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SYMBIOTICALLY SNAPPER SHRIMP *ANCHISTUS CUSTOIDES* BRUCE, 1977 (DECAPODA: PALAEMONIDAE) INHABITING THE MANTLE CAVITY OF THE PEN SHELL (BIVALVIA: PINNIDAE)

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Abstract: Snapper shrimp is a symbiotic organism usually hidden under the rocks, sponges and pen shells in the seagrass and coral habitats. The relationship study within snapper shrimp and pen shell was conducted from Merambong shoal, one of the biggest seagrass beds in peninsular Malaysia. A total of 40 individual pen shells were collected randomly and four species of pen shells were identified. 40 *Anchistus custoides* were found inhabiting symbiotically in the mantle cavity of the pen shell as solitary males and females and heterosexual pairs. Pen shell, *Pinna bicolor* and *Atrina vexillum* recorded the highest average SH 217.79±53.15 mm, SV 2.62±1.36 dm^3 and SH 164.10-224.78 mm with the SV 1.18±0.43 dm^3, respectively compared to the other species. The size of *Anchistus custoides* ranged from 15.00 to 20.00 mm in length and it was determined to be female due to the presence of eggs in the pleopods. The length of the cephalothorax and its length were highly related (r_s=0.563, p≤0.01, N=40) and found wider in females. A little difference in size between the left and right chela in males of identical length was noticed, although the left chela is much bigger than the right. The significant relationship (r_s=0.450, p≤0.01, N=40) between the pen shell length and shrimp (male-female) length revealed that the size of the shell is important to be hosted the snapper shrimp in the shell cavity.

Keywords: Merambong shoal, pen shell, shell volume, snapper shrimp, symbiotic.

Introduction

The relationship between decapods and molluscs, known as symbiosis is a one-of-a-kind chance to investigate the evolutionary methods used by marine invertebrates (García-Ulloa *et al*., 2019). Some invertebrates included small decapods living in or on other organisms such as marine molluscs, anemones, polychaetes and echinoderms (Nizinski, 1989; Itani *et al*., 2002; Baeza *et al*., 2011; Baeza *et al*., 2013). They are an attractive method to determine the development of connections between decapods and molluscs since Pontonnid shrimps have developed a number of adaptations to deal with a symbiotic existence (Peiró & Mantelatto, 2011). These associations may arise when a guest organism is looking for a haven from its predators, investigates a new food niche or uses its host as a place for reproduction (Baeza, 2010). However, because of the different environmental conditions that impede direct observation and identification of the specific types of association, the relationship between a pen shell and a snapper shrimp is frequently not clearly defined (Radda & Milat, 2009; Overstreet & Lotz, 2016).

Because many species are composed of several different life stages, benthic habitats provide interesting systems for testing the working effect of different spatial scales (Munguia, 2004). Previous research has used the benthic creatures connected to the pen shells

(*Pinna bicolor*) as a model system to analyse the communities found in patchy environments (Keough, 1984a; 1984b; Butler, 1987). There have been reports of symbiosis between several different species of pontoniids and a diverse group of host taxa, which includes invertebrates such as corals, jellyfish, sponges and molluscs (Lee & Ko, 2011; Olliff, 2013; Dobson *et al*., 2014; García-Ulloa *et al*., 2019; Chow *et al*., 2021). One of the most well-researched symbiosis types involves bivalve hosts colonised by tiny shrimp (Baeza, 2008; Aucoin & Himmelman, 2010; Baeza *et al*., 2013). Important indications used to estimate the symbiotic relationship between a host and a guest are decapods' physical, sexual and reproductive characteristics in proportion to the size of the host (Baeza *et al*., 2015). In tropical and subtropical climates where they are found, the Pontoniinae family has more than 600 species that live within the first 100 meters of depth (De Grave & Fransen, 2013). The shells of many different kinds of marine bivalves, particularly those belonging to the Pinnidae family, served as homes for pontoniid shrimps (Kennedy *et al*., 2001; Aucoin & Himmelman, 2010). Many marine bivalves, particularly those belonging to the Pinnidae family are home to pontoniid shrimps inside their shells (Richardson *et al*., 1997; Kennedy *et al*., 2001; Rabaoui *et al*., 2008; Aucoin & Himmelman, 2010).

In the region known as the Indo-Pacific, the Pinnidae family may be found from southeastern Africa through Melanesia and New Zealand, all the way north to Japan and New South Wales and New Zealand (Butler & Keough, 1981; Poutiers, 1998). Additionally, pen shells may be found in the seas of the Mediterranean and the United States (Rosewater, 1961; 1982; Butler, 1987; Zavodnik *et al*., 1991; Butler *et al*., 1993; Munguia, 2004). The pen shell is an endemic species in the Mediterranean Sea (Katsanevakis, 2004) and the biggest Mediterranean bivalve as well as one of the largest in the world, reaching lengths of up to 120 cm (Zavodnik *et al*., 1991). In contrast, the horse mussel, known as *Atrina zelandica* is a huge pinnid bivalve that feeds on suspended particles and is found randomly spread around the coast of New Zealand (Cummings *et al*., 1998). In Malaysia, the distribution of pen shells (*Pinna* and *Atrina*) was reported at Merambong shoal and Tanjung Adang shoal (Johor), Bagan Panchor (Perak) and Merchang Lagoon (Terengganu) (Idris *et al*., 2008; 2009; Idris *et al*., 2012).

There is currently no extensive documentation on the symbiosis between the shrimp *Anchistus custoides* Bruce, 1977 (Decapoda: Palaemonidae) that occupy the mantle chamber of the shell (Bivalvia: Pinnidae) of Malaysian pen shells that have been reported. In Malaysia, most pen-shell studies focus on distribution and ecology (Idris *et al*., 2008; 2009). Most of the publications reported in the Indo-Pacific, Mediterranean and American regions a long time ago (Zavodnik, 1967; Butler & Brewster, 1979; Butler & Keough, 1981; Scheltema, 1983; Butler, 1987; Butler *et al*., 1993; Šiletić & Peharda, 2003; Katsanevakis, 2004). This study focuses on discovering symbiotic snapper shrimps in the pen shell mantle cavity, which may eventually help to know the characteristics and habitat information of snapper shrimp living inside the pen shell cavity in the seagrass bed of Malaysia.

