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The effects of concentration and supplementation time of natural and synthetic sources of astaxanthin on the colouration of the prawn *Penaeus monodon*

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ABSTRACT

The purpose of this study was to quantify and model the combined effects of dietary concentration of astaxanthin (32.5–102.5 ppm) and time of supplementation (29–69 days) on the growth, survival, and colouration of *Penaeus monodon* (Black tiger prawn), using natural (*Haematococcus pluvialis*) and synthetic (Carophyll Pink®) astaxanthin. A model was used to determine the optimal combination of concentration and supplementation time required to obtain a commercial colour standard, based on the minimum quantity of astaxanthin required for each source. The provision of astaxanthin, as either natural or synthetic, at any concentration or supplementation time, had no effect on growth or survival. However, the colour of boiled prawns supplemented with natural astaxanthin had a small, but significant, improvement in colour (CIE2000 $L^*a^*b^*$ colour distances between 0.74 ± 0.22 and 1.79 ± 0.63), when compared with supplementation with synthetic astaxanthin. The astaxanthin concentration and supplementation time also had a significant effect on the colour of boiled prawns, where higher concentrations of astaxanthin for longer supplementation times resulted in improved colouration up until a plateau of 98 ppm for 66 days for natural astaxanthin, and 90 ppm for 63 days for synthetic astaxanthin. An additional model was used to predict the minimum usage of astaxanthin for each source during three annual commercial production cycles (Christmas, Summer, and Restock). Compared with the current commercial standard, the natural source would require 21.0%, 21.6%, and 21.3% less total astaxanthin, and the synthetic source would require 0.7%, 0.2%, and 0.3% less total astaxanthin, for the Christmas, Summer, and Restock production cycles, respectively. The predictive models developed here were able to explain the combined effects of dietary concentration of astaxanthin, time of supplementation, and effects of source on the colouration of boiled prawns, thereby predicting the minimum usage required at the commercial pond scale.

1. Introduction

The crustacean aquaculture industry is dominated by the production of prawns, which make up approximately 71% of the global market resulting in a yearly production of 4.9 million tonnes [1]. To accommodate this production, the industry relies on formulated feeds designed to maximise growth, development, and survival of the animals. An essential component in the feed of prawns is carotenoids, a group of fat-soluble pigments [2]. In prawns, carotenoids increase growth [3,4] and survival [5,6], and improve stress resistance [7,8] and reproductive capacity [9,10], but are primarily used to enhance colouration [11,12].

Colouration of the prawn integument directly affects market price, where prawns are graded based on their colour, and darker, redder prawns increase perceived quality and consumer acceptability [13–15]. Prawns cannot synthesize carotenoids *de novo*, therefore, the colour is dependent on the type, quantity, and duration of supplementation of carotenoids that are included in the feed [16,17]. The predominant carotenoid associated with the pigmentation of prawns is the red carotenoid astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), which is converted from other carotenoids, consumed as part of the diet, through major metabolic pathways of the digestive system [18–22]. Astaxanthin accounts for up to 98% of the total carotenoids present in the integument of prawns [23,24], and has the most effective tissue deposition compared to other carotenoids when included at similar, or lower, dosages (reviewed in Wade et al. [16]). For example, 125 mg kg^{-1} of β-carotene supplemented for eight weeks was required to obtain a similar colouration as 50 mg kg^{-1} of astaxanthin supplemented for four weeks in *Penaeus monodon* [21]. In *P. japonicus*, tissue deposition of

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astaxanthin was 43% and 23% higher than canthaxanthin and β-carotene, respectively, when included at the same concentration in feed [25]. Therefore, the primary, and preferred, source of carotenoids for the purpose of colour modification in prawn production systems is astaxanthin, and synthetic astaxanthin accounts for > 95% of market use [26]. However, consumer demands for natural products are driving the industry to identify natural, alternative sources of astaxanthin [27–29].

There are fundamental differences in stereochemistry, esterification, and method of production, between natural and synthetic astaxanthin. For stereochemistry, ratios of the (3R,3′R), (3R,3′S), and (3S,3′S) optical isomers differ between the sources of astaxanthin [30], where natural astaxanthin has a ratio of 1:2:22, while synthetic astaxanthin has a ratio of 1:2:1 of these isomers [31]. For esterification, between 95 and 100% of the natural astaxanthin molecules are esterified, where one or two fatty acids are attached to either end of the molecule, while synthetic astaxanthin is non-esterified or free, meaning that it contains no fatty acids [26,31,32]. Finally, for method of production, synthetic astaxanthin is produced through the chemical synthesis of the petrochemicals [32,33], while natural astaxanthin is synthesized in nature by bacteria (*i.e., Paracoccus carotinifaciens* and *Agrobacterium aurantiacum*), fungi (*i.e., Xanthophyllomyces* spp.), yeast (*i.e., Phaffia rhodozyma*), and microalgae (*i.e., Neochloris wimmeri* and *Haematococcus* spp.) [17,28], and bioaccumulates in higher organisms through the food chain [34]. Among these natural sources, the microalga *Haematococcus pluvialis* has the highest concentrations of astaxanthin of up to 3.8% on a dry weight basis [28,35,36]. Notably, *H. pluvialis* has two life-cycle stages, a vegetative growth stage and a thick-walled encysted stage, which occurs when *H. pluvialis* is stressed [37]. Although research into the use of *H. pluvialis* as a source of dietary pigment has focused on encysted biomass [38,39], vegetative *H. pluvialis* has received little attention. An essential step to develop vegetative *H. pluvialis* as a commercial product for prawn production systems is to demonstrate the minimum dietary quantity required to obtain a targeted colour, by quantifying the optimal combination of the concentration of vegetative *H. pluvialis* and, therefore, astaxanthin, in the feed and the time of supplementation.

