Effects of Methylene Blue and/or Clove Oil on Nile Tilapia Immune Response and Water Bacterial Load Through Transportation

Nemetallah[1], B. R., E. A. Omer[2], T. M. Srour[2], M. R. Latif[2]
[1]Physiology Dept., Fac. of Veterinary Medicine, Edfina, Alex. Univ.
[2]Animal and Fish Production Dept., Fac. of Agric., Saba Basha, Alex. Univ.

ABSTRACT

The effect of methylene blue (MB), clove oil (CO) and the combination of them on immune response of Nile tilapia (*Oreochromis niloticus*) and the total water bacterial count of the transport water were studied. Fish of mean weight of 20 ±5 g/fish were placed in 30 L polyethylene bags filled with 10 L of water at a density of 25 fish/bag (50g/L). Plain transport water served as control group, meanwhile, the other treatments of transport water were treated with 3 mg/L, 40 g/L and 3 mg/L + 40 g/L of MB, CO and MB + CO, respectively in duplicate groups. Transportation was through paved roads for 3 hours. Mortalities were recorded three hours after arrival. Results indicated that the lowest value of plasma cortisol level was recorded by the fish group in which their transport water was treated with MB followed by MB + CO group then the control and finally CO group with significant differences among each other. Glucose level also was significantly lowest in the blood of fish transported in water treated with MB and it was significantly the highest in CO group. Differences among the groups in relation to packed cell volume (PVC) and mortality percent were insignificant (P>0.05). After 3 hours of transport, the control group recorded a significantly ($P < 0.05$) higher water bacterial count than in other test groups as well as before transport with insignificant differences among them. The results suggested that the use of methylene blue at 3 mg/L in transport water may reduce stress and total bacterial count in transport water and increase the survival percent of fish during transportation.

Key words: transport water, methylene blue, clove oil, cortisol, glocuse, Nile tilapia

INTRODUCTION

Modern aquaculture practices frequently expose fish to a variety of acute stressors. Stress was defined as "the nonspecific response of the body to any demand made upon it" (Selye, 1973). Additionally, the major response to stress refers to aspects of whole-animal performance such as changes in growth, condition, overall resistance to disease, metabolic scope for activity, behavior, and ultimately survival (Wedemeyer and McLeay, 1981; Wedemeyer *et al.*, 1990).

Fish (brood stock, fingerlings and fry) transport is one of these stressors (Barton, 1997; Barton, 2000a). Fish are transported for a number of reasons for husbandry purposes including collection and movement of broodstock, movement of hatchery fish to potential release sites (stocking

and supplementation). There is no doubt that the transport of fish can result in intensive stress that have the potential to negatively effect on fish performance, survival and also energy household (Chandroo *et al.*, 2004). Fish survival in a good state of health during transport is influenced by a number of factors, or combination of factors: quality of fish, oxygen, pH, carbon dioxide and ammonia, temperature, density and activity of transported fish. One method commonly used to minimize or mitigate the effects of stress on fish is the use of anaesthetics (McFarland, 1959; Berka, 1986). Anaesthetics are used to aid in the handling of fish during practices that include enumeration, pathological analyses, hormonal implants or injections, vaccinations, stripping, transfer, and hauling (Carmichael and Tomasso, 1988; Brown, 1993). Notably, the higher stocking density during transport may be considered the main reason for stress. In addition, during transport, stress may also be caused by low levels of oxygen or poor water quality due to inadequate water exchange that causes accumulation of excreted carbon dioxide and ammonia (Erikson *et al.*, 1997).

In fisheries and aquaculture, there are many instances requiring some form of sedation or anesthesia, in order to facilitate handling of fish without injuring or stressing them excessively (Summerfelt and Smith, 1990; Ross and Ross, 1999). Clove oil has become a popular fish anaesthetic to overcome the stress caused by aquaculture procedures such as fish handling and transport (Steven *et al.*, 2004). Clove oil is extracted from buds, leaves and stems of clove tree (*Eugenia aromatica*; the active compound is eugenol) and it has been traditionally used as topical anaesthetic for toothaches, headaches and joint pain (Soto and Burhanuddin, 1995).

On the other hand, a constraint on disease control is the relatively limited number of therapeutic agents available for the control of bacterial disease. Fish transport is often the final step in any hatchery operation. There is a need to produce healthy tilapia fry or fingerlings harvested from hatcheries to be delivered to grow-out farms. The ultimate goal of this transport operation is to provide fish in good condition that will survive after stocking in grow-out ponds. Hatchery owners use methylene blue to prevent the proliferation of bacteria during fish transport. Experience and research have shown that fish transport can be improved by the addition of certain chemicals such as methylene blue (Fajardo, 2002). This is to prevent the possible spread and outbreak of the disease in the new environment where the fingerlings will be delivered.

Methylene blue raises the oxygen consumption of cells. This means that the hydrogen to be oxidized is passed on to the oxygen. Thus, while disinfection results from this, methylene blue also acts as an inhibitor of

bacteria and fungi, and is well tolerated by most fish species (Schaperclaus, 1992). The therapeutic action of methylene blue on bacteria and other parasites is probably due to its binding effect with cytoplasmic structures within the cell and also its interference with oxidation-reduction processes.

