The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.).

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Abstract

This study investigated the effect on egg quality of dietary supplementation of Atlantic cod broodstock with the carotenoid astaxanthin (ASTA). Duplicate groups of farm-reared Atlantic cod broodstock were fed either a control diet with no added ASTA, or an ASTA supplemented diet (73.7 mg/kg dry weight; Carophyll Pink®) for 2 months prior to peak spawning. The results indicated that ASTA uptake into eggs from the broodstock diet was highly efficient. Fish fed the diet supplemented with ASTA produced fewer batches of eggs, but the mean number per batch of eggs spawned/kg female was higher, and numbers of floating eggs and numbers of fertilised eggs per kg female in each batch were also significantly improved. A correlation between the egg ASTA content and fertilisation success of individual batches was identified. This improvement in egg quality demonstrated the potential value of ASTA supplementation of broodstock diets for cod. ASTA supplementation produced a 20% increase in the number of eggs per batch spawned, a 37% increase in the number per batch of floating eggs per kg female and a 47% increase in the number per batch of fertilised eggs per kg female. These results clearly demonstrate significant benefits of ASTA supplementation of cod broodstock feeds in terms of improved egg quality and larval production.

Keywords: Atlantic cod, egg quality, astaxanthin, broodstock nutrition

1. Introduction

In recent years catches from cod commercial fisheries have been in serious decline, resulting in an increased interest in cod culture. As a result, global cod culture has increased from 169 t in 2000 to 3812 t in 2004, with a trend towards further increase in the future (FAO, 2006). In order to provide sufficient numbers of good quality fish to establish a sustainable cod production, farms need a year round supply of high quality larval cod. To provide high quality larvae, commercial cod hatcheries need a reliable supply of good quality eggs. The quality of eggs is important because poor quality eggs result in increased larval mortality and deformities during egg and larval rearing which results in reduced production efficiency as well as fish health and welfare problems. At present it is generally accepted that the best source of eggs comes from wild caught fish, as these fish tend to produce better quality eggs and larvae than farmed broodstock. Therefore, most commercial hatcheries currently rely heavily on eggs from wild-caught rather than farmed broodstock. However, reliance on wild broodstock presents a number of problems, including the risk of pathogen introduction, limited potential for stock improvement by selective breeding and raises concerns over the long term sustainability of a cod industry heavily reliant on wild caught broodstock. Little is known about the causes of poor egg quality in farmed cod and further work is needed to understand factors controlling egg quality in this species.

A number of studies have been carried out on other species of farmed fish and numerous parameters have been reported to influence egg quality such as broodstock nutrition, environmental conditions and husbandry practices (Bromage, 1995; Bruce et al., 1999; Brown et al., 2003). If nutritional factors are responsible for quality problems then manipulation of broodstock diets should provide a practical means of

improving egg quality via supplementation with essential nutrients. Nutrition is especially important for cod broodstock because farm reared fish may be conditioned for spawning in tanks and fed formulated feeds over a period of several years. Nutritional input, in both the short and long term, is therefore relevant to fish of both farmed and wild origin.

The influence of nutrient availability on reproductive physiology and broodstock performance in fish has been reviewed previously (Hardy, 1985; Bromage, 1995; Pavlov et al., 2004). These studies have investigated the effects of a number of nutrient supplements including polyunsaturated fatty acids, vitamins C and E, and the carotenoid pigment astaxanthin. In cod, differences in carotenoid pigment concentration have previously been identified between wild and farmed cod broodstock (Salze et al., 2005). These nutritional differences were correlated with differences in egg quality, suggesting that sub-optimal levels of carotenoid pigment may cause some egg quality problems in farmed cod (Salze et al., 2005). For example, Salze et al. (2005) found that carotenoid concentrations were lower in eggs from farmed cod than eggs from wild cod. Similarly, Grung et al. (1993) also found lower concentrations of carotenoid pigment in eggs from farmed cod than wild cod and demonstrated that dietary carotenoid supplementation resulted in an increased carotenoid concentration in the eggs. Numerous functions have been proposed for carotenoids in fish eggs and include UV protection, provitamin A activity, improved respiratory function (Craik, 1985; Mikulin, 2000) and antioxidant protection against free-radical damage (Edge et al., 1997). These findings suggest that carotenoids are important in ensuring normal embryonic development and could also affect hatching rates and larval survival (Torrissen, 1984; Craik, 1985; George et al., 2001). Caretonoids are also a source of pigmentation in the embryo (Pan et al., 2001) and

