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ASCORBIC ACID ENRICHMENT AND RETENTION IN *DAPHNIA CARINATA* AND *CERIODAPHNIA CORNUTA*

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Abstract

Four experiments were conducted to study the ascorbic acid enrichment of cladocerans, *Daphnia carinata* and *Ceriodaphnia cornuta.* Retention of ascorbic acid was also evaluated in relation to post enrichment temperature and stocking density. For this cladocerans *Daphnia carinata* and *Ceriodaphnia cornuta* were cultured in cattle manure, poultry dropping and mustard oil cake in (1:1:1) and were subjected to dose and time dependent ascorbic acid enrichment using four oil emulsions *viz.* D1 (10%), D2 (20%), D3 (30%) and D4 (40%). Results showed that ascorbic acid levels in *Daphnia* was enhanced up to 5-8 folds in 6h of enrichment and declined ($P<0.05$) significantly after that. In *Ceriodaphnia,* ascorbic acid content was recorded up to 12-15 folds higher in 20h of enrichment and a significant ($P<0.05$) decline was observed in ascorbic acid content after that regardless of the treatment which indicated that enrichment and retention was diet independent in *Daphnia* and *Ceriodaphnia*. In starvation experiments, significant ($P<0.05$) correlations were established between temperature of fish tanks and the leaching of ascorbic acid from enriched *Daphnia* and *Ceriodaphnia* in 2h of starvation. In density dependent retention experiments, loss of ascorbic acid was significant ($P<0.05$) in 2h of starvation and was not significantly influenced by the stocking densities of both *Daphnia* and *Ceriodaphnia.*

Key words: Ascorbyl palmitate, *Daphnia carinata,* Enrichment oil emulsion, Ascorbic acid, *Ceriodaphnia cornuta*

Introduction

Vitamins are deemed to be essential nutrients for satisfying the larval fish nutritional requirements and ensure their normal growth (Rønnestad et al., 1999, Halver, 2002). Ascorbic acid (AA) is a water-soluble vitamin and is indispensable for the proper growth of many aquaculture species (Dabrowski 1990 a, b). Ascorbic acid is essential in the synthesis of collagen and cartilage in vertebrates (Halver, 2002). It works as an antioxidant (Hwang and Lin, 2002), immunostimulant (Cuesta et al., 2002) and protects fish against stress (Henrique et al., 2002). The zero endogenous synthesis of ascorbic acid in fish and crustaceans can be traced to the absence of gulonolactone oxidase, an enzyme involved in the last step of ascorbic acid synthesis from glucuronic acid (Dabrowski 1992a). Therefore, larval fishes and crustaceans have to satisfy their ascorbic acid requirements from pelleted diet or *via* enriched zooplankton. For cultured

juveniles and adult fishes delivery of ascorbic acid is generally aided by its incorporation into pellet feeds (Soliman et al. 1986), while live feeds such as rotifers, *Artemia,* algae and daphnids are the vehicle of choice for larval fishes (Merchie et al. 1995a, b, c). Live foods *viz*. *Artemia,* copepods, rotifers and cladocerans cultured under controlled conditions are deficient in vitamins C. Thus, their boosting is essential in order to meet the requirement of larval fishes (Takeuchi *et al*., 1998; Brown *et al.*, 1992; Lubzens, (1987). Various enrichment products are used to enhance the ascorbic acid content in different live feed species (Merchie *et al*., 1997; Takeuchi *et al*., 1998; Helland *et al*., 2000). Use of oil enrichment emulsions containing ascorbyl palmitate (AP), a stable form of ascorbic acid is an established method of ascorbic acid enrichment for rotifers and *Artemia* in commercial hatcheries world wide (Merchie *et al*., 1995a, b).

Water flea *Daphnia carinata and Ceriodaphnia cornuta* are choiceable live food organisms of many aquacultural species in Indian subcontinent. Nutrient composition of both *Daphnia* and *Ceriodaphnia* can be readily manipulated by their diets. Moreover, they act as an intermediary of essential nutrients transferring from microdiets (e.g. algae, yeast or artificial emulsions) to the larval fishes. Very little information is available about ascorbic acid contents of *Daphnia carinata* and *Ceriodaphnia cornuta* cultured under controlled conditions and their boosting *via* short term enrichment protocols using oil emulsions. The present investigations aim to improve the ascorbic acid content of *Daphnia carinata* and *Ceriodaphnia cornuta* by enriching them with ascorbyl palmitate oil emulsions for different hours. The retention capacities of both daphnids were studied when they were subjected to different temperatures and stocking densities post enrichment.

2. Materials and Methods

2.1. Culture of *Daphnia carinata* and *Ceriodaphnia cornuta*

Daphnia carinata and *Ceriodaphnia cornuta* were cultured in outdoor cemented tanks (165L) using fresh cattle manure, mustard oil cake and poultry droppings (1:1:1) at the rate of 0.526 kg m^{-3}. All manures were decomposed for 12 days. On day-13, *Daphnia* (>500µm) and *Ceriodaphnia* (>300µm) were inoculated at the rate of 20 l^{-1} in the culture tanks. On day-15 of inoculum, *Daphnia* (500-699µm) and *Ceriodaphnia* (212-299µm) were harvested by filtering through plankton net. Harvested zooplankton were analysed under microscope for their purity and were cleaned twice with double distilled water. Cleaned *Daphnia* and *Ceriodaphnia* were used for different enrichment experiments.

2.2. Preparation of enrichment diets

Enrichment diets for Ascorbic acid were prepared by following the method of Merchie *et al.* (1995a). Four enrichment diets Diet-1 (D-1), Diet-2 (D-2), Diet-3 (D-3) and Diet-4 (D-4) were prepared using various doses of Ascorbic acid. Ingredients were taken as percent of total diet (Table 1).