Therefore, the present study aimed to determine the effects of methylene blue and/or clove oil on Nile tilapia (*Oreochromis niloticus*) immune response and water bacterial load through transportation.

MATERIALS AND METHODS

The Nile tilapia, *Oreochromis niloticus*, used in the present study (initial weight 20±5g,) were caught from earth ponds in Abides Station Research Center, University of Alexandria, Egypt.

The methods of fish transport in sealed bags were previously described by Pecha *et al.*, 1983.

Two water additives were tested, methylene blue (MB) and clove oil (CO), in the transport water .Four treatments were used as follows: Treatment 1 (control- no chemicals added), Treatment 2 (3 mg/L of MB), Treatment 3 (40 g/L of CO) and Treatment 4 (3 mg/L MB + 40 g/L CO).

Fish were placed in 30 l polyethylene bags with 10 Lof water at a density of 25 fish per bag (50g/L). Bags were then inflated with oxygen immediately after mixing the chemicals in the water, tied with rubber strings and packed in Styrofoam boxes befor butting fish. Each treatment was replicated two times. Transportation was through paved roads for 3h.

Methylene blue was added at a concentration of 3 mg/L according to Subasinghe (1992). Pure CO (obtained from a local pharmacy) was first dissolved in 95% ethanol (to which no additives added) at 1:10 ratio according to Cho and Heath (2000) then added to water to a final concentration of 40 g/L. (Pirhonen and Schreck, 2003).

The behavior of fish in transport bags during fish transportation and three hours after unloading in aquaria was monitored carefully in the terms of swimming speed, respiration and body balance in the water.

Mortality % was recorded after the end of the transportation period and 3 hours after putting in aquaria.

Designated bags of each treatment were opened after 3 hours for water quality monitoring and collection of water samples for bacterial counting. Water sampling was done by getting 100 ml of water using a 25 ml pipette before transport and from the polyethylene bags immediately after bags were opened.

Serial dilution of bacterial suspensions taken from the water samples was up to 10^{-7} in sterile distilled water. A volume of 0.01 ml from suspension of 10^{-5}, 10^{-6} and 10^{-7} dilutions were placed in Petri plates containing Tryticasein Soy Agar. The cover of the Petri pleats was marked into three divisions corresponding to the designated serial dilution. Each Petri plate was replicated three times. The Petri pleats were incubated in an inverted position at 37°C for 24 h. The average count of colonies from the designated of each of the replicates was taken as mean. The number of colony forming units per ml (cfu/ml) of bacterial suspension was computed by adopting the formula:

For 5^{th} dilution = mean × 100 × 10^5
For 6^{th} dilution = mean × 100 × 10^6
For 7^{th} dilution = mean × 100 × 10^7

Where:

Mean = average count of the designated dilution in the three Petri plates.

100 = its reciprocal value correspond to the volume of bacterial suspension plated in one designated division (that is 1/100 of total volume of bacterial suspension from each serially diluted sampled).

On arrival to the lab. (3 hours transport), 5 fish were sampled from each bag for blood withdrawal. Vacutainer tubes (Becton-Dickinson, Rutherford, NJ, USA) with heparin as anticoagulant were used to collect blood from the caudal artery and vein. After thorough mixing, a haematocrit tube was filled for later evaluation. The blood samples were kept on ice until brought back to the laboratory.

Haematocrit of each fish was determined in duplicate using the micro haematocrit method (Brown, 1988). Plasma was separated by centrifugation (3000 × g, 10 min) and stored frozen for later analysis of cortisol by radioimmunoassay (Redding *et al.*, 1984). For serum, blood samples were withdrawn from caudal veins without anticoagulant and left in Eppendorf tubes for 1 hour at room temperature then centrifuged to separate serum.

Cortisol was measured with an enzyme-linked immunoassay method (ELISA, kit 55050, Human, Germany) using a microplate reader, intra-assay coefficient of variation was 8.4 %; the analytic sensitivity of the assay was 1.1ng/dl. When serum volume from some fish was insufficient, fish samples from the same bags were pooled to obtain 25 µL of serum for the assay. Blood glucose was measured according to Gomes *et al.* (2005) using the digital advantage blood glucose system (Roche, Germany).

As forth histology, tissue samples from gills for all treatment groups were collected after transportation. Representative samples of fish were

fixed in 10% formalin and processed for preparation of histological sections using hemotoxylin and eosin stain.

All data were statistically analyzed with ANOVA using SAS package for the IBM-PC (SAS User's Guide, 1988). Duncan's multiple range tests were used to resolve the differences among treatment means (Steel and Torrie, 1980). The results are presented as means±standard error and the differences between treatment means were considered significant at $P < 0.05$.

RESULTS

Plasma cortisol levels in different groups are shown in (Table 1). Addition of clove oil (CO) to transport water significantly elevated cortisol level in transported fish. Addition of MB to transport water significantly ($P < 0.05$) reduced plasma cortisol level as compared to levels in control fish and group exposed to CO. Combined addition of MB and CO to transport water resulted in plasma cortisol level significantly ($P < 0.05$) lower than those of control group but higher than values of fish exposed to MB alone.