Materials and Methods

During the time of low tide, a total of 40 different individuals of pen shells were collected at random from the Merambong shoal in the Johor Strait (N1° 19' 55.62" E103° 35' 57.75") (Figure 1). Merambong shoal is a sandy area with *Enhalus acoroides, Halophila ovalis, Thalassia hemprichii* and *Halodule uninervis* growing in seagrass meadows. Specimens were removed from their natural habitat using a hand scoop and specimens were placed into a plastic bag containing 10% formalin and labelled. Specimens were transported to the laboratory for further examination. In the laboratory, pen-shell specimens were emptied into a washing tray according to the labeled given and washed in distilled water over a 0.5 mm sieve to avoid the shrimp passing through with the running water.

Pen shells adductor muscles were sacrificed by having the back cut off to get the shrimp occupying the shell. The shrimp were identified based on the key features of Anker and De Grave (2016). The number of shrimps found on each pen shell was recorded and preserved in ethanol at a 70% concentration. The works of Richardson *et al*. (1997) were adopted to identify the presence of snapper shrimp inhabiting the pen shell. Using a MITUTOYO digital vernier calliper (±0.01 mm), the total body length (BL) (from the tip of the rostrum to the tip of the telson), cephalothorax length (CL) (from the tip of the rostrum to the posterior end of the carapace) and width of the widest part of the cephalothorax, and length of the right and left chelae (from the base of the dactyl and propodus to the tip of the claw) of the second pereiopods of each shrimp were measured. Morphometric relationships between the characteristic of shrimp (BL and CL) were analysed using Pearson's correlation (Bhujel, 2008).

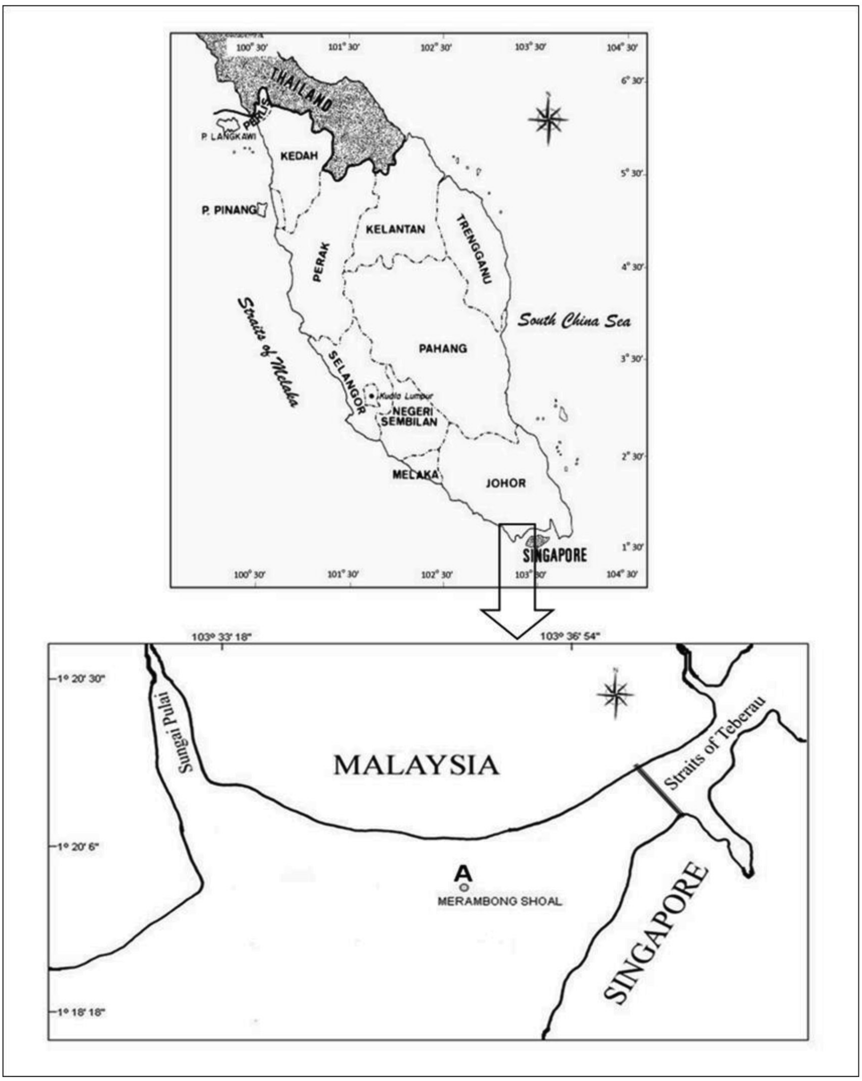

Figure 1: The map displayed the sample regions, including (A) Merambong shoal, which is located off the coast of Southwestern Johor in Malaysia

The shell height (SH) was determined by finding the point that was the farthest along the line perpendicular to the umbo. The shell length (SL) was determined by measuring the length of the perpendicular line to the umbo. When measuring shell width (SW), the distance from left to right was used as the reference point (Figure 2). The estimated volume of each pen shell (SV) was determined by multiplying the three morphometric variables according to Salas-Moya *et al*. (2014), Góngora-Gómez *et al*. (2015) and García-Ulloa *et al*. (2019) in order to comprehend a potential link between the accessible spaces of pen shell for the shrimp.

Results

Snapper shrimp in the pen-shell valve were semi-transparent and pale yellow (Figure 3). The snapper shrimp were found and identified from the Family Palaemoidae and genus *Anchistus* and species *custoides* Bruce, 1977 with 15 to 20 mm length (Figure 4). The broadest cephalothorax and the presence of eggs attached

