Proceedings of Basic and Applied Sciences
ISSN 1857-8179 (paper). ISSN 1857-8187 (online). http://www.anglisticum.mk
Proceedings of the 1st International Conference on New Horizons in Basic and Applied Science, Hurghada – Egypt, Vol 1(1), 2013.

Bacterial Diseases Affecting the Cultured Sepia Officinalis Leading to Increase Mortality Rates in The Laboratory

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Received November 13, 2013; Revised November 14, 2013; Accepted November 15, 2013

Abstract: *The early growth, mortality rates and bacterial infections of the cultured Sepia Officinalis were experimentally studied in the laboratory. Two hundred eighty five sepia larvae were hatched and placed in a 100 liter capacity rectangular glass aquarium (filled with seawater) in the laboratory. The Sepia individuals (285 individuals) were divided into two groups the first fed on a mixture of amphipods, rotifers and artemia and the second group fed only on amphipods to follow their growth and mortality. The second group was observed to grow faster with length 6.76 ± 0.06mm and weight 0.11 ± 0.01gm than the first one. The survival rate was 100% by the end of the first week and decreased gradually by the end of the second week. The recorded mortality rate reached 49% by the day 15th, where they infected with bacterial disease of Vibrio alginolyticus. The clinical signs of the diseased S. Officinalis were lethargic condition, food fasting and multiple skin ulcers with white-gray discoloration were observed and appeared on the body. The main post mortem lesions were congestion of the internal organ, beside the presence of ascetic fluid. The mortality among the diseased Sepia was increased by age; however it may causes death of most individuals by increasing time more than two weeks. The findings of antibiotic sensitivity test cleared that the isolated V. alginolyticus was sensitive to amoxiclav (amoxicillin-clavulanate), streptomycin, ciprofloxacin and chlormphinicol. Controversially, it was resistant to oxytetracycline, tobramycin, gentamycine and enrofloxacin.*

Keywords: *Sepia Officinalis - growth rate - mortality rate - bacterial infections*

Introduction

Sepia officinalis is one of the most easily cultured cephalopods, and has been cultured in aquaria since the late 1960s (Richard, 1971; Pascual, 1978; Boletzky and Hanlon, 1983; Forsythe et al., 1994; Lee et al., 1998; Domingues et al., 2001a, 2001b, 2002, 2003). However, adults of Artemia have been used as food for the cultured cuttlefish with poor results on growth and survival (Domingues *et al*., 2001b). Pinczon du Sel et al. (2000) illustrated that, amphipods are the main prey for *S. officinalis* during the first 3 months. However, caprellid amphipods are small crustaceans that inhabit littoral zones on erect hydrozoans, bryozoans, macroalgae and seagrass (Guerra-García and Tierno de Figueroa, 2009). The life-history traits of 214 gammaridean species were reviewed by Sainte-Marie (1991), who stated that, life-history patterns of gammarid amphipods are influenced by latitude, depth and salinity. Where, gammarids, and amphipods show short life cycles (Cunha *et al.*, 2000). They are considered as an important natural dietary component in a variety of coastal marine finfish, and also can be cultured in controlled conditions (Prato *et al.*, 2006; Aravind *et al***.,** 2007). Samuel and Patterson (2011) illustrated that, the salinity is a major factor controlling the growth rate of cuttlefish regarding that, its optimum degree is ranged between 30-34 ppt. However, they showed a successful growth rates when fed on brine shrimp (*Artemia parthenogenetica*) which was the preferred prey for the first 10 days of culture.

Para-larva fed with mysids had better growth rates from 10th day onwards. Higher survival rate was noted (78%) for Para-larva fed with mysids.

