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Optimal Time to Harvest Brine Shrimp (*Artemia salina*) Nauplii as Live Food for Aquatic Animals

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ABSTRACT

Brine shrimp (*Artemia salina*) are commonly utilized as aquatic live food, but only a few studies have investigated the optimal harvesting time. In the present study, brine shrimp eggs were hatched in artificial seawater and harvested at different periods (22, 24, 26, 28, 30, 32, 34, and 36 h). Harvested nauplii from quadruplicate tanks were analyzed for total carotenoid content, digestive enzyme activities, proximate chemical composition and fatty acids. Economically significant fish were used to test *in vitro* digestibility using their digestive enzymes. Total carotenoid contents significantly decreased with post-hatch time as the equation $y = –0.0108x + 0.6265$ ($p < 0.05$, –65.3% of final value). The trypsin-specific activity was elevated in brine shrimp harvested at 26 to 36 h and was lowest at 24 h ($p < 0.05$). However, there was no difference in the specific activities of chymotrypsin, lipase, and amylase over the eight harvesting periods. Ash contents were significantly lower at 34 to 36 h after hatching, compared to earlier times. Crude lipid contents fluctuated without showing a trend. In the fatty acid profile, levels of highly unsaturated, monounsaturated, polyunsaturated fatty acids, omega-3 and omega-6, were highest at 22 h after hatching. *In vitro* digestibility indicated suitable times for feeding out brine shrimp to guppy (22 h), striped catfish (22 to 24 h), Nile tilapia (22 to 26 h), and swordtail (22 to 30 h). Our investigation of the optimal time for harvesting brine shrimp as live food will help improve the quality of aquaculture produce.

Keywords: *Artemia*, digestive enzyme, fatty acid, *in vitro* digestibility, sea monkeys

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INTRODUCTION

Brine shrimp (*Artemia salina*) are zooplankton that can survive in a salinity range from 9 to 340 g/l (Gajardo & Beardmore, 2012; Sellami et al., 2020). They develop and grow rapidly within 48 h after hatching (Sleet & Brendel, 1983). Brine shrimp nauplii are commonly used as live food in the aquaculture industry, since they are smaller than the mouth size of aquatic animal larvae. In addition, they contain enzymes and nutrients that are necessary for the growth of young aquatic animals (Sellami et al., 2020; Sorgeloos et al., 2001). They are also widely available and easily cultivated at low cost, have a high reproductive rate, and tolerate difficult environmental conditions (McLaughlin et al., 1991; Turan & Mammadov, 2021; Yun et al., 2020).

Brine shrimps are red or orange. Only the keto-carotenoids canthaxanthin and echinenone (in a 19:1 ratio) contribute to their coloring (Krinsky, 1965), which improves the coloration and immune status of fish reared on a diet of *A. salina* (Biswas et al., 2024). Additionally, the digestive enzymes in *A. salina* also affect animals that consume it. They are quickly absorbed by the body, enhancing the digestion, growth, and color of the feeder. In general, brine shrimp comprise five main digestive enzymes: protease, trypsin, and chymotrypsin contribute to the digestion of protein; amylase contributes to the digestion of carbohydrates; and lipase contributes to the digestion of fat (Solorzano et al., 2009).

Many fish fry require highly unsaturated fatty acids (HUFAs) for normal growth and survival (Rainuzzo et al., 1997). As fish fry cannot synthesize this fatty acid sufficiently, they require additional fatty acids. The composition of fatty acids in brine shrimp has been studied in depth. Since they can be deficient in certain essential fatty acids necessary for fish larvae, an enrichment protocol is always applied to improve their lipid profile (Choi et al., 2021; Morshedi et al., 2022; Pham et al., 2023). Therefore, the fatty acid composition of a supply of brine shrimp must be determined, as well as the development of their fatty acid profiles up to harvesting.

