Evaluation of Green Tea Enriched *Artemia* sp. as Food and Antioxidant Activity Enhancer for Freshwater Ornamental Fishes

January 2014

Gwen Manero ANUEVO

Evaluation of Green Tea Enriched *Artemia* sp. as Food and Antioxidant Activity Enhancer for Freshwater Ornamental Fishes

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Agricultural Science

(Doctoral Program in Bioindustrial Sciences)

Gwen Manero ANUEVO

Abstract

Freshwater ornamental farming may be regarded as one of the emerging industry nowadays due to high demand of ornamental fishes as decoration or as food. Neon tetra *Paracheirodon innesi* and guppy *Poecilia reticulata* are among the popular freshwater aquarium fish around the world because of their small size, attractive color and ease to culture. With high demands on export/import, production becomes intense and cost of production also expands. Significant proportion of the total operational costs of production goes to high quality feeds to ensure better nutrition and survival. In larval hatcheries, *Artemia* nauplii are commonly used as food and vehicle of delivering nutrition and chemotherapeutants to produce good quality fish commodities. With emerging environmental risks of using artificial and synthetic drugs, interest in the utilization of plant extract as alternative drug for aquatic animals receives growing interests. The whole paper was conducted to evaluate the application of green tea enriched *Artemia* nauplii as food and antioxidant activity enhancer for freshwater ornamental fishes. Chapter 2 evaluated the improvement on the quality of *Artemia* nauplii in terms of total polyphenol content (TPC), total carotenoid (TC) and antioxidant activity through green tea extract enrichment via bioencapsulation technique. Amount of green tea polyphenols with high antioxidant activity was observed after 6-hours enrichment using instar II *Artemia* nauplii. In Chapter 3, instar II *Artemia* nauplii enriched with green tea extract (GTE) was fed to guppy fry to determine its effect on growth, antioxidant activity and stress resistance. Based on the result, guppy fry fed GTE-enriched instar II *Artemia* nauplii have higher TPC and antioxidant activity which improved their resistance against salinity stress compared to control. To prevent additional feed preparation in hatcheries, Chapter 4 was conducted to determine the influence of fish size on growth performance and stress resistance of ornamental fishes like guppies and neon tetras if fed with GTE-enriched instar II *Artemia* nauplii. After 10 days of feeding, control-fed and GTE-fed guppy fry have better

specific growth rate (SGR) than neon tetra juvenile and guppy adult. But GTE-enriched fed guppy fry samples are more resistant when exposed to salinity stress. To further assess the applicability of GTE-enriched instar II *Artemia* nauplii to neon tetra and guppy, their biochemical and digestive enzyme composition was determined in Chapter 5. During the 10-days feeding period no significant difference was observed on their total lipid and total protein content. Enrichment of green tea extract to *Artemia* nauplii did not also affect the protease and α-amylase composition of neon tetra and guppy. This result is a good indicator when green tea is used because it can improve the stress resistance of aquatic fish, but does not alter their biochemical and enzyme composition. This study as a whole presents a simple, economical and environment-friendly method of improving the growth and resistance of freshwater fishes against stressful conditions.

List of Tables

Page

Table 2.1 C*a*, C*b* and total carotenoid of green tea enriched *Artemia salina* nauplii extracted with acetone over time. 24

Table 2.2 C*a*, C*b* and total carotenoid of green tea enriched *Artemia salina* nauplii extracted with acetone at different naupliar stages. 24

Table 3.1 Growth performance of guppy fry fed unenriched and green tea extract (GTE) instar II *Artemia* nauplii 36

Table 3.2 Total polyphenol content, diphenyl picrylhydrazyl **(**DPPH) free radical scavenging activity and total carotenoids of instar II *Artemia* nauplii enriched without (control) and with green tea extract (GTE) for 6-hours. 37

Table 5.1 Biochemical composition of neon tetra and guppy samples before and after the 10-day feeding period. 64

Table 5.2. Total proteolytic and amylase activities of *Artemia* nauplii, neon tetra and guppy samples determined at 37^{o}C 64

List of Figures

Page

Figure 1.1A Life cycle of *Artemia* 4

Figure 1.1B Delivery of essential nutrients, therapuetants, pigments etc. to target fish larvae using *Artemia* nauplii via bioencapsulation technique. 6

Figure 1.2A Principal tea producing countries in 2011. 7

Figure 1.2B Structure of tea catechins and polyphenols 8

Figure 1.2C The mechanism of a typical phenol antioxidant. 9

Figure 2.1 Total polyphenol content (TPC) of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. 23

Figure 2.2 DPPH free radical scavenging activity of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. 25

Figure 2.3 Total antioxidant (TA) activity of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. 26

Figure 3.1 Photomicrograph showing the gut region of instar II *Artemia* nauplii after 6-hours enrichment without (a) and with green tea extract (b) at 3.50% salinity. 33

Figure 3.2 Total polyphenol content (TPC) of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii. 38

Figure 3.3 Diphenyl picrylhydrazyl (DPPH) free radical scavenging activity of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii. 39

Figure 3.4 Cumulative mortality (%) of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii against salinity stress (3.50% salinity). 40

Figure 4.1 % Specific growth rate (% SGR day^{-1}) of guppy fry, neon tetra juvenile and guppy adult fed with unenriched and green tea extract (GTE)-enriched instar II *Artemia* nauplii for 10 days. 50

Figure 4.2 Total polyphenol content (TPC) of guppy fry, neon tetra juvenile and guppy adult fed with unenriched and green tea extract (GTE)-enriched Instar II *Artemia* nauplii for 10 days. 52

Page

Figure 4.3 DPPH free radical scavenging activity of guppy fry, neon tetra juvenile and guppy adult fed with unenriched and green tea extract (GTE)-enriched instar II *Artemia* nauplii for 10 days. 53

Figure 4.4 Cumulative mortality (%) of guppy fry fed with unenriched (control) and green tea extract (GTE)-enriched instar II *Artemia* nauplii for 10 days against 3.50% salinity. 54

Figure 4.5 Cumulative mortality (%) of guppy adult fed with unenriched (control) and green tea extract (GTE)-enriched instar II *Artemia* nauplii for 10 days against 3.50% salinity. 54

Figure 4.6 Cumulative mortality (%) of neon tetra juvenile fed with unenriched (control) and green tea extract (GTE)-enriched instar II *Artemia* nauplii for 10 days against 1.50% salinity. 55

Figure 5.1 Protease activities of unenriched (control) and GTE-enriched *Artemia* nauplii at different pH. 65

Figure 5.2 Protease activities at different pH in digestive tract of neon tetra fed unenriched (control) and GTE-enriched *Artemia* nauplii. 66

Figure 5.3 Protease activities at different pH in digestive tract of guppy fed unenriched (control) and GTE-enriched *Artemia* nauplii. 66

Table of Contents

		Page
Abstract		i
List of Tables		iii
List of Figures		iv
Chapter 1.	**General Introduction**	1
	1.1. Artemia	2
	1.2. Green tea	6
	1.3. Freshwater Ornamental Production	10
	1.4. Aim and Outline of Thesis	11
Chapter 2.	**Improvement of total polyphenol content, total carotenoid and antioxidant activity of *Artemia salina* nauplii with green tea extract**	14
	2.1. Introduction	15
	2.2. Materials and Methods	17
	2.3. Results	20
	2.4. Discussion	27
Chapter 3.	**Effect of green tea enriched *Artemia* nauplii on growth performance, antioxidant activity and stress resistance of guppy *Poecillia reticulata* fry**	29
	3.1. Introduction	30
	3.2. Materials and Methods	31
	3.3. Results	35
	3.4. Discussion	40

		Page
Chapter 4.	**Influence of fish size on growth performance and stress resistance of freshwater ornamental fish fed green tea enriched *Artemia* nauplii**	44
	4.1. Introduction	45
	4.2. Materials and Methods	46
	4.3. Results	49
	4.4. Discussion	55
Chapter 5.	**Biochemical and digestive enzyme composition of neon tetra *Paracheirodon innesi* and guppy *Poecilia reticulata* fed green tea extract enriched *Artemia* nauplii**	58
	5.1. Introduction	59
	5.2. Materials and Methods	60
	5.3. Results	63
	5.4. Discussion	67
Chapter 6.	**General Discussion and Conclusion**	70
Acknowledgement		79
References		80

CHAPTER 1

General Introduction

1.1. *Artemia* sp

Taxonomy

*Artemia salina (*L.*)* is a primitive aquatic arthropod (salt lakes) with an age of about 100 million years. It was first reported from Urmia Lake in 982 by an unknown Iranian geographer (Asem 2008), and then in 1756, Schlösser pictured both sexes clearly. Linnaeus (1758) described it as *Cancer salinus* but 61 years later, Leach (1819) transferred it to *Artemia salina* (Asem *et al*. 2010). The taxonomic status of the genus *Artemia* is as follows (Martin & Davis 2001; Asem *et al*. 2010):

Subphylum: Crustacea Brünnich, 1772

Class: Branchiopoda Latreille, 1817

Subclass: Sarsostraca Tasch, 1969

Order: Anostraca Sars, 1867

Family: Artemiidae Grochowski, 1896

Genus: *Artemia* Leach, 1819

- *A. salina* (Linnaeus, 1758): Mediterranean area
- *A. monica* Verrill, 1869: USA (Mono Lake; California)
- *A. urmiana* Günther, 1899: Iran (Urmia Lake; West Azerbaijan Province)
- *A. franciscana* Kellogg, 1906: America, Caribbean and Pacific islands
- *A. persimilis* Piccinelli & Prosdocimi, 1968: South America
- *A. sinica* Cai, 1989: Central and Eastern Asia
- *A. tibetiana* Abatzopoulos, Zhang & Sorgeloos,1998: China (Tibet)
- *Artemia* sp. Pilla & Beardmore, 1994: Kazakhstan
- Parthenogenetic population(s) of *Artemia*:Europe, Africa, Asia and Australia

Life cycle of *Artemia*

The life cycle of *Artemia* sp. is summarized in **Figure 1.1A**. In its natural environment at certain moments of the year *Artemia* produces cysts that float at the water surface and that are thrown ashore by wind and waves. These cysts are metabolically inactive and do not further develop as long as they are kept dry. The dehydrated cysts measures between 200-270 microns and an average weight of 3.5 micrograms (http://www.fao.org/docrep/003/w3732e).

Upon immersion of cysts in seawater, the biconcave-shaped cysts hydrate, become spherical, and within the shell the embryo resumes its interrupted metabolism. After about 20 h the outer membrane of the cyst bursts ("breaking") and the embryo appears, surrounded by the hatching membrane. While the embryo hangs underneath the empty shell ("umbrella" stage) the development of the nauplius is completed and within a short period of time the hatching membrane is ruptured ("hatching") and the free-swimming nauplius is born.

After about 8-h, first larval stage (instar I) nauplii are produced, then after 12-h molts into second larval stage (instar II) and is now able to take up exogenous particles (Benjits *et al*. 1975; Rodrigues *et al*. 2011). The larva grows through 15 molts and becomes differentiated into male or female (at least for sexual species) after the tenth molt (Criel & MacRae 2002). The maximum *Artemia* length is usually around 8-10mm for males and 10-12mm for females and their thickness approximates 4mm (including the legs) for both sexes (Criel & MacRae 2002).

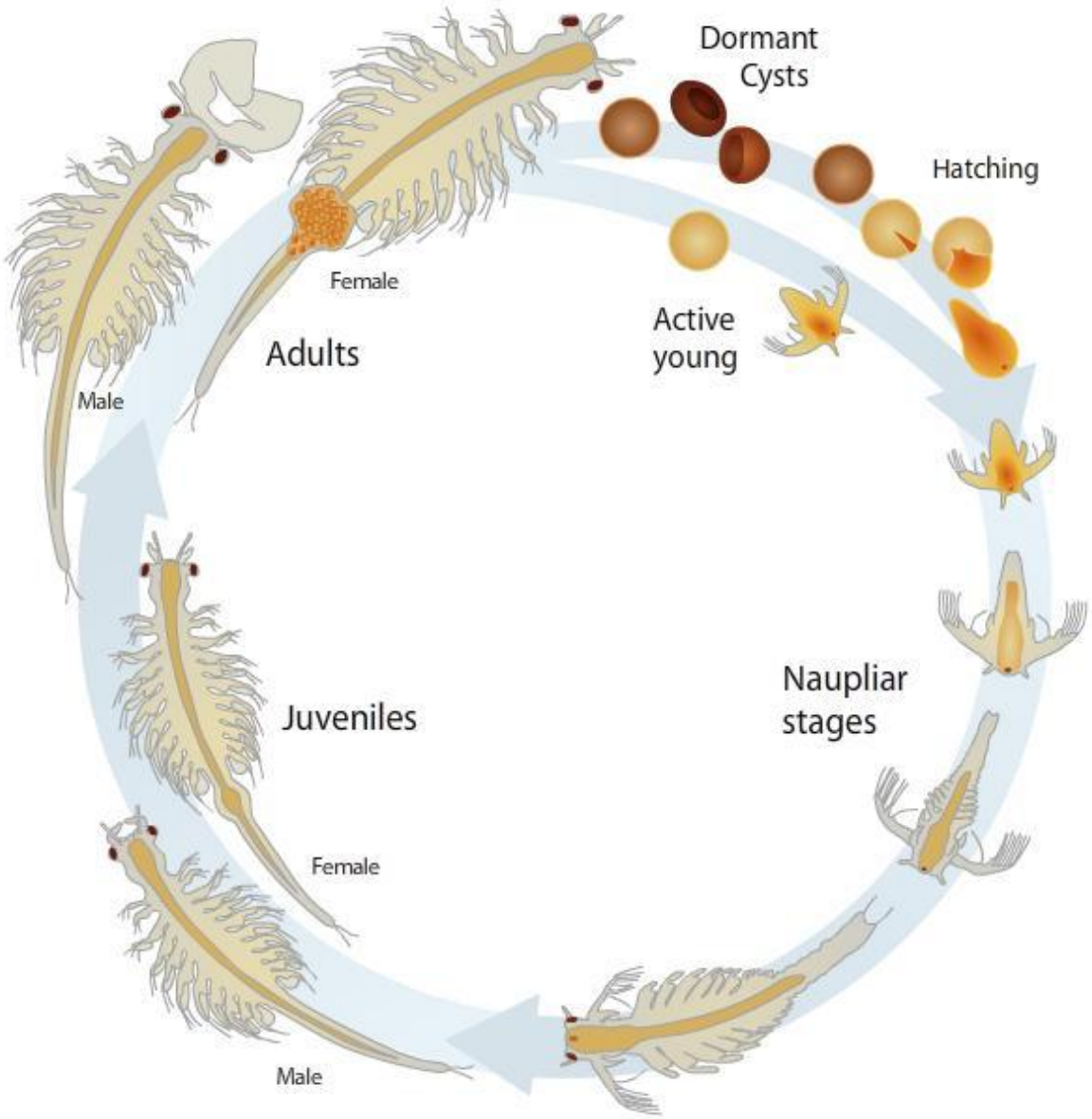

Figure 1.1A Life cycle of *Artemia* (www.flytrapcare.com)

Importance of *Artemia* in aquaculture production

Artemia nauplii are used extensively worldwide as live food for the larval stages of commercially important freshwater and marine fish species due to their availability, low cost, ease of culture and biochemical composition (Kolkovski *et al*. 1997; Sorgeloos *et al*. 1998; Smith *et al*. 2004). Though, there have been much effort to develop larval or postlarval artificial feeds, live food organism like *Artemia* are still preferred in hatcheries. One major nutritional difference between live food organisms and compounded feeds is the digestive enzymes in live food organisms. These enzymes of dietary origin (exogenous enzymes) play an important role in promoting the digestion and even growth of fish, especially in stomachless fish larvae or in larvae initially without stomachs (Dabrowski 1979, 1982; Chen & Lin 1992). Since *Artemia* are nonselective feeders, they can be enriched or fortified with nutrient supplements to enhance their nutritional value or with the medications to provide health benefits for the animals to which they are fed (Lavens & Sorgeloos 1996; Touraki *et al*. 1996; Rodriguez *et al*. 2011).

Despite the improved production of fish larvae through better husbandry and increased knowledge of larval nutrient requirements, stable productions are, nevertheless, limited by the outbreak of bacterial infections during the early life stages (Roiha 2010). Bacterial infections can cause up to 100% mortality that may result to economic loss. An easy and straightforward way of confronting bacterial diseases is to provide the proper chemotherapeutants to the fish larvae through the food chain by using bioencapsulation process (Touraki *et al*. 1996). Bioencapsulation (**Figure 1.1B**) is defined as the process by which live food organisms are enriched with specific nutrients or drug molecules and fed to the target organisms (Aruvalsu *et al*. 2012). Drugs that were successfully delivered using this technique are oxytetracycline (Touraki *et al*. 1995; Gomez-Gil *et al*. 2001; Langdon *et al*.

2003), oxolinic acid (Yahyazadeh *et al*. 2007; Touraki & Niopas, 2012) florfenicol (Roiha *et al*. 2010) and metronidazole (Rodriguez *et al*. 2011).

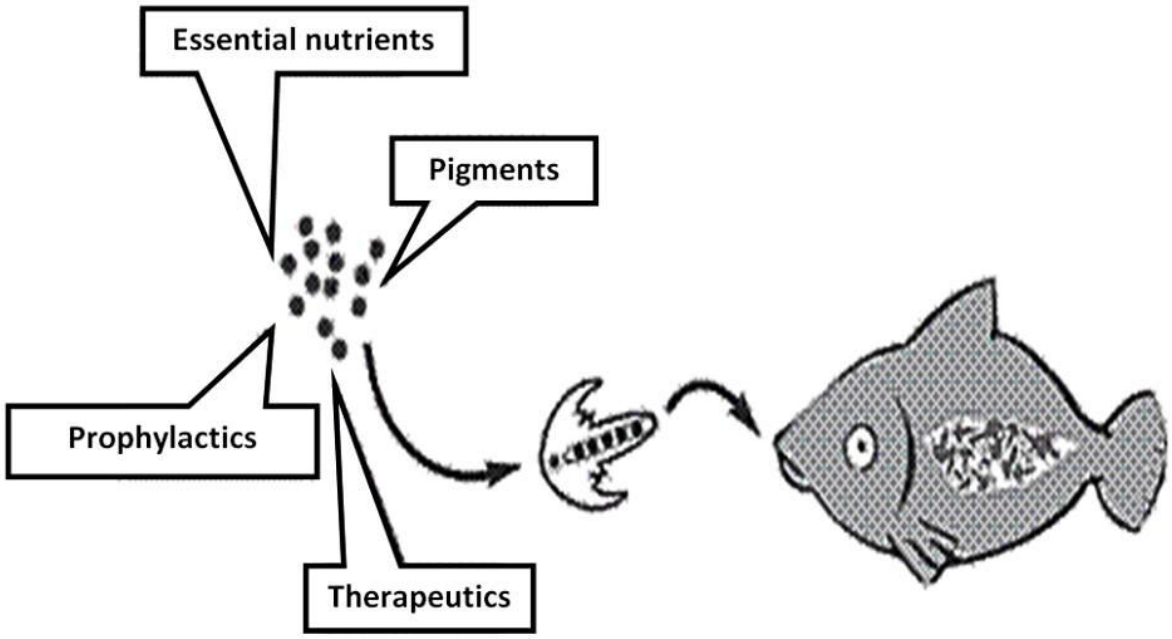

Figure 1.1B Delivery of essential nutrients, therapuetants, pigments etc. to target fish larvae using *Artemia* nauplii via bioencapsulation technique.
(http://images.engormix.com/e_articles/European_finfish05.gif)

1.2. Green Tea

Apart from water, *Camellia sinensis* (L.) is probably the most widely consumed beverage in the world (Muktar & Ahmad 2000; Anesini *et al*. 2008). The annual production of tea is around 4.7 million tonnes per year as of 2011 with China as the leading producer (**Figure 1.2A** FAOSTAT). Tea comes from the leaves of the plant *Camellia sinensis* a member of the *Theaceae* family and is broadly categorized into three types green, oolong and black tea

based on the level of fermentation. During production of green tea, unlike the other two varieties, the leaves are rolled immediately after harvest with heat to inactive the polyphenol oxidase that is capable of oxidizing tea catechins and thus it is less fermented (Cheng 2006).

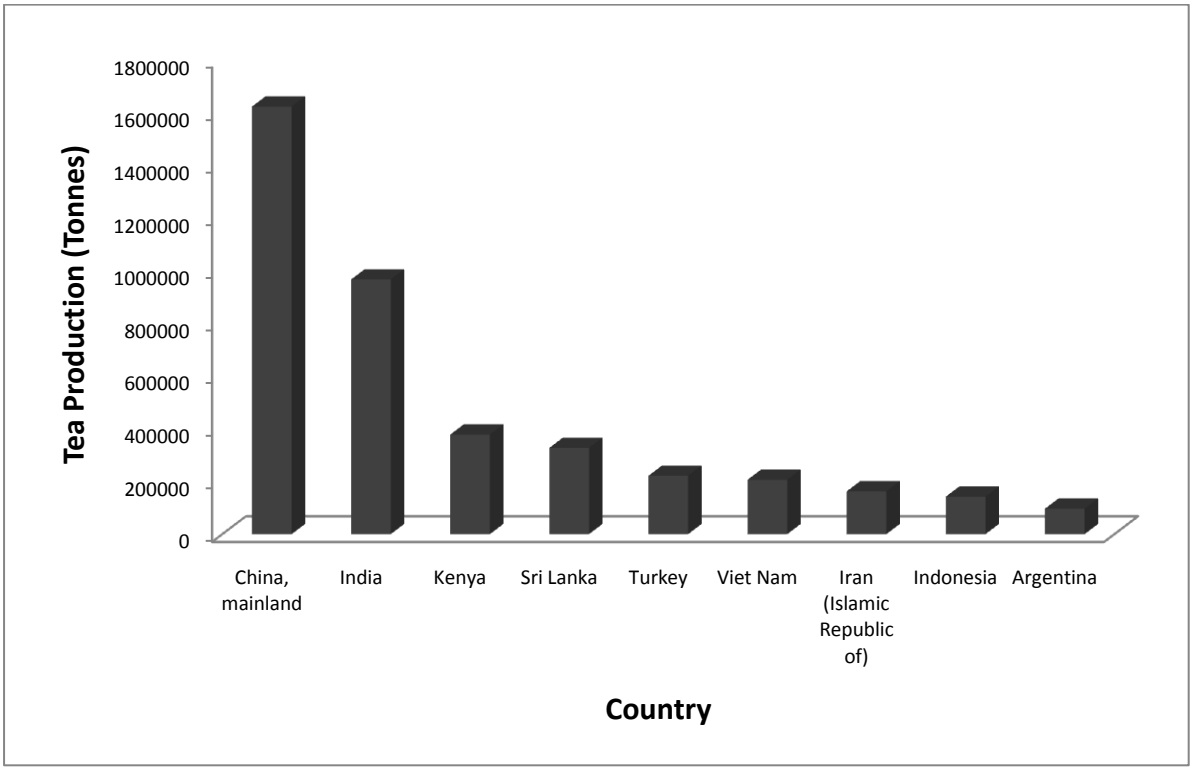

Figure 1.2A Principal tea producing countries in 2011 (FAOSTAT).

