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Effect of consumption of the nutrient-dense, freshwater small fish *Amblypharyngodon mola* on biochemical indicators of vitamin A status in Bangladeshi children: a randomised, controlled study of efficacy

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In Bangladesh, some commonly consumed, indigenous, freshwater small fish species (eaten whole with bone, head and eyes) such as mola (*Amblypharyngodon mola*) are nutrient-dense, containing preformed vitamin A as retinol and especially 3,4-dehydroretinol. The objective of the present randomised, controlled efficacy study was to evaluate the effects of mola on biochemical indicators of vitamin A status. Children (*n* 196), aged 3–7 years, with serum retinol 0·36–0·75 μmol/l, were randomly allocated to one of three treatment groups to receive a daily test meal (6 d/week for 9 weeks) of rice and vegetable curry (no vitamin A) *ad libitum* and 50 g fish curry consisting of: (1) mola, 600 retinol activity equivalents (RAE) (using 40 % biological activity of 3,4-dehydroretinol isomers) (experimental group, *n* 66); (2) rui (*Labeo rohita*), a large fish (no vitamin A), with added retinyl palmitate, 600 RAE (positive control group, *n* 65); or (3) rui, 0 RAE (negative control group, *n* 65). The nutrient compositions of the dishes were analysed. After 9 weeks, no significant treatment effects were observed for serum retinol (*P*=0·52) and retinol-binding protein (*P*=0·81) in the experimental group compared with the negative control, whereas the positive control improved significantly (*P*<0·001). The present results do not suggest conversion of the large amount of 3,4-dehydroretinol in mola curry to retinol. Further research on the functional effect of mola in humans is needed. Mola is a nutrient-dense animal-source food, rich in haem Fe, Zn and especially Ca, thus consumption of mola in Bangladesh should continue to be encouraged.

Freshwater small fish: Vitamin A: Efficacy study: Serum retinol: Bangladesh

Vitamin A deficiency is a major public health problem in many developing countries, especially among children and women of reproductive age[1]. In Bangladesh, it has been estimated that 30·8 % of preschool-aged children have vitamin A deficiency (serum retinol <0·7 μmol/l)[1]. The immediate causes are primarily an inadequate dietary intake of vitamin A, and/or a poor bioefficacy of provitamin A carotenoids from plant-based diets and low intakes of animal-source foods – such as liver, eggs and dairy products containing preformed vitamin A (as retinol and retinyl esters) of high bioavailability[2–5]. However, non-nutritional factors, including infections, may also play a role[4–6].

Dietary diversification and modification – which aims at enhancing the variety, content and bioavailability of micronutrients in diets – has received little attention but is an essential strategy to combat and prevent several nutrient deficiencies, including vitamin A, simultaneously[7,8]. Moreover, this strategy is long term, sustainable, culturally acceptable, and can reach all household members in populations at risk[7,8].

Very little focus has been on the role that nutrient-dense small fish can play in controlling and preventing micronutrient deficiencies. Whole small fish with bone is a commonly consumed food by the poor populations in developing countries with extensive fisheries resources, including Bangladesh. Moreover, small fish is culturally acceptable, relatively cheap and well liked by most household members, also children.

In Bangladesh, where vegetables and fish are a part of the everyday rice-based diet, the majority of the vitamin A intake (85 %) stems from leafy and non-leafy vegetables[9,10]. Some commonly consumed, indigenous, freshwater small fish species have been found to contain high contents of preformed vitamin A in the form of retinol and 3,4-dehydroretinol isomers (termed vitamin A in the present paper), with the relative amounts varying with the species[11,12]. In addition to being an excellent source of animal protein, some small fish, eaten whole with bone, contribute greatly to the Ca intake as well as to intakes of other nutrients such as Fe and Zn[10,11,13–16].

Abbreviations: CRP, C-reactive protein; ICDDR,B, International Centre for Diarrhoeal Disease Research, Bangladesh; RBP, retinol-binding protein; RDR, relative dose–response; RE, retinol equivalents; SRM, standard reference material.