The standard incubation period for brine shrimp cysts is 24 to 48 h (Sorgeloos et al., 1986), but the appropriate harvesting time of the nauplii has not been thoroughly investigated. Proximate chemical composition assessment indicated that feeding out *Artemia* nauplii should take place less than 48 h post-hatching, ideally as soon as they hatch (Sanders, 2008). The current knowledge should be expanded to provide the most effective nutrition for aquatic animals. Thus, this study aims to determine the optimal period for harvesting brine shrimp to raise aquatic animals without an enrichment protocol. Total carotenoid content, digestive enzyme activities, fatty acid profile, and proximate chemical composition were used as assessment criteria. To select the appropriate harvesting time, enzymes extracted from the guppy (*Poecilia reticulata*), swordtail (*Xiphophorus hellerii*), Nile tilapia (*Oreochromis niloticus*) and striped catfish (*Pangasianodon hypophthalmus*), which are economically important species, were used in *in vitro* digestibility tests. The results of these *in vitro* assays

bear significant relationships with *in vivo* digestibility, can be analyzed quickly, and are reliable (Hahor et al., 2022). The current investigation results can direct a practical protocol for harvesting brine shrimp nauplii in live-feed fish farming. Subsequently, aquatic animals will grow healthily if this live food is fed.

MATERIALS AND METHODS

Animal Ethics

This research was authorized by the Prince of Songkla University Institutional Animal Care and Use Committee (Project Code 2024-SCI15-067). Animal rearing, sampling, and euthanasia were based on the "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes" of the National Research Council of Thailand (Application No. U1-06514-2560).

Brine Shrimp Rearing and Collection

Containers were designed for brine shrimp rearing by dividing a 6 L plastic bottle (15 cm long, 12.5 cm in width, and 35 cm in height) into two parts. The upper part (roughly conical) was covered with a black plastic bag 11 cm long and served as the container for hatching brine shrimp cysts. The lower part of the bottle served as the base for the upper part, which was inverted and placed inside the lower part. Approximately 4 g of cysts (Snow Mountain Eagle, Tianjin, China) were hatched in a quadruplicate set of plastic containers, holding 2 L of 30 g/l artificial seawater within a pH range of 8 to 9. A two-way oxygen pump supplied aeration. Incubation proceeded under a 12-h dark/12-h light photoperiod. Under our incubation conditions, the nauplii broke out of the cysts after 15 to 20 h, passing into the Instar I phase. Therefore, the harvesting period began at 22 h and ended at 36 h, when the brine shrimp reached the Instar II phase. At that point, suspension feeding would normally start. Before sampling the nauplii at 22, 24, 26, 28, 30, 32, 34 and 36 h, empty and unhatched cysts and envelopes of hatched cysts were removed using a strainer so that only nauplii ($n = 4$) were collected. Before determining total carotenoid contents, digestive enzyme activities, chemical compositions, and *in vitro* protein digestibility, specimens were rinsed in distilled water to remove seawater and any contaminants.

Total Carotenoid Determination

The extraction technique outlined in Thongprajukaew et al. (2014) was used to extract total carotenoids. For three days, mixing at least once daily, a known weight (~3 mg) of ground, dried material was extracted using 1 ml of acetone at 4°C in the dark. For 10 min, the mixture was centrifuged at 5,000 × *g*. The extinction coefficient, E (1%, 1 cm) = 1,900, was used to determine the total carotenoid content after the absorbance of the supernatant was measured at 474 nm (Foss et al., 1984).

Digestive Enzyme Activities

Enzyme Extraction and Protein Quantification

The sampled brine shrimp were extracted using a tissue homogenizer (THP-220; Omni International, Kennesaw, GA, USA) at a ratio of 1:3 (w/v) in cold 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.5). For 30 min, the homogenate was centrifuged at 15,000 × *g* at 4°C. Prior to analysis, supernatants were gathered and stored at −20°C. Using bovine serum albumin as the protein standard, the standard method of Lowry et al. (1951) was used to determine the protein concentration in the crude enzyme extracts.

Digestive Enzyme Assays

The activities of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), lipase (EC 3.1.1.3), and α-amylase (EC 3.2.1.1) were assessed using crude enzyme extracts. Trypsin and chymotrypsin activities were evaluated using *N*-benzoyl-*L*-Arg-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrates, respectively, in accordance with the method of Rungruangsak-Torrissen et al. (2006). The outcomes were compared with the typical *p*-nitroanilide linear range. Using *p*-nitrophenyl palmitate as a substrate and comparing the results to the linear range of standard *p*-nitrophenol, lipase activity was evaluated using the Winkler and Stuckmann (1979) method. Using soluble starch as a substrate, Areekijseree et al.'s (2004) method assessed the activity of α-amylase and contrasted it with the linear range of regular maltose. By dividing each sample's amylase activity by its trypsin activity, the amylase/trypsin ratios were determined.