Vibrio is one of the most important bacterial pathogens which cause economic losses of marine fishes. This bacterium is normally found in marine water and the disease occurs when fish is exposed to this bacterial pathogen in the existence of stress factors (Austin and Austin, 2007). Earlier reports reveal that *Vibrio harveyi* and *V. alginolyticus* were the predominant isolates from coral reef fish and shell fish at Ruesri Bay (Thongchankaew et al., 2011). *V. alginolyticus* had categorized as one of the seven vibrio fish pathogens (Austin and Austin, 1987) and are frequently isolated from outbreaks and mortalities in many marine fish species including Carpet Shell Clam (*Ruditapes decussatus*) Larvae in Spain **(**Gomez-Leon et al., 2005)**,** gilthead sea bream (*Sparas aurata*) (Akaayli et al., 2008), silver sea bream (*Sparas aurata*) in Hong Kong, Gilt head sea bream (*Sparas aurata*) in Spain, cultured black sea bream (*Mylio macrocephalus*) fry in Japan (Austin and Austin, 2007) and sturgeon (*Acipenser baerii*) in Siberia (Costinar et al., 2010).

The infection with *V. alginolyticus* showed lethargy, food fasting, external hemorrhages, loss of skin coloration, depressed abdomen and the mortalities reached more than 50% among the infected fish. In post mortem examination, hemorrhagic liver and ascetic fluid in the intestine was detected (Alcaide et al., 2001; Toranzo et al., 2005 and Martins et al., 2010). *V. alginolyticus* is a gram-negative rod or slightly curved rods and has been recognized as an opportunistic pathogen in humans as well as marine animals (Zhao et al., 2010). The present study is aimed to evaluate bacterial diseases which infect the cultured cuttlefish (*Sepia officinalis*) during the first two weeks of hatching with reference to the used food alternative under the controlled conditions in the laboratory aquaria. Also it is aimed to determine if it was pathogenic or non-pathogenic bacteria.

Materials and Methods

Laboratory examination *of Sepia_Officinalis*

Two hundred eighty five sepia larvae were hatched and placed in a 100 liter capacity rectangular glass aquarium (filled with seawater) in the laboratory of the National Institute of Oceanography and Fisheries, Alexandria branch, Egypt during January 2013. The eggs of *S. Officinalis* were collected from Abu Qir area on the Mediterranean Sea and put in the glass aquarium to follow hatching process and their growth rates (Samuel and Patterson, 2011 & Baeza-Rojano *et al.*, 2010). Continuous flow of well aerated sea water was used. Water temperature (°C), salinity, dissolved oxygen and pH were measured daily inside the tanks using multi-parameter survivor 4. The water temperature was controlled in the tanks using heaters and the water temperature ranged between 20 and 21°C. The above mentioned parameters are listed in Table (1). In addition, total ammonia was determined and the dissolved oxygen (DO) concentration was measured using a digital dissolved oxygen meter (HI 9142 - Hanna instruments Inc., USA). *Sepia* individuals were divided into two groups the first group (group 1) fed on a mixture of amphipods, rotifers and artemia (with equal ratios). The second group (group 2) fed only on amphipods however; the measurements were calculated for the length (using a vernier caliper of 0.01 mm accuracy) and weight (using sensitive balance) of each individual. Survival and mortality rates of *S. Officinalis* were also counted and isolated to determine and identify the bacterial diseases and infections (Chen et al., 1995).

Table (1): Some oceanographic parameter of seawater inside the used aquarium.

Physical parameter	Group 1	Group 2	Control seawater
Temperature (°C)	20.8	20.6	23
DO	4.4	4.7	5.8
pH	9.1	9	7.7
Salinity (‰)	38	38	38
conductivity	108	92	-
NH4-N (mg/l)	0.178	0.103	0.0003

Early larval stages were photographed with a camera linked to a binocular stereoscopic microscope and their dimensions were measured in micron using micrometer while the other late stages dimensions were measured in millimeters using a vernier caliper of 0.01 mm accuracy, standard deviations (SD) are given in parentheses (Baeza-Rojano *et al.*, 2010). The young larvae *S. Officinalis* were hatched after period of time lasts 35 days in the laboratory under the controlled conditions and feeding processes lasts 16 days with the mentioned food types.