2.3. Enrichment of *Daphnia* and *Ceriodaphnia* with Ascorbic acid

Daphnia and *Ceriodaphnia* were harvested and introduced into the glass bottles (1L) fitted with air pumps containing 500ml of ascorbic acid enrichment diets D-1, D-2, D-3 and D-4 in triplicates. Four experiments were conducted for ascorbic acid enrichment of both *Daphnia* and *Ceriodaphnia* using various diets and for different hours. *Daphnia* were enriched for 0, 6, 12 and 18h in experiment 1; 0, 1, 2, 4 and 6h in experiment 2; 2, 4, 6, 8 and 10h in experiment 3 and 0, 2, 4, 6, 8 and 10h in experiment 4. *Ceriodaphnia* were enriched for 6, 12, 18 and 24h; 14, 16, 18, 20, 22 and 24h; 18, 19, 20, 21, 22 and 23h and 14, 16, 18, 20, 22 and 24h in experiments-1, 2, 3 and 4, respectively. *Daphnia* and *Ceriodaphnia* kept in ascorbic acid enrichment mediums were collected at various hours and cleaned properly with double distilled water to remove any traces of ascorbic acid. Cleaned samples were blot dried twice and immediately frozen at -20°C for further analysis.

2.4. Post enrichment ascorbic acid retention in *Daphnia* and *Ceriodaphnia*

To evaluate the effects of temperature and stocking density of organism on ascorbic acid retention in enriched zooplankton, *Daphnia* and *Ceriodaphnia* were enriched for 6 and 20h, respectively in glass bottles (1L) containing various doses of ascorbic acid (D1, D-2, D-3 and D-4). Enriched *Daphnia* and *Ceriodaphnia* were transferred to circular fish rearing tanks covered with black paper (3L) kept in laboratory water baths pre adjusted at required temperatures, i.e. 20, 30 and 40°C in different experiments. *Daphnia* and *Ceriodaphnia* samples (100mL) were collected at every 20min and the process was continued up to 2h. Three replicates were used for the study. Plankton samples were passed through plankton net (500µm) and washed with distilled water twice. Samples were blot dried properly and immediately stored at -20°C for ascorbic acid analysis. Effects of stocking density on leaching of ascorbic acid in enriched daphnids were studied. For this, *Daphnia* were enriched at densities of 1000, 2000 and 3000 L^{-1} for 6h and *Ceriodaphnia* were enriched at densities of 2000, 4000 and 6000 L^{-1} for 20h in glass bottles and were transferred to circular fish rearing tanks covered with black paper (3L) keeping the same densities. At designated hours, sampling was done as described above in temperature based retention studies.

2.5. Estimation of ascorbic acid in *Daphnia carinata* and *Ceriodaphnia cornuta*

Ascorbic acid content was analyzed by the method of Dabrowski and Hinterleitner (1989). Briefly, pre-weighed tissue samples were homogenized using a motor driven Teflon homogenizer (Remi scientific, India) in 1:10 (w/v) ice-cold 250 mM perchloric acid ($HClO_4$) containing 5% trichloroacetic acid (TCA) and 0.08% ethylene diamine tetra acetic acid (EDTA). The homogenate was centrifuged at 27000xg for 30min at 4ºC. 25 µl of 1% $KBrO_3$ was added and mixture was incubated at 37°C for 1h. After incubation, 25 µl of 0.2% dichlorophenolindophenol (DCIP) was added and the mixture was again incubated at 37°C for 1 h. 100µl of 2% thiourea (in 5% meta-phosphoric acid) was added followed by addition of an equal volume of 2% 2, 4-dinitro phenyl hydrazine (DNPH) in 12M H_2SO_4. Samples were incubated for 3h at 60°C in preheated oven incubator. After 3h, 0.5 mL of ice-cold 18M H_2SO_4 was

added to each sample in ice cold bath. Samples were centrifuged at 11300xg for 3 min at 4ºC. 250µL of aliquots was used in a 96 well Elisa plate preparations and the absorbance was recorded at 524 nm using a fluorometer (Labtech, Australia). Blank was used in triplicate in the same plate. Standard (20-200 µg/mL^{-1}) was prepared with ascorbic acid (1mg mL^{-1})

2.6. Statistical analysis

Experiments were done in triplicates for both *Daphnia carinata* and *Ceriodaphnia cornuta*. All data were statistically analysed using one way analysis of variance (ANOVA) and student's t-test. Statistical significance was accepted at $P<0.05$ levels.

3. Results

3.1. Enrichment of *Daphnia carinata* and *Ceriodaphnia cornuta* with ascorbic acid

Four experiments were conducted with *Daphnia carinata*. In experiment 1, *Daphnia* were enriched for 18h with various enrichment diets. Ascorbic acid content was measured at every 6h of enrichment. Initial ascorbic acid concentration of *Daphnia* was 346.29µg g^{-1} DW. It was significantly ($P<0.05$) increased by 8-fold in D-1 and 4-fold in D-2, D-3 and D-4 fed *Daphnia* after 6h of enrichment compared to the initial ascorbic acid concentration. After 12h of enrichment, ascorbic acid content was lost by 60, 10, 20 and 20% in D-1, D-2, D-3 and D-4 treatments, respectively compared to the ascorbic acid concentration of the respective group after 6h of enrichment. Loss in ascorbic acid content was significant with maximum value in D-1 fed *Daphnia* followed by D-3, D-4 and D-3 treatments. After 18h, Ascorbic acid content was further decreased by 10% in D-1 and 20% in D-2, D-3, and D-4 diets fed *Daphnia* compared to the ascorbic acid concentration of the respective group after 12h of enrichment. At 18h, loss in AA content was significantly lower compared to other three diets. However no significant difference was observed in ascorbic acid content of *Daphnia* fed with D-2, D-3 and D-4 diets at 18h of enrichment (Fig.1.A). In experiment 2, Daphnia were enriched for 1, 2, 4 and 6h. In D-1 fed *Daphnia*, Ascorbic acid content was significantly ($P<0.05$) higher compared to other three enrichment diets in at enrichment hours. Initial ascorbic acid concentration was 337.58µg g^{-1} DW. After 1, 2, 4h of enrichment, significantly ($P<0.05$) increased ascorbic acid concentration was observed in all the treatments. After 6h of enrichment, ascorbic acid concentration was increased by 7-fold in D-1 which was significantly ($P<0.05$) higher to 6-fold in D-2, D-3 and D-4 fed *Daphnia* treatments compared to the initial ascorbic acid concentration (Fig.1.B). In experiment 3, enrichment was conducted for 12h. Initial ascorbic acid concentration of *Daphnia* was 337.08µg g^{-1} DW. Ascorbic acid concentration of *Daphnia* was increased by 6.5, 6, 5 and 5-fold in D-1, D-2, D-3 and D-4 treatments, respectively after 6h of enrichment compared to the initial ascorbic acid concentration. A decreasing trend in the Ascorbic acid concentration was observed from 6h onwards and it was continued up to 12h of enrichment. After 12h of