may be involved in photoreception processes (Rønnestad et al., 1998). Supplementation of broodstock diets with ASTA has also been shown to improve egg quality in red sea bream and yellowtail (Watanabe and Miki, 1993; Verakunpiriya et al., 1997). Dietary carotenoid supplements have also shown a positive relationship between egg pigmentation and fertilisation as well as survival of rainbow trout eggs (Harris, 1984; Craik, 1985) while Svensson et al. (2006) found the colouration of female *G. flavescens* was strongly related to the carotenoid content of the eggs. At the present time there are no reports of the effects of carotenoid supplementation on egg quality in cod. The aim of the experiment reported here was to evaluate the effect of short-term supplementation of ASTA in broodstock diets on a number of egg quality parameters in farmed cod. Duplicate groups of farmed cod broodstock were fed either a control diet, with no ASTA supplement, or an ASTA supplemented diet, for two months prior to peak spawning. Egg numbers were expressed in terms of female biomass to permit comparisons between stocks. The astaxanthin content of eggs was carried out to examine the effects of dietary treatment on astaxanthin content.

2. Materials and methods

2.1 Fish husbandry and diets

The experimental design used two treatment groups of Atlantic cod (*Gadus morhua*) broodstock each housed in duplicate tanks. The control group was fed an unsupplemented diet with no added ASTA throughout the spawning period while the treatment group was fed an ASTA supplemented feed, at a measured inclusion level of 73.7 mg/kg dry weight, for two months prior to the peak-spawning date. The

broodstock were farm-reared fish and were allocated to four fibreglass $7m^3$ tanks in November 2005. Tanks were supplied with seawater at 40 L/min in a flow-though system. The average water temperature during the experimental period was 8^oC and the average salinity was 33 ‰. In January 2006, fish were weighed individually, screened by ultrasound to determine gender and state of maturation and reallocated so that each tank contained a similar number and biomass of males and females. After allocation each group contained 34 or 35 males and 35 or 36 females. The biomass in each tank was; unsupplemented 1, 89.4 kg, unsupplemented 2, 89.0 kg, ASTA treatment 1, 91.5 kg and ASTA treatment 2, 90.0 kg. The average individual fish weight in each tank was 1.29 kg.

The basal feed used was a commercially available moist feed formulation (Vitalis® Marine Broodstock Mix, Skretting, Wincham, UK), specially prepared to contain no added ASTA. The feed was prepared by the addition of water (0.7 L/kg dry mix). For the supplemented feed, Carophyll Pink (DSM, Basle, Switzerland), with a nominal ASTA content of 10% w/w, was added as a source of ASTA at a rate of 1g/kg dry mix. The concentration of ASTA in the feed, as measured by HPLC, was 73.7 mg/kg dry weight. Fish were fed to satiation twice daily.

2.2 Egg quality assessment

The spawning period was regarded as the period from 1st March to 31st May 2006, and the peak spawning date was 15th April 2006. Each day during the 92 day spawning period, egg batches were collected and egg quality was assessed using standard techniques to measure total egg production, floating egg production and fertilisation rate. Dropout (number of sinking (unfertilised eggs)) within each tank was measured, over a 24h period, on five different dates. Samples of floating eggs

(good quality and mainly fertilised eggs) were collected on 14 different dates for hatch rate determination and fertilisation rate. Astaxanthin analysis was carried out on floating eggs collected from each tank on 11 different dates during the course of the spawning period.

2.3 Measurement of astaxanthin concentration in feed and eggs

Carotenoid pigments, including astaxanthin, were extracted from cod eggs largely using the method of Barua et al. (1993). Eleven samples of 20 eggs were collected from each of the four tanks over the spawning period and stored in chloroform/methanol (2:1 v/v) with 0.01% (w/v) BHT. The values presented for astaxanthin are average values for each tank (n =11). Total lipid was extracted from the egg samples by the method of Folch et al. (1957). Samples of egg total lipid (10 mg) were evaporated to dryness under oxygen-free nitrogen, and re-dissolved in 500 µL of isohexane. Total carotenoid pigment was measured spectrophotometrically at 470 nm using an $E_{1\%}$ (w/v) of 2100. Separation and quantification of astaxanthin was carried out using a Lichrosorb 5µ Silica 60 column (4.0 x 125 mm, Phenomenex, Macclesfield, U.K.). The chromatographic system was equipped with a Waters Model 510 pump and astaxanthin was detected at 470 nm using a Waters 486 multiwavelength UV/vis detector (Millipore U.K., Watford). An isocratic solvent system was used containing iso-hexane/acetone (86:14, v/v) at a flow rate 1 mL/min.