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One of these small fish species is mola (*Amblypharyngodon mola*), which is well liked and considered very tasty[17]. Mola is found in inland bodies of water, such as ponds, rivers, beels (floodplain depressions and lakes), canals and paddy fields in Bangladesh and some other Asian countries[18]. Vitamin A in mola is found mainly in the eyes and viscera, with the larger proportion in the eyes[12,19]. Although the viscera are normally removed, mola is eaten either with or without the head and eyes[12]. In a study in rats, receiving equal amounts of retinol per d for 15 d, either from cooked whole mola or as pure retinyl acetate, the amount of liver retinol was higher in those fed mola[19]. Zafri & Ahmad[19] suggested that some dehydroretinol in mola might have been converted to retinol[19]. In the rats fed mola, both retinol and dehydroretinol (determined by a colorimetric method) were found in the liver[19].

To our knowledge, no studies have investigated the effect of the consumption of vitamin A-rich small fish on vitamin A status in humans. We therefore conducted a randomised, controlled efficacy study to evaluate the effect of mola on vitamin A status – as measured by the biochemical indicators, serum concentrations of retinol and retinol-binding protein (RBP), and the results of the relative dose–response (RDR) test – after intake of a daily test meal with mola for 9 weeks by Bangladeshi children with marginal serum retinol concentration ($0.36-0.75$ μmol/l). Moreover, the nutrient compositions – macronutrients, vitamin A compounds and some minerals – of the dishes in the test meals were analysed, and the contributions of the dishes with fish to the US daily Dietary Reference Intakes of Fe, Zn and Ca were evaluated.

Materials and methods

Study population and screening

Children aged 3–7 years of both sexes were identified from four selected neighbouring camps in an urban slum in Mirpur, a suburb of Dhaka, Bangladesh, through a house-to-house survey. This slum area and age group were chosen as a high prevalence of low serum retinol concentration (<0.7 μmol/l) (57 %, *n* 129 out of *n* 226) was found in children (aged 3–5 years)[20], even though the area is covered by the national programme of biannual supplementation of high-dose vitamin A capsules to children (aged 12–59 months). The children (*n* 579) were screened for serum retinol concentrations of $0.35-0.70$ μmol/l between early March and late April 2002, anticipating that about one-third of the children would fall in this group. Anthelmintic treatment was given to each child, before blood collection at screening, as previously described[6,21].

Children who had received vitamin A supplements within the preceding 6 months were excluded. Severely undernourished children and those with serious illnesses, including clinical signs of vitamin A deficiency, were excluded and referred for diagnosis and treatment, as previously described[6,21]. At the time of blood collection, the children were apparently healthy, showing no signs and symptoms of acute illness such as fever, acute diarrhoea, dysentery, pneumonia or acute respiratory-tract infection. Children with serum retinol concentrations <0.35 μmol/l received an oral dose of vitamin A (retinyl palmitate, 60 mg (200 000 international units)), and were excluded from further study. The slum area, data collection and characteristics of the screened population have been previously reported[6,21].

Of the children screened, 196 were identified as having serum retinol concentrations of $0.36-0.75$ μmol/l and they were all invited at baseline (the first day of the feeding trial) (Fig. 1) to participate in the efficacy study, conducted from late April to early July 2002. This group of children was selected, as serum retinol concentrations due to homeostatic regulation[22] are more likely to increase in response to vitamin A intake.

Before carrying out the efficacy study, it was presented to and accepted by the community leaders. Written or oral informed consent was obtained from each child's parent or legal guardian. The study protocol was approved by the Research Review Committee and Ethical Review Committee, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). All children and their family members had access free of charge to the health services, including medicines, given by the physicians at the health clinic at the project facilities. Records were kept of diseases and medicines provided to the study children. Family members or caregivers who accompanied the children to the feeding were offered a free meal, separately. At completion of the feeding trial, all children received an oral dose of vitamin A (retinyl palmitate, 60 mg (200 000 international units)) and the parent or legal guardian was informed about the results of the faeces examination and advised to give anthelmintic treatment, if necessary. All staff of the study received anthelmintic treatment before the feeding trial.

