Contribution to the study on sound production in clownfishes (Perciformes, Pomacentridae): a multidisciplinary approach

Orphal Colleye

Thesis submitted to obtain the degree of
Doctor in Sciences (Biology)
Academic year 2010-2011

Université de Liège
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Cover page (images from the upper left corner to the bottom right corner):
Amphiprion polymnus (Cook D.C.), *A. akindynos* (Fenner R.), *A. akallopisos* (Adams M.J.), *Premnas biaculeatus* (Honeycutt K.), *A. nigripes* (Rusconi G.), *A. chrysogaster* (Adams M.J.), *A. melanopus* (Patzner R.), *A. perideraion* (Patzner R.), *A. frenatus* (Colleye O.), *A. percula* (Patzner R.), *A. clarkii* (Petersen J.), *A. clarkii* (Patzner R.), *A. latifasciatus* (Plantard L.), *A. ocellaris* (Kitson D.), *A. chrysopterus* (Parmentier E.)

While standing in the water, breast high, admiring this splendid zoophyte [sea anemone], I noticed a very pretty little fish which hovered in the water close by, and nearly over the anemone. This fish was six inches long, the head bright orange, and the body vertically banded with broad rings of opaque white and orange alternatively, three bands of each . . .
I made several attempts to catch it; but it always eluded my efforts -- not darting away, however, as might be expected, but always returning presently to the same spot. Wandering about in search of shells and animals, I visited from time to time the place where the anemone was fixed, and each time, in spite of all my disturbance of it, I found the little fish there also.

Dr. Cuthbert Collingwood (1868): *Rambles of a Naturalist on the Shores and Waters of the China Sea.*

ABSTRACT

Clownfishes (*Amphiprion* spp.) are brightly colored fishes that are members of the Pomacentridae family. They are well known for their mutualistic relationship with tropical sea anemones. These fishes live in social groups in which there is a size-based dominance hierarchy. In this structure where sex is socially controlled, agonistic interactions are numerous and serve to maintain size differences between individuals adjacent in rank. Several studies have reported that vocalizations are associated with agonistic interactions but precise data are lacking and further investigations are needed. The nature of the sound-producing mechanisms also remained unresolved, only resting on few assumptions. Thereby, the main aim of the present thesis is (1) to determine the fundamental components of the acoustic communication in clownfishes, and (2) to explain the mechanisms of sound production. In order to achieve these objectives, the research has been divided into three different axes.

Firstly, the study of the acoustic behaviors shows that no acoustic signal is associated with reproductive activities in clownfishes. Sound recordings during agonistic interactions indicate that these fishes produce two types of sounds. Aggressive sounds are produced during chases and threat displays while submissive (or head shaking) sounds are emitted in reaction to aggressive acts by dominant. Both types of sounds show size-related intraspecific differences in dominant frequency and pulse duration: smaller individuals produce higher frequency and shorter duration pulses than larger individuals, and inversely. Consequently, these sonic features might be useful cues for individual recognition and maintenance of cohesion within the group.

Secondly, the study of the sound-producing mechanism highlights that aggressive sounds are initiated by buccal jaw teeth snapping caused by rapid mouth closure attributed to a sonic ligament. It appears that the swimbladder does not function as a resonator that amplifies and changes the quality of sounds. This structure is a highly damped sound source prevented from prolonged resonant vibrations. On the other hand, the rib cage might be the major acoustic radiator and its resonant properties might explain the size-related variations observed in pulse duration and dominant frequency.

Thirdly, the comparison of aggressive sounds among fourteen clownfish species indicates that the same relationship between fish size and both dominant frequency and pulse duration is spread over the entire group (*i.e.* tribe Amphiprionini). These results highlight all species use a highly conservative mechanism of vocalization. Pulse period appears to be the most variable acoustic feature and could be involved in species-specific recognition, as well as pulse duration and dominant frequency in a lesser extent through their relationship with body size.

Although sound production appears to be restricted to some agonistic behaviors, these sounds seem to constitute an integral part of the peculiar way of life of clownfishes. The aggressive and submissive sounds would also result from two different mechanisms.

RÉSUMÉ

Les poissons clowns (*Amphiprion* spp.), bien connus pour leur relation de mutualisme avec les anémones de mer, font partie de la famille des Pomacentridae. Ces poissons vivent en groupes hiérarchisés sur la base de la taille. Dans cette structure sociale impliquant le respect de la hiérarchie et de la territorialité, les interactions agonistiques sont nombreuses et ont pour but de maintenir des différences de tailles entre les individus de rang adjacent. Plusieurs études ont indiqué la présence de sons au cours des interactions agonistiques mais celles-ci souffrent d'un manque de caractérisation et des investigations plus poussées s'avèrent nécessaires. Seules quelques hypothèses du mécanisme producteur de sons ont été formulées, mais jamais vérifiées. Ainsi, la présente thèse vise à (1) étudier les composantes fondamentales de la communication acoustique chez les poissons clowns, et (2) comprendre les mécanismes de production de sons. Afin de répondre à ces objectifs, la recherche a été divisée en trois volets principaux.

Premièrement, l'étude des comportements acoustiques montre qu'aucun son n'est associé aux différentes activités liées à la reproduction chez les poissons clowns. Les enregistrements au cours des interactions agonistiques indiquent que ces poissons produisent deux types de sons. Les sons d'agression sont émis durant les poursuites et autres comportements de menace alors que les sons de soumission sont produits en réponse à une agression de la part d'un dominant. Ces deux types de sons montrent des variations intraspécifiques liées à la taille : les petits individus produisent des pulsations de courtes durées associées à des fréquences élevées, et inversement. Par conséquent, ces caractéristiques acoustiques pourraient être des signaux utiles dans la reconnaissance de l'individu et le maintien de la cohésion au sein du groupe.

Deuxièmement, l'étude du mécanisme de production des sons d'agression montre qu'ils résultent du claquement des dents portées par les mâchoires buccales suite à la fermeture rapide de la bouche produite par l'action d'un ligament sonique. Il apparaît que la vessie natatoire ne fonctionne pas comme un résonateur qui amplifie et change la qualité des sons. Cette structure est une source sonore hautement amortie et dépourvue de vibrations prolongées. En revanche, la cage thoracique pourrait être le principal radiateur acoustique et ses propriétés résonantes pourraient expliquer les variations liées à la taille observées dans la durée et la fréquence des pulsations.

Troisièmement, la comparaison des sons d'agression entre 14 espèces de poissons clowns indique que la même relation entre la taille du poisson et la fréquence dominante ainsi qu'entre la taille et la durée des pulsations s'applique à l'ensemble du groupe (*i.e.* la tribu des Amphiprionini). Ces résultats révèlent que les espèces utilisent toutes un mécanisme très conservé. La période des pulsations semble être la caractéristique acoustique majeure pour l'identification des espèces. La durée des pulsations et la fréquence dominante seraient également impliquées en raison de leurs variations liées à la taille.

Bien que la production de sons ne soit associée qu'à quelques comportements agonistiques, les sons semblent être indispensable au mode de vie particulier des poissons clowns. En outre, ces sons d'agression et de soumission résulteraient de deux mécanismes différents.

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Tout d'abord, je tiens à exprimer toute ma gratitude à Monsieur le Professeur **Pierre Vandewalle** pour m'avoir accueilli dans son service il y a maintenant six ans, époque à laquelle je terminais ma licence en sciences biologiques. Je tiens à le remercier tout particulièrement pour sa confiance, son soutien ainsi que ses précieux conseils qui ont contribué à la réalisation de ce travail. Enfin, je ne le remercierai jamais assez de m'avoir offert l'opportunité de découvrir à deux reprises Tuléar et son Grand Récif.

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"Domo arigato gozaimass" to Professor **Masaru Nakamura** (Sesoko Station, Okinawa) alias Nakamura sensei for the great interest in my research, for helping to collect fishes, and for providing hospitality and laboratory facilities. I am also grateful to all his lab members (**Alam-san**, **Suwa-san**, **Pandit-san**, **Kojima-san**, **Nozu-san**, **Murata-san**, **Nakajima-san**) for the great time we had together during my unforgettable stay at Sesoko Station. Likewise, I would like to extend my warm thanks to **Kenji Iwao** and **Riki-san** (Akajima Marine Science Laboratory) for their technical support and their help when I lived on this tiny Japanese island named "Aka".

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cette parenthèse à leur sujet sans lancer notre bon vieux refrain qui sera quelque peu modifié pour l'occasion : "It was LEGEN… wait for it… DARY !!! "

Je me dois également de réserver quelques lignes de ces remerciements à **Sarah**. Je tiens à la remercier pour m'avoir toujours soutenu, pour avoir fait preuve d'une immense patience face à toutes mes impatiences et mes sauts d'humeurs, pour ses précieux conseils et pour les inombrables conversations où elle m'a écouté sans relâche ni rouspétance.

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FOREWORD

Clownfishes are brightly colored fishes that became literally famous to the large public in 2003 following the release of “Finding Nemo”, an American computer-animated film produced by Pixar Animation Studios. Yet, it was in the mid-20th century with the advent of SCUBA diving that clownfishes began to be known worldwide. Numerous investigations conducted by naturalists and marine scientists have contributed to underwater discoveries, among them the fascinating natural history of clownfishes.

These fishes are especially well known for their outstanding symbiosis with tropical sea anemones, which was first reported in 1868. This relationship has received a great deal of attention, with numerous investigations focusing on the nature of the symbiosis and the immunity mechanisms which enable the fishes to live unharmed among the stinging tentacles of sea anemones. Admittedly, the intimate relationship between clownfishes and their invertebrate hosts is the more glamorous aspect of their general biology, but the considerable emphasis which has been placed on this topic has tended to obscure other equally interesting areas of research.

To a lesser extent, some attention has also been turned to other aspects of the life history of clownfishes. Among these, some studies highlighted the fact that sound production might play an important role in their behavior. Although clownfishes were reported to produce sounds as early as 1930, the sound-producing mechanism has remained obscure, only resting on hypotheses without experimental verification. Additionally, others studies documented that clownfishes are able to produce different types of sounds during different behavioral context. Unfortunately, the lack of detailed comparisons between acoustic parameters measured and the precision of data presented (small sample sizes) emphasize the need for further investigations in order to understand the aim and scope of sound production in clownfishes.

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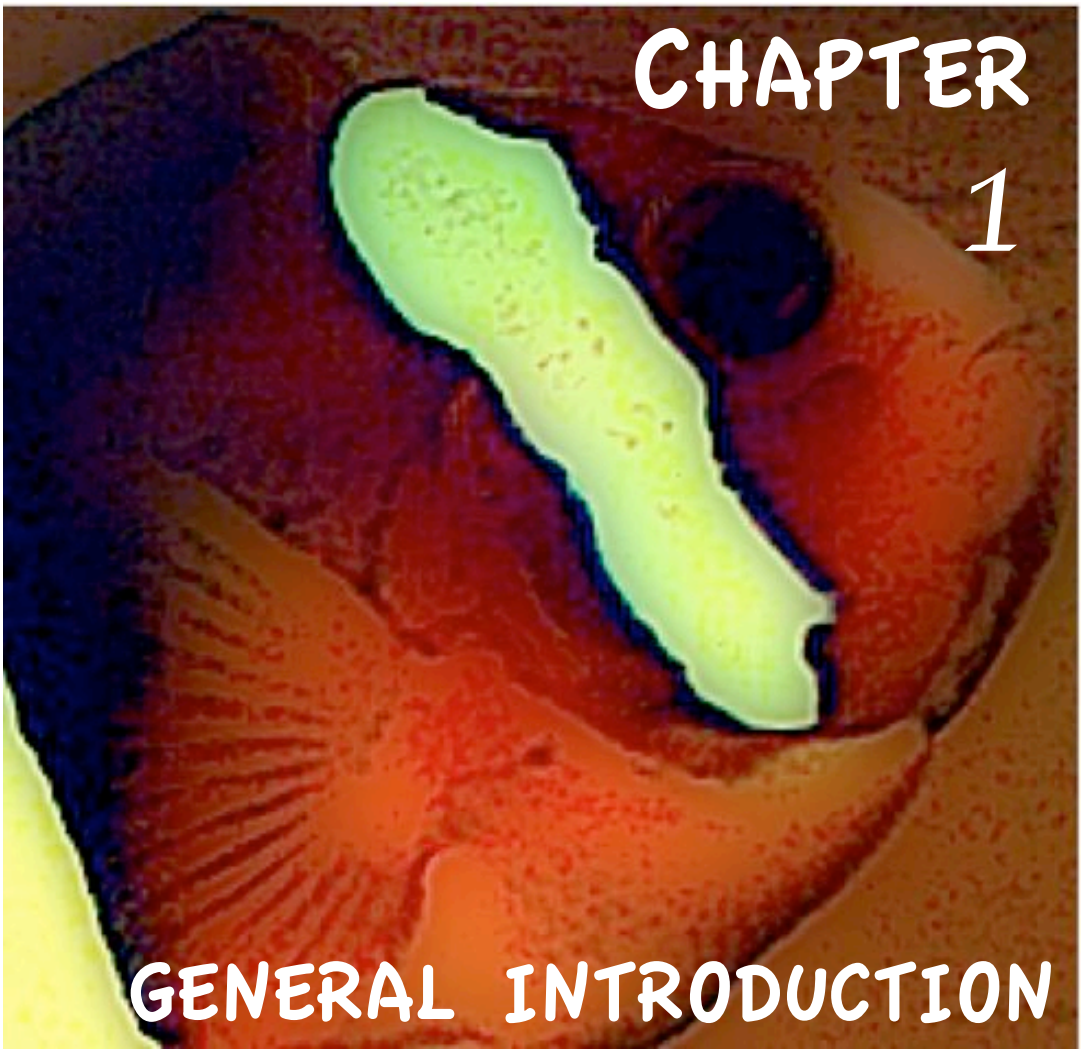

CHAPTER
1
GENERAL INTRODUCTION

1.1. THE CLOWNFISHES

The clownfishes[1] are widely distributed in tropical Indo-West Pacific regions (Figure 1.1), living in coral reef environment (Allen 1972, Fautin & Allen 1992). They can also be found where warm, tropical waters are carried by currents, such as the East coast of Japan (as far North as the latitude of Tokyo for example) (Fautin & Allen 1992). Papua New Guinea, and more precisely the region of Madang, is the most species-rich region of the world where nine species have been reported (Elliott & Mariscal 2001).

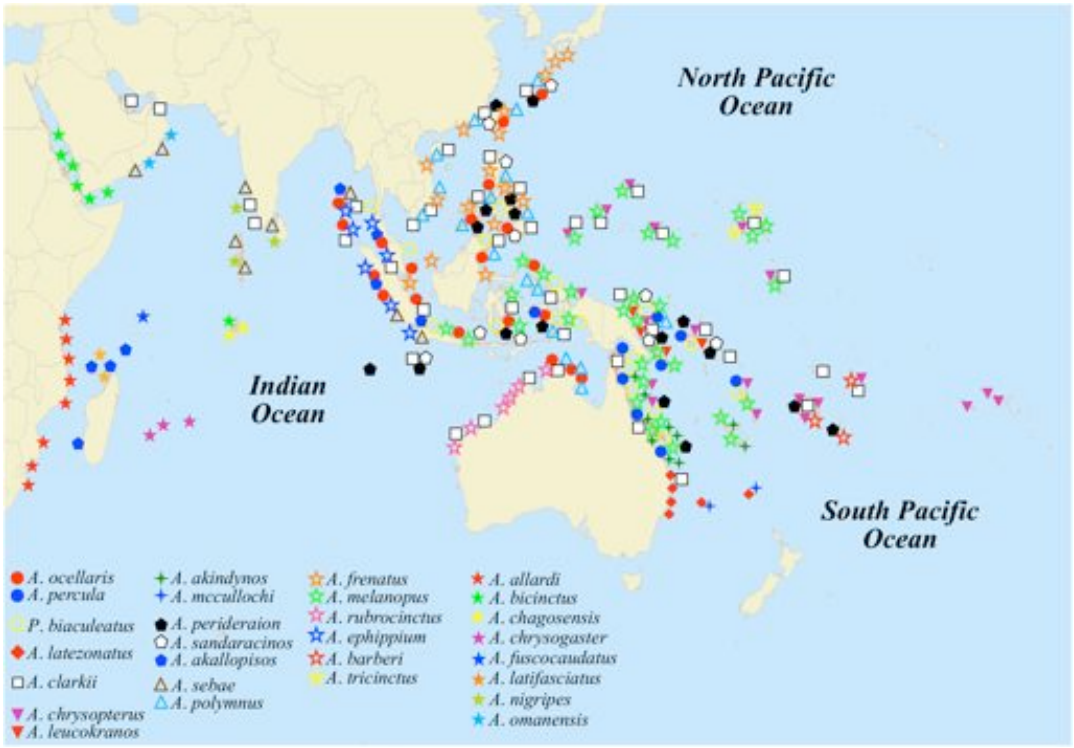


Figure 1.1 Map of the approximate distribution ranges for all clownfish species. Data about the diffusion ranges of the different species were obtained from Allen 1972 and Fautin & Allen 1992.

[1] Note that the term clownfishes instead of anemonefishes will be used throughout the whole manuscript. Anemonefishes may include other fish species than the pomacentrid fishes of the genera *Amphiprion* and *Premnas* (see Randall & Fautin 2002).

1.1.1. Taxonomy and phylogeny

The Pomacentridae, commonly known as damselfishes, are a species-rich family including 28 genera and more than 360 species (Allen 1991, Nelson 2006). According to the last phylogenetic hypothesis (Cooper *et al*. 2009), the family could be divided into five subfamilies (Figure 1.2): Stegastinae (8 genera), Lepidozyginae (1 genus), Chrominae (2 genera), Abudefdufinae (1 genus) and Pomacentrinae (16 genera).

The clownfishes are a group of 29 known species[2] (Allen 1972, Fautin & Allen 1992, Allen *et al*. 2008). These fishes are members of the Pomacentrinae subfamily. Traditional taxonomic studies based on morphological characters (meristic characters, body depth and shape, caudal fin shape, type of teeth, number of white vertical bars; see Allen 1972) have divided the clownfishes into two genera: the monotypic genus *Premnas*, which includes *P. biaculeatus* and the genus *Amphiprion* (Figure 1.3), which includes 28 species that were further divided into four subgenera (*Actinicola*, *Paramphiprion*, *Phalerebus* and *Amphiprion*) and two species complexes within the subgenus *Amphiprion* (*ephippium*-complex and *clarkii*-complex). Allen (1972) hypothesized that the members of the subgenus *Amphiprion* (*clarkii*-complex) are the most ancestral group of clownfishes because they are most similar to other pomacentrids in morphology and behavior. They are relatively deep-bodied, good swimmers, and less dependent on their host anemones for shelter. Most species of the *clarkii*-complex are also able to live in symbiosis with three or more species of host sea anemones (Table 1.1). Later, molecular systematic studies have supported a monophyletic origin of the clownfish species (Elliott *et al*. 1999, Santini & Polacco 2006). Their results allow some hypothesis about the lifestyle and origin of the ancestral clownfish. The more specialized clownfish species such as *A*. *percula* and *A*. *ocellaris* (being slender-bodied with a rounded caudal fin) were assigned to a basal position within the clade. Their analysis supported the progression from specialist to generalist; host

[2] Note that *Amphiprion thiellei* as a valid species was considered to be provisional since its description relies only on two specimens obtained from a pet dealer.

generalization is thus a derived trait that evolved in the clade including *A. clarkii*. Despite all these observations, their evolutionary history still remains poorly explained.

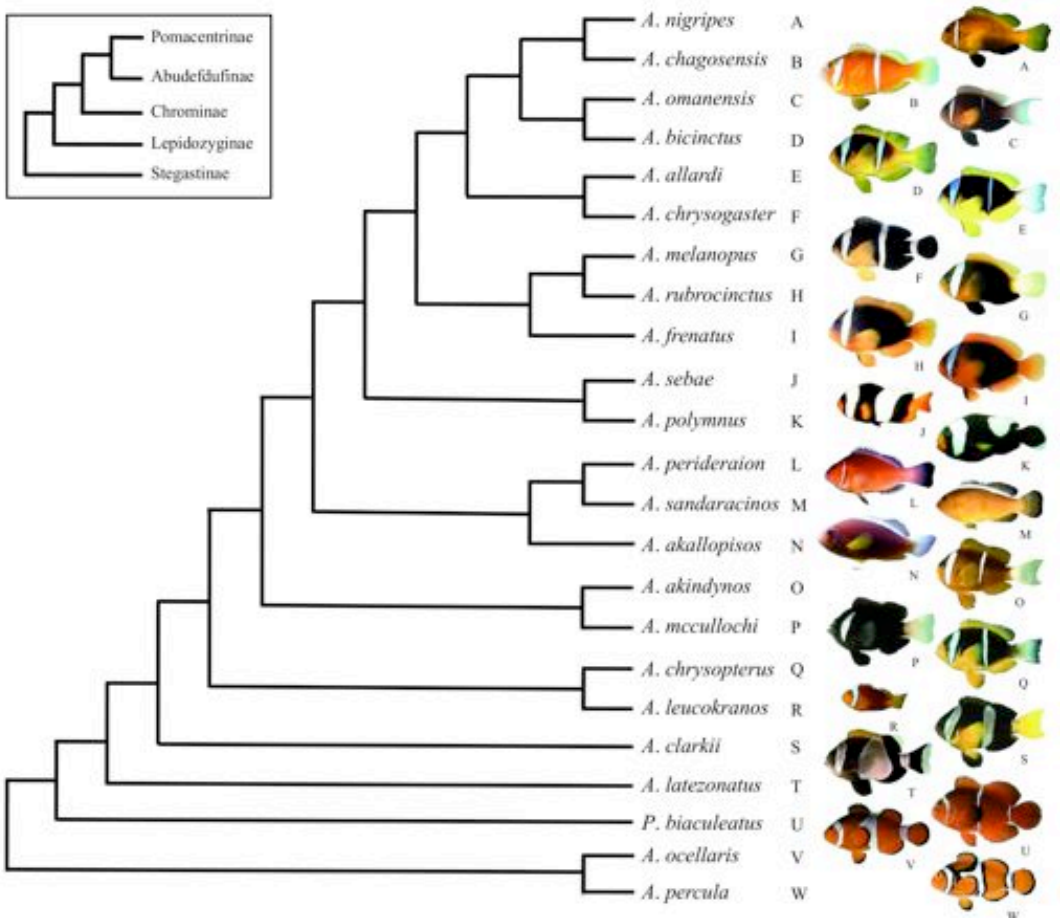


Figure 1.2 Phylogenetic relationships among 23 species of clownfishes (modified from Santini & Polacco 2006). Open square in left upper corner shows a summary of the phylogenetic relationships among the Pomacentridae subfamilies (Cooper *et al*. 2009).

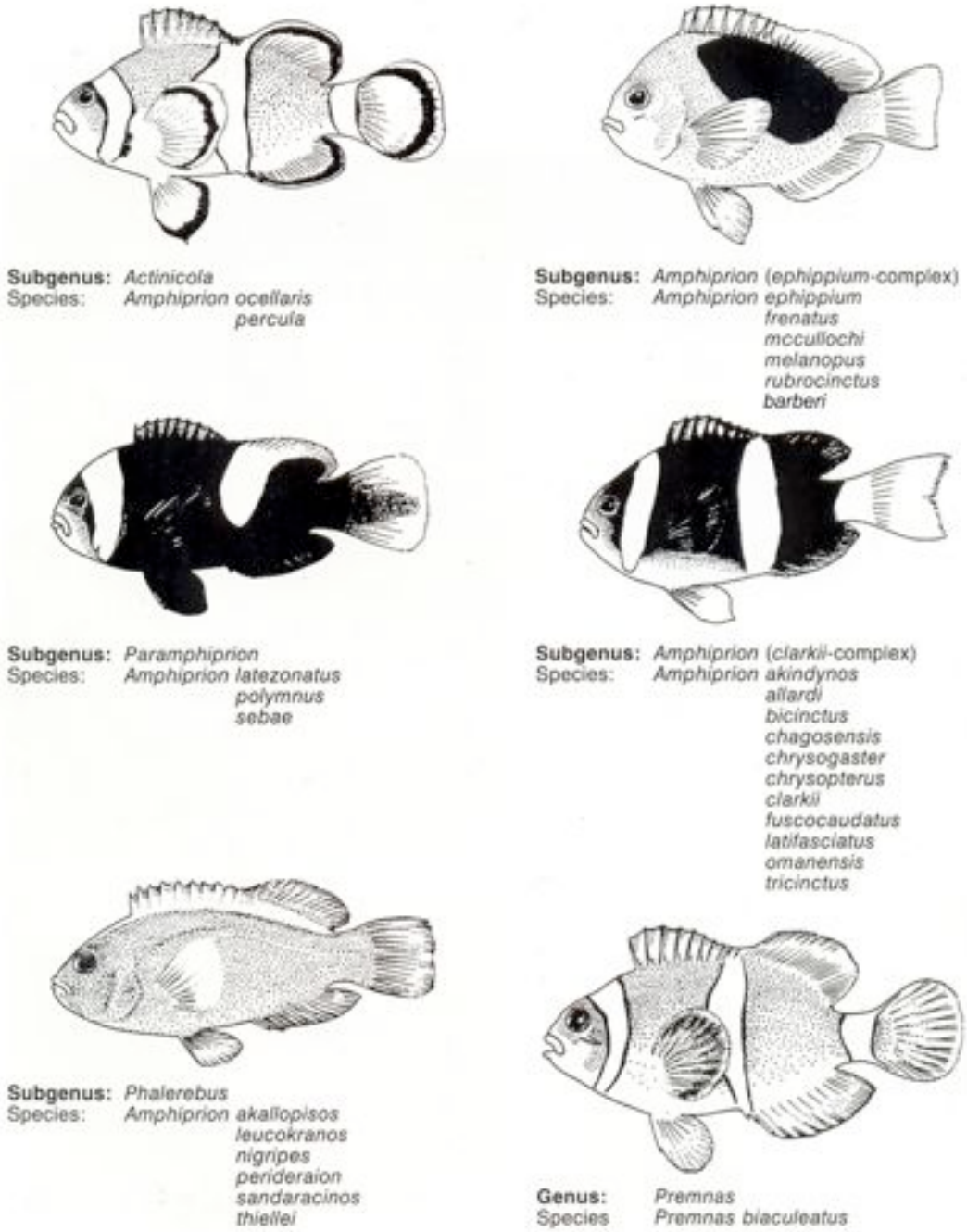


Figure 1.3 The five subgenera of *Amphiprion* and the genus *Premnas* (modified from Allen 1991 and Allen *et al*. 2008).

1.1.2. Morphological characters of clownfishes

The following characters constitute a sufficient diagnostic for differentiating a clownfish (Allen 1972, Nelson 2006; see Figure 1.4) : (1) the dorsal fin is composed of 10 spines (rarely nine or 11) and usually 14-20 soft rays ; (2) suborbitals, preopercular, opercular and interopercular bones have serrated margins and/or sculptured with radiating striae (hence the genus name *Amphiprion*: from the Greek *amphi*, on both sides, and *priōn*, saw); (3) the buccal jaw teeth are uniserial and usually show a caniniform dentition (although some species have a somewhat incisiform dentition, see chapter 5) ; (4) the snout is mostly naked ; (5) the usual color pattern consists of zero to three white vertical bars on a darker background, which is usually various shades of red, orange, brown or black (three species whithout white bars are currently reported; see Figure 1.2). Adult body size ranges from 60 to 125 mm in standard length (SL = length from the tip of the snout to the posterior edge of the caudal peduncle).

Figure 1.4 *Amphiprion clarkii* adult, 102 mm in SL, Shikoku, Japan (Photo by Randall JE).

1.1.3. The *Amphiprion*-actiniarian host relationship

It is interesting to note how numerous species managed to build links when living in competitive areas with high biodiversity such as coral reef environment. One of the most striking examples is probably the fact that clownfishes are able to live unharmed while bathing among the stinging tentacles of a sea anemone (Mariscal 1970a, Lubbock 1980, Fautin & Allen 1992, Elliott & Mariscal 1996). The basic question about the nature of this association seems to be whether the relationship is a mutualistic one whereby both partners make a profit, or whether it is a form of commensalism in which the fish obtains the benefits while the sea anemone is neither harmed nor benefit.

The primary benefit obtained by the fishes is undoubtedly that of protection. Clownfishes are never encountered in nature without their anemone hosts: this is an obligate association for them. Many of them being poor swimmers with truncate or rounded caudal fin, they rapidly become a potential prey for larger fishes (Mariscal 1970b, Allen 1972, Fautin & Allen 1992). Additionally, clownfishes may obtain at least part of their nourishment by feeding on half-digested food rejected by the sea anemone (Verwey 1930, Mariscal 1966, Allen 1972).

Benefits or detriments to the sea anemone are less obvious. Several of the host species are regularly found in nature without symbiotic fish (Verwey 1930, Allen 1972, Fautin 1991), indicating they are less dependent than the fishes upon the association. However, the resident *Amphiprion* can provide a certain degree of protection for its host from the various coelenterate-feeding fishes which inhabit coral reefs (Allen 1972, Godwin & Fautin 1992, Fautin & Allen 1992). Allen (1972) reported that the butterflyfish *Chaetodon auriga* repeatedly attempted to nip at the tentacles of a sea anemone, but was seldom successful due to the territorial defense of the resident clownfish *A. tricinctus*. Another minor benefit is clownfishes may also rid their hosts of the presence of parasitic copepods at the surface of the tentacles (Mariscal 1970c). Scott & Francisco (2006) also revealed that resident clownfishes may feed upon the

gametes being released by their host, which highlights a detriment to the sea anemone but this phenomenon only occurred during food shortage.
Although many biologists have considered this a strictly one-side relationship, and even if the benefits derived by the fish seem to outweigh those gained by the sea anemone; the symbiosis between clownfish and sea anemone is a good illustration of mutualism. Overall, both partners obviously profit by living together, protecting one another from predators.

Of nearly 1000 species of sea anemones distributed all around the world, only 10 represent host to clownfishes (Table 1.1). About a half of clownfish species are specialized to live with a single or a few species of sea anemones while the rest is more generalist and able to live with several host species (Allen 1972, Fautin & Allen 1992, Elliott & Mariscal 2001). Only *A. clarkii* naturally occurs with all 10 host species. Conversely, *Entacmaea quadricolor*, *Heteractis crispa*, *H. magnifica* and possibly *Stichodactyla mertensii* all host between 12 and 14 fish species whereas *Cryptodendrum adhaesivum* and *H. malu* play host to fish of only one species. This specificity has at least four components (Fautin 1991, Fautin & Allen 1992): 1) conditioned or innate preferences by the fish (being the shorter-lived and mobile partner, and thus responsible for the pattern), 2) environmental and ecological requirements (only species that occur in the same geographical area and have similar ecological preferences – sand or reef, deep or shallow – can live together), 3) competition by fish for hosts (the most host specific fishes are those that are usually competitively superior for preferred anemones), and 4) chance.

Regarding this particular relationship, the prime concern was how clownfishes manage to survive in a deadly environment to most fish species. Some hypotheses to account for this invulnerability have been advanced [but reasons for discarding them always existed]: 1) the skin of clownfishes is impenetrable to nematocysts [it may be slightly thinner than that of other damselfishes, and an unacclimated clownfish can be killed by its host's sting (Mariscal 1966)], and 2) while a fish is present, the anemone does not discharge its nematocysts [this cannot explain the invulnerability because an actinian can sting and capture prey while harboring clownfish (Mariscal

1966)]. This implies that the fish, rather than the host, is responsible for its invulnerability to being stung.

Actually, it has been convincingly demonstrated that the source of protection resides in the mucus coating of the fish (Davenport & Norris 1958). From this point of view, two theories confront each other. On the one hand, some authors (Davenport & Norris 1958, Schlichter 1972, Schlichter 1976) proposed that this mucus originates from the host actinian and is transferred to the fish through acclimation behavior. The fish is thereby chemically camouflaged; so basically a fish in anemone's clothing. The fish's behavior of frequently returning to its anemone may serve to maintain its protection. According to this theory, it is their behavior that allows clownfishes to live in this peculiar habitat. On the other hand, Lubbock (1980) stated that the protective mucus originates from the fish itself. He did not find anemone mucus in appreciable amounts on resident clownfish, so anemone mucus on fish may be the result of the protection rather than its cause. Denaturation did not make clownfish mucus more stimulatory to nematocysts, which led Lubbock to conclude that fish mucus do not inhibit nematocyst discharge but it evolved to lack components that stimulate firing. According to this interpretation, the secret to clownfishes peculiar habitat is their unusual biochemistry.

Overall, there is probably truth on both sides even if the question is still open. Although all clownfishes are closely related and share an unusual habitat, they vary in some aspects of their biology, including how many fish occupy a single anemone and which hosts and how many host species they occupy (Table 1.1). Similarly, adaptation may differ among species or combinations of species with behavior and biochemistry probably both playing roles. Fautin & Allen (1992) believed that for fish living with many types of hosts such as *A*. *clarkii*, behavior is likely to be more important to adaptation, whereas for host-specific fish such as *A*. *frenatus*, biochemistry is probably the more significant factor. Brooks & Mariscal (1984) provided evidence that both fish and anemone may be active in forming the symbiosis. The average acclimation time following prolonged separation of *A. clarkii* from its host *Macrodactyla*

doreensis was significantly reduced if the fish had been kept with a surrogate sea anemone made of rubber bands glued to a Petri dish. Thus, it would appear that the fish does produce specific protective mucus in presence of an anemone, which is altered or augmented by a real actinian.

Table 1.1 Host specificity patterns for 29 species of clownfishes (*Premnas* (*P*) and *Amphiprion* (*A*)) and ten species of host sea anemones

Fish species	Anemone species[1]									
	Ca	Eq	Md	Hm	Hc	Ha	Hu	Sh	Sg	Sm
A. barberi[*]										
A. chagosensis[*]										
A. thiellei[*]										
P. biaculeatus		x								
A. frenatus		x								
A. fuscocaudatus										x
A. latezonatus					x					
A. latifasciatus										x
A. mccullochi		x								
A. nigripes				x						
A. sebae								x		
A. akallopisos				x						x
A. ephippium		x			x					
A. omanensis		x			x					
A. polymnus					x			x		
A. rubrocinctus		x							x	
A. sandaracinos					x					x
A. allardi		x				x				x
A. leucokranos				x	x					x
A. melanopus		x		x	x					
A. ocellaris				x					x	x
A. percula				x	x				x	
A. perideraion			x	x	x				x	
A. tricinctus		x			x	x				x
A. bicinctus		x		x	x	x			x	
A. chrysogaster			x	x		x		x		x
A. akindynos		x		x	x	x		x		x
A. chrysopterus		x		x	x	x		x		x
A. clarkii	x	x	x	x	x	x	x	x	x	x

[1] Ca = *Cryptodendrum adhaesivum*, Eq = *Entacmaea quadricolor*, Md = *Macrodactyla doreensis*, Hm = *Heteractis magnifica*, Hc = *H. crispa*, Ha = *H. aurora*, Hu = *H. malu*, Sh = *Stichodactyla haddoni*, Sg = *S. gigantea*, Sm = *S. mertensii*. [*]Host anemone species unknown.

Data about association between host anemone species and fish species were obtained from Fautin & Allen 1992 and Allen *et al.* 2008.

1.1.4. The social structure and sex reversal of clownfishes

Clownfishes live in social groups composed of a breeding pair and between zero to four non-breeders, depending on species and host size (Fricke 1979, Buston 2003). These fishes are typically protandrous hermaphrodite in which sex change is from male to female. Casadevall *et al*. (2009) proposed a schematization summarizing the development of gonads from immature to female status in clownfishes (Figure 1.5).

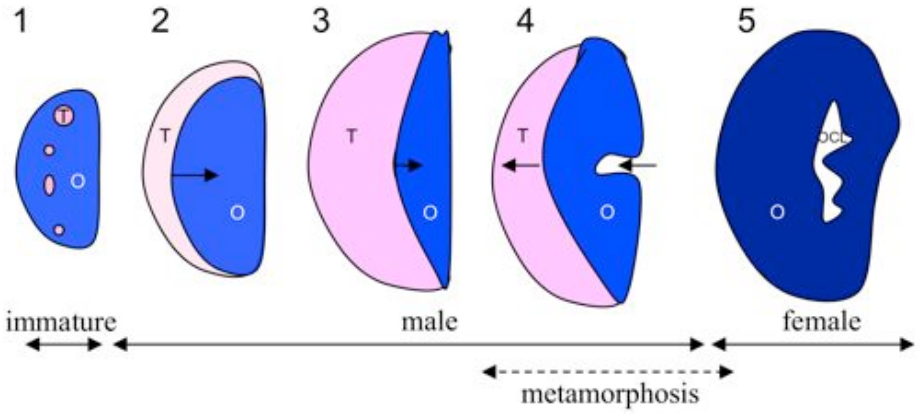


Figure 1.5 Schematization (in transversal section) of the development of gonads from immature to female status in *Amphiprion akallopisos*. Metamorphosis corresponds to the sex inversion. O: Ovarian tissue, OCL: Ovarian Central Lumen; T: Testicular tissue. 1) immature juvenile, 2) subadult male, 3) mature male, 4) sex-change process, 5) functional female (Casadevall *et al*. 2009).

The sex reversal is prevented by social dominance of the larger female over the male (Fricke & Fricke 1977, Moyer & Nakazono 1978). The largest and socially dominant individual is the breeding female (rank 1) whose gonads are functioning ovaries with remnants of degenerate testicular tissue (Fricke 1979, Godwin & Thomas 1993, Casadevall *et al*. 2009).

The breeding male is the second largest (rank 2) with gonads that are functioning testes but also possess non-functioning or latent ovarian cells (Fricke 1979, Godwin & Thomas 1993, Casadevall *et al*. 2009), and the non-breeders get progressively smaller as the hierarchy descends (ranks 3-6). If the dominant female of a group dies, the male changes sex and becomes the

breeding female (its gonads cease to function as testes and the egg producing cells become active). Simultaneously, the largest of the non-breeding individuals becomes the breeding male (Fricke & Fricke 1977, Fricke 1979). This breeding system is thought to have evolved as a mechanism to allow for relatively expeditious pair formation given that sea anemones are generally sparsely distributed and movement among sea anemones by clownfishes is considered to be both rare and risky (Allen 1972, Hattori 1991).

Within this social structure, subordinates benefit from settling in an anemone and queuing for breeding positions, their chances of success being determined by their rank within the group (Buston 2004a). Dominants, however, gain no benefits from subordinates, which are potential challengers for their position. This asymmetry generates evolutionary conflict over subordinate group members, and dominants occasionally evict or kill subordinates that are of similar size to themselves (Allen 1972, Buston 2004b). However, clownfishes have evolved a ploy in order to prevent such conflicts. They adjust their size according to their position in the group hierarchy, maintaining a well-defined size difference with respect to individuals above them in social rank. Hence, a subordinate would never present a threat to its immediate dominant (Buston 2003). Aggressive displays are responsible for maintaining size differences within the hierarchy (Fricke 1979). The dominant female attacks all group members, preferably the two larger ones that are the most likely candidates to take over its position, whereas the functional beta male displays the highest rate of intragroup aggressive acts (Figure 1.6A). In reaction to aggressive acts, group members exhibit an appeasement behavior. The functional beta male most frequently directs it at the alpha female, although many appeasements are also used by the next higher-ranked individual (Figure 1.6B). Overall, the social structure of clownfishes is based on a size-based dominance hierarchy, in which body size ratios are well defined between each dominant and its immediate subordinate in rank. For instance, the growth of individuals in *A. percula* is regulated so that body size ratios between each dominant and its immediate subordinate be about 1.26 (Buston & Cant 2006).

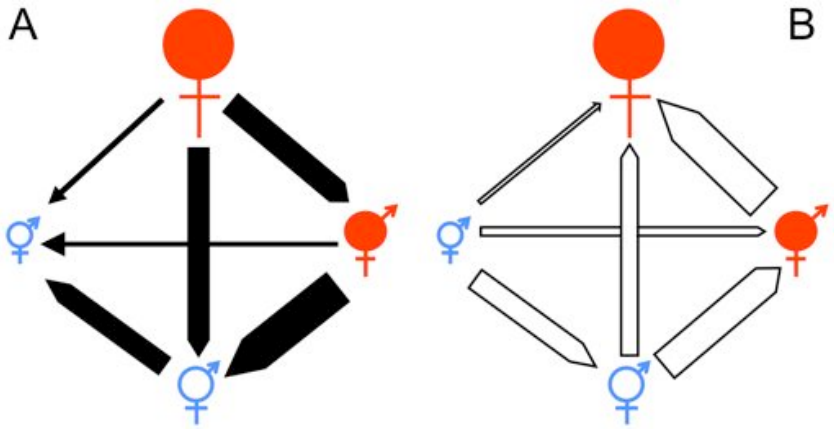


Figure 1.6 Aggressive acts in a group. Red symbol = functional breeding individual. Receiver of aggressive act shown by arrow. (A) Distribution of aggressive acts in % of the total of observed intragroup interactions (= width of arrow). (B) Distribution of appeasement behavior among group members. Treatment of the data is the same as in A (modified from Fricke 1979).

1.1.5. Courtship, spawning and incubation

Spawning occurs throughout most of the year, although there may be seasonal peaks of activity (Allen 1972, Ross 1978). In subtropical or warm temperate seas, reproductive activity is generally restricted to spring and summer, when water temperatures are the highest because cold winter waters inhibit it (Moyer & Bell 1976, Fautin & Allen 1992). Spawning usually occurred within six days before and after the full moon (Allen 1972, Fautin & Allen 1992). Moonlight may serve to maintain a high level of alertness in the male, which assumes most of the nest guarding duties. Moreover, moonlight may stimulate newly hatched larvae to swim towards the surface because they are photopositive (Allen 1972), thereby facilitating their subsequent dispersal by waves.