Green tea antioxidants

Tea polyphenols are natural antioxidants and considered to be responsible for the anticarcinogenic and antimutagenic properties of tea, as well as protective action against cardiovascular diseases (Tanizawa *et al*. 1984; Shahidi & Wanasundara 1992; Tijburg *et al*. 1997; Wiseman *et al*. 1997; Farhoosh 2007). The high antioxidant capacity of tea extracts are due to the presence of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate (**Figure 1.2B**) (Salah *et al*. 1995). Dry leaf of green tea contains about 8-15% of catechins (Goto *et al*. 1996; Horzic *et al*. 2012).

Green tea has been reported to have higher antioxidant activity and a greater level of polyphenols compared to black tea (Koo & Cho 2004). They have even stronger antioxidant capacity compared to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and DL-α-tocopherol; and the toxicity of tea polyphenols is lower than that of BHA, BHT and DL- α tocopherol (Chen & Wan 1994; Farhoosh 2007).

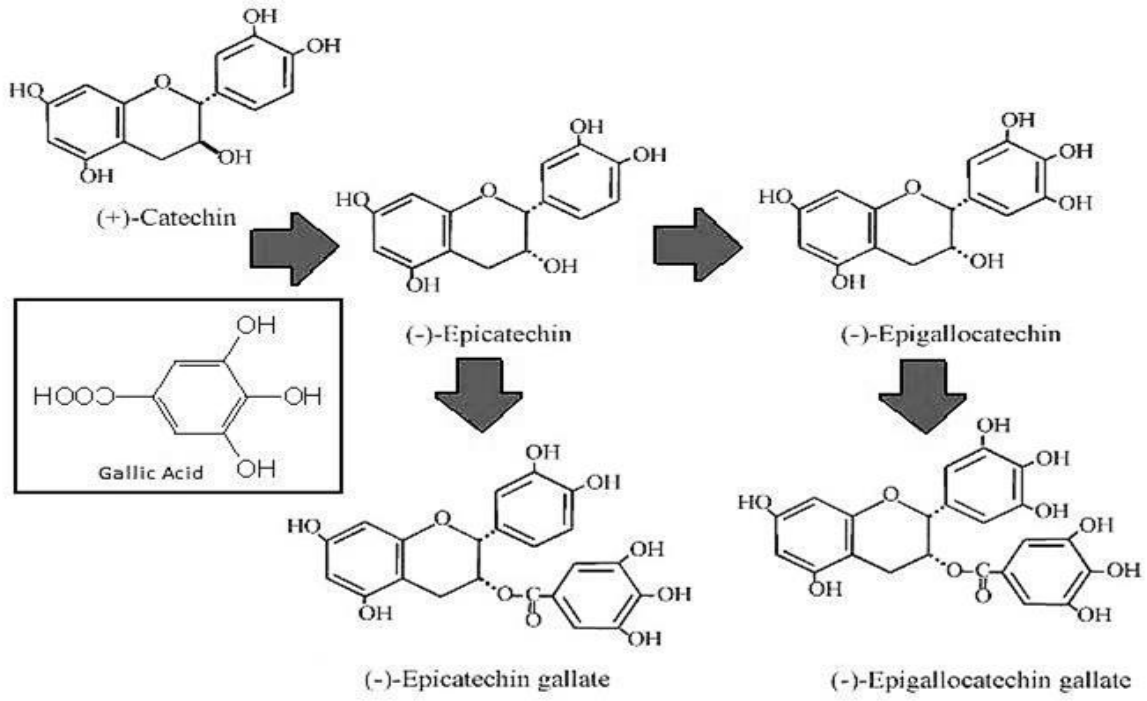

Figure 1.2B Structure of tea catechins and polyphenols (Higdon & Frei 2003).

Green tea extract polyphenols can act as antioxidant by quenching free radicals, inhibiting formation of free radicals and/or interrupting the propagation of free radicals and by acting as chelators for metal ions (Graham 1992; Chen & Chan 1996). The hydroxyl group present on the polyphenolic compounds in green tea can interrupt propagation of free radical autooxidation chain by donating a hydrogen atom to stabilize the free radical (Kaur & Kapoor 2001). The mechanism of a typical phenol antioxidant is illustrated in **Figure 1.2C**.

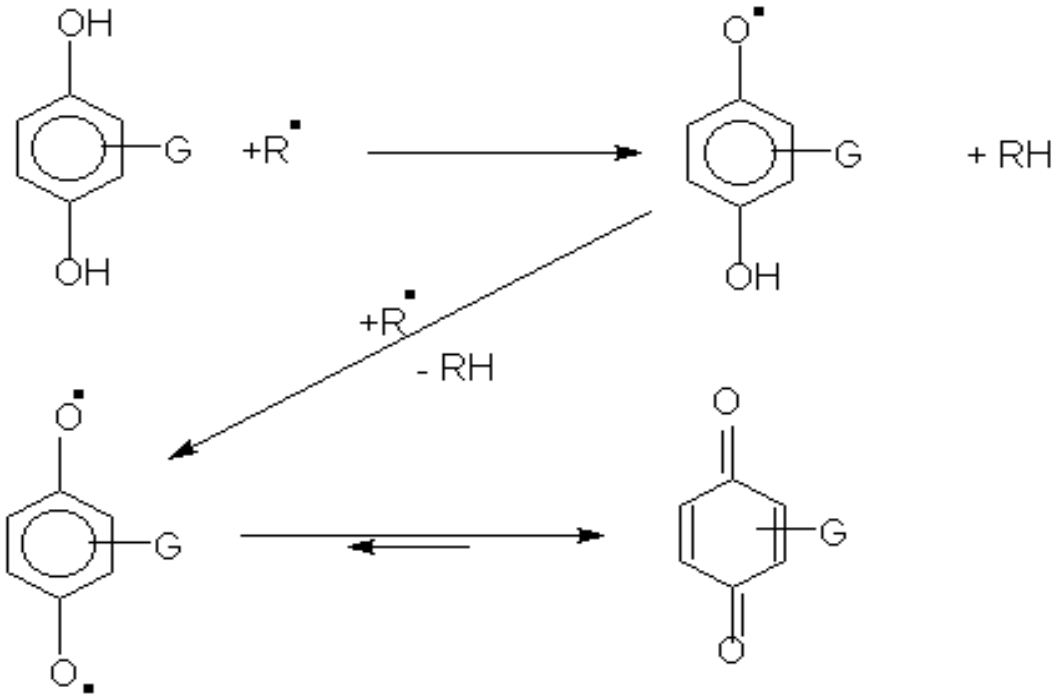

Figure 1.2C The mechanism of a typical phenol antioxidant. (http://journeytoforever.org/biofuel_library/chemoils.html)

Application of green tea in aquaculture

Many studies in the past have indicated green tea to be a potent antioxidant. The antioxidant properties have been applied to food industries, pharmacological studies, cosmetics and recently in aquaculture. Compared to other industries, application of green tea in aquaculture still lacks enough information for it to be established as effective antioxidant. Previous studies utilized green tea as immunostimulant by incorporating it in fish diet that can increase disease resistance, improve survival rate, growth rate and antioxidant system (Sheikhzadeh *et al*. 2011). However, this method can only be used when the receiving animal starts feeding on formulated diets.

1.3. Freshwater Ornamental Production

Freshwater ornamental farming maybe regarded as one of the emerging industry nowadays due high demand of ornamental fishes as decoration or as food. Since 1985, the international trade in exports of ornamentals followed an increase trend with an average growth rate of approximately 14% per year (Aruvalsu *et al*. 2012). In 1998, statistical reports to FAO shows 257 million US$ import values for ornamental fish (www.fao.org/fishery/topic/13611/en).

Because majority (>90%) of freshwater ornamental fish are capture based, industry NGO's have taken steps to educate importers, retailers and consumers on the proper handling to ornamental fish to minimize environmental risks (www.fao.org/fishery/topic/13611/en). Cultivation can help sustain the ornamental fish industry, restore exploited and minimize future use conflicts (HBOI 2000; Tlusty 2002).

Improvement of stress resistance must also be addressed since unlike food fish; exposure of ornamental fish to stresses does not end in the production period at the aquaculture farm. Their marketing involves shipment of densely packed fish, stressful conditions that often last for about 2 days (Dagar *et al*. 2010). At their new location, they are often exposed to new conditions and are relocated several times until reaching their final destination, often a home aquarium. The new environment poses environmental challenges and also exposure to different infectious diseases. Fish that have low stress resistance cannot withstand the stresses encountered during the transportation process, leading to relatively high post-shipment mortality within a week of arrival (Lim *et al*. 2002).

Guppy

Guppy is probably one of the easiest fishes to breed in captivity and is also the number one freshwater ornamental fish species imported into the United States accounting for

approximately 25% of the freshwater ornamentals fishes (Chapman *et al*. 1997). Guppies are live-bearing that are native of streams and rivers of South America (Hoade 1997). Though guppies are freshwater aquatic animals, they can survive for two to four hours after direct transfer to full strength sea water (Hopkins 2005). Wild guppies feed on algal remains, diatoms, invertebrates, plant fragments, mineral particles, aquatic insect larvae, and others.

Neon Tetra

Neon tetra, *Paracheirodon innesi* is one of the most popular among the aquarium hobbyist and fetches good price in ornamental fish market. Neon tetra belongs to the genus *Paracheirodon* in the family *Characidae*. They inhabit the streams of the Rio Putumaya in Peruvian Amazon near Iquitoes and Yarrapa River. In past, during a single month, an average of 1.8 millions of neon tetra, with an estimated value of US$ 1, 75,000 were imported into United States for aquarium trade (Chapman *et al*. 1997; Sanaye *et al*. 2012). Wild neon tetras are omnivorous and feed on plant matter as well as o crustaceans, worms and small insects. In captivity, neon tetras are considered quite difficult to spawn because it requires perfect-water conditions. Majority of neon tetras found in pet shops are wild caught or bred by large breeding companies (http://www.aquaticcommunity.com/tetrafish/neontetra.php).

1.4. Aim and Outline of the Thesis

To satisfy the demand of commercial ornamental fish trade, aquaculturist shift to intensive production. During intensification, especially in larval stages, proper nutrition and medication must be provided substantially to minimize development of microbial diseases to obtain higher survival rate. In hatchery production, an easy and straightforward way of confronting this is to provide proper chemotherapeutants to fish larvae through the food chain by using bioencapsulation technique (Touraki *et al*. 1996). But since majority of exporters are from developing countries, economic costs are usually taken into consideration. Use of

chemotherapeutants is limited because they are expensive and sometimes not available in some countries. Also, with emerging environmental risks, interest in utilization of plant extract as an alternative drug for aquatic animals receives growing interest.

The plant (plant products) to be used must cheap, locally available or have the capability to be produced in the same country as the farms to enhance local economy. Among numerous plants, green tea as plant material probably has many advantages. Green tea as the second most consumed beverage around the world is not only available in all countries, but safe to use as well for aquatic animals. The high amount of polyphenols, especially catechins have long been proven to have many beneficial side effects on human, but limited on aquaculture. Presently, no data was recorded on the effect of green tea enrichment on *Artemia* nauplii as well as its direct application on freshwater ornamental fishes. The general objective of this study was to evaluate green tea enriched *Artemia* nauplii as food and antioxidant activity enhancer for freshwater ornamental fishes.

In Chapter 1, an overview is presented about the importance of *Artemia* nauplii in aquaculture, green tea polyphenols and its antioxidant activity and the possible application in freshwater ornamental production.

Chapter 2 incorporated green tea extract to *Artemia* nauplii to improve its total polyphenol content, total carotenoids and antioxidant activity via bioencapsulation technique. Best enrichment condition was determined according to time exposure of *Artemia* nauplii to green tea extract and based on different naupliar stages of *Artemia*. Green tea extract enriched *Artemia* nauplii were applied to guppy fry (Chapter 3) and influence of fish size (Chapter 4) was evaluated for its effect on growth performance and resistance against salinity stress.

Chapter 5 was conducted to make sure that feeding of green tea extract enriched *Artemia* nauplii only enhanced the antioxidant activity and not affect the biochemical and digestive enzyme composition of neon tetra and guppy.

In chapter 6, the main results obtained in the previous chapters were discussed and conclusion was drawn throughout the thesis based on the framework of the research objectives. Additionally, the perspectives for further research are outlined.

CHAPTER 2

Improvement of total polyphenol content, total carotenoid and antioxidant activity of *Artemia salina* nauplii with green tea extract

2.1. Introduction

Artemia nauplii are used extensively worldwide as live food for the larval stages of commercially important freshwater and marine fish species due to their availability, low cost, ease of culture and biochemical composition (Kolkovski *et al*. 1997; Sorgeloos *et al*. 1998; Smith *et al*. 2004). The non-selective feeding behavior of *Artemia* also makes this organism a good biological carrier for transferring essential nutrients to predator larvae using bioencapsulation technique (Leager *et al*. 1986; Citarasu *et al*. 1998; Immanuel *et al*. 2001, 2004). The naupliar stages of *Artemia* are normally used in bioencapsulation because they can effectively filter feed small particulate up to 30 µm in size (Dobbleleir *et al*. 1980; Smith *et al*. 2004). During bioencapsulation, desired essential nutrients dissolved in water are ingested by nauplii with minimal amount of leakage when given to the fish or shrimp larvae. Thus, this technique is also developed not only for improving the quality of *Artemia* but for delivering water soluble antibacterial agents like oxytetracycline (Touraki *et al*. 1995; Gomez-Gil *et al*. 2001; Langdon *et al*. 2003), oxolinic acid (Yahyazadeh *et al*. 2007; Touraki & Niopas 2012) florfenicol (Roiha *et al*. 2010) and metronidazole (Rodriguez *et al*. 2011) to treat fishes affected by bacterial diseases in larval hatcheries. With emerging environmental risks of using artificial or synthetic drugs, interest in the utilization of plant extract as an alternative drug for aquatic animals increases.

Plants contain phenolic compounds that have outstanding antioxidant and free radical scavenging properties (Hrelia *et al*. 2002). Antioxidants have shown to have multiple functional and remedial properties that include anti-radical, anti-carcinogenic, anti-inflammatory oxidative stress reduction, and cardio protection (Chan 2010). In addition to polyphenols, carotenoids present in plants were reported to serve as antioxidants and as source of vitamin A for animals (Ong & Tee 1992; Miki 1991; Briton 1995; Ni *et al*. 2008).

Among the numerous plants exhibiting potential source of natural antioxidants, tea is an excellent source of polyphenol antioxidants (Hrelia *et al*. 2002). Tea is a well-consumed beverage worldwide mainly because of the many beneficial health effects it has on human. Compared to black tea, green tea has been reported to have higher antioxidant capacity and a greater level of polyphenols (Koo & Cho 2004). Aside from polyphenols, green tea also contains fibers, proteins, carbohydrates, fat, peptides, minerals and organic acid (Sato & Miyata 2000; Bae & Lee 2010). In aquaculture application, previous studies utilized green tea as immunostimulant by incorporating it in fish diet that can increase disease resistance, and improve survival rate, growth rate and antioxidant system (Sheikhzadeh 2011). However, this method can only be used at the stage when the receiving animal starts feeding on formulated diets.

For fish larvae, delivery of natural antioxidants from green tea can be done by first extracting them from the green tea leaves using water, then enrich the *Artemia* nauplii through bioencapsulation, and finally feed the nauplii to the larvae. The advantages of this method are its simplicity, lower economic cost and eco-friendliness. However, before giving it as a food it is important to know first if green tea extract was indeed enriched to *Artemia* nauplii during bioencapsulation. The aim of this paper was to determine the improvement of green tea extract to the total polyphenol content, total carotenoid and antioxidant capacity of *Artemia* nauplii. The study also determines the best condition of enriching green tea antioxidants to *Artemia* nauplii.

2.2. Materials and Methods

2.2.1. Sample preparation of green tea extract

A pack of commercial green tea leaves was purchased from a local supermarket in Tsukuba City, Japan. Tea sample was pulverized using coffee grinder and sieved at 120 µm. Green tea-water-infusion was prepared based on the method used by Komes *et al*. (2010). Tea powder was mixed with distilled water (1 mg/mL, w/v) at 80^{o}C and slowly stirred for 5 minutes. After extraction, the solution was cooled to room temperature and sieved at 120 µm. Salinity of tea extract was adjusted to 3.50% by adding artificial seawater powder and reserved for enrichment experiment.

2.2.2. Hatching of *Artemia*

Artemia salina cysts were obtained from a commercial supplier (A&A Marine) which originally came from Salt Lake, Utah USA. Hatching of cysts were carried out in 2 g cysts/300 mL of 3.50% artificial sea water (Benijts *et al*. 1975). The 24h-incubation was conducted in thermo-controlled room set at 25^{o}C with light and vigorous aeration. Nauplii were separated from cyst shell for enrichment with green tea.

2.2.3. Bioencapsulation of *Artemia* nauplii with green tea water extract

2.2.3.1. Green tea bioencapsulation over time

Newly hatched *Artemia* nauplii were enriched for 24 hours with green tea water extract prepared as mentioned above. Samples were collected after 0, 3, 6, 12 and 24 hour's enrichment period. Enriched *Artemia* nauplii were sieved at 120 µm, washed with tap water, and then with distilled water, blot dried in paper towel and finally kept at -30^{o}C for further analysis.

2.2.3.2. Green tea bioencapsulation using different naupliar stages of *Artemia*

Newly hatched *Artemia,* instar I and instar II naupliar stages (typically 0, 8 and 12 hours after hatching) were enriched in previously prepared green tea extract (GTE) at 3.50% salinity (Benjits *et al*. 1975; Rodrigues *et al*. 2011). Enrichment period was conducted for 6 hours, for it showed high results on a previous study by the team (not published), at thermo-controlled chamber set at 25^{o}C with light and vigorous aeration. Enriched *Artemia* nauplii were collected by sieving with 120-µm sieve, washed with tap water, then with distilled water, blot dried in paper towel and finally kept at -30^{o}C for further analysis.

2.2.4. Determination of total polyphenol content (TPC)

Extraction of TPC from *Artemia* nauplii was based on the method used by Anesini *et al*. (2008) where about 0.2 g of each sample was weighed in an extraction tube, and 5 mL of 70% methanol at 70^{o}C was added. The extract was mixed using a vortex mixer and heated at 70^{o}C for 10 min. After cooling to room temperature, the extract was centrifuged at 200 g for 10 min. The supernatant was decanted in a graduated tube. The extraction step was repeated and extracts were pooled and the volume was adjusted to 10 mL with cold 70% methanol. Estimation of TPC was done by colorimetric assay based on procedures described by Komes *et al*. (2010). Sample (50 µL) was mixed with 2.5 mL Folin Ciocalteu's phenol reagent, 0.75 mL of 20% sodium carbonate and diluted to 5 mL. After 2 hours, the absorbance of blue color solution was read at 725 nm using UV-VIS spectrophotometer (Ultrospec 3300 Pro, Amersham Biosciences). Amount of TPC was calculated using linear regression with gallic acid as standard reference (0-1000 mg L^{-1}). The results are expressed as mg gallic acid equivalents (GAE) per g of dry sample.

2.2.5. Determination total carotenoids

Extraction of pigment using acetone in nauplii was based on the method used by Buyukapar & Yanar (2007), where about 10 mL acetone and 2 g of anhydrous sodium sulfate were added to 1.0 g of *Artemia* sample. The solution was mixed with a vortex mixer, and was then centrifuged at 5,000 rpm for 5 minutes and stored for three (3) days at 4^{o}C in a refrigerator. After three (3) days of extraction, absorption of extract was measured at 470, 645 and 662 nm using UV-VIS spectrophotometer. The concentrations of C*a*, C*b* and total carotenoids were determined according to the equations reported by Lichtenthaler & Wellburn (1983) as follows:

$$C_a\ (\text{mg L}^{-1}) = 11.75\ Abs_{662} - 2.350\ Abs_{645}$$

$$C_b\ (\text{mg L}^{-1}) = 18.61\ Abs_{645} - 3.960\ Abs_{662}$$

$$\text{Total carotenoids (mg L}^{-1}) = 1000\ Abs_{470} - 2.270\ C_a - 81.4\ C_b/227$$

2.2.6. Determination of antioxidant activity

2.2.6.1.1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay

Scavenging activities of the extracts on the stable free radical DPPH were assayed based on the modified Blois method used by Bae & Lee (2010) in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample. A volume of 0.5ml of the sample extract was mixed with 4.5 mL of 0.41 mM DPPH solution in absolute ethanol. The mixture was kept for 30 minutes and then the absorbance was measured at 517 nm in UV-VIS spectrophotometer. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH scavenging activity was calculated using linear regression with L-ascorbic acid as standard reference (0-1000 mg L^{-1}). The results are expressed as mg vitamin C equivalents (VCE) per g of dry sample.

2.2.6.2 Total antioxidant (TA) activity

TA activity of the samples was analyzed according to the method used by Prieto *et al*. (1999) and Prasad *et al*. (2009). In brief, a 0.1 mL-aliquot of the sample was mixed with 1 mL of the reagent solution (0.6 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and then incubated at 95^{o}C for 90 min. After the samples were cooled to 25^{o}C, the absorbance was measured at 695 nm against a blank. The blank contained 1 mL of the reagent solution without the sample. The total antioxidant activity was expressed as the absorbance of the sample. The higher absorbance value indicates the higher antioxidant activity. The total antioxidant activity was calculated using linear regression with L-ascorbic acid as standard reference (0-1000 mg L^{-1}). The results are expressed as mg vitamin C equivalents (VCE) per g of dry sample.

2.2.7. Statistical Analyses

Differences between treatments were performed using one-way analysis of variance ANOVA with Duncan's multiple range tests. The level of significance for all analyses was $p<0.05$. All treatments were conducted in triplicate unless specified. Results are all reported as dry matter and presented as mean ± standard error mean.

2.3. Results

Total polyphenol content

Results of green tea bioencapsulation on TPC of *Artemia* nauplii over time and using different naupliar stages are presented in **Figure 2.1A** and **2.1B**. TPC of newly hatched *Artemia* enriched over time for 24-hrs increases significantly ($p<0.05$) after 3-hrs and 6-hrs. At these enrichment periods, GTE-enriched nauplii are significantly ($p<0.05$) higher than their control. At 12-hrs and 24-hrs, results of TPC did not significantly differ from results

obtained at 3-hrs (control and GTE-enriched) and 6-hrs control. The highest TPC was observed at GTE-enriched nauplii after 6-hrs of enrichment amounted to 8.44 ± 0.65 (mg GAE g^{-1} $sample^{-1}$).

TPC of *Artemia* enriched for 6hrs increases significantly (p<0.05) with the age of nauplii. Instar II nauplii contains the highest amount of TPC with 18.78 ± 0.45 (mg GAE g^{-1} $sample^{-1}$) and 20.08 ± 0.97 (mg GAE g^{-1} $sample^{-1}$) for control and GTE-enriched, respectively. However, no significant difference was observed between control and GTE-enriched nauplii at all stages.