Outcomes and sample sizes

The primary outcome was serum retinol concentration and secondary outcomes were serum RBP concentration and the results of the RDR test at the 9 weeks' endpoint (the day after the last day of the feeding trial). To have a 90 % chance of detecting a difference in the mean serum retinol concentration of 0.15 μmol/l between the experimental and negative control group at the 5 % level of significance, with an assumed standard deviation of 0.20 μmol/l[23], thirty-seven children were required in each group. To account for potential dropouts, sixty-five to sixty-six children in each of the three groups – in total all the identified children – were enrolled.

The RDR test was performed on a random subsample of sixteen children in each group at endpoint, taking into account potential dropouts. In the negative control group, it was assumed that 73 % of the children would have a positive RDR test value. It was estimated that a sample size of thirteen children in each group would be sufficient to have an 80 % chance of detecting a difference in the positive RDR test value of 58 percentage points between the experimental and negative control group at the 5 % level of significance.

Treatment groups

The children were randomly allocated to one of three treatment groups, using a random number table. The children in each group received a daily test meal (6 d/week for 9 weeks)
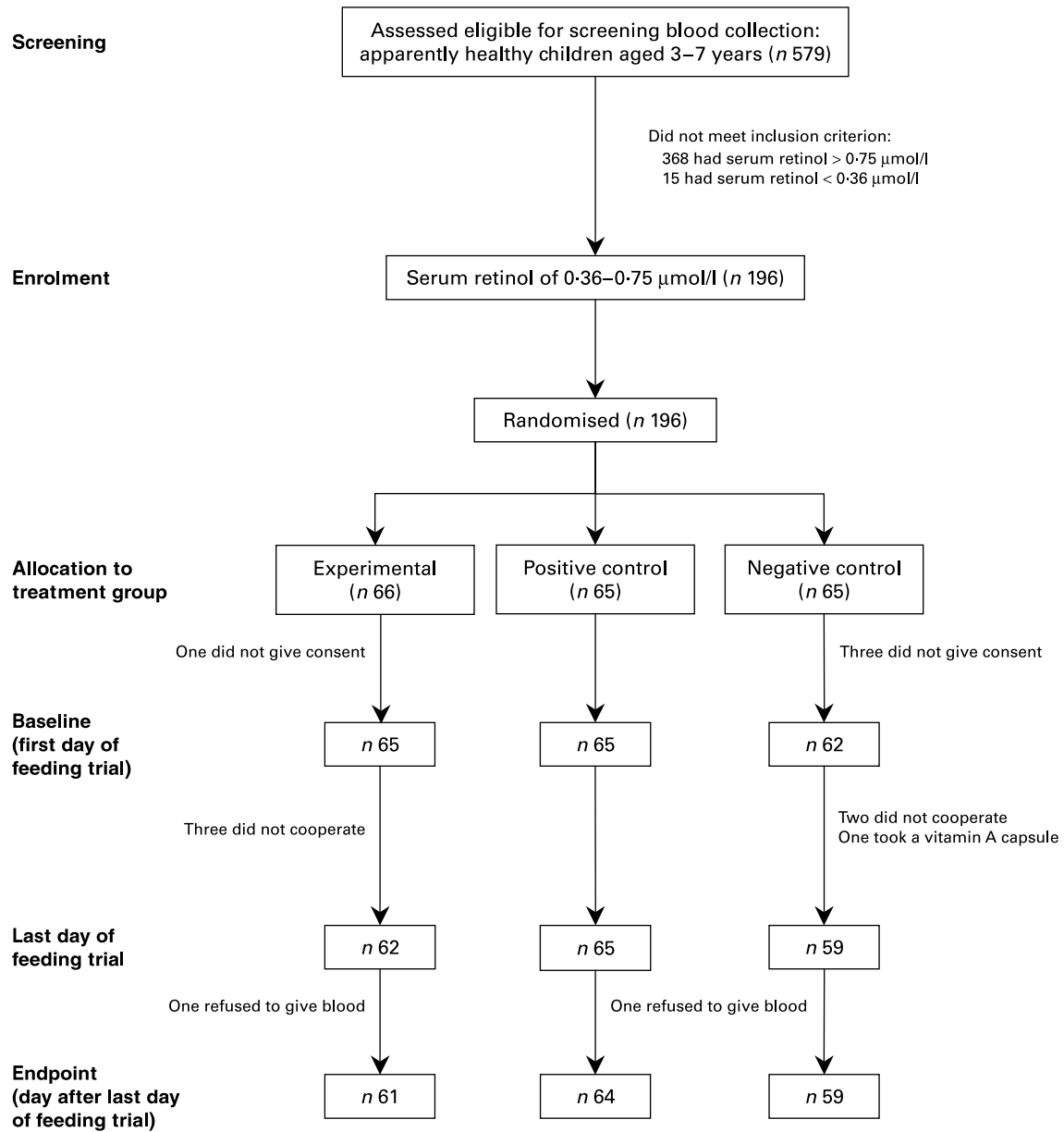
Fig. 1. Diagram of the flow of the children through each stage of the efficacy study. Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry. Each child was served one test meal per d consisting of 50 g fish curry and the same rice and non-leafy vegetable curry *ad libitum* during the feeding trial (9 weeks, 6 d/week).