Courtship is generally stereotyped and ritualized (Allen 1972, Fautin & Allen 1992). Several days prior to spawning, the male selects the nesting site that is frequently situated on a rock positioned next to the sea anemone. Social interactions increase between mates as expressed by chasing, fin-erection, and rapid side by side swimming. Initially the male spends considerable time

clearing algae and debris from the nesting site. Increased nest cleaning activity indicates that spawning will soon take place. On the day of spawning, both fish vigorously clean the nest and much chasing occurs.

Spawning occurs most often during morning hours, and generally lasts from about 30 minutes to more than two hours. The female starts to lay eggs while swimming a roughly circular path around the nest with her belly just brushing the nest surface. The male follows closely behind and fertilizes the spawn.

Throughout incubation, the nest is meticulously guarded and cared for by the male during 7-8 days. Other fishes are aggressively chased from its vicinity, especially potential egg-eaters such as wrasses. The male frequently visits the nest to fan the eggs with his pectoral fins and to remove dead eggs and debris with its mouth (Allen 1972).

1.2. SOUND PRODUCTION IN TELEOST FISHES

Although sound propagation in shallow water may result in signal degradation over short distances (Mann 2006), sound is probably the most effective channel to communicate under water. It offers advantages over other sensory channels. Chemical signals have low production costs, go round barriers and can be carried for long distances in the water currents, but locating the pheromone source can be challenging. Visual signals are dependent on light and can be stopped by barriers existing between the sender and the receiver; so they are not sufficient signals for nocturnal species or those living in turbid environment. Hence, acoustic signals have arisen in situations where darkness reduces the roles of olfactory and visual cues or increases the risk of confusion (Beecher 1989, Sayigh *et al*. 1999).

Sound production in fishes has formed an active area of research. Sound generation in Teleost fishes was first mentioned by Aristotle in his *Historia Animalium* in the fourth century BC (Gohlke 1957). However, it took another 2000 years until the first systematic overviews of vocal fishes were written in the mid-19th century by Müller in 1857. Another important contribution was the report of Duffosé (1874), in which many examples of sound production by fishes were described, including several marine species. Over the past 50 years, it has become clear that many fishes use the acoustic channel. Although sound production may be restricted to some fraction of all fish species, it has to be assumed that a large part of species emit sounds in different behavioral contexts (Tavolga 1971). However, the number of studies citing evidence that fishes use sounds for communication[3] is small. Only a few investigations could demonstrate that sounds function as signals and thus change the behavior of the receiver (Valinsky & Rigley 1981, Riggio 1981, Myrberg 1997a).

[3] A communication takes place when a signal produced by a signaler (the sender) results in a change in the behavior of a receiver. Any communication relies on generation, propagation and detection of emitted signals. Therefore, an efficient communication requires the ability to emit signals, the ability of these signals to be transmitted without modifications, their reception and interpretation. The adaptive advantage of such an interaction can be mutual to both participants (cooperation), or in favour of either the sender (deceit) or the receiver (interception).

In Teleost fishes, the ability to produce sounds was developed independently in distant phylogenetic taxa (Schneider 1967). Currently, taxa of more than 50 fish families include species with the ability to emit sounds (Hawkins 1993). The majority of acoustic signals are used in different behavioral contexts such as aggressive behavior (territorial defense, agonistic interactions, feeding competition) or reproductive activities (mate identification and choice, courtship, discouraging male competitors) (Winn 1964, Tavolga 1971, Hawkins 1993). Fishes have evolved a unique diversity of sound-producing mechanisms among vertebrates, including rhythmical vibration of the swimbladder, rubbing of bones or teeth (stridulatory sounds), and strumming of specialized tendons. Yet, studies on the characterization of both acoustic communication and mechanisms involved in sound production were definitely scarce or were done mainly in a few particular species. At present, some mechanisms remain still unexplained, even in well-studied vocalizing taxa such as cobitids, cyprinids and gobiids.

1.2.1. Sound-producing mechanisms in fishes

There exists no commonly accepted classification of sounds or sound-generating mechanisms in fishes. Schneider (1961) differentiated between mechanisms based on physical principle (stridulation) and those based on the swimbladder. Tavolga (1971) distinguished three major types: stridulatory, swimbladder and hydrodynamic sounds. Note that hydrodynamic sounds are produced when objects such as the fins or body of fishes move through the water (Moulton 1960). Schaller and Kratochvil (1981) suggested a classification based primarily on physical principles: drumming muscle apparatus (direct and indirect types), stridulatory, pneumatic and plucking mechanisms. Ladich and Fine (2006) suggested a classification based on morphological structures which have evolved exclusively for acoustical signaling: the main group includes the swimbladder mechanisms with their innumerable variations (intrinsic, extrinsic directly and extrinsic indirectly vibrating mechanisms; see below for details), whereas the second group

comprises the pectoral mechanisms including pectoral spine rubbing, pectoral tendon plucking and pectoral girdle vibrations.

Unfortunately, it is not yet clear what should be regarded as a communication signal because some types of sounds (*i.e*. hydrodynamic sounds or those resulting from feeding, breathing or gas exchange) are unintentionally produced. Yet, sounds resulting from pneumatic mechanisms appear to be intended. For example, eels are able to emit sounds by eructing gas bubbles from the swimbladder via the pneumatic duct (Fish & Mowbray 1970). These emissions could be just functional because they empty the swimbladder gases; but the spectral structure of the sounds does not change over the course of time, which should be the case since the swimbladder emptied. This implies that the eel must compensate for the gas losses during the emission of bubbles and thus the phenomenon is not simply passive. In the herring, sound is produced by bubble release from the anus because the swimbladder is connected to both the alimentary canal and the anal opening. Such sounds might have a communicative function but this needs to be demonstrated (Wahlberg & Westerberg 2003, Wilson *et al*. 2004).

In this context, we suggest to classify sound-producing mechanisms into two general groups. The first group includes stridulatory mechanisms whereas the second group encompasses swimbladder mechanisms.

A. Stridulatory mechanisms

Stridulatory mechanisms are based on friction of skeletal elements such as teeth, fin rays and spines, or bones. Burkenroad (1930) suggested that haemulids and carangids produce sounds by grating pharyngeal teeth. In the triggerfish (Balistidae) the erection of the specialized dorsal fin spines produces a grating sound when moved in their socket (Schneider 1961). Fish (1953) reported sound production by the friction of adjacent bones in the sea horse, which he attributed to the stridulation of the posterior margin of the skull against some vertebral elements. Numerous catfishes (Siluriformes) produce a squeak by friction of the first pectoral spine within the pectoral

girdle (Pfeiffer & Eisenberg 1965, Fine *et al*. 1997, Heyd & Pfeiffer 2000, Parmentier *et al*. 2009a). In these fishes, the base of the pectoral spine has a dorsal process that bears a series of ridges on the lateroventral surface. The dorsal process slides within a groove in the cleithrum; and pressing the ridges against the groove results in the production of a series of short pulses. Overall, the stridulatory sounds are non-harmonic with a wide range of frequencies from less than 100 Hz to more than 8000 Hz (Tavolga 1971).

B. Swimbladder mechanisms

Swimbladders are used in various ways in sound production. The best-known example is rhythmical vibration of the swimbladder by deformation of its wall under the contraction of specialized muscles called drumming or sonic muscles (Demski *et al*. 1973). Sounds generated by swimbladder drumming mechanisms typically possess a harmonic structure (3-5 harmonics at multiples of the fundamental frequency). Swimbladder sounds have a fundamental frequency ranging from 75 to 300 Hz with most sound energy in the first harmonic, and sometimes in the second or third (Ladich 1999). This range of frequencies corresponds to the muscular contraction rate, placing sonic muscles among the fastest in vertebrates (Rome *et al*. 1996, Fine *et al*. 2001). In the oyster toadfish for instance, a 250 Hz contraction rate generates a sound with a 250 Hz fundamental frequency (Fine *et al*. 2001). Sonic muscles of *Bagre marinus*, *Galeichthys felis*, *Prionotus scitiulus*, *Prionotus carolinus*, *Epinephalus guttatus* and *Holocentrus* sp. have been observed to tetanize between 150 and 400 Hz (Tavolga 1962, Tavolga 1964, Connaughton 2004). This enormous speed of the swimbladder muscles is due in part to their extremely fast relaxation rate (Rome & Lindstedt 1998). This characteristic results from numerous morphological and biochemical adaptations, including specialization of protein isoforms (Hamoir & Focant 1981) and the high concentration of intracellular components (Pennypacker *et al*. 1985, Rome *et al*. 1999). The fibers and myofibrils of sonic muscles are also thinner (Fine *et al*. 1993), and possess a more developed sarcoplasmic

reticulum (Hamoir & Focant 1981, Feher *et al*. 1998). This set of characteristics could facilitate rapid flows of metabolites and calcium (Fine *et al*. 1990, Rome *et al*. 1996, Feher *et al*. 1998).
Interestingly, Parmentier *et al*. (2006a) also showed that carapids (Carapidae, Ophidiiformes) do not use fast-contracting muscles, depending instead on a mechanical decoupling. Carapid fishes drive the bladder with a slow muscle that puts the anterior bladder under tension and then trips a release system that excites sound production. The muscle contraction rate is dramatically slower than in other drumming muscles. In *Carapus acus*, the dominant frequency of 340 Hz in each isolated pulse does not correspond to the contraction rate of its sonic muscle, which tetanizes in the vicinity of 10 Hz. On the other hand, the pulse rate was between 4.5 and 6.9 Hz in *Carapus boraborensis* (Parmentier *et al*. 2003, Lagardère *et al*. 2005), which likely corresponds to the muscle contraction rate. In this case, the contraction rate of the muscle does not determine the main frequency of the pulse but the pulse period.

Based on the origin and insertion of sonic muscles, various types of sonic muscles are distinguished.

Intrinsic sonic muscles attach solely to the swimbladder wall (*i.e*. there is no classical origin and insertion). Typically, two bands of muscles are firmly attached along the lateral surfaces of the swimbladder with fine striated fibers running dorsoventrally (Hawkins & Myrberg 1983). Intrinsic muscles are rather seldom found in fishes but they are well described in Batrachoididae. For example (Figure 1.7), these muscles can be short and concentrated on the anterior poles of the swimbladder as in the midshipman *Porichthys notatus* (Bass & Baker 1991) or can even join each other at the caudal end as in the oyster toadfish *Opsanus tau* (Fine *et al*. 1990, Barimo & Fine 1998). For that matter, the intrinsic sonic muscle of *Opsanus tau* has been the subject of numerous anatomical and physiological studies because it is often considered the fastest vertebrate striated muscle (Tavolga 1964, Rome *et al*. 1996).

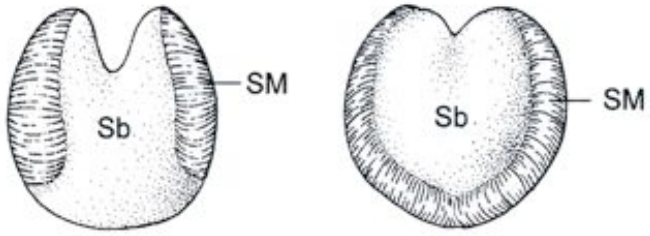


Figure 1.7 Various types of intrinsic sonic swimbladder muscles in two batrachoidid species, *Porichthys notatus* (left) and *Opsanus tau* (right). Sb – swimbladder, SM – sonic muscle (after Bass & Baker 1991).

Extrinsic sonic muscles show a great diversity in their origins and insertions (Jones & Marshall 1953, Demski *et al*. 1973). Generally speaking, these paired muscles insert onto the swimbladder and a neighbouring structure, or insert between two bones of which one is attached to the swimbladder *via* ligaments. This second type of sonic muscles is found in different taxa including Ophidiiformes (Howes 1992, Parmentier *et al*. 2006a), Holocentridae (Carlson & Bass 2000, Parmentier *et al.* 2011) and Sciaenidae (Connaughton *et al*. 1997, Sprague 2000). In some cases, sonic muscles do not make direct contact with the swimbladder but vibrate it indirectly; there are thus direct and indirect swimbladder-vibrating mechanisms. This variation can be illustrated in different catfish families (Siluriformes). Some of them possess indirect vibrating mechanisms consisting of a thin bony plate that attaches to the swimbladder, defined as the elastic spring apparatus (ESA). The elastic spring apparatus is formed by the modified, flexible transverse process of the fourth vertebra (ramus Mülleri). The sonic muscle inserts on the elastic spring apparatus: when it contracts it pulls the elastic spring apparatus forward (acting as protractor muscle), extending the swimbladder; when it relaxes it allows the spring to return to its normal position, the swimbladder is thus caused to pulsate, emitting sound (Alexander 1966). Although the sonic muscle is always inserted on the ramus Mülleri, its origin is usually on the neurocranium as in *Platydoras* and *Arius* (see Figures 1.8A,D) but it also originates on the epaxial musculature near the dorsal fin as in

Synodontis (see Figure 1.8B). On the other hand, the sonic muscle may also act directly when it originates on the transverse process of the fourth vertebra, and inserts on the ventral and ventro-lateral surfaces of the swimbladder as in *Pimelodus* (see Figure 1.8C).

Besides the action of drumming muscles, the swimbladder can be excited by others mechanisms. This structure is thought to function as a drum in some triggerfish species that produce sounds by beating or rubbing pectoral fins against areas of the body wall that cover it (Moulton 1958, Salmon *et al*. 1968). Some authors (Jones & Marshall 1953, Tavolga 1971, Demski *et al*. 1973, Myrberg *et al*. 1993) assumed that the swimbladder could also act as a resonator that amplifies and changes the quality of sounds produced by stridulation, but this hypothesis still requires experimental verification (Ladich & Fine 2006).

1.2.2. Diversity of fish sounds

Generally speaking, the diversity of sounds produced by fishes is associated with differences in the underlying mechanisms of sound production. Nevertheless, it may also result from differences between species of the same family (interspecific differences) and sometimes differences within species (intraspecific or individual differences), as well as variation related to the context of sound production.

Vocal fish produce sounds that commonly comprise low-frequency (most energy below 1000 Hz) pulses that vary in duration, number and repetition rate (Winn 1964, Myrberg *et al*. 1978). These characteristics contrast with the majority of terrestrial vertebrate and insect signals, which contain higher frequencies (> 1 kHz), are tonal and often distinctly frequency modulated.
Such differences may, however, be partly explained by the fact that fishes are not able to modulate their sound output by changes in air flow mediated by vocal membranes and vocal tract resonating structures as in birds and mammals (Fitch & Hauser 2003). Moreover, the majority of fish sounds being produced by sonic swimbladder muscles, the pulsatile low frequency

character of sounds seems to be caused by morphological constraints determined by sonic muscle physiology and swimbladder properties. Sound frequencies cannot be too low because the swimbladder is an inefficient resonator (it will not produce sounds when moved at a low speed), or too high because of limitations in contraction rate of the sonic muscle (Fine *et al*. 2001, Lugli *et al*. 2003).

The diversity of fish sounds is not as remarkable as in other taxa. Most fish show poor amplitude and frequency modulation in their sounds (Crawford *et al*. 1986, Ladich 1997, Lugli *et al*. 1997) and have relatively limited acoustic repertoires. Only few species of fish such as the cichlid *Herotilapia multispinosa* (Brown & Marshall 1978), the batrachoid *Halobatrachus didactylus* (dos Santos *et al*. 2000) or some mormyrid fish of the genus *Pollimyrus* (Crawford 1997) emit more than one or two distinct sound types.
Although the diversity of fish sounds may not be as outstanding as in other taxa, the variability provides sufficient scope for communication. Of particular interest are differences in courtship sounds made by closely related sympatric species that may promote reproductive isolation (Lobel 1998, Hawkins & Amorim 2000, Amorim *et al*. 2008). Differences between individuals of the same species may in turn play a role in sexual selection through male-male competition and female mate choice (choosing the male with the best characteristics; see Winn 1972, Lobel 1998, Amorim *et al*. 2003). Other sources of variability are related to context, including motivation and social status, season, time of day, and sexual dimorphism (Myrberg *et al*. 1965, Fine 1978, Ladich *et al*. 1992a, Connaughton & Taylor 1995, Amorim *et al*. 2004a, Parmentier *et al*. 2010). Fish sound variability is mainly based on temporal patterning of sounds and on frequency variation.

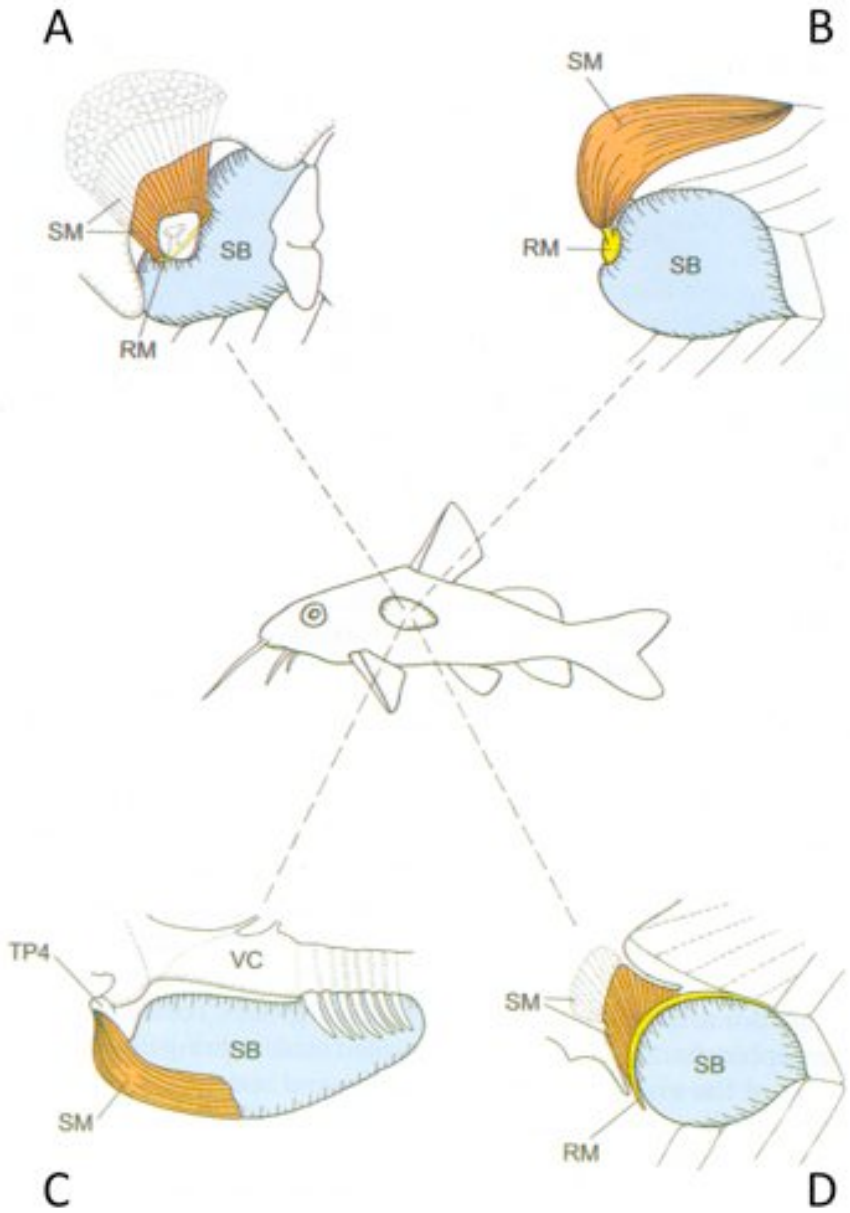


Figure 1.8 Lateral view of the swimbladder-vibrating mechanisms in fishes of four catfish families: A – *Platydoras*, Doradidae (elastic spring apparatus); B – *Synodontis*, Mochokidae (elastic spring apparatus); C – *Pimelodus*, Pimelodidae (drumming muscle mechanism); D – *Arius*, Ariidae (elastic spring apparatus). RM – ramus Mülleri (= elastic spring); SB – swimbladder; SM – sonic (= protractor) muscle; TP4 – transverse process of the 4th vertebra; VC – vertebral column. (modified from Fine & Ladich 2003).

1.3. ACOUSTIC BEHAVIOR OF THE POMACENTRIDAE

Damselfishes are well-known vocal species with at least eight (*Abudebduf*, *Amphiprion*, *Chromis*, *Dascyllus*, *Pomacentrus*, *Plectroglyphidodon*, *Premnas*, *Stegastes*) out of the 28 genera being reported as sound emitters in numerous acoustic studies (Amorim 2006, Parmentier *et al*. 2006b, Maruska *et al*. 2007). Sound production in a species from the basal clade (*Plectroglyphidodon lacrymatus*, Stegastinae subfamily; see Figure 1.2) suggests that pomacentrids are derived from an ancestral taxon capable of sound production (Parmentier *et al*. 2006b). Within this family, sound production mainly occurs during courtship (Spanier 1979, Takemura 1983, Myrberg *et al*. 1986, Lugli *et al*. 1997, Mann & Lobel 1997, Parmentier *et al*. 2010) and agonistic interactions (Myrberg 1972, Santiago & Castro 1997, Parmentier *et al*. 2006b, Parmentier *et al*. 2010). These behaviors are associated with different calls in the pomacentrids *Stegastes partitus* (Myrberg 1972, Myrberg & Spires 1972, Myrberg *et al*. 1993) and *Dascyllus albisella* (Lobel & Mann 1995, Mann & Lobel 1998). On the other hand, a single type of call was identified in other pomacentrid species such as *Chromis viridis*, *Chromis chromis* and *Abudefduf luridus* and (Amorim 1996a, Santiago & Castro 1997, Picciulin *et al*. 2002), although these authors suggested the repertoire could include more calls than those described.

Overall, agonistics sounds (pops, chirps or grunts) in this family consist of broad frequency pulses emitted during chases and threat displays between conspecifics. Chirps are usually referred to multiple pulsed sounds emitted in relatively regular intervals, usually shorter than 100 ms while pops are referred to single (less frequently, double) pulsed sounds emitted at relatively larger or more irregular intervals (Amorim 1996a, Santiago & Castro 1997, Mann & Lobel 1998, Picciulin *et al*. 2002, Parmentier *et al*. 2006b). Grunt sound occurs after a female has entered a male's nest and consists of many irregularly spaced pulses (Kenyon 1994, Myrberg 1972).

The best-characterized courtship sound in damselfishes is the "chirp", produced by the male of several species (*e.g*. *Abudefduf* spp., *Dascyllus* spp.,

Stegastes spp.) primarily during a stereotyped courtship swimming display called the "signal jump", during which the male rises in the water column and then swims down rapidly while producing a pulsed sound (Myrberg 1972, Spanier 1979, Lobel & Mann 1995, Mann & Lobel 1998, Lobel & Kerr 1999). The "signal jump" has been proposed to act as an indicator of male vigor for females choosing mates (Mann & Lobel 1997).

One of the most interesting studies regarding acoustic variability and its role in fish communication comes from reproductive acoustic signals in damselfishes. These studies have included extensive playback experiments in water, which have established the importance of temporal patterning in species recognition. Indeed, the number of pulses in a call and their temporal properties (pulse length, pulse interval) appear to be the most important features in species recognition (Myrberg & Spires 1972, Ha 1973, Myrberg *et al*. 1978, Spanier 1979). In addition, female damselfish might use spectral characteristics of the "chirp" for determining individual identity and male size (Myrberg *et al*. 1993, Lobel & Mann 1995, Myrberg & Lugli 2006).

In summary, pomacentrids mainly emit single or sequences of pops (with one or two pulses) and (multiple-pulsed) chirps during agonistic interactions. A consistent interspecific comparison of agonistic sounds is difficult due to the lack of homogeneity employed by different authors in the acoustical description of sounds and terminology (Amorim 2006). The chirp is the typical courtship sound and shows clear interspecific variability. For example, chirps made by four species of *Stegastes* differ in sound duration, pulse number and pulse repetition rate (Myrberg *et al*. 1978), but chirps produced by *D*. *albisella* are similar to those of *Stegastes* (Lobel & Mann 1995). Therefore, females would need to sample more than one sound and potentially use other cues to discriminate between species because sonic characteristics still overlap substantially between species living in sympatry (Parmentier *et al*. 2009b).

1.3.1. Sound production in clownfishes

Sound production in clownfishes can be traced back as early as 1930 when Verwey stated that *A. akallopisos* and *A. polymnus* were able to produce sounds.

Thereafter, Schneider (1964a) studied sound production in the aquarium of *A. clarkii*, *A. polymnus*, *A. frenatus*, *A. percula* and *A. ocellaris*. He reported that *Amphiprion* species produce sounds when swimming, feeding, associating with anemones, and particularly when fighting for an anemone. He documented three types of sound that he labeled "threatening sounds", "fighting sounds" and "shaking sounds". The sounds, which were clearly audible to the human ear, were mainly associated with agonistic activity. They were emitted by the fish in conjunction with both threat and submissive postures. However, it was difficult to determine which fish (chaser or chased) was responsible for their production.

Unfortunately, these first studies revealed very few detailed data about the physical properties of vocalizations (see Table 1.2).

Allen (1972) recorded sounds for *A. chrysopterus* and *A. perideraion*, both in the field and in the laboratory. Commonly, the recorded sounds consisted of pulses that can be emitted alone or in series. Further insight into the description of sounds was provided since two distinct sounds were differentiated based on their duration, frequency range and repetition of pulses (Table 1.2). Threatening, attacking and submissive fish often delivered a volley of successive "clicks" (usually from 3 to 15 pulses) with an average duration of about 35 to 45 ms. The sound energy of these "clicks" was concentrated below 1 kHz. The second type of sound was recorded only for *A. chrysopterus*, and consisted of muted "grunt" that were usually delivered either individually or in sets of two or three, with about 100 ms in duration. "Grunts" displayed most of the energy concentrated at bout 600 Hz. This call was usually emitted in conjunction with threat postures by resident fish after new specimen (both conspecific and other species) were released in the aquarium.

Takemura (1983) as well as Chen and Mok (1988) studied sound production for seven species of clownfishes (Table 1.2). The former study provided very few information regarding the physical properties of the sounds, whereas the latter study gave quantitative data on the physical parameters of *A. clarkii* and *A. frenatus* sounds. They recorded two types of sounds ("pops" and "chirps") distinguishable by their dominant frequency range and the number of pulses per call. In both species, the pop sound was predominately produced singly although it could be emitted in a set of two. The chirp sound was produced in series, often including 1 to 7 pulses in *A. frenatus* and 1 to 17 pulses in *A. clarkii* (Table 1.2).

More recently, Parmentier *et al*. (2005) showed the geographical variation of the sound production in *A. akallopisos* from populations in Madagascar and Indonesia, a distance of 6500 km. Various acoustic features, including peak frequency, pulse duration and number of peaks per pulse differed between the two populations. This geographic comparison was the first to demonstrate "dialects" in a marine fish species.

This body of literature represents the whole studies about sound production in clownfishes. The main issue with these studies is that authors have adopted different ways of describing sounds which creates confusion, such as in the labeling of sound types, the acoustic parameters measured, and the precision of data presented (including small sample sizes and differences in descriptive statistics given).

Despite the different studies on the sound production in clownfishes, the nature of the sound-producing mechanism has remained unresolved, only resting on few assumptions.

Some authors claimed that sound was produced by rapid up-and-down movements of the opercula and by movements of the mouthbones related to taking food (Verwey 1930, Takemura 1983).

Others implied that sound was produced by grating pharyngeal teeth, and could then be amplified by the swimbladder (Chen & Mok 1988, Rice & Lobel 2003).

In such a context, there existed a need to go further into the functional understanding of the sound producing-mechanism and the different structures involved in sound production because no experimental verification had been provided and numerous questions were still unanswered.

Table 1.2 Sounds produced by clownfishes during aggressive encounters

Species	Sound type	Pulse duration (ms)	# pulses per call	Pulse period (ms)	Dominant frequency (kHz)	Context of sound production	Author
A. clarkii							
A. polymnus	Threatening sounds	25-30	4-12	?	0.6	Fighting over an anemone	Schneider (1964)
A. frenatus	Fighting sounds	45-60	1	?	?		
A. ocellaris	Shaking sounds	?	?	?	?		
A. percula							
A. chrysopterus	Click	35-45	3-15	?	<1	Threat, attack, submission	Allen (1972)
A. perideraion	Click	35-45	3-15	?	<1		
	Grunt	100	1-3	?	0.6		
A. ocellaris		?			?		
A. frenatus		56			0.5		
A. clarkii	?	?	?	?	<8	Threat, dash, bite	Takemura (1983)
A. melanopus		64			?		
A. polymnus		?			?		
A. sandaracinos		?			?		
A. clarkii	Pop	80	1-2		<3	Agonistic displays	Chen & Mok (1988)
	Chirp	50	1-17		<1.5		
A. frenatus	Pop	50	1-2	?	<3		
	Chirp	50	1-7		<1		
A. akallopisos	Pop	7-11	1-15	20-90	875	Agonistic displays	Parmentier *et al.* (2005)
	Chirp	1.7-4.8	5-12	11.2	665		

1.4. AIMS AND THESIS OUTLINE

1.4.1. General aims

The main aims of the present thesis are to determine the fundamental components of the (inter- and intraspecific) acoustic communication, and to describe and explain the mechanisms responsible for sound production in different species of clownfishes. The core of the research project includes the following approaches :

- The first approach consists of identifying and describing as well as possible the sounds produced in different behavioral contexts, namely agonistic interactions and reproductive activities.
- The second approach is interested in describing and understanding the sound-producing mechanism(s). This approach focuses on the aggressive sounds, and it will be undertaken in one species *Amphiprion clarkii*.
- The third approach aims at making comparisons between the variations observed in acoustic features and the morphological differences between species.

1.4.2. Thesis outline

This dissertation is divided into six chapters.

- **Chapter 1**: several generalities regarding the life history of clownfishes and the sound production in fishes are mentioned in the present chapter.

- **Chapter 2**: the specimens studied and their origins are listed. The applied methods are explained here, in order to avoid repetition in the following chapters. These latter only contain a concise material and methods with reference to the second chapter.

The next three chapters correspond to the different approaches of the present thesis (see above), each including a brief introduction, a specific material and methods, the results and a discussion.

- **Chapter 3**: Diversity in the acoustic behavior of clownfishes.

This chapter is divided into three different parts.
The first part focuses on aggressive sounds produced in the context of territorial defense, whereas the second one deals with submissive sounds emitted during agonistic interactions between group members. The overall goal of these first two parts is to determine whether there are differences between individuals in acoustic features, and whether there are different types of sounds associated with threat and submissive postures during interactions.
The third part concerns sound production related to reproductive activities. The interest is to show whether sounds may play a specific role during some reproductive activities (*e.g*. attraction of a partner to the spawning site, synchronization of gamete release).

- **Chapter 4**: Morpho-functional description of the sound-producing mechanism in clownfishes.

This chapter is composed of two different parts and focuses on aggressive sounds in *Amphiprion clarkii*.
The first part consists of a multidisciplinary study combining high-speed video coupled or not with an x-ray system, functional morphology and sound recordings. The main aim is to determine which structure(s) is (are) responsible for sound production and to understand the functional interactions between the structures involved in the mechanism.
The second part aims to give further insight into the understanding of the acoustic waveform, especially regarding the sound radiation. Different experiments are conducted in order to find out the anatomical structure acting as the acoustic radiator responsible for the sound frequency and sound duration variation.

- **Chapter 5**: Interspecific variation of sounds in clownfishes.

This chapter compares the acoustic features in a sizeable number of closely related clownfish species. The main aim is to evaluate the potential role of acoustic communication as a driving force in the evolution of clownfishes.

- **Chapter 6**: General discussion and conclusions.

All findings are discussed in this chapter. The obtained results are synthesized and integrated with data from literature in an attempt to point out the importance of sound production in clownfishes. The last part of this general discussion also contains a concise conclusion.

1.5. APPENDIX

All the results presented in this dissertation have been the subject of scientific publications that have been either published, submitted or in preparation. Data and information have been reworked in order to match the different chapters that form the synthesis of the following papers.

Sound Production in the Clownfish *Amphiprion clarkii*.
Parmentier E, **Colleye O**, Fine ML, Frédérich B, Vandewalle P, Herrel A
Science 2007, 316: 1006-1006.

Hearing abilities in three clownfish species.
Parmentier E, **Colleye O**, Mann D
Journal of Experimental Biology, 212: 2023-2026.

Agonistic sounds in the skunk clownfish *Amphiprion akallopisos*: size-related variation in acoustic features.
Colleye O, Frédérich B, Vandewalle P, Casadevall M, Parmentier E
Journal of Fish Biology, 75: 908-916.

Histological Study of the Sex-Change in the Skunk Clownfish *Amphiprion akallopisos*.

Casadevall M, Delgado E, **Colleye O**, Ber Monserrat S, Parmentier E

The Open Fish Science Journal, 2: 55-58.

Interspecific variation of calls in clownfishes: degree of similarity in closely related species.

Colleye O, Vandewalle P, Lanterbecq D, Eeckhaut I, Lecchini D, Parmentier E

BMC Evolutionary Biology (under review)

Further insight into the sound-producing mechanism of clownfishes: what about the structures involved in sound radiation?

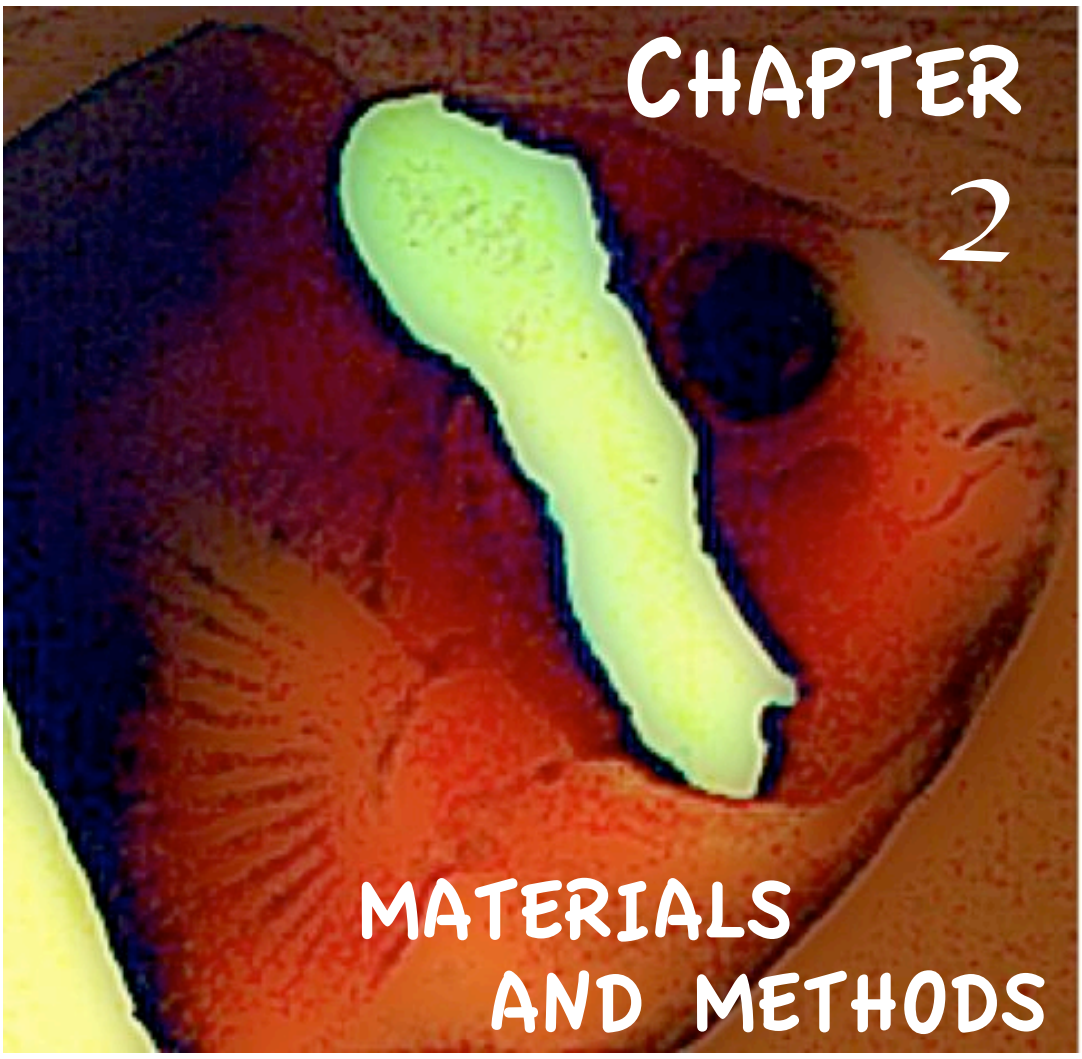
Colleye O, Nakamura M, Frédérich B, Vandewalle P, Parmentier E

(in preparation)

Diversity of sound production in clownfishes: aggressive, submissive and reproductive signals.

Colleye O, Nakamura M, Iwao K, Vandewalle P, Parmentier E

(in preparation)


CHAPTER
2
MATERIALS
AND METHODS

2.1. MATERIAL EXAMINED

All specimens were collected during different fieldworks or were obtained either from aquarium trade or from public Aquaria. Further information about the exact number of specimens collected and used will be given in the different chapters of this dissertation (see also Table 2.1 for summary).

2.1.1. Fieldworks

Fishes were collected during four different fieldworks. The first fieldwork took place at CRIOBE (Centre de Recherches Insulaires et Observatoire de l'Environnement, Opanohu Bay, Moorea, French Polynesia) in July 2007. Two other sampling campaigns occurred at IHSM (Institut Halieutique et des Sciences Marines, University of Toliara, west coast of Madagascar) in October 2007 and June 2010. The fourth fieldwork took place at Sesoko Station, Tropical Biosphere Research Center (University of the Ryukyus, Okinawa, Japan) and at AMSL (Akajima Marine Science Laboratory, Kerama Islands, Okinawa, Japan) from May to August 2009.

2.1.2. Specimens from aquarium trade and public Aquaria

Some specimens were commercially purchased from aquarium trade (Exotic 2000 and Aqua Garden Center, Belgium) or given by the Aquarium of La Rochelle (France) and Oceanopolis (Brest, France). Those institutions manage breeding, hatching and rearing of several species of clownfishes.

2.2. METHODS

2.2.1. Capture and holding of fishes

In the field, all fishes were collected by scuba diving on the fringing reef or in the lagoon. Either they were caught by removing carefully their host sea anemone from its support and by placing it in a bucket, specimens then followed their host; or they were caught using fish net while they hid among the tentacles of their host. Hosts and fishes were transferred to a community tank filled with running seawater maintained at ambient temperature. All fishes were kept under natural photoperiod and fed once daily with food pellets *ad libitum*.

2.2.2. Sound recording

Recordings of sounds were made using different materials depending on the recording conditions:

1. Sound recordings in fish tank were made using two types of hydrophones:

- An Orca hydrophone (sensitivity: −186 dB re. 1 V/µPa) connected *via* an Orca-made amplifier (Orca Instrumentation, France) to a Tascam HD-P2 stereo audio recorder (Wiesbaden, Germany, recording bandwidth: 20 Hz to 20 kHz ± 1.0 dB). This system has a flat frequency response range (±3 dB) between 10.0 and 23.8 kHz.
- A Brüel & Kjaer 8106 hydrophone (Naerum, Denmark, sensitivity: - 173 dB re. 1V/µPa) connected *via* a Nexus™ conditioning amplifier (type 2690, Naerum, Denmark) to a Tascam HD-P2 stereo audio recorder (Wiesbaden, Germany, recording bandwidth: 20 Hz to 20 kHz ± 1.0 dB). This system has a flat frequency response over wide range between 7 Hz and 80 kHz.

The hydrophones were always placed just above the sea anemone (±5 cm) because fishes bathed among the tentacles of their host most of the time.

2. Field recordings were made using a SONY HDD video camera placed in a housing (HC3 series, Ocean Images, Cape Coral, Florida) and coupled with an external hydrophone (High Tech. Inc.) with a flat response of 20 Hz to 20

kHz and a nominal calibration of -164 dB re. 1 V/µPa (Loggerhead Instruments Inc., Sarasota, Florida). Recordings were made by placing the housing at a distance of between 50 cm and 1 m in front of the sea anemone.

On the basis of the experimentation we carried out, different recording methods were used in fish tank:

- Aggressive sounds associated with territory defense were recorded based on published protocol (Parmentier *et al*. 2005). One pair of clownfish (*i.e*. composed of two individuals of different sizes) and its host were placed in the centre of the tank for an acclimation time (~2 days). The resident pair was then challenged by the introduction of conspecific intruder into the tank. Sounds were produced when the intruder approached the territory (the sea anemone) defended by the resident pair. Sexual size dimorphism (see Allen 1972) was used to recognize resident male and female, which enabled the sound emitter to be identified. Recordings lasted approximately 15 min, after which the intruder was removed until the following session.
- Sounds emitted between group members were recorded by placing all individuals of the group and their host in the tank for an acclimation time (~2 days) before being recorded. Each recording session lasted around 45 min during which interactions were observed and noted in order to identify the sound emitter.
- Sounds related to reproduction were recorded using mating pair that has been reared with its host into a separate tank for several years. The different reproductive activities (nest preparation, courtship, spawning and eggs care) were observed and recorded. Each recording session lasted approximately 2 h, and four recording sessions with a 1-hour interval were carried out by day in order to record during the whole daytime from dawn to dusk. Behaviors of male and female were observed and noted.

Sounds emitted between group members as well as sounds related to reproduction were also recorded in the field. In this case, recording sessions (each lasting about 3 to 4 h) were done using the video-sound recording system, at a depth of between 5 and 10 m. This technique enabled us to describe as well as possible the sounds produced during the different behaviors.

2.2.3. Sound analysis

Sounds were digitized at 44.1 kHz (16-bit resolution) and analyzed with AviSoft-SAS Lab Pro 4.33 software (1024 point Hanning windowed fast Fourier transform (FFT)). Recording in small tanks induces potential hazards because of reflections and tank resonance (Akamatsu *et al*. 2002). A relevant equation from Akamatsu *et al*. (2002) was thus used to calculate the resonant frequency of the tanks, and a low pass filter depending on the dimensions of the recording tanks was applied to all sound recordings.

