ประสิทธิศักย์ในการสงบประสาทของน้ำมันกานพลูต่อปลากัดไทย *Betta splendens* Regan,1910

นางสาววริษฐา อังศิริจินดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SEDATIVE EFFICACY OF CLOVE OIL IN SIAMESE FIGHTING FISH *Betta splendens* Regan,1910

Miss Waristha Angsirijinda

A Dissertation Submitted in Partial Fulfillment of the Requirements
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วริษฐา อังศิริจินดา :ประสิทธิศักย์ในการสงบประสาทของน้ำมันกานพลูต่อปลากัดไทย *Betta splendens* Regan,1910 (SEDATIVE EFFICACY OF CLOVE OIL IN SIAMESE FIGHTING FISH *Betta splendens* Regan,1910) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. กิ่งแก้ว วัฒนเสริมกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม รศ. ดร. กำธร ธีรคุปต์, 89 หน้า.

ปัจจุบันตลาดค้าปลาสวยงามมีความต้องการปลากัดเพิ่มสูงขึ้น แต่ยังมีปัญหาหลักในระหว่างการขนส่งคือ พฤติกรรมการก้าวร้าว ของปลากัดที่ ทำให้การขนส่งจำเป็นต้องแยกปลาแต่ละตัวบรรจุในถุงพลาสติกขนาดเล็กทำให้มูลค่าการขนส่งเพิ่มขึ้นและพบการตายระหว่างการขนส่งเพิ่มขึ้น เพื่อแก้ปัญหาดังกล่าวการศึกษานี้จึงทดลองหาความเข้มข้นของน้ำมันกานพลูที่เหมาะสมมาใช้สงบประสาท เพื่อเพิ่มอัตรารอดและความหนาแน่นปลากัดที่บรรจุในถุงเดียวกันสำหรับใช้ขนส่งภายในเวลา 48 ชั่วโมง ร่วมกับข้อมูลด้านพยาธิสภาพเนื้อเยื่อ และศึกษาการฟื้นตัวโดยประเมินค่าจากพฤติกรรมก้าวร้าวและการก่อหวอดก่อนและหลังจากการทดลองจำลองการขนส่ง ผลการศึกษาได้ค่ามัธยฐานความเป็นพิษเฉียบพลันที่ 48 ชั่วโมง มีค่าความเข้มข้น 32.8 ส่วนในล้านส่วน ส่วนความเข้มข้นที่ทำให้ปลาสงบและปลาสลบมีค่า 5 ถึง 20 และ 30 ถึง 50 ส่วนในล้านส่วนตามลำดับ ผลการศึกษาพยาธิภาพที่ระดับความเข้มข้นที่ทำให้ปลาสลบ ของเนื้อเยื่อ เหงือก ตับ ไต มีการเกิดพยาธิสภาพ ส่วนระดับความเข้มข้นที่ทำให้ปลาสงบประสาทในระดับ 10 ถึง 20 ส่วนในล้านส่วน พบการเปลี่ยนแปลงเล็กน้อย ผลการศึกษาหาความหนาแน่นที่เหมาะสม ในการขนส่งปลาชนิดนี้ในถุงปิด อยู่ที่ 15 ตัว ต่อความจุ 2 ลิตร ที่ความเข้มข้นของน้ำมันกานพลูที่ระดับ 20 ส่วนในล้านส่วน เหมาะสำหรับการขนส่ง ภายในเวลา 48 ชั่วโมง เวลาการฟื้นตัวเฉลี่ย มีค่า 10.87 ± 0.82 นาที ผลการศึกษาพฤติกรรมก้าวร้าวไม่พบความแตกต่างอย่างมีนัยสำคัญของค่าเฉลี่ยของระยะเวลาเปิดและจำนวนการเปิดฝาปิดช่องเหงือกระหว่างกลุ่มควบคุมและกลุ่มทดลอง ผลการศึกษาค่าเฉลี่ยพื้นที่และความยาวหวอด ระหว่างกลุ่มควบคุมมากกว่ากลุ่มทดลองเพียงเล็กน้อย ส่วนค่าเฉลี่ยความกว้างของหวอดไม่พบความแตกต่างอย่างมีนัยสำคัญ จากผลการทดลองสรุปได้ว่าน้ำมันกานพลูในระดับความเข้มข้นที่เหมาะสมสามารถนำมาใช้สงบประสาทปลากัด ผลการศึกษาครั้งนี้ อาจช่วยเพิ่มมูลค่าทางเศรษฐกิจต่อวงการค้าปลาสวยงามและหากมีการศึกษากับปลาสวยงามชนิดอื่นของไทยอาจนำมาซึ่งการเพิ่มมูลค่าการส่งออกในอนาคต

สาขาวิชา.....วิทยาศาสตร์ชีวภาพ..... ลายมือชื่อนิสิต .....

ปีการศึกษา...2554…….. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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WARISTHA ANGSIRIJINDA: SEDATIVE EFFICACY OF CLOVE OIL IN SIAMESE FIGHTING FISH *Betta splendens* Regan,1910. ADVISOR: ASSOC. PROF. KINGKAEW WATTANASIRMKIT, Ph.D., CO-ADVISOR: ASSOC. PROF. KUMTHORN THIRAKHUPT, Ph.D. 89 pp.

At present, the demand for the Siamese fighting fish *Beta splendens* in the ornamental fish trade has increased. However, there are many problems arise during the transportation of ornamental fish. The major problem of *B. splendens* transportation comes from the habits of this fish. In general, each fighting fish is loaded in a small plastic bag separately resulting in an increase in transportation costs. Also, there is usually high mortality during transportation. To resolve this problem, the optimum range of sedative dosages was determined in this study in order to increase post-transportation survival and loading density of *B. splendens*. Histological evidence was used to support which concentration was suitable for transportation during 48 hours. Furthermore, the recovery data was assessed on the aggressive behavior and nest building after the end of simulated transport experiment. From the results, the LC_{50} at 48 hours was 32.8 ppm. Clove oil was effective as sedative and anesthetic dosages within 48 hours at concentrations of 5 to 20 ppm and 30 to 50 ppm, respectively. The highest degeneration was found at the anesthetic stages of clove oil whereas the sedative concentrations of clove oil at 15 and 20 ppm caused little impacts in gills, liver and kidney of *B. splendens*. The result from the density experiment showed that clove oil at 20 ppm was suitable dose during 48 hours in closed bags. The optimum density for *B. splendens* transportation was 15 fish/2L. The result of recovery data showed that the mean of recovery time was 10.87 ± 0.82 min. The means of opercular display duration were not significantly different between control and treatment groups as well as the means of opercular display. There was no significant difference in means of bubble nest width between control and treatment groups. However, there were significant differences in means of bubble nest length and bubble nest area. The overall results demonstrate that this species can be successfully anesthetized using sedative doses of clove oil. The result of this study could be valuable for ornamental fish trade. Further investigations on other ornamental species in Thailand should be conducted in order to increase the fish export in the future.

Field of Study : Biological Science Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

Ornamental fish trade in Thailand is a growing industry with increasing export to all over the world. At present, this business generates large amount of income approximately 100 billion baht per year (Wiwatchaisaet, 2000). Generally, there are many problems arise during transportation, resulting in mortality of fish. To save the transportation cost, exporters attempt to increase density of fish with minimal amount of water. Therefore, this situation is concerned in term of economic benefit. In the international ornamental fish market, *Betta splendens* is very popular, and it is known by different names such as fighting fish, Siamese fighting fish and betta fish. The export of male *B. splendens* comprises approximately 10% (3.6 million fish per year) of exported ornamental fish of Thailand (Wiwatchaisaet, 2000). Normally, there are many problems associated with this fish mortality. The major problem of the transportation of *B. splendens* is its habits. Male *B. splendens* have aggressive social fighting behavior, including biting, attacking and chasing. As a result, physical damages or death of *B. splendens* regularly occur during transportation. From this reason, the exporters are unable to load them together in the same water container. Nowadays, each *B. splendens* is loaded in a small plastic bag separately, leading to an increase in transportation cost.

To solve this problem, anesthetics is commonly used to minimize stress and reduce physical damage of fish during transportation. The main purpose of anesthetic use in the ornamental fish trade is to decrease economic loss and increase product quality. In ornamental fish trade, the use of anesthetics at the optimal level should be studied because the dose of anesthetics is species specific (McFaland, 1959). The required optimum dosage of anesthetics is critical, and varies widely between species and size of fish (Coyle, Durborow and Tidwell, 2004).

Presently, many reports mention that clove oil is used as an alternative anesthetic substance in aquaculture. There has been report about sedative efficacy or toxicity of clove oil in *B. splendens*. Hence, this study is aimed to determine the optimal range of sedative and anesthetic dosages of clove oil in order to increase post-transportation survival and loading density of *B. splendens*.

A. Fish transportation

At present, fish transportation has been an essential part of aquacultural trade. In living fish transportation, it is an important step to ensure that there is no mortality resulting from transportation. For ornamental fish consignments, the industry standard for warranty is 5% death on arrival (DOA),and exporters are expected to compensate customers for losses exceeding this standard (Lim *et al*., 2003). To achieve this criteria, a variety of techniques have been developed to manage the quality of water during transportation.

Berka (1986) classified transportation systems for living fish into two types, which are the closed and the open systems. The closed system was a sealed container. Therefore, it was self-contained for the simplest requirements that were water and oxygen for fish to survive in the closed system whereas, the open system consists of water-filled containers in which the requirements for survival were supplied from outside sources such as a small tank with an aerating supply.

The closed system, which is the transportation of fish in sealed polyethylene bags with water and oxygen, has been the common procedure widespread in the world. The most important factor in live-fish transport is an adequate supply of dissolved oxygen (DO). Pure oxygen is used in closed system and DO level in the water is saturated. Therefore, the low oxygen consumption by fish is usually not a problem (Swann,1993). The shipment polyethylene bags provides advantages for the shippers because it reduces the total volume and weight of water during transportation (Cole *et al*., 1990; Swann, 1993). According to Berka (1986), the ratio of the volume of the fish transported per unit water weight ratio of 1:2 to 1:3 was recommended in adult fish.

B. Siamese fighting fish *Betta splendens*

Betta splendens is a native fish in Southeast Asia including Thailand (Smith, 1945). Nowadays, it is very popular ornamental fish worldwide. *B. splendens* are divided into two main groups which are the long finned group for the ornamental objective and the short finned group for the leisure purpose. The females of both groups have the same characters that are duller in color and have shorter fin than those of males. Only the long-finned male Siamese fighting fish is exported. However, the major problem of male *B. splendens* transportation comes from the fish habits because males of this species build bubble nest and take care of developing eggs. Therefore, they also have very aggressive social displays for territory defense. As a result, physical damages or death of *B. splendens* regularly occur during transportation. From previous reason, the exporters could not load them together in the same water container. Nowadays, each fighting fish is loaded in a small plastic bag separately leading to an increase of transportation cost. As mentioned above, a variety of methods have been used to reduce the mortality and cost during transportation. To solve this problem, anesthetics are commonly used to minimize stress and reduce the physical damage to fish during transportation. The main purpose of anesthetic use in the ornamental fish trade is to decrease the economic loss and increase product quality.

According to Harmon (2009), the main purpose of the addition of anesthetics before and during transportation, is to decrease fish metabolic rate, oxygen uptake, carbon dioxide and ammonia production. In ornamental fish trade, the use of anesthetics at a desired and optimal level should be studied because the dose of anesthetics is species specific (McFaland, 1959). The optimal dosage of anesthetics required is critical, and varies. For example, the effect of anesthetic agent at high concentration produces anesthetic stages, resulting in loss of consciousness and subsequently inducing loss of equilibrium, mobility and reflex action on an animal (Summerfelt and Smith, 1990) whereas the low concentration of anesthetic agent produces sedative stages that occur in first stage of anesthesia. Evaluation of the stages of anesthesia and recovery was assessed from criteria outlined in Hikasa *et al*. (1986), which are divided into five stages (1) partial or total loss of reaction in response to external stimuli, (2) partial loss of equilibrium, (3) loss of equilibrium but response to stimuli, (4) complete loss of equilibrium and no response to stimuli, and (5) total loss of gill movement.

Presently, there are several compounds using as an anesthetic substance in fish, such as tricaine methanesulfonate (MS-222), metomidate and benzocaine. However, those anesthetics agent have disadvantages for aquaculture operation. After exposed to MS-222, fish could not be consumed until 21 days withdrawal period (Meinertz *et al*., 1999). Therefore, alternative anesthetic substance from natural sources such as clove oil is needed

because it has several advantages, such as affordable cost, safety and efficacy for using in fish during transportation (Soto and Burhanuddin, 1995; Grush *et al*., 2004)

C. Clove oil

Clove oil is a natural substance derived from the stems, leaves and buds of clove plants *Eugenia aromaticum* which belong to family Myrtaceae (Pruthi, 1980). The first record of using clove oil as traditional medicine was in the Chinese Han period 220-206 BC. Cloves are now cultivated in many parts of tropical region (Farrell, 1990). Moreover, it is used for applications on dentistry or food flavoring classified by the US Food and Drug Administration (FDA) to be a substance that is generally regarded as safe (GRAS) (Anderson *et al*., 1997). Its active primary ingredient is eugenol (4-allyl-2-methoxyphenol), comprising of 70-90% by weight of the clove oil (Isaacs,1983; Keene *et al*.,1998; Briozzo *et al*., 1989). Clove oil contains eugenol (88.58%), eugenol acetate (5.62%), β-caryophyllene (1.39%) and other ingredients less than 0.1%, which are; 2-heptanone(0.93%), ethyl hexanoate (0.66%), humulenol (0.27%), calacorene (0.11%) and calamenene (0.1%) (Prasher *et al*., 2006 ; Chaieb *et al*., 2007).