Carotenoid in diets was extracted after enzymatic digestion with Maxatase enzyme (International Biosynthetics, Rijswijk, Netherlands). Portions of ground diet (1g) were mixed with 10 mL water and 110 mg Maxatase in a 50 mL stoppered glass tube followed by incubation in a water bath at 50^{o}C for 30 min. Samples were then extracted with 5 mL of absolute ethanol and 5 mL of ethyl acetate on a vortex mixer.

The homogenate was centrifuged (1000 x *g*, 5 min) and the supernatant removed to a stoppered glass tube. The pellet was re-extracted in 5 mL of ethyl acetate, centrifuged, and the supernatant combined with the first supernatant. Finally, the pellet was re-extracted in 10 mL of isohexane, centrifuged, and the supernatant combined with the pooled supernatant. The pooled supernatant was dried under N_2 and vacuum desiccated for 2 h before dissolving the residue in 2 mL of isohexane prior to analysis. The astaxanthin was separated and quantified using the HPLC method described above.

2.4 Data analysis

Egg quality indices used for group comparisons included, batch weights of eggs collected, batch weights of floating eggs, fertilisation rate and hatch rate, and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (i.e. floating, fertilised eggs) and hatched eggs. Numbers were calculated in terms of the biomass of female fish to compensate for small differences in broodstock biomass and allow comparison with other stocks. Numbers were calculated from egg batch weight measurements assuming 500 eggs/g. Analysis of variance, or Kruskal-Wallis non-parametric tests, were used to identify differences in egg quality, or biochemical parameters, between individual groups. Group comparisons were made using analysis of variance with tank as a factor nested within each treatment. Where differences were identified, appropriate multiple comparison tests were used to identify differences between the group averages. Spearman's rank test was used to detect any correlation between fatty acid composition and egg quality.

3. Results

201 Total carotenoid pigment concentration in the unsupplemented control diet was 14.8
202 mg/kg and 73.7 mg/kg in the ASTA-supplemented diet. The concentrations measured
203 in the eggs were 0.98 ± 0.48 and 2.79 ± 0.10 ng/egg for the unsupplemented and
204 ASTA supplemented groups, respectively (Fig. 1). A significant correlation was
205 detected between egg astaxanthin content and fertilisation rate (Spearman's r =
206 0.3061, $P < 0.01$) in individual egg batches.
207 Table 1 and Fig. 2 show data on egg production and egg viability in the two treatment
208 groups. In the unsupplemented control group, total production was estimated to be
209 301,032 eggs per kg female. Dropout within the tank was approximately 7% and the
210 number of eggs collected over the season was 280,884 eggs per kg female. A mean of
211 123,022 eggs per kg female (44 % of those collected) were floating eggs evaluated for
212 incubation. The mean fertilisation percentage of floating eggs was 31% and the total
213 number of viable eggs was 42,573 eggs per kg female (15 % of eggs collected). The
214 mean hatch percentage was 11 % of floating eggs incubated, and the total number of
215 hatched eggs was 13,492 per kg female (5 % of collected eggs). The ASTA
216 supplemented group, produced numerically fewer batches of eggs, but the mean
217 number per batch of eggs spawned per kg female was significantly larger ($P < 0.05$).
218 Fertilisation percentages were similar but the weight per batch of floating eggs ($P <$
219 0.01), number per batch of floating eggs per kg female ($P < 0.01$), and number per
220 batch of fertilised eggs/kg female ($P < 0.01$) were all significantly higher in the ASTA
221 supplemented group than in the control group. Cumulative egg production for control
222 broodstock and broodstock fed ASTA are shown in Fig 3. These results show that
223 after 15 days of egg production the broodstock fed an ASTA supplement had
224 produced more eggs than control fish.
225 (Note: a percent is not a rate, a rate denotes units/units time)