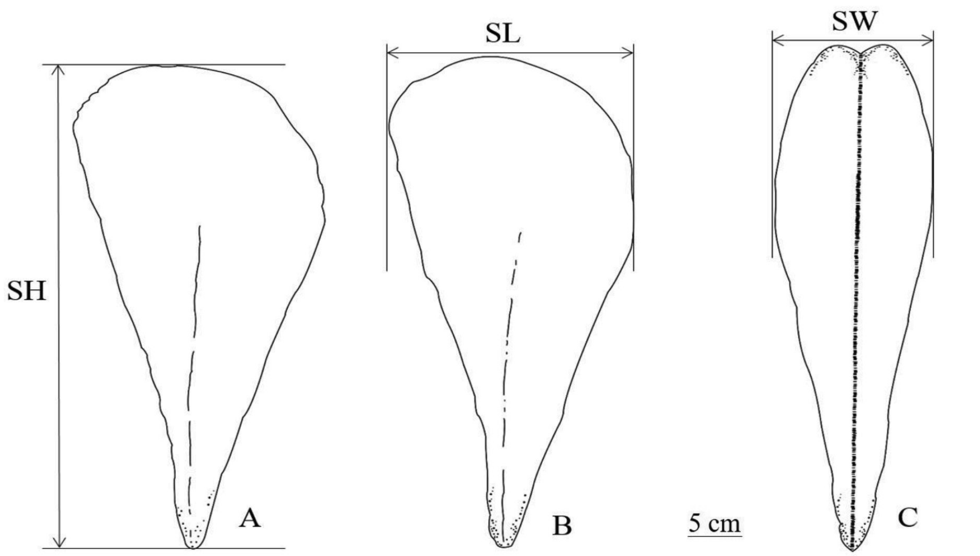

Figure 2: Morphometric measurement of pen shell (A), Shell Height (SH), (B) Shell Length (SL) and (C) Shell Width (SW)

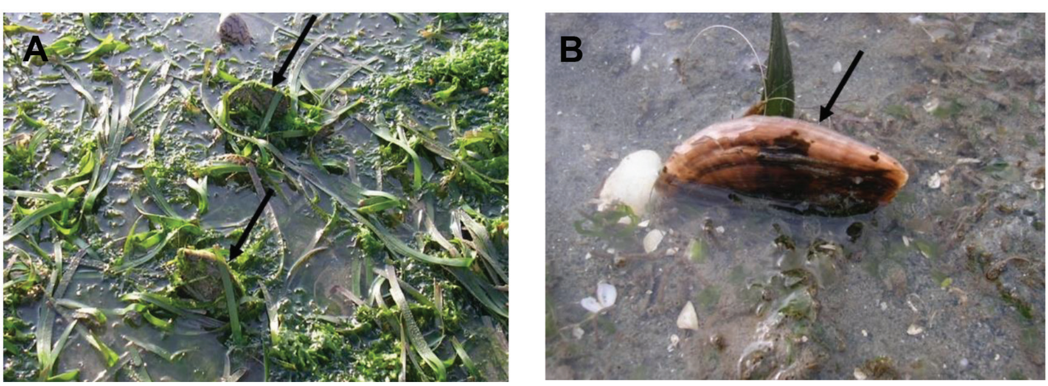

Figure 3: A few examples of pen-shell habitats in the study areas. A - Pen shells (arrow) from Merambong shoal found inhabited with seagrasses and seaweed. B - Pen shell inhabit with spoon grass *Halophila ovalis*

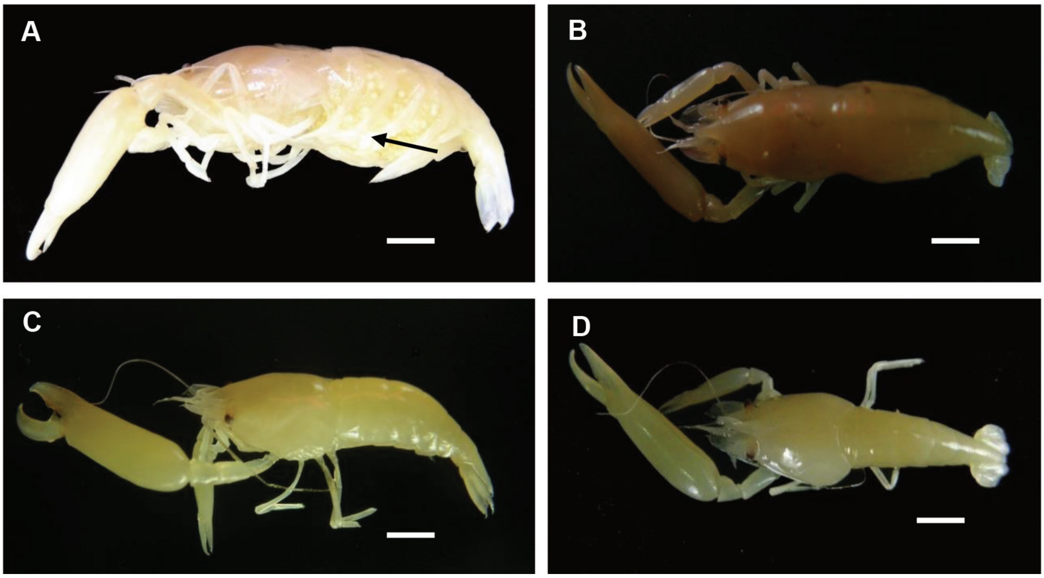

Figure 4: *Anchistus custoides* Bruce, 1977 found in the mantle cavity of the pen shells. (A) and (B) are female with (A) showing the presence of eggs attached to the pleopods and overlaying abdominal pleura (arrow). (C) Male showing pleopods with no overlying pleura and the difference in the size of the chela of the second periopods. Female (B) and male (D), dorsal view. Scale (5 mm)

to the pleopods determined female snapper shrimps. Usually, the width of the cephalothorax distinguishes males and females of *Anchistus custoides* [Figures 4 (B) and (D)]. The breadth of the cephalothorax was substantially connected to its length (r_s=0.563, $p \leq 0.01$, N=40) and females had broader cephalothoraxes [Figure 5 (A)]. The chelae on the second pereiopods of shrimp were not the same size and there was a correlation between the size of the chelae and sex [Figures 5 (B) and (C)]. The left chelae were greater than the right chelae of female and male shrimp. The average size of chelae of males (13.82±1.12 mm) and females (12.20 ±1.95 mm).

During this study, the symbiotic snapper shrimp was observed inhabiting the mantle cavity of the pen shell. Four species of pen shell from two genera (*Pinna* and *Atrina*) have been identified. The genus of *Pinna* comprising of *Pinna bicolor, Pinna muricata* and *Pinna deltodes*. At the same time, *Atrina vexillum* represented the genus *Atrina*. It was found that, out of the 40 pen shells, only 21 pen shells were found to be inhabited by the snapper shrimp (Table 1). A total of 40 individuals of the snapper shrimp have been recorded. *Pinna bicolor* recorded the highest number of shrimps inhabiting the shells with 13 shrimps.