Bacterial Examination

Moribund and clinically diseased *S. Officinalis* were collected from the indoor aquarium and subjected to clinical and post mortem examination according to Amlaker (1970) and Austin and Austin (1987). Water samples were taken in dark brown clean and dry bottles from the indoor aquarium and the seawater (as control sample). Samples for bacterial isolation were taken from ulcers, liver and kidney of moribund and clinically diseased *S. Officinalis* and cultured on plates of tryptic soya agar and Huso-Shotts medium. The two media were prepared with 50% sea water (Chen et al., 1995). The inoculated plates were incubated at 25°C for up to 72 hrs.

Phenotypic characterization

The bacterial colonies were isolated, purified using thiosulphate citrate bile salt sucrose agar (TCBS, Difco) and characterized using phenotypic and biochemical tests as reported by Martins et al., 2010. Commercial miniaturized API 20E galleries (BioMerieux) were also used according to the manufacturer's instructions, but sterile sea water was used as a diluent and 25°C as incubation temperature. The isolates were identified according to diagnostic schemes described by Costinar et al., 2010.

Antibiotic sensitivity test

Drug susceptibility of the isolates was determined by using Kirby-Bauer Disk Diffusion Susceptibility test method (Jorgensen and Turnidge, 2007) and the following antibiotics streptomycin, tobramycin, chlormphenicol, oxytetracycline, amoxiclav, gentamycine, ciprofloxacin and enrofloxacine (from Oxoid Co.) were also used to treat and prevent such bacterial infections.

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Pathogenicity assays

One hundred *S. Officinalis* were acclimated for one week in the indoor aquarium and subdivided into two equal groups each of 50 individuals. The first group was challenged by bath immersion in 3L glass aquarium containing $1.5x10^6$ cells of *V. alginolyticus* ml for 15 minutes (Martins et al., 2010). The second group were submitted to the same procedure without bacteria and used as control. Each group was reared in 50L glass aquarium at water temperature 27 ± 2 °C and observed for 14 days; both clinical signs and numbers of dead individuals were recorded during the observation time.

Results and Discussion

Physic-chemical Oceanographic parameters inside the used aquarium *of Sepia_Officinalis* was controlled inside the aquarium were pH which ranged between 9 and 9.1; salinity which measured 38 ‰ and dissolved oxygen which was ranged between 4.4 and 4.7; while the temperature was controlled between 20.6 °C and 20.8 °C. The total conductivity was ranged between 92 and 108 and the ammonia was ranged between 0.103-0.178 mg/l (Table 1). On the other hand the physical parameters of the seawater were listed in the same table as a control or reference parameters.

Growth and Mortality rates of *Sepia Officinalis* during two week after egg hatching

After one week of hatching and extensive feeding the length was increased to an average ranged between 6.2 ± 0.04mm and 6.3 ± 0.05mm with a total weight calculated from 0.05 ± 0.01gm to 0.06 ± 0.01gm (Table 2). These values were increased by the end of the second week to reach an average from 6.55 ± 0.05mm to 6.76 ± 0.06mm and their weights were ranged between 0.09 ± 0.01gm and 0.11 ± 0.01gm (Figure 1 and 2). During the first week there was no mortality and the survival rate was 100%. Whenever, during the second week, about 140 individuals were dead representing a mortality of 49% and the survival rate reached 51% (145 individuals). However, most of the dead individuals of *Sepia Officinalis* were found floating on the water surface. Some bacterial infections were observed on the dead individuals and isolated to be identified.

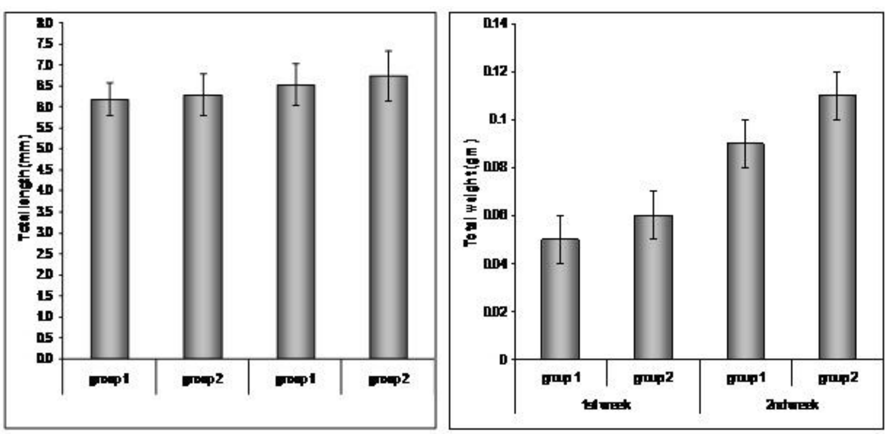

Figure 1. Average length growth and weight of the hatched eggs of *Sepia Officinalis*