In traditional medicine, clove oil is used to treat burns and wound. In Thailand, it has been used as a tropical anesthetic agent for toothache. It has also been widely used in dental clinics to relieve pain (Markowitz *et al*., 1992). In addition, eugenol and clove oil are

used in aromatherapy. Over 1,000,000 lb of clove leaves are imported annually to USA and approximately 80% is converted into eugenol. The soap and detergent industries are a major users at concentrations ranging from 0.05-0.1% (v/v) (Rothenstein *et al*., 1983)

As mentioned in the previous research, eugenol is a major component of clove oil. It has several beneficial properties as antibacterial, antifungal, antiviral, anti-inflammatory and analgesic agents (Keene *et al*., 1998). In addition, in nervous system, eugenol-induced effects related to depressant activity on the central nervous system, such as sedation, reduction of convulsion threshold and hypothermia has also been described (Dallmeier *et al*., 1981). Eugenol also inhibits the conduction of action potential in sciatic nerves of the bullfrog (Kozam, 1977). It has also been reported that eugenol possesses a capsaicin-like action on peripheral endings of primary afferents of the rat urinary bladder (Patacchini *et al*., 1990).

The other ingredients of clove oil is β-carophyllene (Walter, 1973), which shows anti-inflammatory activity in several animal models. This component has a local anesthetic activity compared with related compound, caryophyllene oxide (Gherlardini *et al*., 2001). This compound has significantly inhibiting gastric mucosal injuries induced by necrotizing agents, such as absolute ethanol and 0.6 N HCl. Furthermore, it has cytoprotective effects in rats (Tambe *et al*., 1996)

Previous researches showed that clove oil had an advantage as an anesthetic agent in fish (Hikasa *et al*., 1986 ; Soto and Burhanuddin, 1995; Munday and Wilson, 1997; Anderson *et al*.,1997; Keene *et al*., 1998; Prince and Powell, 2000; Cho and Heath, 2000; Woody *et al*., 2002; Walsh and Pease, 2002; Iversen *et al.,* 2003; Holloway *et al*., 2004; Grush *et al*., 2004; Iversen *et al*., 2009). Hikasa *et al.* (1986) they recommended a dosages of eugenol at 50 to 100 ppm for anesthetic condition in adult common carp (*Cyprinus carpio*). The efficacy of clove oil was also studied in coral reef fish (*Pomacentrus amboinensis)*. Clove oil was effective at lower concentrations than the other anaesthetic chemicals tested, MS-222, 2-phenoxyethanol, benzocaine and quinaldine. Therefore, clove oil may be an effective alternative to quinaldine for anaesthetizing marine fishes. (Munday and Wilson, 1997).

Soto and Burhanuddin (1995) studied the use of clove oil as tool for measuring length and weight of juvenile rabbitfish (*Siganus lineatus*). They found that at dose of 100 mg/l, clove oil had anesthetic effect in the fish at 7.5-12.3 cm total length and 7-38 g in body weight. The actual time required for loss of consciousness (TLC) was 30-45 seconds.

Anderson *et al.* (1997) studied the efficacy of MS-222 and clove oil as an anesthetic agents. Clove oil was as effective as MS-222 in inducing anesthesia in both age-groups of

juvenile and adult rainbow trout (*Onchorhynchus mykiss*). They proposed that clove oil was considered as an alternative to MS-222.

Keene *et al*. (1998) studied the effect of clove oil on juvenile rainbow trout (*Oncorhynchus mykiss*). In their experiment, the estimated 8-96 hr LC_{50} for eugenol was found to be approximately 9 ppm. The concentrations at 40 to 60 ppm of eugenol were found to induce rapid anesthesia with a relatively short time for recovery in juvenile trout.

In telemetric investigation, the concentration of clove oil used for implanting transmitters in the surgical procedures was at 30 mg/l, using with adult rainbow trout (*Oncorhynchus mykiss*) and permitting them to recover within 5 min. Mean time to achieve fourth level anesthesia was 3.7 ± 0.9 min. Average exposure time to the surgery time was 5.8 ± 0.2 min, and average recovery time, the time required to regain equilibrium and full swimming mobility was 4.9 ± 1.0 min (Prince and Powell, 2000).

According to Cho and Heath (2000), clove oil at 20 ppm and tricaine methanesulphonate at 50 ppm were investigated as a fish anesthetic agent by measuring some of the physiological stress responses, such as haematocrit, serum cortisol, serum glucose concentrations, serum lysozyme activity and differential leucocyte counts of the juvenile chinook salmon (*Oncorhynchus tshawytscha*). There were no significant differences for most of the physiological measured between the two anesthetic groups.

Woody *et al*. (2002) reported that clove oil at concentrations of 20, 50 and 80 mg/l used for anaesthetizing sockeye salmon were handled within 3 min and the fish recovered within 10 min. The concentration of 50 mg/l was recommended to apply for fish ranging in length from 400 to 550 mm at water temperatures averaging 9 to 10 °C.

Walsh and Pease (2002) reported that clove oil at concentration of 100 mg/l was effective for large river eels (*Anguilla reinhardtii*) during measuring and tagging procedures at temperatures (17 and 25 °C) and salinities from 0 to 32 g/l.

Iversen *et al*. (2003) reported that clove oil and Aqui-S™ at concentrations 10 mg/l can reduced increases in plasma cortisol compared to control in Atlantic salmon (*Salmo salar*).

Grush *et al.* (2004) reported that the anesthetic effects of eugenol at concentrations of 60 to 100 ppm used in the zebrafish (*Danio rerio*), produced rapid anesthesia with an acceptable short time for recovery compared to MS-222.

Holloway *et al*. (2004) studies that the effect of anaesthesia and euthanasia in trout with eugenol on plasma cortisol, glucose, growth hormone (GH) and two thyroid hormones (tri-iodothyronine (T3) and thyroxine (T4) was compared with tricaine methanesulfonate (MS-222). In fish sampled 10 min after anaesthetizing with 150mg/l of eugenol, cortisol levels were significantly decreased ($P < 0.03$), while there were no differences in either glucose or GH levels.

In term of induction and recovery from anesthesia, the recovery time for fish exposed to eugenol were six to ten times longer than in those exposed to similar concentration of MS-222 (Keene *et al*., 1998).

Peake (1998) found that solution of 60 mg/l of clove oil was optimal as anesthetic for use on walleye (*Stizostedion vitreum*). At this concentration, complete immobilization was 4.3 ± 0.4 min and recovery time occurred in 10.9 ±1.2 min.

Waterstrat (1999) reported that use of clove oil as an anesthetic agent for channel catfish (*Ictalurus punctatus*) fingerlings at 100 mg/l could induce anesthesia within 1 min and fish recovery time was 10-min whereas concentrations of 150 mg/l could prolong recovery time for more than 10 min.

Sladky *et al*. (2001) studied the efficacy of tricaine methanesulfonate compared with eugenol in red pacu (*Piaractus brachypomus*). Eugenol was characterized by more rapid induction, prolonged recovery and a narrow margin of safety. Additionally, Woody *et al*. (2002) reported the recovery time according to levels of concentration of clove oil. They recommended a dosage of clove oil at concentration of 50 mg/l for anaesthetizing sockeye salmon ranging in length from 400 to 550 mm and at water temperature from 9 to10 °C.

Prince and Powell (2000) found that 30 mg/l of clove oil was required for on adult rainbow trout (2.2 ± 0.1 kg BW; 4.6 ± 1.5 cm). Mean time to achieve fourth level anesthetic was 3.7 ± 0.9 min and average recovery time was 4.9 ± 1.0 min.

From previous reasons, the use of anesthetics at a desired and optimal level should be studied because the dose of anesthetics is species specific (McFaland, 1959). The optimum dosage of anesthetics required also varies widely according to the size of fish. Presently, many reports have mentioned that clove oil has been used as an alternative anesthetic substance in aquaculture. At present, there is no report on the sedative efficacy of clove oil in *B. splendens*. Hence, this study was determined the optimum range of sedative dosages of clove oil under simulated transport condition at various loading densities in order to increase post-transportation survival and loading density of *B. splendens*.

Objectives

1. To determine the range of sedative and anesthetic dosages of clove oil including stages of anesthesia in Siamese fighting fish *(Betta splendens)*.

2. To study the effects of sedative and anesthetic dosages of clove oil on gill, liver and kidney of *B. splendens* by comparing the progression of histopathological lesions within 48 hours.

3. To simulate transport based on 48 hours duration with varying density of Siamese fighting fish *B. splendens*.

4. To study the recovery of Siamese fighting fish *B. splendens* after clove oil exposure.

CHAPTER II

SEDATIVE AND ANESTHETIC DOSAGES OF CLOVE OIL INCLUDING STAGES OF ANESTHESIA IN SIAMESE FIGHTING FISH *Betta splendens*

Introduction

The clove oil has been recognized as natural medicine for long century. In traditional medicine, clove oil is used for treating burns and cuts. In dentistry, clove oil has been widely utilized as a relieve pain in dental clinics. In addition, it has been worldwide used as a food flavoring (Curtis, 1990). Presently, in the ornamental fish trade, clove oil is already used as anesthetic agent.

Previous works have characterized the anesthetic dose of clove oil for a number of salmonids, including rainbow trout *Oncorhynchus mykiss* (Anderson *et al*., 1997; Keene *et al*., 1998; Hoskonen and Pirohnen, 2004), sockeye salmon *Oncorhynchus nerka* (Woody *et al*., 2002), Atlantic salmon *Salmo salar* (Chanseau *et al*., 2002; Hoskonen and Pirhonen, 2004) and brown trout *Salmo trutta*, (Hoskonen and Pirhonen, 2004). Most studies have assessed high clove oil concentrations that result in anesthetic effect, loss of equilibrium and loss of reflex activity. However, no study investigates low concentration levels of clove oil to achieve sedation for a long period transportation.

Clove oil can be developed for transportation of *B. splendens* and is becoming commercially available product worldwide. However, the optimum dosages of anesthesia

are varied widely among the species. Thus, the use of clove oil as anesthetic agent should be concerned. Therefore, the safe margin of clove oil for *B. splendens* should be determined by estimating the median lethal concentration of clove oil (LC_{50}) for its practical application in transportation. The acute toxicity bioassay was carried out to determine the LC_{50} value of clove oil. This standard method is composed of the range-finding test and definitive test (ASTM, 1980; FAO, 1982). The range-finding test, provides dose-ranging to determine the concentrations of clove oil that will be used in definitive test. The results of dose-ranging could be clearly determined the optimum range of sedative and anesthetic dosages of clove oil for using in *B. splendens* transportation.

Materials and methods

Experimental organisms

Males, 6-month-old *Betta splendens*, were obtained from a commercial farm in Bangkok. The average weight of each fish was about 1g. Each fish was placed separately in 2-L aquarium and was acclimatized for two weeks prior to the experiment. Fish were maintained on a lighting regimen representative of the local natural environment (12L:12D) and fed with commercial fish pellets (CP Company) twice daily at approximately 3-5% body weight. Tap water was filtered through carbon-resin filters, aerated, and finally used as holding water in this experiment. Fish were fasted for 24 hours prior to the experiment.

Preparation of Clove oil

Because of the incomplete solubility of clove oil in water, it was initially dissolved in ethanol at a ratio of 1 part of clove oil to 10 parts of ethanol (Grush *et al.,* 2004). A fresh stock solution was mixed daily.

The LC50 test

A standard method for acute static toxicity bioassay (ASTM, 1980) was carried out on the fish at the age of six months. This method included the range-finding test and definitive test. A range finding test was carried out to determine the concentration range of clove oil to be used in definitive test. The expected concentrations were between the lowest concentration that caused 100% mortality and the highest concentration that caused 0% mortality. A 10-L experiment aquarium was maintained at temperature of 24± 2 ^{o}C

Five glass aquaria were filled with the test solution for five concentration levels between the highest concentration that caused 0% mortality and lowest concentration that killed all fish while another two aquaria were solvent control. Five *B. splendens* were randomly transferred to each aquarium. Three replicates were designed for each treatment and control. Fish were considered dead when there were no opercular beats observed for 15 minutes. Mortality of fish were observed and recorded every 24 hours throughout the test period of 96 hours.

A definitive test was carried out with five concentration 10, 20, 30, 40 and 50 ppm. Ten *Betta splendens* were randomly transferred to each aquarium. Three replicates were designed for each treatment and control. The total mortality was observed and recorded at 24, 48, 72 and 96 hours after exposure. The median lethal concentration value of each exposed time was calculated by Probit Analysis (Finey, 1971).

Range of sedative and anesthetic dosages

From the studies of sublethal dose, five sets of 2-L experimental aquaria were filled with five concentrations of the test material to obtain concentrations of 1, 5, 10, 15 and 20 ppm, while the two other aquaria were kept free of clove oil as solvent control. In the experiment, static non-renewal water system was used. Three replicates were designed for each treatment and control. Only one *B. splendens* was placed in an aquarium (n=10 individuals per dose) while observations of sedative or anesthetic stages were conducted. These concentrations were classified as sedative or anesthetic doses, respectively (Keene *et al*., 1998).

Evaluation of the stages of anesthesia was assessed from criteria outlined in Hikasa *et al*., (1986). Fish was observed and recorded individually throughout the stages of anesthesia. The duration for each stage was monitored from the first time of exposure to the end of each stage using a digital stopwatch. When each experiment reached stage five of

anesthesia, the fish was transferred to a 2-L recovery aquarium until it fully recovered. The fish mortality was observed for seven days after recovery (Lim *et al*., 2003).

The experiment protocol was submitted to Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Protocol Review No. 1123002.

Statistical Analyses

The statistical analyses were performed using the SPSS 17.0 for Window software. The data of induction and recovery times were expressed as means ± SE. Data were tested for normality (Kolmogorov-Smirnov test). Variations of induction time of *B. splendens* exposed to clove oil at different concentrations were tested using Kruskal-Wallis test followed by Mann-Whitney U- test for comparison at significance level ($p \leq 0.05$).

Results

The total of mortalities of *B. splendens* upon the acute exposure is shown as number of dead fish for test periods at 24, 48, 72 and 96 hours (Table 2-1). The mortality data were estimated for the median lethal concentration (LC_{50}) value using Probit Analysis.

There was no mortality observed in both control and solvent control groups. The concentration that induced 100% mortality of fish after 48 hours was observed at 46 ppm.

Especially, fish exposed to 50-ppm exhibited a 100% mortality rate with the majority of fish dying within the first hour.