of two basal dishes of rice and a non-leafy vegetable curry (vegetable curry), containing no vitamin A, *ad libitum*, as well as a dish of homogenised fish curry (50 g) consisting of: (1) mola (experimental group, *n* 66); (2) rui (*Labeo rohita*), a carp species (large fish) containing no vitamin A, with added retinyl palmitate (positive control group, *n* 65); or (3) rui (negative control group, *n* 65). The fish curry in the positive and negative control groups was the same.

A serving of fish curry (50 g) corresponded to approximately 30 g raw, cleaned and edible mola (whole with bone, head and eyes, and excluding the viscera) or 30 g raw, cleaned and edible rui muscle. A serving of fish curry in the experimental and positive control groups was designed to contain a similar amount of vitamin A of 500 retinol equivalents (RE)/child per test meal per d to meet the daily RDA of

4–6-year-old children[24]. At the time of the study, RE was used as the vitamin A unit.

In the experimental group, the estimated amount of vitamin A in a serving of mola curry was based on a total vitamin A content of 2500 RE/100 g raw, edible parts of mola (whole with bone, head and eyes, and excluding the viscera)[12] as well as a cooking loss of approximately 30 %. The total vitamin A content in the raw, edible parts of mola was based on the following contents of the vitamin A compounds (μg/100 g raw, edible parts)[11] and their biological activities in relation to all-*trans*-retinol: (i) 320 μg all-*trans*-retinol/100 g with a biological activity of 100 %; (ii) 0 μg 13-*cis*-retinol/100 g; (iii) 4990 μg all-*trans*-3,4-dehydroretinol/100 g with a biological activity of 40 %[25]; (iv) 460 μg 13-*cis*-3,4-dehydroretinol/100 g with a biological activity of 40 %[25]; (v) 0 μg β-caro-

tene/100 g. In the positive control group, about 70 μl retinyl palmitate in an oily solution was added to the homogenised rui curry on the plate (in a specific spot, under the study number which was marked on the edge of the plate), just before serving. The vitamin A (all-*trans*-retinol) contents in the samples of the retinyl palmitate solution were verified spectrophotometrically[26] at the Nutritional Biochemistry Laboratory, ICDDR,B, before use.