enrichment, it was decreased by 60% in D-1 and D-2 which was significantly (*P<0.05*) higher to 50% in D-3 and D-4 compared to the Ascorbic acid concentration of respective group found after 6h of enrichment (Fig.1.C). In experiment 4, *Daphnia* were enriched for 10h and ascorbic acid concentration was analyzed at every hour starting from 4th hour of enrichment. Initial Ascorbic acid concentration was 15.14µg g^{-1}DW. After 4h of enrichment, ascorbic acid concentrations were significantly (*P<0.05*) increased by 2-2.5-fold in all the four D-1, D-2, D-3 and D-4 diets compared to the initial ascorbic acid concentration. Among diets, ascorbic acid concentration was not significantly (*P>0.05*) different. ascorbic acid content was increased in 5th hour and up to 6h it was enhanced by 15.65,28.97,14.82 and 9.37 % compared to ascorbic acid content of *Daphnia* after 4h. From 6h onwards, ascorbic acid content was gradually decreased in 7, 8 and 10h of enrichment. After 10h of enrichment, it was decreased by 34.41,41.77,32.55and 28.47 % in D-1, D-2, D-3 and D-4 treatments, respectively compared to the Ascorbic acid concentration of the respective group after 6h of enrichment (Fig.1.D).

Four experiments were conducted with *Ceriodaphnia cornuta.* In experiment-1, *Ceriodaphnia* were enriched for 18h. Ascorbic acid concentration was measured at 6, 12 and 18h of enrichment. Initial Ascorbic acid concentration of *Ceriodaphnia* was 118.79µg g^{-1}DW. It was significantly (*P<0.05*) increased after 6, 12 and 18h of enrichment compared to the initial value. After 18h of enrichment it was increased by 17, 21, 19 and 18-fold in D-1, D-2, D-3 and D-4 diets, respectively compared to the initial ascorbic acid concentration of the respective group (Fig.2.A). In experiment 2, *Ceriodaphnia* were enriched for 24h and ascorbic acid concentration was assayed at every 2h starting from 14h of enrichment. Initial ascorbic acid concentration of *Ceriodaphnia* was 184.27µg g^{-1}DW. Ascorbic acid content was significantly (*P<0.05*) higher after 14, 16, 18 and 20h of enrichment compared to the initial value. After 20h, it was increased by 6-fold in D-1, D-2, D-3 diets fed *Daphnia* and 5-fold in D-4 diet fed *Daphnia* compared to the initial ascorbic acid concentration of the respective group. From 20h onwards, a decreasing trend was observed in all groups. It was decreased by 10% in D-1 diet fed group and 20% in D-2, D-3 and D-4 diets fed groups after 20h compared to the ascorbic acid concentration of respective group at 14h (Fig.2.B). In experiment 3, *Ceriodaphnia* was enriched for 23h and ascorbic acid was measured at every hour starting from 18h of enrichment. Before enrichment, it was observed 185.32µg g^{-1}DW and was significantly *(P<0.05)* increased in 19, 20, 21 and 22h of enrichment compared to the initial concentration of respective groups in previous hour of enrichment. After 22h of enrichment, ascorbic acid content was increased by 6-fold in D-1, D-3 and D-4 diet fed *Daphnia* groups. In D-2 diet fed group it was enhanced by 5-fold in compared to the initial concentration of ascorbic acid and was significantly *(P<0.05)* lower among groups. Ascorbic acid concentration was decreased by 16, 10, 8, and 11% in D-1, D-2, D-3 and D-4 treatments after 23h compared to the ascorbic acid concentration of respective group after 22h of enrichment (Fig.2.C). In experiment 4, *Ceriodaphnia* was fed with all diets of ascorbic acid for 24h and samples were collected at every 2h starting from

14h of enrichment. Initially ascorbic acid content in *Ceriodaphnia* was 93.97µg g^{-1}DW. An increasing trend was observed up to 20h of enrichment regardless of treatments. Ascorbic acid concentrations were 11, 13, 12 and 12-fold higher in D-1, D-2, D-3 and D-4 treatments, respectively after 20h of enrichment compared to the initial ascorbic acid concentration of *Ceriodaphnia cornuta*. Ascorbic acid content decreased gradually from 20h of enrichment up to 24h of enrichment. After 24h of enrichment, it was decreased by 15, 14, 15 and 17% in D-1, D-2, D-3 and D-4 treatments, respectively compared to the ascorbic acid concentration of respective group at 20h of enrichment (Fig.2.D).

3.2 Effects of stocking density on the retention of ascorbic acid after enrichment

Role of stocking density of organisms and tank water temperature of fish rearing tanks on the ascorbic acid retention capacity of zooplankton were evaluated. *Daphnia* and *Ceriodaphnia* were enriched with D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets for 6h and 20h respectively at stocking densities of 1000, 2000 and 3000 organisms L^{-1} for *Daphnia* and 2000, 4000 and 6000 organisms L^{-1} for *Ceriodaphnia*. Ascorbic acid contents of *Daphnia* enriched for 6h at a stocking density of 1000 organisms L^{-1} were 1978.72, 1874.77, 1901.55 and 1937.77µg g^{-1}DW in D-1, D-2, D-3 and D-4 treatments, respectively. The amount of ascorbic acid was significantly *($P<0.05$)* reduced by 40, 37, 32 and 36% in D-1, D-2, D-3 and D-4 enriched *Daphnia* after 2h of introduction in fish tanks (3L) (Fig.3.A). Similar results were obtained when 6h enriched *Daphnia* were enriched at a density of 2000 l^{-1} and introduced in fish tanks at the same rate. The Ascorbic acid contents of *Daphnia* after 6h of enrichment in D-1, D-2, D-3 and D-4 diets were 1821.75, 1841.17, 1846.42, 1785.01µg g^{-1}DW, respectively and reduced significantly (*$P<0.05$*) by 37, 26, 25 and 23% in D-1, D-2, D-3 and D-4 after 2h of starvation in fish tanks (Fig.3.B). The ascorbic acid contents of *Daphnia* enriched for 6h at a density of 3000 L^{-1} were 1956.67, 1941.45, 1901.02 and 1880.76µg g^{-1} DW in D-1, D-2, D-3 and D-4 treatments, respectively. It was significantly (*$P<0.05$*) reduced after 2h of starvation. After 2h, it was reduced by 45, 42, 41 and 39% in D-1, D-2, D-3 and D-4 treatments, respectively compared to the initial ascorbic acid content (Fig.3.C).