Table 2-1: Accumulated number of mortality of *Betta splendens* responded to acute exposure of the clove oil

Clove oil Concentrations (ppm)	**Number of fish**	**Accumulated numbers of mortality**			
		24 hours	**48 hours**	**72 hours**	**96 hours**
0	30	0	0	0	0
10	30	0	0	0	0
20	30	0	0	0	1
30	30	5	7	7	8
40	30	27	28	28	28
50	30	30	30	30	30

Table 2-2: Effective concentrations and 95% confidence limits of *Betta splendens* from Probit analysis program

Probit	Concentration	95% Confidence Limits

	(ppm)	Lower	Upper
0.01	19.99333	13.73156	23.47187
0.02	21.49420	15.87034	24.66915
0.03	22.44645	17.22010	25.43601
0.04	23.16279	18.23085	26.01752
0.05	23.74548	19.04955	26.49399
0.06	24.24144	19.74359	26.90234
0.07	24.67630	20.34974	27.26278
0.08	25.06567	20.89036	27.58762
0.09	25.41978	21.38014	27.88495
0.10	25.74574	21.82922	28.16040
0.15	27.09531	23.66736	29.32201
0.20	28.16790	25.09748	30.27599
0.25	29.08809	26.29593	31.12289
0.30	29.91444	27.34441	31.91120
0.35	30.68019	28.28827	32.66940
0.40	31.40680	29.15595	33.41681
0.45	32.10981	29.96727	34.16810
0.50	32.80167	30.73751	34.93570
0.55	33.49354	31.47969	35.73136
0.60	34.19655	32.20616	36.56750
0.65	34.92316	32.92986	37.45888
0.70	35.68890	33.66586	38.42494
0.75	36.51526	34.43367	39.49392
0.80	37.43545	35.26181	40.71113
0.85	38.50804	36.19861	42.15843
0.90	39.85761	37.34424	44.01255
0.91	40.18357	37.61657	44.46476
0.92	40.53768	37.91078	44.95765
0.93	40.92704	38.23249	45.50140
0.94	41.36190	38.58976	46.11071
0.95	41.85786	38.99488	46.80799
0.96	42.44055	39.46800	47.63005
0.97	43.15690	40.04593	48.64437
0.98	44.10915	40.80883	49.99809
0.99	45.61002	42.00125	52.14173

Table 2-3 : Signs and stages of anesthesia in fish

Stage	Descriptor	Behavioral response
0	Normal	Reactive to external stimuli, opercular rate and muscle tone normal
1	Sedation	Partial or total loss of reaction to external stimuli, equilibrium is normal
2	Partial loss of equilibrium	Partial loss of muscle tone, erratic swimming
3	Total loss of equilibrium	Total loss of muscle tone and equilibrium
4	Anesthesia	Loss of reflex reactivity, slow opercular rate
5	Medullary collapse	Opercular movements cease; cardiac arrest follows fish death

The non-observed effect concentration (NOEC) and the lowest-observed effect concentration (LOEC) were based on the definitive test results as shown in (Table 2-2), and the maximum acceptable toxicant concentration (MATC) was estimated as a median value between NOEC and LOEC (Saragues, 1990). Therefore, the maximum acceptable toxicant

concentration was estimated at 21 ppm. This concentration might be considered to be a safety guideline for using in simulated transportation in the next experiment.

Table 2-4: Induction time to sedative stage1 or sedative stage 2 of *Betta splendens* exposed to different concentrations of clove oil during 48 hours

Clove oil Concentration (ppm)	Stages								
	Minutes			Hours					
	10	20	30	1	6	8	12	24	48
20	S1	S1	S1	S1	S1	S1/S2	S1/S2	S1/S2	S1/S2
15	S1	S1	S1	S1	S1	S1	S1	S1	S1/S2
10	n	n	S1	S1	S1	S1	S1	S1	S1
5	n	n	n	n	S1	S1	S1	S1	n

n indicates presence of stage 0 (normal) , S1 indicates presence of sedative stage 1

S2 indicates presence of sedative stage 2

The estimated LC_{50} at 48 hour of clove oil on *B. splendens* for acute toxicity test was 32.8 ppm. In addition, clove oil was effective as sedative dosage at concentrations of 5 to 20 ppm within 48 hours as shown in (Table 2-4) whereas the fish treated with concentrations of 30 to 50 ppm reached anesthetic stage with 80 to 100 % mortality. Therefore, this study recommends sedative concentrations for transport.

Table 2-5: Means ± SE of induction time to sedative stage1 and sedative stage 2 of *Betta splendens* exposed to different concentrations of clove oil

Clove oil Concentrations (ppm)	**Induction time (min)**			
	Stage 1	**N**	**Stage2**	**N**
20	12.35 ±1.15[a]	30	NR	30
15	14.65 ±1.15[a]	29	NR	30
10	35.40 ±3.56[b]	30	NR	30
5	NR	30	NR	30

NR indicates 'no response' was observed during the 60 minutes of exposure time. The means followed by different superscript in the same column are significantly different at $p \leq 0.05$ level using Mann-Whitney U- test. N indicates sample size.

Table 2-5 shows the comparison of mean induction time of *B. splendens* exposed to clove oil at different concentrations. The dose response appears to have a different mean of induction time in each concentration. Fish treated with lower dosages resulted in increasing induction time whereas the mean induction time decreased at the higher dosages of clove oil.

Table 2-6: Means ± SE of recovery time to recovery stage5 of *Betta splendens* exposed to different concentrations of clove oil

Clove oil concentrations (ppm)	**Recovery time (min)**		**7 days survival rate (%)**	
	Stage 5	**N**		**N**
20	11.33 ±1.18	24	100%	24
15	5.13 ±1.41	27	100%	27
10	P	30	100%	30

P indicates "presence of recovery stage" observed less than 1 minute of exposure time.

N indicates sample size.

At 5 ppm, fish treated with clove oil reached sedation within 8 to 48-h, and 73% recovered to normal before 48 hours. Fish exposed to 10, 15 and 20 ppm of clove oil reached sedation stage 1 within 35.40, 14.65 and 12.35 min, respectively. The means of induction times to sedative stage 1 were not significantly different between treatment groups exposed to 15 and 20 ppm at $p \leq 0.05$ whereas the means of induction times for *B. splendens* treated with 10 ppm of clove oil were significantly different among groups at $p \leq 0.05$.

Fish exposed to 15 and 20 ppm of clove oil reached recovery to normal within 5.13 and 11.33 min, respectively. The comparison of means recovery time of *B. splendens* exposed to clove oil at different concentrations was shown in Table 2-6.

The dose response appears to have a different mean of recovery time in each concentration. Fish treated with lower dosages resulted in decreasing recovery time whereas the mean induction time increased at the higher dosages of clove oil. Especially, during the 7-days of recovery time after treatment with clove oil, no mortality was found at sedative dosages of 5 to 20 ppm.

Discussion

Acute toxicity test in *B. splendens* revealed the LC_{50} at 48 hours of clove oil at 32.8 ppm. In comparison with other fish species, Keene *et al*. (1998) who studied the effect of clove oil on juvenile rainbow trout (*O. mykiss*) reported that the estimated 8-96 hr LC_{50} of eugenol was found to be approximately 9 ppm. Grush *et al*. (2004) studied the anesthetic effect of clove oil in the zebrafish (*Danio rerio*) and reported the estimated 96 hr LC_{50} of eugenol was 21 ppm. Also, the sedative effect to anesthesia was markedly different between species. From previous researches, concentrations of clove oil ranging from 5 to 9 ppm had sedative effect with rapid induction time in sub-adult of the largemouth bass. This dosage was effective for the fish species for transportation (Cooke *et al*., 2004). Keene *et al*. (1998) reported that eugenol at levels of 2 to 5 ppm were used to sedate juvenile of the

rainbow trout (*Oncorhynchus mykiss*) for 6 to 8 hours on the purpose of transportation. The observation was different from this study since *B. spendens* exposed to low doses of clove oil at levels of 2 to 4 ppm had no sedative effect throughout of the test period during 48 hours. It is possible to explain that the proper dosage required is critical and varies widely between the species (Coyle *et al*., 2004). Therefore, this was the reason for studying the efficacy of clove oil in *B. splendens*.

This study supports to other reports on dose response of this anesthetic substance. For example, fish exposed to the high doses of clove oil required a longer recovery time than those exposed to lower doses. The red Pacu *Piaractus brachypomus* exposed to 200 mg/l reached for fully recovery longer than those exposed to 100 mg/l. On the contrary, fish exposed to the high doses of clove oil required a shorter induction time. The red Pacu *P. brachypomus* were exposed to eugenol at 200 mg/l reached for induction of 186.3±124.6 seconds shorter than those exposed to eugenol at 100 mg/l (Sladky *et al*., 2001). The overall results of this experiment demonstrated that increasing doses of clove oil resulted in increase in period of recovery times whereas increasing doses of clove oil resulted in the decrease in induction times. Therefore, this study aimed to examine the response of this species to anesthetic and sedative dosages, prior to its recommendation for using in transportation propose.

Differences in the tolerance of various species to anesthetic exposure have been reported. Many fish species in previous researches could tolerate higher dosages. A dose of 40 ppm of clove oil was found to obtain complete immobilization in Rainbow trout *O. mykiss* (47.4±0.45g) at 3 minute exposed time (Pirhonen and Schreck, 2003). Rainbow trout survived at a concentration of 60 ppm (Wagner *et al*., 2002) and long-finned eel *Angulla reinhardtii* survived at a concentration of 100 ppm (Walsh and Pease, 2002). Moreover, the concentration of 50 ppm was recommended for anesthetizing the red Pacu *Piaractus brachypomus* (Sladky *et al*., 2001) and the sockeye salmon (*Oncorhynchus nerka*) ranging in length from 400 to 550 mm (Woody *et al*., 2002). On the other hand, this study showed that *B. splendens* exposed to clove oil at a concentration of 50 ppm exhibited 100% mortality within one hour.

This study agrees with previous reports of prolonged anesthetic effect of clove oil because clove oil or eugenol is coat gill structures, which could be particularly important when it persists on gill epithelia. Consequently, there is prolonged for sustained anesthetic effects (Sladky *et al*., 2001). Thus, clove oil probably affected on the gills of *B. spendens*. From previous reason, this study should be required to ensure that it does not damage or destruct the gills by exposure to clove oil at sedative doses. Furthermore, the effects on

histopathological alteration in gills of clove oil of this fish species should be investigated, including other vital organ, such as liver and kidney. These data will be valuable for early recognition of toxicity from clove oil in Thai fish. The knowledge from this study can be used to determine the suitability and safety of using clove oil in *B. spendens* transportation.

CHAPTER III

EFFECTS OF SEDATIVE AND ANESTHETIC DOSAGES OF CLOVE OIL ON HISTOPATHOLOGICAL CHANGES IN GILL, LIVER AND KIDNEY OF SIAMESE FIGHTING FISH *Betta splendens*

Introduction

Clove oil is derived from the stems, leaves and buds of *Eugenia aromaticum*. This natural substance has beneficial properties such as antibacterial, antifungal, antiviral, anti-inflammation and analgesic agents (Keene *et al*., 1998). In traditional medicine, it has also been employed for centuries as a topical analgesic in dentistry. In North America, it is widely used as herbicides for the control of weeds. Moreover, clove oil has recently been discovered as an effective fish anesthetic (Soto and Burhanuddin, 1995).

The primary active ingredient of clove oil is eugenol, which comprises of 70-80% of its by weight of clove oil (Isaacs, 1983). The toxic effects of eugenol have also been reported in several animals. According to Manabe *et al*. (1987), eugenol and related compounds produced a high affinity for plasma membrane because of their lipid solubility. Suzuki *et al*. (1985) found that eugenol induced superoxide production in neutrophils of Guinea pigs. Thompson *et al*. (1991) reported cytotoxicity effects of eugenol after exposure at concentration of 1 mM in rat hepatocytes. Sladky *et al*. (2001) reported that high concentration of eugenol induced and caused high concentration levels of carbon dioxide

and glucose in the blood resulting in circulatory failure in *Piaractus brachypomus*. The US National Toxicology Program (2000) reported that methyl eugenol, a component of clove oil, produced evidence of carcinogenicity in male mice at concentration of 3,000 ppm dietary for 103 weeks.

In general, there is a relationship between dosages and toxic effect. The fish treated with higher exposure concentrations experience more toxic effects than those treated with lower doses. However, some tolerant fish did not demonstrate toxic effect (no-appearance effect). Therefore, several previous researchers have used histological studies to detect the toxic effects of chemical exposure in animals in order to confirm the toxic effects that occur in their tissues. Presently, the impact of toxic effects on fish have also been reported by histopathological studies in several vital organs such as gills, livers and kidneys. According to Mallatt (1985), gills, which participate in many important functions in fish, represent the major sites for gas exchange and excretion of nitrogenous waste and are always in direct contact with water. Consequently, these substances can pass the thin layers of epithelium and enter the blood circulation via sinus of secondary lamellar. The liver is a target organ of toxic chemicals because it has a lot of blood supply and its function involves metabolism, biotransformation and storage of nutrients such as glycogen and lipid. In fish, the kidney performs an important function. It is a major site for clearing the blood by filtration and

excretion of metabolic products. When the liver and kidneys are damaged, there is an effect on homeostasis and the survival of the organisms

This study attempts to determine the optimum range of sedative dosages of clove oil by using histological evidence in order to support the efficacy and safety of concentrations. The concentrations which show less or no histopathological lesions, and represent as minimal toxic or no toxic dose could be used to sedate this fish species during simulated transport experiment.

Materials and Methods

Fish supply and maintenance

Males *Betta splendens* at the age of six months were obtained from a commercial farm in Bangkok. The average weight of each fish was about 1g. Each fish was placed separately in a two liter aquarium and was acclimatized for two weeks prior to the experiment. Fish were maintained on a lighting regimen representative of the local natural environment (12L:12D) and fed with commercial fish pellets (CP Company) twice daily at approximately 3-5% of their body weight. Tap water was filtered through carbon-resin filters, aerated, and finally used as holding water in this experiment. Fish were fasted for 24 hours prior to the experiment.

Preparation of clove oil stock solution

Because of the incomplete solubility of clove oil in water, it will be initially dissolved in ethanol at a ration of one part clove oil to ten parts ethanol (Grush *et al*., 2004). The maximum amount of ethanol used in any experiment is 400 ppm (0.04 %).