Chemical Composition Analysis

The methods described in the Association of Official Analytical Chemists (2005) recommendations were used in the proximate composition analysis to determine the amounts of moisture, crude protein, crude fat, and ash. Brine shrimp nauplii were dried for 24 h at 105°C in a hot air oven (WOF155; Wisd Laboratory Instruments, Wertheim, Germany) to determine the moisture content. The crude protein level was ascertained using a Kjeldahl analyzer (KjeltecTM 8100; Foss, Höganäs, Sweden). Petroleum ether was used as the solvent in a Soxhlet extraction equipment (Soxtec™ 8000; Foss, Suzhou, China) to measure the crude lipid content. Samples were burned for 2 h at 600°C in a muffle furnace (E30-HT; Thai Furnaces Engineering, Lampang, Thailand) to quantify the ash content gravimetrically.

Fatty Acid Measurement

Brine shrimp nauplii samples weighing 50 mg were extracted. The methanolysis of fatty acids in the extract was conducted through direct transesterification. In a reaction vial, 1

ml of the precisely weighed sample was mixed with 0.5 M KOH in methanol solution (RCI Labscan, Bangkok, Thailand). The mixture was vortexed for 1 min, followed by sonication for 20 min and hydrolyzed in a thermoreactor at 100°C for 20 min. Then, 0.4 ml of HCl (Merck, Darmstadt, Germany)/methanol was added at a 4:1 v/v ratio to the reaction vial, vortexed for 1 min, and then placed in the thermoreactor for another 20 min at 100°C. Once the time had elapsed, the mixture was cooled to room temperature, and the fatty acids were extracted with 2 ml of deionized water and 3 ml of petroleum ether (RCI Labscan, Bangkok, Thailand), vortexed for 1 min, and left to separate. After passing through a 0.22 µm membrane filter, the upper phase—petroleum ether and fatty acid—was gathered in a vial. Fatty acids were extracted using petroleum ether once more, and the resulting extracts were mixed. Nitrogen gas was evaporated at all extraction stages until they were completely dry.

The fatty acid residue was dissolved using 0.6 ml of heptane solvent (RCI Labscan, Bangkok, Thailand) in an injection vial, and 1.0 µl of the solution was injected for analysis by gas chromatograph-flame ionization detector (GC-FID, 7890A; Agilent Technologies, CA, USA). The carrier was helium gas, which was employed at a steady flow rate of 1 ml/min. The FID gases were the nitrogen makeup at 25 ml/min, H_2 at 30 ml/min, and air zero at 300 ml/min. All samples were injected using a split mode (25:1 ratio) with an injector. Temperature settings for the FID detector were 290°C and 300°C, respectively. The oven temperature was first kept at 140°C for 5 min, then raised to 210°C at a rate of 10°C/min, held there for 5 min, and finally raised to 250°C at a rate of 5°C/min, held there for 8 min. The fatty acid concentration was calculated using the following equation:

$$C_t = \frac{A_t \times C_{std} \times V_{solvent}}{A_{std} \times Wt} \times 1{,}000$$

where, C_t = Fatty acids interest content (mg/g); A_t = Peak areas of the fatty acid interests; C_{std} = Reference solution concentration (Methyl heptadecanoate, C17: 0) (mg/ml); A_{std} = Peak areas of reference solution (Methyl heptadecanoate, C17: 0); $V_{solvent}$ = Volume of heptane solvent that dissolves the sample (ml); Wt = Sample weight (mg).

In Vitro Digestibility

Extraction of Fish Digestive Enzymes

Nile tilapia (5.73 ± 0.05 g), striped catfish (3.52 ± 0.02 g), swordtails (0.16 ± 0.01 g), and guppy (0.18 ± 0.01 g) were bought from a private farm in Songkhla Province, Thailand. The intestines of the fish were removed and placed on ice. They were then combined with 0.2 M Na_2HPO_4-NaH_2PO_4 buffer at pH 8 (1:3 w/v), and the digestive enzymes were extracted as detailed for brine shrimp.