The negative control group was included because changes in serum retinol concentration over time may be related to factors other than mola. The positive control group, receiving synthetic all-*trans*-retinol, was included in order to determine the maximal effect on the serum retinol concentration. Rice and vegetable curry were given *ad libitum* to allow for differences in food intake of children of different ages.

Colour-coding and blinding

A different colour code (blue, green or red) was used for each of the three treatment groups, which were fed in separate colour-coded rooms. All feeding equipment and record material were colour-coded. For the feeding, each child had a colour-coded photo identity card with the child's study number as well as the name of the fieldworker responsible for the child and contact to her/his household. This card was kept at the project facilities.

The children were not informed about which of the three fish curries was given to their group. The mola and rui curries had different colours; the mola curry was dark-green, whereas the rui curry had a yellow tinge. However, their consistencies were comparable, a semi-liquid paste. Thus, the children may not have been blinded to the treatment groups. The feeding staff was not blinded to the treatment groups, as they had access to the cooking facilities and were involved in the preparation and distribution of the test meals. However, the laboratory staff conducting the blood and stool analyses was blinded to the treatment groups. Thus, the present study may not have been blinded – a study that is not blinded may lead to biased results[27].

Efficacy study

At baseline, clinical examination including anthropometry and the RDR test, on a random subsample of fifteen children in each group, was undertaken. During the feeding trial, the test meal was served around lunchtime, replacing one of the meals at home, 6 d/week for 9 weeks. The amount of each dish consumed per child per d was determined. Samples of each dish were collected and the nutrient contents analysed. Attendance and reasons for absence were recorded daily. At endpoint, clinical examination, blood collection including the RDR test and faeces collection were undertaken. The RDR test at endpoint was undertaken on different random subsamples in each group than those from baseline.

Composition of dishes

The dishes in the test meals were developed and tested by K. K., in collaboration with the chief dietitian, ICDDR,B. During a pilot study, the dishes were also tested in children in the study area, and based on their feedback, adjustments were made to the recipes to ensure acceptability. Throughout the feeding trial, the same four dishes – a mola curry, a rui curry, rice and a vegetable curry – were cooked each day in a traditional Bangladeshi way, using standardised recipes. All foods in the test meals other than mola were non-vitamin A-containing foods. The mola and rui curry recipes differed only with respect to the fish species used and contained in addition onion, soyabean oil, freshly ground garlic and ginger, turmeric powder, homogenised roasted chilli seeds and iodised salt (in order of descending amounts). The amount of added oil in the two fish curries was 7·6 g per 50 g serving of fish curry. The vegetable curry consisted of potato, cucumber, bottle gourd, roasted lentil (mug dal), onion, soyabean oil, freshly ground garlic and ginger, turmeric powder, homogenised roasted chilli seeds and iodised salt. The amount of added oil was 4 g per 100 g vegetable curry.

Food supply

Mola and rui were collected, cleaned, packed, stored and transported in collaboration with the Mymensingh Aquaculture Extension Project, Bangladesh, which is supported by Danish International Development Assistance. Mola was collected between November 2001 and January 2002 and rui between March and May 2002 in the Kishoreganj district, about 200 km north-east of Dhaka. Mola was collected mainly from ponds and a few from open bodies of water. Rui was collected mainly from rivers and a few from ponds. The weight of mola was about 1–5 g/fish and the length 3–9 cm/fish. The weight of rui was about 0·5–4 kg/fish and the length 30–70 cm/fish. Mola was cleaned by removing the fins, tail, viscera and gills (only for mola of big size), rubbed with salt and washed several times with water. The head, including the eyes, were not removed. Thus, the raw, cleaned and edible parts of mola included the head with eyes, muscle, bone and skin. Rui was cleaned by removing the head, fins, tail, viscera, gills and scales and washed with water. Thus, the raw, cleaned rui included the muscle, skin and bone. Rui was cut in three to five pieces, depending on the size of the fish, before packaging and storage.