When using the video-sound recordings system, sounds and their associated behaviors were analyzed on the basis of the videos and field observations. Behaviors associated with sound production were described and sounds were extracted in .wav files using the AoA audio extractor setup freeware (version 1.2.5). Sounds were digitized at 44.1 kHz (16-bit resolution), low-pass filtered at 1 kHz and analyzed using AviSoft-SAS Lab Pro 4.33 software (1024 point Hanning windowed fast Fourier transform (FFT)). Only sounds with a good signal to noise ratio[1] were included in the analyses.

Temporal features were measured from the oscillograms whereas frequency parameters were obtained from power spectra (filter bandwidth 300 Hz, FFT size point 256, time overlap 96.87% and a flat top window). Generally speaking, the recorded sounds were composed of a train of pulses, or a series of sounds being composed of several pulses. The following sonic features were measured depending of the type of sounds: number of sounds

[1] The ratio of signal power to the noise power compares the level of a desired signal to the level of background noise. The higher the ratio, the less obtrusive the background noise is.

per train, number of pulses per sound, sound duration in ms, pulse duration in ms, sound period in ms (the average peak to peak interval between consecutive sounds in a train), pulse period in ms (the average peak to peak interval between consecutive pulse units in a series) and dominant frequency in Hz (frequency component with the most energy) (see Figures 2.1 and 2.2). Note that sounds produced simultaneously by several individuals were excluded from acoustic analyses.

2.2.4. Preservation of specimens for study

After sounds had been recorded, most of the specimens were euthanized by an overdose of MS-222 (ethyl 3-aminobenzoic acid methanesulfonate salt) in a seawater solution. Specimens were then fixed in 7% buffered seawater formalin for approximately 2 weeks. Fixation is known to produce shrinkage of the different tissues (Shields & Carlson 1996, Ajah & Nunoo 2003). As a rule, standard length (SL) of specimens was always measured before fixation. After fixation and washing, specimens were transferred to a 70% ethanol solution for preservation.

2.2.5. *In toto* clearing and staining

Osteology was studied on *in toto* cleared and stained specimens (see chapter 4). Alizarin red S was used following the method of Taylor and Van Dyke (1985). Note that the whole protocol was not applied. Cartilage staining was omitted because only juvenile and adult specimens were used. Examination of the specimens was done using a Leica M10 stereoscopic microscope (Leica Camera, Leica, Wetzlar, Germany) coupled to a camera Lucida for drawing. As an aid to the drawings, dissections (*e.g*. removal of some bony structures such as opercular bone or suspensorium) were performed. Drawings figure all bony elements that are visible on the cleared and stained specimens.

2.2.6. Dissections

Dissections were performed for the study of both hard (bones) and soft tissues (muscles and ligaments). Examination of the specimens was done using a Leica M10 stereoscopic microscope equipped with a camera lucida for drawing (see chapter 4). The combination of *in toto* staining (see paragraph 2.2.5) and dissection revealed to be a highly valuable tool for comparing morphological and anatomical structures between species. Dissections of freshly killed specimens were used to study the mobility of articulations and ligaments, as well as to infer possible muscle functions. Using fresh specimens enabled us to suggest functional hypotheses based on cautious interpretations.

2.2.7. Serial sectioning

Serial histological cross sections were made in order to study the detailed anatomy of different structures. Whatever the sample examined, it was fixed in Bouin's solution, dehydrated with ethanol, decalcified as necessary (depending on the structure), embedded in paraffin wax (Paraplast® brand) and serially cut with a Reichert microtome. Slice thickness and staining were different according to the material examined:

1. Gonads were removed from 14 individuals of *A*. *akallopisos* (see chapter 3) and histological examination of cross sections (8 µm) was undertaken to attest the sex of each individual. Slices were stained using Masson's trichrome method (Ganter & Jolles 1970).
2. Three specimens of *A*. *clarkii* (see chapter 4) were selected for serial sectioning in order to observe the detailed anatomy of their swimbladder. Cross-sections (15 µm) were stained with haematoxylin-eosin (Gabe 1976).

Finally, the different sections were observed with a Leica DM1000 microscope coupled with a digital camera (Canon PowerShot S50).

2.2.8. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to compare the buccal teeth of different species (see chapter 5). After having been removed from the fish, the buccal jaws were minutely cleaned off using whet clamps and little brush. After dehydration, samples were critically point-dried with CO_2 using a Leica EM CPD030, and platinum coated using a Balzers SCD 030. The material was then examined using a Jeol JSM-840 A scanning electron microscope.

2.2.9. High-speed video and X-ray

High-speed video recordings coupled or not with an X-ray system, were used in order to determine and to quantify fish movements during sound production (see chapter 4 for more details about material characteristics and the methodology used).

2.2.10. Impact hammer and laser vibrometer

In order to understand the structure acting as the major acoustic source in the sound-producing mechanism, and to parcel out the contribution of this structure to sound properties, we utilized a piezoelectric impact hammer that provides a measure of the input force, coupled with a laser vibrometer that measures the vibration velocity and frequency of a surface of interest (see chapter 4 for more details about material characteristics and the methodology used).

Table 2.1 Summary of the number of specimens of the different clownfish species used in the experiments

Species	Methods								
	Sound recordings			Alizarin red staining	Histological cross section	SEM	High-speed video	X-ray video	Impact Hammer & laser vibrometer
	aggressive	submissive	reproductive						
A. akallopisos	14				14 (gonads)	3 (teeth)			
A. akindynos	2		2						
A. clarkii	18		2	6	3 (swimbladder)		3	2	6
A. chrysogaster	1								
A. chrysopterus	1								
A. frenatus	6	9				3 (teeth)			
A. latifasciatus	1								
A. melanopus	2		2						
A. nigripes	2								
A. ocellaris	4					3 (teeth)			
A. percula	2		2						
A. polymnus	2								
A. perideraion	2		2						
P. biaculeatus	1								

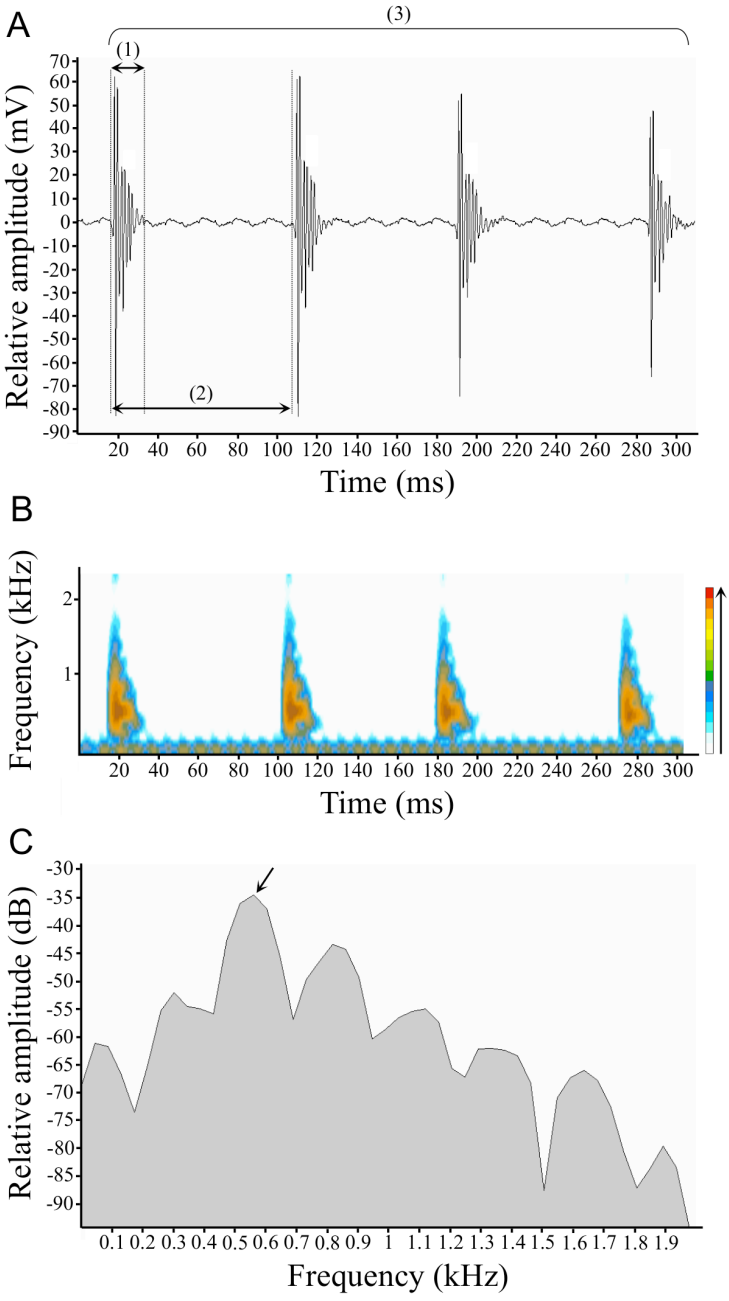


Figure 2.1 (A) Oscillogram and (B) sonogram of aggressive sounds (pops) produced by a 83 mm *Amphiprion clarkii*. Acoustic features measured in the signal: (1) pulse duration, (2) pulse period and (3) number of pulses in a sound. The colour scale corresponds to the intensity associated with the different frequencies. (C) Power spectrum of one pulse showing the dominant frequency (→).

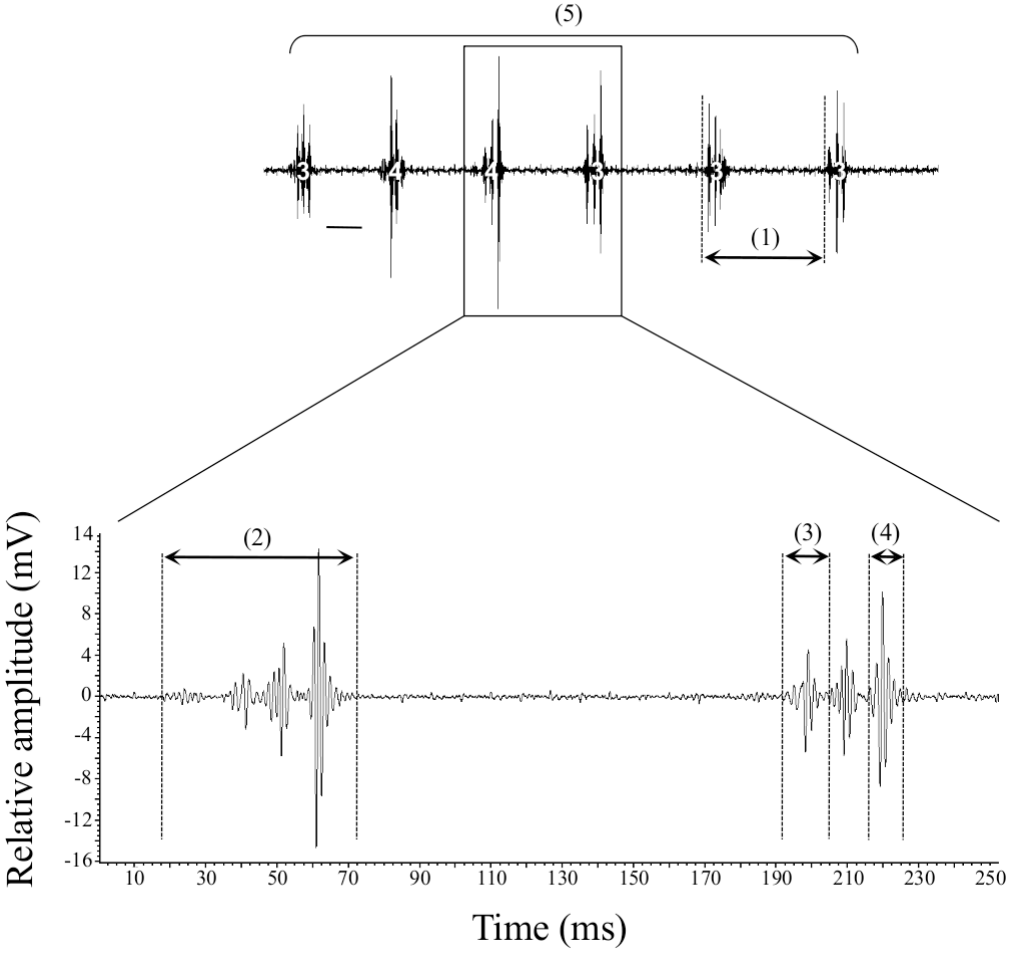
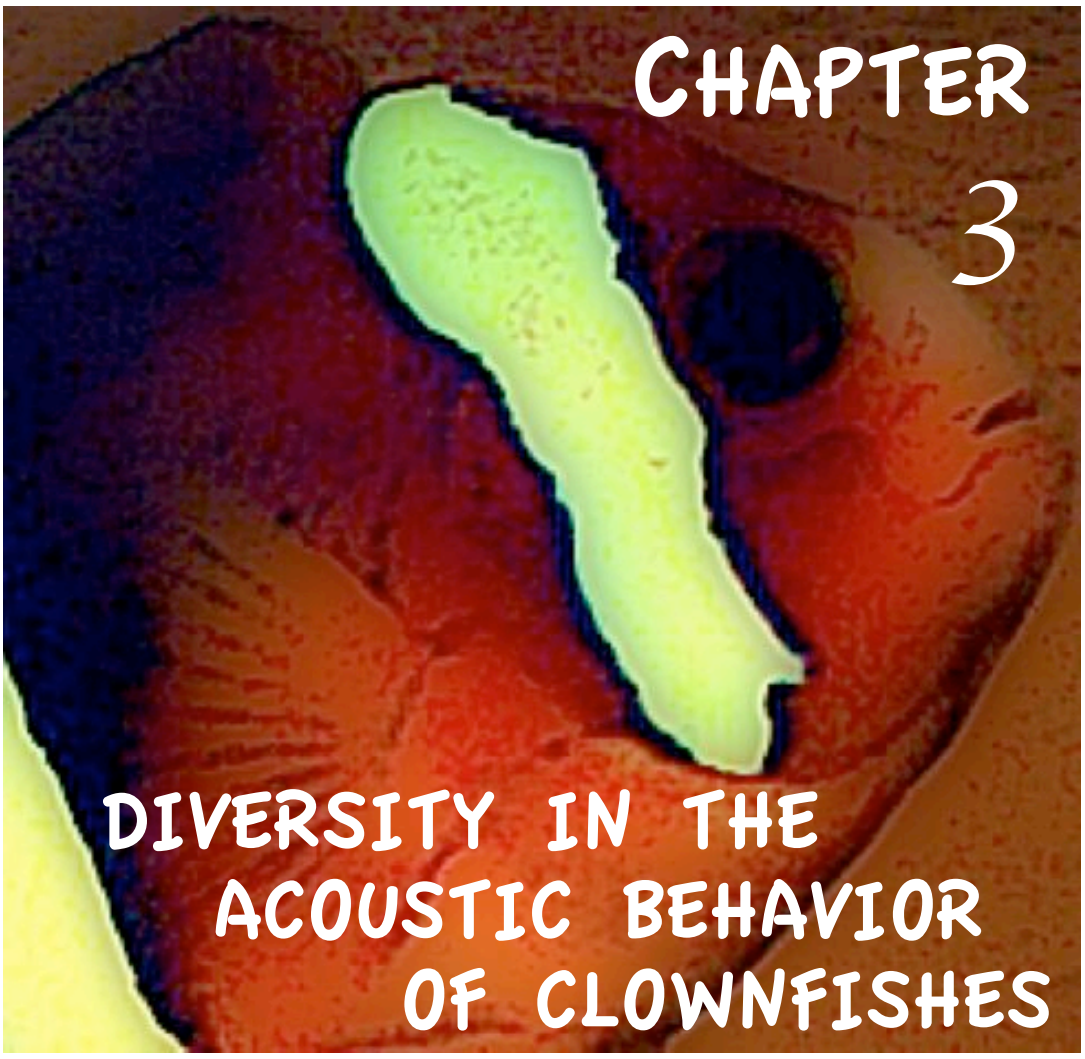


Figure 2.2 Oscillogram of submissive sounds (chirps) produced by a 81 mm *Amphiprion frenatus*. Acoustic features measured in the signal: (1) sound period, (2) sound duration, (3) pulse period, (4) pulse duration and (5) number of sounds in a train. Numbers in white correspond to the pulse number in each sound (a series of six different sounds is shown). Scale bar = 50 ms.


CHAPTER
3
DIVERSITY IN THE
ACOUSTIC BEHAVIOR
OF CLOWNFISHES

3.1. AGGRESSIVE SOUNDS AND AGONISTIC BEHAVIOR *

3.1.1. Introduction

Defending oneself or resources such as food, mate or space (territory) is a widespread behavior with a survival value for the lives of animals. Before physical interactions occur, defensive and offensive interactions often begin with different types of signals such as visual, olfactory or acoustical signals. In these agonistic contexts, producing signals may represent an economic way for solving disputes over resources which otherwise end at much higher costs (injury or death).

Acoustic displays related to agonistic interactions have been observed in representatives of more than 30 families of fishes (see Ladich & Myrberg 2006). Sounds may be used in different behavioral contexts: distressful or disturbance situations (*e.g*. being attacked by predators or simply handled in hand; Fish & Mowbray 1970, Ladich 1997, Heyd & Pfeiffer 2000, Parmentier *et al*. 2009a), competitive feeding (Ladich 1988, Amorim & Hawkins 2000), and competition for space and sometimes mates (Myrberg 1972, Ladich 1989, Ladich 1998). Generally speaking, low levels of aggression are observed during competitive feeding whereas higher levels of aggression are usually displayed while competing for space and mates. Acoustic displays may consist of one type of sound such as croaking in gouramis (Ladich 1998), or several types indicating different levels of aggression or different meanings (Myrberg 1972, Ladich 1989).

Agonistic interactions are mostly used for the monopolization of territories, the majority of fishes aggressively display toward conspecifics while establishing and maintaining territories (Myrberg 1972, Ladich 1989). These aggressive encounters may occur in different ways: either they consist of simply chasing away an intruder, or they can be more complex by involving a

* Slightly modified from: **Colleye O, Frédérich B, Vandewalle P, Casadevall M, Parmentier E.** 2009. Agonistic sounds in the skunk clownfish *Amphiprion akallopisos*: size-related variation in acoustic features. *J. Fish Biol.* **75**: 908-916.

series of different behavioral displays. Aggressive interactions usually begin with the low cost displays such as visual threats or sounds, followed by more costly bites and attacks, likely leading to severe injury or death. In such a context, fishes try to gain information about fighting abilities of the opponents by assessing signals, with the purpose of solving conflicts without escalated to fighting (Morris *et al*. 1995).

Clownfishes live in social groups in which there is a size-based dominance hierarchy (see paragraph 1.1.4). Within each group, numerous agonistic interactions occur and they appear to play an important role by maintaining size differences between individuals adjacent in rank (Fricke 1979, Buston 2003). Interspecific encounters may also occur when the territorial boundaries of two species are adjacent, but they are relatively scarce and of low intensity. On the other hand, intraspecific encounters are common and sometimes rather severe in their intensity. Larger fishes chase smaller ones, and the smallest one is the recipient of numerous charges (Fricke 1979). The fins and abdomen are frequent targets.

Clownfishes were reported to produce sounds during agonistic interactions (Schneider 1964a, Allen 1972, Takemura 1983, Chen & Mok 1988, Parmentier *et al*. 2005). Schneider (1964a) documented "threatening sounds" emitted by fishes in conjunction with threat postures. Likewise, Allen (1972) reported that the charges were accompanied by vocalizations. Thereby, these observations emphasize the fact that sounds could play an important role in the aggressive behavior of clownfishes.

The present study aims to seek if differences in acoustic features of aggressive sounds exist between individuals of different sizes and of different sexual status (non-breeder, male and female) in the skunk clownfish *Amphiprion akallopisos*.

3.1.2. Material and methods

Sound recording and sound analysis

Fourteen *Amphiprion akallopisos* (SL: 28–90 mm) were collected by scuba diving in the lagoon in front of Toliara (23°36′S - 43°66′E; Mozambique Channel, west coast of Madagascar) in October 2007. Fish and their host (*Heteractis magnifica*) were maintained in an outdoor community tank (3.5 × 0.7 × 0.2 m) filled with running seawater at a constant temperature of 26°C. All fish were kept under natural photoperiod and fed once daily with food pellets *ad libitum*.

Sounds were recorded using an Orca hydrophone in a smaller glass tank (0.8 × 0.35 × 0.35 m) at 26°C. Sounds were emitted in a context of territory defense (see paragraph 2.2.2 for details on procedures and material characteristics). Sound analyses were done based on the procedure mentioned in chapter 2 (see paragraph 2.2.3 for details and Figure 2.1). A low pass filter of 3.17 kHz was applied to all sound recordings.

After sounds had been recorded for all specimens, fish were killed with an overdose of MS-222 in solution. Gonads were removed for the production of serial histological sections in order to attest the sex of each individual (see paragraph 2.2.7 for more details). The gonads were categorized according to the study of Hattori & Yanagisawa (1991) and Casadevall *et al*. (2009). Among the collected fish, there were 12 breeders (six females and six males) and two non-breeders.

Statistical analyses

Correlation analyses were used to examine changes in all acoustic characteristics across SL. The data used in these analyses were mean values of all recorded sounds for each individual. Two statistical analyses were then performed to test the influence of sex on sonic features. First, a full ANCOVA was run to test differences between males and females for the sonic variables correlated with SL. Note that non-breeders were not included in this statistical test due to the small sample size (only two individuals). In this test, sonic variables are considered as variates, SL as a covariate and sex is the grouping factor. Secondly, sonic variables not correlated with SL, which failed the test

for normal distributions (Shapiro-Wilk *W* test), were analyzed using a non-parametric Kruskal–Wallis one-way ANOVA by ranks with subsequent Dunn's test for pair-wise comparisons to test differences between sexes (non-breeders, males and females). Statistical analyses were carried out with Statistica 7.1. Results are presented as means ± standard deviation (S.D.). Significance level was determined at $P<0.05$.

Mean ± S.D. values were calculated for each acoustic feature for all individuals. Overall means, S.D. and range values were subsequently calculated using each individual mean values for each variable. In order to compare between-individuals with within-individuals variability for each acoustic feature, the within-individuals coefficient of variance ($C.V._w$ = S.D./mean) was calculated and compared with the between-individuals coefficient of variation ($C.V._b$). The $C.V._b$ was obtained by dividing the overall S.D. by the respective overall mean. The ratio $C.V._b/C.V._w$ was then calculated to obtain a measure of relative between-individuals variability for each acoustic feature. When this ratio assumes values larger than one, it suggests that an acoustic feature could be used as a cue for individual recognition (Bee *et al*. 2001, Christie *et al*. 2004, Amorim & Vasconcelos 2008). Differences between individuals for each acoustic variable were tested using a Kruskal-Wallis analysis due to the lack of homogeneity of variance.

3.1.3. Results

Aggressive sounds (*n* = 1818 pulses analyzed; see Table 3.1) were produced by individuals of the different sexual status, which displayed charge-and-chase when another specimen approached the sea anemone in which they dwelled. Each aggressive sound consisted of a single pulse unit that can be emitted alone or in series (2-14 pulses, $\bar{x}$ = 3.7±0.56, *n* = 14 individuals; see Figure 2.1 in chapter 2). Pulse period averaged 75.9 ms and pulse duration ranged from 5.9 to 18.3 ms ($\bar{x}$ = 12.7±4.09, *n* = 14 individuals). Peak frequency was 691±204 Hz (*n* = 14 individuals) and most sound energy ranged from 415 to 1012 Hz.

Correlation analyses revealed that dominant frequency and pulse duration were highly correlated with SL. The most striking changes were a significant decrease in dominant frequency (r = −0.97, P<0.0001; Figure 3.1A) and a significant increase in pulse duration (r = 0.97, P<0.0001; Figure 3.1B) with increasing SL. Pulse period was correlated with increasing SL (r = 0.62, P=0.01; Figure 3.1C). This sonic feature is equal to the pulse duration plus the interpulse interval. This correlation needs to be carefully interpreted because pulse period was also significantly correlated with pulse duration (r = 0.66, P=0.01), whereas interpulse interval was not correlated with increasing SL (r = 0.04, P=0.8910). The number of pulses per train did not change significantly (r = 0.06, P=0.82; Figure 3.1D) across SL.

A comparison of male and female values using SL as a covariate showed that the dominant frequency (ANCOVA, test for common slopes: $F_{1,8}$=1.309, P=0.286; test for intercepts: $F_{1,9}$=0.281, P=0.609) and pulse duration (ANCOVA, test for common slopes: $F_{1,8}$=0.362, P=0.564; test for intercepts: $F_{1,9}$=0.716, P=0.419) did not differ between sexes.

Kruskal–Wallis one-way ANOVA revealed that means were significantly different between sexes for pulse period (H=6.305, d.f.=2, P=0.0428), but not for number of pulses per train due to high overlap (H=1.559, d.f.=2, P=0.4586). Pair-wise comparisons showed that pulse periods were higher in females (Dunn's test, P<0.05; Table 3.1) than in both males and non-breeders, which showed no significant differences between them (Dunn's test, P>0.05; Table 3.1).

Table 3.1 Summary (mean ± S.D.) of the four acoustic features analyzed from 14 *Amphiprion akallopisos*

Individual (n)	Pulse duration (ms)		Dominant frequency (Hz)		Pulse period (ms)		Number of pulses per sound	
	mean ± S.D.	n	mean ± S.D.	n	mean ± S.D.	n	mean ± S.D.	n
Non-breeders (2)	7.2 ± 1.3	181	974 ± 100	181	72.5 ± 10.3	119	3.8 ± 1.5	43
Males (6)	11.5 ± 2.0	781	775 ± 123	781	72.9 ± 12.1	470	4.3 ± 2.6	154
Females (6)	16.4 ± 2.7	856	493 ± 85	856	80.4 ± 14.4	451	3.5 ± 1.8	169

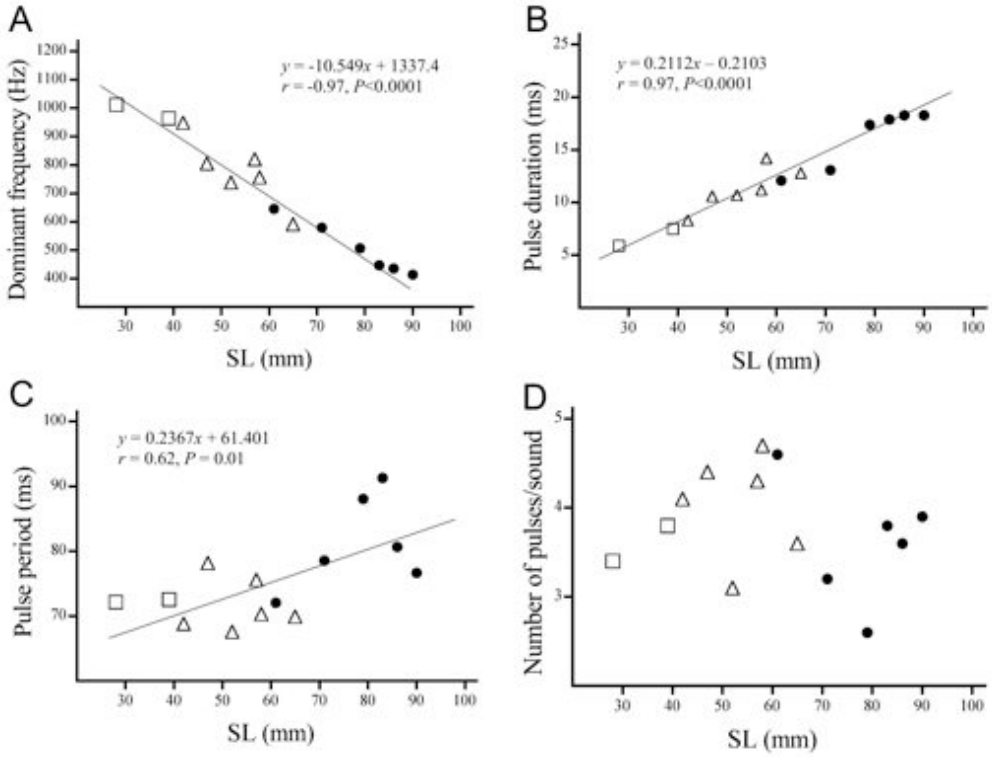


Figure 3.1 Influence of standard length (SL) on acoustic variables in *Amphiprion akallopisos* (□ = non-breeders, △ = males, ● = females). Correlations of (A) dominant frequency, (B) pulse duration, (C) pulse period and (D) number of pulses per sound against SL. Fish ranged from 28 to 90 mm in SL (n = 14). Results are expressed as mean values of all recorded pulses for each individual.

Individuality

Aggressive sounds presented some acoustic features that displayed $C.V._w$ = 0.10 (Table 3.2), suggesting a strong homogeneity of these variables. All the acoustic features analyzed had $C.V._b/C.V._w$ ratios > 1, showing a higher variability among than within individuals. Consistently, the Kruskal-Wallis analyses revealed significant differences among individuals for all features (Table 3.2), indicating that these acoustic variables can potentially provide recognition cues to identify the sound emitter. The larger relative between-individuals variability (larger $C.V._b/C.V._w$ ratios) corresponded to the dominant frequency and the pulse duration (Table 3.2).

Table 3.2 Means, ± S.D., range, within-individuals variability ($C.V._w$) and between-individuals variability ($C.V._b$) for the four acoustic features analyzed from 14 *Amphiprion akallopisos*

Acoustic variables	Overall mean ± S.D. (range)	$C.V._w$ (mean)	$C.V._w$ (range)	$C.V._b$	$C.V._b / C.V._w$	H*	*p*-value
Dominant frequency (Hz)	663 ± 199 (346–1207)	0.10	0.07-0.21	0.30	2.73	1577	<0.001
Pulse duration (ms)	13.4 ± 3.9 (3.8-22.9)	0.10	0.06-0.18	0.29	2.90	1614	<0.001
Pulse period (ms)	75.9 ± 13.4 (32.4-121.9)	0.15	0.09-0.21	0.23	1.53	203.9	<0.001
Number of pulses per sound	3.9 ± 2.2 (2-14)	0.50	0.30-0.68	0.56	1.12	25.55	0.0195

*Results of Kruskall-Wallis test (d.f. = 13, n = 1818) comparing differences between all individuals for each acoustic feature.

3.1.4. Discussion

Some evidences attest that dominant frequency and pulse duration of aggressive sounds are strongly related to fish size in *A. akallopisos*: larger individuals produce lower frequency and longer duration pulses than smaller ones. In the croaking gourami *Trichopsis vittata* for example, sound duration increases during ontogeny whereas dominant frequency decreases (Henglmüller & Ladich 1999). In the grey gurnard *Eutrigla gurnardus*, sound production changes from small juveniles to large adults during competitive feeding (Amorim & Hawkins 2005). In catfishes (Siluriformes) (Fine & Ladich 2003), weakfishes (Sciaenidae) (Connaughton *et al*. 2000), damselfishes (Pomacentridae) (Myrberg *et al*. 1993, Lobel & Mann 1995), gouramis (Osphronemidae) (Ladich *et al*. 1992b) and pearlfishes (Carapidae) (Parmentier *et al*. 2006a), pulse duration increases and dominant frequency decreases in larger fishes. The same kind of relationship has been observed in *A. akallopisos* in which differences in both sound characteristics are related to fish size, and not to sexual status. Size and sex are, however, extremely dependent on each other due to the existing size dimorphism between males and females (Allen 1972). Besides carrying information related to the size, dominant frequency and pulse duration could therefore be signals conveying information on the identity of the emitter.

Within each group, a well-defined size difference is maintained between individuals adjacent in rank (Fricke 1979, Buston 2003). Aggressive displays are responsible for maintaining size differences within the hierarchy (Fricke 1979, Buston 2003), avoiding the generation of evolutionary conflict over subordinates who would become a threat to their dominants by challenging them for their rank (Buston 2003). Sounds are associated with agonistic interactions (Allen 1972, Parmentier *et al*. 2005); they could therefore play a role in the group by conveying a signal for allowing fish size assessment. Additionally, sounds could possess a deterrent function by giving a potential reminder signal of social rank during interactions.

Clear differences were found among aggressive sounds attributed to different individuals. All acoustic variables were significantly more variable between than within individuals and thus could all potentially provide cues to identify individuals. Furthermore, the most important variables to allow individual identification are dominant frequency and pulse duration (Table 3.2).

Since dominant frequency and pulse duration are size-dependent, temporal and spectral differences in aggressive sounds are likely to be detected by members within the group. Teleost fishes such as *Gobius niger* (Gobiidae) and *Sparus annularis* (Sparidae) are able to discriminate tonal sounds differing in frequency of approximately 10%; the frequency discrimination ability at 400 Hz is approximately 40 Hz (Fay 1988). In *A. akallopisos*, sounds emitted by non-breeders, males and females differ in dominant frequencies by >10% (Table 3.1). Such a capacity to discriminate frequency differences has already been underlined in damselfish; females of *Stegastes partitus* preferentially respond to lower frequency chirps of a larger male than those from a smaller fish (Myrberg *et al*. 1986). In *Abudefduf saxatilis*, fish size has a significant effect on auditory sensitivity (Egner & Mann 2005); all fish are most sensitive to the lower frequencies (100–400 Hz) but the larger fish are more likely to respond also to higher frequencies (1000–1600 Hz). The same kind of effect of fish size on hearing abilities was observed in three different clownfish species (Parmentier *et al*. 2009c); although the best hearing sensitivity is at around 100 Hz, small fishes were found to be sensitive to a larger frequency interval (100-450 Hz), which means they are more sensitive to the frequencies emitted by larger conspecifics.

Some behavioral experiments have also shown that fishes are able to respond selectively to sounds differing in their temporal pattern. Some sunfishes of the genus *Lepomis* could recognize conspecific grunts based on their temporal structure (Gerald 1971). There is also evidence from further behavioral experiments that Pomacentrids would be able to distinguish temporal differences of sounds between at least 5 and 10 ms (Myrberg *et al*. 1978). Although differences in pulse duration are somewhat low among

individuals of different sex in *A*. *akallopisos* (Table 3.1), it is likely they could detect them. However, the lack of literature on hearing discrimination abilities of temporal pattern in clownfishes only allows speculations, suggesting that more research on hearing sensitivity would need to be done.

Differences in pulse period have been observed between females and both males and non-breeders, which might reflect a difference in motivation. Motivation is known for playing a role in damselfishes, regarding their sounds produced during aggression. In *Dascyllus albisella* and *D*. *flavicaudus* for example, aggressive sounds are different according to whether they are emitted towards conspecifics or heterospecifics, being multiple-pulsed or single-pulsed, respectively (Mann & Lobel 1998, Parmentier *et al*. 2010). In *Pomacentrus partitus*, the frequency of sounds by a territorial resident was relatively low at the territorial border, but it rapidly increased as intruders approached the residence (Riggio 1981). In *A*. *akallopisos*, motivation seems to be characterized by a higher number of pulses per train and a shorter pulse period (pers. obs.). Such variations in pulse period and number of pulses per sound might be related to the position within the group hierarchy.

The present study demonstrates that dominant frequency and pulse duration of aggressive sounds are morphologically determined signals related to fish size. This relationship is of significant importance because clownfishes live in social group that display a size-based dominance hierarchy. Both acoustic variables convey information on the size of the emitter. The present results also suggest that there is enough information in the aggressive sounds of clownfishes to promote individual recognition (see Amorim & Vasconcelos 2008).

3.2. SUBMISSIVE SOUNDS AND AGONISTIC INTERACTIONS *

3.2.1. Introduction

Clownfishes produce aggressive sounds while displaying charge-and-chase towards another specimen during agonistic interactions (Allen 1972, Parmentier *et al*. 2005; paragraph 3.1.3). Among the agonistic behaviors, it is known that all clownfish species have also evolved ritualized submissive postures, which presumably serve to circumvent physical injury during intraspecific quarreling (Allen 1972).

Several authors (Schneider 1964a, Allen 1972, Fricke 1974) have highlighted the existence of a typical behavior (commonly known as "head shaking") as a reaction to aggressive interactions (Figure 3.2). The form of this pattern (a lateral quivering of the body that begins at the head and continues posteriorly) was considered as a nonaggressive state.

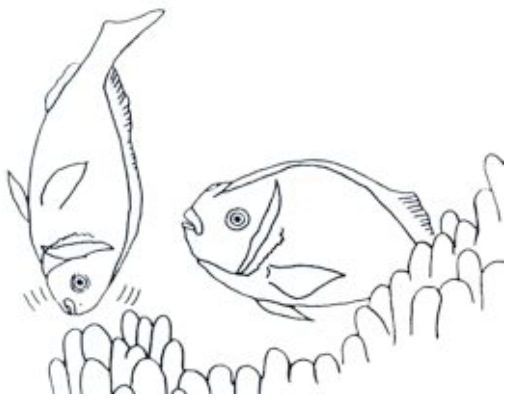


Figure 3.2 Head shaking movements exhibited by *Amphiprion perideraion* ("wiggle" lines indicate head shaking; modified from Allen 1972).

* Slightly modified from: **Colleye O, Nakamura M, Iwao K, Vandewalle P, Parmentier E.** Diversity of sound production in clownfishes: aggressive, submissive and reproductive signals. (in preparation)

Schneider (1964a) defined three types of sounds among which the "shaking sound" was typical and emitted by fishes in conjunction with submissive postures. Allen (1972) reported the presence of head shaking movements associated with sound production during interactions between group members. Unfortunately, the lack of useful physical characteristics of acoustic parameters and the small sample sizes of the behavioral observations required further investigations 1) to be able to differentiate these sounds from aggressive ones and 2) to understand the scope of this acoustic signal within a social group of clownfishes.

The present study aims to record and to analyze submissive sounds produced during agonistic interactions between group members in the tomato clownfish *Amphiprion frenatus*. The objective was to seek if differences in acoustic features exist between individuals of different sizes, and of different social ranks. These data should help to determine the importance of submissive sounds in the social structure of clownfishes.

3.2.2. Material and methods

Sound recording and sound analysis

Three groups being composed of four individuals of *Amphiprion frenatus* (SL: 44-112 mm) were collected by scuba diving on the fringing reef around Nakijin village (26°40'N - 127°59'E; Okinawa, Japan) during May and June 2009. All fish were then brought back with their host (*Entecmaea quadricolor*) to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus where they were transferred to a community tank (3.5 × 2.0 × 1.2 m) filled with running seawater at ambient temperature (28 to 30.5°C). All fish were kept under natural photoperiod and fed once daily with food pellets *ad libitum*.

Recordings were made using a Brüel & Kjaer 8106 hydrophone in a smaller glass tank (1.2 × 0.5 × 0.6 m) filled with running seawater maintained at 28°C by means of a GEX cooler system (type GXC-201x, Osaka, Japan) for recording

under standardized conditions (see paragraph 2.2.2 for details on recording procedures and material characteristics). Sounds were analyzed according to the procedure mentioned in chapter 2 (see paragraph 2.2.3 for details and Figure 2.2), and a low pass filter of 2.05 kHz was applied to all sound recordings. Note that fish also produced aggressive sounds during interactions (see Figure 3.3 in results), but this type of sounds was not taken into account in the analyses. Here, the main interest was to investigate and to compare the acoustic features of submissive sounds between group members.

The social rank of each individual was attested using size differences (see paragraph 1.1.4 for more details). Basically, groups were composed of a breeding pair and two non-breeders (Table 3.3).

Table 3.3 Standard length (SL) and size order of each member in the different groups of *Amphiprion frenatus*

Size order	SL (mm)		
	Group 1	Group 2	Group 3
α (female)	105	110	112
β (male)	76	81	83
γ (non-breeder)	63	65	75
δ (non-breeder)	44	50	53

Statistical analyses

Correlation analyses were used to examine changes in all acoustic characteristics across SL. The data used in these analyses were mean values of all recorded sounds for each individual. Two statistical analyses were then performed to test the influence of social rank on sonic features. First, a full ANCOVA was run to test differences between social ranks (β, γ, δ; see Table 3.3) for the sonic variables correlated with SL. In this test, sonic variables are considered as variates, SL as a covariate and social rank is the grouping factor. Secondly, sonic variables not correlated with SL, which failed the test for normal distributions (Shapiro-Wilk *W* test), were analyzed using a non-

parametric Kruskal–Wallis one-way ANOVA by ranks with subsequent Dunn's test for pair-wise comparisons to test differences between social ranks. All statistical analyses were carried out with Statistica 7.1.Results are presented as means ± standard deviation (S.D.). Significance level was determined at $P<0.05$.

The within-individuals coefficient of variance ($C.V._w$ = S.D./mean) was calculated and compared with the between-individuals coefficient of variation ($C.V._b$ = overall S.D./overall mean) to obtain a measure of relative between-individuals variability for each acoustic feature (see paragraph 3.1.2 for more details). Note that this statistical analysis was run between individuals from a same group, and not between all individuals as a whole.

3.2.3. Results

Submissive sounds (n = 100 pulse units analyzed for each individual in each group; Table 3.4) were produced when subordinates displayed submissive posture as a reaction to charge and chase by dominants. It means that these sounds were never recorded for the dominant females (rank 1) during this study. Submissive sounds were always associated with head shaking movements, even if fish could sometimes carry out these movements without vocalizing. Note that there exist differences between submissive and aggressive sounds (Figure 3.3). Aggressive sounds consist of a single pulse unit that is longer than in submissive sounds, whereas submissive sounds are always composed of several pulse units. When aggressive sounds are produced in series, the pulse period is considerably longer than in submissive sounds, suggesting that each aggressive sound is emitted as a separate unit (Figure 3.3). Submissive sounds were multiple-pulsed (2-6 pulses, $\bar{x}$ = 3.2±0.26, n = 9 individuals) that can be produced alone or in series (2-9 sounds, $\bar{x}$ = 3.0±0.56, n = 9 individuals). Sound period averaged 197.0 ms and sound duration ranged from 23.5 to 50.6 ms ($\bar{x}$ = 35.9±9.59, n = 9 individuals). Pulse period averaged 11.8 ms and pulse duration ranged from 4.7 to 10.3 ms ($\bar{x}$ = 7.9±2.15, n = 9 individuals). Pulses had peak frequency of

591±115 Hz (n = 9 individuals) and most sound energy ranged from 454 to 778 Hz.