In Vitro Protein Digestibility

For use as substrates, nauplii samples were pulverized, freeze-dried for 48 h, and then sieved. According to Thongprajukaew et al. (2011), the enzymatic reactions were carried out. The reaction mixtures included 125 µl of dialyzed crude enzyme extract, 50 µl of 0.5% chloramphenicol, 10 ml of 50 mM Na_2HPO_4-NaH_2PO_4 buffer at pH 8, and 5 mg of dried brine shrimp nauplii. The mixtures were agitated at 200 rpm for 24 h while incubated at 25 °C. The increase in released reactive amino groups of cleaved peptides at 420 nm was used as a spectrophotometric measure of *in vitro* protein digestibility. For comparison, *DL*-alanine linear ranges were employed.

Statistical Analysis

A desired power test of 0.8 (Cohen, 1988) was used to establish the minimum sample size ($n = 4$) using R 3.6.0 Software. Our completely randomized design experiment consisted of four distinct tanks. The collected results were displayed as means and standard error of the mean (SEM). Version 22 of the Statistical Package for Social Sciences (SPSS Inc., Chicago, USA) was used to calculate all statistical values. Duncan's multiple range test was used as a *post hoc* test in a one-way analysis of variance to assess differences in means between treatment groups. If the *p*-value was less than 0.05, the hypothesis was disproved.

RESULTS AND DISCUSSION

Total carotenoid contents of brine shrimp nauplii are illustrated in Figure 1. Their concentrations significantly decreased with post-hatch time ($y = –0.0108x + 0.6265$, $r = –0.853$, $p = 0.007$). Our results corroborate the observation made by Gilchrist and Green (1960) that carotenoid content substantially declines during development. Generally, the only keto-carotenoids found in brine shrimp are canthaxanthin and echinenone, which are present in a 19:1 ratio (Krinsky, 1965). Quantitative changes in *cis*- and, to a lesser extent, all-*trans*-canthaxanthin are strongly connected to the elementary carotenoid in specific developmental stages (Nelis et al., 1988). No feed was available to the nauplii during our trial. Hence, there were no effects of dietary nutrients. In aquaculture, brine shrimp are essentially fed microalgae with high carotenoid contents, such as *Haematococcus pluvialis*, *Isochrysis galbana*, *Myrmecia incisa, Dunaliella salina*, and *Spirulina* sp. Apart from their positive impacts on fish larvae, carotenoids have also been shown to enhance the growth and health of brine shrimp, improving growth, survival rate, antioxidant capacity, and immunity (Gui et al., 2022).

The progressive change in carotenoid content and caloric value is associated with the breakdown and excretion of the yolk sac of newly hatched *Artemia* nauplii (Sanders, 2008; Treece, 2000). Only trypsin activity among all protein-digesting enzymes examined was impacted by post-hatch time (Table 1, $p < 0.05$). Specific activity of chymotrypsin was

not affected ($p > 0.05$). Since trypsin is a key enzyme in the development and digestion of proteins, substantial changes in its activity between 24 and 32 h may be related to some physiological responses. Also, trypsin activates itself and certain zymogens involved in the intestinal digestion of proteins (Moraes & Almeidam, 2020). However, based on dry matter, crude protein contents did not change with time post-hatching (Table 2, $p > 0.05$). In this study, empty, unhatched cysts and hatched cyst envelopes were removed before samples were collected, since the non-protein nitrogen contents from chitinous materials could result in false-positive results when crude protein is analyzed using the Kjeldahl method.