Experimental design

From previous results, clove oil was effective as sedative dosage at concentrations of five to 20 ppm whereas the fish treated with concentrations of 30 to 50 ppm reached anesthetic stage within 48 hours. However, at five ppm, fish treated with clove oil reached sedation within eight to 48 hours, and 73% recovered to normal before 48 hours while all of fish treated with concentration of 50 ppm reached anesthetic stage, and 100% mortality occurred within two hours. From these reasons, in this experiment the range of test concentrations was from 10 to 40 ppm.

In treatment groups, five sets of 2-L experimental aquaria were filled with five concentrations of test material to obtain concentrations of 10, 15, 20, 30 and 40 ppm, while another two aquaria were kept free of clove oil as solvent controls. Twelve *B. splendens* were separately placed in the aquarium. After exposure times of 12, 24 and 48 hours of each group, four *B. splendens* were randomly collected for histopathological study of the gills, livers and kidneys.

Histological analysis

Histopathological analyses were performed using a light microscope and Image software. Approximately 20 serial sections of the gills were produced from each fish for analysis. According to Zodrow *et al*. (2004) and Frías-Espericueta *et al*. (2009), the histopathological lesions were determined based on severity of changes compared to control sections. The degree of histological damage observed in each treatment were scored according to the percentage of the total fields with histological damage found per the total observed in the four samples of each treatment (- = no tissue damage in any field on the slides, + = mild histopathological damage presented <25% of the fields on the slides, ++ = moderate damage presented >75% of the fields on the slides; and +++ = all fields of the slides displayed severe damage compared to the control sections).

The experiment protocol was submitted to Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Protocol Review No. 1123002.

Results

Effects of clove oil on gills

Control fish

Control groups showed normal appearance. Normal gills structure consists of gill arches. Each arch compose of gill filaments which have rows of secondary lamellae. The lamella compose of epithelium with different cell types, and the epithelial cells cover both 1^0 and 2^0 lamellae. The chloride cell (CC) is identified as large epithelial cell with light cytoplasm at the base of lamellae. Pillar cells are also present and separate the capillary channels in secondary lamellae (Figure.3-1 A, B and Figure.3-2 A).

Exposed fish

Treated gill showed several lesions. Fish treated with concentrations from 10 to 20 ppm reached sedative stages within 48 hours, the most common gill changes at sedative doses were epithelial hyperplasia whereas fish treated at concentrations of 30 and 40 ppm reached anesthetic stages within 48 hours. The results of gill alterations in *B. splendens* exposed to different concentrations of clove oil within 48 hours are summarized in Table3-1.

Table 3-1 Gill alterations in *Betta splendens* exposed to different concentrations of clove oil within 48 hours

Gill Lesions	**Clove oil concentration (ppm)**						
	Anesthetic				**Sedative**		
Time(h)	**40** **12h**	**40** **24h**	**30** **24h**	**30** **48h**	**20** **48h**	**15** **48h**	**Control groups**
Epithelial hyperplasia	+++	+++	+++	+++	+	+	-
Epithelial lifting	++	++	+	+	+	+	-
Fusion of the secondary lamellae	+	+	+	+	+	+	-
Blood congestion/Dilation of blood vessels	+	+	+	+	+	+	-

*sample size=30 individuals/dose

Score values: (-) = None, (+) = mild, (++) = moderate and (+++) = severe

In gills of exposed fish, structural alterations mostly occurred mainly between secondary lamellae. The histopathological evidence in gills of exposed fish with anesthetic doses were shown in moderated and severe degree. The general lesions were fusion of the secondary lamellae and necrosis.

At anesthetic concentration of 40 ppm, the gill lesion showed epithelial hyperplasia with severe damage (Figure 3-2 D). Epithelial lifting was observed in moderate degree whereas fusion of the secondary lamellae, dilation of blood vessels and blood congestion were experienced in mild degrees. Epithelial lifting occurred to epithelial cells of the secondary lamellae. Patterns of this lesion were also occurred on gills of exposed fish from both anesthetic doses. However, the degree of lesions observed was more than in 30 ppm. In addition, complete fusion of secondary lamellae occurred in only one specimen exposed to this concentration (Figure 3-2 F). The capillary congestion and dilation of the blood vessel of secondary lamellae were observed in the gills of exposed fish of both anesthetic doses (Figure 3-1E, F). One specimen showed well-outlined dilation of arterial blood vessels (aneurium) in gill lamellae (Figure 3-2 E).

Fish exposed to 30 ppm of clove oil showed severe hyperplasia and moderate lifting of epithelium (Figure 3-1 C) respectively, whereas fusion of the secondary lamellae, dilation and congestion of blood vessels showed mild degrees. Some of fish exposed to 30 ppm showed partial to complete fusion of secondary lamellae as shown in Table 3-1 and Figure 3-1 D.

The degeneration exhibited at sedative doses with mild degrees as shown in Table 3-2 and Figure 3-2. The general lesions epithelial hyperplasia (Figure 3-2 B,C) , epithelial

lifting and fusion of the secondary lamellae were found in some of the lamellae. Histopathological evidence in gills of the fish exposed to 15 and 20 ppm, the most frequent lesions observed were hyperplasia, lifting of epithelium with mild degrees. Only one specimen showed lamellae fusion in some area of the secondary lamellae at 48 hours. Concentration of ten ppm of clove oil did not have any histological lesions on the gills.

Figure 3-1

Photomicrographs of the gill of *Betta splendens*

(H&E staining)

A Photomicrograph of the control gill, showing normal structure of the primary lamella (1) and secondary lamella (2). Scale Bar =20 microns

B Photomicrograph of ethanol solvent control gill, showing normal structure of the primary lamella (1) and secondary lamella (2). Scale Bar =20 microns

C Photomicrograph of the treated gill tissue at 30 ppm, showing epithelial lifting which is characterized by a lifting of the outer layer of the lamellar epithelium with the space (black arrow). Scale Bar =20 microns

D Photomicrograph of the treated gill tissue at 30 ppm, showing fusion of two secondary lamellae (white arrow). Scale Bar =20 microns

E Photomicrograph of the treated gill tissue at 40 ppm, showing dilation of blood vessels (black arrow). Scale Bar =20 microns

F Photomicrograph of the treated gill tissue at 40 ppm, showing blood congestion (black arrow). Scale Bar =20 microns

Figure 3-1

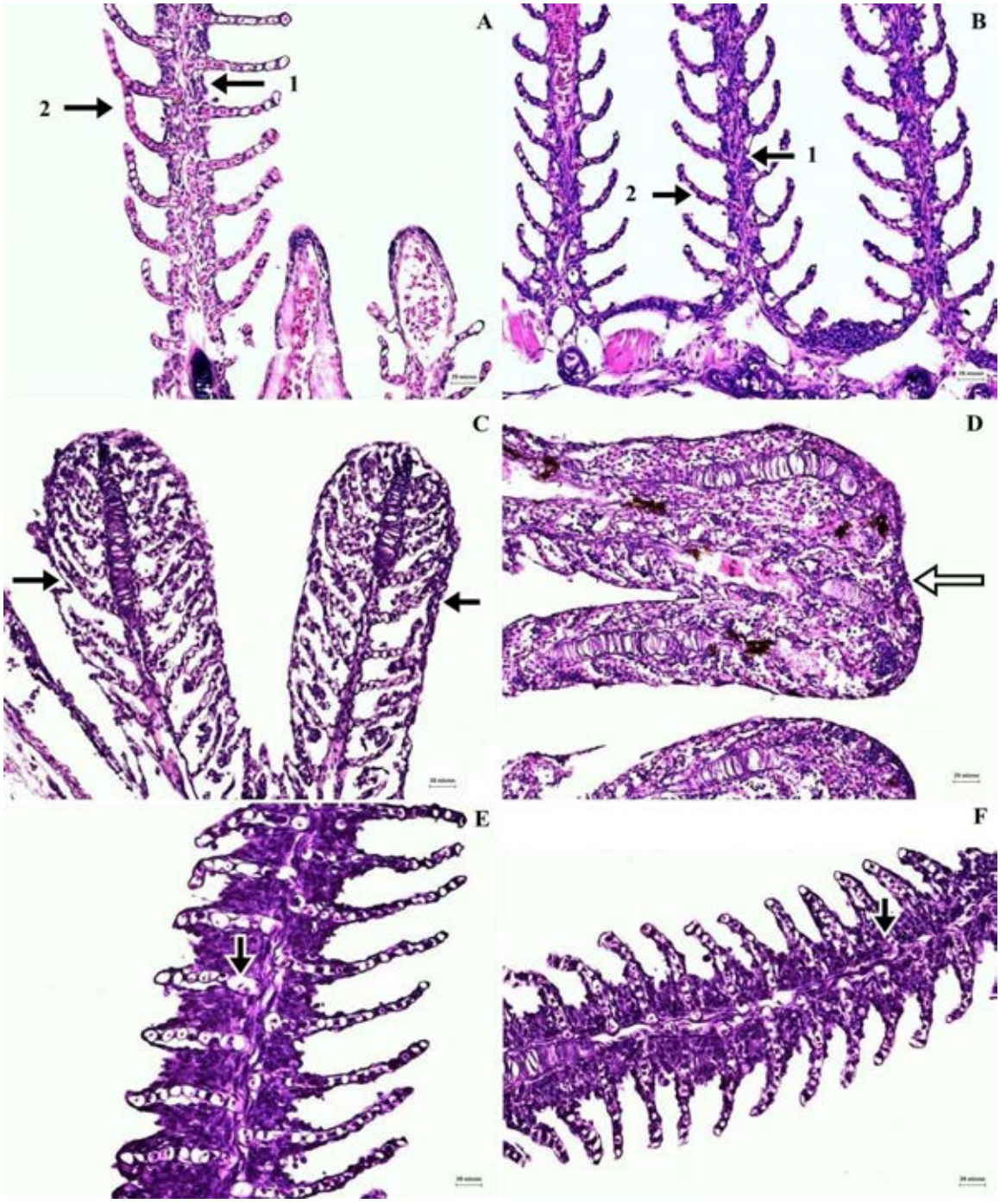


Figure 3-2

Photomicrographs of gill the of *Betta splendens*

(H&E staining)

A Photomicrograph of the control gill, showing normal structure of the primary lamella (1), secondary lamella (2) and chloride cell (white arrow). Scale Bar =20 microns

B Photomicrograph of the treated gill tissue at 15 ppm, showing epithelial hyperplasia (enlargement of tissue by increasing in number of cells) of secondary lamella (black arrow). Scale Bar =20 microns

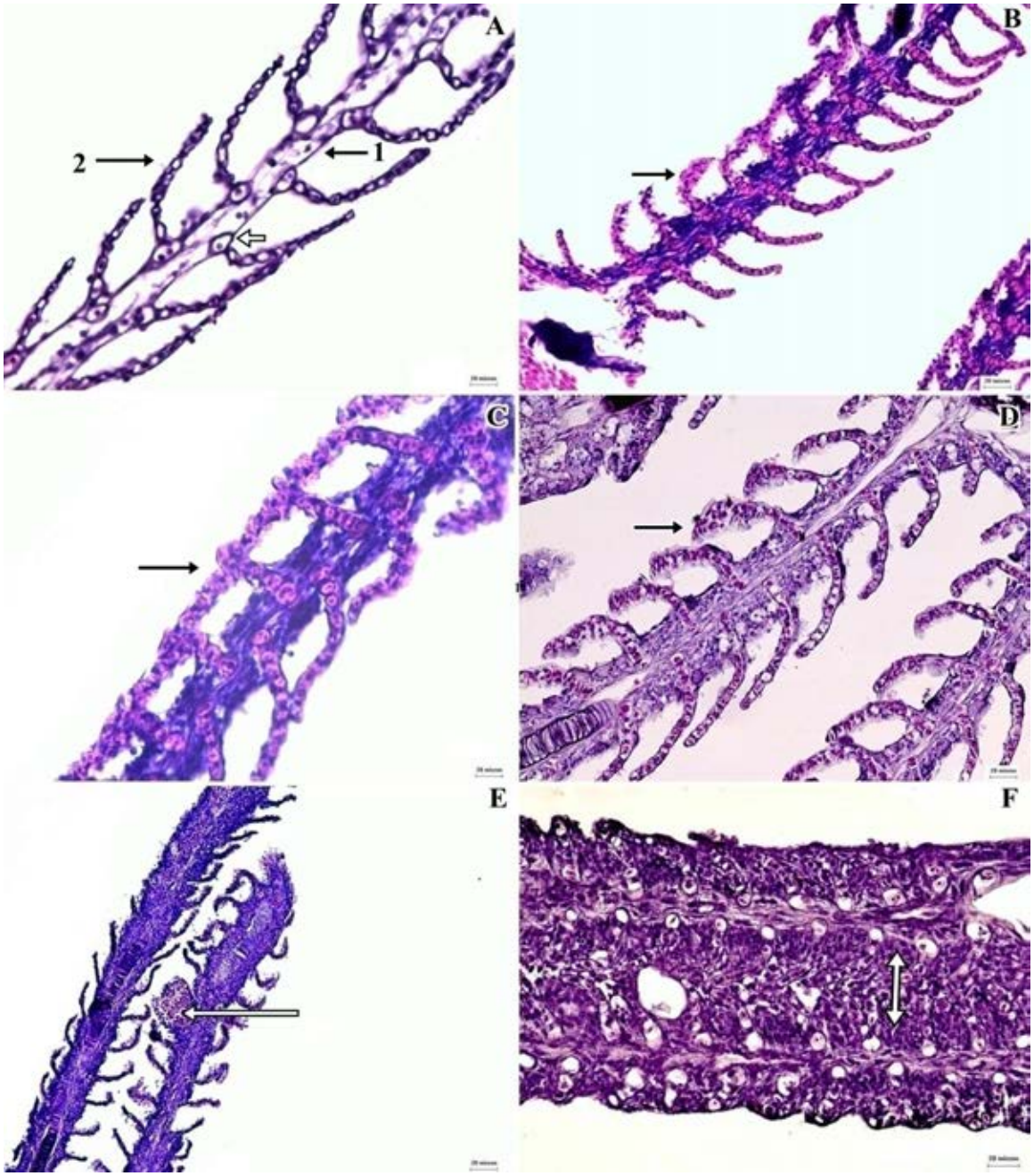
C Photomicrograph of the treated gill tissue at 20 ppm, showing epithelial hyperplasia (black arrow) of secondary lamella. Scale Bar =20 microns

D Photomicrograph of the treated gill tissue at 40 ppm, showing epithelial hyperplasia (black arrow) of secondary lamella. Scale Bar =20 microns

E Photomicrograph of the treated gill tissue at 40 ppm, showing aneurism (well-outlined dilations of arterial blood vessels) in gill lamellae (white arrow). Scale Bar =20 microns

F Photomicrograph of the treated gill tissue at 40 ppm, showing complete fusion of two secondary lamellae (white arrow heads). Scale Bar =20 microns

Figure 3-2



Effects of clove oil on liver

Control fish

Normal hepatocytes have nuclei with dispersed chromatin concentrated close to the nuclear membrane and one or more nucleoli. The nucleus of hepatocytes is generally a single, centrally located, spherical with a clear and dark nucleolus. Binuclear cells have been observed in normal liver cells. The cytoplasm of these cells is granular and vacuolated. The hepatocytes are arranged as irregular cord-like structures in histological sections, which are limited by sinusoids at basal side and canaliculi at the apical side and sinusoid at the basal part of cell (Figure 3-3 A and B and Figure 3-4 A).