Pinna muricata with recorded the higher SH averaged 283.94±34.5 mm while the lowest was *Pinna bicolor* 216.79±53.15 mm. *Pinna muricata* also recorded the highest average value for SL141.88±11.8 mm and *Pinna deltodes* recorded the lowest 105.50±27.0 mm. The highest SW was recorded by *Atrina vexillum* at 44.83±6.42 mm and *Pinna deltodes* recorded the lowest at 25.77±8.10 mm (Table 2). Based on the measurements for shell volume (SV) found, *Pinna bicolor* recorded a wider space value compared to other pen shells with the value of 2.62±1.36 dm^3. Therefore, the shrimp that inhabits *Pinna bicolor* are more numerous than the others because of the space provided. Part of the shrimp found inhabiting the pen shell solitary male (n=4) and solitary female (n=2). Typically, they occur as adult pairs inhabiting the mantle cavity.

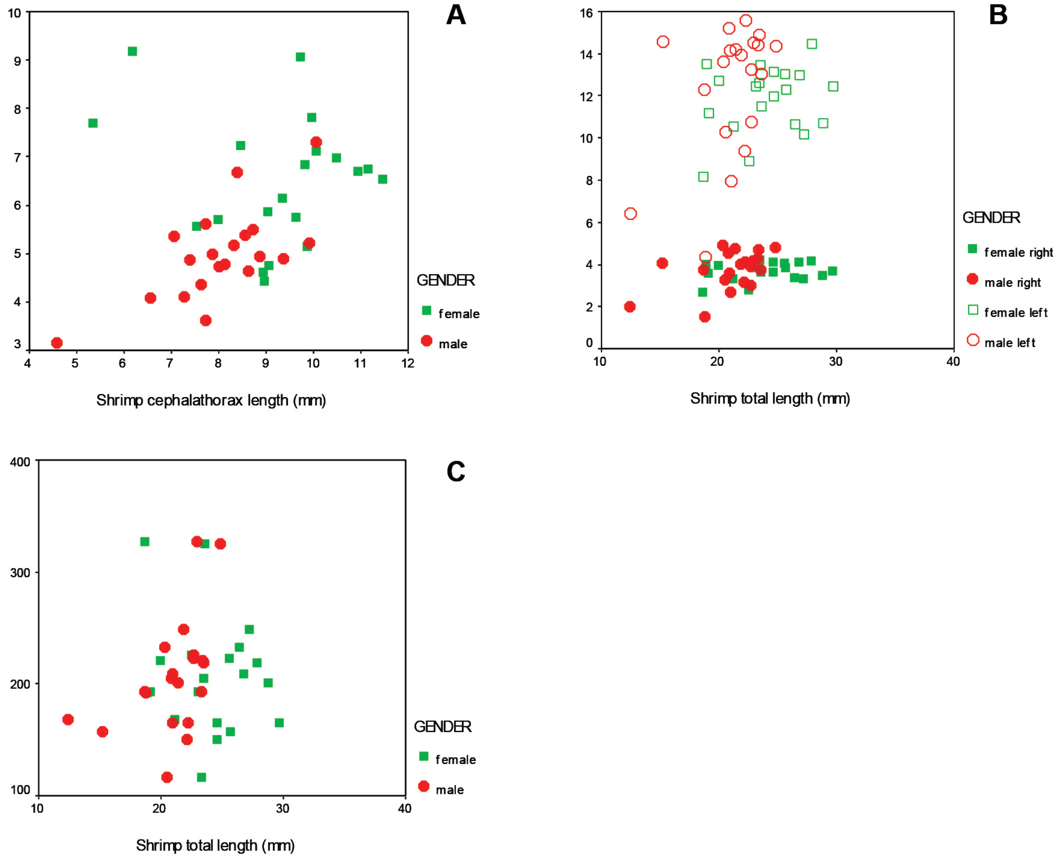

Figure 5: The proportion of breadth to the length between the cephalothorax of male and female *Anchistus custoides* (A), the correlation between the length of the right chela of the second pereiopod (represented by closed symbols) and the left chela of the second pereiopod (represented by open symbols) and the total body length of female and male pen shell inhabitants (B) and a comparison of the length of the female and male *Anchistus custoides* shells as well as their overall length (C)

Table 1: Distribution of snapper shrimp *Anchistus custoides* Bruce, 1977 in the mantle cavity of pen shells

Pen Shell Species	Number of Pen Shells Inhabited by Shrimps	Shrimp			
		Male	Female	Male and Female	Total Number of Shrimps
Pinna bicolor	7	0	1	6+6	13
Pinna muricata	4	1	0	3+3	7
Pinna deltodes	5	1	0	4+4	9
Atrina vexillum	7	2	1	4+4	11

Table 2: Means and ranges of shell measurements of four species of pen shells collected from Merambong shoal

Species	N	Shell Height (SH) (mm)		Shell Length (SL) (mm)		Shell Width (SW) (mm)		Shell Volume (SV) (dm^3)	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
Pinna bicolor	15	150.11-326.90	216.79± 53.15	65.35-150.89	107.62± 31.77	16.12-36.25	28.45± 8.16	0.19-5.18	2.62± 1.36
Pinna muricata	5	246.85-288.84	283.94± 34.51	127.57-153.59	141.88± 11.80	25.63-33.25	29.15± 4.18	0.95-1.41	1.16± 0.20
Pinna deltodes	10	115.57-276.81	203.44± 48.10	61.62-127.90	105.50± 20.27	15.36-32.12	25.77± 8.10	0.25-1.17	0.62± 0.36
Atrina vexillum	10	164.10-224.78	192.65± 22.74	107.83-155.61	131.07± 16.55	38.16-61.09	44.83± 6.42	0.74-1.52	1.18± 0.43

Note: N = number of samples

Studies found that the left chela of a female was somewhat bigger than the right chela, even though both were smaller than that of a male of comparable length, whose left chela was much larger than the right. A substantial correlation (r_s=0.450, p≤0.01, N=40) was obtained between the total length of the pen shells and the total length of the male and female shrimp [Figure 5 (C)]. This association suggests that the biggest individuals occurred in the bigger pen shell while the smallest individuals inhabited the smaller size of the pen shell.