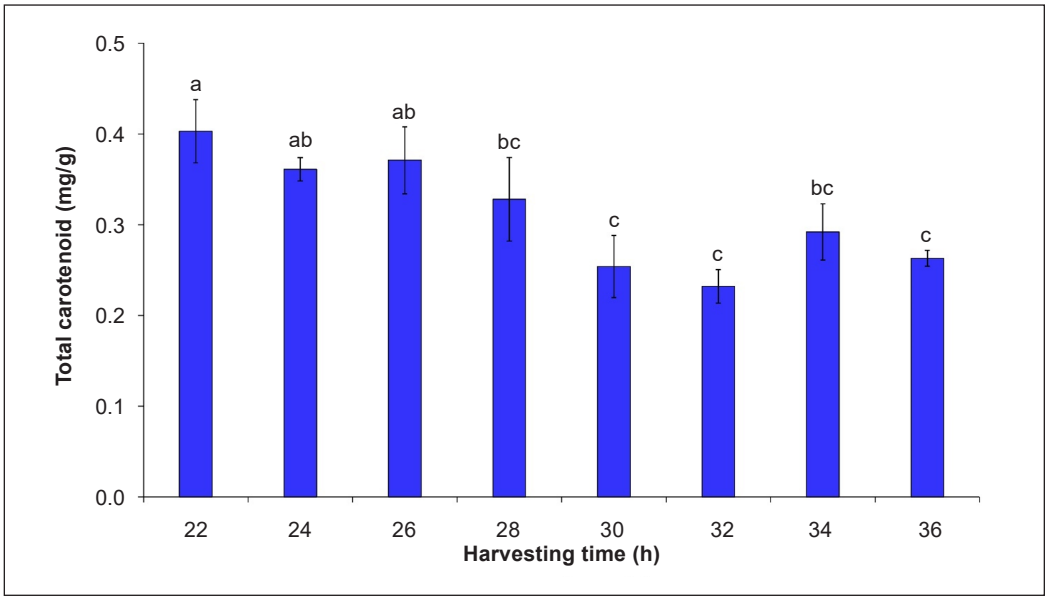


Figure 1. Total carotenoid contents ($n = 4$, based on dry weight) of brine shrimp collected at various harvesting times. Significant differences between treatments are shown by different superscripts ($p < 0.05$)

Table 1
Digestive enzyme activities of brine shrimp nauplii collected at various harvesting times (n = 4)

Digestive enzyme	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
Amylase (U/mg protein)	1.63	1.26	1.93	1.56	1.84	1.86	1.63	1.97	0.08	0.452
Trypsin (mU/mg protein)	6.76^{bc}	4.95^{c}	7.86^{ab}	8.59^{ab}	8.60^{ab}	9.45^{a}	6.44^{bc}	7.70^{ab}	0.51	0.021
Chymotrypsin (mU/mg protein)	5.77	3.74	6.75	7.41	6.74	7.66	6.50	5.65	0.44	0.052
Lipase (mU/mg protein)	1.67	1.61	0.87	1.18	1.40	1.01	0.82	1.00	0.12	0.156
Amylase/trypsin ratio	214	236	231	219	208	203	246	260	7.06	0.373

Note. $^{a–c}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

Table 2
The proximate chemical compositions of brine shrimp nauplii collected at various harvesting times (n = 4)

Component	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
Moisture (% FW)	88.1	90.9	89.2	89.9	87.6	91.1	91.7	92.5	0.61	0.072
Crude protein (% DW)	53.2	51.6	53.6	53.0	54.1	54.5	53.1	53.4	0.27	0.138
Crude lipid (% DW)	12.5^{bc}	12.5^{bc}	13.0^{ab}	12.2^{bcd}	11.1^{d}	11.3^{cd}	14.2^{a}	13.4^{ab}	0.36	0.005
Ash (% DW)	9.49^{a}	9.88^{a}	10.1^{a}	10.7^{a}	10.8^{a}	9.51^{a}	6.57^{b}	6.74^{b}	0.58	0.005

Note. FW = fresh weight, DW = dry weight, $^{a–d}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

No variations occurred in the activity of the carbohydrate-digesting amylase and the marker of carbohydrate utilization per protein unit, the amylase/trypsin ratio (Table 1, $p > 0.05$). Similarly, the lipase activity, a lipid-digesting enzyme, was unaffected by post-hatch time (Table 1, $p > 0.05$). Crude lipid contents fluctuated with no clear change trend (Table 2, $p < 0.05$), indicating some changes in lipid breakdown during the 36-h investigation. The fatty acid analysis showed that post-hatch time did not affect levels of the fatty acids C15:0, C16:0, C17:0, C20:0 and C23:0, total saturated fatty acids (SFAs), and arachidonic acid/eicosapentaenoic acid (ARA/EPA) (Table 3, $p > 0.05$). Nevertheless, the fatty acid levels

C20:1*n*9 significantly increased ($p < 0.05$) with time post-hatching. Meanwhile, the concentrations of all other fatty acids were significantly higher in brine shrimp nauplii at 22 h ($p < 0.05$), including C14:0, C16:1*n*7, C17:1*n*7, C18:1*n*9t, C18:1*n*9c, C18:2*n*6t, C18:2*n*6c, C18:3*n*3, C20:4*n*6, C20:5*n*3, total unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), total *n*-3, total *n*-6, *n*-3/*n*-6, total *n*-9, and HUFAs.