226

227

228 **4. Discussion**

229 A previous study that measured cod egg pigment concentrations identified higher
230 levels of ASTA in eggs from wild cod broodstock compared to farmed broodstock
231 held in the same hatchery (Salze et al., 2005). This study showed that wild eggs
232 contained around 3 times more ASTA than the farmed eggs and that the fertilisation
233 percentage in the latter was about half that seen in the wild eggs. In the present study,
234 short term supplementation of cod broodstock diets with ASTA, for a period of two
235 months prior to peak spawning, increased concentrations of carotenoids in the eggs,
236 by around 3-fold, indicating efficient and rapid uptake. Whilst fish fed the diet
237 supplemented with ASTA produced fewer batches of eggs, the mean number per
238 batch of eggs spawned/kg female was significantly higher (by 20%) and the numbers
239 of floating eggs and numbers of fertilised eggs per kg female in each batch were also
240 significantly improved (by 37 and 47%. respectively). In addition, a correlation
241 between the ASTA content of the eggs and fertilisation success of individual batches
242 was identified.

243 These findings confirm that addition of ASTA to the cod broodstock diets results
244 in uptake and deposition into eggs and provides significant improvements in egg
245 quality, similar to those found in other fish species. The efficient transfer of
246 astaxanthin from broodstock to egg has been shown previously, in both cod and
247 salmonids, (Grung et al., 1993; Torrissen, 1984) although improved egg quality has
248 not been consistently observed in salmonids (Christiansen and Torrissen, 1997;
249 Choubert et al., 1998). However, in marine species, including red sea bream and
250 yellowtail, the addition of synthetic ASTA or krill lipid to broodstock diets was found

to clearly improve a number of egg quality parameters (Watanabe et al., 1991; Watanabe and Miki, 1993). In red sea bream the percentage of buoyant and hatched eggs as well as the percentage of normal larvae was significantly increased in eggs from broodstock fed an ASTA supplemented diet (Watanabe and Kiron, 1995). Supplementation of broodstock feeds with specific nutrients, particularly specific fatty acids and fat-soluble micronutrients, including carotenoids, can lead to an increase in levels of these nutrients in the developing eggs and, in the case of sea bass, sea bream, yellowtail and halibut, these have been shown to have a measurable impact on egg quality (Ashton et al., 1993; Verakunpiriya et al., 1997; Czesny and Dabrowski 1998; Gallagher et al., 1998; Sargent et al., 2002).

In addition to the benefits reported in fin fish there is also evidence from studies on crustacean and echinoderm culture that suggest similar benefits of carotenoid supplementation of broodstock diets. Inclusion of dietary carotenoids was shown to improve egg and larval production in the edible sea urchin *Lytechinus variegates,* (George et al., 2001). Supplementation with highly unsaturated fatty acids (HUFA) and 50 mg/kg ASTA resulted in increased total egg production and egg production/female in cultured *Penaeus monodon* broodstock (Huang et al., 2008). Similarly, survival of *Penaeus vannamei* nauplii was increased following a carotenoid supplement while broodstock diets lacking carotenoid resulted in reduced larval feed intake, increased deformities and reduced survival (Wyban et al., 1997).

More than 600 naturally occurring carotenoids have been identified in vegetables, fruits and seafoods although they mostly originate in plants, photosynthetic bacteria and algae where they are accessory pigments in photosynthesis and photoprotection (Isler, 1981). One explanation for the beneficial effects of ASTA on cod egg quality could be that astaxanthin acts as a fertilisation hormone and improves fertilisation by

276 stimulating and attracting spermatozoa (Hartmann et al., 1947). However, the ability
277 of carotenoid pigments to absorb light and, thereby, quench or inactivate singlet
278 oxygen and free radicals, is a more likely reason for their nutritional efficacy (Mayne,
279 1996). The mechanism by which the damaging effects of light, (UV and visible) and
280 the subsequent generation of reactive oxygen species is attenuated, is a consequence
281 of the conjugated polyene structure of carotenoids that allows sequestration and
282 inactivation of these harmful molecules (Nishigaki et al., 1994). This action of
283 carotenoids on control of damaging free radicals has lead to intervention studies in
284 human conditions that have a pro-oxidant aetiology including heart disease, cancer,
285 stroke, cataract, macular degeneration and immune modulation (Mayne, 1996). In
286 natural spawning of cod, the eggs are released into the upper layers of the oceans, that
287 are both highly illuminated and oxygen-rich, presenting an ideal environment for free
288 radical generation. Thus, the improvements observed in egg and larval quality in
289 farmed cod, when diets are supplemented with ASTA, could be explained by better
290 antioxidant protection both in the diet and in the eggs and larvae themselves (Cowey
291 et al., 1985; Pangantihon-Kuhlmann et al., 1998).
292 A further explanation for the efficacy of ASTA supplementation might be related
293 to stress reduction and enhancement of immune function. Larval fish, both in the wild
294 and in hatcheries, can be subjected to both osmotic and thermal fluctuations as well as
295 to pathogenic challenge. In tiger prawn (*Penaeus monodon),* studies have shown that
296 dietary astaxanthin supplementation can improve resistance to both osmotic stress, in
297 the form of salinity fluctuation, and thermal stress as reduction in temperature from 27
298 to 5^{o}C (Merchie et al., 1998; Chien et al., 2003). The postulated mechanism for
299 improved stress resistance was related to the increased energy production required to
300 respond to stress that would generate more oxygen radicals that could be attenuated