Total carotenoids

Total carotenoids of the acetone extract *Artemia* nauplii enriched with GTE over time and at different naupliar stages are summarized in **Table 1** and **Table 2**, respectively. Control *Artemia* nauplii generally increase over time for 24-hrs while no trend can be found in GTE-enriched samples. Development of bright orange color of *Artemia* nauplii is probably affected by the green color of GTE. The total carotenoid content of different naupliar stages of *Artemia* enriched for 6-hrs increases also with age of nauplii for both control and GTE-enriched nauplii. Total carotenoids of instar II nauplii for control (112.63 ± 2.00 mg g^{-1} $sample^{-1}$) and GTE-enriched (115.34 ± 4.86 mg g^{-1} $sample^{-1}$) are significantly (p<0.05) higher than newly hatched and Instar I nauplii. Although total carotenoids of GTE-enriched are higher than control no significant difference was observed. In this case, chlorophylls (*a* and *b*) from GTE may have a contribution to the total carotenoid values.

Antioxidant activities

In the present study, antioxidant capacities of polyphenols extracted with 70% methanol were determined by DPPH-activity and TAA. Figure **2.2A** shows the DPPH-activity of *Artemia*

nauplii enriched with GTE over time for 24-hrs. Generally, DPPH free radical scavenging activity of GTE-enriched nauplii is higher than their control at all time. Highest DPPH free radical scavenging activity was observed after 6-hrs enrichment on GTE-enriched nauplii which contains 42.79 ± 2.36 (mg VCE g^{-1} $sample^{-1}$). The trend of DPPH free radical scavenging activity is similar with TPC, where highest value was observed at GTE-enriched nauplii after 6-hrs enrichment.

The DPPH free radical scavenging activity of GTE-enriched nauplii of the different naupliar stages of *Artemia* (see **Figure 2.2B**) are significantly ($p<0.05$) higher than their corresponding control. Highest results were observed at GTE-enriched nauplii of instar I and instar II containing 71.64 ± 2.50 (mg VCE g^{-1} $sample^{-1}$) and 73.04 ± 1.95 (mg VCE g^{-1} $sample^{-1}$), respectively.

TA activity of *Artemia* nauplii over time and at different naupliar stages are shown in **Figure 2.3A and B**. TA activities of GTE-enriched *Artemia* are higher than their corresponding control within 24-hrs enrichment, but no significant difference was observed. Unlike DPPH free radical scavenging activity, highest TA activity was observed significantly ($p<0.05$) after 24-hrs enrichment for control (34.00 ± 2.92 mg VCE g^{-1} $sample^{-1}$) and GTE-enriched (37.21± 0.95 mg VCE g^{-1} $sample^{-1}$). This result was probably influenced by the amount of total carotenoids, wherein, it also increases over time.

Enrichment using different naupliar stages also shows that GTE-enriched nauplii are higher than their control. Instar II of GTE-enriched nauplii contains highest TA activity of 18.08 ± 1.08 (mg VCE g^{-1} $sample^{-1}$), which is significantly ($p<0.05$) higher than other samples.

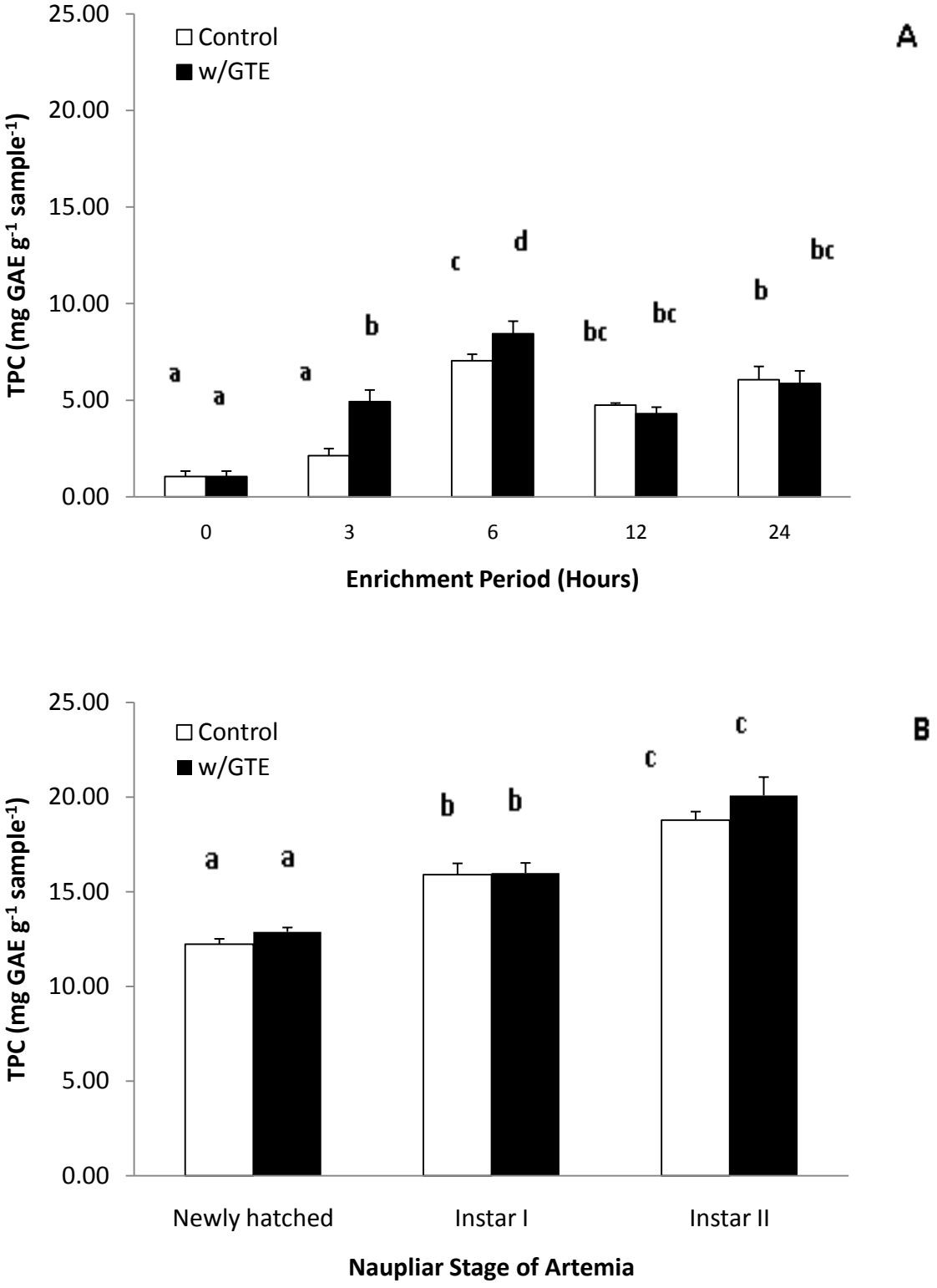

Figure 2.1 Total polyphenol content (TPC) of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. Each value is expressed as mean ± standard error mean (n=3). Values with different letter are significantly different (p<0.05) between the samples.

Table 2.1 C*a*, C*b* and total carotenoid of green tea enriched *Artemia salina* nauplii extracted with acetone over time.

Naupliar Stages of *Artemia*	C*a* (x 10^{-2}) (mg g^{-1} $sample^{-1}$)		C*b* (x10^{-2}) (mg g^{-1} $sample^{-1}$)		Total Carotenoid (mg g^{-1} $sample^{-1}$)	
	Control	w/GTE	Control	w/GTE	Control	w/GTE
Newly Hatched	0.03 ± 0.03^{a}	0.53 ± 0.04^{b}	0.12 ± 0.10^{a}	0.38 ± 0.82^{ab}	83.46 ± 1.41^{a}	92.72 ± 2.21^{ab}
Instar I	$0.18 \pm 0.0.06^{a}$	1.15 ± 0.02^{c}	$0.38 \pm 0.1.80^{ab}$	0.44 ± 0.03^{ab}	92.43 ± 3.98^{ab}	99.79 ± 1.82^{b}
Instar II	0.07 ± 0.00^{a}	1.43 ± 0.08^{d}	0.16 ± 0.60^{a}	0.71 ± 1.79^{c}	112.62 ± 1.98^{c}	115.34 ± 4.86^{c}

Each value is expressed as mean ± standard error mean (n=3). Values with different letter in column are significantly different (p<0.05) between the samples.

Table 2.2 C*a*, C*b* and total carotenoid of green tea enriched *Artemia salina* nauplii extracted with acetone at different naupliar stages.

Enrichment Period	C*a* (x 10^{-2}) (mg g^{-1} $sample^{-1}$)		C*b* (x10^{-2}) (mg g^{-1} $sample^{-1}$)		Total Carotenoid (mg g^{-1} $sample^{-1}$)	
	Control	w/GTE	Control	w/GTE	Control	w/GTE
0-Hour	0.16 ± 0.08^{a}	0.16 ± 0.08^{a}	0.39 ± 0.16^{a}	0.39 ± 0.16^{a}	71.88 ± 1.78^{ab}	71.88 ± 1.78^{ab}
3-Hour	2.31 ± 0.15^{b}	4.29 ± 0.15^{bc}	3.81 ± 0.29^{ab}	6.69 ± 0.24^{bc}	69.67 ± 1.95^{a}	84.32 ± 1.03^{cd}
6-Hour	3.22 ± 0.14^{b}	7.11 ± 2.65^{d}	5.40 ± 0.23^{ab}	10.92 ± 4.30^{c}	81.15 ± 1.91^{bcd}	83.37 ± 2.02^{cd}
12-Hour	4.31 ± 1.02^{bc}	4.09 ± 0.55^{bc}	7.24 ± 1.733^{bc}	5.96 ± 1.31^{ab}	87.14 ± 2.49^{d}	74.97 ± 7.92^{abc}
24-Hour	5.64 ± 1.45^{bc}	4.06 ± 8.57^{bc}	8.35 ± 2.54^{bc}	4.81 ± 1.61^{ab}	81.74 ± 1.82^{bcd}	77.81 ± 1.61^{c}

Each value is expressed as mean ± standard error mean (n=3). Values with different letter in column are significantly different (p<0.05) between the samples.

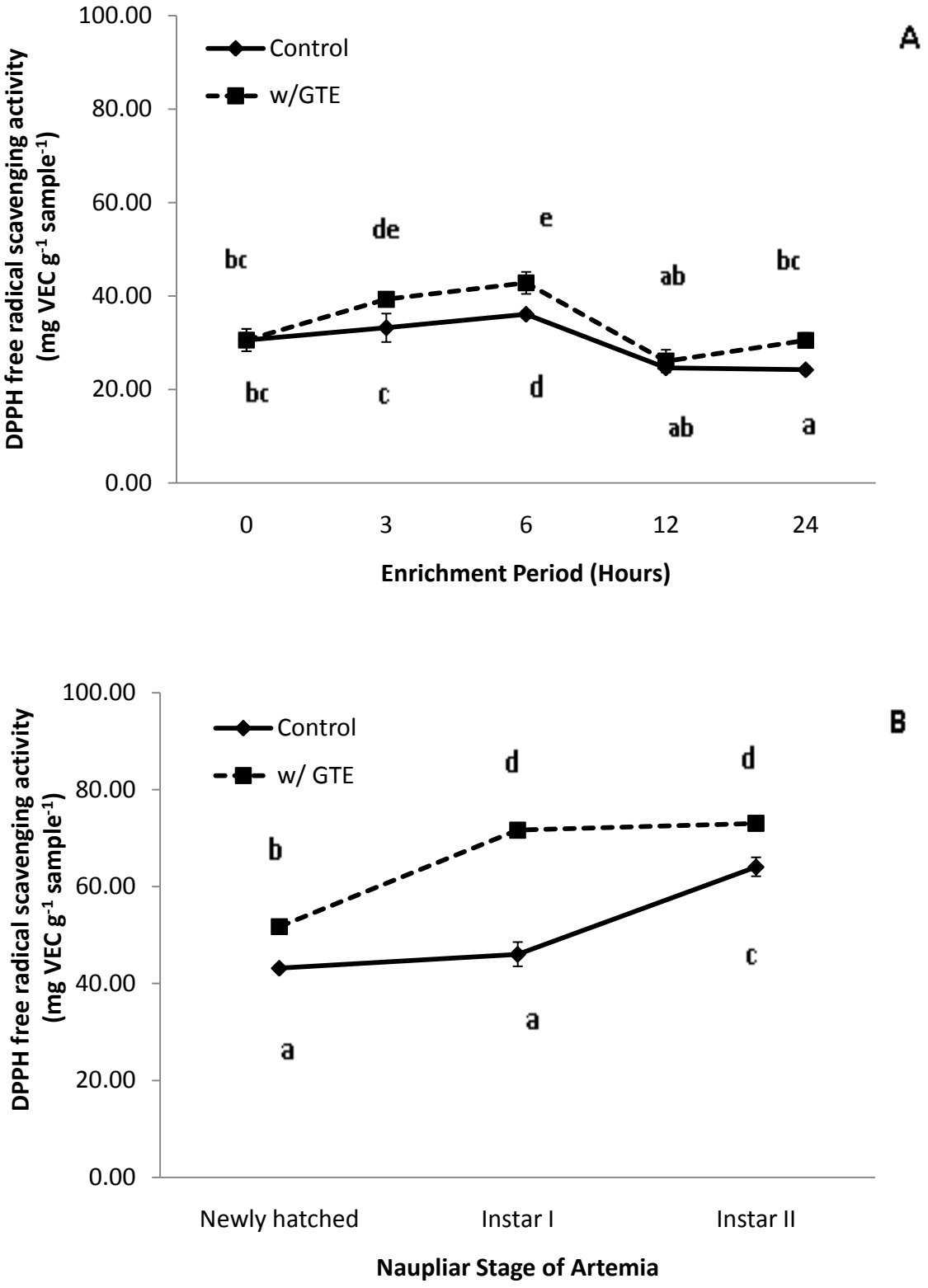

Figure 2.2 DPPH free radical scavenging activity of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. Each value is expressed as mean ± standard error mean (n=3). Values with different letter are significantly different (p<0.05) between the samples.

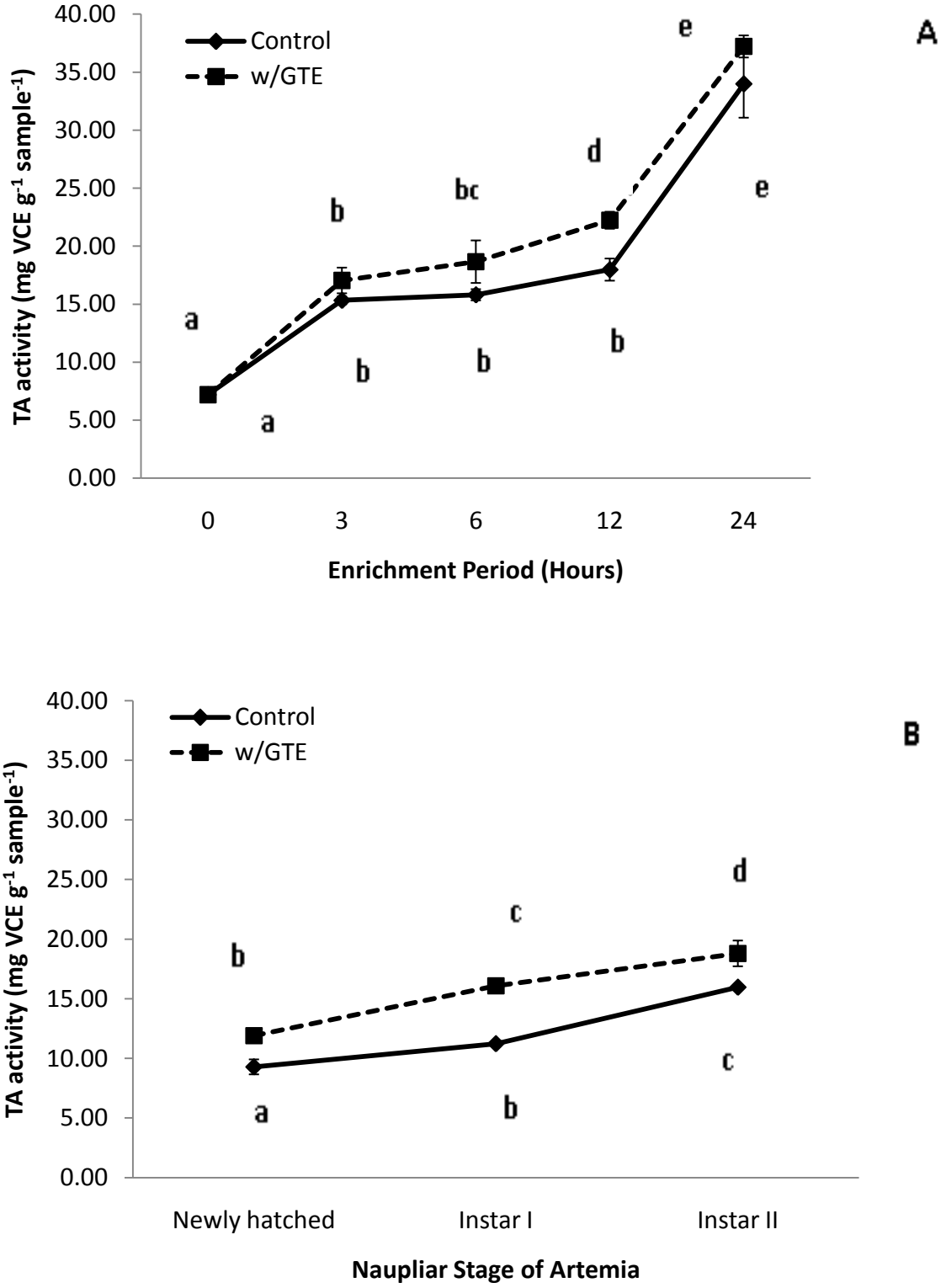

Figure 2.3 Total antioxidant (TA) activity of green tea enriched *Artemia salina* nauplii A) over time B) at different naupliar stages. Each value is expressed as mean ± standard error mean (n=3). Values with different letter are significantly different (p<0.05) between the samples.

2.4. Discussion

In the current study, bioencapsulation of green tea is not only done for the enhancement of antioxidant content of *Artemia* nauplii, but also, it is a way of preventing bacterial transfer carried by live food. The amount of 1 mg/mL (w/v) of green tea powder was proven by Sornsanit *et al*. (2002) to treat *Vibrio*-infected shrimp culture water. *Vibrio* is known to cause mortality in larvae, post larvae, and juveniles of up to 100% of the affected population (Sunaryanto & Mariyam 1987; Sarjito *et al*. 2012).

Presently, there are no data in literature about the antioxidant activities of *Artemia* nauplii, i.e. on what causes it. In plants, on the other hand, it is known that the antioxidant activity of plant materials was due to the presence of phenolics, carotenoids and flavonoids (Barros *et al*. 2007; Prasad *et al*. 2009). Polyphenol compounds play an important role in adsorbing and neutralizing free radicals, quenching singlet oxygen, and decomposing peroxides by donating their hydrogen (Ksouri *et al*. 2007). Without antioxidants like polyphenol, this group of radicals may interact with biological systems in a clearly cytotoxic manner (Barros *et al*. 2007).

In the present study, bioencapsulation of green tea extract to *Artemia* nauplii shows that amount TPC was more associated to age of nauplii than time of exposure. Based on the result of enrichment over time, TPC values increases proportionally with time up to 6-hrs, drops after 12-hrs, then increase again after 24-hrs. This result was obtained probably because newly hatched *Artemia* are non-feeding nauplii and the high amount of TPC up to 6-hrs is due to the nutrition from the stored egg yolk and the TPC attached to the cuticle. The drop in TPC after 12-hrs enrichment is perhaps because the nutrition stored at egg yolk was already utilized by the nauplii. And after 24-hrs TPC increased again, because at this time nauplii already molted to instar II and starts to filter feed.

The TPC of different naupliar stages enriched for 6-hrs proportionally increases with age of nauplii. Higher results using instar II nauplii can be attributed to the fact that at this stage, nauplii already develop a functional digestive system and can obtain their sustenance and other extraneous substances by filter feeding on particles from the water (Makridis & Vadstein 1999; Rodriguez *et al*. 2011). This result agrees with the results obtained by Rodriguez *et al*. (2011) where instar II *Artemia* nauplii also accumulated highest levels of metronidazole than instar I. Instar II nauplii was also used by Immanuel *et al*. (2004) for *Odonus niger* lipid enrichment.

Generally, there was no significant difference observed in TPC between control and GTE-enriched samples regardless of length of exposure and age of nauplii (except for 3-hrs and 6-hrs), but their DPPH free radical scavenging activity and TA activity was statistically different. The improved DPPH free radical scavenging activity and TA activity on GTE-enriched samples can be attributed to polyphenols transferred from green tea to *Artemia* nauplii during bioencapsulation. The carotenoids present in *Artemia* nauplii also contributes to the antioxidant capacity. For control *Artemia* nauplii, the antioxidant activity of the sample is due to carotenoids present in the sample.

In conclusion, enrichment of green tea in *Artemia* nauplii using bioencapsulation technique can improve its total polyphenol, total carotenoids and antioxidant activities. But the best result was obtained when instar II was used in the 6-hr enrichment. With the current results, it will be interesting to investigate the effect of these GTE-enriched *Artemia* nauplii when applied to fish or shrimp larvae to improve their antioxidant capacity against stressful conditions.

CHAPTER 3

Effect of green tea enriched *Artemia* nauplii on growth performance, antioxidant activity and stress resistance of guppy *Poecilia reticulata* fry

3.1. Introduction

In commercial aquaculture, success in the hatchery production of fish fingerlings for stocking in the grow-out production system is largely dependent on the availability of suitable live food organisms for feeding fish larvae, fry and fingerlings (Salma *et al*. 2013). Though there have been many efforts to use artificial diets, live foods are still preferred due to the digestive enzymes they contain. These enzymes play an important role in promoting the digestion and even growth of fish, especially in stomachless fish larvae or in larvae initially without stomachs (Dabrowski 1979; 1982; Chen & Lin 1992). Among the live food organisms currently used, *Artemia* are ideal because of their availability, low cost, ease of culture and biochemical composition (Kolkovski *et al*. 1997; Sorgeloos *et al*. 1998; Smith *et al*. 2004).

Also, since *Artemia* are nonselective feeders, they can be enriched with nutrient supplements to enhance their nutritional value or with medications to provide health benefits for the animals to which they are to be fed (Lavens & Sorgeloos 1996; Touraki *et al*. 1996; Rodriguez *et al*. 2011). With the increasing demand for utilization of natural products over synthetic drugs, *Artemia* nauplii can be used as vehicle to deliver plant extract to promote growth and survival of the receiving aquatic animals. Plant extracts contain high antioxidants that can enhance the animal's antioxidant capacity which could consequently increase its resistance against stress (Pan *et al*. 2003; Chien & Shiau 2005; Angeles *et al*. 2009).

Plants contain phenolic compounds that have outstanding antioxidant and free radical scavenging properties (Hrelia *et al*. 2002). Antioxidants have shown to have multiple functional and remedial properties that include anti-radical, anti-carcinogenic, anti-inflammatory oxidative stress reduction, and cardio protection (Chan 2010). In addition to polyphenols, carotenoids present in plants were reported to serve as antioxidants and as source of vitamin A for animals (Ong &Tee 1992; Miki 1991; Briton 1995; Ni *et al*. 2008).