Exposed fish

Treated groups showed several lesions. The most common changes at sedative doses were cloudy swelling hepatocytes, but the liver architecture was intact with normal capsule. The highest degeneration was found at anesthetic doses of clove oil. The shape of hepatocytes became irregular while many nuclei exhibited shrinkage and pycnosis. The scores of liver lesions after exposure to clove oil is shown in Table 3-2.

Table 3-2 Liver alterations in *Betta splendens* exposed to different concentrations of clove oil within 48 hours

Liver Lesions	**Clove oil concentration (ppm)**						
	Anesthetic				**sedative**		
Time(h)	**40 12h**	**40 24h**	**30 24h**	**30 48h**	**20 48h**	**15 48h**	**Control groups**
Swelling	+	+	++	+++	+	+	-
Dilation	+	+	+	+	-	-	-
Fatty fusion	++	+++	+	+	-	-	-
Necrosis	++	++	++	++	-	-	-

*sample size=30 individuals/dose

Score values: (-) = None, (+) = mild, (++) = moderate and (+++) = severe

In liver of exposed fish, structural alterations of hepatocytes occurred mostly around blood vessels and sinusoids. The histopathological evidence in liver of fish exposed with anesthetic doses of clove oil shown in moderate and severe degrees. The general lesions were fatty change with lipid vacuole fusion and necrosis.

At anesthetic concentration of 40 ppm, the exposed liver lesion showed cellular swelling, dilation of blood vessels with mild degrees whereas both lipid droplets accumulation and necrosis were experience in moderate degrees. Cellular swelling was

also occurred in hepatocytes of exposed fish of both anesthetic doses. Moreover, the pattern of this lesion was found in all specimens (Figure 3-3 F). Lipid droplets accumulation, a very large vacuoles were often seen and giving the liver a patchy appearance in the cytoplasm. In addition, the large vacuole in the cell forced the nuclei to the peripheral of the hepatocytes (Figure 3-4 D). These results indicated that fish expose to dosages of 40 ppm had more distinct vacuolated hepatocytes. The other evidence showed in a degenerative process of nuclei. There were many regions in the liver where cells had loss of cellular contents, necrosis and nuclei with irregular shapes and pycnotic nucleus (Figure 3-3 F).

At concentration of 30 ppm, liver lesions were cellular swelling and necrosis with moderate degrees (Figure 3-4 E). Other alterations included dilate sinusoid and fatty changes in some areas to mild degrees (Figure 3-4 C). The focal necrosis with karyolysis and pycnotic nucleus was noticed in many cells. More than 2-3 hepatocytes showed fatty change with lipid vacuole in cytoplasm (Figure 3-4 B).

The degeneration occurred at sedative doses with mild degrees. The general lesions of fish exposed to 15 and 20 ppm exhibited only cellular swelling in some area of the liver tissue with mild degrees (Figure 3-3 C and D). However, these lesions was absent in fish exposed to the concentration less than 10 ppm.

Figure 3-3

Photomicrographs of the liver of *Betta splendens*

(H&E staining)

A Photomicrograph of the control liver, showing normal structure of hepatocyte (black arrow) and sinusoid (white arrow). Scale Bar =20 microns

B Photomicrograph of ethanol solvent control liver, showing normal structure of hepatocyte (black arrow) and sinusoid (white arrow). Scale Bar =20 microns

C Photomicrograph of the treated liver tissue at 15 ppm, showing cellular swelling (black arrow). Scale Bar =20 microns

D Photomicrograph of the treated liver tissue at 20 ppm, showing cellular swelling (black arrow). Scale Bar =20 microns

E Photomicrograph of the treated liver tissue at 30 ppm, showing cellular swelling (black arrow). Scale Bar =20 microns

F Photomicrograph of the treated liver tissue at 40 ppm, showing cellular swelling (black arrow). Scale Bar =20 microns

Figure 3-3

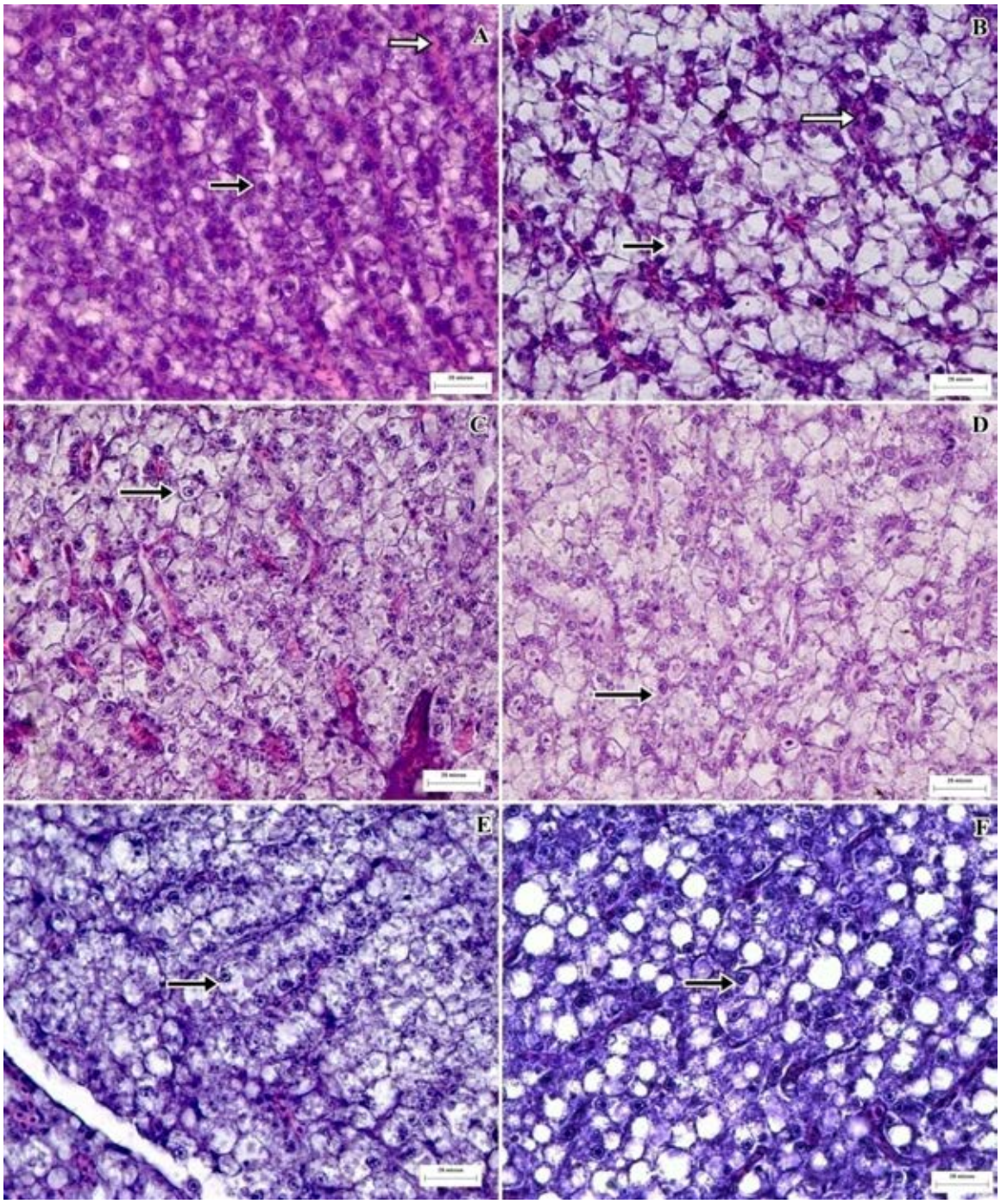


Figure 3-4

Photomicrographs of the liver of *Betta splendens*

(H&E staining)

A Photomicrograph of the control liver, showing normal structure of hepatocytes (black arrow) and sinusoid (white arrow). Scale Bar =20 microns

B Photomicrograph of the treated liver tissue at 30 ppm, showing mild fat accumulation (black arrow). Scale Bar =20 microns

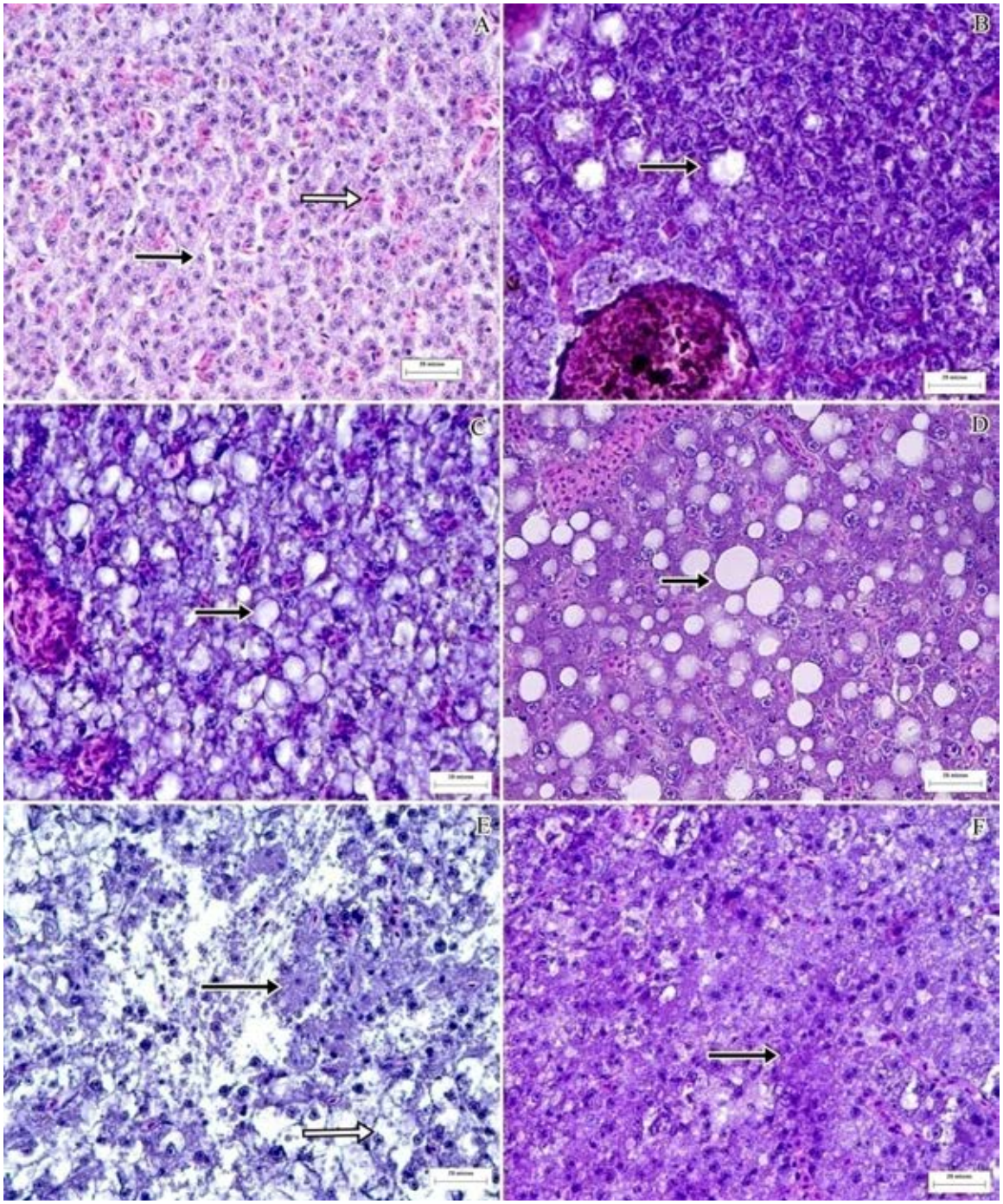
C Photomicrograph of the treated liver tissue at 30 ppm, showing fat accumulation (black arrow). Scale Bar =20 microns

D Photomicrograph of the treated liver tissue at 40 ppm, showing severe cellular fat accumulation (black arrow). Scale Bar =20 microns

E Photomicrograph of the treated liver tissue at 30 ppm, showing cellular swelling (white arrow) and necrosis (black arrow). Scale Bar =20 microns

F Photomicrograph of the treated liver tissue at 40 ppm, showing necrosis (black arrow). Scale Bar =20 microns

Figure 3-4



Effects of clove oil on kidney

Control fish

The histology of both control groups showed normal appearance of nephrons and hemopoietic tissues. The structural details of the kidney of control groups are shown in Figure 3-5.

Experimental groups

Treated groups showed several lesions. The most common kidney changes at sedative doses were tubular swelling. Moreover, the kidney architecture was intact with normal capsule. The highest degeneration was found at anesthetic doses of clove oil. Moreover, in fish exposed to the concentration less than 10 ppm did not have any effects.