The mean glucose level in control fish group exposed to transport stress for 3 hours was 78.9 mg/dl (Table 1). Addition of CO to transport water significantly ($P < 0.05$) increased glucose level above that of control group. Addition of MB to transport water significantly ($P < 0.05$) reduced glucose level in transported fish. Combined addition of MB and CO resulted in plasma glucose level which was not significantly ($P > 0.05$) different from that of control fish

Table (2) depicted the PCV values in fish exposed to transport stress for 3 hours in different groups. Basel PCV in control group was 26.75%. Addition of CO, MB or combined addition of MB and CO to transport water did not affect PCV values in treated fish.

After 3 hours of transport no mortality was observed in different treated groups or in the control group. At 6 hours after exposure to transport the mortality (%) was 6% in control group. Mortality percentage was reduced to 2% in fish group exposed to transport water containing MB. Clove oil increased mortality percentage to 8%. Combined use of MB and CO resulted in 4% mortality (Table 3). However, the differences among treatments were insignificant ($P > 0.05$).

Bacterial count in transport water was around 5×10^5 cfu/ml before fish transport (Table 4). The control group (without treatments) recorded significantly ($P < 0.05$) higher bacterial count in transport water compared to that recorded in transport water before transport or other treatments after transport. Otherwise, insignificant ($P > 0.05$) differences were found among

the groups of before transport, MB, CO and MB + CO. Numerically, the transport water treated with MB achieved the lowest bacterial count compared to other treatments. Combined use of MB and CO acted nearly similar to CO.

According to observation on fish that transported in plastic bags it was detected that freely fish swimming transported in bags water indicating that O_2 level in bags was quite enough and the water quality was suitable. Fish in plastic bags containing CO added to its water settle to the bottom of the bag and showed sluggish movement indicating sedation, many of them lost equilibrium and tried to swim upward to the water surface. After exposure to 3 hours of transport, fish in the CO group were affected since most of them were gulping air on the water surface with minimal fin movements.

Table (1). Plasma cortisol and glucose levels of Nile tilapia (*O. niloticus*) exposed to transport stress for 3 hours.

Treatment	Cortisol level (ng/dl)	Glucose level (mg/dl)
Control	77.85±2.00^{c}	78.90±1.00^{b}
Methylene blue (MB)	62.10±0.88^{a}	62.50±0.23^{a}
Clove oil (CO)	91.90±1.26^{d}	89.60±0.72^{c}
CO + MB	72.15±0.82^{b}	80.20±0.71^{b}

Means ± standard errors with the same superscripts in the column are not significantly different ($P < 0.05$).

Table (2). Packed cell volume (PCV) of Nile tilapia (*O. niloticus*) exposed to transport stress for 3 hours.

Treatment	PCV %
Control	26.75±2.61^{a}
Methylene blue (MB)	26.30±0.37^{a}
Clove oil (CO)	33.94±2.91^{a}
CO+ MB	29.29±0.71^{a}

Means ± standard errors with the same superscripts in the column are not significantly different ($P < 0.05$).

Table (3). Mortality % in Nile tilapia fish (*O. niloticus*) 6 hours after exposure to transport stress after 6 hours.

Treatment	Mortality (%)
Control	6.0±2.00[a]
Methylene blue (MB)	2.0±2.00[a]
Clove oil (CO)	8.0±4.00[a]
CO+ MB	4.0±0.00[a]

Means ± standard errors with the same superscripts in the column are not significantly different ($P < 0.05$).

Table (4). Water bacterial count (cfu/ml) before and 3 hours after transport of Nile tilapia (*O. niloticus*).

Treatment	Bacterial count (cfu/ml)
Before transport	$(5 \times 10^5) \pm 014142.14^b$
Control (without treatments)	$(2 \times 10^7) \pm 707106.78^a$
Methylene blue (MB)	$(2 \times 10^5) \pm 021213.20^b$
Clove oil (CO)	$(8 \times 10^5) \pm 212132.03^b$
CO+ MB	$(9 \times 10^5) \pm 070710.68^b$

Means ± standard errors with the same superscripts in the column are not significantly different ($P < 0.05$).

Fish in bags containing MB and CO added to its water were much less affected and were similar in their behavior to fish in control drug free plastic bags.

Fig. (1) Shows histological section in a gill filament of *O. niloticus* fish before transport. The secondary lamellae are long, filled with blood cells and tapering towards the free ends.

Gills of fish exposed to transport stress without addition of MB or CO showed some morphological changes as bulging of secondary lamellae ends, and lymphocytic aggregations at their bases indicating the response of fish gills to stress (Fig. 2).

Fig. (3) Shows histological section in gills of fish exposed to transport in water containing MB. Secondary lamellae appear elongated with one layer of blood cells. The lamellae are not engorged with blood cells and the

lymphocytic aggregation at the base of the secondary lamellae is much less than in those of fish exposed to CO added to transport water.