Discussion

A number of studies have highlighted the relevance of the host-guest size association between shrimps and the various types of bivalve organisms. It was the first time that reported the snapper shrimp *Anchistus custoides* resided in the mantle cavity of pen shells in Malaysia seagrass habitat. In the sea of Hong Kong, it was observed that adult pairs of the pontoniine shrimps *Anchistus custos* and *Conchodytes monodactylus* inhabit *P. bicolor* (Morton, 1987). In another situation, a shrimp, *Conchodytes nipponensis* (DeHann) was found living in the mantle cavity of a specimen of *A. pectinata* from Korea (Lee & Ko, 2013) and Dutch New Guinea (Rosewater, 1961). Garcial-Ulloa *et al.* (2019) discovered that the pearl oyster shrimp *Pontonia margarita* lived in the mantle cavity of the *Pinna rugosa*.

This study found that the mantle cavity of the pen shell contained both male and female snapper shrimp. According to Richardson *et al*. (1997), female snapper shrimp may be distinguished by the presence of eggs connected to the pleopods or in their absence, by the wider look of the cephalothorax and the evident extent of the overlapping abdominal pleural partly covering the pleopods. Gracia-Ulloa *et al*. (2019) reported the female cephalothorax length was the widest compared to male cephalothorax length with an average of 9.48±0.16 mm and 5.05±0.76 mm, respectively, being similar to the cephalothorax length average (females = 8.21 ± 2.46 mm; males = 6.39±2.02 mm) reported by Cabrera-Pena and Solano-Lopez (1996). A similar observation was recorded from this study with an average of 8.80 ±0.71 mm and 6.27±0.37 mm for females and males.

Richardson *et al.* (1997) reported that the chelae of the second pereiopods of *P. pinnophylax* are of unequal size and there is also a difference in size of chelae in relationship to sex. The female's right claw is slightly larger than the left, although both are smaller than those of male *P. pinnophylax* of similar length and whose right chela is substantially larger than the left. Richardson *et al.* (1997) found that the chelae of the second pereiopods of *P. pinnophylax* are not similar and there is also a size disparity between the chelae and the gander. Although the right claw of the female *P. pinnophylax* is marginally bigger than the left, both claws are much shorter than those of male *P. pinnophylax* of comparable length, whose right chela is significantly larger than the left. The presence study found the left chelae of *Anchistus custoides* was greater than right chelae and the average length of male chelae was greater than female. According to García-Ulloa *et al.* (2019), the maximum chelae length of the second pereiopod of *P. margarita* females was longer than their male counterparts with an average of 15.61±1.94 mm and 12.65±3.03 mm.

During the study conducted on the Merambong shoal, pen shell was found living in muddy sand areas and associated with seagrasses. Seagrass areas provide an important source of food for aquatic life. In the Sungai Pulai estuary, (Hossain *et al.*, 2018) reported ten species of seagrasses inhabiting the sandy-muddy area while Arina *et al.* (2020) recorded seven species (*Cymodocea serrulata*, *Halophila ovalis*, *Halodule pinifolia*, *Enhalus acoroides*, *Thalassia hemprichii*, *Halodule uninervis* and *Syringodium isoetifolium*) of seagrasses inhabiting the sandy-mud area in Merambong shoal. According to Idris *et al.* (2009), a total of seven species of pen shell have been recorded at Merambong shoal but from this study, only four species of pen shell have been recorded.

From the study conducted, it was found that the presence of *Anchistus custoides* inhabiting four species of pen shells. *Pinna bicolor* recorded higher space than three other species with a range size of 150.11-326.90 mm and an average of 216.79±53.15 mm with the SV 2.62±1.36 dm^3. García-Ulloa *et al*. (2019) reported the presence of shrimp in pen shells with SH ranging from 198 to 271 mm. However, there was no association between the size of the shrimp and the host shell. This research found that the average estimated volume for each rugose pen shell (1.26±0.76 dm^3) was lower than what was reported for *A. tuberculosa* (3.55±0.76 dm^3) by Góngora-Gómez *et al.* (2015) using the same morphometric computation. This would imply that the SV of the *Pinna bicolor* was big enough to accommodate not only a single *Anchistus custoides* but also a male and female. However, Baeza *et al.* (2013) conclude that a shell length of <175 mm is insufficient for the symbiotic shrimp. Aucoin and Himmelman (2010) came to a similar conclusion when they investigated the development of *Pontonia* sp. with its host, the pin shell *Pinna carnea*.

The investigations of the shrimps indicated that they react either violently or defensively along the shell edge, and they might serve as a warning to any curious predatory fish prevalent within the canopy of the seagrass meadow (Bell & Harmelin-Vivien, 1982; 1983). On the other hand, it is unclear whether the *Anchistus custoides* benefit from the association. In a study of the zoea development of *Pontonia pinnophylax*. Calafiore *et al.* (1991) found that the development from zoea stage VIII to the post-larval stage only occurred in the presence of adult mussels. In the absence of *Pinna*, the zoea continued to grow but they did not transform into juvenile shrimps.

According to Richardson *et al.* (1999), the snapper shrimp, pontoniine demonstrated a strong affinity for shade and they rapidly became immobilised in even a weak current flow. Therefore, the presence of these shrimp inside pinnids would provide them with the necessary shelter, shade and protection. The shrimp may get some of their nutrients from the pen shells. When *Pinna* is being fed in suspension, there is never a break in the creation of pseudofaeces on its part. In most cases, the contractions of the adductor muscle are responsible for the expulsion of pseudofeces; however, it seems that this is not the case in *Pinna* (Yonge, 1953). It is currently unclear whether or if shrimp are there, what function they play or what kind of influence they have on the pen shell. The presence of shrimp at the shell border and patrolling the mantle margin may operate as a first line of defense, inhibiting predatory fish from grazing on the posterior edge of the mantle and shell (Richardson *et al.,* 1999). The precise nature of the interaction between the two species has not been scientifically established in this research. This is because *Anchistus custoides* represents the last link in a series of species that are all at risk of extinction. In the coastal seas of Johor, Malaysia, there is a kind of shrimp that lives inside of a pen shell, which is also an endangered bivalve.

Conclusion

A pair and single of adult snapper shrimp *Anchistus custoides* Bruce, 1977 with the ranges size of 15 to 20 mm in length have been recorded inhabiting the mantle cavity of pen shell in Merambong shoal seagrass beds. *Pinna bicolor* and *Atrina vexillum* recorded the highest number of shrimp inhabiting the shell with the average SH 150.11-326.90 mm with the SV 2.62±1.36 dm^3 and SH 164.10-224.78 mm with the SV 1.18±0.43 dm^3, respectively. It was observed that the shrimp was living symbiotically with the pen shells. Female shrimps were identified by the presence of eggs attached to the pleopods and the female size was greater than the male specimens.