Crustaceans lose energy during molting and use stored energy for growth, reducing certain fatty acid levels. Molting is a crucial biological process closely related to crustaceans' growth and organ development. It requires significant energy to absorb water and swell the outer shell (Huang et al., 2015; Jung et al., 2013). Not only does this involve the metabolism of nutrients (particularly fats and fatty acids), but the molting process demands energy production to maintain energy balance and survival post-molt (Fang et al., 2021; Huang et al., 2015; Wang et al., 2014). While certain fatty acids, such as C18:0 and C20:1*n*9, increase depending on the rearing conditions (Peykaran Mana et al., 2014), brine shrimp nauplii molt twice within 24 h after hatching (Benijts et al., 1976). When they molt, the depletion of energy and nutrients for growth could reduce their nutritional value compared to younger nauplii. The proportion of fatty acids is lower in Instar–II+ *Artemia* than in Instar–I (McKay & Jeffs, 2023). The balance of nutrients, such as UFAs and PUFAs, is important to establish when determining the optimal harvesting time to

Table 3
Fatty acid profiles of brine shrimp nauplii collected at various harvesting times (n = 4)

Fatty acid	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
C14:0	2.75^{a}	2.76^{a}	2.68^{a}	2.73^{a}	2.44^{b}	2.43^{b}	2.35^{b}	2.42^{b}	0.27	0.009
C15:0	0.39	0.38	0.39	0.39	0.36	0.36	0.36	0.38	0.11	0.288
C16:0	16.5	16.5	16.3	16.7	15.2	15.3	15.1	15.7	0.65	0.098
C16:1*n*7	17.1^{a}	16.8^{a}	15.8^{ab}	14.9^{bc}	13.1^{d}	13.2^{d}	12.8^{d}	13.6^{cd}	0.73	0.001
C17:0	0.66	0.66	0.67	0.66	0.63	0.64	0.67	0.68	0.16	0.809
C17:1*n*7	0.30^{a}	0.28^{a}	0.21^{b}	0.15^{c}	0.12^{d}	0.12^{d}	0.11^{d}	0.11^{d}	0.08	0.005
C18:0	4.68^{b}	4.76^{b}	4.91^{b}	5.13^{ab}	4.75^{b}	4.91^{b}	5.08^{b}	5.53^{a}	0.37	0.023
C18:1*n*9t	18.8^{a}	18.6^{a}	17.5^{ab}	16.6^{bc}	14.8^{d}	15.0^{cd}	14.9^{cd}	16.0^{bcd}	0.74	0.002
C18:1*n*9c	11.5^{a}	11.4^{ab}	10.9^{ab}	10.3^{bc}	9.29^{c}	9.51^{c}	9.66^{c}	10.4^{abc}	0.62	0.009
C18:2*n*6t	3.94^{a}	3.65^{a}	2.65^{b}	1.66^{c}	1.24^{d}	1.09^{d}	0.95^{d}	1.11^{d}	0.28	0.005
C18:2*n*6c	0.86^{a}	0.79^{b}	0.53^{c}	0.30^{d}	0.22^{e}	0.19^{ef}	0.15^{f}	0.16^{ef}	0.13	0.005
C18:3*n*3	12.8^{a}	11.8^{b}	7.73^{c}	4.01^{d}	2.96^{e}	2.38^{ef}	1.96^{f}	2.15^{ef}	0.53	0.005
C20:0	0.14	0.14	0.14	0.14	0.13	0.13	0.14	0.15	0.07	0.171
C20:1*n*9	0.89^{d}	1.02^{d}	1.47^{c}	1.98^{b}	1.99^{b}	2.11^{ab}	2.19^{a}	2.22^{a}	0.20	0.005
C20:4*n*6	1.29^{a}	1.18^{a}	0.77^{b}	0.42^{c}	0.15^{d}	0.29^{cd}	0.24^{cd}	0.27^{cd}	0.23	0.005
C20:5*n*3	13.4^{a}	12.3^{b}	7.88^{c}	3.92^{d}	3.05^{de}	2.55^{e}	2.05^{e}	2.30^{e}	0.59	0.005
C22:1*n*9	0.22^{bc}	0.21^{c}	0.21^{c}	0.27^{ab}	0.23^{bc}	0.22^{bc}	0.25^{bc}	0.30^{a}	0.11	0.017
C23:0	0.99	1.06	1.09	1.04	0.99	1.00	1.01	1.01	0.21	0.504
SFAs	26.1	26.3	26.2	26.8	24.4	24.8	24.7	25.9	0.85	0.229
UFAs	81.1^{a}	78.0^{a}	65.6^{b}	54.4^{c}	47.2^{d}	46.6^{d}	45.3^{d}	48.7^{d}	1.31	0.005
MUFAs	48.9^{a}	48.4^{ab}	46.1^{abc}	44.1^{bcd}	39.5^{d}	40.1^{cd}	39.9^{d}	42.7^{cd}	1.22	0.004
PUFAs	32.3^{a}	29.7^{b}	19.6^{c}	10.3^{d}	7.63^{d}	6.50^{d}	5.36^{d}	5.99^{d}	0.84	0.005
Total *n*-3	26.2^{a}	23.8^{b}	15.6^{c}	7.93^{d}	6.01^{de}	4.93^{e}	4.01^{e}	4.45^{e}	0.76	0.005
Total *n*-6	6.10^{a}	5.62^{b}	3.95^{c}	2.37^{d}	1.62^{e}	1.57^{e}	1.34^{e}	1.54^{e}	0.31	0.005
n-3/*n*-6	31.5^{a}	31.4^{a}	30.0^{b}	29.1^{bc}	26.1^{c}	26.8^{c}	27.0^{c}	29.0^{bc}	0.94	0.019
n-9	4.29^{a}	4.24^{a}	3.95^{ab}	3.32^{abc}	3.72^{ab}	3.15^{bc}	2.98^{bc}	2.90^{c}	0.40	0.002
HUFAs	14.7^{a}	13.4^{b}	8.65^{c}	4.33^{d}	3.20^{de}	2.84^{e}	2.30^{e}	2.57^{e}	0.59	0.005
ARA/EPA	0.10	0.10	0.10	0.11	0.05	0.11	0.12	0.12	0.11	0.428