by the presence of ASTA. Astaxanthin supplementation has been shown to improve health and immune function in salmon and rainbow trout although the exact mechanism is not known (Christiansen et al., 1995; Thompson et al., 1995). However, a study using spleen cell suspensions, isolated from mice fed control or ASTA supplemented diets, showed enhanced T-dependent antigen specific humoral immune responses in the supplemented mouse cells (Jyonouchi et al., 1995a). Similar immune enhancement, via modulation of T-dependent antibody responses, has also been observed in humans supplemented with ASTA by the same authors (Jyonouchi et al., 1995b).

The benefits of ASTA supplementation seen in the present study suggests that hatcheries should check the status of their cod broodstock with regard to dietary ASTA concentrations in the pre-spawning period. If necessary, short term supplementation should be used to boost these nutrients prior to spawning. If the status of the broodstock is unknown, the hatchery should consider sending samples of eggs for analysis at the start of each spawning period. Such tests would assess the nutritional status of eggs prior to spawning, thus allowing corrective action to be taken before spawning commences. More information on the ASTA status of eggs from commercial broodstock is required, and should be assessed in relation to egg quality. Records of egg quality in standard form (e.g. no of fertilised eggs per kg female) are necessary to allow effective comparisons between eggs from different broodstock populations.

Future studies should aim to determine the most efficient forms, concentration of ASTA and other carotenoids and duration of supplementation required for optimal response. More information is also required on the role of environmental conditions, husbandry and behavioural interactions in relation to spawning of cod broodstock.

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467 maturation diet improves nauplii quality. World Aquacult. 28, 59-62.
468
469

Table 1. Egg production and egg quality indicators. Egg numbers are expressed as numbers per kg female.

Parameter	Control	ASTA supplemented
Total no. of eggs produced/ kg female	301032 ± 46235	335795 ± 19947
Mean no. per batch of eggs produced/kg female	4548 ± 409	5454 ± 820*
Total weight of eggs collected (g)	27054 ± 3441	30065 ± 3215
No. of batches collected	66 ± 4.2	62 ± 5.7
Mean wt. of collected egg batches	409 ± 26	490 ± 97
Total no. of collected eggs/ kg female	280884 ± 44355	311279 ± 13453
Mean no. per batch of eggs collected/kg female	4244 ± 400	5052 ± 678
Total weight of floating eggs (g)	11923 ± 1762	14764 ± 2343
Mean wt. per batch of floating eggs (g)	189 ± 42.4	259 ± 72.1**
Total no. of floating eggs/kg female	123022 ± 14629	152859 ± 14407
Mean no. per batch of floating eggs/kg female	1928 ± 417	2615 ± 494**
Mean fertilisation rate (% floating eggs)	31.5 ± 5.0	33.0 ± 1.4

Total no. of fertilised eggs/ kg female	42573 ± 2334	57484 ± 4236
No. of batches with fertilised eggs	61.5 ± 6.4	56.5 ± 6.4
Mean no. per batch of fertilised eggs/kg female	698 ± 110	1028 ± 191**
Mean percent hatch (% floating eggs)	11.0 ± 1.4	13.5 ± 0.7
Total no. of hatched larvae/ kg female	13492 ± 2906	20645 ± 3299
Mean no. per batch of hatched larvae/kg female	212.4 ± 66.0	354 ± 89.9

Values are mean ± SD, n = 2. Significant differences in mean weights or numbers per batch between the control and ASTA supplemented groups are shown as * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$).