Ascorbic acid contents of *Ceriodaphnia* enriched for 20h at a density of 2000 L^{-1} in D-1, D-2, D-3 and D-4 diets were 1211.43, 1297.53, 1281.0 and 1337.17µg g^{-1}DW, respectively. It was significantly *($P<0.05$)* reduced by 35% in D-1 and 34% in D-2, D-3 and D-4 treatments compared to their initial ascorbic acid contents after 2h of starvation in fish tanks (Fig.4.A). The initial ascorbic acid contents of *Ceriodaphnia* kept at a density of 4000 L^{-1}in D-1, D-2, D-3 and D-4 diets for 20h were 1185.97, 1299.63, 1417.76, 1498.08µg g^{-1}DW, respectively. It was reduced by 20, 21, 23 and 28% in D-1, D-2, D-3 and D-4 treatments, respectively after 2h of starvation in fish tanks (Fig.4.B). Ascorbic acid contents of *Ceriodaphnia* enriched for 20h at a density of 6000 L^{-1} in D-1, D-2, D-3 and D-4 diets were 1082.81, 1324.88, 1303.5 and 1444.07µg g^{-1}DW, respectively. It was reduced by 20% in all the *Ceriodaphnia*

regardless of the treatment after 2h of fasting compared to the initial ascorbic acid content (Fig.4.C). Among all the stocking densities, loss of ascorbic acid was significant at 2000 organisms L^{-1}, at 4000 and 6000 organisms L^{-1}, ascorbic acid loss was not significantly different thus the appropriate stocking density ranges from 4000 to 6000 organisms L^{-1}.

3.3 Effects of water temperature on the retention of ascorbic acid after enrichment

Effects of water temperature of fish tanks on ascorbic acid retention capacity of enriched *Daphnia* and *Ceriodaphnia* were evaluated. In experiment-1, the ascorbic acid contents of *Daphnia* after 6h of enrichment at 28 ºC in D-1, D-2, D-3 and D-4 diets were 1938.3, 1988.17, 1982.24 and 1982.92µg g^{-1} DW, respectively. It was reduced by 35, 35, 34 and 33% in D-1, D-2, D-3 and D-4 enriched *Daphnia* after 2h of feeding in empty fish tanks maintained at 20°C (Fig.5.A). In experiment-2, the ascorbic acid contents were 1972.43, 1988.18, 1938.83 and 2044.35µg g^{-1} DW in D-1, D-2, D-3 and D-4, respectively after 6h of enrichment. It was decreased by 30, 31, 19 and 32% in D-1, D-2, D-3 and D-4 enriched *Daphnia*, respectively after 2h of introduction at 30°C (Fig.5.B). In experiment-3, ascorbic acid contents were significantly ($P<0.05$) lost by 40, 32, 36 and 34% in D-1, D-2, D-3 and D-4 enriched *Daphnia*, respectively when kept at a temperature of 40 °C for 2h. Initial ascorbic acid contents were 1962.45, 1950.37, 2040.15 and 1956.15µg g^{-1} DW after 6h of enrichment in D-1, D-2, D-3 and D-4 diets respectively (Fig.5.C)

In experiment-1, the ascorbic acid contents of *Ceriodaphnia* were 1147.12, 1082.02, 1104.07 and 1115.36µg g^{-1}DW in D-1, D-2, D-3 and D-4 diets, respectively after 20h of enrichment at 29 °C. After 2h of starvation at 20°C in simulated fish tanks, it was decreased by 30, 28, 29 and 31% in D-1, D-2, D-3 and D-4 enriched *Ceriodaphnia*, respectively compared to their initial ascorbic acid contents (Fig.6.A). In experiment-2, the ascorbic acid contents of *Ceriodaphnia* were 1304.62, 1325.88, 1419.6 and 1388.88µg g^{-1}DW in D-1, D-2, D-3 and D-4 diets, respectively after 20h of enrichment and was reduced by 21, 20 19 and 20% in D-1, D-2, D-3 and D-4 fed *Ceriodaphnia* when introduced in fish tanks maintained at 30°C after 2 h (Fig.6.B).

In experiment-3, *Ceriodaphnia* were enriched in D-1, D-2, D-3 and D-4 diets and their ascorbic acid content were 1362.90, 1341.90, 1255.27 and 1314.86µg g^{-1}DW, respectively after 20h. These enriched *Ceriodaphnia* were introduced in fish tanks maintained at 40°C and their ascorbic acid content was lost by 27% in D-1 and D-2 enriched *Ceriodaphnia*, 24 and 26% in D-3 and D-4 enriched *Ceriodaphnia* after 2h of introduction (Fig.6.C).