Exposure of fish to transport water containing CO resulted in histological changes in the gills. The secondary lamellae appeared much shorter with bulged ends there was engorgement of blood cells in the secondary lamellae (Figs. 4 and 5). Lymphocytic infiltration appeared at the base of the lamellae in the form of blue plaques.

Combined addition of CO and MB resulted in distention of the primary lamellae which appeared filled with blood cells; some secondary lamellae appeared filled with basophilic cells at their base with degenerative changes in the secondary lamellae attached to the base of the primary lamellae while some of the secondary lamellae had swollen ends (Fig. 6).

Fig. (1): Histological section: gill of Nile tilapia, *O. niloticus* fish before transport. Showing normal morphology of primary and secondary lamellae. (H and E stain, x 250).

Fig. (2): Histological section: gill of Nile tilapia, *O. niloticus* fish after transport without addition of methylene blue (3 mg/L) or clove oil. (H and E stain, x 250). Note: 1) Lymphocytic aggregation at S.L bases. 2: Bulging of S.L ends.

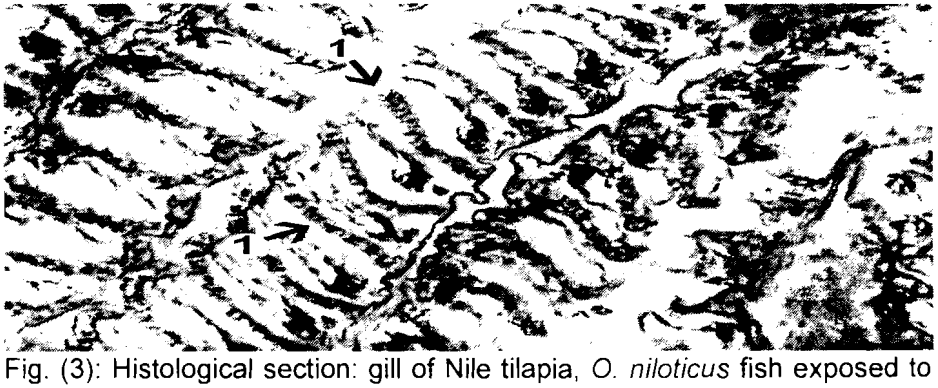

Fig. (3): Histological section: gill of Nile tilapia, *O. niloticus* fish exposed to transport water containing methylene blue (3 mg/L). (H and E stain, x 250). Note: 1) S.L appeared with one layer of blood cells.

Fig. (4): Histological section: gill of Nile tilapia, *O. niloticus* fish exposed to transport water containing clove oil (40g/L). (H and E stain, x 250).
Note: 1) Secondary lamellae appeared engorged with blood cells.

Fig. (5): Histological section: gill of Nile tilapia, *O. niloticus* fish exposed to transport water containing clove oil (40g/L). (H and E stain, x 250).
Note: 1) Secondary lamellae (S.L) appeared with bulged ends.
2) S.L much Shorter.

Fig. (6): Histological section: gill of Nile tilapia, *O. niloticus* fish exposure to transport water containing combined of methylene blue (3 mg/L) and clove oil (40 g/L). (H and E stain, x 250).
Note: 1) distention of primary lamellae.
2) S.L had swollen ends.

DISCUSSION

In the present study the cortisol levels of fish exposed to transport stress was around (77.85 ng/dl) which is higher than cortisol levels of unstressed tilapia (<10 ng/dl) Foo and Lam (1993).

Failure to suppress stress induced via activation of the hypothalamo pituitary interrenal axis results in a release of cortisol which in turn causes various physiological responses, the purpose of which is to help the fish overcome or compensate for the stress. Severe or chronic stress is often associated with poor performance and has long been associated with immunosuppression in cultured fish (Pickering and Duston, 1983; Thomas and Lewis, 1987; Maule *et al.,* 1989).

Addition of CO (40 g/L) to water used in fish transport significantly increased plasma cortisol levels. This result contradicts previous reports which indicated that CO suppressed plasma cortisol levels in channel catfish (*Ictlurus puncatatus*) (Small, 2003). Wagner *et al.,* (2003) have suggested that low concentration of CO may facilitate fish transport. Therefore, CO may be more appropriate for use in commercial aquaculture situations, where anesthetics may be used in large quantities by unskilled laborers and released in natural water bodies. Furthermore, the longer recovery time exhibited by fish anesthetized with CO (Anderson *et al.,* 1997; Munday and Wilson, 1997; Prince and Powell, 2000; Sladky *et al.,* 2001) may be an additional advantage in activities such as morphological evaluations, acquisition of tissue biopsies and strip-spawning, where long

handling periods outside the water are involved (Rodri´guez-Gutierrez and Esquivel-Herrera, 1995). In the last several years, CO has been recognized as an effective anaesthetic for sedating fish for a number of invasive and noninvasive fisheries management and research procedures (Soto and Burhanuddin, 1995; Anderson *et al.*, 1997; Keene *et al.*, 1998; Prince and Powell, 2000; Srivastava *et al.*, 2003).