The results of kidney alterations in *B. splendens* exposed to different concentrations of clove oil within 48 hours are summarized in Table 3-3.

Table 3-3 Kidney alterations in *Betta splendens* exposed to different concentrations of clove oil within 48 hours

Kidney Lesions	**Clove oil concentration (ppm)**						
	Anesthetic				**Sedative**		
Time(h)	**40 12h**	**40 24h**	**30 24h**	**30 48h**	**20 48h**	**15 48h**	**Control groups**
Swelling	+	+	+	+	+	+	-
Dilation	++	++	+	+	+	-	-
Fatty fusion	-	-	-	-	-	-	-
Necrosis	++	++	+	+	+	-	-

*sample size=30 individuals/dose

Score values: (-) = None, (+) = mild, (++) = moderate and (+++) = severe

The kidney of exposed fish, structural lesions occurred mostly around blood vessels. The histopathological evidence in kidney of exposed fish with anesthetic doses of clove oil were shown in moderate degrees. Most lesions had more distinct dilation of Bowman's space and necrosis of tubular cells. The other histological alterations were cellular swelling and shrunken of renal tubule as shown in Figure 3-5 C, D, E, F.

At anesthetic concentration, fish exposed of 40 ppm, the kidney lesion showed cellular swelling with mild degrees. This lesion was also occurred on renal tubule of exposed fish from both anesthetic doses (Figure 3-5 F). Dilation of Bowman's space was observed in moderate degrees whereas tubular necrosis was experienced in mild degrees. Degeneration of Bowman's capsules and renal tubules were more distinct in this concentration. Some glomeruli appeared shrunken with congested capillaries and there was an increased space within the Bowman's capsule whereas the renal corpuscles exhibited moderate damage including breakdown of glomerular blood capillaries (Figure 3-5 D). The other evidences showed in a degenerative process of nuclei, necrotic tubular cells and nuclei with irregular shape and pycnotic nucleus were also noted as shown in Table 3-3 and Figure 3-5 F.

At concentration of 30 ppm, the kidney lesions showed cellular swelling, dilation of Bowman's space and tubular necrosis with mild degrees. Some renal tubules dilated and had necrosis. The shape of renal tubule became irregular, many nuclei exhibited shrinkage and pycnosis. Reduction and occlusion in tubule lumen were observed along with reduction of tubule lumens (Figure 3-5 E). Furthermore, glomeruli were distorted in some specimens. The congestion distortion of capillaries and enlargement of Bowman's space were also observed as shown in Table 3-3.

The histological evidence in kidney of exposed fish with sedative doses from concentrations 15 and 20 ppm of clove oil exhibited few kidney morphological changes. The kidney of the fish exposed to concentration of 20 ppm showed cellular swelling and dilation of Bowman's space with mild degree whereas tubular swelling was seen in mild degree of fish exposed to 15 ppm. Moreover, effects were absent in fish exposed to the concentration less than 10 ppm as shown in Table 3-3.

Figure 3-5

Photomicrographs of the kidney of *Betta spledens*

(H&E staining)

A Photomicrograph of the control kidney, showing normal structure of renal corpuscle (r) and renal tubule (t). Scale Bar =20 microns

B Photomicrograph of ethanol solvent control kidney, showing normal structure of renal tubule (t). Scale Bar =20 microns

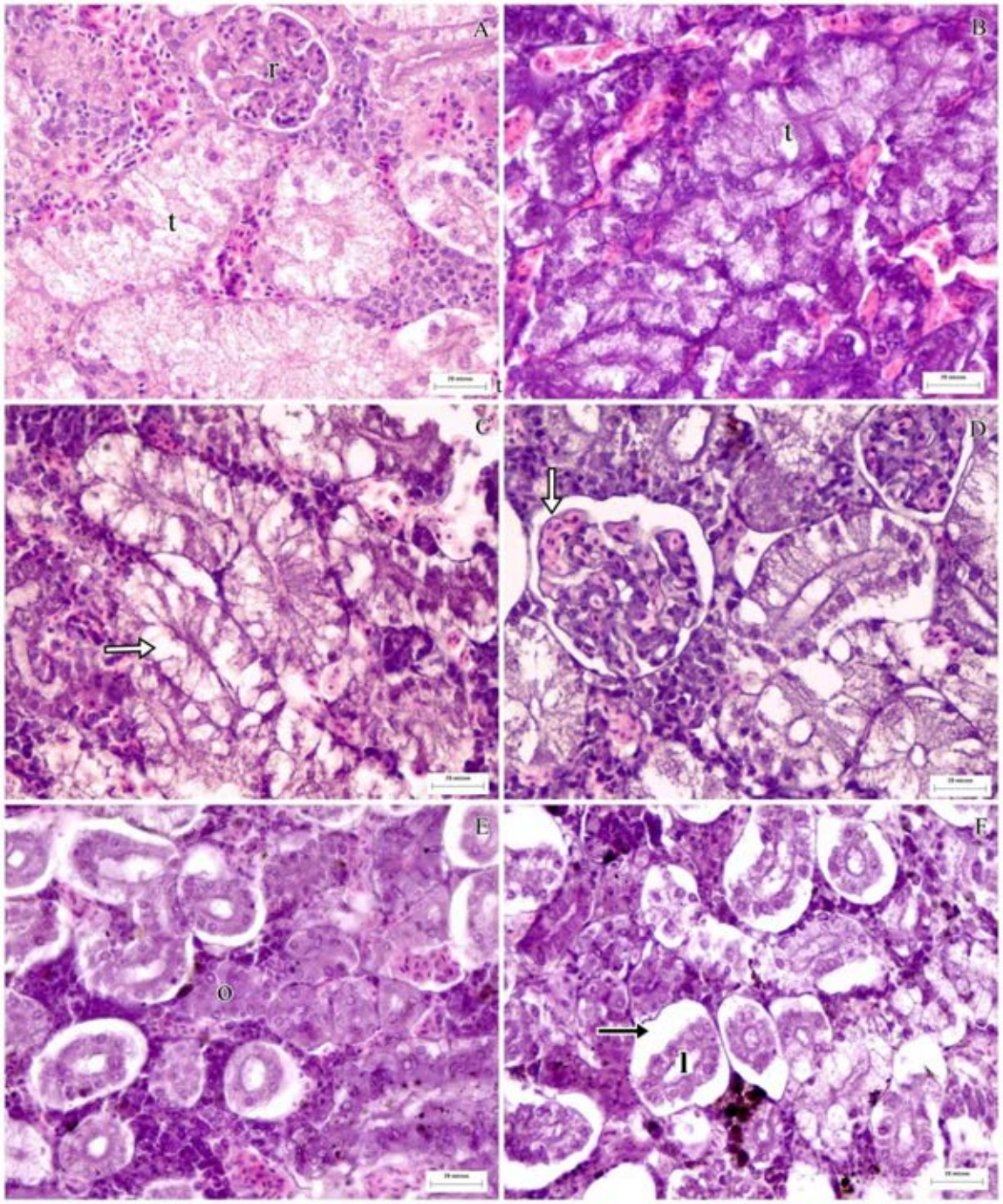
C Photomicrograph of the treated kidney tissue at 20 ppm, showing hydropic swelling (white arrow). Scale Bar =20 microns

D Photomicrograph of the treated kidney tissue at 40 ppm, showing dilated capillaries in glomerulus (white arrow). Scale Bar =20 microns

E Photomicrograph of the treated kidney tissue at 30 ppm, showing occlusion of tubular lumen (o). Scale Bar =20 microns

F Photomicrograph of the treated kidney tissue at 40 ppm, showing the narrowing of tubular lumen (l) and tubular contraction (black arrow). Scale Bar =20 microns

Figure 3-5



Discussion

Previous researches showed that clove oil or eugenol has an advantage as an anesthetic agent in fish (Soto and Burhanuddin,1995 ; Anderson *et al*.,1997; Keene *et al*., 1998; Mylonas *et al*., 2005).

Some studies reported the toxic effects of clove oil or components in oil of clove (Manabe *et al*., 1987; Suzuki *et al*., 1985; Thompson *et al*., 1991; Sladky *et al*., 2001). There are few reports in toxicological studies of clove oil. This research presents the first evidence of histopathological study of clove oil on the Siamese fighting fish *B. splendens*.

This study agrees with previous reports of histopathological effects on gills because gills are the first organ to be directly exposed with water. Therefore, gills, which participate in many important functions in fish such as respiration, osmoregulation and excretion, show certain responses to particular chemical exposures.

Several reports have described gill lesions relevant to exposure in several fish species. Moreover, the severity of damage to the gills depends on the concentration of the toxicant and the period of exposure. Toxic conditions resulted in two groups of lesions in gills. The first group was the result of the direct effects and the second group was the result of defensive responses of the fish (Mallatt, 1985).

According to Morgan and Tovell (1973), alterations such as lifting, hyperplasia of the epithelial cells and fusion of secondary lamellae, were defensive responses of fish. They

increased the distance across which waterborne irritants had to diffuse to reach the bloodstream. The result was a barrier to the entrance of toxicants (Mallatt, 1985; Hinton and Laurén,1990).

However, several previous reports have well documented the ability of fish to regenerate apparently normal tissue and organs following injury (Goss, 1969; Reimschuessel *et al*., 1990, 1993; Lombarte *et al*., 1993; Chamie and Reimschuessel, 1994; Rokokous *et al*., 1994; Oliver, 1915).

The gill's ability to return to a normal state is remarkable (Speare *et al*., 1999; Darwish *et al*., 2002; Tort *et al*., (2002a; 2002b); Bowker, 2006). Speare *et al*. (1999) studied gills morphology of rainbow trout by exposure to hydrogen peroxide. The gills lamellar regeneration occurred during healing process. Darwish *et al*. (2002) observed that the ability of severely damaged gills to return to normal was about six to eight days after exposure to potassium permanganate in channel catfish. Tort *et al*. (2002a; 2002b) indicated the restoration of gill epithelial tissue in both walleye and cutthroat trout by exposure to hydrogen peroxide as a treatment. As in other reports, Bowker (2006) reported detected scattered epithelial separation (mild to severe) and hypertrophy of gill epithelium (mild to severe), and concluded that such pathologies were typically reversible. These alterations occurred as tubular swelling, lifting, hyperplasia of the epithelial cells and fusion of secondary lamellae, all of which were considered as reversible changes.

Like other organs, the kidney's ability to repair sublethal toxic injury has been well documented in the mammals for more than a century (Cuppage and Tate, 1967; Ishmael *et al*., 1982; Reimschuessel *et al*., 1990). Fish, especially, it retained the ability to form new nephrons as they grow throughout their adult life (Yasatake and Wales, 1983). Moreover, renal tubular regeneration following toxicity has been described in several fish species. Reimschuessel *et al*. (1990) reported that the nephron of gold fish (*Carassius auratus*), like that of the mammal, could regenerate the proximal tubule epithelium following toxicant induced injury. Augusto *et al.* (1996) reported on the nephrotoxicity effect of getamicin in the Nile tilapia (*Oreochromis nilotica*) and also on the capacity of this species for nephroneogenesis. van Dyk *et al.* (2007) studied the liver of the (*Oreochromis mossambicus*) that was exposed to cadmium and zinc over a lengthy period of 672-hours. The fish showed regenerative responses after exposure to the 5% and 10% metal concentrations.

The results of histopathological changes in this study showed that clove oil at sedative doses had little impact on gills, the liver and kidney of *B. splendens*. Therefore, this study recommends sedative concentrations between 15 to 20 ppm to be used for the transportation of *B. splendens*.

CHAPTER IV

SEDATIVE EFFECTS OF CLOVE OIL IN SIAMESE FIGHTING FISH *Betta splendens* UNDER SIMULATED TRANSPORT CONDITION AT VARIOUS LOADING DENSITIES

Introduction

The ornamental fish trade in Thailand and many countries is a growing industry, in part due to increasing exports. At present, this business in Thailand generates an income of approximately 100 billion baht per year. There are many problems during transportation resulting in mortality of fish, especially regarding the duration of the trip and fish density. To save the cost of transportation, exporters attempt to increase density of fish with minimal water in plastic bags. In the international ornamental fish market, *Betta splendens* is very popular, known by different names such as fighting fish, Siamese fighting fish and *Betta* fish. The export of male *Betta splendens* comprises approximately 10% (approximately 3.6 million fish per year) of the exported ornamental fish of Thailand. The major problem of *B. splendens* transportation comes from the habits of the males. Males Siamese fighting fish have very aggressive social behaviors including biting, attacking and chasing.

As a result, physical damages or death of *B. splendens* regularly occur during transportation. Therefore, exporters cannot load males together in the same water container. Nowadays, each fighting fish is loaded in a small plastic bag separately leading to an

increase in transportation cost. As mentioned above, a variety of methods have been used to reduce the mortality and cost in transportation.

To solve this problem, anesthetics are commonly used to minimize stress and reduce the physical damage to fish during transportation. The main purpose of anesthetic use in the ornamental fish trade is to decrease the economic loss and increase product quality. In the ornamental fish trade, the use of anesthetics at a desired and optimal level should be studied because the dose of anesthetics is species specific. The optimum dosage of anesthetics required is critical, and varies widely between the species and size of fish.

Presently, many reports mentioned that clove oil is used as an alternative anesthetic substance in aquaculture. At present, there is no report on the sedative efficacy of clove oil on *B. splendens*. This study has determined the optimum range of sedative dosages of clove oil under simulated transport condition at various loading densities in order to increase post-transportation survival and the optimum loading density of *B. splendens*.

Materials and methods

Experimental organisms

Six month old *Betta splendens* males were obtained from a commercial farm in Bangkok. The average weight of each fish was about 1g. Each fish was placed separately in a two liter aquarium and was acclimatized for two weeks prior to the experiment. The fish were maintained on a lighting regimen representative of the local natural environment (12L:12D) and fed with commercial fish pellets (CP Company) twice daily at approximately 3-5% of their body weight. Tap water was filtered through carbon-resin filters, aerated and finally used as holding water in this experiment. Fish were fasted for 48 hours prior to the experiment. Clove oil was used to sedating the fish obtained from our previous experiments to induce sedation, 15 ppm to light sedation and 20 ppm for deep sedation. At concentration of 15 ppm, some fish treated with clove oil reached recovery before 48 hours. Therefore, the optimal dosage used for inducing sedation within 48 hours was selected at concentration of 20 ppm.