4. Discussion

The nutritional value of two important cladocerans was evaluated in the present study. In *Daphnia* and *Ceriodaphnia*, ascorbic acid contents ranged from 267-356µg g^{-1}DW and 100-243µg g^{-1}DW, respectively before enrichment. Merchie *et al*. (1995b) also

observed that ascorbic acid content of adult *Artemia* varied from 256-341µg g^{-1}DW before enrichment. The value indicated the presence of trace amount of inherent ascorbic acid compared to the fairly high ascorbic acid requirement of carp larvae (Mahajan and Aggrawal 1980; Gouillou-Coustans *et al*., 1998). Thus, it becomes vital to enrich the *Daphnia* and *Ceriodaphnia* with ascorbic acid exogenously to meet the ascorbic acid requirement of different stages of fishes. Elevation of ascorbic acid levels in live food *via* enrichment has improved the commercial larviculture of several species (Merchie *et al*., 1995b, 1997). Ascorbic acid concentrations of *Artemia* increased 4-fold from 0.15 to 0.6mg g^{-1} DW, using *Isochrysis* spp (Ritar *et al*., 2004). Tamaru, 1998, observed a 60-fold increase in ascorbic acid concentration in *Artemia* enriched with algae, which increased the stress resistance of newly hatched lobster larvae. Use of oil emulsions containing up to 20-30% ascorbyl palmitate (AP) for enrichment was demonstrated by Hapette and Poulet (1990a, b) and Merchie *et al*. (1995a). Merchie *et al*. (1995a) used AP emulsions in a dose dependent manner for the enrichment of *Artemia* nauplii and found a 10-12-fold enhancement in ascorbic acid levels. In the present investigation, enrichment trials conducted with *Daphnia* and *Ceriodaphnia* also confirmed the earlier findings. Ascorbic acid content was enhanced by 8-10-fold in 6h enriched *Daphnia* and 10-12-fold in 12h enriched *Ceriodaphnia* regardless of doses. In the present study, ascorbyl palmitate used for the ascorbic acid enrichment was quickly assimilated by both *Daphnia* and *Ceriodaphnia*. Merchie *et al*. (1995a, b) and Sorgeloos *et al*. (1998) also observed quick assimilation of ascorbyl palmitate in *Artemia* after 12h enrichment. In case of *Daphnia*, highest concentration of ascorbic acid was found after 6h of enrichment. This implicates the readily availability of ascorbic acid to *Daphnia* when supplied in the form of ascorbyl palmitate. Gradual decrease in ascorbic acid content after 6h of enrichment in *Daphnia* and 12h in *Ceriodaphnia* suggested the assimilating capacity of these two important fish food organisms.

Our results are supported by the findings of Merchie *et al*. (1995b) who enriched *Artemia* nauplii with varying concentrations of ascorbyl palmitate (10, 20 and 30%) and found variation in their ascorbic acid concentrations (600, 1400 and 2300µg g^{-1} in respective diets). They also observed that there was a sudden decrease in ascorbic acid concentration after a continuous enrichment of 12h in *Artemia*. Merchie *et al*. (1995b) showed that *Artemia* is able to assimilate the ascorbic acid when supplied in the bound ascorbyl palmitate form. Retention of ascorbic acid after enrichment in zooplankton is essential for optimizing the availability of digestible form of ascorbic acid for different stages of fish. Our results confirmed a rapid loss of ascorbic acid during 2h fasting at different temperatures. At 30°C the loss of ascorbic acid (25%) in enriched *Daphnia* and *Ceriodaphnia* was significantly ($P<0.05$) lower compared to the loss of ascorbic acid at 20 and 40°C (35-50%). This might be due to the stability of ascorbic acid at this temperature. Our results were also supported by the study of Hapette and Poulet (1990b). They studied that ascorbic acid content was reduced at rate of 70mg g^{-1} in enriched copepod *Calanus helgolandicus* during 8 days of starvation at 15°C. Rotifers and *Artemia* enriched with ascorbyl palmitate emulsions also retained

their ascorbic acid concentration over 24h of nonfeeding periods (Merchie *et al.*, 1995a). In our study, no direct relation was found between ascorbic acid enrichment and its retention with the density of *Daphnia* and *Ceriodaphnia*.

5. Conclusion

It may be concluded from the above study that the optimum duration for ascorbic acid enrichment of *Daphnia carinata* is 6h and for *Ceriodaphnia cornuta*, it is 20h. For both the cladocerans, 20% ascorbic acid ascorbyl palmitate emulsion is advisable for their commercial enrichment strategies. Stocking density of both the cladocerans was not significantly correlated to the retention of ascorbic acid after enrichment but the optimal stocking density after enrichment for *Daphnia* ranged from 2000-3000 $L^{-1}$and for *Ceriodaphnia* it ranged from 4000 to 6000 organism L^{-1}. 30°C is the optimum temperature for feeding of the enriched *Daphnia* and *Ceriodaphnia* to larval fishes and for optimal retention of ascorbic acid within enriched cladocerans.

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Table legends

Table.1. Preparation of ascorbic acid enrichment emulsions using ascorbyl palmitate (ingredients are in % by weight of total diet for 100mg)

Ingredients	Diets			
	D-1 (10%)	D-2 (20%)	D-3 (30%	D-4 (40%)
Cod liver oil	10	10	10	10
Tween- 80	10	10	10	10
Yeast extract powder	10	10	10	10
Ascorbyl palmitate	10	20	30	40
Dechlorinated water	60	50	40	30

Figure Legends

Fig.1. Fig shows the Ascorbic acid enrichment of *Daphnia carinata* (500 µm-699 µm) in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets for (A) 0, 6, 12 and 18h, (B) 0, 1, 2, 4 and 6h, (C) 0, 4, 6, 8, 10 and 12h.

Fig.2. Ascorbic acid enrichment of *Ceriodaphnia cornuta* in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets for (A) 0, 6, 12 and 18 hours, (B) for 14, 16, 18, 20, 22 and 24 h,(C) for 18, 19, 20, 21, 22 and 23 and (D) for 14, 16, 18, 20, 22 and 24 h.

Fig.3. Fig shows the density dependent leaching of ascorbic acid in 6h enriched *Daphnia carinata* (500 µm-699 µm) in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets and fed to larval fishes at a rate of- A) 1000 orgL^{-1}, (B) 2000 orgL^{-1}, (C) 3000 orgL^{-1}.

Fig.4. Fig shows the density dependent leaching of ascorbic acid in 6h enriched *Daphnia carinata* (500 µm-699 µm) in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets and fed to larval fishes at a temperature of- A) 20 °C, (B) 30 °C, (C) 40 °C.

Fig.5. Fig shows the density dependent leaching of ascorbic acid in 12h enriched *Ceriodaphnia cornuta* (500 µm-699 µm) in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets and fed to larval fishes at a rate of- A) 2000 orgL^{-1}, (B) 4000 orgL^{-1}, (C) 6000 orgL^{-1}.