*Note***.** SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, ARA = arachidonic acid, EPA = eicosapentaenoic acid, $^{a–f}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

ensure the nutrient content of nauplii is complete and suitable for fish larvae and juvenile aquatic animals.

Ash contents in *Artemia* range from 4.20 to 21.4%, and their contents increase with molting (Léger et al., 1986). In the current investigation, ash contents were substantially reduced in brine shrimp nauplii harvested at 34 to 36 h after hatching compared to earlier times (Table 2, $p < 0.05$). This probably indicates a loss of minerals after starvation.

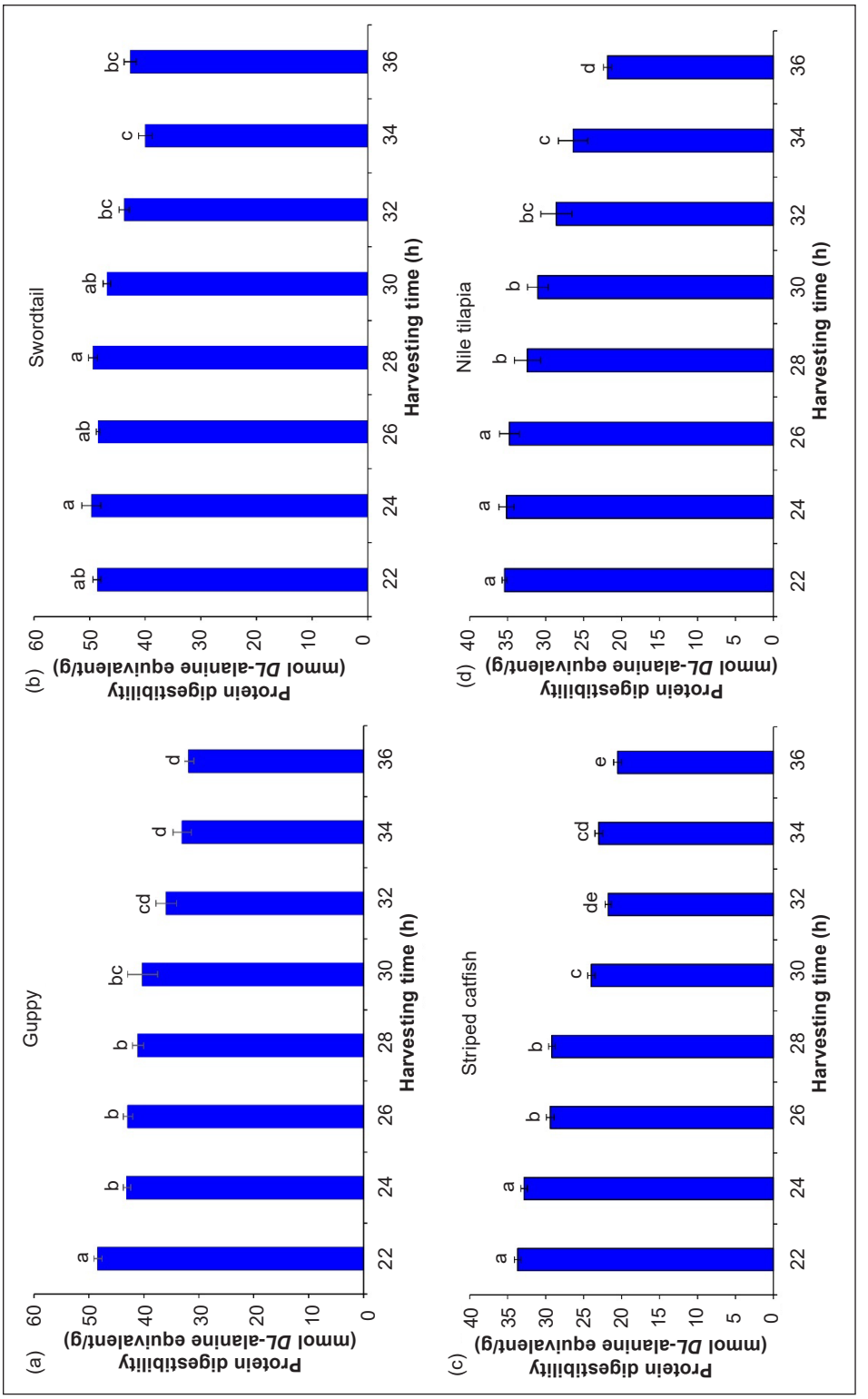


Figure 2. In vitro digestibility of protein from brine shrimp collected at various harvesting times ($n = 4$). The crude digestive enzymes utilized in digestibility tests were taken from the guppy (a), swordtail (b), striped catfish (c), and Nile tilapia (d). Significant differences between treatments are shown by different superscripts ($p < 0.05$)

Protein digestibility in the ornamental fish (guppy and swordtails) and food fish (striped catfish and Nile tilapia) decreased significantly with post-hatch time (Figure 2, $p < 0.05$). The guppy better utilized protein from brine shrimp nauplii harvested soon after hatching (22 h), whereas striped catfish (22 to 24 h) and Nile tilapia (22 to 26 h) better utilized protein from nauplii harvested 2 to 4 h later. A wider range of harvesting times (22 to 30 h) was suitable for swordtails. As brine shrimp age, there may be changes in their biochemical composition, such as an increase in chitin content. This limits larval fish's