Therefore, the aim of this study was to quantify and model the combined effects of the dietary concentration of astaxanthin and time of supplementation on prawn colouration using vegetative *H. pluvialis* as a source of natural astaxanthin and a commercially-available synthetic astaxanthin. This model was subsequently used to (1) determine the combinations of concentrations and supplementation times for each source of astaxanthin that provided the desired colouration, and (2) establish the optimal combination of concentration and supplementation time based on the minimum quantity of astaxanthin required for each source to provide the desired colour according to commercial standards.

2. Methods

2.1. Experimental design

Astaxanthin as vegetative *H. pluvialis* (provided by MBD Industries Ltd., Townsville, Australia; henceforth referred to as 'natural') and as Carophyll® pink (10% CWS; DSM Nutritional Products Ltd., Basel, Switzerland; henceforth referred to as 'synthetic'), was used as a dietary supplement for the prawn, *Penaeus monodon*, to determine the effects of dietary concentration of astaxanthin and time of supplementation on boiled prawn colouration. Due to the potential interaction between concentration and supplementation time, a central composite design was used, providing a framework to model non-linear relationships from the data [40]. For two factors, the design consisted of factorial points (n = 4 tanks) and axial points (n = 4 tanks), which are all at an equal distance away from a central point (n = 5 tanks; Fig. 1). The values for each of the concentration and supplementation time combinations were based around a design incorporating the hypothesised optimal, which was the commercially-recommended concentration of 80 ppm at a supplementation time of 56 days (8 weeks). Therefore, the dietary concentration of astaxanthin and supplementation time of the feed treatments ranged between 32.5 and 102.5 ppm and between 29 and 69 days, respectively (Fig. 1). In addition, a feed treatment without the addition of astaxanthin was also included as a negative control (n = 4 tanks).

2.2. Feed formulation

A basal feed formulation was produced and used for all treatments (Table 1). All dry feed ingredients were milled and passed through a 630 μm sieve. Milled, dry ingredients were mixed in an upright mixer (A200; Hobart Food, Silverwater, Australia) for 10 min. Fish oil was then added and the ingredients were mixed for an additional 10 min. Finally, water (30% of dry batch weight) was added to the mixture during a further 10 min of mixing. The resulting dough was screw pressed through dies No.6, No.8, and No. 10 (Dolly; La Monferrina, Rome, Italy) to produce 2.0, 2.5, and 3.0 mm pellets, respectively. The pellets were steamed for 15 min in a steam oven (SGD-52; Federal Hospitality Equipment, Sydney, Australia) and then dried in an oven for 12 h at 60 °C (SG10; Solar Dryers Australia, Bellingen, Australia). All feed was stored at −20 °C and used within five weeks of manufacture.

The basal feed was analysed for the proportion of crude protein, total lipids, ash content, and moisture content (n = 3 for each set of analysis, Table 1). Crude protein was estimated by quantifying nitrogen in the basal feed using an elemental analyser (OEA Laboratory Ltd., UK) and multiplying this by a conversion factor of 6.25 (AOAC 990.03). Total lipid concentration was quantified gravimetrically by extracting samples with a dichloromethane:methanol (2:1, v/v) solution [41]. The ash content was determined by the combustion of 1 g samples at 550 °C (152C; S.E.M. (S.A.), Magill, SA, Australia) over 6 h. Finally, the moisture content was determined by drying the samples in a moisture balance (MS70, A&D Company Ltd., Tokyo, Japan) at 110 °C.

The basal feed formulation was used as the negative control treatment and for each treatment that included natural or synthetic astaxanthin, the astaxanthin was added as a substitute for the equivalent fraction of wheat flour. The quantity of astaxanthin to be included in each treatment was determined by quantifying the concentration of astaxanthin in each source. Samples from each source of astaxanthin (n = 3) were extracted using enzymatic hydrolysis [42] and analysed using high-performance liquid chromatography (Varian ProStar System; Agilent, Santa Clara USA). The natural source (as *H. pluvialis*) had an astaxanthin concentration of 2.30 ± 0.03% and the synthetic source (as Carophyll® pink) had an astaxanthin concentration of 10.03 ± 0.05%.

2.3. Feeding trial

Prawns (mean weight = 6.91 ± 0.04 g) were sourced from Pacific Reef Fisheries (Ayr, Australia) and randomly stocked into 70 L red plastic experimental tanks (n = 10 prawns per tank), which measured 400 mm (l) × 300 mm (w) × 300 mm (h) and had a water volume of 60 L. Red tanks were used as they produce prawns with an intermediate colour when boiled [43,44]. The tanks were maintained with aeration and a constant flow of three tank volumes per hour. The experimental tanks were covered to reduce the occurrence of algal growth, which can provide an additional source of carotenoids [44], and suspended in three 10,000 L parabolic tanks, which acted as temperature-controlled water baths. The parabolic tanks were part of a recirculating aquaculture system consisting of one 20,000 L underground sump, a propeller bead filter (PBF-50S; Aquaculture Systems Technologies, New Orleans, USA), a high-output UV steriliser (E150S; Emperor Aquatics, Pottstown, USA), two water chillers (TAC 600 SSD; Toyesi Pty. Ltd., Kings Park, Australia), and four biofilters with a filter media (Kaldnes Type C1 Media; Aquasonic Pty. Ltd., Wauchope, Australia) volume of