Adult snapper shrimp *Anchistus custoides* has been found living in the mantle cavity of pen shells in the seagrass beds of Merambong shoal. Their lengths vary from 15 to 20 mm. A pair and a single adult snapper shrimp Anchistus custoides Bruce, 1977 have been reported. *Pinna bicolor* and *Atrina vexillum* reported the maximum number of shrimp occupying the shell, with an average shell height of 150.11-326.90 mm and a shell volume of 2.621.36 dm^3 for *Pinna bicolor* and SH 164.10-224.78 mm and SV 1.18±0.43 dm^3 for *Atrina vexillum*, respectively. It was also discovered that the shrimp lived symbiotically with the pen shells. It was possible to determine whether or not a shrimp was female by seeing whether or not it had eggs attached to its pleopods. Additionally, the size of female specimens was larger than that of male specimens.

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SURAT KETERANGAN
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Jabatan	: Lektor Kepala

Telah melakukan penelitian yang dipublikasi pada bulan Oktober tahun 2022 dengan judul sebagai berikut:
Symbiotically Snapper Shrimp Anchistus Custoides Bruce, 1977 (Decapoda: Palaemonidae) Inhabiting The Mantle Cavity Of The Pen Shell (Bivalvia: Pinnidae)

Adapun penelitian ini sudah mengacu pada prosedur pertimbangan etik dari:

1. *American Fisheries Society* (AFS, 2014) yang berjudul *Guideline for the Use of Fishes in Research* yang menyebutkan bahwa: penelitian dalam kondisi laboratorium baru mengatur tentang hewan percobaan berupa ikan hidup, untuk hewan percobaan berupa udang dan kerang tidak termasuk (hal 43 ; terlampir), dan
2. *Canadian Council on Animal Care* (CCAC, 2005) yang berjudul *Guideline on the Care and Use of Fish in Research, Teaching and Testing* yang menyebutkan bahwa: pedoman tersebut hanya digunakan untuk hewan uji berupa ikan (Kelas: Chondrichthyes, Agnatha, dan Osteichthyes) dan Avertebrata (Kelas: Cephalopoda) (hal 13,14 ; terlampir).

Sedangkan dalam penelitian tersebut menggunakan kerang (*Pinna* dan *Atrina vexillum*) sebagai hewan penelitian. Sehingga penelitian tersebut tidak perlu dilakukan ***Uji Ethical Clearence.***

Demikian Surat Keterangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar** atas nama Dr. Endang Dewi Masithah, Ir., MP.

Surabaya, 27 April 2023

Mengetahui, Dekan FPK Unair	Wakil Dekan III FPK Unair
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Guidelines for the Use of Fishes in Research

Use of Fishes in Research Committee members:
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American Fisheries Society
Bethesda, Maryland
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Studies of early life stages may require very large numbers of individuals. In all cases, studies should be designed to use the fewest animals necessary to reliably answer the questions posed. The use of adequate numbers to establish variance and to ensure reliability is essential so as to prevent needless repetition of the study (ASIH et al. 1987, 1988). A true "replicate" is the smallest experimental unit to which a treatment can be applied independently. Pseudoreplication can result from wrongly treating multiple samples from one experimental unit as multiple experimental units or from using experimental units that are not statistically independent (Heffner et al. 1996). Statistical power analysis can improve designs of experiments (Peterman 1990). Conducting statistical power analyses ensures the development of study designs that have the appropriate statistical power to accomplish research objectives.

2.4 Mortality as an Experimental Endpoint

In laboratory studies, experimental endpoints, other than death of the experimental subjects, should be developed unless mortality is required by the study protocol. The use of mortality as an endpoint is appropriate when one or both of the following criteria are met: (1) Little or no information pertaining to research objectives is available on the species of interest or the experimental variable being imposed (e.g., short-term, limited mortality studies may be used to develop experimental limits for subsequent sublethal studies), and (2) mortality data are required, or at least preferred, by a sponsoring agency to provide a basis for criteria development as part of a regulatory process. Studies that require mortality endpoints include, but are not limited to, those concerning the effects of pathogens and parasites, toxicological research, and physiological tolerance.

2.5 Fish Health Management: Control of Pathogens and Parasites

In laboratory studies involving fishes, healthy subjects are prerequisites for reliable data (Jenkins 2011a), unless an infectious disease is part of the experimental protocol. Fish used in research must be free of any notable microbial presence that could indicate a diseased condition. Fish free from infectious fish pathogens generally will be satisfactory; however, an unrecognized disease condition, even at chronic or nonlethal levels, can seriously confound research results (Lawrence et al. 2012). The source of fish used in research will, in general, influence their health status. Fish raised in captivity have a level of health oversight that will not occur in wild-caught fish. When inquiring about the health status of fish at a culture facility, the researcher can request specific information including any available fish health inspection reports. When fish are brought into a laboratory setting from the wild, the researcher should expect that microorganisms are present. If no disease symptoms are apparent, this is no guarantee that these wild-caught fish are free from problematic disease organisms. Once those fish are in a laboratory setting, the culture conditions and associated stressors will be very different from those in the natural environment, whereby an active disease event can develop. Many laboratories will administer formalin baths to newly arrived fish during an acclimation period (see section 7.3 Acclimation to Laboratory Conditions). The goal is to eliminate external protozoa and monogeneans from the

4. Animal Welfare Considerations

4.1 General Considerations

Research involving living animals, including fishes, must be based on experimental designs and animal care practices that can lead to scientifically valid results. Fishes are acutely sensitive to stress (e.g., Barton and Iwama 1991), and responses may include changes in behavior (e.g., Martins et al. 2012), reduced growth, changes in osmotic status, suppressed immune systems (with consequent disease onset), and altered reproductive capacity (Iwama et al. 2006; Schreck et al. 2001; Schreck 2010). Accordingly, unless the experimental objectives require actions or conditions designed to test responses to stress, fishes should be maintained, handled, and tested under conditions that will not create such responses. The Guidelines addresses the conduct of scientific research and focuses on established facts and the processes through which knowledge is developed. Research plans submitted to IACUCs should address animal care considerations, in addition to the details of research goals, objectives, and procedures. The extent to which IACUCs incorporate personal values concerning animal welfare into their institutional guidelines is determined within each institution.