ability to digest them and reduces absorption. The strong exoskeleton of crustaceans can hinder access to the nutrient-rich tissues contained within (Cara et al., 2003; Luizi et al., 1999; Schipp et al., 1999), and digestive enzymes of small or juvenile fish may not be concentrated enough to digest the complex chitin structure. The simple intestine of small or juvenile fish may also limit their digestive capacity. Insufficient digestive enzyme quantity, activity and variety may further limit the digestion of chitin. The findings of this experiment provide information that could improve the nutritional value of live aquafeed, and the evaluation of the nutrient content of developing brine shrimp could promote more efficient nutrient utilization in fish larvae.

CONCLUSION

The harvesting time had a relatively small effect on overall digestive enzyme activities and chemical composition. However, total carotenoid contents, fatty acid profiles, and *in vitro* protein digestibility showed a clear decreasing trend with harvesting time. The results of this study conflict with the standard practice of feeding out brine shrimp nauplii to aquatic animals at 48 h after hatching. Although the digestibility assays indicated an optimum harvesting range, these data may not be consistent with the evolution of the key nutrients of brine shrimp. Therefore, the nauplii should be fed out 22 h after hatching from their cysts, or as early as possible, depending on the hatching conditions. However, further experiments on feeding brine shrimp to aquatic animals are still needed, as this study was conducted only *in vitro*.

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