Green tea is an excellent source of polyphenol antioxidants, particularly of the group known as green tea catechins (Hrelia *et al*. 2002). Enrichment of green tea to *Artemia* nauplii is an attractive method because it is straightforward, inexpensive and contains low toxins since it is safe for human consumption. Incorporation of green tea extract and green tea powder as dietary supplement was reported by Kono *et al.* (2000), Ishihara (2002), Cho *et al.* (2007), Abdel-Tawwab (2010), Thawonsuwam *et al.* (2010) and Sheikhzadeh (2011). So far, no study was conducted yet on the application of green tea extract to fish fry or fish larvae via *Artemia* nauplii.

Guppy *Poecilia reticulata* is considered as one of the most popular species in the world due to its short generation interval, easiness to breed and wonderful color variations (Whitten 1979; Magurran 1982; Baboli *et al*. 2012). Improvement of guppy production and, at the same time, enhancement of their antioxidant capacity, for better growth performance and survival rate, can be started by supplying guppy fry or larvae with appropriate nutrition.

This study was conducted to provide information regarding the application of GTE-enriched *Artemia* nauplii as food for guppy fry to improve growth, antioxidant activity and stress resistance during production.

3.2. Materials and methods

3.2.1. Sample preparation of green tea extract (GTE)

A pack of commercial green tea leaves was purchased from a local supermarket in Tsukuba City, Japan. Tea sample was pulverized using coffee grinder and sieved at 120 µm. Green tea-infused water was prepared based on the method used by Komes *et al*. (2010). Tea powder was mixed with distilled water (1 mg/mL) at 80^{o}C and slowly stirred for 5 minutes. After extraction, the solution was cooled to room temperature and sieved at120 µm. Salinity

of the tea extract was adjusted by adding 3.50% artificial seawater powder and reserved for enrichment experiment.

3.2.2. Hatching of *Artemia*

Artemia salina cysts were obtained from a commercial supplier (A&A Marine) which originally came from Salt Lake, Utah, USA. Hatching of cysts were carried out with having 2 g cysts/300 mL of 3.50% artificial sea water (Benijts *et al*. 1975). The 24 h-incubation was conducted in a thermo-controlled room set at 25^{o}C with light and vigorous aeration. Newly hatched nauplii were separated from cyst shell and transferred to another container containing 1 L of 3.50% salinity water. Nauplii were incubated for another 12 hours to molt into the second stage (instar II).

3.2.3. Enrichment procedure

The instar II *Artemia* nauplii were separated from container using a 120 µm sieve and transferred to a previously prepared GTE at 3.50% salinity. Enrichment period was conducted for 6 hours, for it showed high results on a previous study conducted by the team (not published), at thermo-controlled chamber set at 25^{o}C with light and aeration. Enriched *Artemia* nauplii were collected, sieved at 120 µm, washed with tap water, and then with distilled water, blot dried in paper towel and finally kept at -30^{o}C. Presence of GTE in the gut of *Artemia* nauplii was observed under light microscope (**Figure 3.1**).

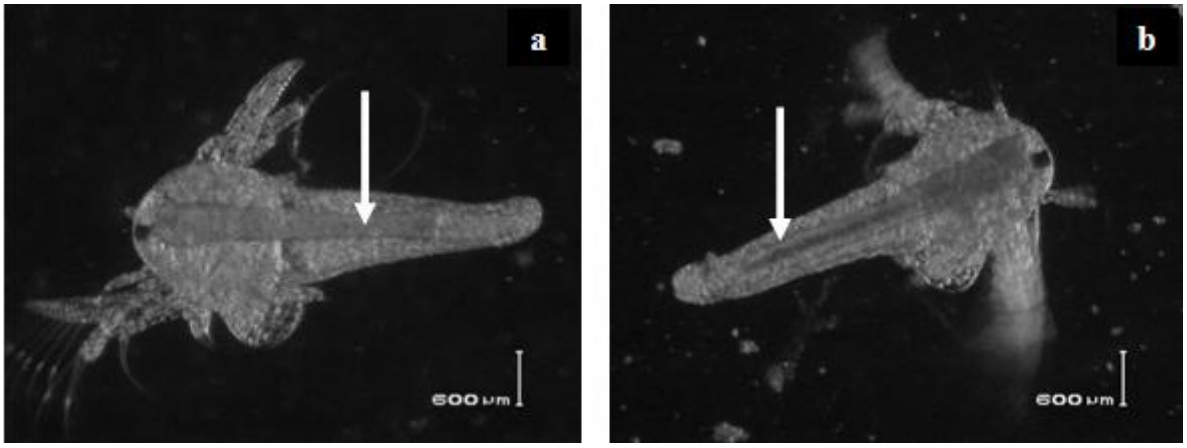

Figure 3.1 Photomicrograph showing the gut region of instar II *Artemia* nauplii after 6-hours enrichment without (a) and with green tea extract (b) at 3.50% salinity.

3.2.4. Fish culture and feeding experiment

Adult male and female guppy *Poecilia reticulata* were purchased from a commercial ornamental fish shop (Tsukuba City, Japan) and stocked in 250-L aquarium containing freshwater supplied with aeration. They were cultured in laboratory conditions until they produce fry for the experiment.

Day 0-old fry samples obtained from stocked guppies mentioned above were transferred in 10-L aquarium containing 5 L freshwater (10 fish per aquarium). Each aquarium was supplied with compressed air via air-stones using aquarium air pumps at ambient temperature (25^{o}C). Fish samples were fed with unenriched (control) and GTE-enriched instar II *Artemia* nauplii (those that gave highest TPC and antioxidant activities during bioencapsulation). Feeding was done three times a day at 9 am, 12 noon and 4 pm. Before the first feeding of each day, the aquaria were cleaned and one-third of the water was changed. Feeding experiment was done after 0, 10 and 20 days. Three replicates were maintained for every experimental trial.

3.2.5. Growth performance

To determine the effect of control and GTE-enriched *Artemia* nauplii to guppy fry growth, 10 pcs of fish were randomly collected from each treatment before and after the feeding experiment for individual weight and length determination. For weight measurement, collected fish were blot-dried with a paper towel and weighed to the nearest 0.1 mg. Each length was measured from head to tail of the fish using a ruler. Fish samples for each treatment were pooled together and dried at 60^{o}C for 24-hours. Dried samples were grinded and kept in a desiccator until analyses.

Specific growth rate was (SGR) was calculated as (SGR, % day^{-1}) = 100 (ln final weight- ln initial weight)/ total duration of the experiment.

3.2.6. Total polyphenol content (TPC) and antioxidant activity analyses

Control and GTE-enriched instar II *Artemia* nauplii and guppy fish samples were analyzed for total polyphenol content (TPC) and 1,1-diphenyl-1-picrylhydrazyl **(**DPPH) free radical scavenging activity. TPC extraction was based on the method used by Anesini *et al*. (2008) and estimated by colorimetric assay as described by Komes *et al*. (2010) against gallic acid as reference standard and reported as gallic acid equivalents (GAE) per g of dry sample. DPPH free radical scavenging was assayed based on the modified Blois used by Bae & Lee (2010) against vitamin C as reference standard and reported as mg vitamin C equivalents (VCE) per g of dry sample.

3.2.7. Determination total carotenoids in *Artemia* nauplii

Extraction of pigment using acetone in nauplii was based on the method used by Buyukapar & Yanar (2007), where about 10 mL acetone and 2 g of anhydrous sodium sulfate were added to 1.0 g of *Artemia* sample. The solution was mixed with a vortex mixer, and was then

centrifuged at 5,000 rpm for 5 minutes and stored for three (3) days at 4^{o}C in a refrigerator. After three (3) days of extraction, absorption of extract was measured at 470, 645 and 662 nm using UV-VIS spectrophotometer. The concentrations of C*a*, C*b* and total carotenoids were determined according to the equations reported by Lichtenthaler & Wellburn (1983) as follows:

$$C_a \text{ (mg L}^{-1}) = 11.75\ Abs_{662} - 2.350\ Abs_{645}$$

$$C_b \text{ (mg L}^{-1}) = 18.61\ Abs_{645} - 3.960\ Abs_{662}$$

$$\text{Total carotenoids (mg L}^{-1}) = 1000\ Abs_{470} - 2.270\ C_a - 81.4\ C_b/227$$

3.2.8. Stress study

A stress test was used to evaluate the resistance of guppy fry against salinity stress. Guppy fry fed with enriched *Artemia* nauplii were exposed to 3.50% salinity (optimal salinity stress for guppy as reported by Lim *et al*. 2002) at 15 fish /L density. Survival was monitored at regular interval of 1 hour until all fish samples died.

3.2.9. Statistical analyses

Differences between treatments were performed using one-way analysis of variance ANOVA with Duncan's multiple range tests. The level of significance for all analyses was $p<0.05$. All treatments were conducted in triplicate unless specified. Results are all reported as dry matter and presented as mean ± standard error mean.

3.3. Results

Growth performance

Growth performance of guppy fry fed with unenriched and GTE-enriched instar II *Artemia* nauplii are shown in **Table 3.1**. Individual length and weight of guppy fry in all treatments increased significantly ($p<0.05$) with longer feeding period. However, no significant

difference (p>0.05) was observed between guppy samples fed with unenriched and GTE-enriched *Artemia* nauplii. Highest SGR was observed in guppy fish samples fed with GTE-enriched *Artemia* (12.0 ± 0.9 % day $^{-1}$), but no significant difference (p>0.05) was observed in all treatments.

Table 3.1 Growth performances of guppy fry fed unenriched and green tea extract (GTE) instar II *Artemia* nauplii

Items	Day 0		Day 10		Day 20	
	Control	w/ GTE	Control	w/ GTE	Control	w/ GTE
Length (mm)	8 ± 0^{a}	8 ± ^{a}	12 ± 0^{b}	12 ± 0^{b}	16 ± 0^{c}	17 ± 0^{c}
Weight (mg)	3.9 ± 0.2^{a}	3.9 ± 0.2^{a}	13.1 ± 0.4^{b}	13.2 ± 0.5^{b}	35.1 ± 1.3^{c}	37.5 ± 1.9^{c}
SGR (% day^{-1})			11.8 ± 0.7^{a}	12.0 ± 0.9^{a}	10.8 ± 0.4^{a}	11.1 ± 0.3^{a}

Mean (n=10) having the different letter in the same row are significantly different at p<0.05

TPC and antioxidant activities

TPC and antioxidant activity evaluated through DPPH free radical scavenging activity of the methanol extract of unenriched and GTE-enriched *Artemia* nauplii are shown in **Table 3.2**. Although the TPC of GTE-enriched instar II *Artemia* nauplii (18.57 ± 0.41 mg GAE g^{-1} sample^{-1}) is higher than that of the control (15.03± 0.36 mg GAE g^{-1} sample^{-1}), no significant difference (p>0.05) was observed. Values of DPPH free radical scavenging activity, on the other hand, shows that that of GTE-enriched instar II *Artemia* nauplii (89.16 ± 9.01 mg VCE g^{-1} sample^{-1}) is significantly higher (p<0.05) than that of unenriched *Artemia* nauplii (65.42 ± 1.65 mg VCE g^{-1} sample^{-1}) which served as control. Also shown in **Table 3.2** are the values of total carotenoid content present in unenriched and GTE-enriched *Artemia* nauplii. Although the amount of total carotenoid in GTE-enriched *Artemia* is higher than in the control, no significant difference (p>0.05) was found between them.

Table 3.2 Total polyphenol content, diphenyl picrylhydrazyl **(**DPPH) free radical scavenging activity and total carotenoids of instar II *Artemia* nauplii enriched without (control) and with green tea extract (GTE) for 6-hours.

Parameters	*Artemia* nauplii	
	Control	w/GTE
Total polyphenol content (mg GAE g^{-1} $sample^{-1}$)	15.03 ± 0.36^{a}	18.57 ± 0.41^{a}
DPPH free radical scavenging activity (mg VCE g^{-1} $sample^{-1}$)	65.42 ± 1.65^{a}	89.16 ± 9.01^{b}
Total carotenoids (mg g^{-1} $sample^{-1}$)	112.62 ± 1.98^{a}	115.34 ± 9.01^{a}

Mean (n=3) having the different letter in the same row are significantly different at p<0.05.

Before and after feeding experiment, guppy fish samples were analyzed for TPC and results are summarized in **Figure 3.2**. Highest TPC (20.13 ± 1.33 mg GAE g^{-1} $sample^{-1}$) was observed in guppy samples fed with GTE-enriched *Artemia* nauplii for 10-days. This result is significantly higher (p<0.05) compared to the other treatment.

The DPPH free radical scavenging activities (**Figure 3.3**) of guppy fry fed with GTE-enriched *Artemia* nauplii are significantly higher (p<0.05) than their corresponding control. Like TPC, highest DPPH free radical scavenging activity with value of 11.87 ± 0.24 (mg VCE g^{-1} $sample^{-1}$) was also noted on guppy fish samples given with GTE-enriched *Artemia* nauplii for 10 days.

Stress test

Response of guppy fry against salinity stress is summarized in **Figure 3.4**. At day 0, first appearance of mortality (10%) on guppy fry was observed after 1-hour exposure at 3.50% salinity and 100% mortality after 5-hours. The lower mortality was noted on guppy fry samples fed with GTE-enriched instar II *Artemia* for 10 days, where a fish starts to die after 6 hours (10%) and mortality was 100% after 9 hours. At day 20, 10% of guppy fry fed with GTE-enriched *Artemia* died after 5 hours and 100% died after 8-hours of exposure. Control

guppy fry were observed to have faster rate of mortality compared to their corresponding GTE-enriched fed samples.

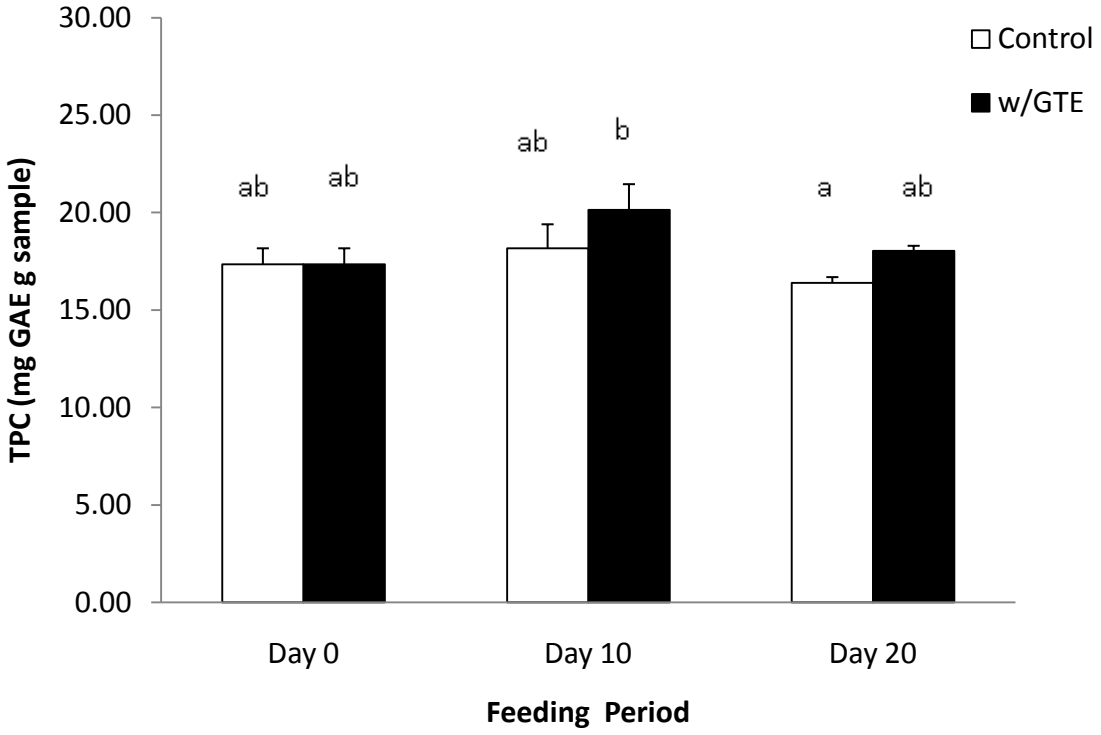

Figure 3.2 Total polyphenol content (TPC) of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii. Each value is expressed as mean ± standard error mean (n=3). Values with different letter are significantly different (p<0.05) between samples. Data are reported in dry matter.

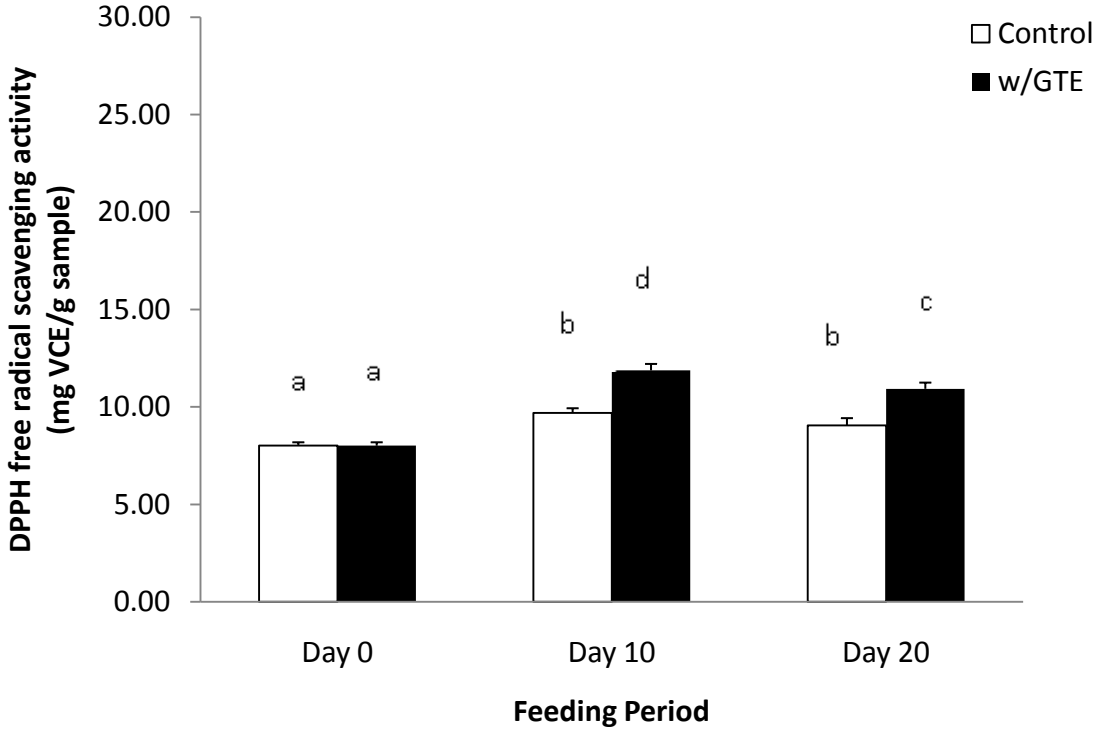

Figure 3.3 Diphenyl picrylhydrazyl **(**DPPH) free radical scavenging activity of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii. Each value is expressed as mean ± standard error mean (n=3). Values with different letter are significantly different (p<0.05) between samples. Data are reported in dry matter.

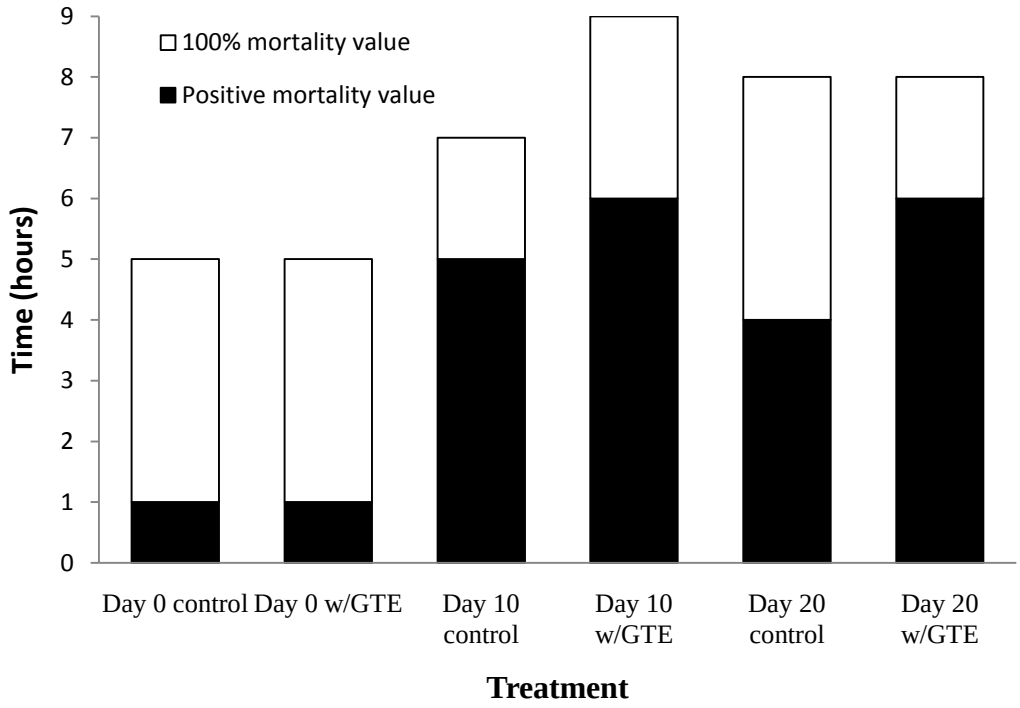

Figure 3.4 Cumulative mortality (%) of guppy fry fed unenriched (control) and green tea extract (GTE) enriched instar II *Artemia* nauplii against salinity stress (3.50% salinity). *n*=15 fish per treatment.

3.4. Discussion

Fry and larval stages of shrimp and fish are commonly fed by *Artemia* nauplii primarily because of nutrition and for effective digestion since at these stages digestive system are not yet fully developed or does not possess sufficient digestive enzyme activity necessary for effective digestion of artificial diets (Langdon 2003). Knowing that, the most logical way of enhancing fry and larval fish's antioxidant capacity for stress resistance is through their food which in this case is *Artemia* nauplii through bioencapsulation. Bioencapsulation is a technique by which live-food organisms are enriched with specific nutrients or drug molecules and fed to the target organisms (Aruvalsu *et al*. 2012).

In this study, bioencapsulation of green tea extract to *Artemia* nauplii was not only done for the enrichment of antioxidant content but, also, it is a way of preventing bacterial transfer

carried by live-food. The amount of 1 mg/mL (w/v) of green tea powder was proven by Sornsanit *et al*. (2002) to treat *Vibrio*-infected shrimp culture water. *Vibrio* is known to cause mortality in larvae, post larvae, and juveniles of up to 100% of the affected population (Sunaryanto & Mariyam 1987; Sarjito *et al*. 2012).

Based on the results of the current study, unenriched and GTE-enriched instar II *Artemia* nauplii when given as food were consumed by guppy fry based on the improved length, weight and SGR (% day^{-1}). But, guppy fry samples fed with GTE-enriched *Artemia* have better SGR compared to their corresponding control. A similar result was also observed by Aruvalsu *et al*. (2012) in freshwater molly (*Poecilla sphenops)* fish fry, where SGR was improved by feeding it with *Artemia* nauplii enriched with herbal plant extract *Vitex negundo*. Also, terrestrial herbal herbs and seaweed extract enriched to 12-day old *Artemia* nauplii improved the SGR of *Penaeus indicus* juveniles after 30 days of feeding (Immanuel *et al*. 2004).