Preparation of Clove oil

Because of the incomplete solubility of clove oil in water, it was initially dissolved in ethanol at a ratio of one part of clove oil to 10 parts of ethanol (Grush *et al*., 2004). A fresh stock solution was mixed daily.

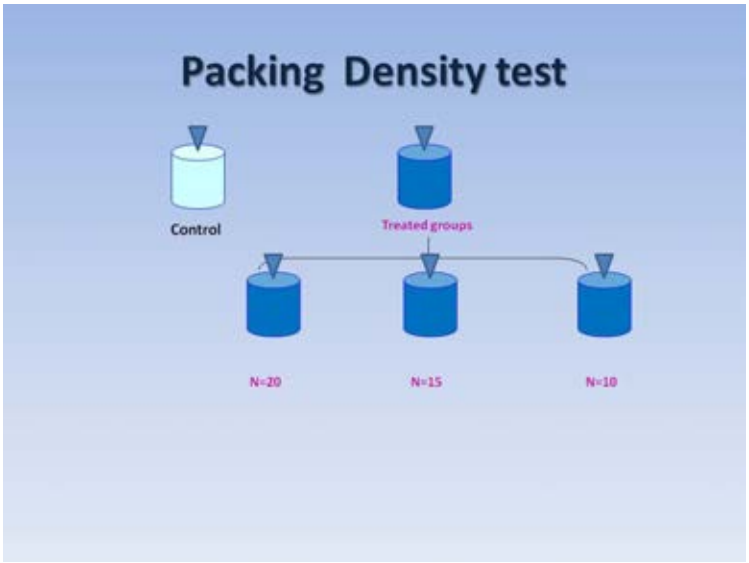


Figure 4-1 Diagram of packing density test

The packing density test

The fish were transferred to polyethylene bags (14 x 25 inch) filled with two liters of water. Each bag was filled with medical-grad oxygen at a 1:3 ration of oxygen to water and sealed with elastic bands. The packing densities were divided into two groups, the treatment groups, each bag containing with 20, 15 and 10 fish/2-L, respectively, and the control group with three replications as shown in Figure 4-1. All bags were placed in a styrofoam box (33x43x30 cm) for preventing sudden change in temperature. This experiment was conducted at room temperature (27-30 ^{o}C) for 48 hours. The mortality was observed and recorded after 48 hours. At the end of density experiment, the bags were opened, and the fish were released into a first recovery aquarium containing aerated water.

After recovery time, the fish were transferred to separated aquariums, and fish mortality was recorded every day for a period of one week.

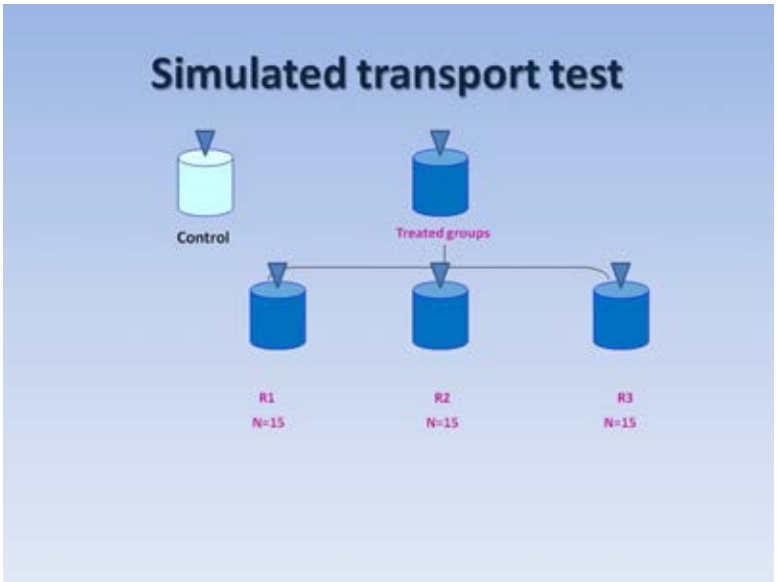


Figure 4-2 Diagram of simulated transport test

The simulated transport test

The simulated transport experiments were divided into two groups, treatment and control groups. In the control group, to avoid fighting, each fish had to be transferred to a small polyethylene bag (4x12 inch) filled with 100 ml of water. These bags were filled with medical-grade oxygen at a 1:3 ration of oxygen to water and sealed with elastic bands. All of the control bags were placed together in a polyethylene bags (14 x 25 inchs). In the treatment group, each bag contained clove oil at a concentrations of 20 ppm with a density of 15 fish/2-L based on a duration of transport time of 48 hours compared to the control group with three replicates as shown in Figure 4-2.

To simulate transport conditions, the bags were floated on the surface of vibrated water using a vibration pump applied to a low-amplitude strain (standard deviation at 50 Hz:

48-h) in tanks that made small quick regular movements. This experiment was conducted at room temperature (27-30 ^{o}C) for 24 hours to simulate transport within country and at temperature of 22±1 ^{o}C to simulate shipment conditions for 24 hours outside of Thailand.

After the end of stimulated-transport, the bags were opened, water samples were collected and fish were transferred into first recovery aquarium containing aerated water. After recovery time, the fish were transferred to separate aquariums as shown in Figure 4-3 and Figure 4-4. The fish survival rates were recorded every day for a period of one week (Lim *et al*., 2003). Water samples were taken before and after transport in order to measure temperature, dissolved oxygen, pH, total ammonia and dissolved CO_2.

Dissolved oxygen concentrations, temperature and pH were monitored using a Cyberscan pd 300 portable digital meter (Eutech Company). Total ammonia was monitored with a commercial test kit (Tetra Werke, Melle, Germany). Concentration of carbon dioxide (titrimetric method) was determined with standard procedures (APHA, 1995).

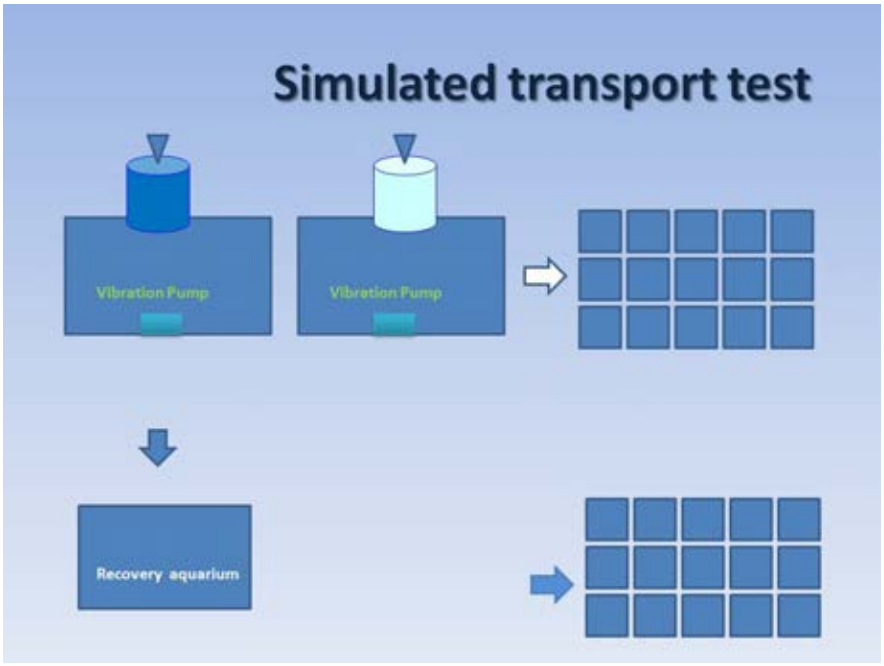


Figure 4-3 Diagram of simulated transport test

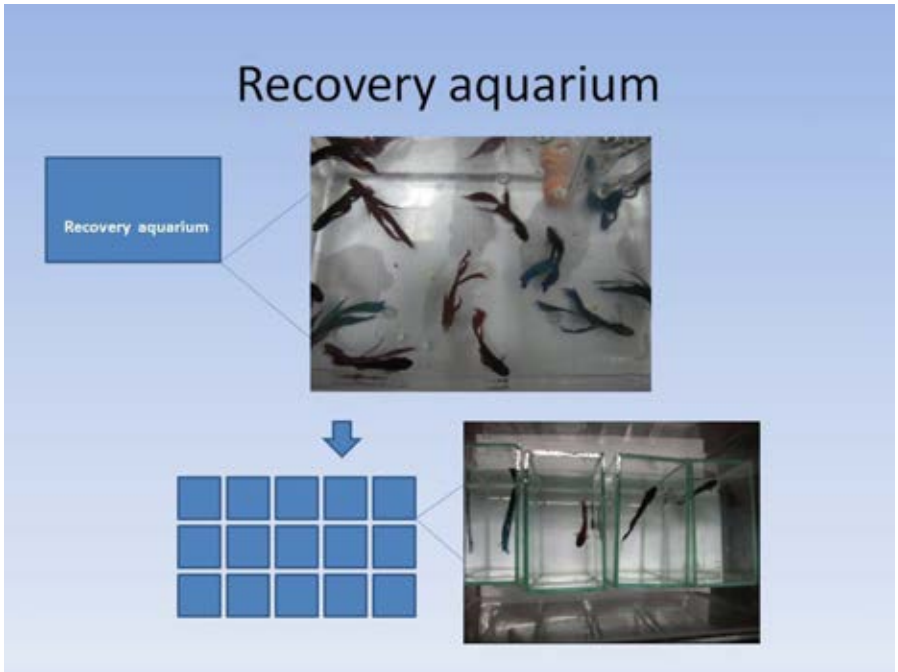


Figure 4-4 Diagram and photographs of recovery aquarium

The recovery of *B. splendens* after clove oil exposure

To study the recovery, the behavior of *B. splendens* was studied before and after exposure to clove oil. The experiments were divided into two parts. The first part evaluated the stages of recovery following criteria outlined in Hikasa *et al.* (1986). The second part assessed the aggressive behavior and nest building of *B. splendens* males following the method of Clotfelter *et al.* (2006) and Jaroensutasinee and Jaroensutasinee (2001).

For the first experiment, All of *B. splendens* were randomly sampled from the stimulated transport experiment group. This group was also used for the second experiment (aggressive behavior and nest building) and the results were compared with the control group with three replicates.

Aggressive behavior and nest building

Aggressive behavior was recorded by tallying the number of branchiostegal displays (opercular erections) as shown in Figure 4-5 (Simpson, 1968). After the first experiment, aggressive behavior was assessed by placing a glass mirror along the wall of the two liter aquarium for ten minutes. The duration (measured in seconds) of aggressive opercula displaying in the one minute period was recorded as shown in Figure 4-6 (Clotfelter *et al.*, 2006). The rate of aggressive displays was calculated by dividing the

number of opercular displays performed by the time spent (in seconds) on the side of the mirror. This number was multiplied by 100 (Snekser *et al*., 2006).

For the nest building experiment, males *B. splendens* were returned to one liter aquariums by the following day and placed in visual contact with females. After 72 hours the aquariums containing males were checked for the presence or absence of a bubble nest. The bubble nest length (b) and width (a) were measured using a vernier calipers. The area (A) of the bubble nest was estimated using the ellipsoid equation $A = \pi ab$ (Clotfelter *et al*., 2006; Jaroensutasinee and Jaroensutasinee, 2001).

Statistical Analyses

The data of recovery time were expressed as means ± SE. The variables were tested for normality using the Kolmogorov-Smirnov test. The data of aggressive behavior (number of opercular displays), bubble nest width and bubble area did not show normal distribution and thus, these data were compared using nonparametric tests followed by the Mann-Whitney U-test for comparison at significant level ($p<0.05$). All statistical analyses were performed using the SPSS 17.0 for Window software.

Figure 4-5 Male *Betta splendens* performed aggressive behavior by showing opercular erection.

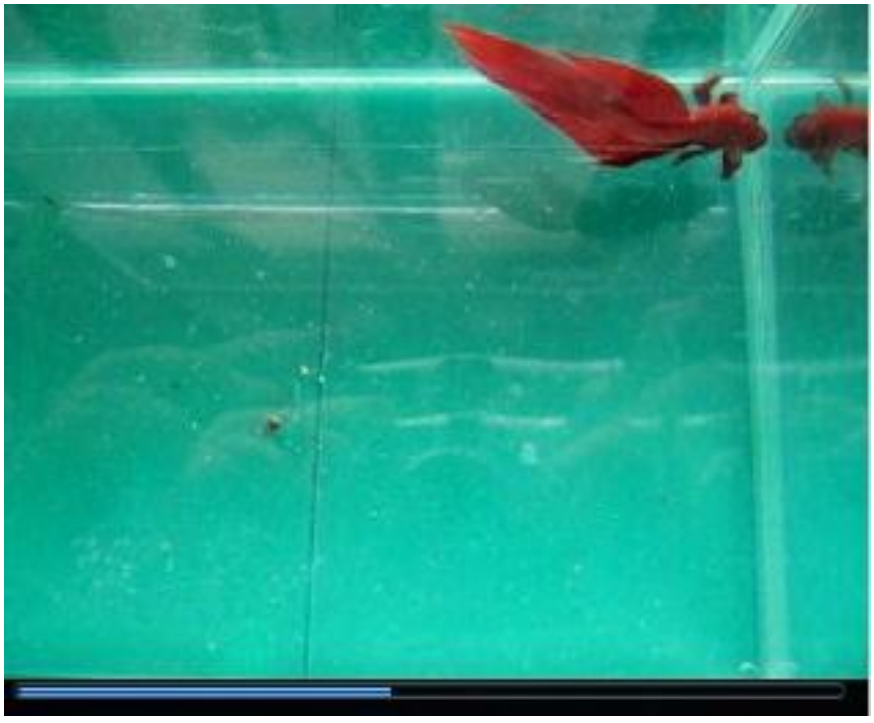


Figure 4-6 The duration of aggressive opercula displaying was recorded by video recorder (measured in seconds).