Correlation analyses revealed that dominant frequency and pulse duration were highly correlated with SL. Dominant frequency significantly decreased (r = −0.98, P<0.0001; Figure 3.4A) whereas pulse duration significantly increased (r = 0.98, P<0.0001; Figure 3.4B) with increasing SL. Pulse period was correlated across SL (r = 0.96, P<0.0001; Figure 3.4C) but this sonic variable was also significantly correlated with pulse duration (r = 0.95, P=0.0001). Additionally, sound duration was correlated with increasing SL (r = 0.97, P<0.0001; Figure 3.4E); this acoustic feature being also significantly correlated with both pulse duration (r = 0.93, P=0.0003) and pulse period (r = 0.98, P<0.0001). Likewise, sound period was correlated with increasing SL (r = 0.86, P=0.0031; Figure 3.4F), being significantly correlated with sound duration (r = 0.82, P=0.0063). The number of pulses per sound did not change significantly (r = 0.10, P=0.7917; Figure 3.4D) across SL, as well as the number of sounds per train (r = 0.06, P=0.8686; Figure 3.4G).

A comparison of social rank values using SL as a covariate showed that the dominant frequency (ANCOVA, test for common slopes: $F_{2,3}$=3.677, P=0.156; test for intercepts: $F_{2,5}$=1.204, P=0.374) and pulse duration (ANCOVA, test for common slopes: $F_{2,3}$=3.644, P=0.175; test for intercepts: $F_{2,5}$=4.204, P=0.137) did not differ between individuals of different social ranks. Thereby, differences between social ranks in these acoustic features exclusively resulted from size differences. Additionally, the influence of fish size on acoustic features was enhanced by comparing them between individuals of the same social rank but from different groups. All the acoustic features were significantly different between individuals of the same rank (Table 3.5), except when these ones had similar SL. In this case, acoustic features did not differ (Dunn's test, P>0.05).

Kruskal–Wallis one-way ANOVA revealed that means were significantly different between social ranks for pulse period (H=6.489, d.f.=2, P=0.0390) and sound duration (H=7.200, d.f.=2, P=0.0273), but not for sound period

(H=3.822, d.f.=2, *P*=0.1479), number of pulses per sound (H=1.898, d.f.=2, *P*=0.3871) and number of sounds per train (H=2.508, d.f.=2, *P*=0.2853).
Pairwise comparisons showed that pulse period and sound duration were higher in rank 2 (Dunn's test, $P<0.05$; Table 3.4) than in rank 4, whereas no significant differences were observed between ranks 2 and 3 (Dunn's test, $P>0.05$; Table 3.4), and between ranks 3 and 4 (Dunn's test, $P>0.05$; Table 3.4) due to considerable overlap.

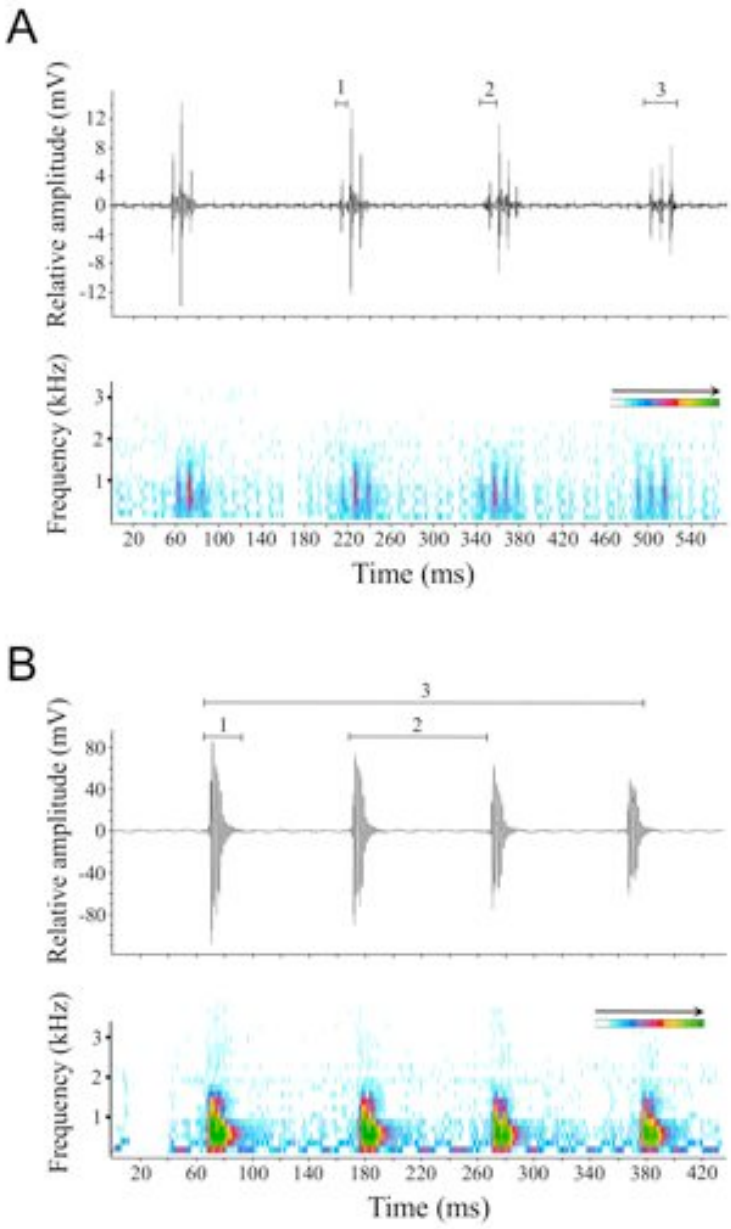


Figure 3.3 Oscillograms and spectrograms of submissive (A) and aggressive (B) sounds produced by a 63 mm SL specimen of *Amphiprion frenatus* showing the differences in (1) pulse duration and (2) pulse period. The sonic variable measured in (3) represents the sound duration in the case of submissive sounds, and the train duration in the case of aggressive sounds. The colour scale corresponds to the intensity associated with the different frequencies, and indicates that aggressive sounds are louder than submissive ones.

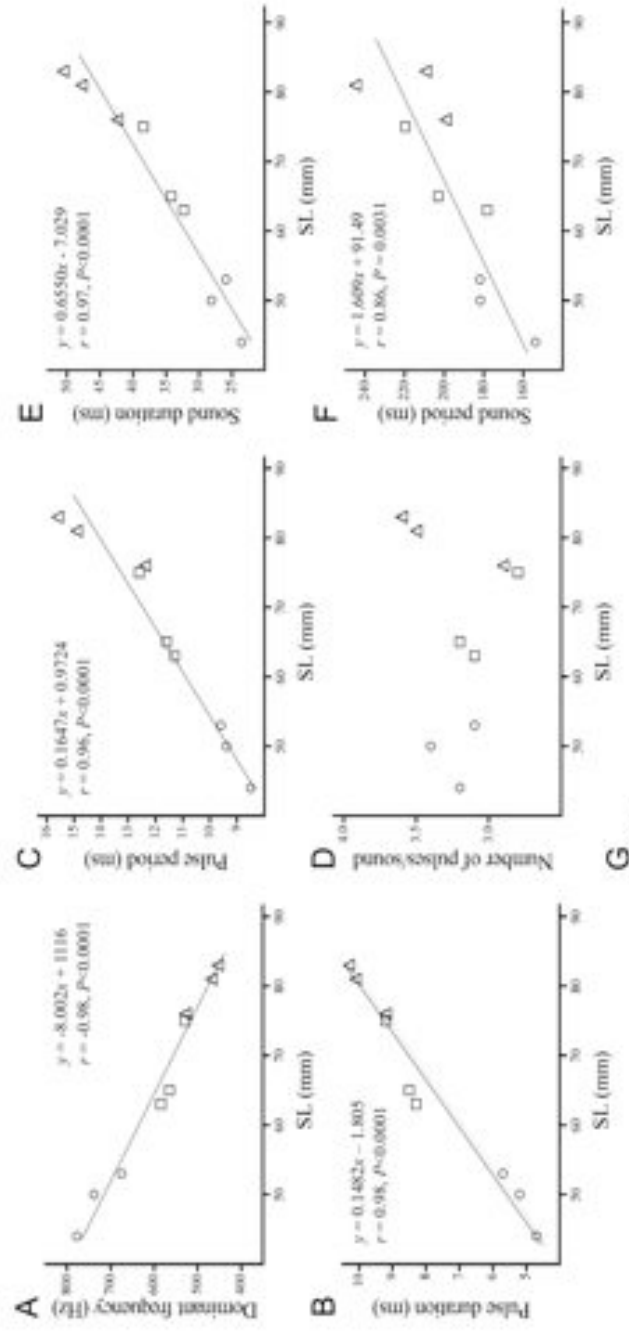


Figure 3.4 Influence of standard length (SL) on acoustic variables in *Amphiprion frenatus* (○ = rank 4, □ = rank 3, △ = rank 2). Correlations of (A) dominant frequency, (B) pulse duration, (C) pulse period, (D) number of pulses per sound, (E) sound duration, (F) sound period and (G) number of sounds per train against SL. Fish ranged from 44 to 112 mm in SL (n = 9). Results are expressed as mean values of all recorded pulses for each individual.

Table 3.4 Summary (mean ± S.D.) of the seven acoustic features analyzed from 9 *Amphiprion frenatus*

Individual (*n*)	Pulse duration (ms)		Dominant frequency (ms)		Pulse period (ms)		Number of pulses per sound		Sound duration (ms)		Sound period (ms)		Number of sounds per train	
	mean ± S.D.	*n*	mean ± S.D.	*n*	mean ± S.D.	*n*	mean ± S.D.	*n*	mean ± S.D.	*n*	mean ± S.D.	*n*	mean ± S.D.	*n*
Rank 2 (3)	9.9 ± 0.9	300	482 ± 54	300	14.3 ± 1.8	20	3.3 ± 0.9	91	46.6 ± 11.9	91	216.2 ± 43.8	56	2.7 ± 0.7	33
Rank 3 (3)	8.7 ± 0.9	300	559 ± 60	300	11.8 ± 1.4	20	3.0 ± 0.7	99	36.6 ± 9.7	98	192.5 ± 33.5	71	3.1 ± 1.3	34
Rank 4 (3)	5.2 ± 0.6	300	731 ± 100	300	9.2 ± 0.9	21	3.2 ± 0.8	96	25.8 ± 7.3	96	172.3 ± 31.3	73	3.7 ± 1.3	27

Table 3.5 Comparison of the acoustic features between individuals of the same social rank but from different groups of *Amphiprion frenatus*. H values are the result of the Kruskal-Wallis test (d.f. = 2, $n = 300$)

Acoustic variables	Social rank	H	*p*-value
Dominant frequency (Hz)	2	108.4	<0.0001
	3	42.11	<0.0001
	4	49.54	<0.0001
Pulse duration (ms)	2	79.16	<0.0001
	3	37.72	<0.0001
	4	139.0	<0.0001
Pulse period (ms)	2	121.5	<0.0001
	3	35.13	<0.0001
	4	64.19	<0.0001
Sound duration (ms)	2	6.418	0.0404
	3	15.18	0.0005
	4	10.27	0.0059
Sound period (ms)	2	9.995	0.0068
	3	22.80	<0.0001
	4	12.31	0.0021

Table 3.6 Means, ± S.D., range, within-individuals variability ($C.V_w$) and between-individuals variability ($C.V_b$) for the seven acoustic features analyzed from 9 *Amphiprion frenatus*

Acoustic variables	Group number	Overall mean ± S.D. (range)	$C.V_w$ (mean)	$C.V_w$ (range)	$C.V_b$	$C.V_b / C.V_w$	H*	*p*-value
Dominant frequency (Hz)	1	631 ± 135 (431–1036)	0.10	0.09-0.15	0.21	1.90	213.7	<0.001
	2	593 ± 139 (431–862)	0.10	0.08-0.12	0.22	2.20	251.0	<0.001
	3	552 ± 105 (345–776)	0.09	0.08-0.11	0.19	2.11	239.9	<0.001
Pulse duration (ms)	1	7.4 ± 2.1 (3.8-11.2)	0.10	0.09-0.11	0.28	2.80	232.4	<0.001
	2	8.5 ± 2.8 (3.6-15.1)	0.10	0.09-0.11	0.34	3.40	250.8	<0.001
	3	9.1 ± 2.8 (4.5-13.9)	0.08	0.06-0.12	0.31	3.87	233.2	<0.001
Pulse period (ms)	1	10.7 ± 2.0 (6.7-15.4)	0.10	0.08-0.12	0.19	1.90	143.8	<0.001
	2	11.9 ± 2.5 (7.4-17.6)	0.10	0.08-0.11	0.21	2.10	167.4	<0.001
	3	12.7 ± 2.6 (8.2-18.4)	0.08	0.05-0.09	0.21	2.62	176.2	<0.001
Number of pulses per sound	1	3.1 ± 0.6 (2-5)	0.20	0.19-0.23	0.21	1.05	5.36	ns
	2	3.4 ± 0.9 (2-6)	0.26	0.21-0.31	0.27	1.04	2.59	ns
	3	3.1 ± 0.8 (2-5)	0.25	0.22-0.29	0.27	1.08	12.86	<0.01
Sound duration (ms)	1	29.2 ± 8.4 (9.8-48.2)	0.22	0.18-0.24	0.29	1.32	59.24	<0.001
	2	36.9 ± 14.1 (16.6-72.4)	0.28	0.22-0.33	0.38	1.36	25.94	<0.001
	3	36.6 ± 14.1 (15.0-73.8)	0.24	0.13-0.35	0.38	1.58	49.90	<0.001
Sound period (ms)	1	176.4 ± 36.9 (92.2-295.3)	0.18	0.16-0.21	0.21	1.17	16.53	<0.01
	2	206.3 ± 40.9 (135.6-280.9)	0.15	0.12-0.18	0.20	1.33	21.07	<0.001
	3	201.7 ± 31.5 (118.3-266.3)	0.13	0.08-0.16	0.16	1.23	16.17	<0.01
Number of sounds per train	1	3.3 ± 1.5 (2-9)	0.39	0.28-0.53	0.44	1.13	4.36	ns
	2	3.2 ± 1.3 (2-7)	0.35	0.30-0.41	0.41	1.17	4.93	ns
	3	2.6 ± 0.8 (2-5)	0.23	0.18-0.27	0.30	1.30	14.39	<0.01

*Results of Kruskal-Wallis test (d.f. = 2, n = 360) comparing differences between individuals of a group for each acoustic feature.

Individuality

Submissive sounds presented some acoustic features that displayed $C.V._w \leq 0.10$ (Table 3.6), suggesting a strong homogeneity of these variables. All the acoustic features analyzed had $C.V._b/C.V._w$ ratios > 1, showing a higher variability among than within individuals. The Kruskal-Wallis analyses revealed significant differences among individuals for almost all features (Table 3.6), indicating that these acoustic variables (except the number of pulses per sound and the number of sounds per train in groups 1 and 2; see Table 3.6) can potentially provide recognition cues to identify the sound emitter. The larger relative between-individuals variability (larger $C.V._b/C.V._w$ ratios) corresponded to the dominant frequency, pulse duration and pulse period (Table 3.6).

3.2.4. Discussion

In clownfishes, different types of sounds such as "threatening" and "shaking" (Schneider 1964a), "click" and "grunt" (Allen 1972) or "pop" and "chirp" (Chen & Mok 1988, Parmentier *et al*. 2005) have been recorded during agonistic interactions between conspecifics. Unfortunately, these terms have been inconsistently applied which create confusion (Amorim 2006). Sufficient data exists, however, to attest that the behavioral posture exhibited by fish during submissive sounds are equivalent to "shaking" sounds described by Schneider (1964a). Furthermore, our observations highlight that submissive sounds are clearly inherent to a different behavior and possess different function from aggressive sounds. Aggressive sounds are produced during chases and threat displays between conspecifics (see paragraph 3.1.3), while submissive sounds are always emitted when subordinates exhibit an appeasement behavior (head shaking movements) in reaction to aggressive displays by higher-ranking individuals. Therefore, both types of sounds seem to be an integral part of the agonistic behavior of clownfishes.

Dominant frequency and pulse duration of submissive sounds display size-

related variation in the different groups of *A*. *frenatus*. The more fish size increases, the more dominant frequency decreases, and the more pulse duration increases. The same relationship has been observed in aggressive sounds for *A*. *akallopisos* (see paragraph 3.1.3). Here, differences in both acoustic acoustic features are related to fish size and not to social status. However, size and social rank are extremely related to each other due to the size-based hierarchy within each group (Fricke 1979, Buston 2003). Dominant frequency and pulse duration could therefore be signals conveying information on the social rank of the emitter within the group. As a result, submissive sounds could possess an appeasement function by expressing the lower rank status during interactions. In the same way, the grey gurnard *E*. *gurnardus* emit knocks during low levels of aggression and grunts mainly while performing frontal displays to opponents (Amorim *et al*. 2004a).

Clear differences were found among submissive sounds attributed to different individuals. All acoustic variables were significantly more variable between than within individuals and thus could all potentially provide cues to identify individuals. Furthermore, the most important variables to allow individual identification are dominant frequency and pulse duration. Pulse period, in a lesser extent, was also consistently important for discriminating among individuals (Table 3.6). In order to be good candidates for individual recognition, these acoustic features should propagate through the environment, and should be detected by the receiver. Sound propagation in shallow water can result in signal degradation over short distances, including sound pressure level and frequency attenuation, and sound duration loss (Mann 2006). The effect of environmental attenuation and signal degradation, however, should not impose a major restriction within a group of clownfishes since all individuals inhabit a restricted territory (the sea anemone) and spend most of the time in close vicinity of their host.

Since dominant frequency and pulse duration of submissive sounds are size-dependent, inter-individual differences could be detected by members within each group. In *A*. *frenatus*, sounds emitted by individuals of different social ranks differ in dominant frequencies and pulse duration by >10%

(Table 3.4; see paragraph 3.1.4 for comparison with other fish species).

No information related to size can be extracted from number of pulses per sound or number of sounds per train. Differences in these acoustic features appear to be related to a difference in motivation. The smallest individuals (rank 4) in *A. frenatus* groups emitted the highest number of sounds per train (Table 3.4). This observation suggests that lower-ranking individuals might produce more sounds in order to limit aggressive acts from dominants or to show their submission.

Acoustic recognition is beneficial when vocal animals defend long-term territories. However, agonistic sounds always occur with visual stimuli and that is why their functional effect is difficult to determine. Under these conditions, playbacks experiments are the most successful tools for studying the functional significance of acoustic signals (Myrberg 1997a).

Myrberg & Riggio (1985) verified that males of the bicolor damselfish *Stegastes partitus* can recognize territorial neighbors based on acoustic cues, probably the dominant frequency that decreased with male size. In addition, males of *S. partitus* use a sound as a "keep-out" signal in territorial maintenance (Myrberg 1997b). Indeed, empty territories, from which the residents had been removed, remained free of conspecific intruders when the resident's sounds were repeatedly transmitted. Such researches on the functional significance of sounds would need to be carried out in clownfishes in order to determine the importance of the acoustical component in comparison with the visual one during agonistic interactions within group.

The present study suggests that there is enough information in the submissive sounds of clownfishes to promote individual recognition. Dominant frequency and pulse duration are morphologically determined signals related to fish size. This relationship is of significant importance because clownfishes live in social group in which there is a size-based dominance hierarchy. Such a relationship between acoustic features and fish size has also been observed for aggressive sounds in *A. akallopisos* (see paragraph 3.1.3). Thus, both types of sounds convey information on the size of the emitter, and probably on their identity (sex and/or social rank).

At present, it would be very interesting to conduct quantitative and qualitative monitoring of sounds within a group in order to determine the importance of acoustic signals during agonistic interactions, and to make the connection with the study of Fricke (1979), which investigated the aggressive dominance in groups of *A*. *akallopisos* by quantifying all agonistic interactions (*i.e*. aggressive acts and appeasement behavior) between group members (see also Figure 1.6).

3.3. REPRODUCTIVE BEHAVIOR AND ACOUSTIC SIGNALS *

3.3.1. Introduction

Courtship signals are used by a variety of animals for mate assessment, mate attraction, synchronization of reproduction, and to provide a species-specific cue to prevent hybridization of closely related sympatric species (Bradbury & Vehrencamp 1998). The production of courtship sounds to attract mates has been described from many marine and freshwater fish species such as gadoids like the Atlantic cod *Gadus morhua* (Nordeide & Kjellsby 1999) and the haddock *Melanogrammus aeglefinus* (Hawkins & Chapman 1966, Hawkins & Amorim 2000); sciaenids such as the weakfish *Cynoscion regalis* (Fish & Mowbray 1970, Connaughton & Taylor 1995); batrachoidids such as the midshipman *Porichthys notatus* (Ibara *et al*. 1983, Bass 1990) and the toadfish *Opsanus tau* (Winn 1964); the striped blenny *Chasmodes bosquianus* (Tavolga 1958); gobiids (Lugli & Torricelli 1999) and cichlids (Brown & Marshall 1978, Lobel 1998). Besides being produced to attract spawn-ready mates to the spawning site, acoustic signals may also be used for signaling the release of gametes. In the haddock *M*. *aeglefinus* for example, fast vocalizations and humming sounds by the male indicates the final stage of courtship and continue during the mating act, when milt and eggs are released into the water column (Hawkins & Amorim 2000). Spawning sounds have also been observed among coral reef fishes. Some ostracids such as the cowfish *Lactoria fornasini* and the trunkfish *Ostracion meleagris* produce sounds during approximately the whole period of gamete release (Moyer 1979, Lobel 1996), whereas the hamlet *Hypoplectrus unicolor* (Serranidae) emits unique spawning sounds just before or during the mating act (Lobel 1992).

* Slightly modified from: **Colleye O, Nakamura M, Iwao K, Vandewalle P, Parmentier E.** Diversity of sound production in clownfishes: aggressive, submissive and reproductive signals. (in preparation)

The damselfishes (Pomacentridae) are one of the best-studied families for use of acoustic communication during courtship (Myrberg 1972, Spanier 1979, Kenyon 1994, Lobel & Mann 1995, Mann & Lobel 1998, Lobel & Kerr 1999, Parmentier *et al*. 2010). Males of several species (*e.g*. *Abudefduf* spp., *Dascyllus* spp., *Stegastes* spp.) produce courtship sounds, either during the signal jump swimming display (Myrberg 1972, Lobel & Mann 1995, Mann & Lobel 1998), during zigzag pattern (Lobel & Kerr, 1999), while a male escorts a female towards the nest (Maruska *et al*. 2007), or when females visit the nest accompanied by males and perform a pseudo-spawning behavior, with both mates passing over the nesting surface (Mann & Lobel 1998, Parmentier *et al*. 2010).

While damselfishes have been examined extensively, studies that describe the courtship sounds in clownfishes are limited in number. To date, sound production during reproductive period has only been reported in three clownfish species (*A*. *ocellaris*, *A*. *frenatus*, *A*. *sandaracinos*) by Takemura (1983). However, these observations need to be carefully considered since the recorded sounds were infrequently emitted and hardly heard, and sometimes they do not seem to be directly related to spawning behavior (Takemura 1983). Therefore, deeper attention must be paid in order to confirm the implication of acoustic signals during reproduction in this group.

Courtship in clownfishes is generally stereotyped and ritualized, and is typically accompanied by different activities such as nest cleaning (preparation of the nest site), spawning and nest care (parental care). The aim of this study was to determine whether clownfishes use sounds to synchronize one or several of their reproductive activities.

3.3.2. Material and methods

Recordings were made both in aquarium and in the field. Depending on the recording conditions, different methods and materials were used (see paragraph 2.2.2).

Recordings in aquarium

In Oceanopolis Aquarium in Brest (France), seven species namely *A. akindynos*, *A. clarkii*, *A. melanopus*, *A. ocellaris*, *A. percula*, *A. perideraion* and *P. biaculeatus* are reared for several years. Each mating pair is maintained in separate glass tanks (0.70 x 0.45 x 0.50 m) filled with running seawater at ambient temperature (26°C). Recordings were made in *A. akindynos*, *A. melanopus*, and *A. percula* during July 2008.

In addition, recordings were made in Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus (Okinawa, Japan). One mating pair of *A. clarkii* was kept in a separate tank (1.2 x 0.5 x 0.6 m) filled with running seawater maintained at 28°C. Recordings were carried out during summer season 2009 (between May and July) because reproduction is limited to this period in this geographical area (see paragraph 1.1.5).

Recordings were made using a Brüel & Kjaer 8106 hydrophone according to the procedure mentioned in chapter 2 (see paragraph 2.2.2).

Field recordings

Field recordings were made on the fringing reef in front of Hizuchi beach (26°11'N – 127°16'E; Akajima, Kerama Islands, Japan). Recording sessions, each lasting from 1 to 4 h, were made in August 2009 at a depth of between 5 and 10 m. Seawater temperature was on average 28°C. Recordings were made by placing the housing in front of the sea anemone. The different behaviors displayed by fishes were analyzed on the basis of the videos and field observations.

Recordings were made using a SONY HDD video camera placed in a housing and coupled with an external hydrophone (see paragraph 2.2.2 for details on material characteristics).

3.3.3. Results

A total of eight spawning events were observed from the five species studied. All reproductive patterns including nest preparation, courtship, spawning and parental care were once observed and recorded in *A. akindynos*, *A. melanopus*, and *A. percula* during July 2008 at Oceanopolis Aquarium. *Amphiprion clarkii* spawned four times between May and July 2009 in Sesoko Station, and all the reproductive activities were observed and recorded. Generally speaking, spawning always occurred in the afternoon from 2:00 to 5:00 p.m., whatever the species. In addition, one entire spawning sequence in *A. perideraion* was observed and recorded in the field for approximately 80 minutes in August 2009 (11:20 to 12:40 a.m.).

The most striking observation was the complete absence of sound production throughout all activities of the reproductive period in the different clownfish species recorded, and whatever the recording environment (aquarium or field).

Nest preparation and social interaction

Basically, the arrival of spawning period was indicated by an increase of cleaning activity by the male, and by the belly of the female that was noticeably distended (especially in *A. percula* and *A. perideraion*). These features became usually distinct three or four days before spawning.

Occasionally, fish chased one another or engaged in fast side by side swimming and belly touching; the female seemed to be the initiator in most of these encounters. Sometimes, the female entered the nest and pressed her belly against the rock (spawning ground). Pecking movement of the male at the surface of nest became more vigorous from about two hours before spawning; this movement was continued until just before spawning.

About 15 minutes before spawning, nest-cleaning activities was more rigorously carried out by the female, which pecked the surface of the spawning ground. Also, she pressed her belly against the substrate. These

activities seemed to aim at making sure of the completion of the spawning ground. At the onset of spawning, the whitish cone-shaped ovipositor of the female was clearly apparent.

Spawning and fertilization

The spawning was carried out in the following way: the female entered the nest, pressed her belly against the spawning ground and swam slowly in a circular path; the male followed closely behind and fertilized the spawn. Locomotion during the spawning passes was achieved by rapid fluttering of the pectoral fins. The male frequently mouthed the eggs during the spawning period. Both fish also nibbled on the tips of the anemone tentacles for preventing the spawn from entering into contact with them.

Parental care

After the spawning, the incubation period took place and was characterized by parental care, which lasted usually six to seven days. The male assumed nearly the full responsibility of tending the nest. Except an initial moderate level of activity at spawning, there were no cleaning activities the first two days. Then, an abrupt increase occurred the next few days until hatching. Two basic nest-caring behavior patterns were observed. Fanning was the most common and was mainly performed by fluttering the pectoral fins. Mouthing the eggs and substrate biting at the periphery of the nest were also exhibited. Dead eggs were regularly removed as indicated by bare patches at the nesting surface.

3.3.4. Discussion

Unlike observations made by Takemura (1983), no acoustical behavior was observed during reproductive activities in clownfishes. Moreover, Takemura's data are doubtful since *A*. *ocellaris*, *A*. *frenatus* and *A*. *sandaracinos* would emit

these types of sounds with high frequency component of more than 2 kHz (Takemura 1983). Parmentier *et al*. (2009c) determined the hearing sensitivity in three different clownfish species (*A*. *frenatus*, *A*. *ocellaris* and *A*. *clarkii*); the frequency range over which they can detect sounds was between 75 and 1800Hz, and they were the most sensitive to frequencies below 200Hz. Therefore, this finding raises the question over the interest of clownfishes to produce sounds they could not detect during reproductive activities. It remains these sounds could just be a by-product of the nest cleaning activities.

In the field, clownfishes spawn on average 1.0 ± 0.5 or 0.6 ± 0.1 times per month depending on whether they live in tropical waters (Allen 1972, Ross 1978) or in more temperate regions (Moyer & Bell 1976, Ochi 1989, Richardson *et al*. 1997). In captivity, the spawning frequency is higher and on average 24.9 ± 5.3 times per year (Hoff 1996, Gordon & Bok 2001). This frequency was observed in captive clownfishes reared at Oceanopolis Aquarium, whose spawning occurred approximately every two weeks. In this context, it could be argued that the captivity modulates some aspects of the behavior (Gordon & Bok 2001) such as sound production during reproduction. However, 1) the pair of *A*. *clarkii* reared at Sesoko Station showed the same spawning frequency (~ every 2 weeks), although its reproduction was restricted to summer season and it was reared under semi-natural conditions (*i.e*. outdoor tank filled with natural seawater and maintained under natural photoperiod); 2) spawning was witnessed in the field for *A*. *perideraion*, and no sound was produced by the mating pair during the reproductive event. Yet, aggressive sounds were heard during this recording session, which indicates that the recording material worked well.
Overall, sound production does not seem involved in the reproductive behavior of clownfishes. This observation might be explained by some particular aspects of the life history of clownfish species.

The reproductive behavior of pomacentrids is subdivided into the following major categories (Reese 1964, Allen 1991): 1) establishment of territory, 2) selection of nest site within the territory, 3) preparation of the nest site, 4) courtship and pair formation, 5) spawning and fertilization, and 6) parental

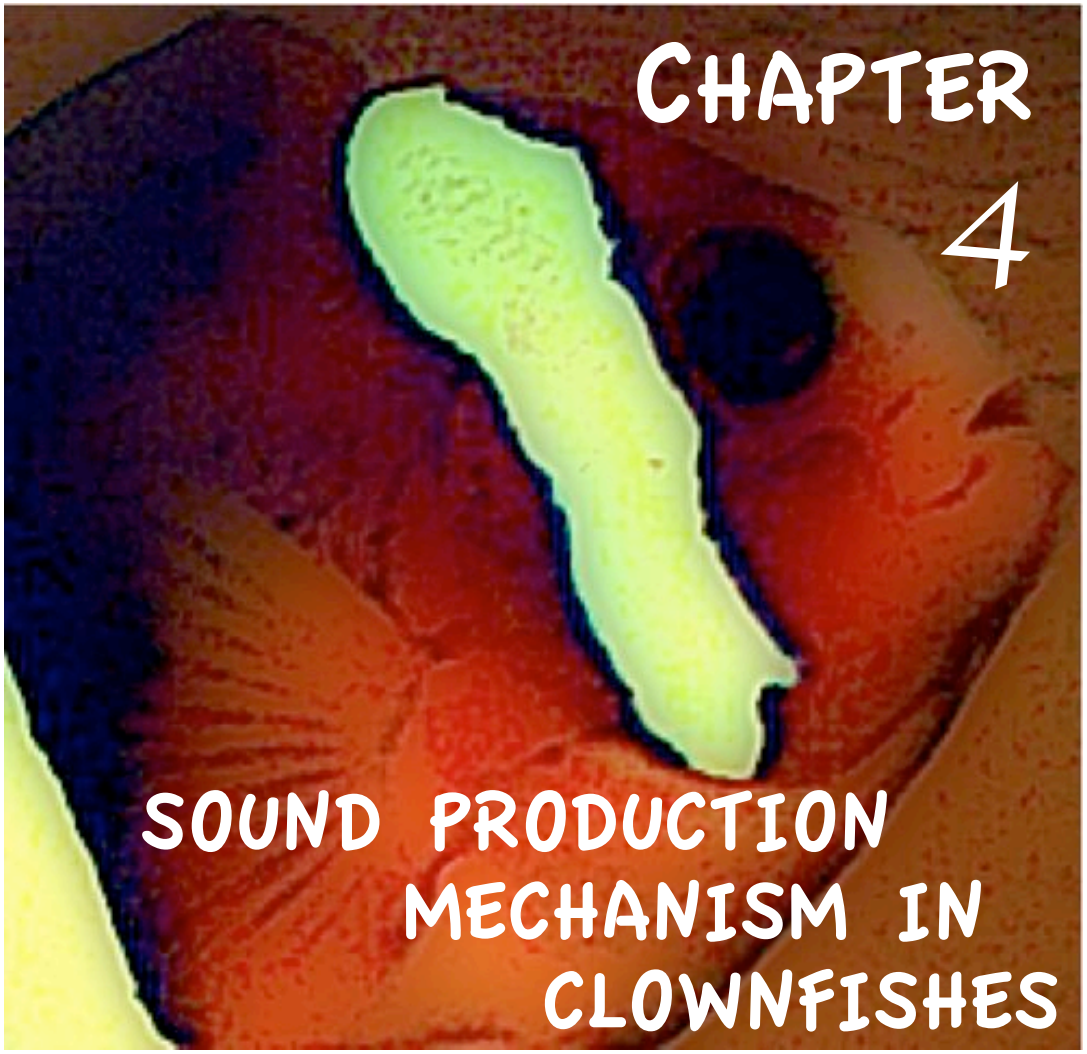
care. Clownfishes conform to this general pattern but are distinctive with regards to formation of permanent pair bonds that usually last for several years in most species (Allen 1972, Fautin & Allen 1992). In other damselfishes, one male may mate with several females during a single spawning episode (Allen 1991, Fautin & Allen 1992). In clownfishes, however, male does not need to exhibit special courtship behavior for attracting female. Pair-bonding is very strong and is correlated by the small size of their territories (centered on actinians) that is, in turn, correlated with the unusual social hierarchy existing in each social group. On the other hand, it seems that other cues such as visual signals might be useful for synchronizing reproductive activities. For example, just before spawning occurs, the female joins the male and becomes more insistent in the nest-cleaning activities; probably in order to convey visual cues about its readiness to spawn. Likewise, it is possible that the male regulates its level of nest-caring activity in response to visual stimuli received when inspecting eggs (Allen 1972). A visual stimulus of this sort would signal the stage of egg development and the need for increased fanning and mouthing activities. Allen (1972) experimentally demonstrated that strong agitation of the eggs is a requisite for hatching. He also noted that there was a pronounced increase in the amount of male nest care on day six of incubation. On that day, the embryos are well developed with one of the most noticeable features being the large eyes with their silvery pupils. Such a feature might serve as an appropriate visual cue.

The present study highlights that clownfishes do not use acoustic signals for mate assessment, mate attraction or synchronization of reproductive patterns. Instead, other cues such as visual and even perhaps chemical signals might be involved in reproductive activities. However, new behavioral tests would need to be run to determine the proper role of such signals during the reproduction of clownfishes.

3.4. CONCLUSION

Sounds produced by clownfishes can be divided into two main categories: aggressive sounds produced in conjunction with threat postures (charge and chase), and submissive sounds always emitted when subordinates exhibited an appeasement behavior (head shaking movements) in reaction to aggressive displays by dominants. Both types of sounds show intraspecific differences related to fish size, highlighting that some acoustic features (*i.e.* dominant frequency and pulse duration) might be useful cues for individual recognition within the group. These observations are of significant importance because the social structure of clownfishes relies on a size-based dominance hierarchy. On the other hand, no acoustic signal is associated with reproductive activities.

Overall, it appears that clownfishes show less diversity in their acoustic behavior in comparison with other pomacentrid species. This is probably due to their peculiar way of life: these fishes form social groups that inhabit a restricted territory (the sea anemone), spend most of the time in close vicinity of their host, and rarely interact with other species on the reef.


CHAPTER
4
SOUND PRODUCTION
MECHANISM IN
CLOWNFISHES

4.1. FUNCTIONAL STUDY OF THE SONIC MECHANISM *

4.1.1. Introduction

Among vertebrates, teleost fishes have evolved the largest diversity of sonic organs involving stridulation of bony structures, plucking of stretched tendons or contraction of intrinsic and extrinsic muscles that excite swimbladder vibration by deformation of its wall (Tavolga 1971, Demski *et al*. 1973, Kratochvil 1978, Fine *et al*. 1997, Parmentier *et al*. 2006a).

Damselfishes (Pomacentridae) are among the best-studied groups in terms of sound production with more than 20 species belonging to eight different genera reported as sound emitter (Takemura 1983, Myrberg *et al*. 1986, Lobel & Mann 1995, Amorim 1996a, Santiago & Castro 1997, Lobel & Kerr 1999, Picciulin *et al*. 2002, Parmentier *et al*. 2005, Parmentier *et al*. 2006b, Maruska *et al*. 2007, Parmentier *et al*. 2010). Within this family, clownfishes (*Amphiprion*) are well known to produce aggressive sounds during agonistic interactions involving conspecifics or heterospecifics (Verwey 1930, Allen 1972, Takemura 1983, Chen & Mok 1988, Parmentier *et al*. 2005).

Although clownfish sounds have been first reported as early as 1930 (Verwey 1930), the mechanism of sound production has remained unexplained. It can be claimed that clownfishes do not produce sounds by stimulating their swimbladder since there are no specialized sonic muscles attached to the bladder in this group (Chen & Mok 1988). Some assumptions were proposed, including notably sound production by pharyngeal teeth grating, which could then be amplified by the swimbladder (Chen & Mok 1988, Rice & Lobel 2003). It was also reported that sound was produced by rapid up-and-down movements of the buccal jaws similar to those of feeding

* Slightly modified from: **Parmentier E, Colleye O, Fine ML, Frédérich B, Vandewalle P, Herrel A.** 2007. Sound Production in the Clownfish *Amphiprion clarkii*. *Science* **316**: 1006-1006.

(Verwey 1930, Takemura 1983). However, these hypotheses have never been attested and still require experimental verification.

Here, we give a functional and morphological description of the sonic mechanism of the aggressive sounds in the yellowtail clownfish *Amphiprion clarkii*, using a multidisciplinary approach that combines functional morphology, high-speed videos, high-speed cineradiography and sound recordings.

4.1.2. Material and methods

Specimen collection

Ten *Amphiprion clarkii* including one mating pair (SL: 90-102 mm) and eight juveniles (SL: 35-60 mm) were provided by the Aquarium of La Rochelle (La Rochelle, France) and the Oceanopolis Aquarium (Brest, France) respectively. The mating pair was isolated in a fish tank (0.4 x 0.5 x 0.4 m) with a clay pot for simulating the sea anemone, whereas all juveniles were kept in another tank (0.8 x 0.4 x 0.5 m). The environmental temperature was 26°C. All fishes were maintained on a 12h:12h light:dark cycle and were fed with food pellets once a day *ad libitum*.

High-speed video during sound production

Three individuals (the mating pair and one juvenile, SL: 60-102 mm) were recorded with the high-speed video camera in the experimental tank (0.4 x 0.5 x 0.4 m). A conspecific intruder was introduced into the aquarium (Figure 4.1) to induce territorial behavior from resident fish, which was usually accompanied by sound production. Three video sequences were produced for each of the tested individual. Three landmarks were used to follow the movements performed by the fish during sound production (Figure 4.2): 1) the neurocranium, 2) the upper jaw tip, and 3) the lower jaw tip.

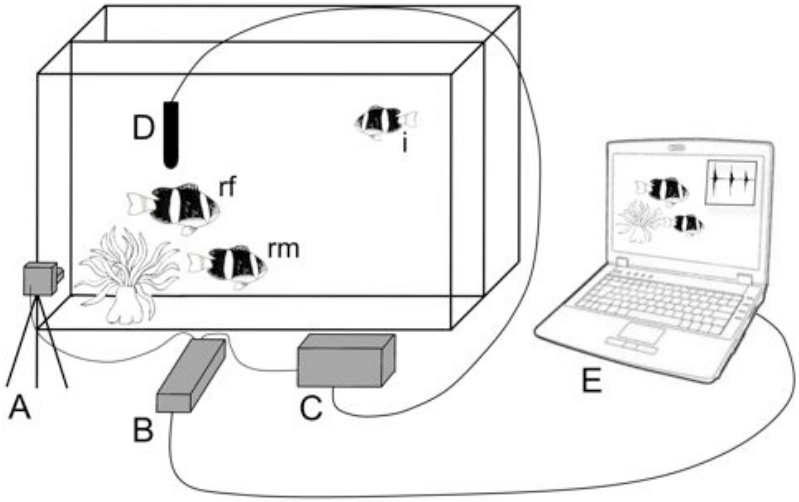


Figure 4.1 Schematic view of the experimental setup used for high-speed video recordings associated to sound production in *Amphiprion clarkii*. A) high-speed camera, B) data acquisition box, C) sound amplifier Nexus, D) hydrophone, E) laptop with the recording software Midas, rf: resident female, rm: resident male and i: intruder.

Specimens were filmed at 200 fps. Videos were recorded in lateral view using a Redlake MotionPro high-speed camera (resolution: 1280 x 1024 pixels, San Diego, CA, USA). This camera was connected to a laptop (Asus v9280S, San Diego, CA, USA), allowing the visualization of fish movements in real time. This imagery system was synchronized with a BK 8106 hydrophone connected to a Nexus™ conditioning amplifier type 2690 (see paragraph 2.2.2 for details on material characteristics) by means of a data acquisition box (Midas, DA Module, BNC Breakout Box, Cambridge, MA, USA). For recording sessions, a wall limiting the depth of field was placed into the experimental tank in order to improve film resolution (see Figure 4.1). The three anatomical landmarks shown in Figure 4.2 were digitized frame-by-frame from the high-speed videos using Midas software (Redlake, version 2.2.0.7). The coordinates of all landmarks were recalculated to a frame of reference moving with the fish (the origin of the referential was digitized on each frame). In this frame, the anterior tip of the base of the anal fin was taken as origin and the horizontal *X*-axis was defined by the straight line passing through this

landmark and the lower tip of the first vertical white band situated on the opercular bone (see Figure 4.2). A buffer memory with a capacity of 2GB was used to record the data after visualization.

