and Huber 1982, Schul 1997, Stumpner 1997, 2002, Nebeling 2000), descending interneurons (DN) with axons projecting to the posterior ganglia possibly being involved in escape response (Wohlers and Huber 1982, Atkins and Pollack 1987), and T-fibers (TN) with ascending and descending axons (Wohlers and Huber 1982, Atkins and Pollack 1987, Stumpner 1996b, 1999b, Schul 1997)

most likely involved in bat detection (Libersat and Hoy 1991, Faure and Hoy 2000a, b), and,

- d) Abdominal interneurons with main input in the prothoracic auditory neuropile, soma in the seventh abdominal ganglion and the axon projecting to the brain; this interneuron has been detected until now only in *Ancistrura nigrovittata* (Stumpner 1999a), *Barbitistes* species (Stumpner unpublished) and *Leptophyes laticauda* (Molina unpublished).

From an evolutionary point of view, the monophyletic origin of Ensifera predicts a high degree of similarities and homologies at the level of the elements involved in the auditory network (Hennig 1988, Schul 1997, Stumpner et al. 1995, Stumpner 1997). But some differences emerge between tettigonioids and grylloids:

- a) The relatively large number of auditory receptors in crickets (55 to 70) (Eibl 1978, Esch et al. 1980) compared to bushcrickets (15 to 61) (Schumacher 1979, Lakes and Schikorski 1990).
- b) The presence in crickets of a second pair of Omega interneurons called “ON2” whose morphology and physiology is completely different from ON1 and without homologous neurons in bushcrickets (Wohlers and Huber 1982, Lewis 1992, Stiedl et al. 1997), and,
- c) The presence of more than two ascending interneurons (AN) in bushcrickets (Stumpner 2001), compared to only two in crickets (Wohlers and Huber 1982), where the existence of three ascending neurons has been suggested only occasionally (Boyd et al. 1984, Stout et al. 1985).

Specific adaptational processes working since the separation of tettigonioids and grylloids (Schul 1997) and the independent evolution of the two groups (Gwynne 1995) have been suggested as possible explanations for the differences observed between them.

The study of the auditory network constitutes itself an additional experimental approach to understanding the evolutive processes within Ensifera, and in this sense, *Ancistrura nigrovittata*, a southern European phaneroterid species with ethological (Heller and Helversen 1986, Dobler et al. 1994a, b) and neurophysiological studies, constitutes a good starting point since:

- a) Single components of the auditory network are known, characterized and identified (Stumpner 1995, 1996a, b, 1997, 1999a, b, 2001, 2002).

- b) Connections between some cells have been suggested (Stumpner 2001, 2002).
- c) Close correlations between behavioral and neuronal data have been found (Stumpner 1997, 1999a), and,
- d) *Ancistrura* and other bushcrickets allow much easier recording, staining and characterization of single auditory receptor cells (Römer 1983, Kalmring et al. 1990a, b, Stumpner 1996a) than in crickets.

Keeping in mind that there is no possibility to monitor simultaneously all the auditory neurons that are involved in the sound communication system, an intracellular recording approach coupled with photodynamic action was used as an experimental technique to study the auditory network in *Ancistrura nigrovittata*. Photodynamic action (because it requires oxygen) is a physical method to ablate cells (Mountcastle and Jay 1993) in which fluorescent dye molecules are excited with a specific wavelength from a light source (e.g. a laser beam). The dye-excited molecules release excess energy reacting with oxygen and producing singlet oxygen and oxygenated free radicals (Spikes and Livingston 1969, Fridovitch 1978, Spikes 1991) with cytotoxic effects that will cause cellular death of only the cells stained (Miller and Selverston 1979, Martin and Logsdon 1987).

In neurobiological studies the photodynamic technique was first successfully applied in crustaceans (Miller and Selverston 1979), using lucifer yellow CH as a sensitizer (Stewart 1978, 1981). Since then, the same technique has been successfully used to answer different behavioral and physiological questions in crickets (Atkins et al. 1984, 1992, Bodnar 1993, Jacobs and Miller 1985, 1988, Selverston et al. 1985), grasshoppers (Heitler 1997), cockroaches (Libersat et al. 1989, Libersat and Mizrahi 1996, Mizrahi and Libersat 2001), flies (Warzecha et al. 1992), crustaceans (Selverston and Miller 1980, Eisen and Marder 1982, Miller and Selverston 1982a, b, Heitler and Fraser 1989, Fraser and Heitler 1991, Harris-Warrick and Flamm 1997), snails (Cohan et al. 1983, Arshavsky et al. 1985, Elliott and Kleindienst 1990, Kemenes et al. 1991, Kemenes and Elliott 1994, Yeoman et al. 1994), and leeches (Peterson 1983, Gu et al. 1989, Camhi and Macagno 1990, Schmidt and Deitmer 1996).

Some of these studies successfully investigated connections between neurons (Selverston and Miller 1980, Eisen and Marder 1982, Miller and Selverston 1982a, b, Peterson 1983, Arshavsky et al. 1985, Selverston et al. 1985, Fraser and Heitler