4.2 Stress

The study of stress has focused on how animals have evolved physiological and behavioral mechanisms to address the challenges of changing environmental conditions and then to permit them to maintain homeostasis, or self-sustaining balance. The set of environmental variables (conditions) best suited for the well-being of each species typically encompasses a specific range for each factor and species (see section 5.7 Facilities for Temporary Holding and Maintenance), as stress responses are species-specific (Schreck 2010). Accordingly, when fishes are maintained within these ranges, a state of homeostatic balance is expected. Deviations from homeostasis characterize a stress response. While many definitions for stress have been proposed, we employ the definition of Schreck (2000) and Schreck et al. (2001): "a physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of insult." When stressed, fish generally attempt to reestablish homeostasis via a process known as "allostasis regulation in which they adjust their physiological function to re-establish a dynamic balance" (Sterling and Eyer 1988). While allostasis is generally adaptive because it helps keep animals alive in the face of a short-term stressor(s), it can be maladaptive over the long term and have negative consequences on growth, reproduction, and immunological health (Schreck 2010). Accordingly, investigators need to understand those factors that might cause stress in their experimental animal(s), the potential consequences, and how stress might be avoided by optimizing experimental conditions.

7. Laboratory Activities

7.1 General Principles

Working with live fishes under laboratory conditions requires attention to many details concerning the requirements for, and limits of tolerance of, the particular species under study. Acceptable physical facilities and an adequate supply of water with good quality must be provided, even if the fishes are to be held for only short periods of time. Although fish may tolerate marginal facilities and conditions for a few hours or even several days, holding them under less than optimal conditions will affect the results of the research. Standards for humane treatment of animals must also be maintained, regardless of the length of time that the fishes are held.

The reader should note that some content of section 7 is not restricted to laboratory activities, but may be applicable to field situations, as well.

7.2 Confinement, Isolation, and Quarantine

Prior to bringing fishes into a laboratory, facilities and plans should be in place to ensure that the fish cannot escape, especially species not native to the watershed, and that the introduced fishes can be isolated physically from fishes already present. Each holding unit should have its own set of nets and other equipment. Facilities and equipment used for previous studies should be disinfected prior to use in new studies, typically with a chlorinated disinfectant or another disinfectant such as Virkon® Aquatic (www.wchemical.com/). If the introduced fishes may carry disease agents, especially pathogens or parasites that are not endemic to the area, quarantine-level facilities should be used. The level of quarantine required will vary with the seriousness of the known or suspected disease agent (see section 2.5 Fish Health Management: Control of Pathogens and Parasites).

Individual fish with suspected ill health should be quarantined from the others so as to negate the potential for spread of potential disease agents. Such fish should be evaluated by an individual with expertise in fish diseases (fish pathologist or veterinarian), and the proper therapeutant should be applied as directed. Providing guidance for the treatment of specific diseases is beyond the scope of this document. The investigator is strongly urged to establish a working relationship with individuals with expertise in fish health with whom they may consult.

Experimentation with nonindigenous fishes, transgenic fishes, or other genetically modified fishes is a special situation that requires additional precautions to preclude their escape. Permitting with site visits by state wildlife agencies may be required for holding nonindigenous species (see section 3.4 Permits and Certificates). The specific barriers may be similar to those used to prevent the escape of disease agents but must be developed to fit the physical characteristics of the laboratory or experimental facility. The USDA has developed

Canadian Council on Animal Care

guidelines on:

the care and use of fish in research, teaching and testing

This document, the CCAC *guidelines on: the care and use of fish in research, teaching and testing*, has been developed by the *ad hoc* subcommittee on fish of the Canadian Council on Animal Care (CCAC) Guidelines Committee.

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the care and use of fish in research, teaching and testing

A. PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer review agency responsible for setting and maintaining standards for the care and use of animals used in research, teaching and testing throughout Canada. In addition to the *Guide to the Care and Use of Experimental Animals*, vol. 1, 2nd ed., 1993 and vol. 2, 1984, which provide the general principles for the care and use of animals, the CCAC also publishes detailed guidelines on issues of current and emerging concerns. The CCAC *guidelines on: the care and use of fish in research, teaching and testing* is the seventh of this series. This document supersedes Chapter I - Fish, *Guide to the Care and Use of Experimental Animals*, vol. 2 (CCAC, 1984).

These guidelines aim to provide information for investigators, animal care committees, facility managers and animal care staff that will assist in improving both the care given to fishes and the manner in which experimental procedures are carried out.

The present document has drawn substantially from the work of organizations listed in Appendix A. Their contributions to the development of these guidelines are gratefully acknowledged.

The guidelines have been developed by the CCAC subcommittee on fish and were reviewed by a total of 69 experts. A preliminary first draft was agreed on by the subcommittee and circulated to experts in June 2002 (including representatives of the organizations listed in Appendix A), and a second draft was circulated for widespread comment in June 2003. A final review was carried out in August 2004 involving all individuals who had previously provided significant input to the development process. The development of these guidelines also involved consultation with the Canadian Association for Laboratory Animal Science (CALAS) and the Canadian Society of Zoologists (CSZ) through workshops held at annual meetings in Québec City (June 2003), Acadia University (May 2004), and Hamilton (June 2004). Consultations were also held at the Aquaculture Association of Canada and AquaNet annual meetings in Québec City (October 2004), and at the CCAC Workshop on the Fish Guidelines in Vancouver (April 2005).

The guidelines have been organized in a format that should facilitate easy access to relevant sections. Early sections provide an ethical overview relevant to the use of fishes in research, teaching and testing. This is followed by a brief overview of regulations and responsibilities relevant to the care and use of fishes in science in Canada. The remainder of the document provides guidelines to assist in caring for fishes in laboratory facilities, followed by guidelines to help in the development and review of experimental protocols. An overview of the CCAC *guidelines on: the care and use of fish in research, teaching and testing* is provided through a summary of the guidelines listed in this document prior to the beginning of the main text.

The refinement of animal care and use guidelines is a continuous process. These guidelines are intended to provide assistance in the implementation of best practices, and should not be viewed as regulations. Where regulatory requirements are involved or where it is absolutely imperative to adhere to a particular guideline, the term *must* has been used.