Fig.6. Fig shows the density dependent leaching of ascorbic acid in 12h enriched *Ceriodaphnia cornuta* (500 µm-699 µm) in D-1 (10%), D-2 (20%), D-3 (30%) and D-4 (40%) diets and fed to larval fishes at a temperature of- A) 20 °C, (B) 30 °C, (C) 40 °C.

Fig. 1.

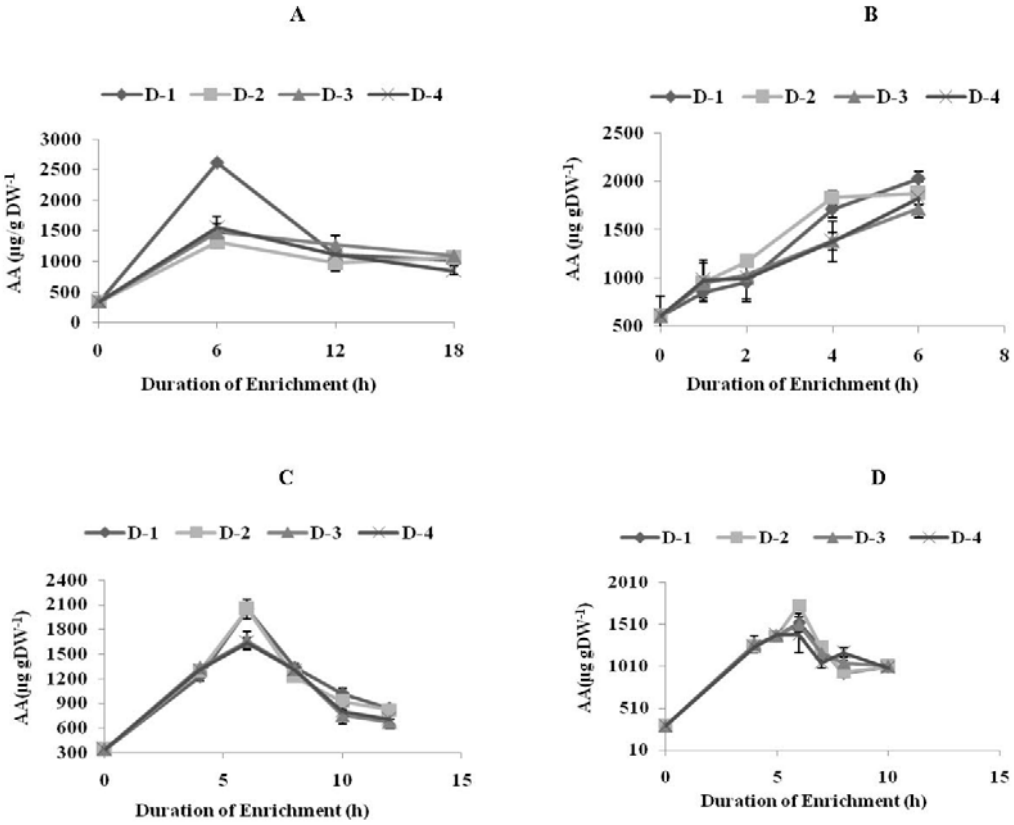

A
D-1
D-2
D-3
D-4
AA (µg/g DW-1
3000
2500
2000
1500
1000
500
0
0
6
12
18
Duration of Enrichment (h)
B
D-1
D-2
D-3
D-4
AA (µg gDW-1)
2500
2000
1500
1000
500
0
2
4
6
8
Duration of Enrichment (h)
C
D-1
D-2
D-3
D-4
AA(µg gDW-1)
2400
2100
1800
1500
1200
900
600
300
0
5
10
15
Duration of Enrichment (h)
D
D-1
D-2
D-3
D-4
AA(µg gDW-1)
2010
1510
1010
510
10
0
5
10
15
Duration of Enrichment (h)

Fig.2.