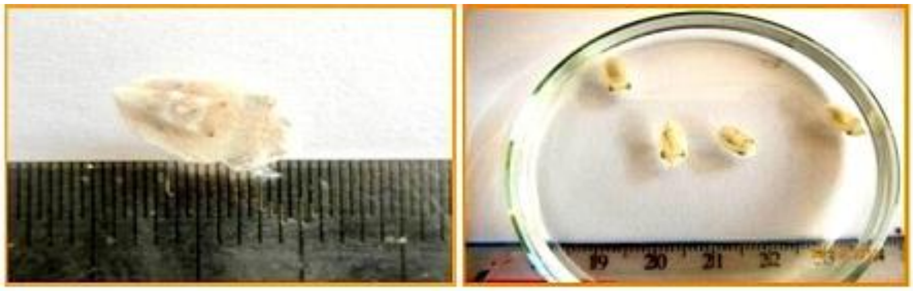

Figure 2. The general length and size of the yong individuals of *Sepia Officinalis* after two weeks

Amphipods (gammarids, caprellids and others) are among the most adaptable species in the world (Woods, 2009). Due to their opportunistic feeding, fast growth and reproductive cycles, their culture would be considerably less expensive compared to mysid culture, which requires a constant supply of *Artemia nauplii* for their culture (Domingues et al., 1998). However, the nutritional value of amphipods is relatively high containing high levels of beneficial polyunsaturated fatty acids, particularly DHA (22:6n−3) and EPA (20:5n−3) (Woods, 2009), which are important for feeding of the cultured *Sepia Officinalis* leading to fast growth and increasing their productivity. Consequently, there are some factors affect the cuttlefish rearing and growth, such as, salinity, dissolved oxygen and the food type (as the caparace hardness of the used food) which may make it difficult for cuttlefish hatchlings to manipulate and digest these prey but the gammarids seem to be good candidates as an alternative diet for *S. officinalis* hatchlings (Domingues et al., 2003). Contrary, the used amphipods (*Cymadusa filosa*) in the present study as a diet illustrated an obvious increase in length and weight with relatively higher values than the used mixture diet (mixture of amphipods, rotifers and artemia). Results obtained from the present study indicate that amphipod (*Cymadusa filosa*) is another good alternative food and prey for the cuttlefish hatchlings during the first weeks of the life cycle. However, the obtained growth rates (0.966-0.06 $mm.d^{-1}$) and weight increase (see table 2 and figure 1) in the second group were considerably high compared to the obtained from the first group (0.936 $mm.d^{-1}$), which have the same oceanographic conditions, but generally lower than the obtained by Baeza-Rojano *et al.* (2010). Whenever, the present study confirms that amphipods seem to be a valid prey for cuttlefish. There are few reports on non-living and/or non-marine food items being fed to the cephalopods in captivity, but these alternative foods were usually added as supplements to the normal diet with only qualitative observations on acceptance and growth (DeRusha et al, 1989).

Table 2. Total length and weight as a growth form of *Sepia Officinalis*

Periods	Daily growth (mm/day)	Total length (mm/week)	Total weight (gm)
1st week	0.886-0.900	6.2 ± 0.04 - 6.3 ± 0.05	0.05 ± 0.01 - 0.06 ± 0.01
2nd week	0.936-0.966	6.55 ± 0.05 - 6.76 ± 0.06	0.09 ± 0.01 - 0.11 ± 0.01

The survival rate is low (51%) by the end of the second week (Table 3), this may be due to the increase of the ammonia values in the water tank as well as the lower dissolved oxygen which may help to infect the young individuals with *V. alginolyticus* bacteria which cause increasing mortality rate. Ammonia increase may cause cuttlefish irritation, leading to the shoot backwards by jet propulsion and can eject ink shortly after hatching (Samuel and Patterson, 2011).