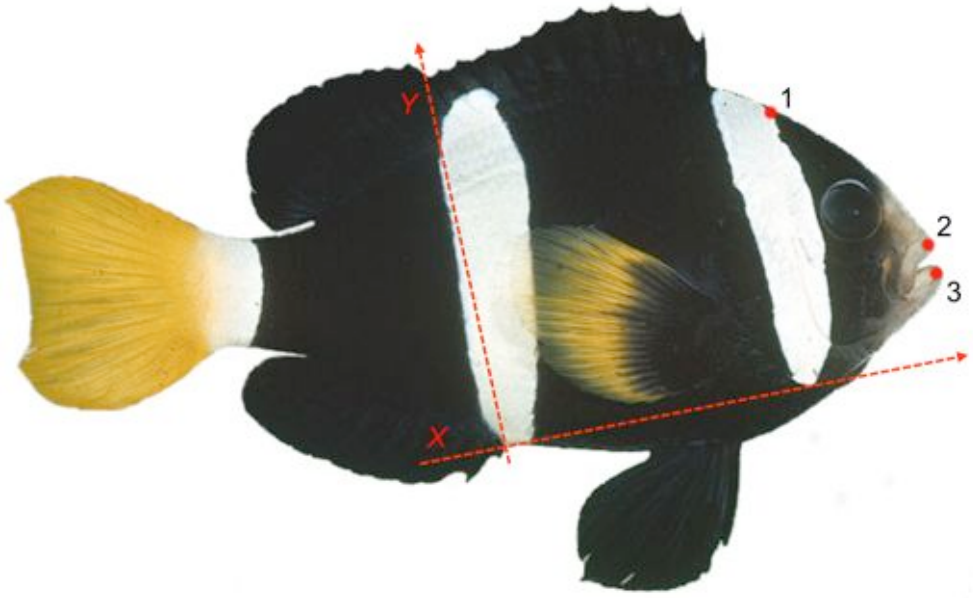


Figure 4.2 Lateral view of *Amphiprion clarkii* showing the three landmarks used to track the sound production movements during the high-speed movies. The landmarks are: 1) the neurocranium, 2) the upper jaw tip, and 3) the lower jaw tip.

X-ray video recording during sound production

For X-ray recordings, only the mating pair of *A. clarkii* was placed into an experimental tank (0.4 x 0.3 x 0.2 m) extended by a narrow corridor (0.3 x 0.2 x 0.1 m). Fish were maintained into this corridor during recordings in order to ensure the specimen was perpendicular to the lens axis. High-speed X-ray videos were recorded using a Philips Optimus X-ray generator (Royal Philips Electronics NV, Eindhoven, The Netherlands) coupled to a 14-inch image intensifier with two zoom modes (10 and 6 inch) and a Redlake Motion Pro camera (resolution: 1280 x 1024 pixels, San Diego, CA, USA). Videos were recorded at 500 fps in lateral view, using the 10-inch zoom function. X-rays were generated at 65 kV.

In order to increase the quantification accuracy of movements of fish bones, small radio-opaque markers (Figure 4.3) were inserted onto different bones. Note that the positions of interest were selected after observation of high-speed videos. Prior to implantation of these markers, fish were anaesthetized with 50 mgl^{-1} MS222 in solution. Each marker implantation was preceded by loading a small lead sphere into the tip of a hypodermic needle (16-gauge on the scale of the Stubs). The needle was then inserted subcutaneously, touching the bones at specific position of interest. Next, the lead marker was released from the hypodermic needle by pushing a steel wire through the back of the needle while simultaneously retracting it. In this way, 5 markers were inserted and used to track the movements of 1) the neurocranium, 2) the lower jaw, 3) the basihyal bone, 4) the hyoid bar, and 5) the cleithrum bone in each individual. Finally, the position of the markers was checked by taking X-ray images (see Figure 4.3). For each individual, three sequences were analyzed. All landmarks were digitized frame-by-frame from the X-ray videos using Midas software (Redlake, version 2.2.0.7). The digitization and recalculation of reference frame was done in the same way as for high-speed video recordings. A radio-opaque marker inserted on the second pterygiophore of the anal fin was taken as origin, and the horizontal *X*-axis passed by this landmark and the landmark 5 (see Figure 4.3).

Sound recording experiments

Three *A. clarkii* (SL: 55-102 mm) specimens were used in these experiments. Sounds were recorded using an Orca hydrophone placed in the experimental tank (0.8 × 0.4 × 0.5 m) at 26°C. After being first recorded in a context of territory defense (see paragraph 2.2.2 for details on procedures and material characteristics), the three individuals were anaesthetized by placing them into a dedicated container with 50 mgl^{-1} of MS-222 in seawater.

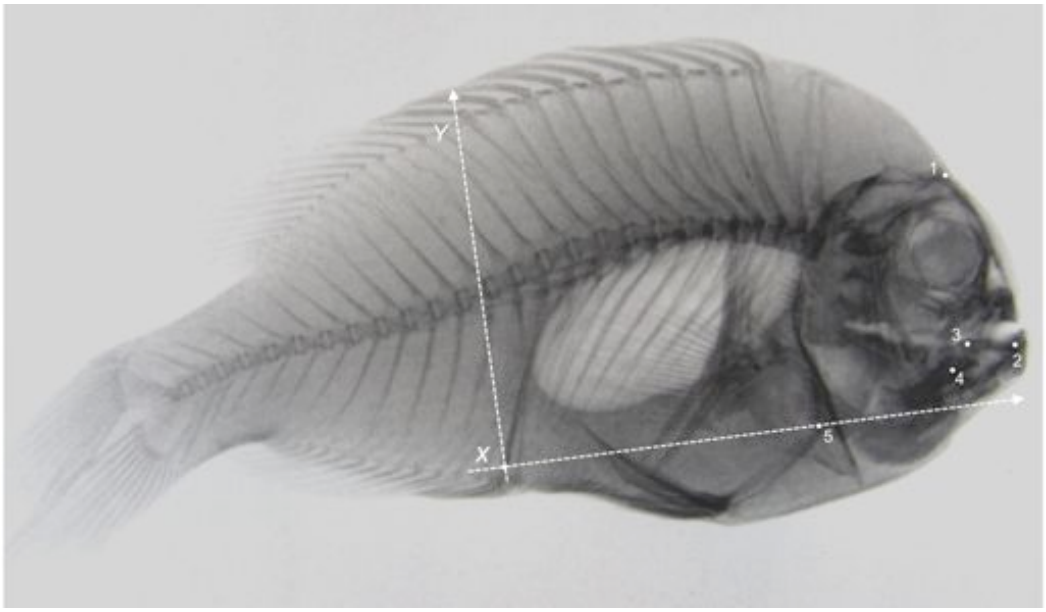


Figure 4.3 Position of the inserted radio-opaque markers on a lateral X-ray image of *Amphiprion clarkii* (SL = 102 mm). The markers are: 1) the neurocranium, 2) the lower jaw, 3) the basihyal bone, 4) the hyoid bar, and 5) the cleithrum bone.

Based on analyses of high-speed video recordings, sound seems to be produced when buccal jaws came into contact (see results, paragraph 4.1.3), thus two underlying experiments were carried out to validate the sonic mechanism.

(1) Natural sounds produced by fish were compared with sounds obtained after cutting upper and lower jaw teeth of the same fish.

(2) A ligament joining the hyoid bar to the internal part of the mandible was transected in order to observe how it could be involved in the sound-producing mechanism.

After a three hours time recovery, fish were again recorded by introducing a conspecific intruder into the tank. Sound analyses were done based on the procedure described in chapter 2 (see paragraph 2.2.3 for details). A low pass filter of 2.45 kHz was applied to all sound recordings.

The *W* Shapiro-Wilk statistic was used for testing the normal distribution of acoustic data. Non-parametric Wilcoxon matched-pairs signed rank test was

then used to compare acoustic features for each of the experimental conditions that we have tested. Statistical analyses were carried out with Statistica 7.1. Results are presented as mean ± S.D. Significance level was determined at $P<0.05$.

Morphological study

Eight *A. clarkii* (SL: 35-60 mm) specimens were euthanized with an overdose of MS-222 (500 mgl^{-1}). Three were fixed in 7% buffered seawater formalin for approximately 2 weeks before being transferred to 70% ethanol for storage. Then, they were *in toto* stained with Alizarin Red S according to Taylor and Van Dyke's method (Taylor & Van Dyke 1985) in order to observe osseous structures. The other specimens were dissected and examined with a Wild M10 binocular microscope (Leica, Wetzlar, Germany) equipped with a camera lucida. The osteology nomenclature follows the description of Barel *et al*. (1976) and Emery (1973). The terminology of muscles follows that of Winterbottom (1974).

4.1.3. Results

Description of movements during sound production

The sounds obtained during the high-speed video recordings were composed of pulses, which were mainly emitted in series. These series were generally composed of 2-8 pulses. The means for pulse duration and pulse period were 26.2 ± 5.6 ms and 34.6 ± 14.9 ms, respectively. The dominant frequency ranged from 450 to 960 Hz with a mean of 685 ± 179 Hz.

The high-speed video recordings showed that sounds were accompanied by a series of movements including a mouth opening, a neurocranium elevation, and finally a fast mouth closing. Synchronization of sound pulses with video images indicated that sound was produced during mouth closing, precisely when buccal jaws came into contact (see Movie S1 in supplementary

material). No other discernable movement was observed during sound production. Overall, all skeletal structures moving during sound production were located at the level of fish head (Figure 4.4).

The experiment using high-speed video coupled with the X-ray system confirmed and complemented these results (Figure 4.5). A lowering of the hyoid bar and the anterior part of the branchial basket (*i.e*. the basihyal bone) was also observed. This downward displacement took place simultaneously with the neurocranium elevation, the mouth opening (lower jaw depression), and the backward movement of the pectoral girdle (*i.e*. the cleithrum bone) (see red line on Figure 4.5). Then, a mouth closing (lower jaw elevation) occured while all the other structures were still moving in the same direction.
The neurocranium elevation was immediately followed by its lowering. Likewise, a forward displacement of the pectoral girdle immediately followed its backward motion. These movements appeared to be constant.
On the other hand, the movements of the hyoid apparatus (24.1 m/s^2, n = 38 frames) and the lower jaw (39.5 m/s^2, n = 25 frames) displayed an acceleration (see black arrows on Figure 4.5). Moreover, the hyoid apparatus and the lower jaw retained their lowered and elevated position during the whole sound duration, before returning to their original position simultaneously when the sound ended. Inversely, the neurocranium and the pectoral girdle were already returned to their original position at the end of the sound duration.

Overall, sounds were accompanied by rapid (< 30 ms) movements that included a mouth opening, a neurocranium elevation, a hyoid lowering, a backward motion of the pectoral girdle, and lastly a lower jaws closing (see Movie S2 in supplementary material). Sounds were typically produced when the hyoid apparatus was rapidly lowered and the mouth quickly and simultaneously closed (see blue arrows on Figure 4.5).

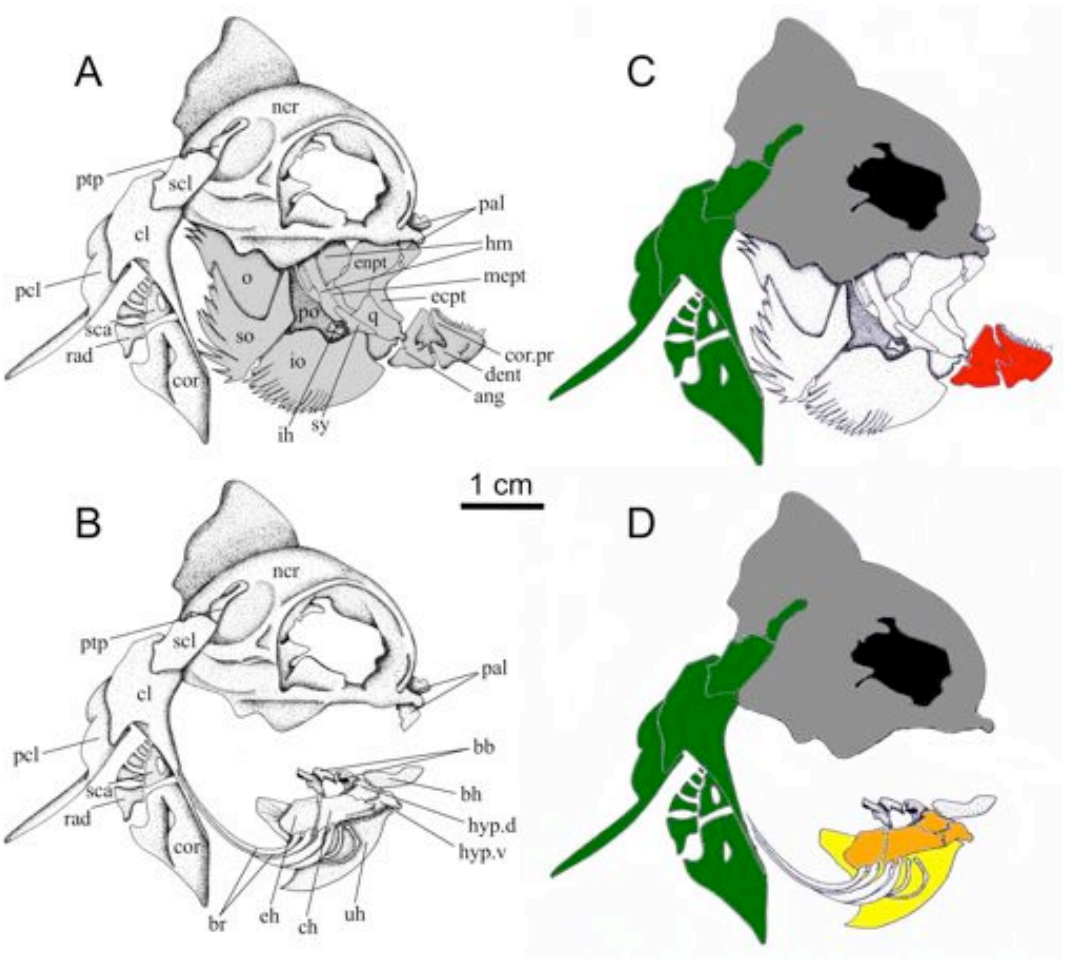


Figure 4.4 Right lateral view of the neurocranium and the pectoral girdle in *Amphiprion clarkii*. (A) Inner view of the left opercular series, suspensorium and lower jaw in grey. (B) Right lateral view of the hyoid apparatus when the opercular series and suspensorium have been removed. (C) and (D) present some color structures in order to facilitate the understanding of the Figure 4.6. Grey: neurocranium, Green: pectoral girdle, Red: lower jaw, Yellow: urohyal, Orange: hyoid bar.
Osteology labelling: ang, Angulo-articular; bb, Basibranchial; bh, Basihyal; br, Branchiostegal rays; ch, Ceratohyal; cl, Cleithrum; cor, Coracoid; cor. pr, Coronoid process; dent, Dentary; ecpt, Ectopterygoid; eh, Epihyal; enpt, Entopterygoid; hbr, Hypobranchial; hm, Hyomandibular; hyp. d, Hypohyal dorsal; hyp. v, Hypohyal ventral; ih, Interhyal; io, Interopercular; mept, Metapterygoid; ncr, Neurocranium; o, Opercular; pal, Palatine; pcl, Postcleithrum; po, Preopercular; ptp, Posttemporal; q, Quadrate; rad, Radial; sca, Scapula; scl, Supracleithrum; so, Subopercular; sy, Symplectic; uh, Urohyal.

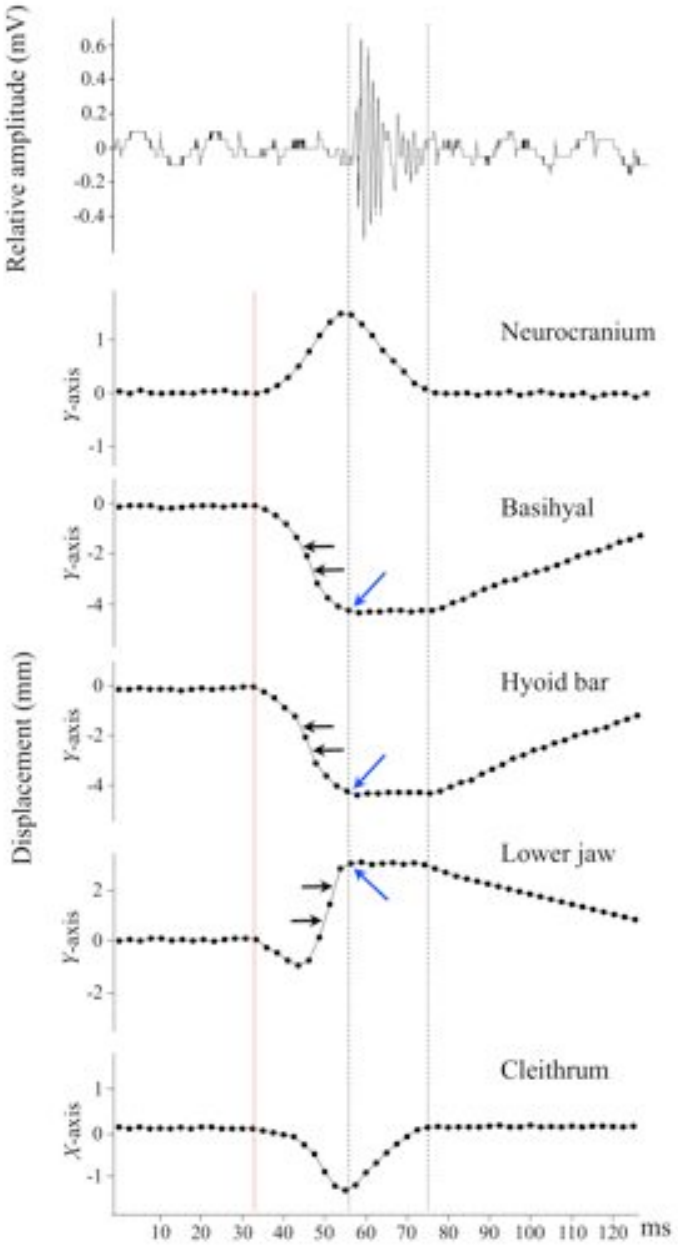


Figure 4.5 Sound production in *Amphiprion clarkii*. (A) Oscillogram of one pulse. (B) Movements of the neurocranium, the hyoid apparatus, the lower jaw and the cleithrum during the production of one pulse. Movements are quantified using X-ray video recordings. The red line indicates the beginning of the sonic movements; the blue arrows, the pulse onset; and the double dotted lines, the pulse duration.

Functional morphology

Manipulations of freshly dead specimens showed that low-amplitude elevations of the neurocranium depressed the lower jaw and the branchial basket *via* the isometric contraction of the sternohyoideus and protractor hyoidei muscles. This phenomenon is well known in the majority of adult teleost fishes, and usually occurs during feeding and breathing processes (see Ballintijn 1969, Osse 1969, Lauder 1983, Vandewalle *et al*. 1992, Vandewalle *et al*. 1995, Konow & Bellwood 2005, Van Wassenbergh *et al*. 2005).

A higher amplitude elevation of the neurocranium did not accentuate the mouth opening, but rather forced the mouth to close by a previously unknown mechanism.

Dissections showed the presence of a ligament (Figures 4.6 and 4.7), which connects the ceratohyal bone to the coronoid process of the angulo-articular bone (see also Stiassny 1981). As shown in Figure 4.6B, this ligament is responsible for the rapid lower jaw elevation leading to mouth closing (see Movie S3 in supplementary material). Basically, the function of the ligament can be compared to a drawbridge chain. The hyoid depression stretches down and backward the ligament that, in turn, exerts traction on the lower jaw. At a certain moment during the hyoid bar lowering, the ligament is led to pass by the articulation between the quadrate and the lower jaw (Figure 4.6B). Beyond this moment, the ligament passes over the articulation and forces the mandible to turn around its articulation by acting as a cord, while the hyoid bar is still lowering.

Sound recording experiments

Comparisons of sounds before and after cutting upper and lower jaw teeth indicated significant differences in pulse duration. It resulted in shorter pulses decreasing from 24.2 to 15.1 (Wilcoxon matched-pairs signed rank test, $P<0.0001$, $n = 45$ pulses), without the typical low-amplitude high frequency onset (Figure 4.8). Despite this difference in the oscillogram, dominant

frequency did not display significant differences between both experimental conditions (691 *vs* 681, Wilcoxon matched-pairs signed rank test, P=0.7383, n = 45 pulses). These results supported the fact that intact sounds were initiated by teeth collisions.

Additionally, transecting right and left sonic ligaments muted the fish. They still displayed territorial behavior towards intruders by chasing and charging them but no sounds were emitted, which supported the fact that the ligament is involved in the sound-producing mechanism.

Overall, these experiments showed that aggressive sounds in the clownfish are initiated by buccal teeth collisions caused by rapid jaw closure attributed to a sonic ligament between the hyoid bar and the internal part of the mandible.

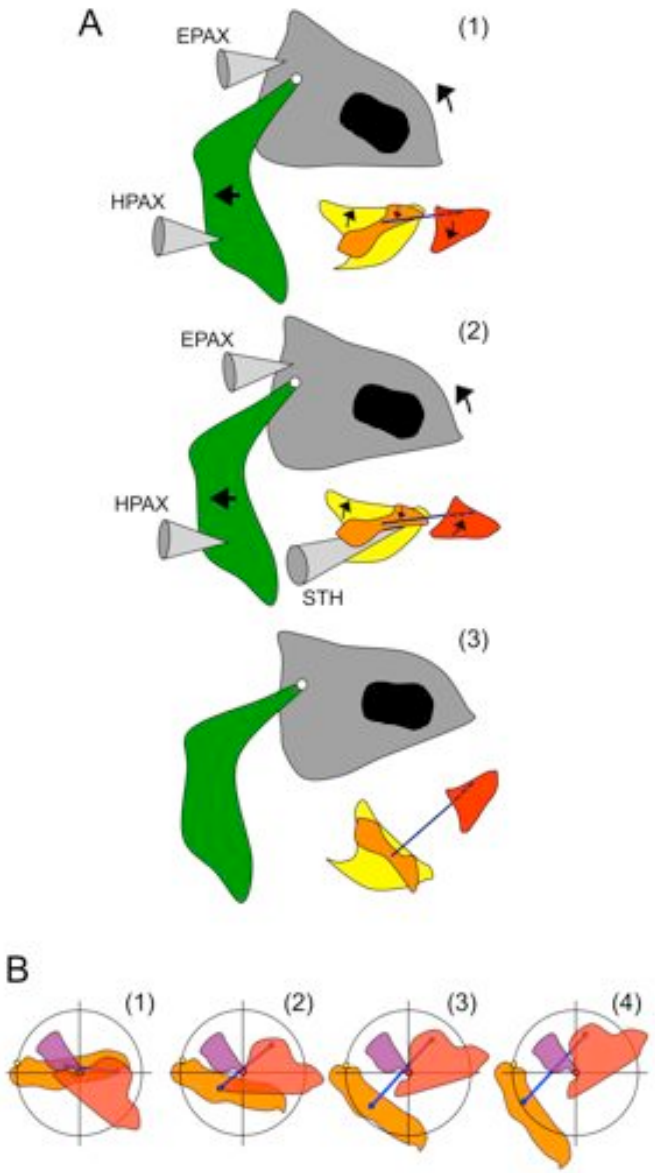


Figure 4.6 (A) Schematic representation of skull kinematics in *Amphiprion clarkii* illustrating the different phases of sound production. Sound production occurs in phase 3 when the mouth is closed. Colour coding: Grey: neurocranium, Green: pectoral girdle, Yellow: urohyal bone, Orange: hyoid bar, Red: lower jaw, Blue: sonic ligament. Arrows indicate displacement. Cones represent inferred contraction of a muscle group. Myology labelling: EPAX : epaxialis muscle, HPAX: hypaxialis muscle, STH: sternohyoideus muscle. (B) Schematic representation showing the function of the sonic ligament (Blue) in mouth closure. Colour coding as in (A), except Purple: quadrate bone.

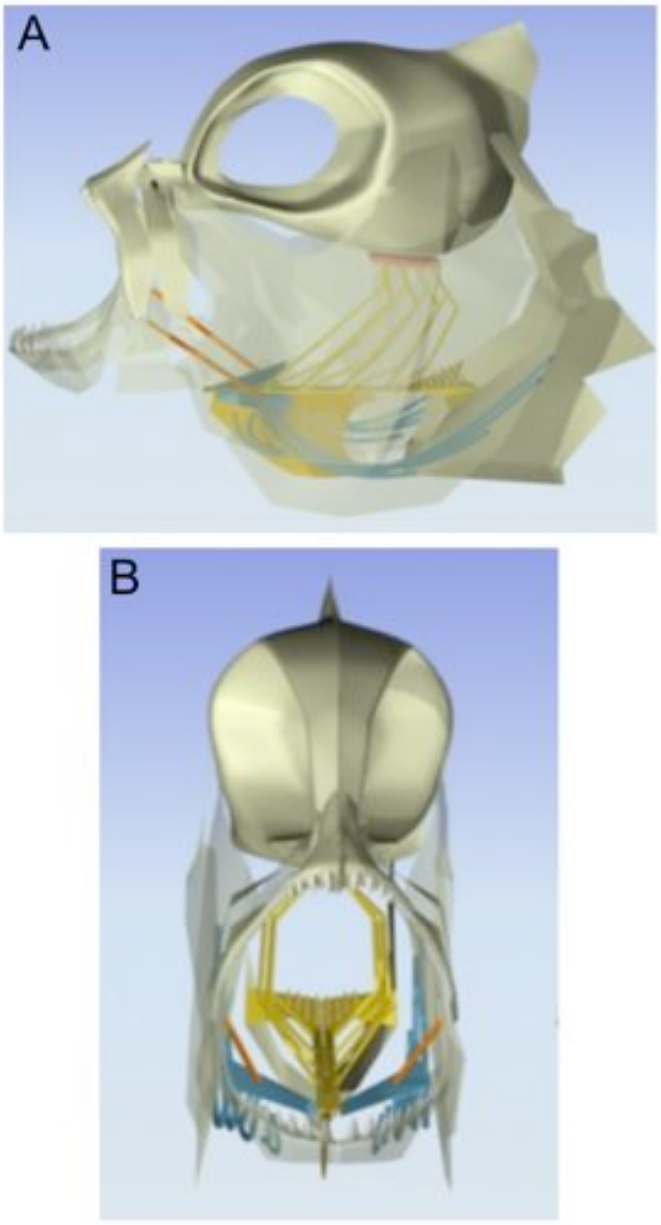


Figure 4.7 (A) Left lateral and (B) frontal 3D representation showing the position of the sonic ligaments in the buccal cavity of *Amphiprion clarkii*. Colour coding: Grey: neurocranium, pectoral girdle, opercular series, suspensorium and buccal jaws, Orange: sonic ligaments, Yellow: branchial basket, Blue: Hyoid bar and branchiostegal rays.

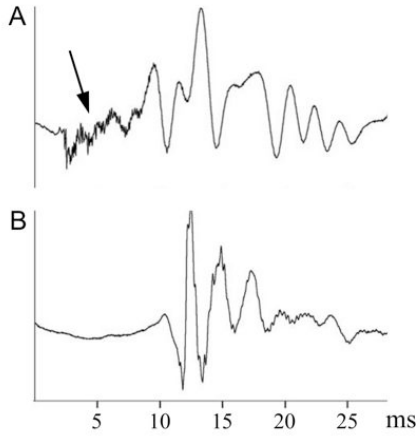


Figure 4.8 Oscillogram of a pulse produced by *Amphiprion clarkii*. (A) sound from an intact fish, (B) sound from the same fish after the upper and lower jaw teeth were cut. The bottom pulse is shorter and deprived of the low-amplitude high frequency onset (arrow).

4.1.4. Discussion

Fish skulls are morphologically complex and highly kinetic systems (Osse 1969, Vandewalle 1978, Ferry-Graham & Lauder 2001, Konow & Bellwood 2005). Numerous studies have examined the associations between feeding apparatus, functional morphology, biomechanics and prey-capture kinematics in teleost fishes. It is well known that fishes are able to perform a wide variety of movements during feeding such as jaw protrusion, lower jaw depression, hyoid depression or suspensorium abduction (Osse 1969, Elshoud-Oldenhave & Osse 1976, Vandewalle 1978, Lauder 1983, Motta 1984, Aerts 1991, Vandewalle *et al*. 1992, Vandewalle *et al*. 1995, Ferry-Graham & Lauder 2001, Wainwright & Bellwood 2002, Herrel *et al*. 2005, Konow & Bellwood 2005, Van Wassenbergh *et al*. 2005).

Studies on the characterization of both acoustic communication and mechanisms involved in sound production have only been done in a few fish families (Tavolga 1962, Winn & Marshall 1963, Schneider 1967, Daugherty & Marshall 1976, Fine *et al*. 1997, Fine *et al*. 2001, Parmentier *et al*. 2006a, Longrie *et al*. 2009). Although the damselfishes (Pomacentridae) are one of the most intensely studied families in terms of sound production, the sound-

producing mechanism has remained unclear. In clownfishes, it has been hypothesized that sound production involves rapid up-and-down movements of the mouthbones similar to those of taking food (Verwey 1930, Takemura 1983), or it can result from the action of the pharyngeal jaws grating amplified by the swimbladder (Luh & Mok 1986, Chen & Mok 1988, Rice & Lobel 2003).

According to our results, it appears that the first hypothesis was founded because fish performs such movements while producing sounds. On the other hand, it clearly appears that the second assumption is not responsible for the sound production in clownfishes. The hyoid apparatus and thus the floor of the pharyngeal cavity being totally depressed during sound production (see Movie S3 in supplementary material), lower and upper pharyngeal jaws are prevented from coming into contact. Thereby, fish cannot produce aggressive sounds by grating their pharyngeal jaws.

Additionally, the acoustic features of clownfish sounds are not congruent with the characteristics of sounds resulting from pharyngeal teeth grating. Indeed, pharyngeal teeth grating produces high frequency stridulations that spread on a wide range of frequencies from below 100 Hz to over 8000 Hz, but the predominant frequencies are in the 1000-4000 Hz range (Fish 1954, Moulton 1958, Tavolga 1971). On the other hand, clownfish sounds show a relatively wide range of frequencies with the peak frequency typically below 1 kHz.

Although it was not verified experimentally, the lowering of the hyoid apparatus would be due to the neurocranium elevation and the backward motion of the pectoral girdle. These movements imply the action of different muscles: the epaxialis and hypaxialis muscles that insert on the neurocranium and the pectoral girdle respectively (Lauder & Lanyon 1980, Lauder & Liem 1981; see also Figure 4.6A). The hyoid lowering might also be enhanced by the sternohyoideus muscle. This large muscle originates on the anterior surface of the ventral region of the cleithrum bone and attaches to the urohyal bone that connects to the medial hyoid elements by a pair of ligaments. It is known that this muscle acts to retract and depress the branchial basket (Aerts 1991, Vandewalle *et al*. 1992, De Visser & Barel 1996).

In clownfishes, sounds are initiated by teeth collisions caused by rapid jaw closure attributed to a sonic ligament between the hyoid bar and the internal part of the mandible. According to our observations, the hyoid lowering is responsible for the mouth closing leading to sound production. Although it was not experimentally verified by electromyography, note that the adductor mandibulae muscles could also act in conjunction with the ligament because these muscles are known to be responsible for mouth closing (Ferry-Graham & Lauder 2001).

The presence of the ligament between the hyoid bar and the internal part of the mandible (*i.e*. the sonic ligament in the present study) is interpreted as a synapomorphy uniting the members of the Pomacentridae family (Stiassny 1981), in which many of whom are known to be prolific sound producers. Numerous pomacentrid species produce the same kind of broadband pulsed sounds during chases and threat display between conspecifics (see Parmentier *et al*. 2006b), which suggests that a basic pattern is present for the sound production in pomacentrids.

Thereby, the homologous ligament mechanism is likely involved in sound production throughout this large family. The genus *Plectroglyphidodon* is placed in a basal position in the damselfish cladogram (Tang 2001, Jang-Liaw *et al*. 2002, Quenouille *et al*. 2004), and the presence of sounds in *Plectroglyphidodon lacrymatus* suggests that pomacentrids are derived from an ancestral taxon capable of sound production. The absence of sound production in some species would indicate a character lost secondarily (Parmentier *et al*. 2006b). By its relationships with the lower jaw and due to the induced movements, it is hard to believe that this ligament is only involved in sound production. Hence, it might perform a function for which it was not originally selected or adapted. This functional movement might be an exaptation of the feeding mechanism, with the sound-producing species using the ligament as a novel skeletal adaptation for performing a new function in another context.

4.2. FURTHER INSIGHT INTO THE STRUCURE RESPONSIBLE FOR THE SOUND RADIATION *

4.2.1. Introduction

Although the sound-producing movements have been determined in the clownfish *Amphiprion clarkii*, the mechanism still remains incomplete. Indeed, only the onset of the sound was highlighted. There exists a need to go further into the understanding, especially regarding the anatomical structure involved in sound radiation. Indeed, dominant frequency and pulse duration are known to be signals directly related to body size in clownfishes (see chapter 3). Such variations suggest that both acoustic features are subject to a morphological constraint.

Classically, the character of sounds changes during the development of an organism, mainly in relation to larger body size, and probably to the development of the sound-generating structures. This general phenomenon has been widely investigated in many groups of animals and is largely based on resonance. For example, Würdinger (1970) showed that in the greylag goose *Anser anser*, the dominant frequency of sounds is correlated negatively to the size of the tympaniform membranes, while main frequencies are know to decrease with increasing body size in frogs (Davies & Halliday 1978, Ryan 1985). Same kinds of correlation are also known in several groups of fishes that produce pulsed sounds. In the grey gurnard *Eutrigla gurnardus* for example, sound production changes from small juveniles to large adults during competitive feeding (Amorim & Hawkins 2005). In the croaking gourami *Trichopsis vittata*, sound duration increases during ontogeny whereas dominant frequency decreases (Henglmüller & Ladich 1999). In weakfishes (Connaughton *et al*. 2000), pearlfishes (Parmentier *et al*. 2006a), damselfishes (Myrberg *et al*. 1993, Lobel & Mann 1995) and clownfishes

* Slightly modified from: **Colleye O, Nakamura M, Frédérich B, Vandewalle P, Parmentier E.** Further insight into the sound-producing mechanism of clownfishes: what about the structure involved in sound radiation? (in preparation).

(Parmentier *et al*. 2009c, see chapter 3), pulse duration increases and dominant frequency decreases in larger fishes. Grey gurnards, weakfishes and pearlfishes produce sounds by contracting sonic muscles attached to the swimbladder. In this view, variations in peak frequencies and pulse durations may result from muscle-scaling effects (Wainwright & Barton 1995), as larger fishes with larger sound-producing muscles would take longer to complete a muscle twitch, resulting in longer pulse durations and lower peak frequencies (Connaughton *et al*. 2000). On the other hand, croaking gouramis generate stridulatory sounds by two enhanced pad-like tendons of the fourth and fifth pectoral fin ray (Kratochvil 1978). Henglmüller & Ladich (1999) suggested that the frequency changes during the ontogeny of croaking gouramis are probably related to the growth of the suprabranchial chamber (*i.e*. an airbreathing cavity dorsally of the gills in all labyrinth fishes); this structure is thought to be the main resonating structure in this species. The swimbladder has also been thought to function as a resonator that amplifies and changes the quality of sounds produced by stridulation in grunts and triggerfishes (Burkenroad 1930, Salmon *et al*. 1968), but this hypothesis has never been experimentally verified. Thereby, such examples raise the question about the resonating structure involved in the sound-producing mechanism of clownfishes.

The present study aims to seek the structure(s) responsible for the size-related variations in sound duration and frequency. More precisely, we conducted a series of experiments in order to find out the acoustic radiator in the sound production of the yellowtail clownfish *Amphiprion clarkii*.

4.2.2. Material and methods

Capture and holding of fish

Eighteen *Amphiprion clarkii* (SL: 55-108 mm) were collected by scuba diving on the fringing reef around Nakijin village (26°40’23’’N - 127°59’48’’E, Okinawa, Japan) during May and June 2009. All fish were then brought back

with their host (*Heteractis magnifica*) to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus. Then, they were transferred to a community tank (3.5 × 2.0 × 1.2 m) filled with running seawater at ambient temperature (28 to 30.5°C). This community tank was compartmentalized for separating fish into pairs. In addition to sexual size dimorphism, coloration of the caudal fin (Hattori & Yanagisawa 1991) was used to identify male and female. All fish were kept under natural photoperiod and fed once daily with food pellets *ad libitum*.

Sound recording and sound analysis

Sounds were recorded using a Brüel & Kjaer 8106 hydrophone in a smaller glass tank (1.2 × 0.5 × 0.6 m) filled with running seawater maintained at 28°C by means of a GEX cooler system (type GXC-201x, Osaka, Japan) for recording under standardized conditions. Sounds were emitted in a context of territory defense (see paragraph 2.2.2 for details on procedures and material characteristics). Sound analyses were done based on the procedure mentioned in chapter 2 (see paragraph 2.2.3 for details and Figure 2.1). A low pass filter of 2.05 kHz was applied to all sound recordings. In addition to the usual sonic features measured, the number of oscillation cycles and the Q_{3dB} (quotient of the stimulation frequency that produced the maximal amplitude response divided by the bandwidth, or frequency range, across which the amplitude of sound produced by the resonator was within 3 dB of the maximal amplitude) (Bradbury & Vehrenkamp 1998) were calculated to determine the effects of the swimbladder filling experiment (see below) on these two sonic characteristics.

Recording experiments: effects of anaesthesia and swimbladder filling

The effects of anaesthesia (negative control) and swimbladder filling on the acoustic features of aggressive sounds were examined.

Nine specimens were anaesthetized by placing them into a dedicated container with 50 mgl^{-1} of MS-222 in seawater. Fish were considered deeply anaesthetized when they turned upside down. Then, they were placed in the recording tank, and after a three hours time recovery, fish were again recorded by introducing a conspecific intruder into the tank.

At five days interval, the implication of the swimbladder in sound modulation was tested by rapidly filling it with physiological liquid (NaCl 9‰) under general anaesthesia in 50 mgl^{-1} MS-222. Once deeply anaesthetized, fish were placed on wet cotton in a dissecting tray. Two hypodermic needles (27-gauge; 0.4 mm × 20 mm) were inserted subcutaneously into the swimbladder. The first needle was coupled to a 5 ml syringe for filling the swimbladder while the second one was used for draining the overflow, indicating when the swimbladder was totally filled. Fish were then placed in the recording tank for a recovery period of three hours. Aggressive sounds were again recorded by challenging fish with conspecific intruders.

Swimbladder volume estimation

Swimbladder volume was determined using a cast method (adapted from Barnett & Bellwood 2005). Ten specimens were euthanized with MS-222 (500 mgl^{-1}) and were fixed in 7% buffered seawater formalin for approximately 2 weeks before being transferred to 70% ethanol for storage. Then, they were transversally sectioned at the level of the base of the anal fin (Figure 4.8) to expose the posterior part of the swimbladder. After punching the swimbladder wall, an internal cast was formed by injecting specific silicone rubber (Vandamme®, Liège, Belgium) using a 5 ml syringe. Care was taken to inject silicone with constant pressure until the entire swimbladder was full. The silicone was left to partially set for 1 hour with the fish maintained on ice before being transferred to 70% ethanol. After the silicone had set for one night, the swimbladder cast was removed. The volume of the silicone cast was determined by water displacement in a 500 ml volumetric flask filled with

water to the graduation mark. The displaced water volume was measured using a 1 ml volumetric pipette (accuracy: 0.01 ml). Triplicate measurements were taken for each silicone cast on separate days and the mean was calculated. The coefficient of variation between triplicates was less than 2%. Then, the calculated volume was used to obtain the equivalent radius of a sphere. This radius was used to calculate the resonant frequency (F) of the swimbladder according to the formula for an underwater bubble (Minnaert 1933, Weston 1967):

$$F = \left(\frac{1}{2\pi R}\right)\sqrt{\frac{3\gamma P}{\rho}}$$

where R is radius (cm); γ, ratio of specific heats (=1.402); P, pressure (atmospheric pressure + hydrostatic pressure); ρ, water density. To compare with natural sounds emitted by fish, the resonant frequency was calculated for a depth of 30 cm because fish were mostly situated at this depth, swimming among the tentacles of the sea anemone, just above its oral disc.

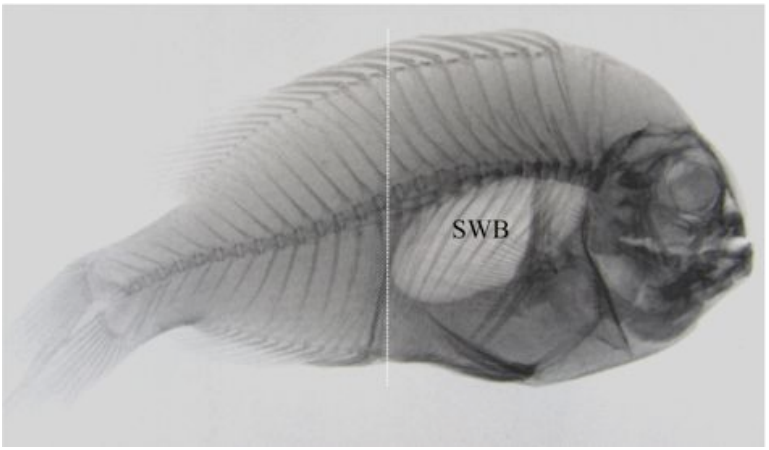


Figure 4.9 X-ray picture in lateral view of *Amphiprion clarkii*. The vertical dotted line shows the place where fish were transversally sectioned for exposing the posterior part of the swimbladder (SWB).