Two fieldworkers, with experience from previous fish research studies, collected and packed all the fish, after training by K. K. and the extension manager, Mymensingh Aquaculture Extension Project, who also coordinated and supervised the work. After the fish were caught, they were transported by motorbike to a permanent site for cleaning. All mola was cleaned by the same five trained local women and for rui by the same two to five trained male fish retailers from a local fish market. Immediately after cleaning the fish, the excess water was drained using a colander, before being packed and weighed in freezer plastic bags for food (Coop, Denmark) in fixed amounts (500 g mola and 1060 g rui, taking into account a bone weight of 5·7 % (60 g) of the total weight). Each bag was weighed to the nearest ±2 g using a calibrated kitchen scale (model HR2388; Philips, Budapest, Hungary) and labelled with the date, amount and place of collection. The bags were put in black plastic bags in amounts (3000 g mola and 6360 g rui) for the daily recipes and stored in a freezer (−20°C) at the Mymensingh Aquaculture Extension Project Kishoregonj district office, within 3–8 h of collection. From collection to freezing, the fish

were kept in cool boxes containing ice and ice packs. Records of the daily fish collection were kept. All the fish was later transported in big, insulated ice boxes to an ice cream factory in Dhaka where it was kept in a freezer room (-22 to $-27°C$), before being transported in cool boxes periodically to the project facilities where it was stored ($-20°C$) until use. All freezers used were connected to a stabiliser and a generator to maintain the temperature in times of electricity irregularities. The freezer temperature was recorded daily and was always $< -20°C$. The weights of the frozen bags were checked at the time of cooking, and each bag was within ± 100 g of the planned weight.

The rice used was the common local variety, Pajam, which was supplied by the Bangladesh Rice Research Institute, Gazipur, Bangladesh. It was grown in the T. Aman season (August–November) 2001 in Mymensingh district, Bangladesh. After harvesting at the end of November, the paddy was stored in gunny bags and just before the start of the feeding trial it was soaked, parboiled, dried and milled using standardised procedures.

Bottle gourd and cucumber were supplied daily and potato, lentils, onion, ginger, garlic, turmeric powder, chilli seeds, oil and salt were supplied twice weekly by a local vendor. To ensure similar qualities of the ingredients from day to day, a quality checklist – defined maturity, texture, freshness, colour, size, shape, country of origin and brand name – was used.

Preparation of dishes

The test meals were prepared (between 06.00 and 12.00 hours) in the kitchen at the project facilities using standardised procedures. The frozen bags of raw, cleaned mola and rui were taken out of the freezer, wiped with a towel, covered with aluminium foil and subsequently thawed in a refrigerator for 1 and 2 d, respectively, before being cooked. The refrigerator was connected to a stabiliser and a generator and the temperature was recorded twice daily and was always maximum $+5°C$. The whole content of the fish bags, including the thawing water in the bag, was used for cooking. Before cooking the rui curry, the raw, cleaned rui was steamed in a fixed amount of water (which was later used for cooking the rui curry) and the muscles were separated from the bones by use of hands. The bones were discarded. All ingredients used for the rui and mola curry were mixed before cooking. The mola curry was cooked gently, by simmering for 15 min, initially 5 min without the lid and 10 min with the lid on, and subsequently homogenised in a blender to ensure a uniform distribution of the vitamin A. The rui curry was also homogenised in order to have a similar consistency. Rice was steamed in a fixed amount of water, i.e. full absorption of the water by the grains.