These positive reports on SGR regardless of fish samples simply show the feasibility of using plant extracts to target aquatic animals through *Artemia* nauplii. The advantage of the present study is that the method used is simpler and safer since it utilizes water extract compared to ethanol and methanol which are commonly used for extracting bioactive compounds from plants. Also, utilization of green tea is rather practical to use for a fish farmer since it can be found locally at a cheaper value.

On the other hand, a previous study conducted by Kono *et al*. (2000) shows that incorporation of green tea extract or green tea powder on fish diet of yellowtail (*Seriola quinqueradiata)* and ayu (*Plecoglossus altevelis)* decreases their growth. There was also lower growth rate observed to juvenile olive flounder (*Paralichthys olivaceus)* when diet was incorporated with green tea. However, on another study, a 0.5 g GT/kg diet resulted to higher growth rate compared to control in Nile tilapia (Abdel-Tawwab 2010). From those studies, it

could be concluded that the effect of green tea when incorporated in fish diets depend on the species of fish.

In plants, it is known that the antioxidant activity of plant materials was due to the presence of phenolics, carotenoids and flavonoids (Barros *et al*. 2007; Prasad *et al*. 2009). Polyphenol compounds play an important role in adsorbing and neutralizing free radicals, quenching singlet oxygen, and decomposing peroxides by donating their hydrogen (Ksouri *et al*. 2007). Without antioxidants like polyphenol, this group of radicals may interact with biological systems in a clearly cytotoxic manner (Barros *et al*. 2007).

In the current study, survival of guppy fry against salinity stress was influenced by the presence of polyphenols ingested by guppy from GTE-enriched instar II *Artemia* nauplii. Guppy fry samples fed for 10 days have the highest resistance to salinity. This could be attributed to their high amount of TPC and DPPH free radical scavenging activity values, thus we could say that their resistance against salinity stress was improved by the presence of polyphenols they have ingested from the green tea extract.

At day 20, decrease in TPC and DPPH free radical scavenging activity of guppy fry fed with unenriched and GTE-enriched instar II *Artemia* nauplii samples could be one of the reasons why their survival against salinity stress was lower. At this stage, guppies are already bigger and the amount of TPC consumed is insufficient to enhance their antioxidant activities. To improve the TPC amount on older guppy, green tea concentration could be increased, use older stages of *Artemia* nauplii so that they can ingest more amount of TPC and/or incorporate green tea extract on artificial diets.

The amount of total carotenoids inherent in *A*rtemia nauplii probably contributed as well to the antioxidant activity of the guppy fry samples that's why even for control samples there were improvement on stress resistance compared to the initial samples.

In conclusion, instar II *Artemia* nauplii unenriched and GTE-enriched improved the growth performance of guppy fry better during the 20-days feeding period. Also, GTE-enriched-instar II-*Artemia* nauplii-fed guppy fry contains higher TPC and DPPH free radical scavenging activities which resulted to better resistance against salinity stress. However, better specific growth rate and survival was observed on guppy fry samples fed with GTE-enriched *Artemia* nauplii with less feeding time (i.e. 10 days of feeding). It is hoped that this baseline information will be of potential use in freshwater ornamental production where stressful condition is extended up to export/import trading.

CHAPTER 4

Influence of fish size on growth performance and stress resistance of freshwater ornamental fish fed green tea enriched *Artemia* nauplii

4.1. Introduction

Neon tetra *Paracheirodon innesi* and guppy *Poecilia reticulata* are among the popular freshwater aquarium fishes around the world because of their small size, attractive color, and ease to culture. Starting from production to trading, these fishes are subjected to different forms of stress such as chemical, physical, or biological stress and sudden change of oxygen that may result in the formation of excessive amounts of singlet oxygen and free radicals (Pan *et al*. 2003). Formed free radicals (superoxide, hydroxyl, and lipid peroxides) may interact with biological systems in a clearly cytotoxic manner (Barros *et al*. 2007).

To minimize these kinds of scenarios, the antioxidant capacity of the animals should be enhanced to increase their resistance to stress by providing them with nutritious food that contains high amounts of antioxidants (Chien & Shiau 2005). In larval production, *Artemia* nauplii are used extensively worldwide as live food because of their availability, low cost, ease of culture, and biochemical composition (Smith *et al*. 2004). Although there has been much effort to develop larval or postlarval artificial feeds, live food organisms such as *Artemia* are still preferred in hatcheries because of their digestive enzymes. Because *Artemia* are nonselective feeders, they can be enriched or fortified with nutrient supplements or medication to provide health benefits to which they are fed (Rodriguez *et al*. 2011). But because of emerging environmental risks of using chemotherapeutants to improve survival rate, the demand for using natural antioxidants increases. The best-known sources of antioxidants are plants that contain polyphenols with outstanding antioxidant effects and have the ability to donate hydrogen or electrons in a biological system to prevent damages caused by free radicals (Hrelia *et al*. 2002).

Among the plants, green tea is the excellent source of natural antioxidant because it possesses polyphenols, especially catechins and phenolic acids, which have been considered the main

players for their beneficial effects on human health (Zuo *et al*. 2002). Green tea is widely available and biocompatible because it is the second most consumed beverage apart from water consumed by humans (Anesini *et al*. 2008).

In the earlier chapter, feeding of green tea extract (GTE)–enriched *Artemia* nauplii to guppy fry for 10 days improved their antioxidant activity and resistance to salinity stress. To prevent additional feed preparation in hatcheries, this study was conducted to determine if *Artemia* nauplii enriched with green tea can also enhance the growth, the antioxidant activities, and the stress resistance of different sizes of freshwater ornamental fishes such as guppies (fry and adult stages) and neon tetras (juvenile stage) during the 10-day feeding period.

4.2. Materials and methods

4.2.1. Sample preparation of green tea extract (GTE)

A pack of commercial green tea leaves was purchased from a local supermarket in Tsukuba City, Japan. The tea sample was pulverized using a coffee grinder and was sieved at 120 µm. Green tea–infused water was prepared based on the method used byKomes *et al*. (2010). Tea powder was mixed with distilled water (1 mg ml^{-1}) at 80°C and slowly stirred for 5 minutes. After extraction, the solution was cooled to room temperature and was sieved at 120 µm. The salinity of the tea extract was adjusted by adding 3.50% artificial seawater powder and was reserved for the enrichment part of the study.

4.2.2. Hatching of *Artemia*

Artemia salina cysts were obtained from a commercial supplier (A&A Marine), which originally came from Salt Lake, Utah, USA. Hatching of cysts was carried out in 2 g cysts per 300 ml of 3.50% artificial seawater (Benijts *et al*. 1975). The 24-hour incubation was conducted at room temperature with light and vigorous aeration. Newly hatched nauplii were

separated from cyst shells and transferred to another container containing 1 L of 3.50% salinity water. Nauplii were incubated for another 12 hours to molt into the second stage (instar II).

4.2.3. Enrichment procedure

The instar II *Artemia* nauplii were separated from the container using a 120 µm siever and were transferred to a previously prepared GTE at 3.50% salinity. The enrichment period was conducted for 6 hours, for it showed high results on a previous study by the team (not published), at room temperature with light and aeration. Enrichment of *Artemia* nauplii without GTE served as control. Enriched *Artemia* nauplii were collected, sieved at 120 µm, washed with tap water and then with distilled water, blot-dried in paper towel, and kept at −30°C.

4.2.4. Fish samples

Adult female guppies (32–38 mm) and juvenile neon tetras (10–15 mm) were purchased from a commercial ornamental fish shop (Tsukuba City, Japan) and stocked in a 250 L aquarium containing freshwater with aeration. Day-0-old guppy fry (8 mm) samples were collected from the adult guppies maintained under laboratory conditions. All fish samples were acclimatized to laboratory conditions and fed with the control instar II *Artemia* nauplii for 5 days before the start of the experiment.

4.2.5. Feeding experiment

After measuring the weight, fish samples were transferred to experimental tanks containing 10 fish per 10 L of freshwater at room temperature with mild aeration to maintain dissolved oxygen. Fish samples were fed ad libitum with *Artemia* nauplii (control and GTE-enriched) three times a day at 9:00 AM, 12:00 noon, and 4:00 PM. Before the first feeding of the day, the

aquaria were cleaned, and one-third of the water was changed. The feeding experiment was conducted for 10 days. Three replicates were conducted for each treatment.

4.2.6. Growth performance

To determine the effect of feeding with the control and the GTE-enriched *Artemia* nauplii to fish growth performances, five pieces of fish were randomly collected from each aquarium before and after the feeding experiment for individual weight. Collected fishes were blot-dried on a paper towel and weighed to the nearest 0.1 mg. Fish samples from each treatment were pooled together and dried at 60°C for 24 hours. Dried samples were ground and kept in a desiccator until analyses.

Specific growth rate was (SGR) was calculated as (SGR, % day^{-1}) = 100 (ln final weight- ln initial weight)/ total duration of the experiment.

4.2.7. Total polyphenol content (TPC) and antioxidant activity analyses

Fish samples collected from growth performance determination were analyzed for total polyphenol content (TPC) and 1,1-diphenyl-1-picrylhydrazyl **(**DPPH) free radical scavenging activity. TPC extraction was based on the method used by Anesini *et al*. (2008) and estimated by colorimetric assay as described by Komes *et al*. (2010) against gallic acid as reference standard and reported as gallic acid equivalents (GAE) per g of dry sample. DPPH free radical scavenging activity was assayed based on the Blois-modified procedure used by Bae and Lee (2010) against vitamin C as reference standard and was reported as milligram vitamin C equivalents (VCE) per gram of dry sample.

4.2.8. Stress study

A stress test was used to evaluate the resistance of fish against salinity. This salinity stress test was suggested to be more stable than exposure to other parameters such as temperature,

pH, and ammonia (Lim *et al*. 2002). After 10 days of feeding, 15 pcs of neon tetras (juvenile) and guppies (fry and adult) were exposed to 1.50% (1 L) and 3.50% (1 L) salinities (optimal salinity stress for neon tetras and guppies as reported by Lim *et al*. 2002), respectively. Mortality was monitored at a regular interval (30 minutes) until all fish samples died. The cumulative mortality was calculated by summing the mortality counts noted for the whole run.

4.2.9. Statistical analyses

Differences between SGR, TPC, and DPPH free radical scavenging activity were performed using one-way analysis of variance (ANOVA) with Duncan's multiple-range tests. The level of significance used for all analyses was $p<0.05$. All treatments were conducted in triplicate unless specified. Results were all reported as dry matter and presented as mean ± standard error mean.

4.3. Results

Growth performance

The SGR (% day^{-1}) for guppy fry, neon tetra juveniles, and guppy adults are shown in **Figure 4.1.** Results showed that the SGR (% day^{-1}) of guppy fry both fed with the control and the enriched *Artemia* nauplii are significantly higher ($p < 0.05$) than those of neon tetra juveniles and guppy adults. Between the control- and the GTE-enriched-*Artemia*-fed fish samples, no statistical difference was found in all sizes.

.

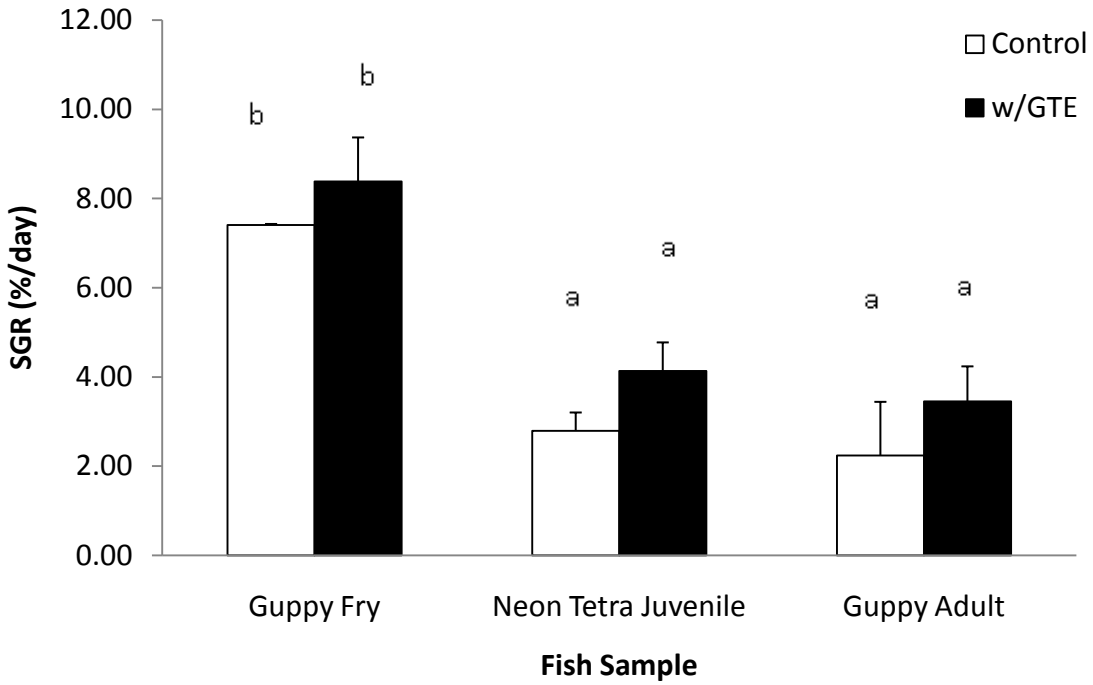

Figure 4.1 The % specific growth rate (% SGR day^{-1}) of guppy fry, neon tetra juveniles, and guppy adults fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days. Each value is expressed as mean ± standard error mean (n = 3). Values with different letters are significantly different ($p < 0.05$) between samples.

Total polyphenol content and antioxidant capacities

TPC of fish samples is shown in **Figure 4.2**. Data show that TPC values of guppy fry fish samples (for control and GTE-enriched) are significantly higher than those of neon tetra juveniles and guppy adults. The highest TPC was observed in guppy fry fed with GTE-enriched *Artemia* (18.52 ± 0.30 mg GAE g^{-1} $sample^{-1}$). The TPC of neon tetra juvenile samples fed with GTE-enriched *Artemia* nauplii was significantly higher ($p < 0.05$) than that of their control-fed counterparts. In the case of guppies (fry and adult), although the values of TPC on samples fed with GTE-enriched *Artemia* were higher, no significant difference ($p > 0.05$) was found when compared with their control-fed counterparts.

The DPPH free radical scavenging activity of fish samples are shown in **Figure 4.3**. The trend was similar to TPC, where the highest value was observed in guppy fry fed with GTE-enriched *Artemia* (11.48 ± 0.46 mg VCE g^{-1} $sample^{-1}$). No significant difference ($p > 0.05$) was found on the values for the guppies fed with either the control or the GTE-enriched *Artemia* nauplii, while for neon tetra juvenile samples, the values for fish samples fed with GTE-enriched *Artemia* were significantly higher ($p < 0.05$) than their control-fed counterparts.

Stress Test

Figures 4.4–6 show the % cumulative mortality of fish samples exposed at 1.50% and 3.50% salinities for neon tetras and guppies, respectively. Data show that guppy fry (**Figure 4.4**) samples fed with nonenriched and GTE-enriched instar II *Artemia* nauplii have better resistance to salinity stress compared with neon tetra juveniles (**Figure 4.5**) and guppy adults (**Figure 4.6**). Neon tetras are shown to be more sensitive than guppies in terms of salinity stress because the first sign of mortality was observed after 30 minutes of exposure and 100% mortality after only 150 minutes. As for the guppies, results show that fry samples have higher salt tolerance than those in the adult stage. The first sign of mortality was observed after 300 minutes and 180 minutes for fry and adults, respectively. A 100% mortality for guppy fry was observed after 600 minutes, and for adults, after 480 minutes upon exposure to 3.50% salinity. Between guppy fry samples fed with nonenriched and GTE-enriched *Artemia* nauplii, results showed that fish samples given *Artemia* nauplii enriched with GTE have better survival than those given control *Artemia* nauplii under salinity stressful conditions.

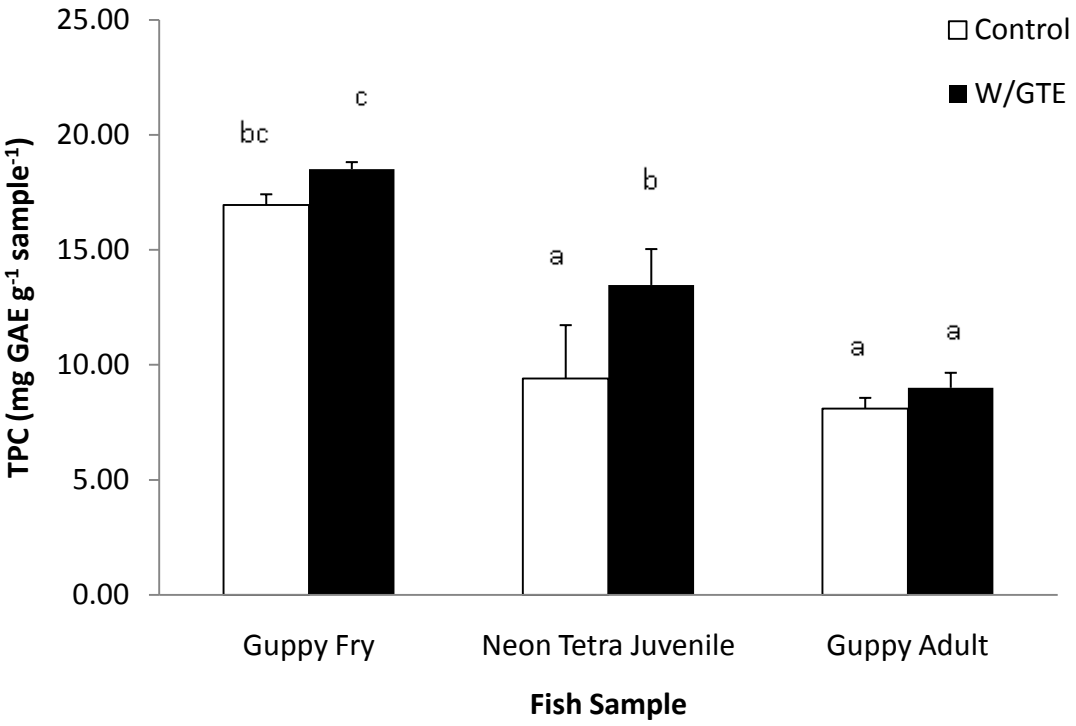

Figure 4.2 Total polyphenol content (TPC) guppy fry, neon tetra juveniles, and guppy adults fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days. Each value is expressed as mean ± standard error mean (n = 3). Values with different letters are significantly different ($p < 0.05$) between samples. Data are reported in dry matter.

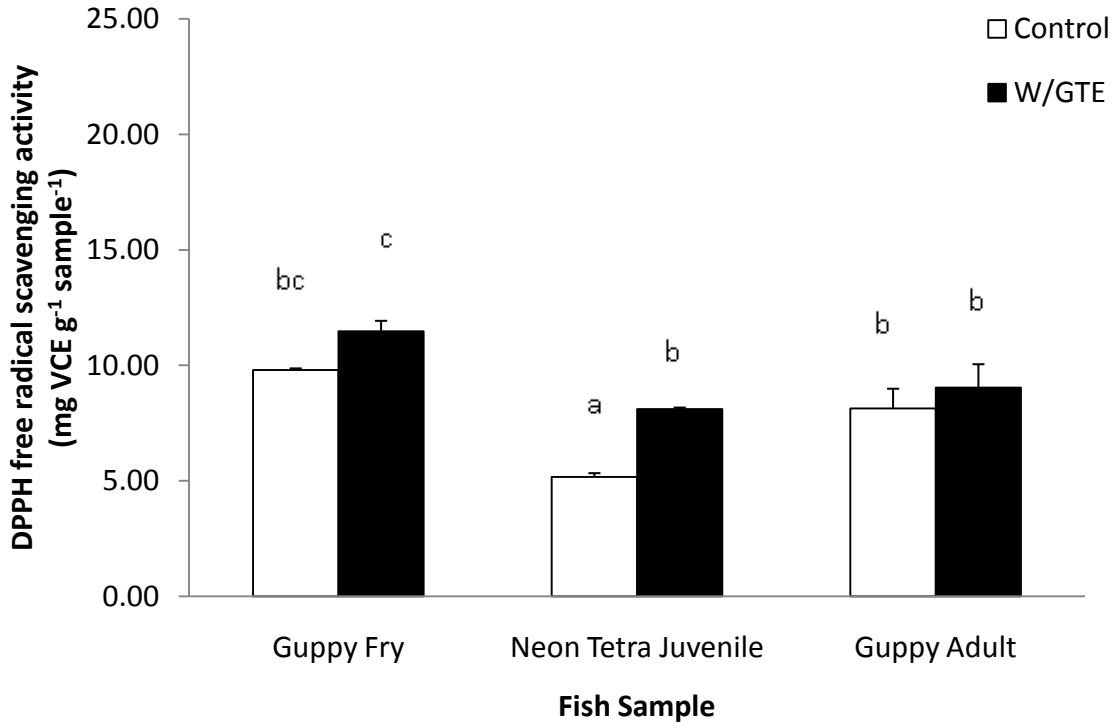

Figure 4.3 DPPH free radical scavenging activity of guppy fry, neon tetra juveniles, and guppy adults fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days. Each value is expressed as mean ± standard error mean (n = 3). Values with different letters are significantly different ($p < 0.05$) between samples. Data are reported in dry matter.

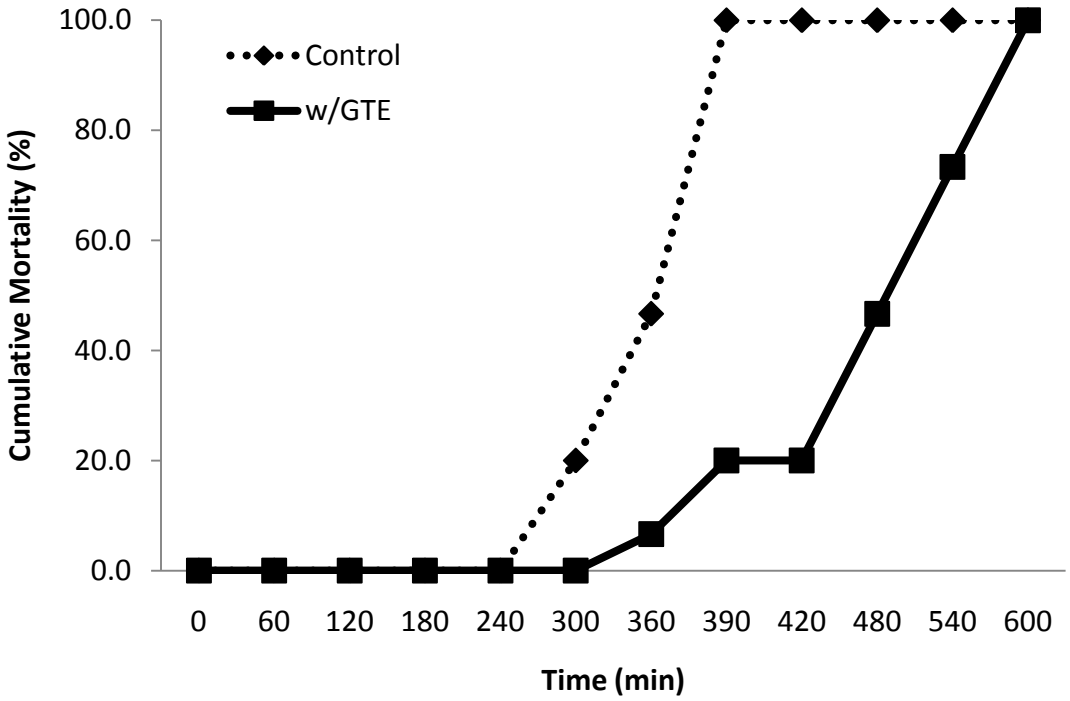

Figure 4.4 The % cumulative mortality (CM) of guppy fry fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days against 3.50% salinity (*n* = 15).