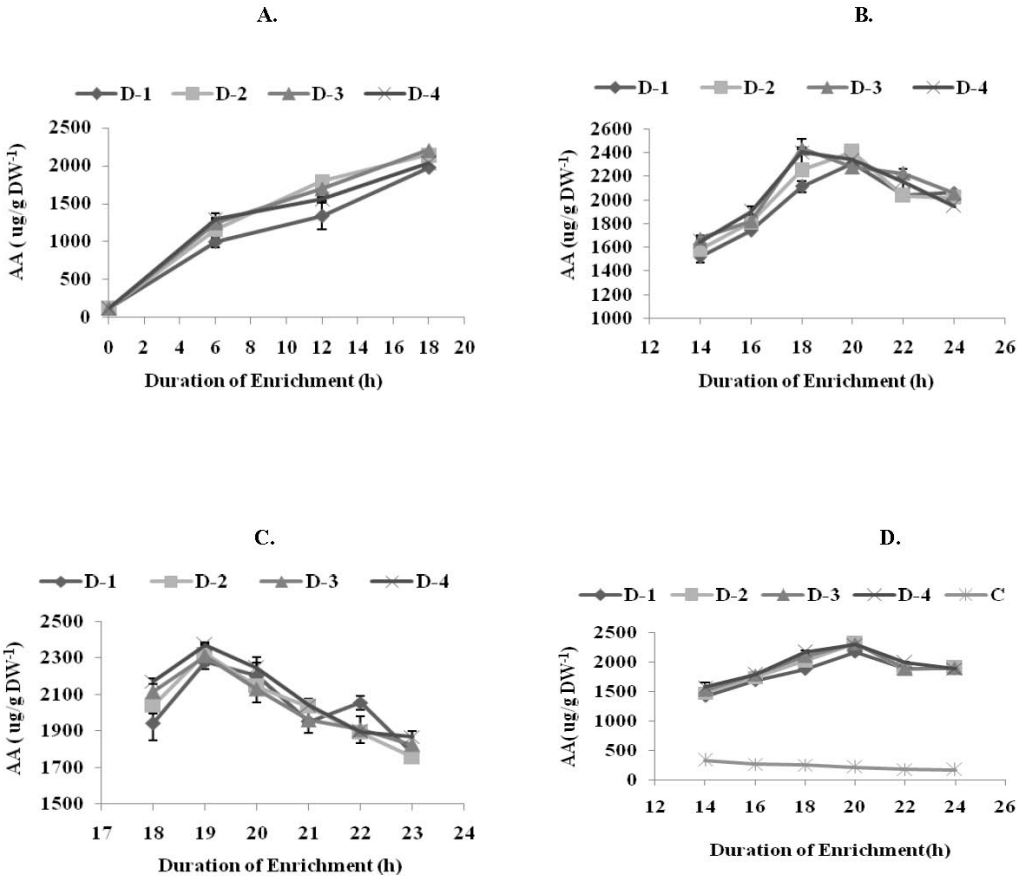

A.
D-1
D-2
D-3
D-4
AA (ug/g DW-1)
Duration of Enrichment (h)
B.
AA (ug/g DW-1)
Duration of Enrichment (h)
C.
AA (ug/g DW-1)
Duration of Enrichment (h)
D.
C
AA(ug/g DW-1)
Duration of Enrichment(h)

Fig.3.

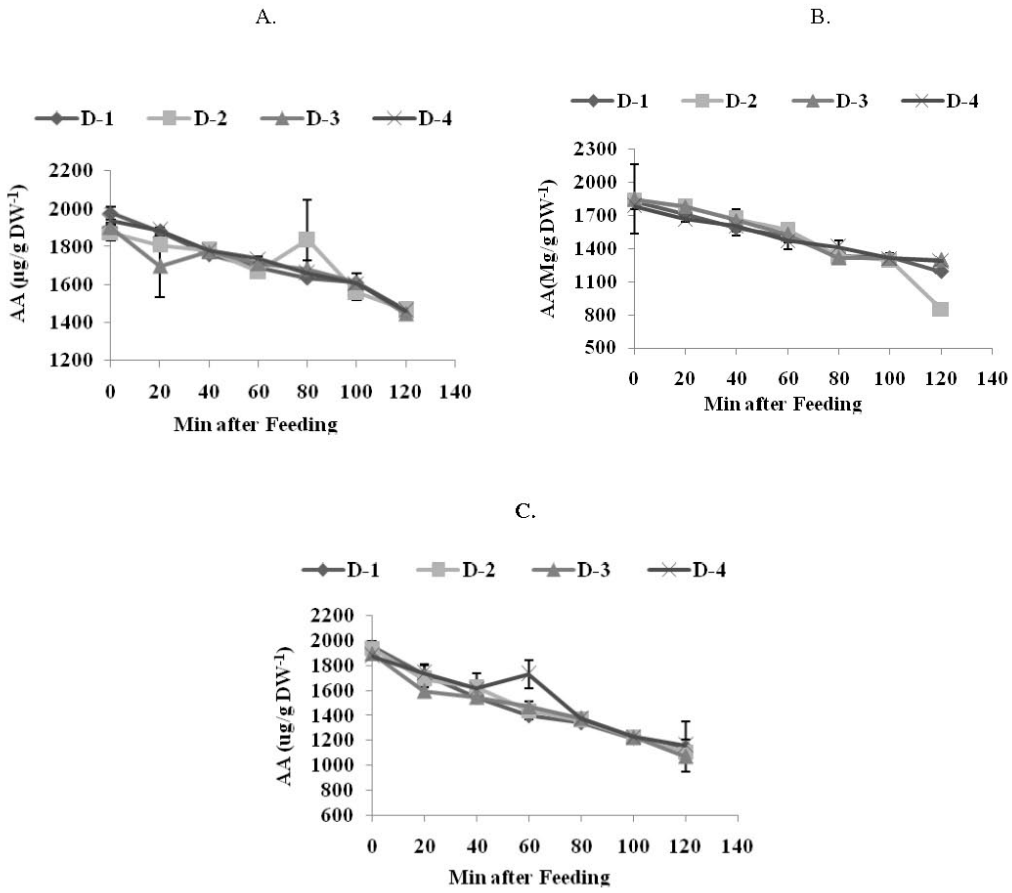


Fig.4.