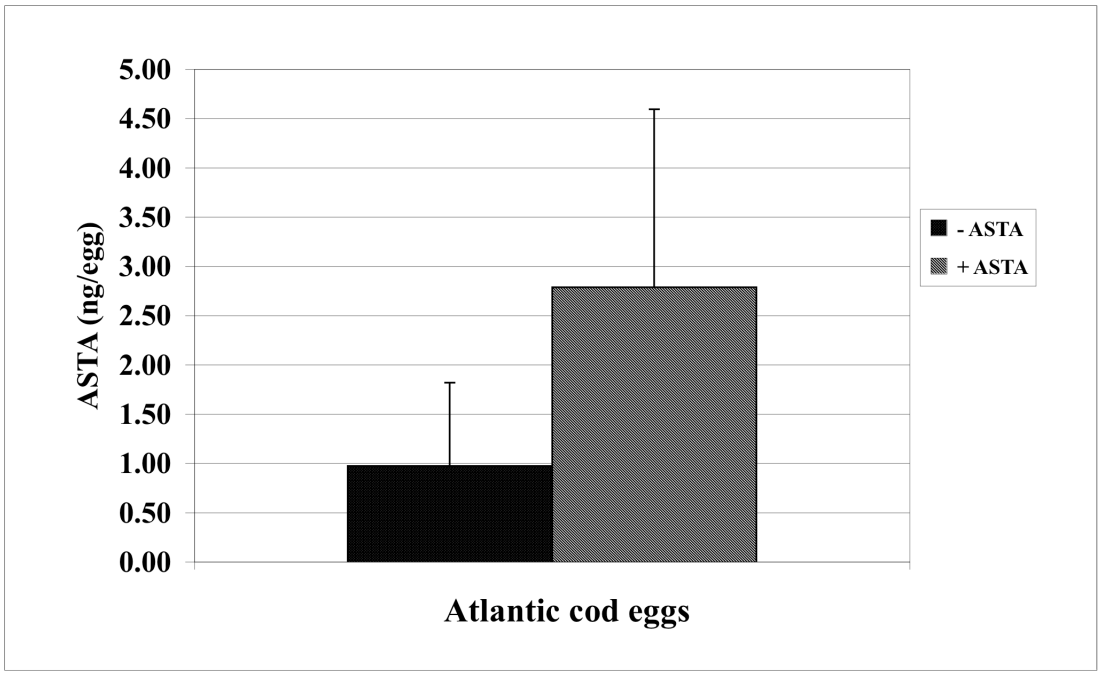
Figure legends

Figure 1. Astaxanthin content of eggs from control cod broodstock and broodstock fed an astaxanthin supplemented diet for two months prior to peak spawning. Values are ng astaxanthin/egg (mean ± SD, n = 2).

Figure 2. Egg production and egg quality parameters in cod broodstock fed a diet with and without added astaxanthin. Differences in the mean number of eggs spawned, mean number of floating eggs and mean number of fertilised eggs were statistically significant ($P < 0.05$).

Figure 3. Cumulative egg production, over the 90 day spawning period, from control broodstock and broodstock fed an astaxanthin supplemented diet.