B. INTRODUCTION

The greatest challenge in providing *guidelines on: the care and use of fish* is the wide variety of fishes used in Canada and the diversity of their habits, behavior, life history, and environmental and husbandry requirements. In addition, the scientific information required to define the preferred conditions for fish well-being is limited. While considerable research has been conducted on culture strategies and environmental and water quality requirements, such studies have generally been aimed at determining conditions that optimize production in aquaculture systems, rather than improving the welfare of fishes, and have not usually addressed the difference between *tolerance* and *preference* (Fisher, 2000).

An important consideration in these guidelines is the naturally high mortality rates of juveniles in species whose ecological strategies include the generation of large numbers of progeny to ensure adequate survival in the wild. In addition, many experimental populations of species with usually high survival contain individuals that will not thrive to adulthood even under the best environmental conditions. In some situations, a population-based (or a group of study fish) approach to well-being may be appropriate, but individuals that are not likely to thrive should be euthanized as soon as they are identified.

Another consideration for these guidelines is the general acceptance by the public of the current killing methods used in harvesting wild fishes or in recreational angling. In general, the public appears to be willing to accept these killing methods for food production but not when fishes are used for research. These guidelines accept that for research, teaching, and testing use of any animal, including fishes, more emphasis will be placed on individual well-being than is generally accepted for the commercial harvesting or production of animals for food. It is recognized, however, that in some instances investigators may obtain fishes from people involved in commercial or recreational harvesting and have little influence over the capture methods.

These guidelines apply to fishes held in facilities for research, teaching and testing, as well as to fishes that are studied in their natural habitats.

1. Definition of Fish

For the purpose of these guidelines, fishes are defined as all bony and cartilaginous fish genera (classes Chondrichthyes [cartilaginous fishes], Agnatha, and Osteichthyes [bony fishes]). Fish eggs, embryos or larvae that have not developed beyond exclusive reliance on their own yolk nutrients are not covered by these guidelines. Similarly, invertebrates (except cephalopods) are not covered under the CCAC system of surveillance, but institutions are encouraged to foster respect for these animals by ensuring that holding facilities and levels of husbandry meet standards equivalent to those used for fishes.

2. Rationale for Guidelines on the Care and Use of Fish

The use of fishes as experimental subjects has increased substantially over the past two decades. This increase in use is a result of the rapid development of the aquaculture industry, requirements for testing involving fishes as indicators of environmental change, and the use of fishes as a replacement for mammals in biomedical, pharmacological and genetic research (DeTolla *et al.*, 1995; Fabacher & Little, 2000). The trend toward the use of fishes as a replacement for studies that would previously have used mammals as experimental subjects is not discouraged. However, it must also be recognized that fishes have the capacity to perceive noxious stimuli. Noxious stimuli are those stimuli that are damaging or potentially damaging to normal tissue (e.g., mechanical pressure, extremes of temperature and corrosive chemicals). Whether or not fishes have the capacity to experience any of the adverse states usually associated with pain in mammals is subject to a great deal of debate in the scientific literature (FAWC, 1996; FSBI, 2002; Rose, 2002; Braithwaite & Huntingford, 2004). Nonetheless, fishes are capable of behavioral, physiological and hormonal responses to stressors (including noxious stimuli) which can be detrimental to their well-being. These CCAC guidelines both support the leadership role that Canadians play in fish research, and ensure that the welfare of fishes is carefully considered during the use of fishes for research, teaching and testing, recognizing that better welfare will result in better science.

3. Ethical Overview

Guideline 1:

Fishes used in research, teaching and testing must be treated with the respect accorded to other vertebrate species.

The CCAC's surveillance system for animals used in research, teaching and testing is based on the principles of humane science, i.e. the Three Rs of Russell and Burch (Russell & Burch, 1959) - Reduction, Replacement and Refinement. For the CCAC, these principles are laid out in its *policy statement on: ethics of animal investigation* (CCAC, 1989). The *ethics of animal investigation* applies to all species covered by the CCAC system, i.e. all vertebrates and cephalopods.

In addition, the CCAC system takes a "moral stewardship" approach to the use of animals in science as explained in the CCAC Experimental Animal User Training Core Topics - Module 2, Ethics in Animal Experimentation (http://www.ccac.ca/en/CCAC_Programs/ETCC/Module02/toc.html).
The first guideline statement in the CCAC *guidelines on: institutional animal user training* (CCAC, 1999a) states, "Institutions must strive through their training programs to sustain an institutional culture of respect for animal life".

3.1 Principles of the Three Rs

According to the CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989), it is the responsibility of the local animal care committee (ACC) to ensure that fishes are used only if the investigator's best efforts to find a non-animal model have failed.

As for any other species covered by the CCAC system, investigators using fishes are required to use the most humane methods on the smallest number of animals necessary to obtain valid information. This requires the use of a sound research strategy, including: identification of key experiments that determine whether a particular line of enquiry is worth pursuing; use of pilot studies; staging of *in vitro* to *in vivo* experiments where possible; and implementation of staged increase in test stimuli where possible (Balls *et al.*, 1995). The numbers and species of animals required depend on the questions to be explored. Field studies, aquaculture studies and laboratory studies require different statistical designs; field studies and aquaculture production typically require the use of larger numbers of animals. The life stage of the fishes used in each study will also affect the numbers of animals needed. Studies of early life stages typically require large numbers of individuals. In all cases, studies should be designed to use the fewest animals necessary. Heffner *et al.* (1996) and Festing *et al.* (2002) provide discussions on the appropriate treatment of samples and experimental units. Investigators are encouraged to consult with a statistician to develop study designs that have the appropriate statistical power to accomplish the research objectives (Nickum *et al.*, 2004).

The CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989) also requires adherence to the following principles:

- animals must be maintained in a manner that provides for their optimal health and well-being, consistent with the demands imposed by the experimental protocol;
- animals must not be subjected to pain and/or distress that is avoidable and that is not required by the nature of the relevent protocol;
- expert opinion must attest to the potential value of studies with all animals, including fishes (e.g., scientific merit for research, see CCAC *policy statement on: the importance of independent scientific merit of animal based research projects* [CCAC, 2000a]; pedagogical value for teaching; and the appropriateness of the method to provide data for testing according to current regulatory requirements);
- if pain or distress is a justified component of

Zavodnik, D. (1967). Contribution to the ecology of *Pinna nobilis* L. (Mollusca: Bivalvia) in the northern Adriatic. *Thalassia Jugosl, 3*, 93-102.

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