Morphological study

Specimens, whose swimbladder was cast, were dissected and examined with a Leica M10 binocular microscope (Leica Camera, Leica, Wetzlar, Germany) coupled to a camera Lucida. Three specimens were cleared and stained with Alizarin Red S according to Taylor and Van Dyke's method (Taylor & Van Dyke 1985) in order to visualize osseous structures. The location of the swimbladder in the body cavity, and its position in relation to surrounding structures (vertebrae, ribs, parapophyses) were described. The osteology nomenclature follows that of Patterson and Johnson (1995).
In addition, three smaller specimens (SL: 35-41 mm) were purchased from a pet store and euthanized with an overdose of MS-222 (500 mgl^{-1}). They were placed in a Bouin's fixative solution in order to make histological cross sections (see paragraph 2.2.7 for more details).

Sound resonance experiments

Two experiments were done in order to determine the structure acting as the acoustic radiator.
(1) Three specimens (SL: 76-97 mm) were euthanized with an overdose of MS-222 (500 mgl^{-1}). They were dissected under a Leica M10 binocular microscope in order to expose the anterior part of the vertebral column and the skull (Figure 4.10). Then, specimens were carefully skeletonised with boiling water for removing all remnants of flesh tissue and oven dried at 60°C. To assess the role of the buccal jaws in sound radiation, specimens were placed in a dissecting tray and maintained in the lateral position using modeling clay. The lower jaws were struck with a miniature modal analysis impact hammer (PCB model 086E80, Depew, NY, USA; X100 gain; with a steel tip, transducer sensitivity 22.62 mVN^{-1}) (see Figure 4.10). Sound produced by striking the buccal jaws was recorded with a Sennheiser ME62 microphone (frequency range 20 Hz–20 kHz ± 2.5 dB, sensitivity 32 $mVPa^{-1}$ ± 2.5 dB) coupled with a Sennheiser K6 modulatory system onto a Tascam recorder.

The microphone was positioned 3 cm above the specimens. All recordings were made within a sound-proof booth.

(2) Three specimens (SL: 69-96 mm) were euthanized with an overdose of MS-222 (500 mgl^{-1}). They were dissected under a Leica M10 binocular microscope. Swimbladder and surrounding structures (ribs and vertebrae) were completely exposed on one side by incision of the body musculature (Figure 4.11). Then, specimens were placed in a dissecting tray and two series of hits were done with the miniature impact hammer (X100 gain; with a vinyl tip cover, transducer sensitivity 20.80 mVN^{-1}). Firstly, fish were struck at the level of the fourth rib; vibrations were measured with a Polytec laser Doppler vibrometer (LDV) including Vibrometer Controller (OFV 5000) and a Vibrometer Sensor Head (OFV 505, sensitivity $5mms^{-1}V^{-1}$) with the laser beam focussing on retro-reflective laser discs placed on the sixth rib (Figure 4.11A). Secondly, fish were placed, belly up in a support, in order to stimulate directly the swimbladder on the anterior part. A retro-reflective laser disc was placed on the posterior part to measure vibration of the swimbladder wall (Figure 4.11B). Sounds resulting from the different series of hits were recorded using the same microphone as for buccal jaws experiment. All recordings were made within a sound-proof booth. All data were captured using a data acquisition box (Midas, DA Module, BNC Breakout Box, Cambridge, MA, USA). Velocity and duration were measured for the vibration trace, as well as duration for hammer force trace for each fish using LabVIEW SignalExpress 3.0, and peak frequency with the highest energy was measured from the vibration trace. In both experiments, the following sonic variables were analyzed: sound duration (ms) was measured from oscillograms whereas dominant frequency (Hz), relative intensity (dB rel.) and Q_{3dB} were obtained from power spectra.

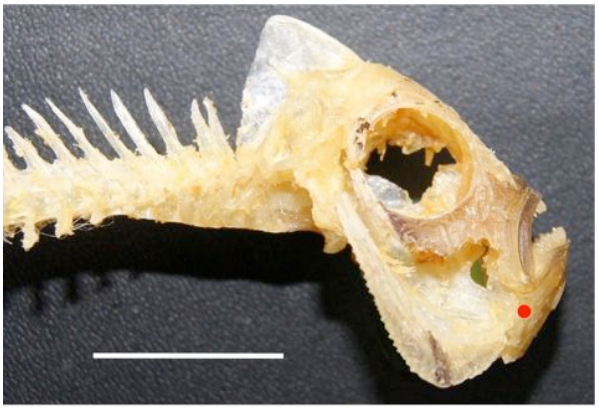


Figure 4.10 Right lateral view of the skull and the anterior part of the vertebral column of a dead specimen of *Amphiprion clarkii* (SL = 85 mm). The specimen has been skeletonized with boiling water for removing flesh tissue and oven dried at 60°C. The red dot indicates the site struck with the impact hammer. Scale bar = 1 cm.

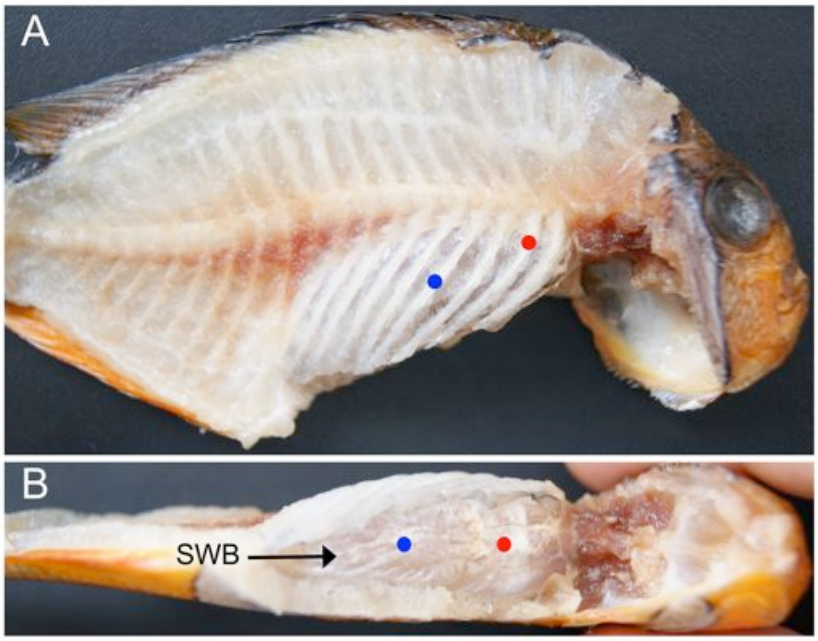


Figure 4.11 (A) Right lateral view of a freshly dead specimen of *Amphiprion clarkii* (SL = 96 mm) whose body musculature has been dissected. The red dot indicates the site struck with the impact hammer at the level of the fourth rib, and the blue dot is the laser target disc located on the sixth rib where vibration was measured. (B) Ventral view of the same specimen illustrating the site where swimbladder (SWB) was directly struck with the impact hammer (red dot), and the laser target disc where vibration was measured (blue dot).

Statistical analyses

Shapiro-Wilk *W* statistic was used for testing the normal distribution of acoustic data. Non-parametric Friedman's test with subsequent Dunn's test for pairwise comparisons was used to compare acoustic features for each of the experimental conditions (*i.e*. before anaesthesia, after anaesthesia and after swimbladder filling; 100 pulses were analyzed per individual for each conditions tested). Additionally, non-parametric Wilcoxon matched-pairs signed rank test was used to compare data about the Q_{3dB} and the number of oscillation cycles before and after swimbladder filling.

ANCOVA was run to compare linear regressions of peak frequencies of natural sounds produced by fish and the resonant frequencies calculated on the basis of a bubble-like swimbladder of equivalent volume against SL. Note that these values were first ln-transformed because they were exponentially related to fish size.

ANCOVA was run to compare linear regressions resulting from the effects of variable force hammer hits on the measured parameters in fish of different size. Then, non-parametric Kruskal–Wallis one-way ANOVA by ranks with subsequent Dunn's test for pairwise comparisons was used to compare data between fish.

All statistical analyses were carried out with Statistica 7.1. Results are presented as means ± S.D. Significance level was determined at $P<0.05$.

4.2.3. Results

Sounds were composed of short pulses (< 20 ms) emitted alone or in series, and in a narrow band of low frequencies (< 1 kHz). The sound of 55-108 mm (SL) fish ranged in pulse period from a mean of 90.0 to 128.6 ms, and in number of pulses per sound from 2.9 to 5.5 pulses. Pulse duration increased from a mean of 10.8 to 19.7 ms whereas dominant frequency decreased from 700 to 340 Hz (Table 4.1).

Table 4.1 Summary (mean ± S.D.) of the four sonic features analyzed from 18 *Amphiprion clarkii* alive

SL (mm)	Pulse duration (ms)	Dominant frequency (Hz)	Pulse period (ms)	Number of pulses
	mean ± S.D.	mean ± S.D.	mean ± S.D.	mean ± S.D.
55	10.8 ± 1.3	700 ± 77	114.9 ± 10.1	3.7 ± 1.6
72	13.0 ± 0.7	553 ± 63	104.6 ± 19.3	4.4 ± 3.3
75	13.5 ± 0.9	537 ± 46	125.1 ± 23.5	3.3 ± 1.6
76	13.7 ± 0.8	530 ± 61	95.4 ± 15.2	5.5 ± 2.0
76	13.6 ± 0.9	531 ± 57	110.9 ± 14.5	4.2 ± 2.4
81	14.8 ± 0.6	500 ± 54	90.0 ± 13.3	4.3 ± 1.6
82	14.9 ± 0.7	502 ± 56	101.4 ± 12.4	6.4 ± 2.6
83	14.8 ± 0.9	503 ± 59	101.6 ± 20.4	5.0 ± 1.9
83	14.9 ± 0.8	497 ± 59	103.2 ± 16.4	3.9 ± 2.1
85	15.1 ± 0.6	495 ± 65	128.0 ± 15.5	2.9 ± 1.1
85	14.9 ± 0.8	493 ± 54	118.5 ± 15.4	4.9 ± 2.1
87	15.2 ± 0.8	482 ± 69	106.9 ± 12.9	4.8 ± 1.7
90	15.3 ± 0.9	433 ± 52	128.2 ± 20.3	4.6 ± 1.9
90	15.5 ± 0.9	431 ± 43	128.6 ± 13.5	5.5 ± 2.6
94	15.9 ± 0.7	426 ± 42	115.2 ± 20.2	4.4 ± 2.5
95	15.8 ± 0.7	425 ± 35	106.9 ± 11.9	3.4 ± 1.8
97	15.9 ± 0.9	415 ± 43	124.6 ± 14.3	4.1 ± 2.1
108	19.7 ± 0.9	340 ± 29	120.5 ± 16.3	5.0 ± 2.1

Effects of swimbladder filling on acoustic features

The comparison of experimental conditions (*i.e.* before anaesthesia, after anaesthesia, after swimbladder filling) using Friedman's test revealed that experiments induce significant differences in some acoustic features such as pulse duration (χ^2=13.56, d.f.=2, P=0.0003; Figure 4.12A) and dominant frequency (χ^2=13.56, d.f.=2, P=0.0003; Figure 4.12B), but not in pulse period (χ^2=4.667, d.f.=2, P=0.1066; Figure 4.12C) and number of pulses per sound (χ^2=1.556, d.f.=2, P=0.5690; Figure 4.12D). Pairwise comparisons showed that pulse duration (15.04 *vs* 15.01 ms, Dunn's test, P>0.05; Figure 4.12A), dominant frequency (484.7 *vs* 483.1 Hz, Dunn's test, P>0.05; Figure 4.12B), pulse period (110.8 *vs* 113.2 ms, Dunn's test, P>0.05; Figure 4.12C) and

number of pulses (4.7 *vs* 4.7, Dunn's test, $P>0.05$; Figure 4.12D) were not affected by anaesthesia. On the other hand, the swimbladder filling induced a significant decrease in pulse duration of about 3 ms (2.93±0.07, n = 9 individuals) from 15.0 to 12.1 ms (Dunn's test, $P<0.001$; Figure 4.12A and 4.13B), and a significantly increase in dominant frequency of around 105 Hz (104.6±2.71, n = 9 individuals) from 485 to 589 Hz (Dunn's test, $P<0.001$; Figure 4.12B and 4.13D). Overall, it clearly appears that the swimbladder filling specifically acted on size-related acoustic features.

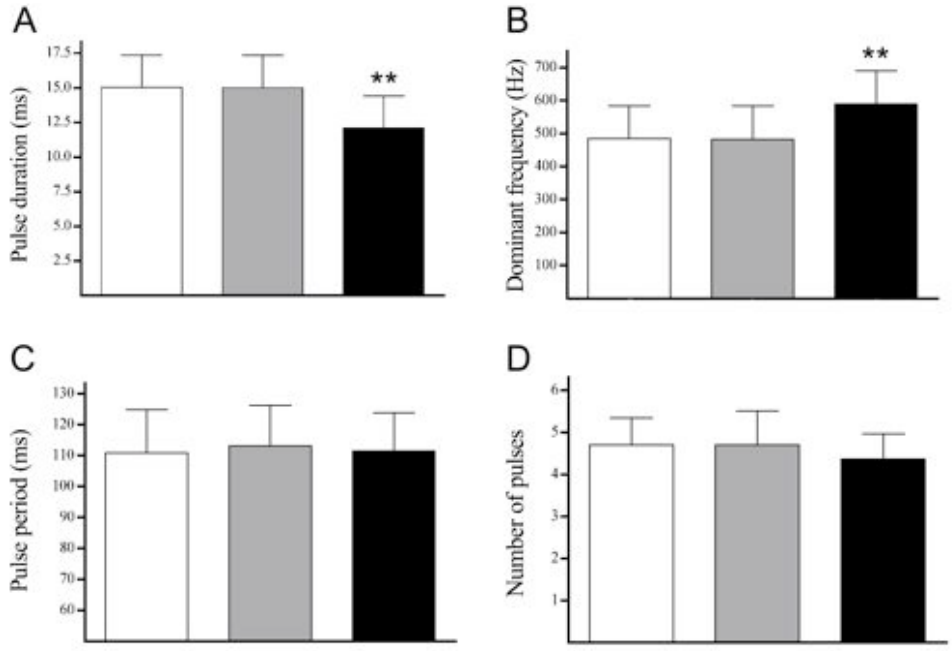


Figure 4.12 Effects of anaesthesia and swimbladder filling on the acoustic features in *Amphiprion clarkii*. The measured acoustic features were: (A) pulse duration, (B) dominant frequency, (C) pulse period and (D) number of pulses. Fish (n = 9) ranged in standard length from 55 to 108 mm and all recordings were made at 28°C. Asterisks refer to the significance level of the Dunn's test (**, $P<0.001$). White, grey and black bars represent values (means ± S.D.) before anaesthesia, after anaesthesia and after swimbladder filling, respectively.

Deeper attention to the oscillograms showed changes in the sound waveform after filling the swimbladder. Although the number of oscillation cycles did not vary (6.53 *vs* 6.55; Wilcoxon matched-pairs signed rank test, P=0.4961), all recorded pulses exhibited a waveform with a decrease in the period of oscillation (Figure 4.13B). This observation matched with the upward shift observed in peak frequency (see white arrows on Figure 4.13C,D). Such changes in acoustic features indicate that sound radiation appeared to be affected by the swimbladder filling. These results were also supported by the change in Q_{3dB}, decreasing from 4.1 to 3.7 (Wilcoxon matched-pairs signed rank test, P=0.0039) further to the swimbladder filling.

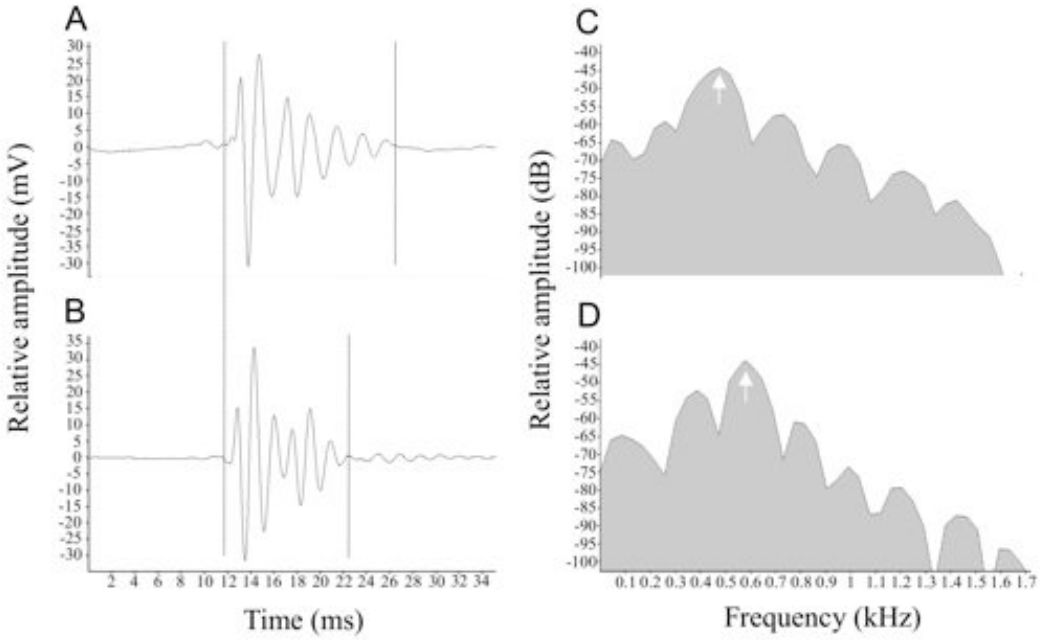


Figure 4.13 Oscillogram of one pulse showing the difference in sound waveform between an intact fish (A) and with the swimbladder filled (B). The double dotted lines indicate the pulse duration. Power spectrum of the same pulses showing the peak frequency (see white arrows) in an intact fish (C) and when the swimbladder was filled (D).

Swimbladder volume and resonance frequency

The swimbladder volume was exponentially related to fish size (r = 0.99, P<0.0001; Figure 4.14A): the more fish size increased, the more swimbladder volume increased. Natural dominant frequencies produced by fish decreased with an octave from 700 Hz in a 55 mm SL individual with a 0.49 cm^3 swimbladder volume, to 340 Hz in a 108 mm SL individual with a 3.85 cm^3 swimbladder volume (Figure 4.14A, Table 4.1). Then, frequencies emitted by fish were compared with the resonant frequency calculated on the basis of the radius of a bubble-like swimbladder of equivalent volume (Figure 4.14B). Although values were rather close and displayed the same scattering plot (ANCOVA, test for common slopes: $F_{1,16}$=0.0059, P=0.9398), the calculated resonant frequency values were inferior to natural dominant frequencies emitted by fish. This finding strongly suggests that sound frequency is not determined by the natural resonant frequency of the swimbladder.

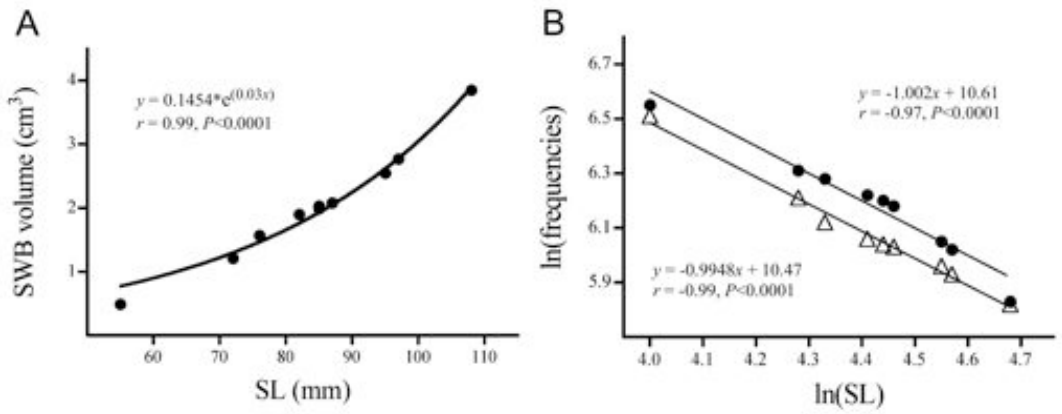


Figure 4.14 (A) Relationship between swimbladder volume and fish size (SL) in *Amphiprion clarkii*. Fish (n = 10) ranged in SL from 55 to 108 mm. The significance level was determined at P<0.05. (B) Comparison between peak frequencies of natural sounds (filled circles) and calculated resonant frequencies (open triangles) in *Amphiprion clarkii*. Note that data were ln-transformed because they were exponentially related to SL. Resonant frequencies were calculated for a depth of 30 cm. The significance level was determined at P<0.05. Analyse of covariance shows that both linear models do not differ (ANCOVA, test for common slopes: $F_{1,16}$=0.0059, P=0.9398).

Morphological study

Generally speaking, the swimbladder of *A. clarkii* is a thin-walled sac located in the dorsal portion of the body cavity and surrounded by bony structures such as vertebrae and ribs. *Amphiprion clarkii* possesses 11 precaudal vertebrae (Figure 4.15). The first two each possess a pair of epineural ribs, the second being the longest. From the third to the eleventh, the vertebrae possess pairs of ribs and intermuscular bones as well. From the sixth to the eleventh vertebrae, there are pairs of ventral parapohyses on which ribs articulate (Figure 4.15). These ribs are dorsally closely attached to the serosa of the abdominal cavity. The anterodorsal part of the swimbladder leans against the vertebral column. From the second to the seventh vertebrae, there is a groove at the midline that makes the swimbladder anteriorly bilobate (Figure 4.16). Both lobes surround the ventral apophysis of the first vertebra (onto which the retractor dorsalis muscle inserts). The dorsocaudal part of the swimbladder is connected to the last four precaudal vertebrae *via* the ventral parapohyses that are more and more elongated (Figure 4.15). The swimbladder is laterally delimited by the different pairs of ribs onto which the wall is attached (Figure 4.16). The posterior part of the swimbladder presses against the second pterygiophore of the anal fin (Figure 4.15). The swimbladder wall is very thin (~20 µm) and histological cross section cannot clearly identify all of its layers (Figure 4.17). The tunica interna of the swimbladder is a cuboidal epithelium. The tunica externa contains a thicker fibrous layer, which is more developed ventrally. At this level, the tunica externa is also doubled by the coelomic epithelium lining the abdominal cavity. Moreover, the tunica externa is also connected to a second fibrous layer lining the myosepta in relation with the ribs (Figure 4.17C). As a result, the movements of the ribs have a direct influence on the whole fibrous layer.

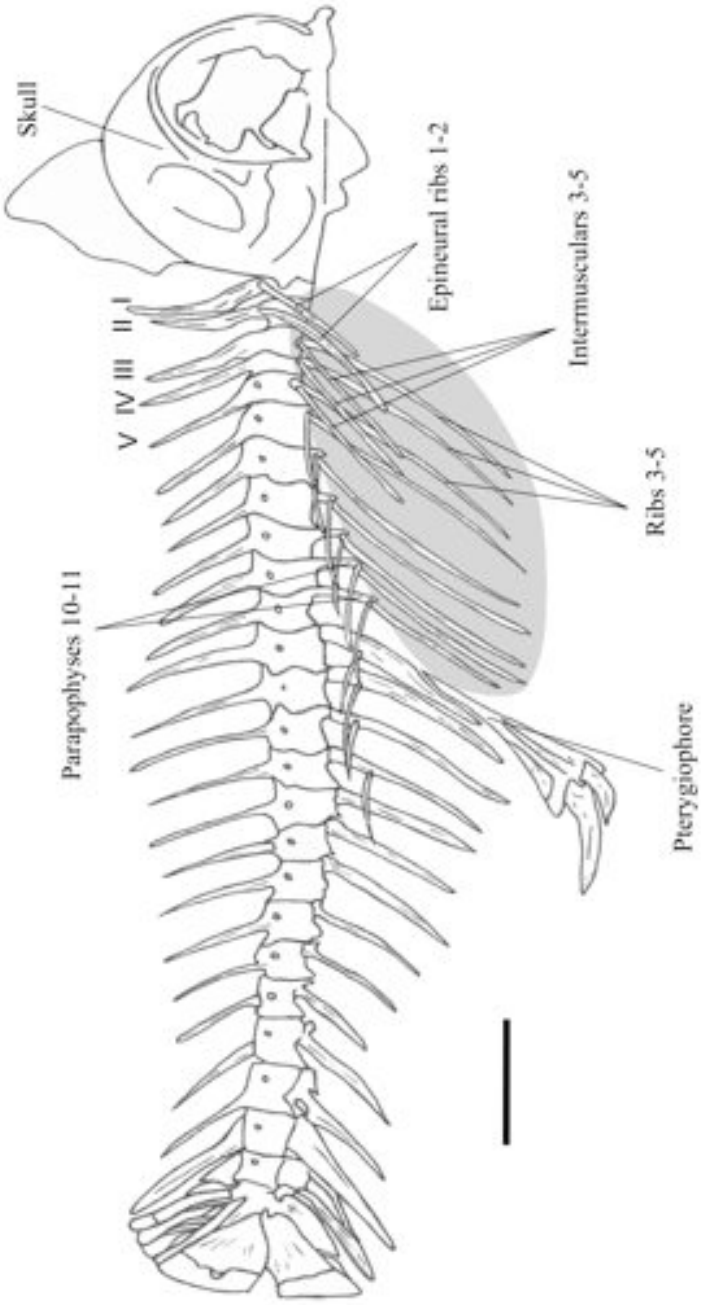


Figure 4.15 Lateral view of the skull, axial skeleton and hypural structure in *Amphiprion clarkii* showing the location of the swimbladder (grey) in relation to the rib cage. Latin numbers refer to the first five vertebrae. Scale bar = 5 mm.

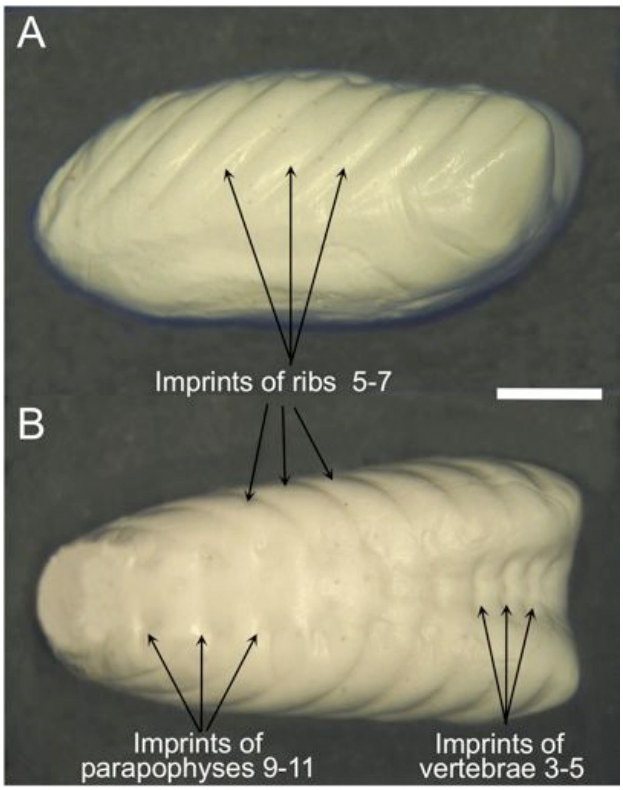


Figure 4.16 Internal silicone cast of the swimbladder in *Amphiprion clarkii*. (A) Lateral view showing the imprints of the ribs. (B) Dorsal view with the imprints of the parapophyses and vertebrae. A midline groove is formed in the anterodorsal part because the swimbladder leans against the vertebral column. Scale bar = 5 mm.

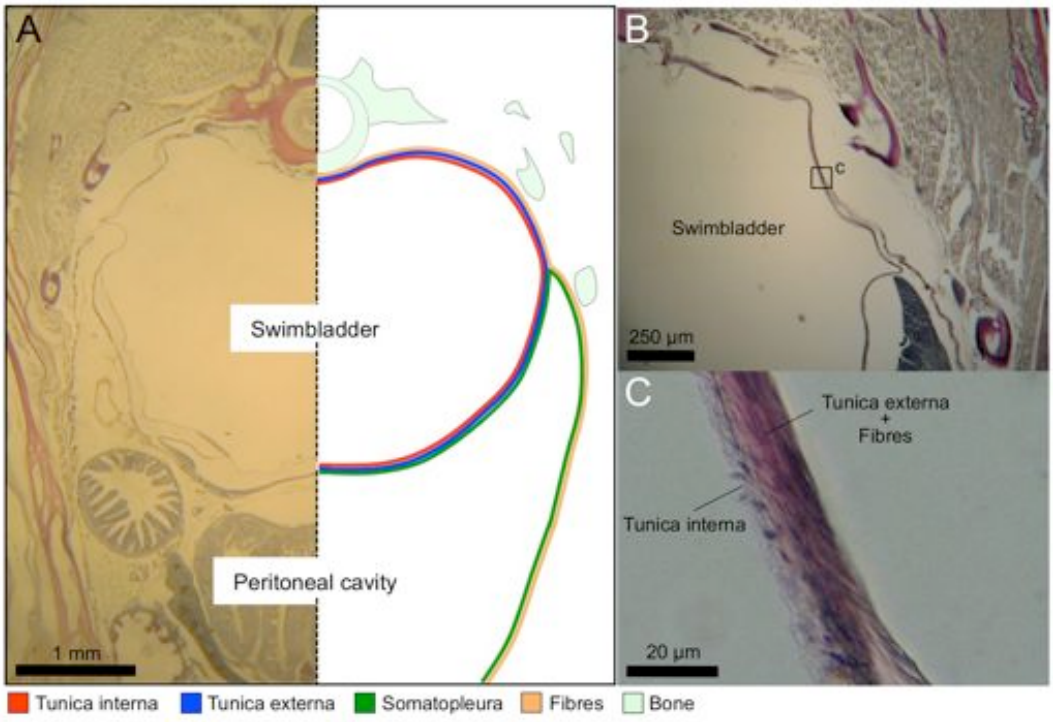


Figure 4.17 Cross-section of *Amphiprion clarkii* at the level of the abdominal cavity. The schematic drawing in (A) helps to distinguish the different swimbladder tissues. The tunica externa is connected to a fibrous layer lining the myosepta tissues as shown in (B) and (C). (C): enlargement of c.

Sound resonance experiments: buccal jaws

Striking the buccal jaws using the impact hammer generated a waveform with an asymmetrical half-cycle with a shorter rise than fall time (6.3 ms and 8.5 ms, respectively, n = 45; Figure 4.18). The rise time represents the period during which the hammer was transferring energy to the jaws. It started with a relatively low force as pressure on the jaws increased and then continued with a steeper slope. The fall time represents the time when the hammer bounced back from the jaws surface. The slope of the fall time decreased just before return to the baseline.

The sound waveform induced by the hammer strike on buccal jaws displayed differences with that of natural sounds emitted by fish. It suddenly disappeared into the background noise without displaying decay (see Figure 4.18).

Comparison of hammer and sound traces indicated that the onset of the hammer trace and sound waveform was closely aligned (Figure 4.18). Generally speaking, hammer duration (rise and fall time) closely corresponded to sound duration.

Changes in hammer force (*i.e*. harder hits) caused significant changes in sound parameters (Figure 4.19). Sound duration increased from 8.9 to 13.5 ms (r = 0.87, P<0.0001, n = 45), and sound amplitude increased from -64.6 to -58.1 dB relative (r = 0.87, P<0.0001, n = 45) with harder hits. Inversely, peak frequency dropped from 1650 to 1100 Hz (r = -0.84, P<0.0001, n = 45), and Q_{3dB} decreased from 11.2 to 5.2 (r = -0.87, P<0.0001, n = 45) with harder hits.

Comparisons of values calculated from regressions of variable force hammer strikes in three specimens of different size showed that hammer strikes had the same effects on sound duration (ANCOVA, test for common slopes: $F_{2,39}$=0.1842, P=0.8325), sound amplitude (ANCOVA, test for common slopes: $F_{2,39}$=0.3754, P=0.6894), sound frequency (ANCOVA, test for common slopes: $F_{2,39}$=0.1678, P=0.8461) and Q_{3dB} (ANCOVA, test for common slopes: $F_{2,39}$=2.9250, P=0.0652), whatever the fish size. In addition, there were no significant differences between individuals of different size in sound duration

(H=1.155, d.f.=2, *P*=0.5613), sound amplitude (H=1.140, d.f.=2, *P*=0.5656), sound frequency (H=1.754, d.f.=2, *P*=0.4161) and Q_{3dB} (H=0.932, d.f.=2, *P*=0.6275), which suggest that buccal jaws might maintain similar acoustic properties as they increased in size.

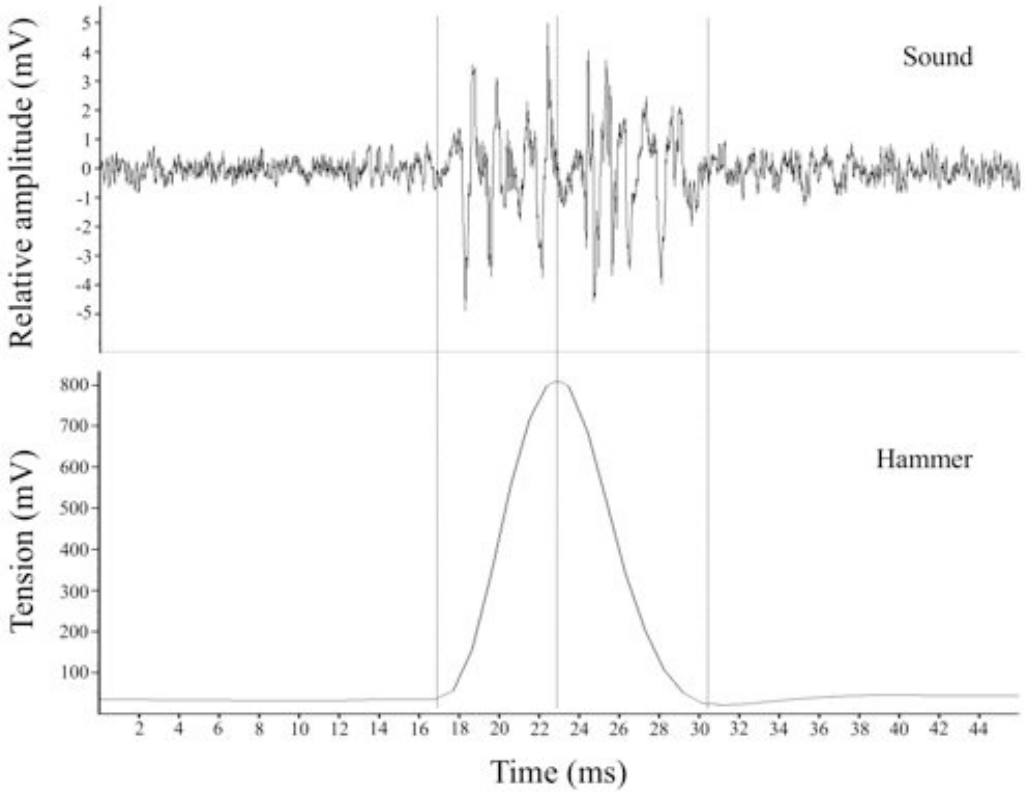


Figure 4.18 Waveform of a hammer strike (8 mV, 0.35 N) on buccal jaws and induced sound for a 85 mm SL specimen of *Amphiprion clarkii*. Cursors mark the beginning, peak and end of the hammer strike.

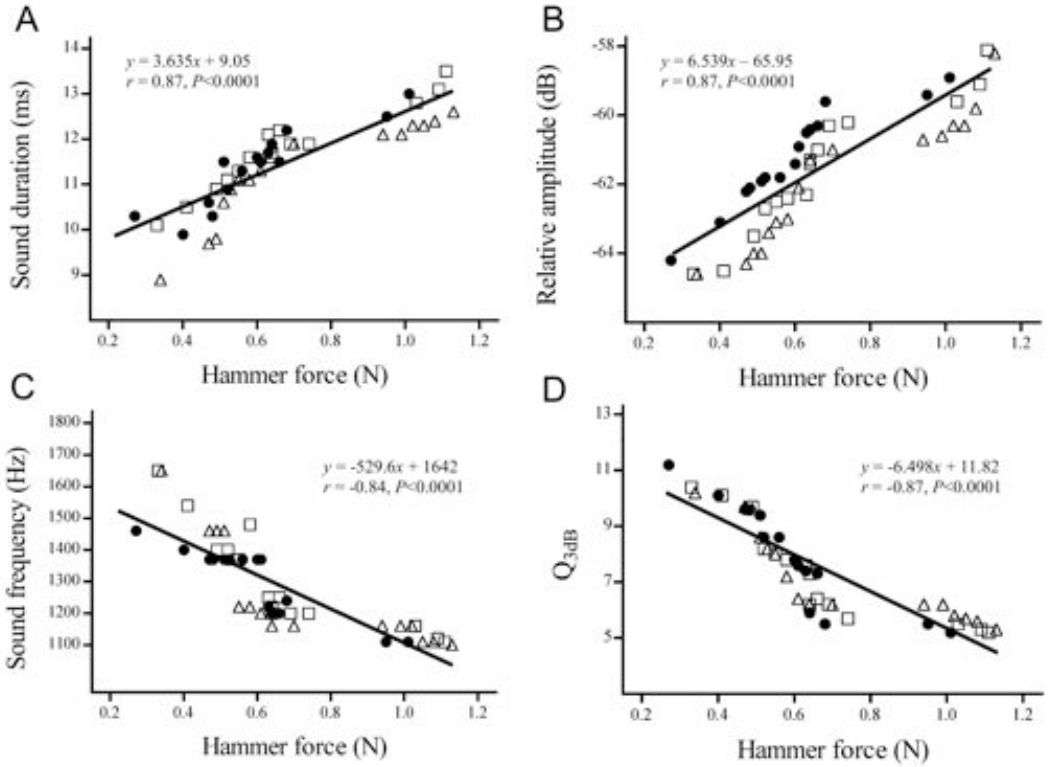


Figure 4.19 Comparisons of (A) sound duration, (B) relative amplitude, (C) sound frequency and (D) Q_{3dB} for sounds induced by variable force hammer hits on buccal jaws in fish (n = 3) of different size (● = 97 mm SL, □ = 85 mm SL, △ = 76 mm SL).

Sound resonance experiments: ribs and swimbladder

A typical hammer strike (10 mV, 0.48 N), the velocity of the ribs vibration and the induced sound waveform are represented in Figure 4.20 for a 96 mm SL specimen of *A. clarkii*. The hammer waveform was an asymmetrical half-cycle with a shorter rise than fall time (6.1 ms and 8.7 ms, respectively, n = 45).

The ribs vibration displayed a sinusoidal pattern that began with a negative deflection (N1), indicating that the ribs were pushed in (*i.e.* away from the laser sensor; Figure 4.20). It was followed by a longer and greater positive peak (P1). Generally speaking, the signal was composed of three oscillation cycles that became progressively longer and greater, whereupon there was

vibration decay, indicating that the ribs lost velocity. N1 vibration was approximately two-thirds as long as P1 (6.7 ms and 8.6 ms, respectively, n = 45; Figure 4.20). Likewise, N1 velocity was approximately half as big as P1 velocity (11.2 mms^{-1} and 24.8 mms^{-1}, respectively, n = 45). Moreover, the frequency spectrum obtained from the vibration of the ribs highlighted a very low peak frequency (24.84±7.82 Hz, n = 45; Figure 4.21).
Interestingly, striking the ribs with the impact hammer produced a sound waveform that exhibited a progressive damping effect (Figure 4.20) as observed for natural sounds emitted by fish.

Comparison of hammer, velocity and sound indicated that the onset of the traces corresponded closely (Figure 4.20). The peaks of the hammer trace and N1 vibration were closely aligned, and the end of the rebound of the hammer trace almost coincided with the peak P1, suggesting that expanding ribs were pushing the hammer back. The highest peaks of the sound waveform occurred within the rise time of the hammer hit as the ribs were compressed. As the force of the hammer declined and the ribs expanded, the positive acoustic pressure of the signal declined. The remaining sound waveform occurred between N2 and P3, with the sound decaying to background level at the peak P3. The vibration decay of the ribs (after P3 peak) did not result in audible sound. Therefore, the ribs could still vibrating weakly without producing sounds, or at least without producing detectable sounds.

Changes in hammer force (*i.e*. harder hits) caused significant changes in ribs vibration (duration and velocity) and sound parameters (Figure 4.22). The total duration N1+P1 for the first cycle of vibration decreased from 26 to 7 ms (r = -0.86, P<0.0001, n = 45), whereas N1+P1 velocity increased from 11.5 to 73.1 mms^{-1} (r = 0.94, P<0.0001, n = 45) with increasing hammer force, indicating that harder hits transferred higher frequency energy to the ribs.
Sound duration increased from 26.1 to 39.3 ms (r = 0.37, P=0.0114, n = 45), and sound amplitude increased from -83.7 to -75.1 dB relative (r = 0.86, P<0.0001, n = 45) with harder hits, which transferred more energy to the ribs. Inversely, peak frequency dropped from 900 to 300 Hz (r = -0.49, P=0.0006, n

= 45), and Q_{3dB} decreased from 22.5 to 7.5 (r = -0.51, P=0.0004, n = 45) with harder hits.

Comparisons of values calculated from regressions of variable force hammer strikes in three specimens of different size showed that hammer strikes had the same effects on N1+P1 duration (ANCOVA, test for common slopes: $F_{2,39}$=0.8813, P=0.4223) and N1+P1 velocity (ANCOVA, test for common slopes: $F_{2,39}$=1.4883, P=0.2383), whatever the fish size.

The effects of variable force hammer hits on sound duration (ANCOVA, test for common slopes: $F_{2,39}$=0.8002, P=0.4565), sound amplitude (ANCOVA, test for common slopes: $F_{2,39}$=1.7003, P=0.1959), sound frequency (ANCOVA, test for common slopes: $F_{2,39}$=2.3959, P=0.1044) and Q_{3dB} (ANCOVA, test for common slopes: $F_{2,39}$=3.0142, P=0.0606) were similar whatever fish size.