The elevated levels of cortisol due to addition of CO coincide with the increase in mortality rate of transported fish observed in the present study. Thomas and Robertson (1991) suggest that an "adequate" corticosteroid stress response may be essential for recovery from severe or prolonged stressors, as cited by Ledingham and Watt (1983) who reported increased post-surgery mortality in animals anesthetized with metomidate. If fish are too heavily sedated, loose equilibrium, and cease swimming, they may die from suffocation if they all settle to the bottom, or experience mechanical injury from hitting the tank walls (Cooke and Bunt, 2004). Wagner *et al.* (2003) recommended that low concentrations of CO may assist fish transport, but at present there is only one preliminary study that actually examines low levels of CC Cooke *et al* (2000) evaluated the response of adult rainbow trout transported using four CO concentrations by activity radio telemetry. Clove oil showed promise for this purpose, but most of the concentrations tested resulted in total or partial loss of equilibrium (Cooke and Bunt, 2004).

Circulating levels of cortisol is commonly used as indicator of the degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Ruane *et al.* (1999) and Barton (2000b) both showed that brown trout (*Salmo trutta*) exhibited greater cortisol increases after brief handling and short-term confinement, respectively, than did rainbow trout (*Oncorhynchus mykiss*). This difference was also consistent with glucose responses between these two species. Similarly, both McDonald *et al* (1993) and Barton (2000a) found that lake trout (*Sal velinus namaycush*) cortisol increased after transportation in plastic bag and return to basal levels after 24 h (Gomes *et al* , 2003). On the other hand, some temperate fishes such as salmonids species present a significant increase in cortisol levels after 2 h transportation and maintain their cortisol elevated for more than 48 h (Barton, 2000a) When stress disturbs the ability of a fish to osmoregulate, the ionic composition of its blood comes to resemble that of the surrounding medium (Pickering, 1993).

Elevated glucose levels in fish exposed to CO in transport water coincide with higher levels of cortisol in that fish group. Increased hepatic glycogenolysis during stress leading to depletion of liver glycogen levels and hyperglycemia was reported by Paxton *et al.* (1984) and Vijayan *et al*

(1990). Stress also stimulates catecholamines release which stimulates glycogenolysis in the liver (Fabbri *et al.*, 1998; Mommsen *et al.*, 1999). Histology examination of gill of CO treated group shown destructive changes. Total bacteria count in water exposed to CO in transport water was significantly reduced. Hassanein and Desheesh (1998) reported that CO has in vitro and in vivo bactericidal effect on *E. amylovora*. Anaesthetizing fish prior to transport (Carmichael *et al.*, 1984) can reduce metabolic rate and hence oxygen demand, reduces general activity, increase ease of handling, and mitigate the stress response.

In the present study the group of fish transported in water treated with MB (3 mg/L) achieved the lowest mortality percent. This result is in agreement with previous experienced research which has shown that fish transport can be improved by addition of MB (Fajardo, 2002). Methylene blue is known as a disinfecting agent and is also excellent agent against methemoglobin intoxication (Schaperclaus, 1992). Methylene blue treated group was significantly reduced total bacterial count than that in control post transport with insignificant differences with other treatments. According to Van Duijn (1973), MB may be used for the treatment of IchthyoPhthiriosis (white spot disease), skin and gill flukes, velvet disease, *Costiasis*, coral fish disease, *Chilodonelliasis* and as palliative medicine in all cases of disease of the gills, where fishes suffer from difficulty in breathing.

According to Schaperclaus (1992) MB acts as an inhibitor of bacteria and fungi and is well tolerated by most fish species. Additionally, bath treatment with MB is often used to control Psedodactylogyrus in tank-reared eel culture in China. According to Anderson (1992), MB can be used for the treatment of integumentary mycosis for all ages of freshwater fishes in ponds and raceways at an application rate of 2 to 2.5 mg/L in permanent bath .At a concentration of 2-3 ppm, it can be used as an indefinite bath for fish at all ages (Subasinghe, 1992) . At 1-2 ppm, it can be used for the treatment of *Icthyopthirius multifilis* (Tonguthai and Chanatchakool, 1992).

In the present work cortisol levels in fish transported in water containing MB was lowest among the treated group indicating reduced effect of transport stress on fish in this group. This result is supported by the reduction in glucose levels in the same MB treated group and the least destructive changes in the gills. According to Alapide-Tendencia and de la Peña (2001), the control of disease is particularly difficult because fish are often farmed in system where production is dependent on natural environmental conditions. Changes and deterioration in the aquatic environment cause most of the bacterial disease encountered and

environmental effects give rise to many other adverse culture conditions. A constraint on disease control is the relatively limited number of therapeutic agents available for the control of bacterial disease.

Addition of MB to transport water containing CO ameliorated the drastic effects of CO on transported fish as indicated by lowered cortisol levels and reduced histopatholgical changes in gill histology. This effect expressed itself in decreasing the mortality rate in that group, although the data in the present study greatly recommends MB in fish transport yet very few studies have been published in this topic and it deserve further investigation.

CONCLUSION

Methylene blue in a dose of 3 mg/L could be used in transport fish water to reduce fish stress, mortality and water bacterial count through Nile tilapia transportation.