Results

The results from the packing density experiments, the optimum density for *B. splendens* transportation in closed bags was 15 fish/2L because at this density there was no fish mortality in any replicates during one week period. The means of recovery times to normal are show in Table 4-1. These results demonstrate that this species can be successfully anesthetized using sedative doses under simulated transport conditions.

Table 4-1. Means ± SE of recovery time to recovery stage 5 of *Betta splendens* exposed to different concentrations of clove oil

Clove oil concentrations (20ppm)	Recovery time (min)		7 days survival rate (%)	
	Stage 5	N		N
Treatment group	10.87 ± 0.82	45	100%	45
R1	14.13 ± 1.59	15	100%	15
R2	9.40 ± 1.03	15	100%	15
R3	9.00 ± 1.29	15	100%	15

N = sample sizes, R = replicate

Aggressive behavior

Two measures of aggression, the duration and numbers of opercular displays given toward the mirror stimulus, were investigated. The means of the number of opercular displays were not significantly different between control and treatment groups (Mann-Whitney U-test: Z=-0.426, P=0.670). Furthermore, the means of the duration of opercular displays were not significantly different between control and treatment groups (T=0.277, P=0.783). The means of the duration and number of opercular displays of the treatment groups and those of the control are showed in Table 4-2.

Table 4-2. Number of opercular display, duration of opercular display and rate of opercular display of *Betta splendens* after exposed to clove oil

Measurement Mean ± SE(S)	Number of opercular display	Duration of opercular display	N	Rate of opercular display
Control group	8.60±0.78	17.20±1.63	15	51.33%
Treatment group	8.44±0.69	16.52±1.40	36	55%
R1	6.90±1.09	19.18±3.12	11	41.56%
R2	9.08±1.37	15.83±2.56	12	59.84%
R3	9.15±1.10	14.92±1.66	13	63.61%

N = sample sizes, R = replicate , S= seconds

For the rate of opercular displays, male *B. splendens* fish in the treatment groups showed higher rates of opercular displays (55%) more than those in the control group (51.33%).

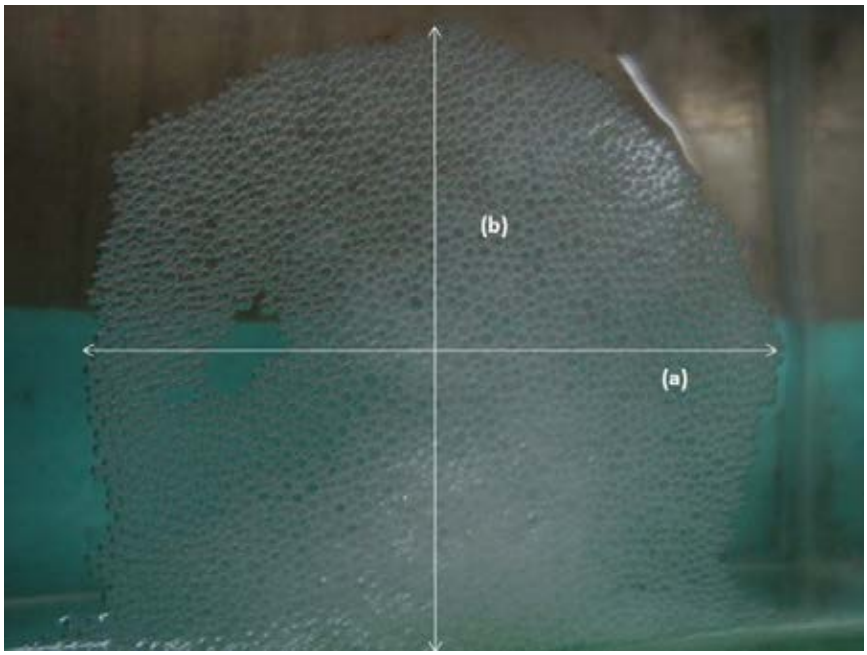


Figure 4-7 Bubble nest of male *Betta splendens*, (a)= bubble nest width, (b)= bubble nest length

Bubble Nest building

All of the treatment groups (n=45) constructed bubble nests two weeks after simulated transport experiments, 45 (100%). The means of the bubble nest widths, lengths and areas two week after the recovery period are shown in Table 4-3 and Figure 4-7.

Data for bubble nest area using Mann-Whitney U-test, showed that there were significant differences between the control and treatment groups (Z= -2.695, P= 0.007).

However, there was not a significant difference in means of bubble nest width (Mann-Whitney U-test: $p>0.05$). This experiment indicates there were no effects of clove oil on the ability of a male *Betta* to construct a bubble nest at a two week period after the end of the simulated transport experiment.

Table 4-3 Means ± SE of bubble nest width, bubble nest length, and bubble nest area of *Betta splendens* after exposure to clove oil

Measurement		Bubble nest Width (cm)	Bubble nest Length (cm)	Bubble nest area $(cm)^2$
	N	Mean ± SE		
Control group	45	4.49 ± 0.27[a]	10.17 ± 0 .41[a]	72.19±5.68[a]
Treatment group	45	3.93 ± 0.36[a]	8.25 ± 0.46[b]	54.26±6.62[b]
R1	15	2.56 ± 0.51[a]	8.68 ± 0.82[a]	
R2	15	4.64 ± 0.61[b]	7.28 ± 0.90[a]	
R3	15	4.58 ± 0.62[b]	8.80 ± 0.64[a]	

The mean followed by different superscript in the same column are significantly different at $p \leq 0.05$ level using Mann-Whitney U- test. N = sample sizes, R = replicate

Water quality

After the end of transport experiment, the means of dissolved oxygen (DO) level were higher in the treatment group that was treated with clove oil than in the control group (6.82±0.05 mg/l and 4.8±0.50 mg/l, respectively. The DO decreased in control group, this decrease was significant ($p<0.05$) between the control group and treatment groups. After 48 hours, the pH for the control and treatment groups were 6.28 and 5.47, respectively. Total ammonia in the treatment group was ranged from 0.25 to 1.5 mg/l. At the end of a 48 hours transport experiment, the means of CO_2 in the control and treatment groups were 14.00±1.89 mg/l and 6.67±0.76 mg/l, respectively.

Discussion

Previous researches show that clove oil (eugenol) has an advantage as an anesthetic agent in fish (Soto and Burhanuddin, 1995; Anderson *et al*., 1997; Keene *et al*., 1998; Mylonas *et al*., 2005; Iversen *et al*., 2009). This work presents the first evidence of efficacy of sedative dosages of clove oil on the Siamese fighting fish, *B. splendens.* This study agrees with few previous reports of the sedative effect of clove oil on fish which it exhibited reduced activity and interaction, but was able to maintain equilibrium, swimming capacity and avoid physical damage. Eugenol at levels of two to five ppm was used to sedate juveniles of the rainbow trout, *Oncorhynchus mykiss* for six to eight hours for the

purpose of transport (Keene *et al*., 1998). Concentrations of clove oil ranging from five to nine ppm had sedative effects with rapid induction time in sub-adult of the largemouth bass. This dosage was effective for fish transport (Cooke *et al*., 2004).

From previous studies, the estimated LC_{50} at 48 hours of clove oil for acute toxicity test was 32.8 ppm. Clove oil was effective as sedative and anesthetic dosages at concentrations of five to 20 ppm and 30 to 50 ppm, respectively, within 48 hours. The sedative concentrations of clove oil at 20 and 15 ppm caused fewer lesions in the gills of *B. splendens* whereas lesions in gills were not found on fish exposed to ten ppm (Angsirijinda *et al*., 2011). Therefore, this study used sedative concentrations between 10 to 20 ppm.

Results of this study showed that clove oil induced anesthetic effects on *B. splendens* in terms of increased sedation during transport which may associate with the decrease in metabolic rate of the fish and oxygen consumption in the water. This study is consistent with previous reports in term of DO decline in control group. According to Harmon (2009), anesthetics are widely used to slow the fish's rate of metabolism, thus reducing oxygen uptake during transportation. Wedemeyer (1996) reported that MS-222 at 10 mg/l could reduce oxygen consumption rates of spring Chinook salmon, *Oncorhynchus tshawytscha*. It was also reported that oxygen consumption rate was 210 mg kg^{-1} $1h^{-1}$ in control transportation tank and 190mg kg^{-1} $1h^{-1}$ in treated tank. The other aspects

concerned besides clove oil effectiveness, sedative effects and loading density in transport should be body size and weight of the fish.

In conclusion, the result from the packing density experiment, clove oil at the concentration of 20 ppm was effective as sedative dosage for *B. splendens* during 48 hours in closed bags. The optimum density for *B. splendens* transportation in closed bags was 15 fish/2L. There was no fish mortality during one week. The result of recovery test showed that all of exposed fish could recovery to normal stage and having behavior at two weeks, indicated that were no effects of clove oil both on the ability of a male *B. splendens* to construct a bubble nest and have aggressive behavior at a two-week period after the end of the simulated transport experiment. This experiment indicated that there were no effects of clove oil both on the ability of a male *B. splendens* to construct a bubble nest and have aggressive behavior at a two week period after the end of the simulated transport experiment. Results of this study could potentially allow higher transport densities to ornamental fish trade markets. It could have a positive impact on the economics of *B. splendens* farms in Thailand through the increase of potential export in the future.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

At present, the demand for *Betta splendens* in the ornamental fish trade has increased. However, there are many problems arise during the transportation of ornamental fish. The major problem of *B. splendens* transportation comes from the habits of this fish. In general, each fighting fish is loaded in a small plastic bag separately resulting in an increase in transportation costs. Also, there is usually high mortality during transportation and after arrival.

To resolve this problem, the optimum range of sedative dosages of clove oil was determined in this study in order to increase loading density and post-transportation survival of *B. splendens* in the closed bags system. This study showed the first evidence of efficacy of sedative dosages of clove oil on the Siamese fighting fish, *B. splendens.* The results demonstrated that this species could be successfully anesthetized using sedative doses under simulated transport conditions at various loading densities. In addition, the safe margin of clove oil for *B. splendens* estimated by the median lethal concentration of clove oil (LC_{50}) for its practical application in transportation was investigated.

Acute toxicity test in *B. splendens* revealed that the LC_{50}at 48 hours of clove oil was 32.8 ppm. In comparison with other fish species, Keene *et al*. (1998) who studied the effect

of clove oil derivative (eugenol) on juvenile rainbow trout (*O. mykiss*) reported that the estimated 8-96 hr LC_{50} was found to be approximately 9 ppm. Grush *et al*. (2004) reported that the anesthetic of clove oil derivative (eugenol) in the zebrafish (*Danio rerio*) estimated at 96 hours LC_{50} was 21 ppm. Also, the sedative effect to anesthesia was markedly different between species. From previous researches, concentrations of clove oil ranging from 5 to 9 ppm had sedative effect with rapid induction time in sub-adult of the largemouth bass. This dosage was effective for this fish species for transportation (Cooke *et al*., 2004). Keene *et al*. (1998) reported that eugenol at levels of 2 to 5 ppm were used to sedate juvenile of the rainbow trout (*Oncorhynchus mykiss*) for 6 to 8 hours on the purpose of transportation but in *B. spendens* exposed to low doses of clove oil at levels of 2 to 4 ppm had no sedative effect throughout of the test period of 48 hours. In this experiment, concentrations of clove oil were effective as sedative dosage at concentrations of 5 to 20 ppm within 48 hours. It is possible to explain that the proper dosage of anesthetics required is critical and varies widely between the species (Coyle *et al*., 2004). Therefore, this was the reason for studying the efficacy of clove oil in *B. splendens*.

More important, this work presents the first evidence on the impacts of clove oil on gills, liver and kidney on Siamese fighting fish *B. splendens*. However, the results of histopathological changes showed that it had little impacts on gills, liver and kidney because of using clove oil in low doses. Moreover, several previous reports have well

documented in the ability of fish to regenerate apparently normal tissues and organs following injury (Goss, 1969; Reimschuessel *et al*., 1990, 1993; Lombarte *et al*., 1993; Chamie and Reimschuessel, 1994; Rokokous *et al*., 1994; Oliver, 1915). Thus, this study suggests that clove oil is relatively safer in terms of impacts on gills, liver and kidney of this fish species. Therefore, this study recommends the sedative concentrations between 10 to 20 ppm to be used for transportation based on the experimental condition.

From the packing density experiment, fish were packed in sealed plastic bags for 48 hours with three densities: 20, 15 and 10 fish/2L. Fish mortality was monitored for a period of one week after 48 hours of packing. In addition, recovery data, including aggressive behavior and nest building of *B. splendens* was assessed at two weeks after the recovery period.

From the results, a clove oil concentration of 20 ppm was a suitable dose for *B. splendens* during 48 hours transportation. The optimum density for *B. splendens* transportation in closed bags was 15 fish/2L because at this density there was no fish mortality in any replicates. Moreover, at this density there was no fish mortality during one week after recovery period. The mean of recovery times to normal was 10.87± 0.82 min. The means of opercular display durations were not significantly different between control and treatment groups as well as the means of opercular display. The data of bubble nest area showed that there were significant differences between the control and treatment

groups. However, there was no significant difference in means of bubble nest width ($p>0.05$). This experiment indicated that there were no effects of clove oil both on the ability of a male *B. splendens* to construct a bubble nest and display aggressive behavior at two-week period after the end of the simulated transport experiment.

In conclusion, the estimated LC_{50} at 48h of clove oil for acute toxicity test was 32.8 ppm. Clove oil was effective as sedative and anesthetic dosages at concentrations of 5 to 20 ppm and 30 to 50 ppm, respectively within 48 hours. The highest degeneration was found at the anesthetic stages of clove oil. However, the sedative concentrations of clove oil at 20 and 15 ppm caused little impacts in gills, liver and kidney of *B.* splendens. The result from the packing density experiment showed that clove oil concentration of 20 ppm was effective for *B. splendens* during 48 hour in closed bags. The optimum density for *B. splendens* transportation in closed bags was 15 fish/2L. There was no fish mortality during one week. The result of recovery showed that all of exposed fish recovered to normal stage within two weeks after the end of simulated transport experiment.

Results of this study are valuable for ornamental fish trade. Further investigations on other ornamental species in Thailand should be conducted in order to increase the fish export in the future.

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