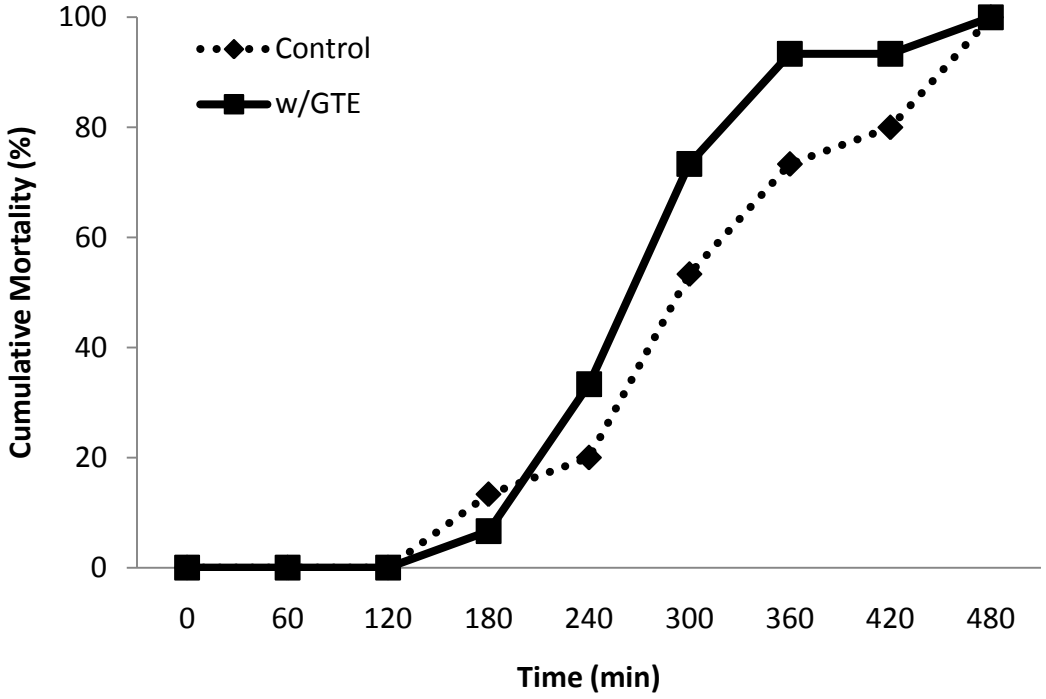

Figure 4.5 The % cumulative mortality (CM) of guppy adults fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days against 3.50% salinity (*n* = 15).

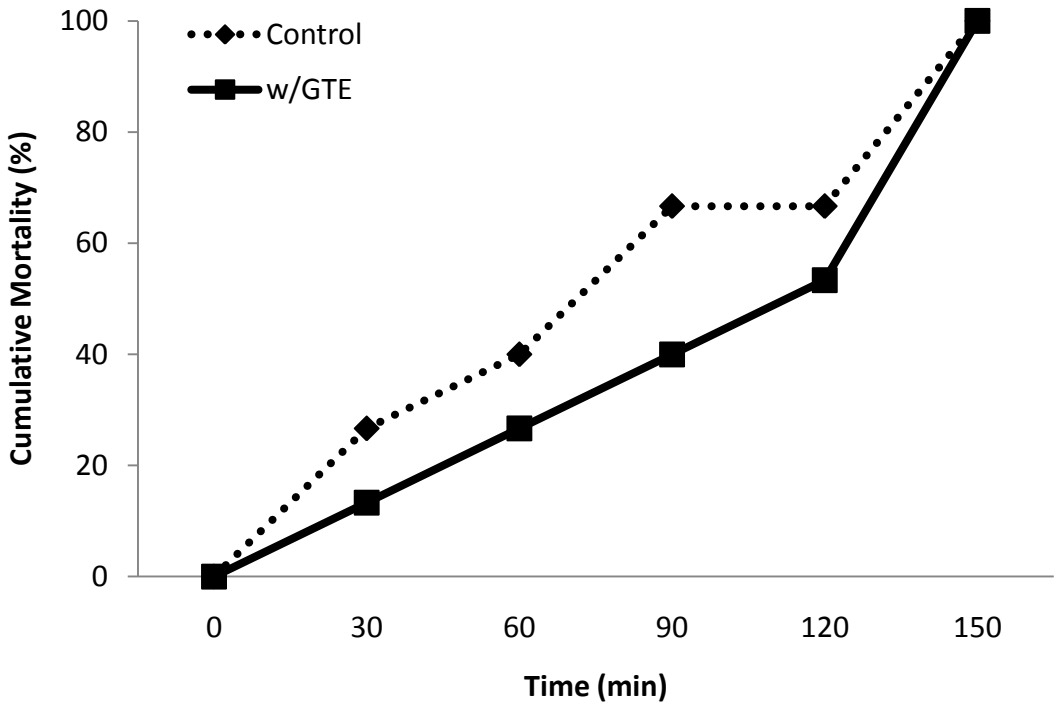

Figure 4.6 The % cumulative mortality (CM) of neon tetra juveniles fed with the control and the GTE-enriched instar II *Artemia* nauplii for 10 days against 1.50% salinity (n = 15).

4.4. Discussion

The results of the present study show that the nonenriched and GTE-enriched instar II *Artemia* nauplii can enhance the growth of neon tetra juveniles and guppy adults, but the best result was observed when given to guppy fry. In a previous study conducted by Aruvalsu *et al*. (2012), freshwater fish molly *Poecilia sphenops* fry obtained maximum SGR when fed with *Artemia* nauplii enriched with herbal extract of *Vitex negundo* Linn. as opposed to those fed with nonenriched *Artemia* nauplii. The same result was obtained by Immanuel *et al*. (2004), where *Penaeus indicus* juveniles fed with *Artemia* nauplii enriched with terrestrial herbs and seaweed powder had higher SGR than their control-fed counterparts. In this study, the SGR of guppy fry fed with GTE-enriched instar II *Artemia* nauplii was higher than their corresponding control-fed counterparts, but no significant difference (p > 0.05) was determined between them. The improvement in SGR of fish samples fed with GTE-enriched

Artemia nauplii was due to the components found in green tea such as polyphenols, protein, carbohydrates, fats, peptides, and organic acid (Sato & Miyata 2000; Bae & Lee 2010).

After 10 days of feeding, data show that the amounts of TPC and DPPH free radical scavenging activities of fish samples fed with GTE-enriched *Artemia* nauplii are higher than their corresponding control-fed counterparts. The additional value could be attributed to the amount of polyphenols obtained from GTE ingested by *Artemia* nauplii during enrichment, which was later given as food to the fish. Although no significant difference (except in neon tetra) was observed on TPC values between fish samples fed with the control and those fed with the GTE-enriched instar II *Artemia* nauplii, the latter group exhibited better survival upon exposure to salinity stress. This was due to the higher value of their DPPH free radical scavenging activity. This scavenging activity supports the presence of polyphenols from GTE that accumulated in their body during feeding, which enhances the fishes' antioxidant activity. DPPH free radical scavenging activity values represent how fast the polyphenols present in the animal's body absorb or neutralize free radicals during stressful conditions (Ksouri *et al*. 2007). In addition, the increased amount of TPC and DPPH free radical scavenging activity in fish enhances their physiological response, which includes the functioning of osmoregulatory organs, the maintenance of plasma osmolality, ion uptake, and the secretion as well steroidogenesis against acute increase in salinity (Dagar *et al*. 2010).

Control-fed fish samples were also observed to contain TPC and DPPH free radical scavenging activity, which could be attributed to the total carotenoid present in *Artemia*. Like polyphenols, carotenoids are reported to serve as antioxidants and as a source of vitamin A for animals (Ni *et al*. 2008). It was also observed that only neon tetra juveniles fed with GTE-enriched *Artemia* nauplii exhibit higher amounts of TPC and DPPH free radical scavenging activity, which is significantly different compared with its control-fed counterparts. This

result was probably caused by guppies and neon tetras having different abilities to use the amount of nutrients from GTE.

Based on the stress test, data show that guppy fry have better tolerance to salinity stress compared with neon tetra juveniles and guppy adults basically because of higher values of TPC and DPPH free radical scavenging activity. Insignificant increase in guppy adults could probably be due to the amount of TPC obtained from GTE-enriched instar II *Artemia* nauplii not being high enough to enhance their antioxidant activity. On the other hand, the nutrient from the yolk of guppy fry may have an additional role on their survival against salinity stress.

Aside from herbal plant extract, there have been studies conducted previously on improving growth and stress resistance through *Artemia* nauplii enrichment such as vitamin C for freshwater prawn (Merchie *et al*. 1995) and *Poecilia reticulata* (Lim *et al*. 2002), stressol and cod liver oil for *Penaeus indicus* postlarvae (Citarasu *et al*. 1998), and *Odonus niger* liver oil for *Penaeus monodon* postlarvae (Immanuel *et al*. 2004). The advantage, however, of using GTE for the enrichment of *Artemia* nauplii as antioxidant enhancer is its availability and economic value. In addition, the simplicity of the extraction and enrichment procedures allows a hobbyist or a first-time aquaculturist to conduct the procedures on their own.

In conclusion, the supplementation of instar II *Artemia* nauplii was able to improve the growth of fish samples. However, feeding with GTE-enriched instar II *Artemia* nauplii can only enhance the antioxidant activity of fish from fry to juvenile stages as observed in guppy fry and neon tetra juveniles. The current procedure, therefore, may be used as an alternative method for improving the survival of fish samples in stressful conditions encountered during larval production and during transportation. It is, thus, recommended to test GTE-enriched *Artemia* nauplii to other fish commodities to further assess its applicability to aquaculture.

CHAPTER 5

Biochemical and digestive enzyme composition of neon tetra *Paracheirodon innesi* and guppy *Poecilia reticulata* fed green tea extract enriched *Artemia* nauplii

5. 1. Introduction

Neon tetra *Paracheirodon innesi* and guppy *Poecilia reticulata* are among the popular freshwater aquarium fish around the world because of their small size, attractive color and ease to culture. As the commercial demand of ornamental freshwater production increases, cost of production also expands due to intensification that may lead to microbial diseases and improper nutrition. Food nutrient value and fish health are the major important factors that consume production cost (Yousefian *et al*. 2012). The best way to maximize food intake of a given fish is to characterize the composition of its digestive enzymes. From the profile of digestive enzymes, it is possible to predict then the ability of a species to use different nutrients (Hofer & Kock 1989; Furne *et al*. 2005). It was reported by Lemieux *et al*. (1999) that digestive protease activity is correlated to enhanced food conversion efficiency and growth rate in Atlantic cod.

In larval production, *Artemia* nauplii are commonly used because of nutrition and as a vehicle for delivering nutrients and therapuetants to targeted fish. In the previous chapter (4), *Artemia* nauplii were enriched with green tea extract through bioencapsulation and were fed to neon tetra and guppy fish. Based on the results, fish samples fed green tea extract have better resistance when they were exposed to osmotic stress.

Since green tea enriched in *Artemia* nauplii was able to enhance the antioxidant activity of the fish, it is also interesting to determine its direct effect on their nutrition. The objective of the present work was to analyze the biochemical and digestive enzyme composition of neon tetra and guppy fed green tea enriched *Artemia* nauplii.

5.2. Materials and methods

5.2.1. Fish samples

Adult (32-38 mm) and juvenile neon tetra (10-15 mm) were purchased from a commercial ornamental fish shop (Tsukuba City, Japan) and stock in 250-L aquarium containing aged tap water. They were acclimatized at laboratory condition for five days before the start of feeding experiment.

5.2.2. Sample preparation of green tea extract (GTE)

A pack of commercial green tea leaves was purchased from a local supermarket in Tsukuba City, Japan. Tea sample was pulverized using coffee grinder and sieved at 120 µm. Green tea-water-infusion was prepared based on the method used by Komes *et al*. (2010). Tea powder was mixed with distilled water (1 mg/mL) at 80^{o}C and slowly stirred for 5 minutes. After extraction, the solution was cooled to room temperature and sieved at 120 µm. Salinity of tea extract was adjusted by adding 3.50% artificial seawater powder and reserved for enrichment experiment.

5.2.3. Hatching of *Artemia*

Artemia salina cysts were obtained from a commercial supplier (A&A Marine) which originally came from Salt Lake, Utah USA. Hatching of cysts were carried out in 2 g cysts/300 mL of 3.50% artificial sea water (Benijts *et al*. 1975). The 24h-incubation was conducted in thermo-controlled room set at 25^{o}C with light and vigorous aeration. Newly hatched nauplii were separated from cyst shell and transferred to another container containing 1 L of 3.50% salinity water. Nauplii were incubated for another 12 hours to molt into second stage (instar II).

5.2.4. Enrichment procedure

The instar II *Artemia* nauplii were separated from container using a 120 µm siever and transferred to previously prepared GTE at 3.50% salinity. Enrichment period was conducted for 6 hours for it showed high results on a previous study by the team (not published), at thermo-controlled chamber with light and aeration at room temperature. Enriched *Artemia* nauplii were collected, sieved at 120 µm, washed with tap water, and then with distilled water, blot dried in paper towel and finally kept at -30^{o}C.

5.2.5. Feeding experiment

Fish samples were culture in experimental tanks containing 10 fish /10-L of aged tap water at ambient temperature (25^{o}C) with mild aeration to maintain dissolved oxygen under laboratory conditions. The entire experiment was conducted with three replicates for each treatment. Fish samples were fed ad libitum with *Artemia* nauplii (unenriched and GTE-enriched) three times a day at 9 am, 12 noon and 4 pm. Before the first feeding, the aquaria were cleaned and one-third of the water was changed daily. Feeding experiment was conducted for 10 days. At the end of feeding period, fish samples (5 pcs aquarium^{-1}) were starved for 24 hours, killed and their complete digestive tracts were removed and immediately stored at -30^{o}C until analyzed.

5.2.6. Biochemical composition

After feeding experiment, 5 fish aquarium^{-1} were pooled together and dried at 60^{o}C overnight in an oven. Dried samples of *Artemia* nauplii and fish samples were analyzed for total lipid and total protein. Total lipid was extracted with 2:1 chloroform: methanol mixture and purified according to the method of Folch (1957). Determination of the lipid content was carried out gravimetrically. Extraction of the total protein was conducted according to the

Martone (1980), where 0.2 g of the sample was digested in 25.0 mL of 1M sodium hydroxide solution in a boiling water–bath for 30 min. The digest was diluted ten-fold (v/v) with water and protein was estimated based on Lowry method (1957) against bovine serum albumin as standard.

5.2.7. Enzyme assay

Digestive tract of starved fish samples were homogenized with cold distilled water (250 mg/mL). After homogenization the samples were centrifuge at 12,000 rpm for 10 minutes at 4^{o}C and the supernatant was removed for further analysis. During preparation the homogenates were continuously kept on ice.

Total protease activity was estimated by casein digestion assay following the methods by Walter (1984) used by Furne *et al*. (2005). The enzymatic determination was made using several pH values over the physiological range of the digestive tract. The buffers used were KCl-HCl 0.1 M (pH 1.5), glycine-HCl 0.2 M (pH 3.0), citrate 0.1 M-phosphate 0.2 M (pH 4.0 and 7.0), Tri-HCl 0.1 M (pH 8.5 and 9.0) and glycine-NaOH 0.1 M (pH 10.0). The enzymatic reaction mixtures were composed of casein at 1% (w/v) in water (0.25 mL), buffer (0.25 mL) and extract (0.1 mL). It was covered for 1 hour at 37^{o}C. The reaction was stopped by the addition of 0.6 mL of trichloroacetic acid at 8% (w/v). After being kept for 1 hour at 2^{o}C, the sample was centrifuged at 1,800 x g for 10 min, and absorbance of the supernatant measured at 280 nm. The samples were assayed in triplicate. L-tyrosine was used as a standard. One unit of total proteolytic activity was defined as the quantity of enzyme that released one μmol of tyrosine mL^{-1} min^{-1}.

Amylase activity was evaluated using 1% starch solution in 20 mM sodium phosphate buffer pH 6.9, containing 6.0 mM NaCl as substrate (Worthington, 1993). A total of 0.5 mL of substrate solution was added to 0.5 mL of enzyme preparation followed by 3 min of

incubation. This was followed by addition of 0.5 mL of dinitrosalicylic acid and incubation in boiling water bath for 5 min. Absorbance value at 540 nm was recorded. Maltose was used as a standard, and the activity unit of α-amylase was defined as the quantity of enzyme that produce one *u*mol of maltose mL^{-1} min^{-1}.

Protein content of the supernatant solutions was determined by Lowry method (1957) using bovine serum albumin as the standard.

5.2.8. Statistical Analyses

Differences between treatments were performed using one-way analysis of variance ANOVA with Duncan's multiple range tests. The level of significance for all analyses was $p<0.05$. All treatments were conducted in triplicate unless specified. Results are all reported as dry matter and presented as mean ± standard error mean.

5.3. Results

In **Table 5.1** the biochemical composition of neon tetra and guppy samples are shown. Values of total lipid for neon tetra show a higher significant difference between day 0 and day 10, but not between control and GTE-enriched samples. While total lipid of guppy samples after day-10 is lower than the initial, no significant difference was found statistically. In terms of total protein, no significant difference was found among treatments in both neon tetra and guppy samples.

The total proteolytic activities of *Artemia* nauplii are higher in unenriched (control) samples than GTE-enriched nauplii (**Table 5.2**). The digestive tract of neon tetra also shows high proteolytic activities in control than samples fed GTE-enriched *Artemia* nauplii. While guppy samples fed with GTE-enriched *Artemia* nauplii have higher total proteolytic activity

than its control. The amylase activities of control samples are higher than GTE-enriched for *Artemia nauplii*, neon tetra and guppy.

Table 5.1 Biochemical composition of neon tetra and guppy samples before and after the 10-day feeding period. Results are reported as dry matter and presented as mean ± standard error mean (n=3).

Biochemical Composition	Day 0		Day 10	
	Control	w/GTE	Control	w/GTE
Neon tetra				
Total Lipid (%)	5.67 ± 0.00^{a}	5.67 ± 0.00^{a}	11.40 ± 0.48^{b}	10.10 ± 0.65^{b}
Total Protein (%)	42.31 ± 0.71^{a}	42.31 ± 0.71^{a}	42.41 ± 0.83^{a}	42.13 ± 0.88^{a}
Guppy				
Total Lipid (%)	16.89 ± 2.21^{a}	16.89 ± 2.21^{a}	15.07 ± 4.61^{a}	15.59 ± 0.74^{a}
Total Protein (%)	35.99 ± 2.58^{a}	35.99 ± 2.58^{a}	37.29 ± 1.59^{a}	38.87 ± 5.32^{a}

Values within a row with different letter are significantly different (p<0.05) between samples**.**

Table 5.2. Total proteolytic and amylase activities of *Artemia* nauplii, neon tetra and guppy samples determined at 37^{o}C. Results are presented as mean ± standard error mean (n=3).

Samples	Total Proteolytic Activity (U/mg protein)	Amylase Activity (U/mg protein)
Artemia nauplii		
Control	0.028 ± 0.002	0.846 ± 0.071
w/GTE	0.019 ± 0.001	0.513 ± 0.040
Neon tetra		
Control	0.019 ± 0.000	0.863 ± 0.093
w/GTE	0.017 ± 0.000	0.763 ± 0.025
Guppy		
Control	0.008 ± 0.000	0.366 ± 0.026
w/GTE	0.009 ± 0.000	0.361 ± 0.033

Total proteolytic activity was obtained as the sum of those determined at pH 1.5, 3.0, 4.0, 7.0, 8.5, 9.0 and 10.0.

Amylase activity was determined at pH 6.9.

Figure 5.1 shows the proteolytic activities of *Artemia* nauplii at different pH levels at 37^{o}C. Generally, GTE-enriched samples are lower than control in all pH except at pH 9.0 where its value is almost the same with control. Control samples have higher proteolytic activities in all pH levels.

Figure 5.2 and **Figure 5.3** shows the enzymatic activity of neon tetra and guppy samples determined at different pH values. Lowest proteolytic activity was observed at pH 4.0, 0.001 ± 0.000 (U mg^{-1} protein^{-1}) and 0.002 ± 0.000 (U mg^{-1} protein^{-1}) for control and GTE-enriched fed samples, respectively. The highest proteolytic activity was noted at alkaline pH values (9.0 and 10.0). Higher proteolytic activities were also observed at alkaline pH for guppy samples. Also, proteolytic activities of guppy fed GTE-enriched *Artemia* nauplii are slightly higher than control in all pH values. But generally, GTE-enriched Artemia nauplii did not change the proteolytic activities in digestive tract of the fish samples.

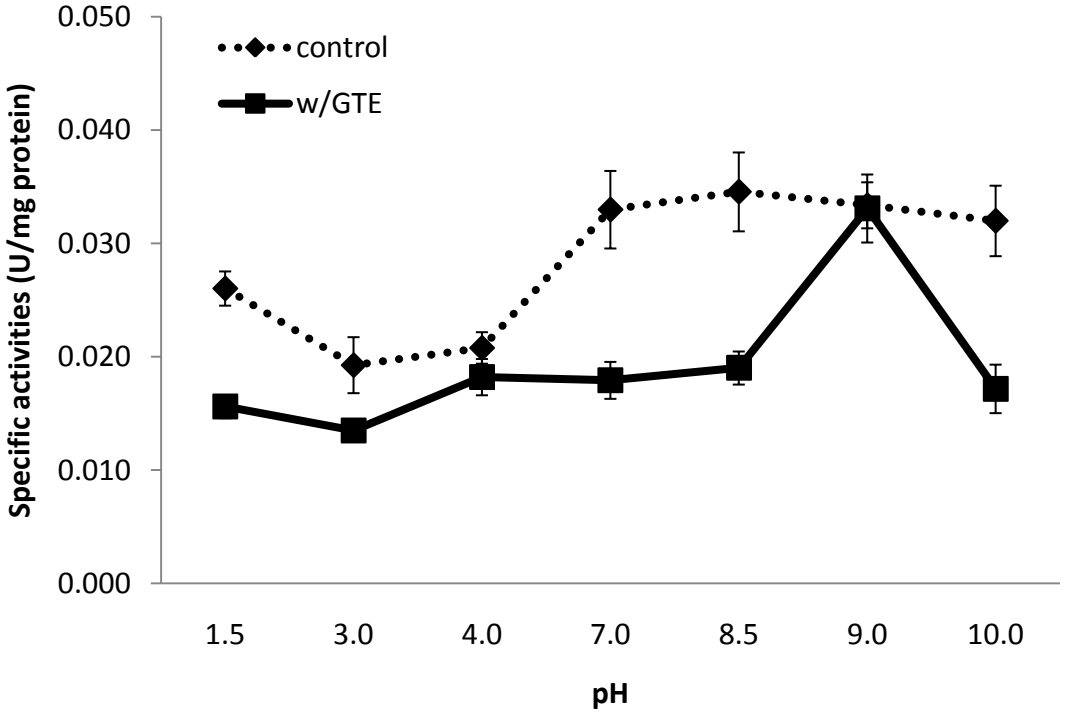

Figure 5.1 Protease activities of unenriched (control) and GTE-enriched *Artemia* nauplii at different pH.