REFERENCES

Alapide-Tendencia, E.V. and L.D. de la Peña. 2001. Bacterial Diseases. In: Liao-Po, G. D., Lavilla, C. R and Cruz-Lacierda, E. R. (eds.). Health Management in Aquacult. Aquacult. Dep., Southeast Asian Fisher. Dev Cen Tigbauan. Iloilo, Philippines.

Anderson, I.G. 1992. The use of chemotherapeutic agents in finfish and shellfish culture in Australia. pp. 493-504. In: Sheriff, M., Subasinghe, R. P., and Arthur, J.R. (eds.). Diseases in Asian Aquacult. I. Fish Health Section, Asian Fishe. Soci., Manila, Philippines.

Anderson, W.G., R.S. McKinley and M. Colavecchia. 1997. The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. North Am. J. Fish. Manage., 17: 301–307.

Barton, B.A. 1997. Stress in Finfish: past, present and future a historical perspective. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Society for Experimental Biol. Seminar Series. Cambridge University Press, Cambridge, England,62: 1–33.

Barton, B.A. 2000a. Stress In: Stickney, R.R. (Ed.), Encyclopedia of Aquaculture, pp. 892–898. John Wiley and Sons, New York, USA.

Barton, B.A. 2000b. Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. N. Am. J. Aquacult., 62: 12–18.

Barton, B.A. and G.K. Iwama. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis., 1: 3–26.

Berka, R. 1986. The transport of live fish: a review. FAO Report. EIFAC Technical Paper 48. United Nations, Rome, 52 pp.

Brown, L.A. 1988. Anesthesia in fish. The Veterinary Clinic of North America, 18: 315–329.

Brown, L.A. 1993. Anesthesia and restraint. In: Stoskopf, M.K. (Ed.), Fish Medicine, pp. 79– 90. WB Saunders, Philadelphia, USA.

Carmichael, G.J. and J.R. Tomasso. 1988. Survey of fish transportation and techniques. Prog. Fish-Cult., 50: 155–159.

Carmichael, G.J., J.R. Tomasso, B.A. Simco and K.B. Davis. 1984. Characterization and alleviation of stress associated with hauling largemouth bass. Trans. Am. Fish. Soc., 113: 778–785.

Chandroo, K.P., I.J. Duncan and R.D. Moccia. 2004. Can fish suffer? Perspectives on sentience, fear and stress. Applied Anim. Behaviour Sci., 86: 225-250.

Cho, G.K. and D.D. Heath. 2000. Comparison of tricaine methane-sulphonate (MS222) and clove oil anaesthesia effects on the physiology of juvenile Chinook salmon *Oncorhynchus tshawytscha* (Walbaum). Aquacult. Res., 31: 537–546.

Cooke, S.J. and C.M. Bunt. 2004. Construction of a junction box for use with an inexpensive, commercially available underwater video camera suitable for aquatic research. North Am. J. Fish. Manage., 24: 253– 257.

Cooke, S.J., J.F. Schreer, D.H. Wahl and D.P. Philipp. 2000. Locomotory dynamics as indicators of stress in fish remote measures utilizing activity transmitters. In: Barton, B., Pottinger, T., Iwama, G., MacKinlay, D. (Eds.), Stress in Fish: New Directions, pp. 77– 86. Proceedings of the International Congress on the Biol. of Fish. University of Aberdeen, Scotland, UK.

Erikson, U., T. Sigholt and A. Seland. 1997. Handling stress and water quality during live transportation and slaughter of Atlantic salmon (*Salmo salar*). Aquacult., 149: 243– 252.

Fabbri, E., A. Capuzzo and T.W. Moon. 1998. The role of circulating catecholamines in the regulation of fish metabolism: an overview. Comparative Biochemistry and Physiology, 120C: 177–192.

Fajardo, L.J. 2002. Effect of zeolitic material on ammonia concentration during fish transport. Undergraduate Thesis. College of Fisher., Cen. Luzon State University, Science City of Muñoz, Nueva Ecija. Philippines.

Foo, J.T. and T.J. Lam. 1993. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. Aquacult., 115: 145-158.

Gomes, L.C., E.C., Chagas, R. Crescêncio, M.A. Pessoa, A.L. Silva, E.S. Carvalho, G.A. Junior, M.V. Brito, and M.S. Porto. 2005. Validation of a simple portable instrument for measurement of blood glucose in four Amazon fishes. *Journal of Aquaculture in the Tropics*, 20: 101–110.

Gomes, L.C., C.A. Araujo-Lima, R. Roubach, A.R. Chippari-Gomes and N.P. Lopes. 2003. Effect of fish density during transportation on stress and mortality of juvenile tambaqui, *Colossoma macropomum*. J. World Aquacult. Soc., 34: 76–84.

Hassanein, F.M. and M.A. Desheesh. 1998. Bactericidal activity of some natural compounds and plant oils in comparison with streptomycin against pear fire blight disease (*Erwinia amylovora*). Alex. Sci. Exch., 19: 25–37.

Keene, J.L., D.G. Noakes, R.D. Moccia and C.G. Soto. 1998. The efficacy of clove oil as an anaesthetic for rainbow trout, *Onchorhynchus mykiss* (Walbaum). Aquacult. Res., 29: 89–101.