Table 3. Survival and mortality rates of *Sepia Officinalis* inside the aquarium

Period	Living Ind.	Survival %	Dead Ind.	Mortality %
1st week	285	100	0	0
2nd week	145	51	140	49

Ind.=individuals

There are few reports on the growth rate of the cuttlefish *Sepia Officinalis* and their mortality. Survival individuals were poor with different food type than amphipods, however, DeRusha, et al. (1989) found that, the survival was poor with crabs and no qualified growth data have been published so far. During the present study, the maximum growth rate reached 6.76 ± 0.06 mm and weighed 0.11 ± 0.01 g by the end of the second week; these results were relatively low when compared with Nabhitabhata and Nilaphat (1999) who found the growth increase to 7.7 mm and 0.18 g during 12 days at maximum. The present data was considered higher than Samuel and Patterson (2011) who studied another species (*Sepia pharaonis*) and measured only 6 mm and weighed 0.0985 g. The small size of the animals in the present study may be due to the difference in the nutritional value of the feed provided. However, the increase of ammonia inside the aquaria is the main reason for increasing mortality rate and the bacterial infections, which led to whiteness of the dorsal parts due to the infection by *V. alginolyticus*.

Clinical signs

The clinical signs of the diseased *Sepia Officinalis* were lethargic condition, food fasting and multiple skin ulcers with white-gray discoloration were observed and appeared on the body (Figure 3a and b) causing skin depigmentation. The main post mortem lesions were congestion of the internal organ, beside the presence of ascetic fluid. The mortality among the diseased *Sepia* was increased by age; however it causes death of all individuals by increasing time more than two weeks. The clinical signs of *V. alginolyticus* infection in *Sepia Officinalis* were lethargic condition, food fasting and multiple skin ulcers with white gray discoloration of the body. These results were in agreement with the findings of Toranzo et al. (2005) and Martins et al. (2010). The onset of the disease may be resulted from the suppression of the immune system due to overcrowdness, increased ammonia and pH values and decreased dissolved oxygen inside the indoor aquarium. Bullock *et al*. (1986) and Suomalainen *et al*. (2005) reported that the sharp increase in the ammonia level, pH, physical contact and the high decrease in the dissolved oxygen are the most possible triggering factors for initiation, establishment and spread of infection in addition to jeopardize animal immune system.

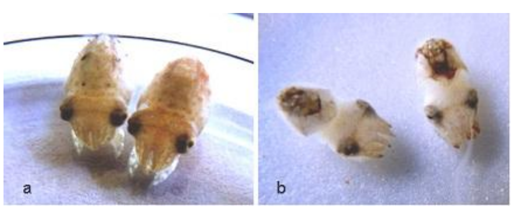

Figure 3. The general shape of *Sepia Officinalis* before (a) and after (b) bacterial infections.

Isolation and characterization of the bacterial strains

Ten bacterial strains were isolated from ulcer, internal organ and water of the aquarium. These colonies of suspected *V. alginolyticus* were rounded, with regular edges, creamy in colour and in some cases adhered strongly to the culture media. The biochemical and physiological characteristics of the isolated bacteria were similar and allowed the presumed identification of the bacteria as *V. alginolyticus*. In fact, all strains were Gram-negative, motile curved rods, cytochrome oxidase and catalase positive. No growth was observed in the absence of NaCl and grown in 8% NaCl. With regard to API20E galleries, H_2S and gelatinase tests gave negative and positive results respectively (Table 4).

Table 4. Biochemical characterization of the isolated bacteria *vibrio alginolyticus*.