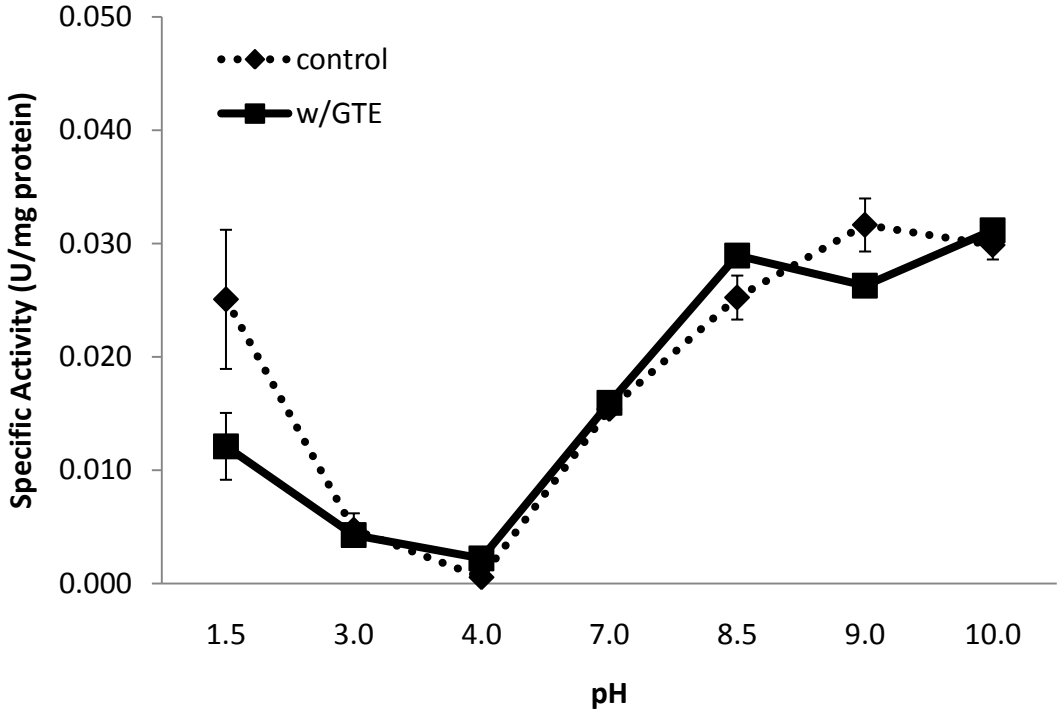

Figure 5.2 Protease activities at different pH in digestive tract of neon tetra fed unenriched (control) and GTE-enriched *Artemia* nauplii.

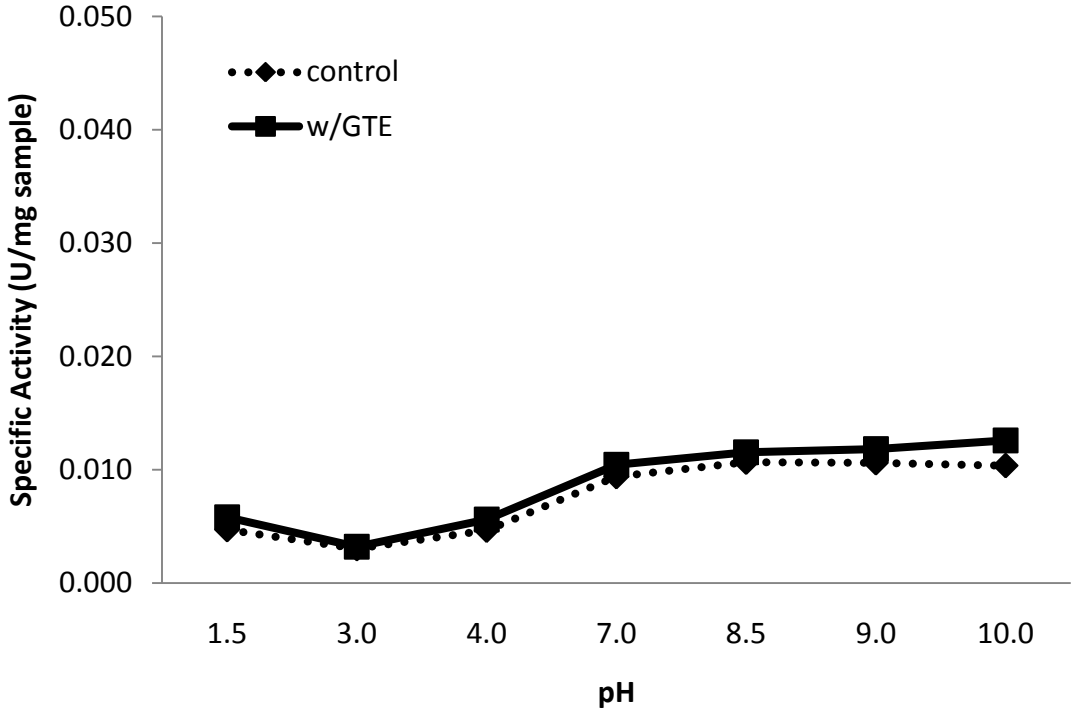

Figure 5.3 Protease activities at different pH in digestive tract of guppy fed unenriched (control) and GTE-enriched *Artemia* nauplii.

5.4. Discussions

Polyphenols are considered as one of the anti-nutrient component together with saponin and phytic acid of plant based products which prevents them to be fully utilized in aquaculture commodities despite their availability. In the current study, despite the fact that green tea contains high amount of polyphenols, green tea extract introduced via *Artemia* nauplii to neon tetra and guppy during the 10-days feeding period did not affect the biochemical composition (total lipid and total protein). The anti-nutrients were probably balanced out by the nutritional component of *Artemia* nauplii or the feeding period is short to observe its probable antinutrivitive effect. Previous study conducted by Kono *et al*. (2000) shows that green tea extract and green tea powder incorporated in fish diets decreases the lipid accumulation of yellow tail and ayu.

Amylase activities of unenriched (control) *Artemia* nauplii are higher than GTE-enriched nauplii. Same scenario was also observed for neon tetra and guppy samples. Amylase activities of neon tetra are higher than guppy samples. Results indicate that the enzyme profiles of neon tetra and guppy were influenced mainly by their food intake.

The result of the present study agrees with previous studies which postulate show that amylase activities are greater in omnivorous (Fange & Grove, 1979; Ugolev *et al*. 1983; Hidalgo *et al*. 1999, and Furne *et al*. 2005). Neon tetras and guppies are omnivorous animals, thus amylase activities expected to be higher since they need them to break down polysaccharides into short-chain sugars (Hidalgo *et al*. 1999; Natalia *et al*. 2004).

Total proteases of GTE-enriched *Artemia* nauplii are almost halfway lower than unenriched samples, but almost no difference was observed between control and GTE-enriched fed *Artemia* nauplii for neon tetra and guppy. This result indicates that green tea enrichment did not alter the enzyme profile of neon tetra and guppy when incorporated in *Artemia* nauplii.

The proteolytic activities of neon tetra and guppy at different pH, is similar to the pattern of *Artemia* nauplii, where high proteolytic activities at acidic condition was observed at pH 1.5 and at alkaline pH at 8.5, 9.0 and 10.0. Higher values were observed for neon tetra than guppy samples.

Proteolytic activities observed in fish at optimum pH 2.0 indicate the presence of pepsins (Clark *et al*. 1985; Hidalgo *et al*. 1999). In this study, *Artemia* nauplii fed neon tetra and guppy has high proteolytic activities at pH 1.5. High pepsin secretion was also reported in eel, tilapia, salmon, sea bass and trout species which are all possess clear stomach region (Jonas *et al*. 1983; Torrisen 1984; Sabapathy & Teo 1993; Hidalgo *et al*. 1999). Guppies and neon tetras are omnivorous which are stomachless, yet have pepsin found on their digestive tracts. Pepsin enzyme was probably from *Artemia* nauplii that were supplemented to them during the 10-days feeding period. The low proteolytic activity observed at pH 3.0 and pH 4.0 also shows the presence of gastric proteases in *Artemia* nauplii, neon tetra and guppy for gastric digestion (Furne *et al*. 2005).

The high proteolytic activities in alkaline pH (8.5-10.0) of *Artemia* nauplii, neon tetra and guppy could be attributed to trypsin, chymotrypsin, collagenases, elastases, carboxypeptidase, which have been reported in different types of fish (Eshel *et al*. 1993; Chiu & Pan, 2002; Chong *et al*. 2002; Furne *et al*. 2005). This result was further supported by other reports which showed that carp (Jonas *et al*. 1983), halibut and turbot (Glass *et al*. 1989), goldfish (Hidalgo *et al*. 1999) and discuss (Chong *et al*. 2002) have maximum protease activities at pH 8.0-10.0.

Despite many studies on enzyme characterization, comparing of enzyme results with other species from different studies is difficult as often highlighted by other researchers because of the difference in sampling and preparation of enzyme extract from tissues (Alarcon *et al*.

1999; Hidalgo *et al*. 1999; Chong *et al*. 2002; Natalia *et al*. 2004). In the present study, enzyme profile of neon tetra and guppy fed unenriched and GTE-enriched *Artemia* nauplii was presented. The existence of proteolytic activities at different pH levels means that *Artemia* nauplii were able to enhance enzymes present on the digestive tract of neon tetra and guppy regardless of their feeding habit.

In summary, no significant difference was observed on biochemical composition of neon tetra and guppy in terms of their total lipid and protein. Also, no major changes occurred in the digestive enzyme composition on the two freshwater ornamental fishes when they fed with GTE-enriched *Artemia* nauplii. With protease enzymes presented at different pH level, future studies should be investigated to determine if these two mentioned ornamental fishes can digest a wider range of ingredients, for the development of cost-effective formulated feed for intensive farming.

CHAPTER 6

General Discussion and Conclusion

Mortalities due to stress events routinely occur in aquaculture that lead to substantial economic losses, especially for ornamental fish production where exposure to stresses does not end in the production period (Dagar 2010). During ornamental fish export business, freight costs range from 30% to more than 100% of the fish cost and in order to cut down the expenses, fish are packed in heavy densities (Lim & Chua 1993; Lim *et al*. 2002). Ornamental fish undergo stress due to handling procedures (e.g., selection, sorting, etc.) prior to packing, loading and unloading processes, crowding, and adverse water quality conditions during transportation which often takes up to 48-hours (Lim *et al*. 2002). Fish with low resistance cannot withstand the stresses encountered during the transportation process, leading to relatively high post-shipment mortality within a week of arrival. This low resistance to stress is due to sub-optimal physiological conditions arising from infectious diseases, nutritional deficiencies, poor water quality, etc. (Lim *et al*. 2002).

Until now, there are still no adequate practical solutions to common stresses experience by fish during the culture period up to trading. And the best way to offset this stressful scenario is to enhance the antioxidant capacity of the aquatic animals from larval production by providing them with high containing antioxidant compounds through their nutrition. Since majority of hatcheries prefers *Artemia* nauplii as live food, this study focused on incorporating green tea extract (which are known to posses high containing antioxidants) through bioencapsulation technique.

Chapter 2 of this paper determined the best condition of incorporating green tea extract (GTE) in optimizing the total polyphenol content, total carotenoid and antioxidant activity of *Artemia* nauplii. One condition was based on contact time where newly hatched *Artemia* nauplii were exposed to green tea extract up to 24-hours. Prior to enrichment study, a preliminary run was conducted to green tea extract alone to determine the stability of polyphenols and its antioxidant activities. In the preliminary run, total polyphenol content

(TPC) and antioxidant activities of green tea extract at 3.50% salinity and with aeration show no significant difference within 24-hours. And generally, the most appropriate enrichment technique for newly hatched Artemia is commonly applied to 24-h period after hatching (Han *et al*. 2000; Makridis *et al*. 2000; Sorgeloos *et al*. 2001; Stewart *et al*. 2000; Woods 2003; Ando *et al*. 2004; Hai *et al*. 2010).

Another condition was exposing different naupliar stages of *Artemia* nauplii for 6-h (time where highest result was observed during preliminary run) enrichment period. The three (3) early naupliar stages were used, since the nutrients of *Artemia* from their yolk are still intact, thus no additional food is needed for their survival.

Results of the enrichment study shows that incorporation of GTE to *Artemia* was more associated to age of nauplii than time of exposure. GTE-enriched instar II *Artemia* nauplii registered the highest TPC, total carotenoid and DPPH free radical scavenging activity. High results can be attributed to the fact that at this stage, nauplii already develop a functional digestive system and can obtain their sustenance and other extraneous substance by filter feeding on particles from the water (Makridis & Vadstein 1999; Rodriguez *et al*. 2011). This result agrees with the results obtained by Rodriguez *et al*. (2011) where instar II *Artemia* nauplii also accumulated highest levels of metronidazole than instar I. Instar II nauplii was also used by Immanuel *et al*. (2004) for *Odonus niger* lipid enrichment. The values of TPC and antioxidant activities obtained using newly hatched and instar I was probably due to the nutrition they obtained from their yolk or to the TPC attached to their cuticle.

Aside from TPC, total carotenoids present in *Artemia* nauplii in all stages also contributes to the antioxidant activity results. Total carotenoids could be the one responsible for the antioxidant capacity of unenriched *Artemia*.

From the results obtained in Chapter 2, GTE-enriched *Instar II* Artemia nauplii were given to guppy fry (Chapter 3) and marketable ornamental fishes (Chapter 4).

In chapter 3, application of GTE-enriched *Artemia* nauplii were fed to guppy fry for 20 days (marketable size for guppy fry) to determine its effect on their growth performance, antioxidant activity and stress resistance against salinity. Salinity stress test was developed by Lim *et al*. (2002) to quantitatively evaluate the guppies' resistance before a stressful condition, such as shipment (Dagar 2010). This stress test was suggested to be more stable than exposure to other parameters such as temperature, pH and ammonia (Lim *et al*. 2003; Dagar 2010).

Success on hatchery production also includes high growth rate and better survival. Overall results show that guppy fry were able to ingest and utilize the nutrients of *Artemia* nauplii as shown on their improved specific growth rate (SPG) from day 0 to day 20 samples. The results obtained on this study aggress with the results obtained by Aruvalsu *et al*. (2012) for freshwater molly *Poecilla sphenops* fish fry. Aruvalsu *et al*. (2012) shows that specific growth rate was improved by feeding with *Artemia* nauplii enriched with herbal plant extract *Vitex negundo*. Results also indicate shifting of live feed organisms from feeding young fish in the industry from *Moina* to *Artemia* was successful. Aquaculturist prefers *Artemia* over *Moina* since the latter is cultured in water enriched with organic manure (unhygienic environment) that might threaten the health of freshwater ornamental fishes due to bacterial diseases that comes along with it (Lim *et al*. 2001, Sales 2003).

Green tea enrichment is also a way of decreasing bacteria present in water since 1 mg/mL concentration was proven by Sornsanit *et al*. (2002) can treat *Vibrio*-infected shrimp culture water. In the field, *Vibrio* can cause mortality in larvae, post larvae, and juveniles up to 100% of affected population.

Survival of guppy fry against salinity stress was influenced by the presence of polyphenols ingested by guppy from GTE-enriched instar II *Artemia* nauplii. High resistance of guppy fry was observed after 10 days of feeding with GTE-enriched *Artemia*. At day 10, TPC and DPPH free radical scavenging activity possesses the highest values, thus we could say that their resistance against salinity stress was influenced by the polyphenols they have ingested from the green tea extract.

At day 20, decrease in TPC and DPPH free radical scavenging activity of guppy fry fed unenriched (control) and GTE-enriched instar II *Artemia* nauplii samples could be the reason why their survival against salinity stress was lower. At these stages, TPC was probably utilized by fish for their growth performance than on enhancing their antioxidant activities. To improve the TPC amount on older guppy, green tea concentration could be increased, used older stages of *Artemia* nauplii so that they can ingest more amount of TPC or incorporate green tea extract on artificial diets.

To prevent many feed preparation in hatcheries or in the field, GTE-enriched *Artemia* nauplii were also applied to marketable size ornamental fishes like neon tetra and adult guppy (Chapter 4). During trading, different sizes of ornamental fish are used, thus the objective of this study was to determine the influence of fish size on growth performance and stress resistance of freshwater ornamental fish fed green tea enriched *Artemia* nauplii. The 10-days short term feeding trial was conducted based on the results obtained in Chapter 3 for guppy fry.

Chapter 4 shows that unenriched and GTE-enriched instar II *Artemia* nauplii can enhance neon tetra and guppy fish samples regardless of their size. In a previous study conducted by Aruvalsu *et al*. (2012), freshwater fish molly *Poecilia sphenops* fry obtained maximum specific growth rate (SGR) when fed with *Artemia* nauplii enriched with herbal extract of

Vitex negundo Linn. than unenriched *Artemia* nauplii. The same result was also obtained by Immanuel *et al*. (2004) where, *Penaeus indicus* juveniles fed with *Artemia* nauplii enriched with terrestrial herbs and seaweed powder has higher SGR than unenriched *Artemia* nauplii. In this case, SGR of guppy fry samples are significantly higher ($p<0.05$) compared to neon tetra juveniles and guppy adults. The SGR of fish samples fed with GTE-enriched instar II *Artemia* nauplii are higher than their corresponding control, but no significant ($p>0.05$) difference was observed between them. The improvement in SGR of fish samples fed with GTE- enriched *Artemia* nauplii was due to the component found in green tea like polyphenols, protein, carbohydrates, fats, peptides and organic acid (Sato & Miyata 2000; Bae & Lee 2010).

Generally, after 10-days of feeding period, no significant difference was observed on TPC values between fish samples fed unenriched (control) and GTE-enriched instar II nauplii but the latter samples have better survival upon exposure to salinity stress. This was due to the high value of their DPPH free radical scavenging activity which supports the presence of polyphenols from green tea extract that were accumulated to their body during feeding which enhances the fish' antioxidant capacity. DPPH free radical scavenging activity demonstrates how fast the polyphenols present in the animal's body absorbed or neutralize free radicals during stressful conditions (Ksouri *et al*, 2007).

With respect to size, guppy fry has higher tolerance to salinity stress compared to neon tetra juveniles and adult guppy basically due to the presence of their high values of TPC and DPPH free radical scavenging activity. For adult guppies, the amount of TPC obtained from GTE-enriched instar II *Artemia* nauplii is probably low enough to enhance their antioxidant activity. Also, the nutrient of guppy fry from its yolk may have an additional role on its survival against salinity stress.

Lastly, since green tea enriched in *Artemia* nauplii was able to enhance the antioxidant activity of fish, it is also interesting to determine its direct effect on their nutrition. Chapter 5 presents the biochemical and digestive enzyme composition of neon tetra and guppy fed green tea enriched *Artemia* nauplii to further test its applicability in freshwater ornamental farming.

The current study shows that despite containing high amount of polyphenols (considered as one of anti-nutrition in aquaculture) green tea extract introduced via *Artemia* nauplii to neon tetra and guppy during the 10-days feeding period did not affect their biochemical composition (total lipid and total protein). The anti-nutrients were probably balanced out by the nutritional component of *Artemia* nauplii or the feeding period is short to observe its probable antinutrivitive effect.

With regards to enzyme composition, amylase activities of unenriched (control) *Artemia* nauplii are higher than GTE-enriched nauplii. Same scenario was also observed for neon tetra and guppy samples. Amylase activities of neon tetra are higher than guppy samples. Results indicate that the enzyme profiles of neon tetra and guppy were influenced mainly by their food intake.

Neon tetras and guppies are omnivorous aquatic animals which feed both plant and meat food. Amylase activities are often reported in herbivorous and omnivorous species because they are needed to break down polysaccharides into short-chain sugars (Hidalgo *et al*. 1999; Natalia *et al*. 2004). The result of the present study agree with previous studies which postulate show that amylase activities are greater in omnivorous than carnivorous fish (Fange & Grove, 1979; Ugolev *et al*. 1983; Hidalgo *et al*. 1999 and Furne *et al*. 2005).

The proteolytic activities of neon tetra and guppy at different pH, is similar to the pattern of *Artemia* nauplii, where high proteolytic activities at acidic condition was observed at pH 1.5

and at alkaline pH at 8.5, 9.0 and 10.0. Higher values were observed for neon tetra than guppy samples.

Proteolytic activities observed in fish at optimum pH 2.0 indicate the presence of pepsins (Clark *et al*. 1985; Hidalgo *et al*. 1999). In this study, *Artemia* nauplii fed neon tetra and guppy has high proteolytic activities at pH 1.5. High pepsin secretion was also reported in eel, tilapia, salmon, sea bass and trout species which are all possess clear stomach region (Jonas *et al*. 1983; Twining *et al*. 1983; Torrisen 1984; Sabapathy & Teo 1993; Yamada *et al*. 1993; Hidalgo *et al*. 1999). Guppies and neon tetras are omnivorous which are stomachless, yet have pepsin found on their digestive tracts. Pepsin enzyme was probably from *Artemia* nauplii that were supplemented to them during the 10-days feeding period. The low proteolytic activity observed at pH 3.0 and pH 4.0 also shows the presence of gastric proteases in *Artemia* nauplii, neon tetra and guppy for gastric digestion (Furne *et al*. 2005).

The high proteolytic activities in alkaline pH (8.5-10.0) of *Artemia* nauplii, neon tetra and guppy could be attributed to trypsin, chymotrypsin, collagenases, elastases, carboxypeptidase, which have been reported in different types of fish (Eshel *et al*. 1993; Chiu & Pan 2002); Chong *et al*. 2002; Furne *et al*. 2005). This result was further supported by other reports which showed that carp (Jonas *et al*. 1983), halibut and turbot (Glass *et al*. 1989), goldfish (Hidalgo *et al*. 1999) and discuss (Chong *et al*. 2002) have maximum protease activities at pH 8.0-10.0.

Conclusion and recommendation

In summary, green tea enrichment using *Artemia* nauplii via bioencapsulation technique could be the best alternative for improving the antioxidant capacity of freshwater ornamental fishes. The simplicity and versatility of the designed enrichment process could cater small scale farmers, hobbyist and beginner aquaculturist. Utilization of green tea as source of natural antioxidants is cheap and free of environmental risks that make it attractable to developing countries who are majority exporters of ornamental fish in the world. Green tea bioencapsulation using *Artemia* nauplii is also a practical method of introducing natural antioxidants to smaller fishes because direct application of tea powder or extract to water, lowers the pH of the environment, which is detrimental to aquatic animals.