Ledingham, I. McA. and I. Watt. 1983. Influence of sedation on mortality in critically ill multiple trauma patients. Lancet 1: 1270.

Maule, A.G., R.A. Tripp, S.L. Kaattari and C.B. Schreck. 1989. Stress alters immune function and disease resistance in Chinook salmon (*Oncorhynchus tshawytscha*). J. Endocrinol., 120: 135–142.

McDonald, D.G., M.D. Goldstein and C. Mitton. 1993. Responses of hatchery-reared brook trout, lake trout, and splake to transport stress. Trans. Am. Fish. Soc., 122: 1127–1138.

McFarland, W.N. 1959. A study of the effects of anesthetics on the behaviour and physiology of fishes. Publ. Inst. Mar. Sci., Univ. Tex. 6: 23–55.

Mommsen, T.P., M.M. Vijayan and T.W. Moon. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biol. and Fisher., 9: 211–268.

Munday, P.L. and S.K. Wilson. 1997. Comparative efficacy of clove oil and other chemicals in anaesthetization of *Pomacentrus amboinensis*, a coral reef fish. J. Fish Biol., 51: 931–938.

Paxton, R., D.H. Gist and B.L. Umminger. 1984. Serum cortisol levels in thermally-acclimated goldfish (*Carassius auratus*) and killifish (*Fundulus heteroclitus*), implications in control of hepatic glycogen metabolism. Comparative Biochemistry and Physiology, 78B: 813–816.

Pecha, O., R. Berka, J.K. Pludku and P. Vacich. 1983. Fry transport in polyethylene bags Ser.Metod.VURH Vodnany, 10: 16 p.

Pickering, A.D. 1993. Endocrine-induced pathology in stressed salmonid fish. Fish. Res., 17: 35–50.

Pickering, A.D. and J. Duston. 1983. Administration of cortisol to brown trout, *Salmo trutta* L., and its effects on the susceptibility to Saprolegnia infection and furunculosis. J. Fish Biol., 23: 163–175

Pirhonen, J. and C.B. Schreck. 2003. Effects of anaesthesia with MS-222, clove oil and CO2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). Aquacult., 220: 507–514.

Prince, A. and C. Powell. 2000. Clove oil as an anesthetic for invasive field procedures on adult rainbow trout. North Am. J. Fish. Manage., 20: 1029–1032.

Redding, J.M., C.B. Schreck, E.K. Birks and R.D. Ewing. 1984. Cortisol and its effects on plasma thyroid hormone and electrolyte concentration in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol., 56: 146– 155.

Rodri´guez-Gutierrez, M. and A. Esquivel-Herrera. 1995. Evaluation of the repeated use of xylocaine as anesthetic for the handling of breeding carp (*Cyprinus carpio*). Aquacult., 129: 431– 436.

Ross, L.G. and B. Ross. 1999. Anaesthetic and Sedative Techniques for Aquatic Animals (2nd ed.) Blackwell, London, UK.

Ruane, N.M., S.E. Wendelaar Bonga and P.H. Balm. 1999. Differences between rainbow trout and brown trout in the regulation of the pituitary-internal axis and physiological performance during confinement. Gen. Comp. Endocrinol., 113: 210–219.

SAS. 1988. SAS/STAT User's Guide, Release 6.03 Edition, SAS Institute Inc., Cary, NC, USA.

Schaperclaus, W. 1992. Fish Diseases. Volume 1. A. A. Balkeena, Rotterdam, Holland.

Selye, H. 1973. The evolution of the stress concept. Am. Sci., 61: 692–699.

Sladky, K.K., C.R. Swanson, M.K. Stoskopf, M.R. Loomis and G.A. Lewbart. 2001. Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetic in red pacu (*Piaractus brachypomus*). Am. J. Vet. Res., 62: 337– 342.

Small, B.C. 2003. Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*. Aquacult., 218: 177–185.

Soto, C.G. and Burhanuddin. 1995. Clove oil as a fish anesthetic for measuring length and weight in rabbit fish (*Siganus lineatus*). Aquacult., 136: 149–152.

Srivastava, S.K., N.S. Nagpure, B. Kushwaha and A.G. Ponniah. 2003. Efficacy of clove oil as an anesthetic agent in fishes. Ind. J. Anim. Sci., 73: 466–467.

Steel, R. G. and J.H. Torrie. 1980. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill, New York, USA.

Steven J.C., D.S. Cory, G. Kenneth, C. Ostranda, L. Bruce and H.W. David. 2004. Behavioral and physiological assessment of low concentrations of clove oil anesthetic for handling and transporting largemouth bass (*Micropterus salmoides*). Aquacult., 239: 509–529.

Subasinghe, R.P. 1992. The use of chemotherapeutic agents in aquaculture in Sri Lanka. pp. 547-554. In, Sheriff, M., Subasinghe, R. P., and Arthur, J. R. (eds.). Diseases in Asian Aquaculture I. Fish Health Section, Asian Fisher. Soci. Manila, Philippines.