Tests	Result	Tests	Result
Colony shape	Round	Colony colour	Creamy
Gram stain	-ve rods	Motility	+ve
Cytochrome oxidase	+ve	Catalase	+ve
Growth in 0% NaCl	-	Growth in 3% NaCl	+
5% NaCl	+	.. .. 8% NaCl	+
API20E			
ONPG	-	GEL	+
ADH	-	Glucose	+
LDC	+	Manitol	+
ODC	+	Inositol	-
CIT	+	Sorbitol	+
H2S	-	Rhaminose	-
URE	-	Sucrose	+
TDA	+	Rafffinose	+
IND	+	Salcine	-
VP	v	Arabinose	-
Lactose	+	Adonitol	+
Malonate	-	Xylose	+

ODC = ornithine decarboxylase, LDC = lysine decarboxylase, ADH = arginine dihydrolase, IND = indole, CIT = citrate, URE = urea hydrolysis, VP = Voges-Proskauer, TDA = tryptophane deaminase, GEL = gelatin hydrolysis, ONPG = Ortho-nitrophenyl b-d-galactopyranoside, H2S = hydrogen sulfide production, V = variable.

The findings of antibiotic sensitivity test cleared that the isolated *V. alginolyticus* was sensitive to amoxiclav (amoxicillin-clavulanate), streptomycin, ciprofloxacin and chlormphinicol. Controversially, it was resistant to oxytetracycline, tobramycin, gentamycine and enrofloxacin (Table 5). The isolated bacteria from naturally infected *Sepia Officinalis* were identified as *V. alginolyticus* due the colony characters, cell morphology, gram stain, biochemical reactions and the API 20E galleries.

Table 5. The antibiotic sensitivity tests of *v. alginolyticus*

Antibiotics	Result
Ciprofloxacin	S
Amoxiclav	S
chlormphenicol	S
Streptomycin	S
Oxytetracycline	R
Tobramycine	R
Gentamycine	R
enrofloxacin	R

S = sensitive R = Resistant

However, these findings were in agreement with that of Martins et al. (2010). Regarding to the antibiotic sensitivity test of *V. alginolyticus*, the isolated strain was sensitive to both of amoxiclav, streptomycin, ciprofloxacin and chlormphinicol (Costinar et al., 2010).

Pathogenicity assays

The experimentally infected (50 individuals) *S. Officinalis* showed lesions similar to those of naturally infected individuals including lethargic condition, food fasting and multiple skin ulcers with skin depigmentation. However, the observed postmortum lesion was congestions of the visceral organs. By the end of observation time (14 days) the mortality of the experimentally infected fish reached 100%. *V. alginolyticus* re-isolated in pure culture from the experimentally infected *S. Officinalis*. The pathogenecity assay showed that. *V. alginolyticus* was pathogenic to *S. Officinalis*. However, the moribund and dead individuals showed clinical signs similar to the signs of those naturally infected individuals including lethargy, off food, skin depigmentation, multiple skin ulcers and visceral congestion in addition to 100% mortality for the experimentally infected individuals. The pathogenicity of *V. alginolyticus* may be related to the extra-cellular toxins which have undesirable effects on the physiological states of the infected individuals, in addition their cytotoxic effects (Sinderman, 1990 and Birkbeck and Gallacher, 1993).

Conclusions

It was concluded that, amphipods (*Cymadusa filosa*) are one of the most adaptable species that can be used as food for the cultured squid (*Sepia Officinalis*) due to its high nutritional value (containing useful polyunsaturated fatty acids), leading to increasing growth.

Salinity, dissolved oxygen, ammonia concentration, food type and the bacterial infections are factors which may affect the cuttlefish rearing and growth. The maximum growth rate reached 6.76 ± 0.06 mm and weighed 0.11 ± 0.01 g by the end of the second week.

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The survival rate is relatively low (51%) by the end of the second week, may be due to the increase of the ammonia values in the water as well as the lower dissolved oxygen. In addition, the infection with *V. alginolyticus* bacteria which may causes increase mortality.

The clinical signs of *V. alginolyticus* infection in *Sepia Officinalis* were lethargic condition, leading to food fasting and multiple skin ulcers with white gray discoloration of the body.

The isolated bacteria *V. alginolyticus* was pathogenic to *S. Officinalis*. This bacterium was sensitive to both of amoxiclav, streptomycin, ciprofloxacin and chlormphinicol which can be used for their treatment.

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