To further establish the capability of GTE-enriched *Artemia* nauplii, it needs to be tested on other freshwater ornamental commodities, at longer feeding period or higher dosage. Also, incorporation of green tea extract or powder in artificial diets for adult stages is recommended. Stress resistance can be tested starting from larval stage up to trading for additional validity of green tea as antioxidant enhancer for aquatic animals.

It is also interesting to know the possible effect of high containing antioxidant on the pigmentation of colorful ornamental fishes.

And lastly, with the given digestive enzyme profile of neon tetra and guppy at different pH, formulation and development of cost-effective formulated feed for intensive farming is highly recommended.

Acknowledgement

I would like to express my special appreciation and thanks to my advisor, Dr. Nakao NOMURA for accepting me to work under his laboratory four years ago. For giving me full support, numerous advises, and words of encouragement and for allowing me to grow as a researcher.

I would also like to thank my committee members, Dr. Yutaka KITAMURA, Dr. Kasumi YAMADA, and Dr. Yingyan YANG for their time and suggestions for the improvement of my dissertation.

I greatly thank the Ministry of Education of Japan (Monbukagakusho) for the scholarship.

Special thanks also to:

My labmates: Kristel, Cons, Yui, Edgar, Amie, Rhea, Shimada-san, Yasui-san, Omiya-san, Bird, Nat, Mo, Katrin, Oui, Jui, Ping, Nanwan, Je, Gaye, Takumi-san and Arvin for the fun and crazy moments

Association of Filipino Scholars in Tsukuba (FAST) for the company

WATANABE family (Doc. Wats, Naho, Yuchan and baby Koshi) for serving as my family here in Japan

Tatay, Nanay, Dods, Orwen, Donabel, Paul, Jay and Wayne for all the love and inspiration

And to the ONE up there for guiding me all these years, LORD this is all for YOU.

References

Abdel-Tawwab M, Ahmad MH, Seden MEA, Sakr SFM (2010) Use of green tea, *Camelia sinensis* L. in practical diet for growth and protection of Nile tilapia, *Oreochromis niloticus* (L), against *Aeromonas hydrophila* infection. Journal of World Aquaculture Society 41: 203-13

Alarcon FJ, Moyano FJ, Diaz M (1999) Effect of inhibitors present in protein sources on digestive proteases of juvenile seabream (*Sparta aurata*). Aquatic Living Resources 12: 233-238

Ando Y, Samoto H, Murayama Y (2004) Positional distribution of DHA and EPA in triacyl-*sn*-glycerols (TAG) of *Artemia franciscana* nauplii enriched with fish oils ethyl esters and TAG. Aquaculture 233: 321-335

Anesini C, Ferraro GE, Filip R (2008) Total polyphenol content and antioxidant capacity of commercially available tea (*Camellia sinensis*) in Argentina. Journal of Agricultural and Food Chemistry 56: 9225-9229

Angeles IP, Chien YH, Yambot AV (2009) Effect of injected astaxanthin on survival, antioxidant capacity, and immune response of the giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) challenged with *Lactococcus garvieae*. Journal of Shellfish Research 28: 931-937

Aruvalsu C, Shobana S, Banu H, Chandhirasekar D, Prabhu D (2012) Bioencapsulation of *Artemia* nauplii with herbal extract for promoting growth of fish fry *Poecilia sphenops* Val. Journal of Modern Biotechnology 1: 37-44

Asem A (2008) Historical record on brine shrimp *Artemia* more than one thousand years ago from Urmia Lake, Iran. Journal of Biological Research 9: 113-114

Asem A, Rastegar-Pouyani N, De Los Ríos-Escalante P (2010) The genus *Artemia* Leach, 1819 (Crustacea: Branchiopoda). I. True and false taxonomical descriptions. Latin American Journal of Aquatic Research 38: 501-506

Baboli MJ, Naderi A, Mohammad GH, Kalantarhormozi S (2012) The potential dried decapsulated *Artemia urmiana* cysts on growth and survival guppy fry, *Poecilia reticulata*. International Journal of Agriculture and Crop Sciences 4: 1391-1393

Bae MS and Lee SC (2010) Effect of deep sea water on the antioxidant activity and catechins content of green tea. Journal of Medicinal Plants Research 4:1662-1667

Barros L, Ferreira MJ, Queiros B, Ferreira I, Baptista P (2007) Total phenols, ascorbic acid, *β*-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidants. Food Chemistry 103: 413-419

Benjits F, Vanvoorden E, Sorgeloos P (1975) Changes in the biochemical composition of the early larval stages of the brine shrimp, *Artemia salina* L. In: Persoone., Jaspers E. (Eds.), *Proceedings- 10th European Symposium on Marine Biology*, vol. 1. Universa Press, Wetterean, Belgium, pp. 1-9

Britton G (1995) Structure and properties of carotenoids in relation to function. The Journal of the Federation of American Societies for Experimental Biology 9: 1551-1558

Buyukacapar HM, Yanar M, Yanar Y (2007) Pigmentation of rainbow trout (*Oncorhynchus mykiss*) with carotenoids from marigold flower (*Tagetes erecta*) and red pepper (*Capsicum annum*). Turkish Journal of Veterinary and Animal Sciences 31:7-12

Clark J, Mcdonald NL, Stark JR (1985) Metabolism in marine flatfish: II. Protein digestion in dover sole (*Solea solea* L.). Comparative Biochemistry and Physiology – Part B Biochemistry and Molecular Biology 81: 217-222

Chan ES, Yim ZH, Phan SH, Mansa RF, Ravindra, P (2010) Encapsulation of herbal aqueous extract through absorption with ca-alginate hydrogel beads. Food and Bioproducts Processing 88: 3195–201

Chapman FA, Fitz-Coy SA, Thunberg EM, Adams CM (1997) United States of America trade in ornamental fish. Journal of the World Aquaculture Society 28:1-10

Chen HY and Lin HF (1992) Effect of different *Artemia* diets on the growth and digestive enzyme activities of early postlarval *Penaeus monodon*. Asian Fisheries Science 5: 73-81

Chen WJ and Wan SQ (1994) Research progress on polyphenols of tea. Natural Products Research and Developments 6: 74-80

Chen ZY and Chan PT (1996) Antioxidative activity of green tea catechins in canola oil. Chemistry and Physics of Lipids 82: 163-172

Cheng TO (2006) All teas are not created equal: the Chinese green tea and cardiovascular health. International Journal of Cardiology 108:301-308

Chien YH and Shiau WC (2005) The effects of dietary supplementation of algae and synthetic astaxanthin on body astaxanthin, survival, growth and low dissolved oxygen stress resistance of kuruma prawn, *Marsupenaeus japonicus* Bate. Journal of Experimental Marine Biology and Ecology 318:201-211

Chiu ST and Pan BS (2002) Digestive protease activities of juvenile and adult eel (*Anguilla japonica*). Aquaculture 205: 141-156

Cho SH, Lee SM, Park BH, Ji SC, Lee J, Bae J (2007) Effect of dietary inclusion of various sources of green tea on growth, body composition and blood chemistry of the juvenile olive flounder, *Paralichthys olivaceus*. Fish Physiology and Biochemistry 33: 49-57

Chong ASC, Hashim R, Lee CY, Ali AB (2002) Partial characterization and activities of proteases from digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture 203: 321-333

Citarasu T, Immanuel G, Peter MM (1998) Effect of feeding *Artemia* enriched with stressol and cod liver oil on growth and stress resistance in the Indian white shrimp *Penaeus indicus* postlarvae. Asian Fisheries Science12: 65-75

Criel G and MacRae T **(**2002) *Artemia* morphology and structure. Reproductive biology of *Artemia*. In: T. Abatzopoulos, J. Beardmore, J. Clegg, P. Sorgeloos (Eds.). *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers

Dabrowski K (1979) The role of proteolytic enzymes in fish digestion. In Styczynska-Jurewicz E, Backiel T, Jaspers E and Persoone G (eds.) Cultivation of fish fry and its live food. European Mariculture Society, Special Publication 4. Belgium: Bredene. 107-126

Dabrowski K (1982) Proteolytic enzyme activity decline in starving fish alevins and larvae. Environemtal Biology of Fishes 7: 73-76

Dagar A, Zilberg D, Cohen Z, Boussiba S and Khozin-Goldberg I (2010) Short-term dietary supplementationwith the microalga *Parietochloris incisa* enhances stress resistance in guppies *Poecilia reticulata*. Aquaculture Research 41: 267-277

Dobbleleir J, Adam N, Bossuyt E, Bruggeman E, Sorgeloos P (1980) New aspects of the use of inert diets for high density culturing of brine shrimp. In: The Brine Shrimp *Artemia*. Volume 3:165-174

Eshel A, Lindner P, Smirnoff P, Newton S, Harpaz S (1993) Comparative study of proteolytic enzymes in the digestive tracts of the European sea bass and hybrid striped bass reared in freshwater. Comparative Biochemistry and Physiology 106A: 627-634

Fange R and Grove D (1979) Digestion. In: Hoar WS, Randall DJ, Brett JT (Eds.). Fish Physiology, Bioenergetics and Growth 8. Academic Press, New York, pp: 161-260

Farhoosh R, Golmovahhed GA, Khodaparast MHH (2007) Antioxidant activity of various extracts of old leaves and black tea wastes (*Camellia sinensis* L.). Food Chemistry 100: 231-236

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry. 226:497-50

Furne M, Hidalgo MC, Lopez A, Garcia-Gallego M, Morales AE, Domezain A, Domezaine J, Sanz A (2005) Digestive enzyme activities in Adriatic Sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. Aquaculture 250: 391-398

Glass HJ, McDonald NL, Moran RM, Stark JR (1989) Digestion of protein I different marine species. Comparative Biochemistry and Physiology 94B: 607-611

Graham HN (1992) Green tea composition, consumption and polyphenol chemistry**.** Preventive Medicine 21: 334-350

Gomez-Gil B, Cabanillas–Ramos J, Paez–Brambila S, Roque A (2001) Standardization of the bioencapsulation of enrofloxacin and oxytetracycline in *Artemia franciscana* Kellog, 1906. Aquaculture 196: 1-12

Goto T, Yoshida Y, Amano I, Horie H (1996) Chemical composition of commercially available Japanese green tea. Foods and Food Ingredients Journal of Japan 170: 46-51

Gulcin I, Elmastas M, Aboul-Enein HY (2007) Determination of antioxidant and radical scavenging activity of basil (*Ocimum basilicum L*. Family *Lamiaceae*) assayed by different methodologies. Phytotheraphy Research 21: 354-361

Hai VN, Buller N, Fotedar R (2010) Encapsulation capacity of *Artemia* nauplii with customized probiotics for use in the cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). Aquaculture Research 41: 893-903

Han K, Geurden I, Sorgeloos P (2000) Enrichment strategies for *Artemia* using emulsions providing different levels of *n*-3 highly unsaturated fatty acids. Aquaculture 183: 335-347

HBOI (2000) Marine ornamental production. www.hboi.edu/aquaculture/marine.html. 1 October 2000

Hidalgo MC, Urea E, Sanz A (1999) Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170: 267-283

Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit. Rev Food Sci Nutr. 43: 89-143

Hrelia S, Bardoni A, Angeloni, C, Leoncini E, Gallina T, Lercker G, Biagi, PL (2002) Green tea extracts can counteract the modification of fatty acid composition induced by doxorubicin in cultured cardiomyocytes. Prostaglandins, Leukotrienes and Essential Fatty Acids 66: 519-524

Hofer R and Kock G (1989) Method for quantitative determination of digestive enzymes in fish larvae. Polskie Archiwum Hydrobiologii 36: 439-441

Immanuel G, Immanuel A, Palavesam M, Peter M (2001) Effects of feeding lipid enriched *Artemia* nauplii on survival, growth, fatty acids and stress resistance of postlarvae *Penaeus indicus* Asian Fisheries Society14: 377-388

Immanuel G, Palavesam A, Sivaram V, Babu M, Marian P (2004) Feeding trashfish *Odonus niger* lipid enriched *A. franciscana* on growth, stress resistance and HUFA requirements of *Penaeus monodon* postlarvae. Aquaculture 237: 301-313

Ishihara N, Arataki T, Tamaru Y, Inoue M, Nishimura A, Aoi N (2002) Influence of green tea polyphenols on feed performance, growth performance and fish body component in yellowtail (*Seriola quinqueradiata*). Japan Journal of Food Chemistry 9: 7-1

Jonas E, Ragyanssszki M, Olah J, Boross L (1983) Proteolytic digestive enzymes of carnivorous (*Silarus glanis* L.), herbivorous (*Hypophthalmiichttys molitirix* Val.) and omnivorous (*Cyprinus carpio* L.) fishes. Aquaculture 30: 145-154

Kaur C and Kapoor HC (2001) Antioxidants in fruits and vegetables - the millennium's health. International Journal of Food Science and Technology 36: 703-725

Kolkovski S, Koven W, Tandler A (1997) The mode of action of *Artemia* in enhancing utilization of microdiet by gilthead seabream *Spanus aurata* larvae. Aquaculture 155: 501-514

Kono M, Furkawa K, Sagesaka YM, Nakagawa K, Fujimoto K (2000) Effect of green tea extract and green tea grounds as dietary supplement on cultured yellow tail and ayu. Nippon Shokuhin Kagaku Kaishi 47: 932-937

Komes D, Horzic D, Belscak A, Ganic K, Vulic I (2010) Green tea preparation and its influence on the content of bioactive compounds. Food Research International 43: 167-176

Koo MW and Cho CH (2004) Pharmacological effects of green tea on the gastrointestinal system. European Journal of Pharmacology 500: 177-85

Ksouri R, Megdiche W, Debez A, Falleh H, Grignon C, Abdelly C (2007) Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritime*. Plant Physiology and Biochemistry 45: 244-249

Langdon C (2003) Microparticle types for delivering nutrients to marine fish larvae. Aquaculture 227: 259-275

Lavens P and Sorgeloos P (eds.) (1996) Manual on the Production and Use of Live Food for Aquaculture. Vol. 36, p. 295. FAO, Fisheries Technical Paper, Rome Italy

Leach WE (1819) Entomostraca, Dictionaire des Science Naturelles 14, page. 524

Leager DA, Bengston KL, Simpson P, Sorgeloos P (1986) The use and nutritional value of *Artemia* as a food source. Oceanography and Marine Biology - An Annual Review 24: 521-623

Lichtenthaler HK,Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochemical Society Transactions 11: 591-592

Lim LC, Dhert P, Chew WY, Dermaux V, Nelis H, Sorgeloos P (2002) Enhancement of stress resistance of the guppy *Poecilia reticulata* through feeding with vitamin C supplement. Journal of the World Aquaculture Society 33: 32-40

Linnaeus C (1758) Systema naturae. Hofniae 1: 634

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193: 265-275

Magurran AE (2005) Evolutionary Ecology: the Trinidadian Guppy. Oxford University Press.

Makridis P and Vadstein O (1999) Food size selectivity of *Artemia franciscana* at three developmental stages. Journal of Plankton Research 21: 2191-2201

Makridis P, Fjellheim AJ, Skjermo J, Vadstein O (2000) Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. Aquaculture 185: 207-218

Martin JW and Davis GE (2001) An updated classification of the Recent Crustacea. Natural History Museum Los Angeles County Science Series 39: 1-124

Miki W (1991) Biological functions and activities of animal carotenoids. Pure and Applied Chemistry 63:141-146

Muktar H and Ahmad N (2000) Tea polyphenols: prevention of cancer and optimizing health. The American Journal of Clinical Nutrition 71: 1698S-1702S

Natalia Y, Hashim R, Ali A, Chong A (2004) Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (Osteoglossidae). Aquaculture 233: 305-320

Ni H, Chen QH, He GQ, Wu GB, Yang YF (2008) Optimization of acidic extraction of astaxanthin from *Phaffia rhodozyma*. Journal of Zhejiang University Science B 9: 51-59

Ong ASH and Tee ES (1992) Natural sources of carotenoids from plants and soils. Methods in Enzymology 213: 142-167

Pan CH, Chien YH, Hunter B (2003) Alterations of antioxidant capacity and hepatopancreatic enzymes in *Penaeus monodon* (Fabricius) juveniles fed diets supplemented with astaxanthin and exposed to *Vibrio damsela* challenge. Journal of the Fishery Society of Taiwan 30:279-290

Prasad KN, Yang B, Dong X, Jiang G, Zhang H, Xie H, Jiang Y (2009) Flavanoid contents and antioxidant activities from *Cinnamun* species. Innovative Food Science and Emerging Technologies 10: 627-632

Preito P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Analytical Biochemistry 269: 337-341

Rainha N, Lima E, Baptista J, Rodrigues C (2011) Antioxidant properties, total phenolic, total carotenoid and chlorophyll content of anatomical parts of *Hypericum foliosum*. Journal of Medicinal Plants Research 5: 1930-1940

Rodriguez L, Livengood EJ, Miles RD, Chapman FA (2011) Uptake of metronidazole in *Artemi*a at different developmental life stages. Journal of Aquatic Animal Health 23:100-102

Roiha IS, Otterlei E, Samuelsen OB (2011) Evaluating bioencapsulation of florfenicol in rotifers (*Brachionus plicatilis*). Aquaculture Research 42: 1110-1116

Sabahpathy U and Teo LH (1994) Some kinetic properties of amylase from the intestine of the rabbitfish, *Siganus canaliculatus* (Park). Comparative Biochemistry and Physiology- Part B Biochemistry and Molecular Biology 109: 139-144

Salah N, Miller NJ, Parganga G, Tifburg L, Bolwell GP, ice-Evan C (1995) Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics 322: 339-346

Salma D, Davoodi R, Shamsaei M, Kamali A (2013) Comparative Effect of Fairy Shrimp and *Artemia* in the Rearing of Blue Gourami, *Trichogaster trichopterus*, Larvae. Annual Review and Research in Biology 3: 70-75

Sanaye SV, Singh H, Tibile RM (2012) Growth and survival of neon tetra, *Paracheirodon innesi* (Myers, 1936) fry fed mixed zooplankton, formulated feed and combination thereof. Scholars Research Library 3: 5665-5668

Shahidi F and Wanasundara PD (1992) Phenolic antioxidants. Critical Reviews in Food Science and Nutrition 32: 67-103

Sarjito, Ningrum NE, Radjasa OK, Prayitno SB (2012) Application of repetitive sequence-based PCR on the richness of *Vibrio* on the tiger shrimp(*Penaeus monodon* Fab.). Journal of Coastal Development 15: 303-309

Sato T and Miyata G (2000) The nutraceutical benefit, Part I: Green tea. Nutrition 16: 315-317

Sheilhzadeh N, Nofouzi K, Delaza A, Oushani AK (2011) Immunodulatory effects of decaffeinated green tea (*Camellia sinensis*) on the immune system of rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology 31: 1268-1269

Smith GG, Ritar AJ, Brown MR (2004) Uptake and metabolism of a particulate form of ascorbic acid by *Artemia* nauplii and juveniles. Aquaculture Nutrition 10: 1-8

Sorgeloos P, Dhert P, Candreva P (2001) Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200:147-159

Sorgeloos P, Coutteau P, Dhert P, Merchie G, Lavens P (1998) Use of brine shrimp, *Artemia* spp., in larval crustacean nutrition: a review. Reviews in Fisheries Sciences 6: 55-68

Sornsanit K, Nomura N, Migo VP, Matsumura M (2002) The use of green tea powder and green tea polyphenols for treatment of *Vibrio*-infected shrimp culture water. Japanese Journal of Water Treatment Biology 38: 111-115

Stewart AB, Spicer AV, Inskeep EK, Dailey RA (2001) Short communication: Steroid hormone enrichment of *Artemia* nauplii. Aquaculture 202: 177-181

Sunaryanto A and Mariyam A (1987) Occurrence of a pathogenic bacteria causing luminescene in *penaeid* larvae in Indonesia hatcheries. Bulletin of the Brackishwater Aquaculture Development Centre 8: 64-70

Tanizawa H, Toda S, Sazuka T, Taniyama T, Hayashi T, Arichi S (1984) Natural antioxidants. I. Antioxidative components of tea leaf (*Thea sinensis L*.). Chemical and Pharmaceutical Bulletin 32: 2011-2014

Thawonsuwan J, Kiron V, Satoh S, Panigrahi A, Verlhac V (2010) Epigallocatechin-3-gallate EGCG affects the antioxidant and immune defense of the rainbow trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry 36: 687-97

Tlusty M (2002) The benefits and risks of aquacultural production for the aquarium trade. Aquaculture 205: 203-219

Tijburg LBM, Mattern T, Folts JD, Weisgerber UM, Katan MB (1997) Tea flavanoids and cardiovascular diseases: a review. Critical Reviews in Food Science and Nutrition 37: 771-785

Torrisen KR and Torrisen OJ (1984) Digestive proteases of Atlantic salmon (*Salmo salar*) from different river strains: development after hatching, rearing temperature effect and effect of sex and maturation. Comparative Biochemistry and Physiology - Part B Biochemistry and Molecular Biology. 77: 15-20

Touraki M, Rigas P, Kastritsis C (1995) Liposome mediated delivery of water soluble antibiotics to the larvae of aquatic animals. Aquaculture 136: 1-10

Touraki M, Mourelatos S, Karamanlidou Gh, Kalaitzopoulou S, Kastritsis C (1996) Bioencapsulation of chemotherapeutics in *Artemia* as a means of prevention and treatment of infectious diseases of marine fish fry. Aquacultural Engineering 15: 133-147

Touraki M and Niopass I (2012) Bioencapsulation and pharmacokinetic study of oxolinic acid in rotifers (*Brachionus plicatilis*),*Artemia franciscana* nauplii and metanauplii. Aquaculture Research 44: 701-712

Ugolev AM, Yegorova VV, Kuz'mina VV, Gruzdkov AA (1983) Comparative molecular characterization of membrane digestion on fish and mammals. Comparative Biochemistry and Physiology. 76B: 627-635

Walter HE (1984) Proteinases: methods with hemoglobin, casein, and azocoll as substrates. In: Bergmeyer HU (Ed), Methods of Enzymatic Analysis Vol V. Verlag Chemie, Weinheim, pp: 270-277.

Whitten WA (1979) Livebearers. Tropical Fish Hobbyist Publications. Neptune City, New Jersey, USA

Wiseman SA, Balentine DA, Frei B (1997) Antioxidants in tea. Critical Reviews in Food Science and Nutrition 37: 705-718

Woods CMC (2003) Effects of varying *Artemia* enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. Aquaculture 220: 537-548

Worthingthon CC (1988) Worthington enzyme manual: enzymes and related biochemicals.

Yahyazadeh M, Eimanfar A, Camargo WN, Soltani M, Mohebbi F (2007) The use of oxolinic acid to enrich *Artemia urniana* from Urnia Lake, Iran. Research Journal of Biological Science 2: 221-224

Zuo Y, Chen H, Deng G (2002) Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta 57: 307-316

http://www.aquaticcommunity.com/tetrafish/neontetra.php

http://www.fao.org/docrep/003/w3732e/w3732e0m.htm#4.1.%20Introduction,%20biology%20and%20ecology%20of%20Artemia

http://www.fao.org/fishery/topic/13611/en

http://www.flytrapcare.com

http://images.engormix.com/e_articles/European_finfish05.gif

http://journeytoforever.org/biofuel_library/chemoils.html