Summerfelt, R.C. and L.S. Smith. 1990. Anaesthesia, surgery, and related techniques. In, Schreck, C.B., Moyle, P.B. (Eds.). Methods for Fish Biol., pp. 213– 272. American Fisher. Soci., Bethesda, Maryland, USA.

Thomas, P. and D.H. Lewis. 1987. Effect of cortisol on immunity in red drum, *Sciaenops ocellatus*. J. Fish Biol., 31A: 123– 127.

Thomas, P. and L. Robertson. 1991. Plasma cortisol and glucose stress responses of red drum (*Sciaenops ocellatus*) to handling and shallow water stressors and anesthesia with MS-222, quinaldine sulfate and metomidate. Aquacult., 96: 69–86.

Tonguthai K. and P. Chanatchakool. 1992. The use of chemotherapeutic agents in Thailand. In, Shariff M, Subasinghe RP, Arthur JR. eds. Diseases in Asian Aquaculture I. p. 555-565. Fish Health Section, Asian Fisher. Soci., Manila. Philippines.

Van Duijn, C.J. 1973. Diseases of Fishes. 3rd edition. Butterworth and Co. Ltd., London. UK.

Vijayan, M.M., J.S. Ballantyne and J.F. Leatherland. 1990. High stocking density alters the energy metabolism of brook charr., *Salvelinus* fontinalis. Aquacult., 88: 371–381.

Wagner, E., R. Arndt and B. Hilton. 2003. Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide. Aquacult., 211: 353–366.

Wedemeyer, G.A., B.A. Barton and D.J. McLeay. 1990. Stress and acclimation. In C. B. Schreck and P. B. Moyle (eds.), Methods for fish biology, pp. 451–489. American Fisher. Soci., Bethesda, Maryland, USA.

Wedemeyer, G.A. and D.J. McLeay. 1981. Methods for determining the tolerance of fishes to environmental stressors. In A. D. Pickering Stress and Fish, pp. 247–275. Academic Press, New York, USA.

Wendelaar Bonga, S.E. (1997). The stress response in fish. Physiol. Rev., 77: 591–625.

الملخص العربى

تأثير أزرق الميثيلين وزيت القرنفل أوخليط بينهما على الاستجابة المناعية لأسماك البلطى النيلى والحمل البكتيرى للمياه أثناء النقل

بهيج رياض نعمة الله[1] وإجلال على عمر[2] وطارق محمد سرور[2] ومينا لطيف رمزى[2]

١- كلية الطب البيطرى بإدفينا - جامعة الإسكندرية

٢- كلية الزراعة (سابا باشا) - جامعة الإسكندرية

تم دراسة أثر أزرق الميثيلين وزيت القرنفل أوخليط بينهما على الاستجابة المناعية لأسماك البلطى النيلى والحمل البكتيرى للمياه أثناء النقل حيث وضعت الأسماك ذات متوسط وزن ٢٠±٥جم فى أكياس بلاستيكية سعتها ٣٠ لتر حيث تم ملئها بـ ١٠ لتر فقط وبكثافة نقل ٢٥ سمكة/كيس (٥٠ جم/لتر). كانت المعاملة الأولى هى المعاملة المعيارية وقد كانت بدون أية إضافات لماء النقل أما باقى المعاملات فكانت عبارة عن إضافة ٣ أو ٤٠ أو ٣+٤٠ ملليجم/لتر من أزرق الميثيلين أوزيت القرنفل أو خليط من أزرق الميثيلين + زيت القرنفل على التوالى إلى ماء النقل فى مكررتين لكل معاملة. تمت عملية نقل الأسماك خلال طرق معبدة لمدة ٣ ساعات وتم مراقبة وتسجيل النسبة المئوية لوفيات الأسماك لمدة ثلاث ساعات بعد الوصول للمعمل. وقد أشارت النتائج إلى تسجيل المعاملة بأزرق الميثيلين أقل المعاملات فيما يتعلق بمستوى كورتيزول بلازما الدم وتلاها فى ذلك المعاملة بخليط أزرق الميثيلين مع زيت القرنفل ثم المعاملة المعيارية وأخيرا معاملة زيت القرنفل مع وجود فروق معنوية فيما بينهما. وقد كان مستوى الجلوكوز فى الدم منخفض معنويا فى الأسماك التى تمت معاملة مياه نقلها بأزرق الميثيلين

بينما كان مرتفع معنويا عند المعاملة بزيت القرنفل. ولم تكن هناك فروقا معنوية سواءا فى حجم الخلية المكتظ (PVC) أو نسبة الوفيات فيما بين المعاملات المختلفة. وقد سجلت المعاملة المعيارية ارتفاعا معنويا فيما يتعلق بالعد البكتيرى مقارنة بباقى المعاملات وأيضا العد البكتيرى قبل النقل والتى لم تكن بينها وبين بعضها فروقا جوهرية. وقد اقترحت النتائج إمكانية استخدام أزرق الميثيلين بجرعة ٣ ملليجم/لتر فى ماء نقل الأسماك للتقليل من الإجهاد والعد البكتيرى فى ماء النقل وزيادة نسبة الإعاشة خلال نقل الأسماك.