521 Figure 1.



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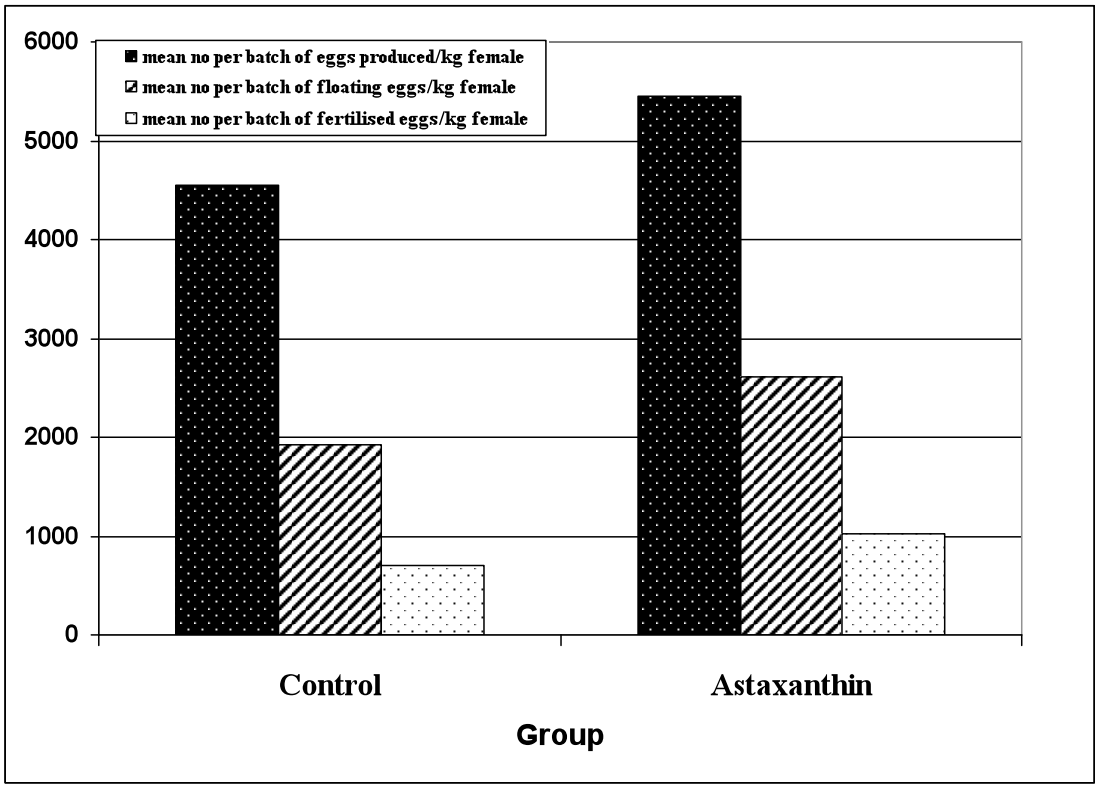
524 Figure 2.



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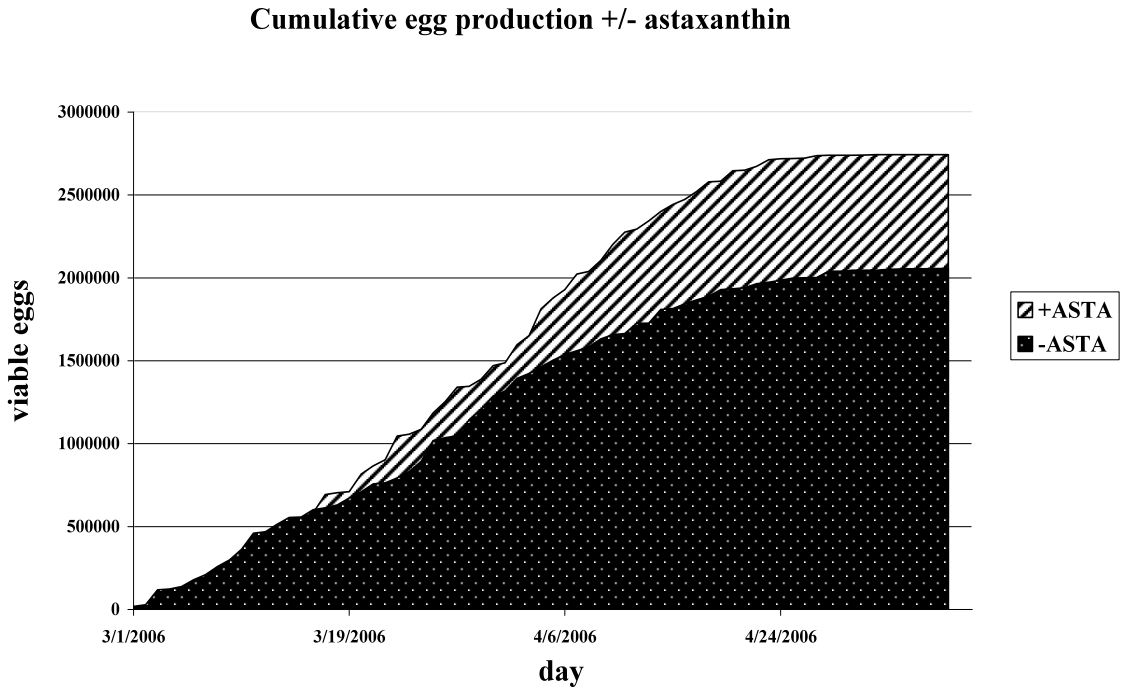
527 Figure 3.



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