In addition, there were no significant differences between individuals of different size in N1+P1 duration (H=1.994, d.f.=2, P=0.3690) and N1+P1 velocity (H=0.281, d.f.=2, P=0.8690), as well as in sound amplitude (H=4.011, d.f.=2, P=0.1346). On the other hand, sound duration (H=34.35, d.f.=2, P<0.001), sound frequency (H=32.69, d.f.=2, P<0.001) and Q_{3dB} (H=32.03, d.f.=2, P<0.001) showed significant changes in relation to fish size, whatever the hammer force (Figure 4.22). This result suggests that the acoustic properties of the ribs might vary with increasing size.

Sound duration and dominant frequency were closely related to fish size, and varied in the same way as natural sounds produced by fish. The more fish size increased, the more sound duration increased and dominant frequency decreased (Figure 4.22). These results were also supported by the frequency spectra for the sound of the small, medium and large fish with SL ranging from 69 to 96 mm (Figure 4.23); the smallest fish actually exhibited the highest frequency energy, whereas the largest one had the lowest frequency energy. In addition, the Q_{3dB} was also related to fish size, being lower in bigger individuals.

Note that the experimental trial consisting in stimulating directly the swimbladder with the impact hammer did not generate usable results. Even a soft hit (~0.10 N) was tricky because it nearly popped the swimbladder. This

latter was pushed in by the hammer but never recovered its initial form, resulting in a deformed swimbladder wall. As a result, the vibration trace at the measurement site was distorted and leveled off. Such results are likely caused by the thinness of the wall.

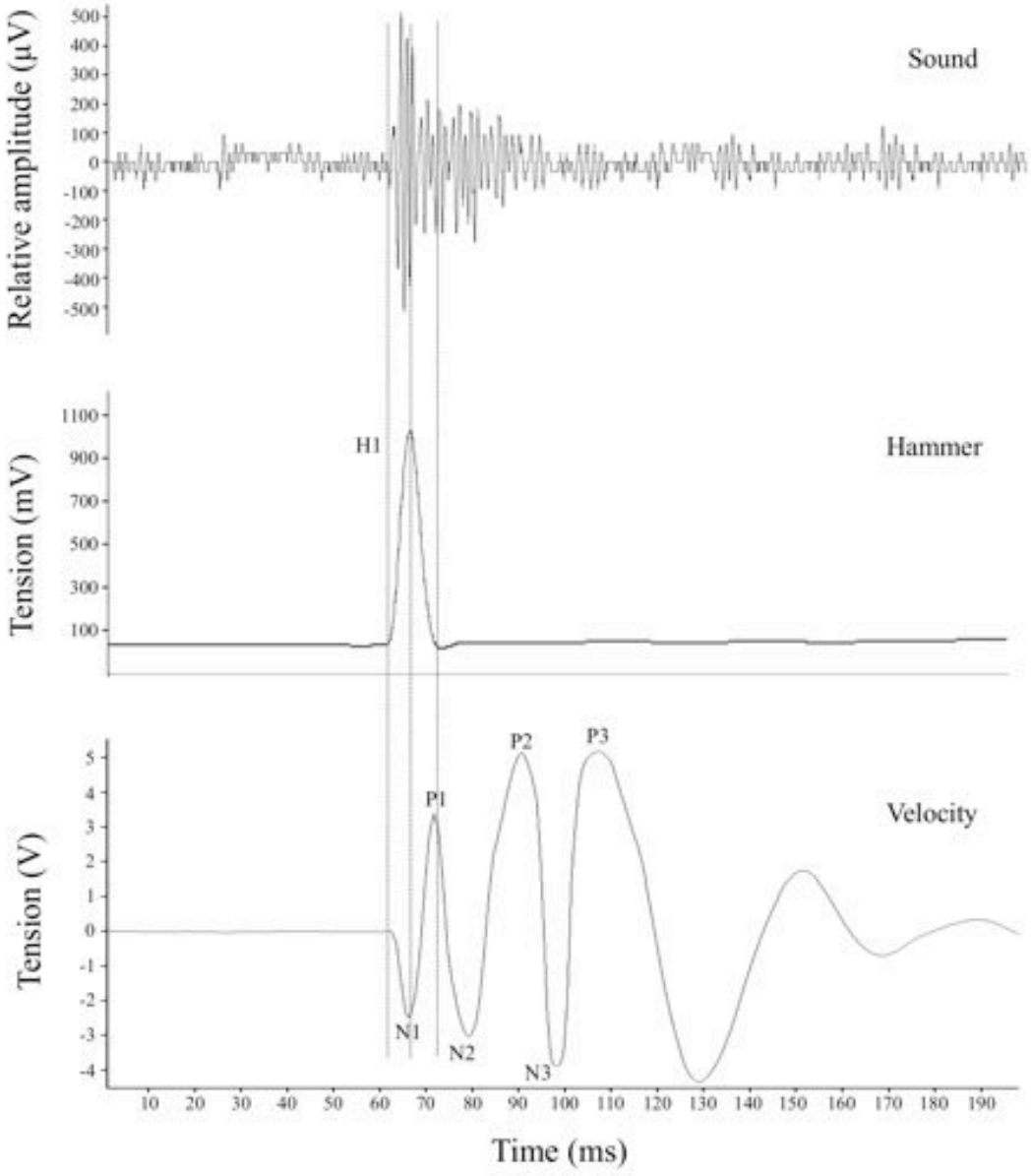


Figure 4.20 Waveform of a hammer strike (10 mV, 0.48 N), the velocity of the rib cage vibration and the sound waveform induced for a 96 mm SL specimen of *Amphiprion clarkii*. Cursors mark the beginning, peak and end of the hammer strike (H1). Note that the highest peaks of the sound waveform are complete by the peak of the hit, which occurs at N1 of vibration when the rib cage is compressed, and that the positive acoustic pressure of the signal declines as the force of the hammer declines and the rib cage expands.

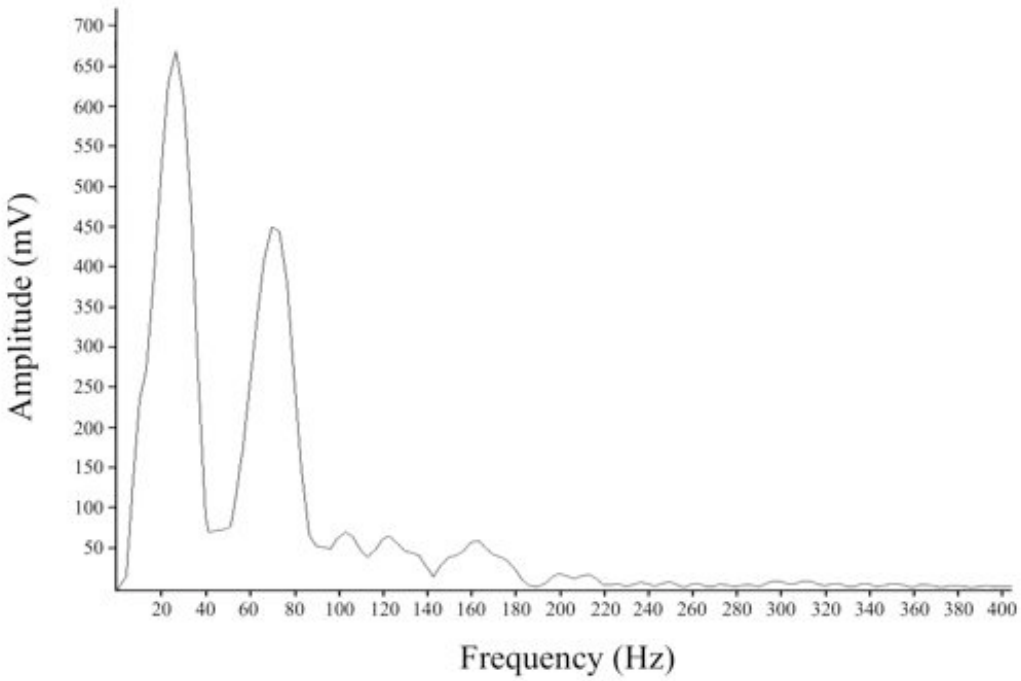


Figure 4.21 Frequency spectrum for a 96 mm SL specimen of *Amphiprion clarkii* showing the peak frequency (~20 Hz) obtained from the vibration of the rib cage induced by a hammer hit of 0.48 N.

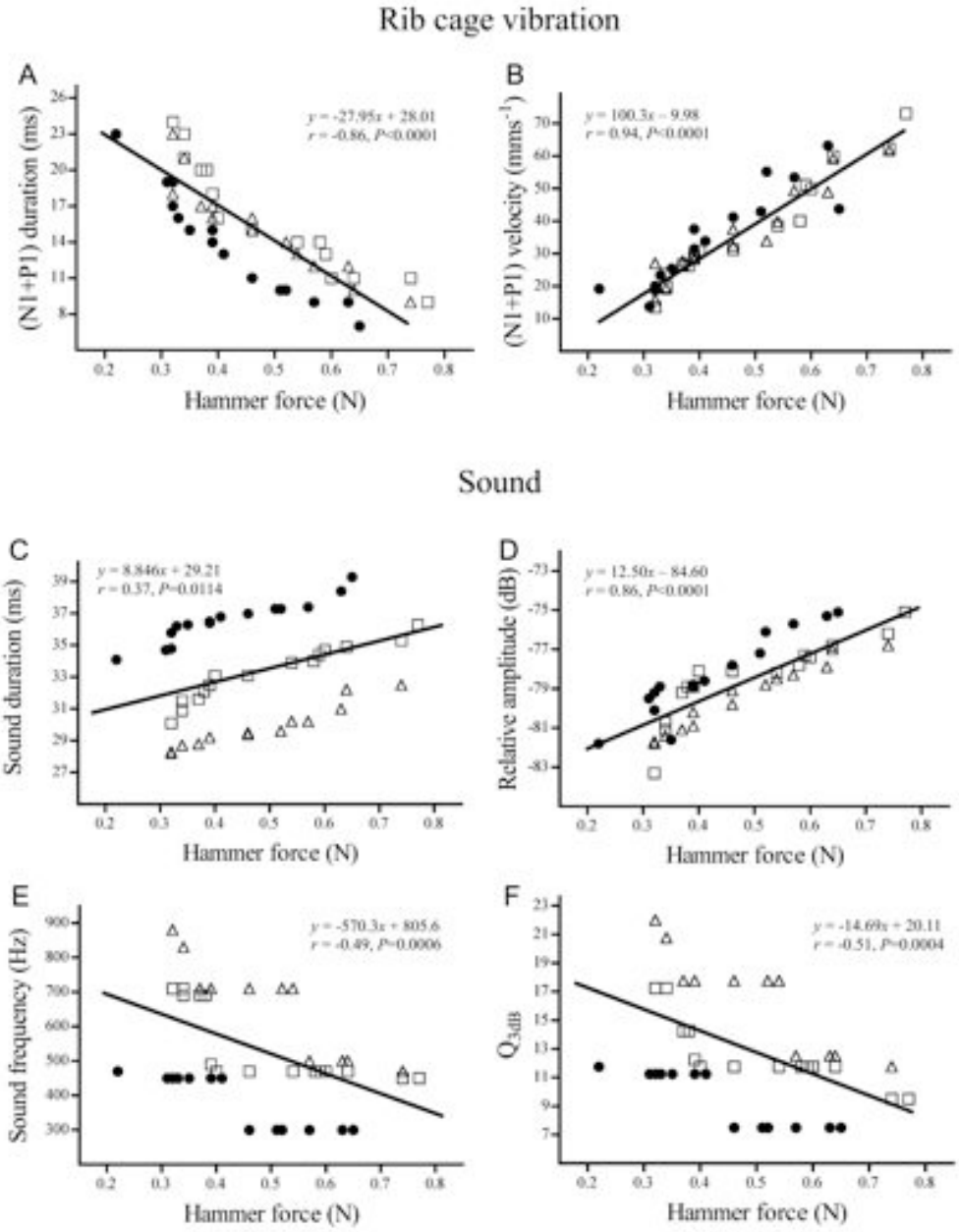


Figure 4.22 Comparisons of (A) N1+P1 duration and (B) N1+P1 velocity of the first cycle of rib cage vibration induced by variable force hammer hits in fish (n = 3) of different size (● = 96 mm SL, □ = 84 mm SL, △ = 69 mm SL). Comparisons of (C) sound duration, (D) relative amplitude, (E) sound frequency and (F) Q_{3dB} for sounds induced by variable force hammer hits on rib cage in the same fish.

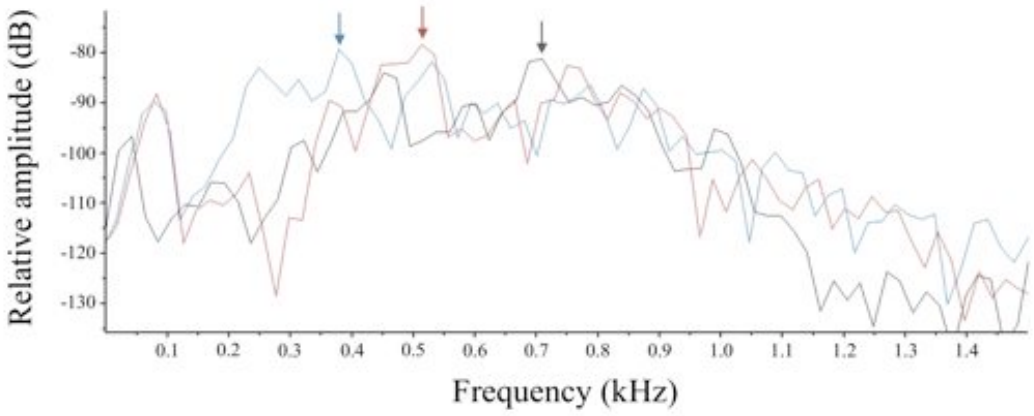


Figure 4.23 Power spectra for sounds induced from a small (69 mm SL), medium (84 mm SL) and large (96 mm SL) individual of *Amphiprion clarkii* by variable force hammer hits. Note the inverse relation between peak frequency and fish size, being the highest for the small fish (black arrow), the lowest for the large fish (blue arrow), and intermediate for the medium fish (red arrow).

4.2.4. Discussion

Acoustical properties of the swimbladder

It has been hypothesized that the swimbladder may act as a resonator and thus change the quality of sounds produced by stridulation in some fish species. Burkenroad (1930) noted that if the swimbladder of the white grunt *Haemulon plumieri* was deflated, the character of the sound became "dry" and lost its "gruntlike" quality. Salmon *et al*. (1968) found sounds were produced by the rubbing of the pectoral fins against the body sides where skin thinly covered evaginations of the swimbladder. In addition, swimbladders have long been considered to function as underwater bubbles that are excited to pulsate at their resonant frequency (Harris 1964, Van Bergeijk 1964). Due to the compressibility of gas in the bladder compared with the surrounding water, an acoustic pressure wave is believed to excite the bladder into vibration.

In clownfishes, the sound-producing mechanism of aggressive sounds is initiated by teeth collisions caused by rapid mouth closure (see paragraph

4.1.3). Sound duration and frequency are known to be morphologically determined signals strongly related to fish size (Parmentier *et al*. 2009c, see also chapter 3), which suggests that both acoustic features are subject to a morphological constraint. Considering the positive relationship existing between fish size and swimbladder volume (Figure 4.14A), it appears that the swimbladder could be the structure responsible for the size-related variations in acoustic features. The energy of the vibrating jaws resulting from the snapping could be transferred through bones to the swimbladder, which may excite it to vibrate. This forced vibration could be possible because the swimbladder wall is rigidly attached to the vertebral column and ribs (Figures 4.16 and 4.17). Moreover, filling the swimbladder with physiological liquid supports this possibility because this experiment specifically modifies size-related acoustic features (Figure 4.13). In the channel catfish *Ictalurus punctatus* for example, removing the gas in the swimbladder did not induce changes in sonic features of sounds produced by pectoral spine locking, suggesting this organ does not play an active role in sound production (Fine *et al*. 1997). Conversely, deflating the swimbladder with a needle significantly modified some acoustic features in the Nile tilapia *Oreochromis niloticus* (Longrie *et al*. 2009). In clownfishes, the swimbladder filling induced pulsed sounds with slight but significant differences in pulse duration and dominant frequency, which imply this structure might play a role in sound radiation.

Calculations of the resonant frequency of the swimbladder in clownfishes using Minnaert's formula (1933) indicate that swimbladder resonance itself does not explain the recorded frequencies (Figure 4.14B). The dominant frequencies of natural sounds are higher than the calculated swimbladder resonant frequencies. It could be due to the nonspherical shape of the swimbladder because the resonance frequency of a bubble of constant volume increases with any deformation of its spherical shape (Weston 1967, Sand & Hawkins 1973). On the other hand, the mechanical properties of the swimbladder wall and the viscosity of the surrounding fish tissue can affect the presumably resonant bubble within (Sand & Hawkins 1973, Love 1978). This notion was supported by Feuillade and Nero (1998) who showed that the

presence of an elastic shell (representing the swimbladder wall) enclosed by a viscous shell (representing the surrounding fish tissue) induces a shift in resonance to a higher frequency.

The use of impact hammer allows deeper investigation of the contribution of the swimbladder as an acoustic source to sound properties. Interpretation of the mechanical events during a hammer strike is complicated because of separation of the hammer strike from the site used to measure displacement (Figure 4.11). Striking the ventral surface of the swimbladder forces it inward and increases the pressure within the bladder, but this manipulation also highlighted that the swimbladder is an inefficient resonator. As a result, the hammer strike creates a deformation of the swimbladder wall, and energy in the compressed bladder does not rebound to cause vibration.

This observation appears to indicate that generalizations about fish swimbladders are incorrect when applied to clownfishes. Inversely, the results of the present study support other recent findings (Fine *et al*. 2009), and indicate that swimbladders are highly damped and thus prevented from prolonged resonant vibrations. An alternative explanation is that swimbladders are inefficient low Q sources with minimal dependence on resonance because of rapid damping by the swimbladder wall (Fine *et al*. 2001, Connaughton *et al*. 2002).

Implication of the rib cage as acoustic radiator?

Striking the buccal jaws with impact hammer generates an acoustic waveform different to that of natural sounds produced by fish; the percussive event suddenly ends up without decaying before the call disappears in the background noise (Figure 4.18). Moreover, sonic characteristics of sounds induced by hammer strike do not display differences between individuals of different sizes (Figure 4.19), suggesting that buccal jaws would maintain similar acoustic properties as they increased in size. Conversely, striking the rib cage with the impact hammer generates sounds with size-related variation in some acoustic features: sound duration and frequency are positively and

negatively related to fish size (Figures 4.22 and 4.23). Such relationships between sonic features and fish size are consistent with those observed in natural sounds, suggesting the resonant properties of the rib cage might be responsible for the size-related variations in acoustic features. In addition, manually induced sounds exhibit an acoustic waveform similar to that of natural sounds (Figure 4.20). Some acoustic features are a bit different to natural sounds, sound duration and the sharpness of tuning (Q values) being both higher. However, these differences may be explained by the elimination of the damping attributable to the body musculature that has been dissected (see Fine *et al*. 2001).

Furthermore, the tight connection between the swimbladder wall and rib cage might explain why clownfish sounds display characteristics of a broadly tuned, highly damped sound source. Clownfish sounds possess low Q (the quality factor Q is a descriptor of the sharpness of tuning) values (Q_{3db}=4.1) and the acoustic waveform of pulses decays rapidly (<20 ms). In the oyster toadfish *Opsanus tau* and the weakfish *Cynoscion regalis* for example, swimbladder sounds are not sharply tuned and display such characteristics (Q_{3db}<2, pulse duration<10 ms; Fine *et al*. 2001, Connaughton *et al*. 2002). In this context, the swimbladder might act as a damper that dissipates the vibrations of the rib cage. Such a damping effect is consistent with the low peak values related to the frequency spectrum obtained from the vibration of the rib cage induced by hammer strikes (Figure 4.21). Likewise, the swimbladder filling increases the pressure inside, and thus changes the tension in the swimbladder wall. Therefore, modifying the physical properties of the swimbladder might affect the resonant properties of the rib cage because the wall is closely attached to the ribs and the first precaudal vertebrae, which might explain the resultant differences in sound duration and frequency.

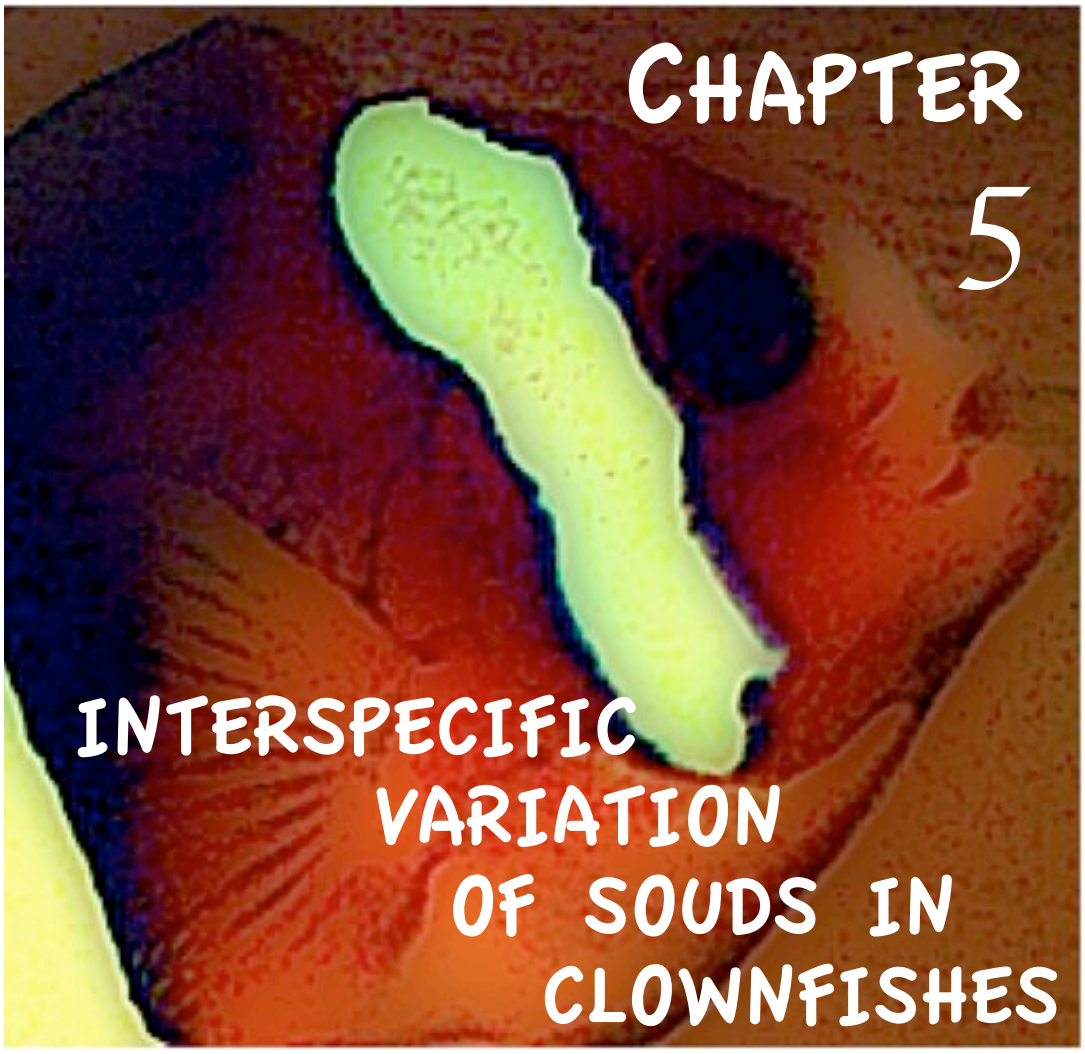
All this set of observations suggests that the rib cage might act as the major acoustic radiator during sound production in clownfishes. This assumption is supported by the fact that ribs are mobile because they articulate with parapophyses (Figure 4.15), and thus could vibrate easily. Thereby, acoustic

waves resulting from jaw vibration are likely transferred to the rib cage *via* the articulation between the skull and vertebral column.

4.3. CONCLUSION

On the basis of their spectral and temporal characteristics, aggressive sounds of clownfishes may be considered as stridulatory sounds produced by a previously unknown mechanism. Sounds are initiated by teeth collisions caused by rapid jaw closure attributed to a sonic ligament that joins the hyoid bar to the internal part of the mandible. The function of the ligament can be compared to a drawbridge chain, which forces the mandible to turn around its articulation by acting as a cord during the lowering of the hyoid apparatus.

Additionally, theories about which the swimbladder might function as a resonator that amplifies and changes the quality of sounds produced by stridulation are incorrect when applied to clownfishes. Indeed, it clearly appears that swimbladder is an inefficient resonator, being highly damped and prevented from prolonged resonant vibrations. On the other hand, the rib cage might be the major acoustic radiator and its resonant properties might explain the size-related variations observed in pulse duration and dominant frequency.


CHAPTER
5
INTERSPECIFIC
VARIATION
OF SOUDS IN
CLOWNFISHES

5.1. INTERSPECIFIC VARIATION OF CALLS IN CLOWNFISHES: DEGREE OF SIMILARITY IN CLOSELY RELATED SPECIES *

5.1.1. Introduction

Acoustic communication provides a good model for studying the evolution of behavior. Generally speaking, acoustic signals used in mate choice and mate recognition can play a role in speciation for sound producing taxa such as anurans (Gerhardt 1998, Ryan *et al*. 2007), insects (Claridge & Morgan 1993, Mendelson & Shaw 2005) and birds (O'Loghlen & Beecher 1999, Safi *et al*. 2006). Variation in acoustic signals can act as pre-zygotic isolating mechanisms (Wells & Henry 1992, Cocroft & Ryan 1995, Zuk *et al*. 2001), the receivers being the selective force on the evolution of the signal (Higgins & Waugaman 2004). The acoustic signal is typically composed of both temporal and spectral components, which may evolve at different rates (Gerhardt 1991). Examining these patterns of acoustic variation among species may be useful for testing the evolutionary history of the characters (Brooks & McLennan 1991, Martins 1996).

Among the sound-producing fish, the coral reef damselfishes (Pomacentridae) are one of the most intensely studied families with sound production being documented for more than 20 species, belonging to eight different genera (Takemura 1983, Santiago & Castro 1997, Parmentier *et al*. 2006b). Within this large and diverse fish family, clownfishes are colorful coral reef fishes well known for their mutualistic relationship with tropical sea anemones that host them (Allen 1972). Recent phylogenetic investigations using both morphological and molecular characters supported the monophyly of the clownfishes belonging to the genera *Amphiprion* and *Premnas* (Elliott *et al*. 1999, Santini & Polacco 2006, Cooper *et al*. 2009). More specifically, on the basis of 23 (out of 29) species and three mitochondrial genes, Santini and

* Slightly modified from: **Colleye O, Vandewalle P, Lanterbecq D, Eeckhaut I, Lecchini D, Parmentier E.** Interspecific variation of calls in Clownfishes: degree of similarity in closely related species. *BMC Evol. Biol.* (under review)

Polacco (2006) proposed a first hypothesis concerning the lifestyle and origin of the ancestral clownfish. They suggested it was a slender-bodied animal with a rounded caudal fin. However, their interpretation of the evolutionary history of this group remains poorly explained.

In clownfish, groups are composed of a breeding pair and between zero to four non-breeders, depending on species and size of host (Fricke 1979, Buston 2003). Within each group, the sex is controlled socially and there is a size-based dominance hierarchy: the breeding female is the largest individual, the breeding male is the second largest and the non-breeders get progressively smaller as the hierarchy descends (Fricke 1979, Buston & Cant 2006). The size hierarchy represents a queue to attain dominant status; individuals only ascend in rank when a higher rank individual disappears, and the smallest fish in the group is always the most recent recruit (Buston 2003, 2004a). Clownfishes are prolific callers, producing sounds during interactions among group members (Takemura 1983, Parmentier *et al*. 2005). In such a system, sounds are not used for mate attraction. However, acoustic signals might confer a higher probability of attaining breeding status. Dominant frequency and pulse duration of the calls being morphologically determined signals related to fish size (Parmentier *et al*. 2009c, see also chapter 3), sounds seem to be important for the live in social group because the hierarchy determines which fish can have access to reproduction (Buston 2003).

Agonistic interactions are involved in daily behavior (Fricke 1979) and sounds are known to be associated with them (Schneider 1964a, Allen 1972, see also chapter 3), suggesting how important acoustic communication is in clownfish group. So, the question arises as to whether this behavior is also important in speciation process. To find out, the first step was to compare the calls of closely related clownfish species in order to evaluate the variation in call characters (see Cocroft & Ryan 1995). *Amphiprion* species produce the same kind of broadband pulsed sounds during agonistic interactions (Chen & Mok 1988, Parmentier *et al*. 2009c), suggesting the differences should not be at the level of the biomechanics. Although sounds are all produced by snapping jaws, other characteristics could display variation among species.

This study analyzed the sounds of 14 different clownfish species. The aim was to seek in this system of communication, which acoustic features are the most important and to evaluate the potential role of acoustic communication as a driving force in the evolution of clownfishes.

5.1.2. Material and methods

Sound recording and sound analysis

Forty-three specimens belonging to 14 species were audio-recorded, and 50 sounds per individual were analyzed. Different methods were used to collect acoustic data. On the one hand, sounds were recorded during fieldworks in the lagoons in front of Toliara (Mozambique Channel, west coast of Madagascar, 23°36'S - 43°66'E), in front of Opunohu Bay (Moorea, French Polynesia, 17°29'S - 149°51'W) and on a fringing reef in front of Sesoko Station (Okinawa, Japan, 26°39'N - 127°57'E). Fishes were collected by scuba diving and were placed with their anemone host in glass tanks filled with running seawater at a constant temperature of 26°C. On the other hand, recordings were also made on fishes maintained in tanks (T = 26°C) in the Aquarium of La Rochelle (France) and in Oceanopolis (France).
In either case, sounds were emitted in a context of territory defense, and were recorded using a Brüel & Kjaer 8106 hydrophone (see paragraph 2.2.2 for details on procedures and material characteristics). Sound analyses were done based on the method mentioned in chapter 2 (see paragraph 2.2.3 for details and Figure 2.1), and a low pass filter defined on the basis of the dimensions of the recording tanks was applied to all sound recordings.

Character reconstruction of acoustic signals

Acoustic characters were obtained from recordings of living fishes. Some acoustic characteristics, such as dominant frequency and pulse duration, vary in a predictable fashion with the size of the calling individual (Parmentier *et*

al. 2009c, see also chapter 3). Because differences in these features between species might simply reflect an effect of differences in body size, the size-frequency and size-duration relationships were taken into account rather than the variables alone. Ideally, these relationships should be determined within each species, but we had limited sample size for some species used in the analyses. Accordingly, all individuals' means were used as data points in a linear regression analysis, and the overall significant slope was used to remove the effects of body size from the among-species comparison. Note that data related to dominant frequency were first ln-transformed because they were exponentially related to fish size. Then, the among-species comparison was made by pooling together individuals belonging to the same species.

Coding of acoustic characters

In this study, we focused on variation in agonistic sounds between closely related species. Usually, phylogenetic studies deal with behavioural characters and score the presence or absence of a given display. However, variation in most acoustic characters is quantitative rather than categorical. Quantitative characters are often used in phylogeny reconstruction but methods of coding these characters continue to be debated (Archie 1985, Pimentel & Riggins 1987). Therefore, variation among species for each character was examined according to published protocol (Cocroft & Ryan 1995). This method is based on the criterion of non-overlap of 95% confidence intervals to define gaps, and divides each acoustic variable into one or more sets of overlapping intervals. Each set was coded as a single character state (see Figure 5.2 in results). This coding method is relevant because it provides a consistent way of comparing character change across species (Cocroft & Ryan 1995).

Morphological study

Sound being initiated by teeth collision (see chapter 4), the buccal dentition of three different species (*A*. *akallopisos*, *A*. *frenatus* and *A*. *ocellaris*) was

studied, with three individuals from each species used to make comparisons among tooth shapes (see paragraph 2.2.8 for more details on samples preparation).

The sternohyoideus muscle is known to retract and depress the branchial basket (Aerts 1991, Vandewalle *et al*. 1992, De Visser & Barel 1996). Thus, this large muscle might play an important role in the sound-producing mechanism of clownfishes because the hyoid depression leads to the mouth closing responsible for sound production. The sternohyoideus muscle of three species (*A*. *akallopisos*, *A*. *clarkii* and *A*. *ocellaris*) was carefully removed from three preserved specimens for each species and weighed. We used the mass of this muscle as a rough indication of its force-producing capacity (see Wainwright *et al*. 2004).

Statistical analyses

The data used in the analyses were mean values of all recorded sounds for each individual. Because we had limited sample size for some species, an analysis of covariance (ANCOVA) was run to test whether acoustic variables related to fish size evolved in a similar way among five different groups. The first four groups corresponded to species for which we have a sufficient sample size (*A*. *akallopisos*, *A*. *clarkii*, *A*. *frenatus* and *A*. *ocellaris*, Table 5.1) while the fifth group was a pool grouping all individuals of the other species with limited sample size (only one or two individuals). The comparison aimed to determine whether the intraspecific size-related variation of the acoustic variables is similar to the interspecific variation (the pool of species). Note that data about dominant frequencies were first ln-transformed because they were exponentially related to fish size. The other two acoustic variables (pulse period and number of pulses per sound) were tested for the assumption of normality (Shapiro-Wilk test), and then they were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance by ranks with subsequent Dunn's test for pairwise comparisons to test differences between

species. Statistical analyses were carried out with Statistica 7.1. Results are presented as means ± S.D. Significance level was determined at $P<0.05$.

5.1.3. Results

Interspecific differences in sounds

Sounds were produced by the 14 species during aggressive interactions. The call of each species consisted of short pulses emitted alone or in series, and in a relatively narrow band of low frequencies (Table 5.1).

Table 5.1 Summary of the acoustic variables recorded for *Amphiprion* and *Premnas* species. Species are presented in ascending size order

Species (*n*)	Pulse duration (ms)	Dominant frequency (Hz)	Pulse period (ms)	Number of pulses per train
	mean ± S.D.	mean ± S.D.	mean ± S.D.	mean ± S.D.
A. percula (2)	8.2 ± 1.9	853 ± 152	88.8 ± 18.3	2.5 ± 0.8
A. nigripes (2)	9.4 ± 1.4	736 ± 123	124.7 ± 18.1	3.8 ± 1.7
A. ocellaris (4)	9.7 ± 1.5	742 ± 124	106.9 ± 21.7	2.2 ± 0.4
A. latifasciatus (1)	10.3 ± 0.8	674 ± 102	123.5 ± 18.2	3.0 ± 1.3
A. akallopisos (11)	12.5 ± 3.4	645 ± 204	73.8 ± 12.4	3.7 ± 2.3
A. perideraion (2)	11.0 ± 1.9	650 ± 86	67.8 ± 18.4	3.2 ± 1.7
A. melanopus (2)	11.6 ± 2.2	602 ± 96	90.2 ± 22.0	2.6 ± 0.7
A. polymnus (2)	13.3 ± 1.9	564 ± 79	97.6 ± 27.4	2.9 ± 1.6
A. akindynos (2)	13.3 ± 1.9	554 ± 106	106.1 ± 15.9	3.2 ± 1.6
A. frenatus (6)	14.3 ± 2.5	521 ± 123	106.9 ± 24.7	2.5 ± 0.8
A. clarkii (6)	15.4 ± 2.9	477 ± 126	109.1 ± 30.7	3.5 ± 1.8
A. chrysogaster (1)	17.7 ± 1.3	420 ± 59	114.0 ± 11.1	2.6 ± 1.3
A. chrysopterus (1)	18.9 ± 1.1	411 ± 77	160.9 ± 24.9	3.1 ± 1.3
P. biaculeatus (1)	20.5 ± 1.6	399 ± 85	123.1 ± 16.0	3.4 ± 1.7

n, number of recorded individuals per species with 50 sounds analyzed per individual

Pulse duration and dominant frequency were highly related to fish size across species (Figure 5.1). The more fish size increased, the more pulse duration increased (r = 0.98, $P<0.0001$; Figure 5.1A), and the more dominant frequency decreased (r = -0.99, $P<0.0001$; Figure 5.1B). The inter-species comparison using fish size as a covariate showed that pulse duration (ANCOVA, test for common slopes: $F_{4,33}=1.812$, $P=0.150$) and dominant frequency (ANCOVA, test for common slopes: $F_{4,33}=1.753$, $P=0.162$) did not

differ among species, all the 14 species being situated on the same slope. Thereby, variation among species in both acoustic features was clearly explained by size dimorphism between clownfish species.

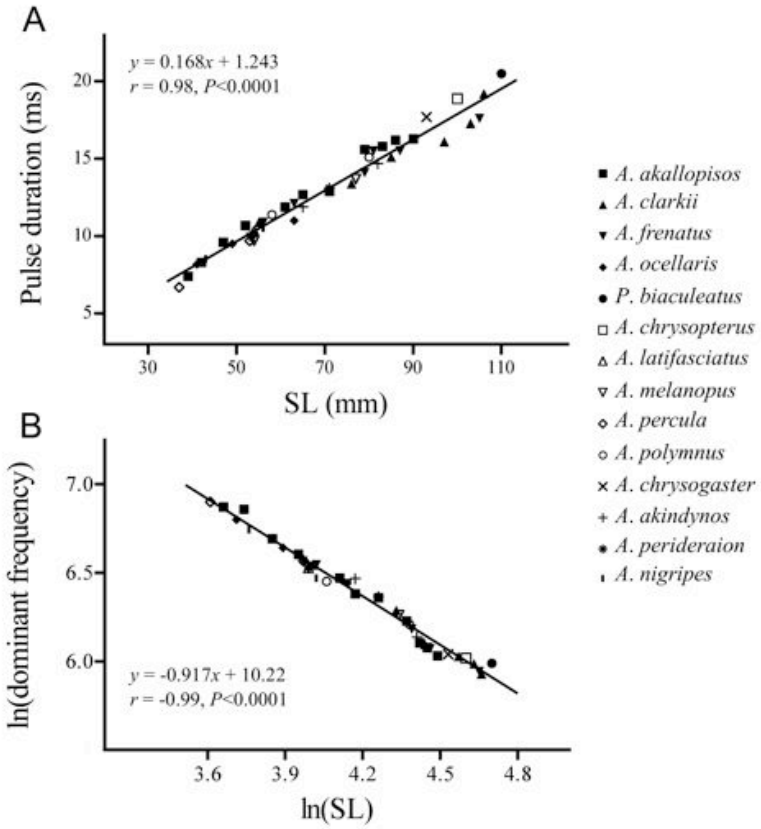


Figure 5.1 Influence of fish size (SL) on acoustic variables in 14 clownfish species. Correlation of (A) pulse duration and (B) dominant frequency against SL. Note that data related to dominant frequency were ln-transformed because they were exponentially related to fish size. Fishes ranged from 37 to 110 mm (n = 43). The significance level was determined at P<0.05. Results are expressed as mean values of 50 recorded pulses for each individual.

Some differences between species still remained for the size-frequency and size-duration relationships even after removing the effect of body size (Figures 5.2A,B). However, this observation needs to be carefully interpreted because deeper attention to pairwise comparisons revealed that pulse duration/body size relationship (H=8.332, d.f.=4, P=0.0801) and dominant

frequency/body size relationship (H=7.276, d.f.=4, *P*=0.1220) were not significantly different between individuals having similar body size (53-54 mm SL) and belonging to five different species (*A. latifasciatus*, *A. melanopus*, *A. ocellaris*, *A. percula* and *A. perideraion*).

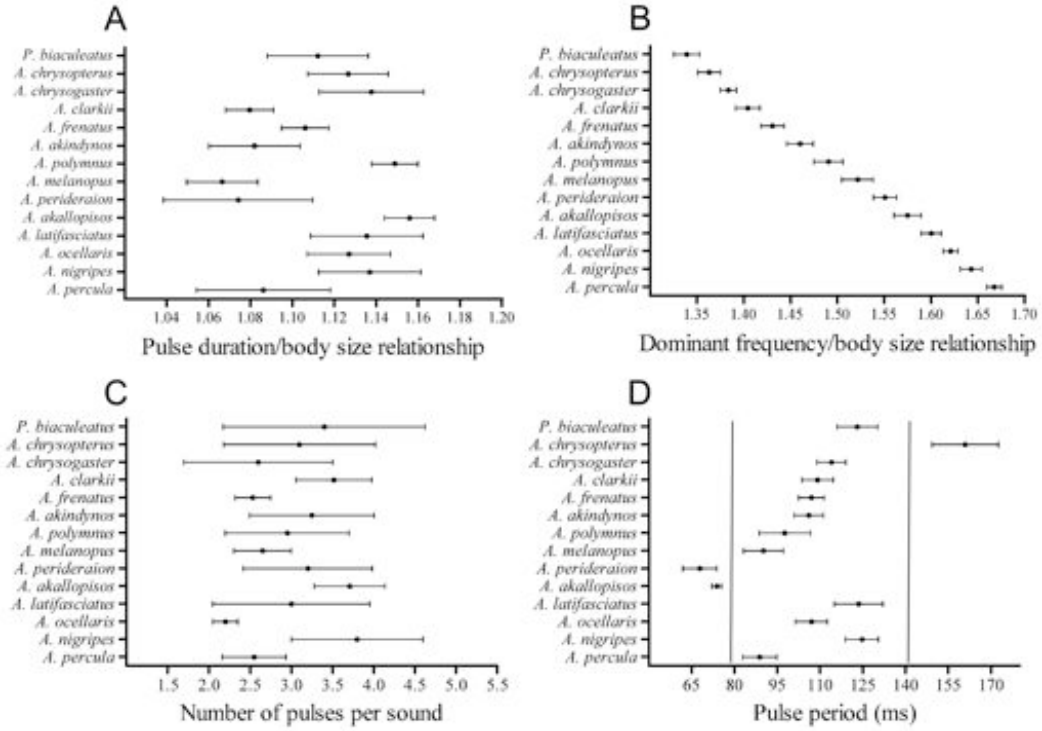


Figure 5.2 Variation of acoustic features in 14 clownfish species. Results are represented as means ± 95% confidence intervals. Vertical lines have been added between sets of overlapping species to indicate gaps.