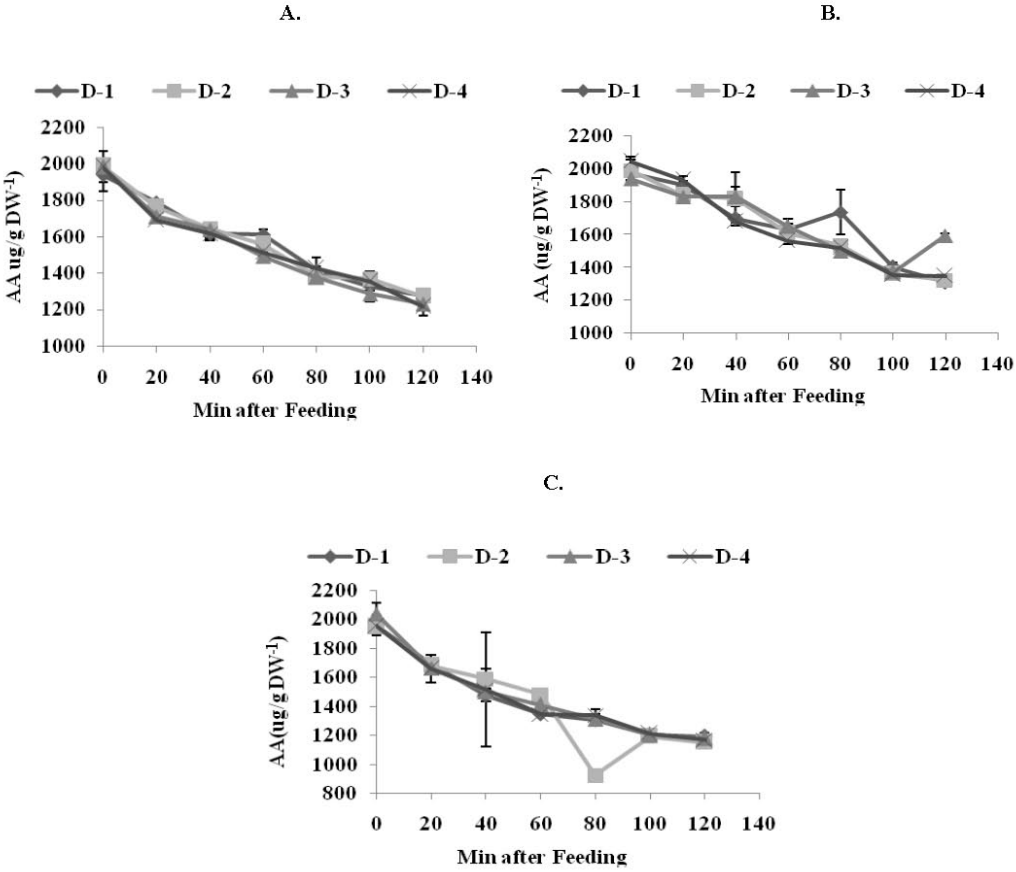

A.
B.
C.
D-1
D-2
D-3
D-4
AA ug/g DW-1)
AA (ug/g DW-1)
AA(ug/g DW-1)
Min after Feeding
0
20
40
60
80
100
120
140
800
1000
1200
1400
1600
1800
2000
2200

Fig.5.

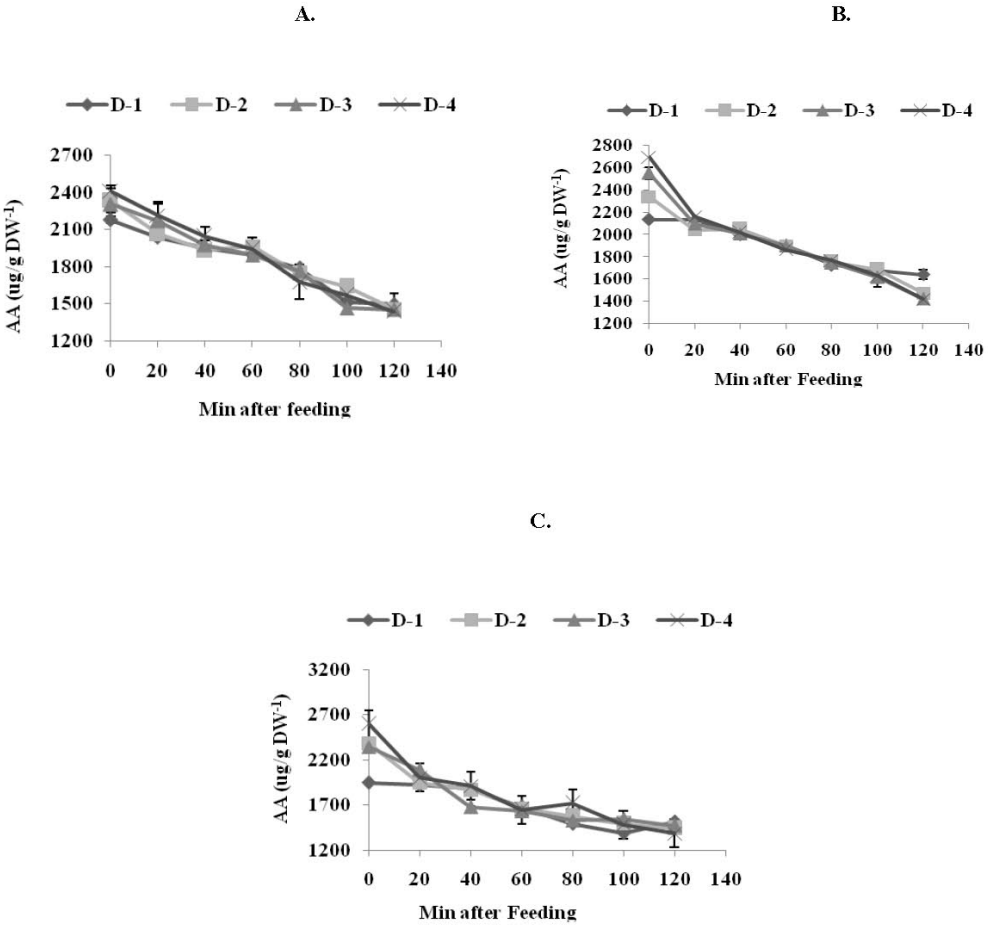
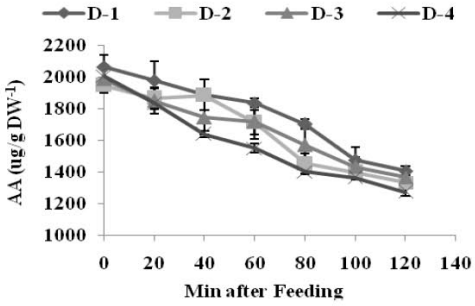
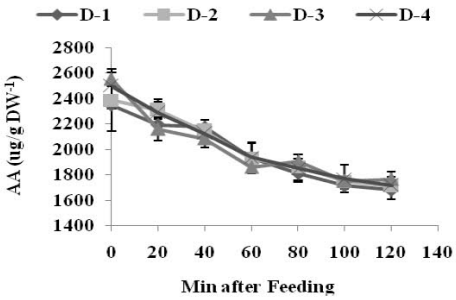


Fig.6

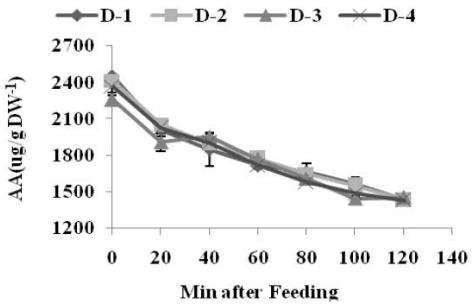
A.


D-1
D-2
D-3
D-4
AA (ug/g DW-1)
2200
2000
1800
1600
1400
1200
1000
0
20
40
60
80
100
120
140
Min after Feeding

B


D-1
D-2
D-3
D-4
AA (ug/g DW-1)
2800
2600
2400
2200
2000
1800
1600
1400
0
20
40
60
80
100
120
140
Min after Feeding

C.


D-1
D-2
D-3
D-4
AA(ug/g DW-1)
2700
2400
2100
1800
1500
1200
0
20
40
60
80
100
120
140
Min after Feeding