The dishes were prepared by three cooks, eight kitchen helpers (women living in the community), one senior nutritionist and two assistant nutritionists, after training by K. K. and senior nutritionist. The senior nutritionist also had the daily overall responsibility for the kitchen, supervised and monitored the kitchen staff and procedures, kept daily records of activities and did the weighing. K. K. was in the kitchen daily to check and discuss the procedures and solve problems. Throughout the feeding trial, the same two kitchen staff weighed the amounts of cleaned ingredients for each dish to the nearest ± 2 g, using a kitchen scale (model HR2388; Philips) that was calibrated daily. To minimise the day-to-day variation in the cooking methods, each dish was cooked by the same cook throughout the feeding trial. On few days, small amounts of boiled water were added to the fish curries before blending and to the vegetable curry, immediately before weighing the individual portions, to ensure the standardised total cooked weights.

Measurement and calculation of dish intake

The amounts of the fish curry, rice and vegetable curry served per child were weighed on separate tarred plates, which were permanently marked with the child's study number and used throughout the feeding trial. The amount of fish curry served per child (50 g) was fixed. Initially, all children were given the same amounts of rice (150 g) and vegetable curry (100 g); subsequently those who asked for more were given more. Additional pre-weighed portions of rice (50 g) and vegetable curry (25 g) were served in cups, transferred to the respective plates which were then labelled with a sticker for later recording of the additional amounts served. The amount of leftover of each dish per child was calculated by subtracting the weight of the plate with leftover from the mean plate weight used for each dish (n 5; $CV \leq 1\%$). Total daily intake of each dish per child was calculated by subtracting the amount of the leftover from the total amount served. In the positive control group, the intake of retinyl palmitate was recorded as either 0 or 100 %.

Throughout the feeding trial, for each group of children, two nutritionists weighed and recorded the amounts of each dish served and left over (to the nearest ± 2 g) using the same two kitchen scales (model HR2388, Philips), which were calibrated daily. A pre-tested form was used for recording, which was cross-checked at the completion of feeding by two nutritionists from another feeding room. The whole process was closely supervised and monitored daily, directly by K. K. and senior nutritionist.

Attendance, test meal distribution, motivation and ensuring compliance

All the children lived in the surrounding area of the project facilities, where the test meals were served (between 12.00 and 15.00 hours). To each household, a female fieldworker (n 8), assisted by a female field attendant (living in the community), was assigned. The fieldworkers and field attendants maintained close contact and motivated the families, through frequent visits, to ensure that the children did not drop out of the study.

Most children were accompanied to and from feeding by their family members or caregivers, or by fieldworkers or attendants, and few came alone. On arrival to the project facilities, three experienced physicians, one assigned to each group, hung the identity card around the child's neck and recorded her/his attendance. The reasons for absence were retrospectively obtained from the family member or caregiver or the child by the fieldworker and physician. Absent days were not compensated for. If the child was unwilling to come or did not show up, the fieldworker or attendant and in a few instances the field administrator or physician visited the

household to motivate the child's attendance. The children had free access to a playroom with a kindergarten teacher and a caregiver before feeding, where they made drawings for their feeding rooms, practised writing the alphabet and played with toys.

Standardised guidelines and procedures for feeding were used. In each room, the feeding was conducted by trained staff, the same two nutritionists who also weighed the dishes, six field attendants or kitchen helpers and two servants. The nutritionists had the overall responsibility for the feeding, including instructing and supervising the feeding staff, checking each child's identity card upon arrival to the room, seating the child on the floor, giving motivation, observing the children, ensuring that no food was switched, serving the dishes and removing the identity card after the child had eaten. The field attendants cared for the children and motivated them to eat the test meal, especially all of the fish curry, as well as constantly observed and ensured that no food was switched. Outside the room, the servants handed the plates and cups with the dishes upon request by the nutritionists as well as served drinking water, offered *ad libitum* after feeding, unless requested before. Hand washing was done before and after feeding. The feeding was conducted continuously, as about only one-third of each group could be accommodated at a time in the room.

To ensure that the children ate all of the fish curry served, they were first served the fish curry and rice. After finishing the fish curry, they were served the vegetable curry and subsequently additional rice and vegetable portions. The few children who did not eat, because they were not feeling well or did not wish to eat for other reasons, were attended to by the physicians and given special care and attention. In a few instances, the family member or caregiver and the fieldworker were also present during the feeding.