The number of pulses broadly overlapped between species (Figure 5.2C, Table 5.1), although there were some differences (H=47.62, d.f.=13, *P*<0.01). Pairwise comparisons showed a few species were significantly different (Dunn's test, *P*<0.01): *A. ocellaris* and *A. nigripes*, *A. ocellaris* and *A. clarkii*, *A. ocellaris* and *A. akallopisos*. Pulse period displayed the most variation between species (H=383.1, d.f.=13, *P*<0.001), but considerable overlap in pairwise comparisons showed that several species were similar (Dunn's test, *P*>0.05; Figure 5.2D, Table 5.1).

Morphology and sounds

Deeper attention was given to three species having different teeth shape. Sound comparisons of these three species based on three specimens having the same size (61-63 mm SL) revealed that the dominant frequency of *A. akallopisos*, *A. ocellaris* and *A. frenatus* was not significantly different (H=0.0207, d.f.=2, *P*=0.9897) and was respectively 646, 627 and 625 Hz. Moreover, the acoustic waveform and the power spectrum exhibited the same pattern despite the different types of teeth: rectangular and incisiform in *A. akallopisos*, conical and caniniform in *A. frenatus* and spatulate in *A. ocellaris* (Figure 5.3).

Comparison of the sternohyoideus mass/body mass ratio between *A. akallopisos*, *A. clarkii* and *A. ocellaris* revealed some significant differences between species (H=7.385, d.f.=2, *P*=0.0249). More precisely, pairwise comparisons showed a higher ratio in *A. akallopisos* than in *A. ocellaris* (Dunn's test, *P*<0.05; Table 5.2), whereas there were no differences between *A. akallopisos* and *A. clarkii*, and between *A. clarkii* and *A. ocellaris* (Dunn's test, *P*>0.05). *Amphiprion ocellaris* presented the lowest SH mass/body mass ratio (Table 5.2) and this species produced sounds with the smallest number of pulses (Figure 5.2C, Table 5.1). Likewise, *A. akallopisos* possessed a shorter pulse period than the two other species (Figure 5.2C, Table 5.1) and the highest SH mass/body mass ratio (Table 5.2).

Table 5.2 Body mass, sternohyoideus muscle (SH) mass and ratio of SH mass to body mass in *Amphiprion akallopisos*, *A. clarkii* and *A. ocellaris*

Species	SL (mm)	Body mass (gr)	SH mass (gr)	SH mass/Body mass (%)
Amphiprion akallopisos 1	79	20.561	0.237	1.15
Amphiprion akallopisos 2	58	8.726	0.099	1.13
Amphiprion akallopisos 3	53	6.017	0.068	1.13
Amphiprion clarkii 1	90	35.172	0.374	1.06
Amphiprion clarkii 2	83	24.051	0.256	1.06
Amphiprion clarkii 3	77	21.395	0.224	1.05
Amphiprion ocellaris 1	73	17.284	0.163	0.94
Amphiprion ocellaris 2	47	4.975	0.047	0.94
Amphiprion ocellaris 3	45	4.237	0.039	0.92

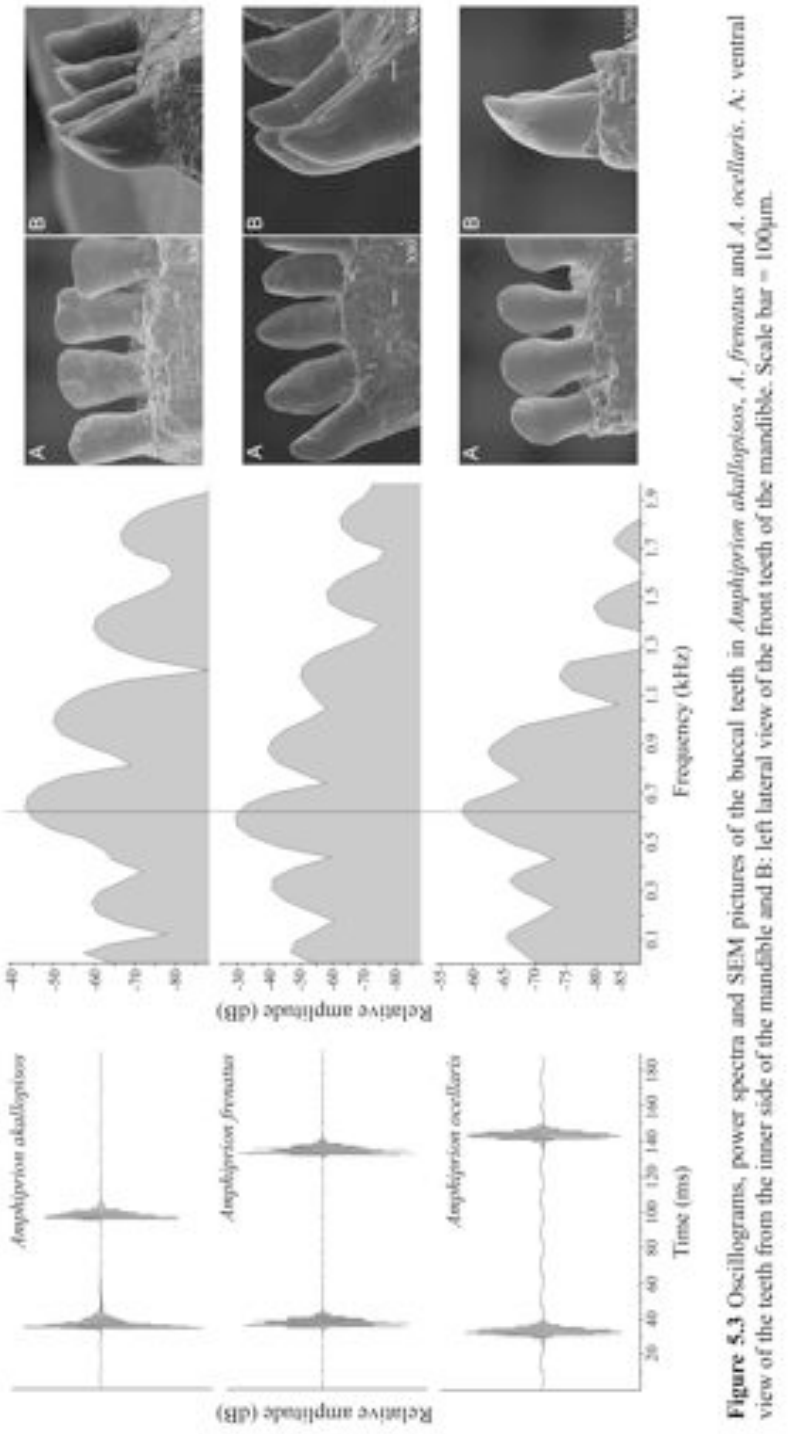


Figure 5.3 Oscillograms, power spectra and SEM pictures of the buccal teeth in *Amphiprion akallopisos*, *A. frenatus* and *A. ocellaris*. A: ventral view of the teeth from the inner side of the mandible and B: left lateral view of the front teeth of the mandible. Scale bar = 100µm.

5.1.4. Discussion

This study is one of the first comparisons of acoustic characteristics in a sizeable number of closely related species of fishes (see also Malavasi *et al*. 2008). The most important insight was found at the level of the relationship between fish size and both dominant frequency and pulse duration. These kinds of relationships were already well known in fishes and have been found in numerous species from different taxa (Ladich *et al*. 1992b, Myrberg *et al*. 1993, Lobel & Mann 1995, Henglmüller & Ladich 1999, Amorim & Hawkins 2005, Parmentier *et al*. 2009c). However, it appears that these relationships come to a higher taxonomic level in the case of clownfishes (*i.e*. spread over the entire tribe Amphiprionini; see Cooper *et al*. 2009) since dominant frequency and pulse duration are strongly predicted by body size among 14 different species. Classically, acoustic characters linked to variation in morphology are more conservative than characters that display variation at the level of physiology (Ryan 1988). The relationship found across all species between fish size and frequency and between fish size and pulse duration highlights they are all subject to a constraint related to morphology. Moreover, different species having different types of teeth (*A. akallopisos*, *A. frenatus* and *A. ocellaris*) and the same size produce sounds that display the same power spectrum and the same oscillogram, demonstrating that teeth shape does not make an important difference in the sounds produced by the snapping jaws. Thereby, all clownfish species seem to use the same mechanism of vocalization, which thus must have remained largely conserved during evolution.

The size influence suggests that all the fourteen species might have a major overlap at the level of pulse length and dominant frequency. The ratio of these sound characteristics to body size suppresses this relationship at the level of pulse duration but not at the level of dominant frequency. Regarding the dominant frequency, the ratio continues to be smaller in bigger species, but highly reduces the overlap between species. For example, some *Amphiprion clarkii,* measuring between 55 and 110 mm in SL, produced a frequency range

that was between 700 and 400 Hz, overlapping also the frequency of smaller *A. ocellaris* (625-900 Hz; see also Parmentier *et al*. 2009c). However, the ratio of dominant frequency/body size was 1.40 in *A. clarkii* and 1.62 in *A. ocellaris*, clearly distinguishing the species. Thus another factor other than body length must be important in determining the absolute frequency values. The thickness of the vibrating jaws or the volume of the rib cage could be the determining factor (see chapter 4). Moreover, the data of this last factor are also correlated with fish size. The question arises whether the fish is able to determine the relationship (or the discrepancy) between the emitter size and the sound frequency it detects. If so, it could help the fish to distinguish whether the emitter is conspecific or not. In the future it would be interesting to compare these size-related relationships between *Amphiprion* and other Pomacentridae species. Comparison with *Pomacentrus partitus* data (Myrberg *et al*. 1993) seems to indicate these fish might also be placed on the same slope.

Variations in sounds are considered pre-zygotic isolating mechanisms leading to speciation (Cocroft & Ryan 1995, Grant & Grant 1996, Slabbekoorn & Smith 2002). In *Amphiprion* species, acoustic properties contribute to the differentiation of species because they always showed differences in at least one of the acoustic characteristics recorded. The fact that all species have the same biomechanics implies these fish do not have many possibilities to develop variations in their calls: they can differ in dominant frequency and pulse duration through their body size, in pulse period and in number of pulses in a call. Body size as a trait of natural selection has already been demonstrated in the speciation event of some stickleback species in which this difference is thought to be an adaptation to alternative foraging habitats (Nagel & Schluter 1998). In damselfishes as well, evolutionary change in body size (*i.e*. gigantism or nanism) could be assumed as a relatively common phenomenon (Frédérich & Sheets 2010, Aguilar-Medrano *et al.* 2011). Premating isolation based on body size could also be important in some *Amphiprion* species. In *Amphiprion akallopisos*, size-dependent aggressive hierarchy exists within groups; all aggressive interactions (biting, chasing,

frontal and lateral display, body jerking) appear to be preferentially directed towards individuals adjacent in ranks (Fricke 1979). In *Amphiprion percula*, rank was the only factor associated with the probability of mortality; low-rank individuals suffer from a higher mortality rate than high-rank individuals. The most likely explanation for this pattern is competition for rank (Buston 2003), preventing smaller fish from having access to reproduction. It means that acoustic communication can be an important factor for mating access and that its characteristics can constitute barriers.

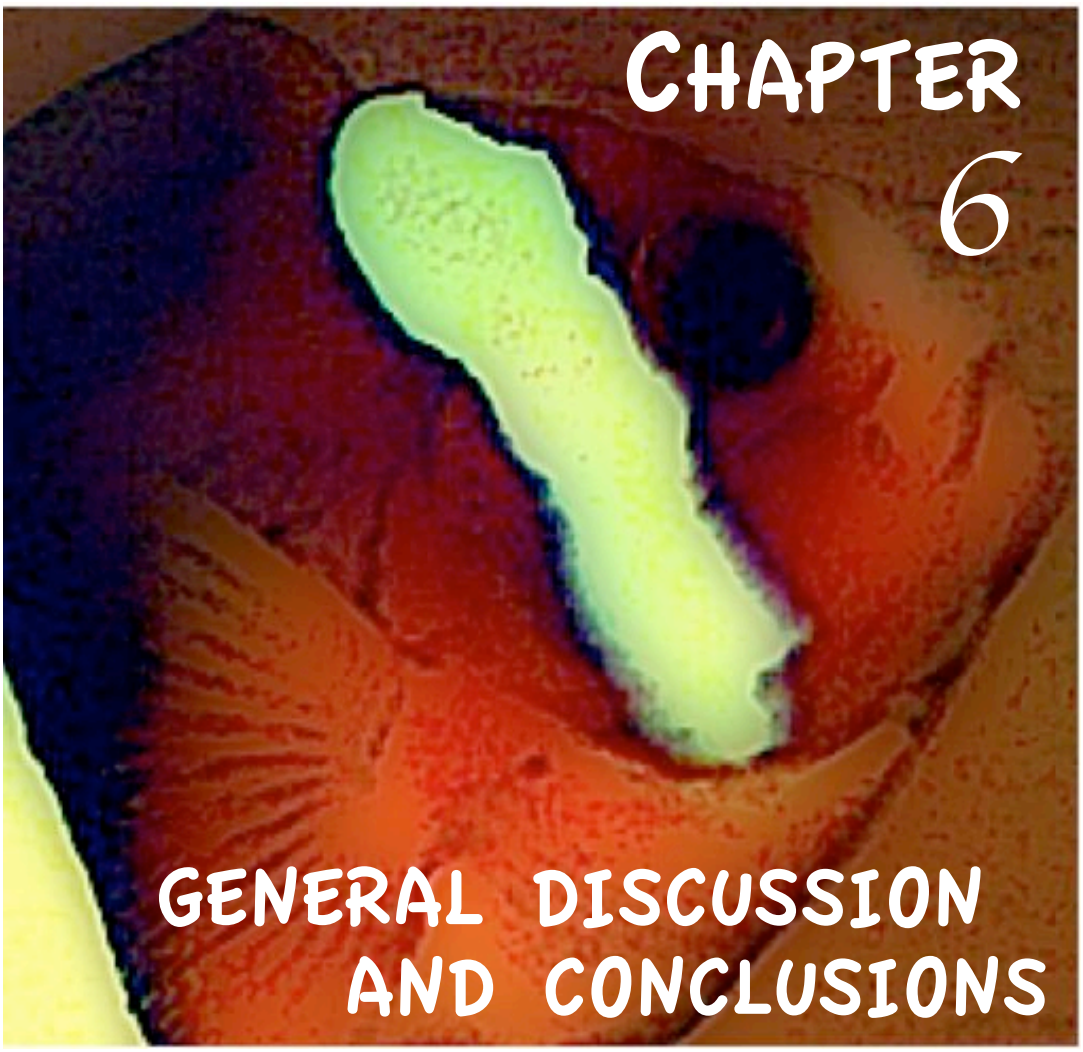
Competition for the limited anemone resource may have resulted in niche partitioning through specialization for different anemone species (Elliott & Mariscal 2001), most clownfish remain in close contact with their hosts and rarely interact with other species on the reef (Allen 1972). However, some clownfish species appear to partition the anemone resource with other species by having a refuge in size (Hattori 2000, Elliott & Mariscal 2001): small *A. sandaracinos* or *A. leucokranos* cohabit with *A. chrysopterus* in the region of Madang (Papua New Guinea), while small *A. perideraion* use the same host sea anemone as *A. clarkii* in the region of Okinawa (Japan). In both cases, the different sizes of cohabiting species imply they possess clear differences in their acoustic repertoire (Table 5.1), size-related call characteristics such as main frequency and pulse length being a by-product of the evolutionary trait. In the Japanese heterospecific groups, small *A. perideraion* are not considered as competitors and should receive less aggressive attention from larger congener *A. clarkii*. Although *A. clarkii* suppresses the growth and reproduction of *A. perideraion* (Hattori 1995), subadult *A. perideraion* are able to mature in heterospecific groups, and change to female when they are the largest among conspecific members. This suggests that *A. perideraion* in heterospecific groups prepare for reproduction before the disappearance or emigration of larger *A. clarkii*. Thus, they adopt a mating strategy that involves waiting for vacated breeding posts because of their low mobility and a low host density (Hattori 2000).

Due to the relative simplicity of many central and peripheral vocal mechanisms, fish typically lack the ability to produce complex and dynamic,

frequency-modulated calls (Rice & Bass 2009). Vocal differences among fish species are usually due to variations in temporal patterning (Malavasi *et al*. 2008, Parmentier *et al*. 2009b). Pulse period has been shown to be the most important acoustic feature involved in species recognition in pomacentrids (Myrberg & Spires 1972, Spanier 1979). Rapid divergence in this character could thus lead to pre-zygotic isolation (see Cocroft & Ryan 1995), differences in the calling characteristics being able to prevent the signaller to be considered as a competitor. Myrberg *et al*. (1978) conducted playback experiments to test the responsiveness of different *Stegastes* species. Although sounds of each species were able to elicit responses of all the other species, males significantly more responded to sounds of their own species than to sounds from congeners. Interestingly, species that cohabit individual sea anemones (*i.e*. *A*. *sandaracinos* or *A*. *leucokranos* with *A*. *chrysopterus* in the region of Madang, or *A*. *perideraion* with *A*. *clarkii* in the region of Okinawa) present a completely different pulse period. On the other hand, pulse period is not systematically significantly different between sympatric species: *A. clarkii*, *A. frenatus* and *A. ocellaris* have the same pulse period range (Figure 5.2D) while living in sympatry on the fringing reef around Sesoko island (Hattori 1991). However, these three species occupy different host species, being *Heteractis crispa* for *A*. *clarkii*, *Entacmaea quadricolor* for *A*. *frenatus* and *Stichodactyla gigantea* for *A*. *ocellaris* (Hattori 1991, 1995), which suggest there are no overlaps in sound parameters among species. Moreover, it remains to explain why some species such as *A*. *akallopisos* and *A*. *perideraion,* for example, present such significant differences in their pulse periods compared to other clownfish species. Comparing the sternohyoideus mass to body mass ratio between different species shows a relationship with the number of pulses: *A*. *ocellaris* possesses the smallest ratio and produces the least number of pulses in a sound, while *A*. *akallopisos* has the highest ratio and emits sounds with the highest number of pulses. Generally speaking, such interspecific variations in this acoustic feature are known to be determined by the central nervous system (Demski *et al*. 1973). However a higher

sternohyoideus mass to body mass ratio could also increase the production of the number of pulses per sound (see Marden 2000).

In summary, although they display differences in their characteristics, sounds appear to be highly conservative in clownfishes because they share the same kind of mechanism. The conservative character can also be due to the way clownfishes live, and the way they use their calls. Sounds are not produced to find mate, restricting the constraints of diversification. However, sounds are highly important because they allow defending the competition to mate access. It means that calling differences can prevent competition between cohabiting species and promote the taxon diversification.


CHAPTER
6
GENERAL DISCUSSION
AND CONCLUSIONS

6.1. DIVERSITY OF THE SONIC REPERTOIRE IN CLOWNFISHES

Generally speaking, the diversity of sounds produced by fishes may result from differences in the underlying mechanisms of sound production. Most fishes have relatively limited acoustic repertoires only including one or two distinct sound types. Sound generation by sonic muscle contraction rate depends on pattern generators in the central nervous system (Bass & Baker 1990). The sound-generating system based on swimbladder mechanism reduces the variation of sounds to differences in intensity, rate and number of muscle contractions. These variations could be associated to muscle fatigue. Generally speaking, muscle fatigue seems to be represented by a decrease in amplitude (Barimo & Fine 1998, Mitchell *et al*. 2008) rather than a lengthening of the period between contractions. However, some vocal fish species present a diverse repertoire of sounds by producing several sound types. Courtship calls of the male haddock *Melanogrammus aeglefinus* can be classified into five categories according to duration and knock interval (short slow knocks, short fast knocks, long slow knocks, long fast knocks and humming; Hawkins & Amorim 2000). Clearly, these sounds provide a rich variation in the time domain, which is associated with specific motor patterns (Hawkins & Rasmussen 1978, Hawkins & Amorim 2000). Males of the mormyrid fish *Pollimyrus adspersus* and *P*. *isidori* produce three different kinds of sounds known as grunts, moans and growls even within a single one courtship vocalization (Crawford *et al*. 1986, Crawford & Huang 1999). Differences between sounds are mainly caused by temporal changes in muscle contraction patterns, although frequency modulations can also be a factor of variation (Crawford *et al*. 1986, Crawford & Huang 1999). The Lusitanian toadfish *Halobatrachus didactylus* emits the boatwhistle advertisement call that is related to the breeding season and at least three other sound types known as grunts, croaks and double croaks during agonistic encounters (dos Santos *et al*. 2000, Amorim 2006). The final section of the boatwhistles often shows frequency modulation with a slight increase of the dominant frequencies. On the other hand, the agonistic calls are based on stereotyped

pulses produced by changing temporal patterns (*i.e*. varying the frequency of pulse production). For instance, croaks result from an increase in the pulse repetition rate typical of grunts (dos Santos *et al*. 2000).

In cichlid fishes, agonistic sounds can be broadly classified into three classes, probably associated with the sound-producing mechanism (Amorim 2006): (1) growls are low frequency pulses, which have been suggested (although not demonstrated) to be produced by the pharyngeal jaw apparatus and then amplified by the swimbladder (Lanzing 1974, Rice & Lobel 2003); (2) "chewing" sounds are broad frequency band stridulatory sounds that can be heard when the fish are not eating and are threatening conspecifics (Lanzing 1974); and (3) thump-like sounds are produced apparently as a result of body movements such as head nodding (Brown & Marshall 1978). Each species of cichlid is capable of emitting sounds belonging to one or two of these three broad sound categories. For example, the rainbow cichlid *Herotilapia multispinosa* emits four distinct sound types (thumps, growls, whoofs and volley sounds) whose the first two types would result from a different sound-producing mechanism (Brown & Marshall 1978). Recently, Longrie *et al*. (2009) showed that the Nile tilapia *Oreochromis niloticus* displays a particular mechanism involving the contraction of a set of bundles (the vesica longitudinalis) situated in the hypaxial musculature, ventro laterally to the swimbladder; this contraction should result in compression of the rib cage and also of the swimbladder.

Damselfishes are well-known vocal species that produce broadband pulsed sounds associated with courtship and agonistic interactions (Myrberg 1972, Spanier 1979, Luh & Mok 1986, Kenyon 1994, Lugli *et al*. 1997, Mann & Lobel 1998, Santiago & Castro 1997, Parmentier *et al*. 2006b). These behaviors are associated with different calls in the pomacentrids *Stegastes partitus* (Myrberg 1972, Myrberg & Spires 1972, Kenyon 1994) and *Dascyllus albisella* (Lobel & Mann 1995, Mann & Lobel 1998). Moreover, the pomacentrid *Dascyllus albisella* shows a great diversity and complexity in its acoustic repertoire by producing pulsed sounds during six different kinds of behavior (signal jump, visiting by females, mating, aggression towards conspecifics and

heterospecifics, and nest cleaning; see Mann & Lobel 1998). Likewise, Parmentier *et al*. (2010) showed that *D*. *flavicaudus* produces sounds during six different behaviors related to courtship and aggressive interactions. Although there exist differences in some acoustic features between calls, all these sounds appear to be constructed on the basis of the same mechanism since they display the same kind of sound spectrum and show few differences in terms of pulse duration, ranging between 13 to 16 ms (see Parmentier *et al*. 2010). Also, differences in the dominant frequency are related to fish size in Pomacentridae (Myrberg *et al*. 1993, Lobel & Mann 1995, Parmentier *et al*. 2009c), while differences in the number of pulses and the pulse period are due to the fish physiology reflecting the behavior and its motivational state (Parmentier *et al*. 2010).

In clownfishes, no acoustic signal is produced during the different reproductive activities, highlighting that sounds are not produced to find mate and restricting the constraints of diversification. The highly conservative sound-producing mechanism in clownfishes implies they do not have many possibilities to develop variations in their calls. Some call characteristics are strongly predicted by body size, indicating they are subject to a morphological constraint. Furthermore, these fishes do not possess specialized sonic muscles attached to the swimbladder, which limit the possibilities of sound modulation. On the other hand, these fishes produce two different types of sounds during agonistic interactions involving conspecifics. This diversity in their acoustic behaviors is probably in agreement with their peculiar way of life. Indeed, they live in social groups that inhabit a restricted and sparsely distributed territory (*i.e*. the sea anemone). They spend most of the time in close vicinity of their host and rarely interact with other species on the reef (Allen 1972). Although they were not recorded in the present study, it is likely that clownfishes produce aggressive sounds towards heterospecifics such as butterflyfishes (*i.e*. coelenterate-feeding fishes) since sound production during heterospecific interactions has already been demonstrated for other pomacentrids such as *D*. *albisella* and *D*. *flavicaudus* (Mann & Lobel 1998, Parmentier *et al*. 2010). Thereby, it could be argued that clownfishes show

little diversity in their acoustic behavior in comparison with other pomacentrid species.

Although the size of the acoustic repertoire is typically limited to two sound types being produce solely during agonistic interactions, sound production seems to represent an integral part of the agonistic behaviors in clownfishes. Indeed, aggressive and submissive sounds are clearly inherent to a specific behavior and possess different functions. Aggressive sounds are emitted during charges and threat displays, whereas submissive sounds are always emitted in conjunction with submissive postures (head shaking movements). Thereby, they could have a deterrent or appeasement function with the purpose of solving conflicts without escalated to fighting. Moreover, both types of sounds can readily be distinguished by human ears, and visually by differences in sound spectra and shape of the temporal envelope (see paragraph 3.2.3), highlighting they should be produced by two different mechanisms. Aggressive sounds result from buccal jaw teeth snapping further to rapid mouth closure (see paragraph 3.1.3). Submissive sounds are emitted in conjunction with a lateral quivering of the body that begins at the head and continues posteriorly. To date, this mechanism is still incompletely understood. However, Schneider (1964a) reported that sounds are produced by the friction of adjacent bones in *Amphiprion* spp., with the probable mechanism being the stridulation of the posterior margin of the skull against some vertebral elements. This possibility is also supported by the spectral and temporal characteristics of submissive sounds that are similar to those of stridulatory sounds (see Tavolga 1971).

Interspecific diversity

One of the most interesting aspects when looking at the diversity of sounds emitted by members of the same family is probably its potential to allow species-specific recognition. Interspecific differences may result from variations in physical characteristics of sound produced. Within the Gadidae family, Hawkins and Rasmussen (1978) compared the agonistic sounds of the

cod (*Gadus morhua*), the haddock (*M. aeglefinus*), the pollack (*Pollachius pollachius*) and the tadpole fish (*Ranicepus raninus*) and showed that all acoustic emissions consist of low-frequency pulses which could be distinguished among species by differences in their temporal structures (*i.e.* pulse duration, number of pulses and pulse repetition rate). Likewise, there exist significant interspecific differences in timing, number and grouping of the pulses between agonistic sounds associated with competitive feeding in four triglid species (Amorim 1996b).

Interestingly, such interspecific differences based on variation in physical characteristics of sounds also exist between closely related species. Crawford *et al*. (1997) demonstrated a clear distinction between the courtship sounds of two mormyrid species *P. adspersus* and *P. isidori*, with significant differences in pulse repetition rate, fundamental frequency and pulse duration. Gerald (1971) reported interspecific differences between the courtship calls of six sunfish species of the genus *Lepomis* (Centrarchidae), with differences in number of sounds per emission, sound duration and pulse repetition rate. Two species of carapids *Carapus boraborensis* and *C. homei* differ in duration of sound sequence and knock period, one emitting long sound with fast knock repetition rate and the other producing brief sound with longer knock period (Parmentier *et al*. 2003). Differences in agonistic vocalizations in three species of croaking gouramis (*Trichopsis vittata*, *T. schalleri* and *T. pumila*) suggest that discrimination is possible based on temporal characteristics such as number of croaks per call, number of double pulses, double pulse period and perhaps on the dominant frequencies and sound pressure levels, although these two last factors seem to depend on fish body mass (Kratochvil 1985, Ladich *et al*. 1992b). Recently, Amorim *et al*. (2008) have compared the courtship acoustic signals among five Lake Malawi cichlid species (*Pseudotropheus* spp.) with overlapping distributions and they have found that males from the five species produce low frequency pulsed sounds that differ in the number and rate of pulse production.

Within the Pomacentridae family, four species of the genus *Stegastes* (*S. planifrons*, *S. leucostictus*, *S. partitus* and *S. dorsopunicans*) are sympatric,

establish nearby territories and share the same reproductive season. They also share a conspicuous courtship display known as the signal jump or dip associated with sound production, and for which there exist species-specific differences in duration, number of pulses and pulse repetition rate (Myrberg *et al*. 1978, Spanier 1979, Lobel & Mann 1995). Testing the responsiveness of the *Stegastes* using playback experiments of dip sounds showed that fishes are fully capable of distinguishing their sounds from similar sounds of the other species; the number of pulses and pulse rate being the key features for species-specific recognition (Myrberg & Spires 1972, Myrberg *et al*. 1978, Spanier 1979).

In clownfishes, comparisons of aggressive sounds among 14 species revealed significant interspecific differences in dominant frequency, pulse duration and pulse period (see chapter 5). Clownfish species rarely interact with other congeners on the reef due to niche partitioning through specialization for different anemone species (Elliott & Mariscal 2001). Yet, some clownfish species are able to partition the anemone resource with other species by having a refuge in size (Hattori 2000, Elliott & Mariscal 2001; see chapter 5). In this context, the size differences of cohabiting species imply they differ in their acoustic repertoire, notably in dominant frequency and pulse duration since these acoustic characteristics are highly related to fish size. Moreover, species that cohabit individual sea anemones present a completely different pulse period. Therefore, all these acoustic characteristics can constitute barriers for promoting species-specific recognition and for preventing competition between cohabiting species. Clownfish sounds are not produced in mate attraction. However, sounds are highly important in the group hierarchy because they are involved in competition to access to breeding status.

Intraspecific diversity

One of the most common intraspecific variation in fish sounds is the inverse relationship existing between dominant frequency and fish size. This

relationship is widespread for many species that produce sounds with repeated pulses such as cichlids (Rowland 1978, Amorim *et al*. 2003, Amorim *et al*. 2004b), mormyrids (Crawford *et al*. 1997) and tigerfishes (Schneider 1964b). In addition to this relationship between dominant frequency and fish size, a positive relationship between pulse duration and fish size is present in other fish species such as croaking gouramis (Ladich *et al*. 1992b), damselfishes (Myrberg *et al*. 1993, Lobel & Mann 1995), weakfishes (Connaughton *et al*. 2000) and pearlfishes (Parmentier *et al*. 2006a). Such a relationship between fish size and dominant frequency and between fish size and pulse duration has also been found in clownfishes (see chapter 5).

From a competitive point of view, being able to judge the opponent's size from the frequency and duration of their sounds is useful because larger individuals are usually dominant, and have more chance of winning contests. An interesting phenomenon is the change in sound characteristics associated with the result of contests. In the grey gurnard *E. gurnardus*, the proportion of interactions accompanied by knocks (less aggressive sounds) increase while those accompanied by grunts (more aggressive) decrease with fish size during competitive feeding, suggesting that smaller fish are more active sound producers and are more aggressive when disputing food, probably because a larger body size give an advantage in capturing prey (Amorim & Hawkins 2005). In the haddock *M. aeglefinus*, the duration of courtship knocking sounds increase with the introduction of another male into a tank previously occupied by only one male and a female, showing the effects of increasing competition between males during spawning (Bremner *et al*. 2002). In the croaking gouramis *T. vittata*, croaking sounds during agonistic displays enhance the probability of winning contests by males (Ladich *et al*. 1992a) and thus of attaining dominance. The acoustic features related to male body mass, such as sound pressure level and dominant frequency, are good predictors of the fight outcome since the sounds of winners have higher sound pressure levels and lower dominant frequencies than losers (Ladich 1998). In the tilapia *Oreochromis mossambicus*, there exist significant differences in sounds produced during male-male encounters, the sounds of the winner

present longer pulse durations and lower dominant frequencies for a given length, and the winner produces more courtship sounds when subsequent court with females occurs (Amorim & Almada 2005).

In clownfishes, the larger and dominant individual of the group produces aggressive sounds with longer pulse durations and lower dominant frequencies (see paragraph 3.1.3, chapter 3), which might explain how it can access to the highest rank associated with breeding status within the hierarchy. The same relationship between fish size and acoustic features has been found for submissive (or head shaking) sounds in a clownfish group. However, these sounds cannot be used to express dominance because they are always produced towards higher-ranking individuals in reaction to aggressive displays (see paragraph 3.2.3, chapter 3). On the other hand, this different sound type may be associated with different levels of motivation or may reflect differences in function. Amorim *et al*. (2004a) demonstrated that the grey gurnard *E. gurnardus* emit knocks during low levels of aggression and grunts mainly while performing frontal displays to opponents. Likewise, clownfishes produce aggressive sounds in conjunction with threat postures and submissive sounds while exhibiting an appeasement behavior (head shaking movements). Therefore, both types of sounds exhibit size-related intraspecific differences in dominant frequency and pulse duration, suggesting that these acoustic features might be useful cues for individual recognition within the group.

In clownfishes, several acoustic variables show very little intra-individual variation (*i.e*. coefficient of variation ≤ 0.1, see paragraph 3.1.2 for more details); these variables are more variable between than within individuals (see paragraphs 3.1.3 and 3.2.3), indicating they could potentially provide cues to identify individuals. The most important variables to allow individual identification are dominant frequency and pulse duration, and pulse period in a lesser extent (see chapter 3). These observations are of significant importance because the social structure of clownfishes relies on a size-based dominance hierarchy in which agonistic interactions consistently occur in order to maintain well-defined size differences between individuals adjacent

in rank (Fricke 1979, Buston 2003). Being associated with agonistic interactions, aggressive and submissive sounds play an important role in the group hierarchy by conveying information for allowing fish size assessment.

6.2. DIVERSITY, WAY OF LIFE AND SOUND IN CLOWNFISHES

Clownfishes are strongly united as a group by their common dependence upon an anemone host (Allen 1972, Buston 2003). This is an obligate association for them because there are dependent on the anemone for shelter and protection from larger carnivores (Mariscal 1970b, Allen 1972, Fautin & Allen 1992). Despite all clownfish species share the same way of life, they diversify into three main ecological axes as various vertebrates: the habitat, the trophic morphology and communication (Streelman & Danley 2003).

Some species are specialized to live with a single or few species of hosts while others are more generalist and able to live with several host species (Table 1.1; Allen 1972, Fautin & Allen 1992, Elliott & Mariscal 2001). There exist some morphological variation in body depth and caudal fin shape, which reflect differences in habitat use. For example, *A. clarkii* is deep-bodied, good swimmer with an emarginated caudal fin shape and less dependent on its host for shelter. Being a superior disperser (Hattori 1995) may explain its ability to occupy any host anemone (Allen 1972). *Amphiprion ocellaris* is slender-bodied with a rounded caudal fin shape; this species is more host specialist by living in association with only few anemone species. Note that *A. ocellaris* is usually found with either *Heteractis magnifica* or with *Stichodactyla gigantea*, both of which are large and strongly stinging anemones (Elliott & Mariscal 1996, Fautin & Allen 1992). The high level of protection received may enable this species to adapt its body and caudal fin shape for feeding instead of escaping predators (Elliott *et al*. 1999).

The majority of clownfish species are omnivorous feeders, feeding mainly on planktonic crustaceans (Allen 1972). The stomach contents of *A. chrysopterus*, *A. melanopus* and *A. tricinctus* reveal they feed mainly on zooplankton consisting of copepods (~ 40%) but they also consume benthic

algae (25-30%) and vagile benthic invertebrates in variable proportion according to each species. These species have a conical-type dentition (Allen 1972). However, some other species such as *A. perideraion* appears to be partly adapted for algal feeding. This species differs from the others by having an incisiform dentition and the benthic algae represent more than 40% of the items found in its stomach contents (Allen 1972). Likewise, Frédérich *et al*. (2009) showed that *A. akallopisos* mainly consumes benthic algae (> 60%) while this species also exhibit an incisiform dentition (Figure 5.3).

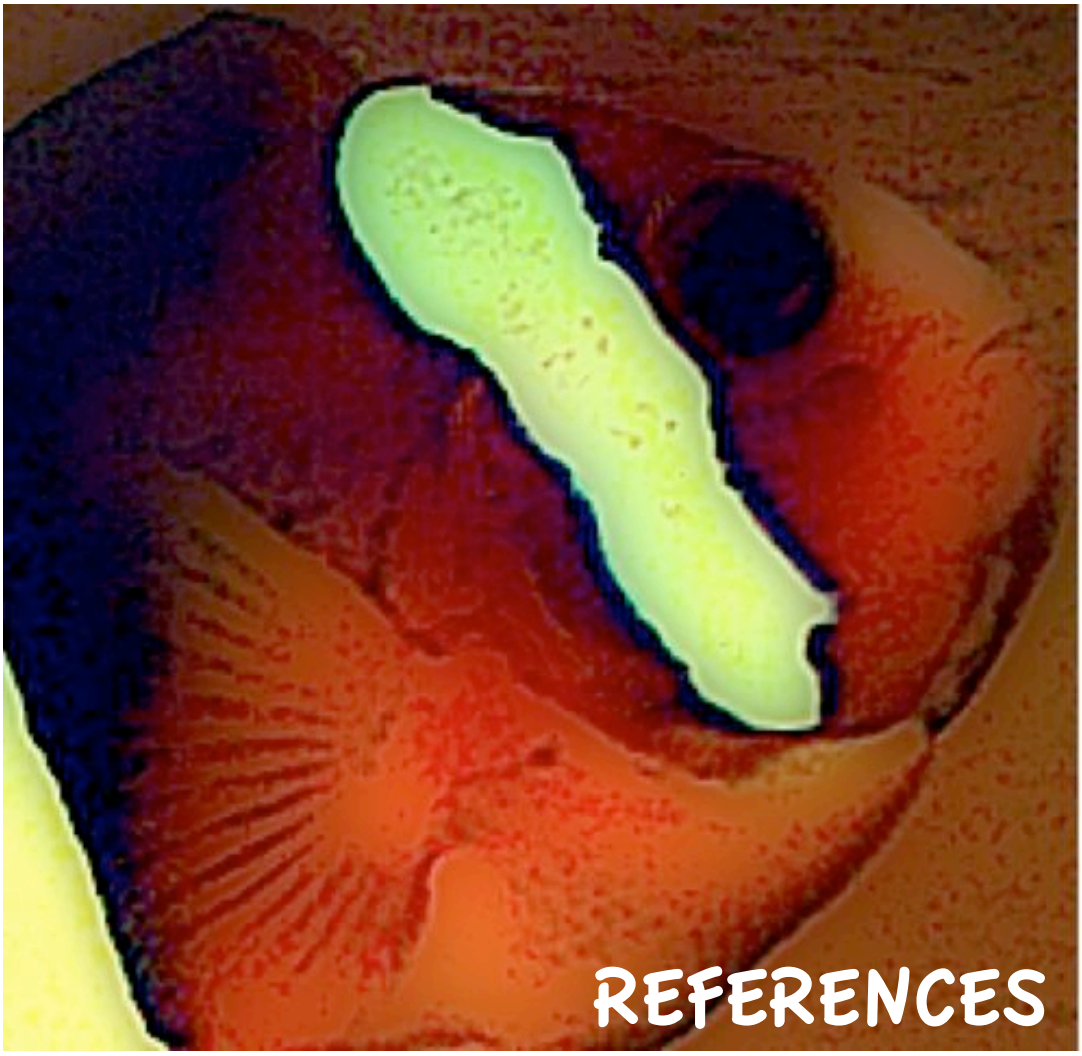
There are clear differences in color pattern among clownfishes. The most striking variation is related to the number of white vertical bars bore by the different species. The usual color pattern varies between zero to three white vertical bars on a darker background, which is usually various shade of red, orange, brown or black (Allen 1972,1991). Some species are also known for the absence of white bars (see Figure 1.2). These differences may play an important role in species recognition since clownfishes appear to be able to distinguish their partner based on visual cues (Fricke 1973). Besides this visual communication, some acoustic features could play a role in species-specific recognition (see chapter 5). Pulse period appears to be the most variable acoustic feature and could be involved in species-specific recognition. In a lesser extent, pulse duration and dominant frequency might be important through their relationship with body size, and because there exists a size dimorphism between clownfish species (Allen 1972). Additionally, sound production seems to be an integral part of the peculiar way of life of clownfish. Indeed, aggressive and submissive sounds appear to be key factors within the size-based dominance hierarchy. Both types of sounds convey important information within the restricted territory (*i.e*. the sea anemone). They present differences in sound spectra and shape of the temporal envelope and would result from two different mechanisms.

6.3. CONCLUSIONS

The objectives were (1) to determine the fundamental components of the acoustic communication, and (2) to describe and explain the mechanisms responsible for sound production in clownfishes.

1. The study of acoustic behaviors shows that no acoustic signal is associated with reproductive activities in clownfishes. On the other hand, theses fishes produce two types of sounds during agonistic interactions. Aggressive sounds are emitted during chases and threat displays whereas submissive (or head shaking) sounds are produced in reaction to aggressive acts. Both types of sounds show size-related intraspecific variation in their dominant frequency and pulse duration: smaller individuals produce higher frequency and shorter duration pulses than larger individuals, and inversely. These sonic features might be useful cues for individual recognition and maintenance of cohesion within the group. The interspecific variation in dominant frequency and pulse duration of aggressive sounds is mainly related to size differences among species. Pulse period appears to be the most variable acoustic feature and could be involved in species-specific recognition.

2. The morpho-functional analysis of the sound-producing mechanism reveals that aggressive sounds are initiated by teeth collisions caused by rapid jaw closure attributed to a sonic ligament that joins the hyoid bar to the internal part of the mandible. It appears that the swimbladder does not function as a resonator that amplifies and changes the quality of sounds. On the other hand, the rib cage might be the major acoustic radiator and its resonant properties might explain size-related variation observed in pulse duration and dominant frequency. Comparison of aggressive sounds among fourteen clownfish species highlights all species use a highly conservative mechanism of vocalization.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