The whole process was closely supervised and monitored daily by K. K. and senior nutritionist. Other investigators made unscheduled spot visits to monitor the activities of the staff and weekly staff meetings were held; problems encountered were discussed and advice given accordingly. The children and family members or caregivers were informed of the importance of eating all of the test meals, not changing the normal diet, not taking vitamin A or multivitamin or mineral supplements and anthelmintic drugs, as well as not seeking medical treatment outside of the study, and if considering doing so, informing and discussing with the physicians.

Clinical examination

Information of the date of the most recent intake of vitamin A capsule as well as signs and symptoms of illness in the children were obtained and they were examined clinically as described in detail previously[6,21]. Height (without shoes) was measured to the nearest 0·1 cm, weight (in light clothing) to the nearest 0·1 kg and mid-upper arm circumference to the nearest 2 mm, and the Z-scores of weight-for-height, weight-for-age and height-for-age were calculated using the US National Center for Health Statistics/WHO international reference population[28], as described in detail previously[6,21]. Children with weight-for-height, weight-for-age and height-for-age Z-scores < -2 were considered wasted, underweight and stunted, respectively[28].

Blood collection

Non-fasting venous blood (4 ml) was collected from each child between 09.00 and 13.00 hours, using trace element-free plastic syringes and stainless steel needles. Blood was injected into evacuated tubes (Venoject II; Terumo Europe NV, Leuven, Belgium), which were wrapped in aluminium foil, transported to the Nutritional Biochemistry Laboratory, ICDDR,B, and centrifuged (1000 g for 10 min at room temperature) within 5 h. Serum was transferred using trace element-free pipettes into cryovials (Simport, Quebec, Canada) and immediately stored at $-20°C$ until analysed for retinol, RBP, Zn, C-reactive protein (CRP) and transthyretin at ICDDR,B.

For the RDR test, fasting venous blood was collected from each child in the early morning (0 h), immediately before giving an oral dose of 3·5 μmol (1000 μg) retinyl palmitate in an oily solution. After vitamin A dosing, a small breakfast, vitamin A free and high in fat, was served. The children were under observation until a second blood sample was taken 5 h after dosing. The RDR test results were calculated using the equation:

$$RDR\,test\,value = ((retinol\,5h - retinol\,0h)/retinol\,5h) \times 100\%$$

A RDR test value $\geq 20\%$ was considered positive and indicative of inadequate liver vitamin A stores[29]. The all-*trans*-retinol contents in the samples of retinyl palmitate solution were verified spectrophotometrically[26] at the Nutritional Biochemistry Laboratory, ICDDR,B, before use.

Socio-economic status and morbidity collected at screening

Socio-economic status data were collected on the day of obtaining the consent for participating in the screening study. Data on the presence or absence of specific symptoms in the child during the previous 2 weeks from the day of blood collection at screening were collected through a recall interview of the mother or caregiver. Diarrhoea was defined as $\geq$ three loose or watery stools in a 24 h period. All data were collected by the fieldworkers, through household visits, using precoded and pretested questionnaires in Bangla, as described in detail previously[6].

Faeces collection and examination

A plastic container was provided by the fieldworker for faeces collection during the collection of the socio-economic status data and before the anthelmintic treatment was undertaken as well as at endpoint. The parent or caregiver was asked to collect a sample of faeces from the child in the early morning of the following day. The fresh faeces sample in normal saline (0·9 % (w/v) aqueous NaCl) was examined microscopically by direct smear, and the presence of trophozoites of *Giardia intestinalis* and *Entamoeba histolytica* was verified. In addition, 1–2 g of fresh faeces were fixed in 10 % (v/v) formalin in normal saline and later processed by a quantitative ether sedimentation technique[30] before microscopic examination to estimate the intensity of helminthic infections of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm, expressed as eggs/g faeces. Faeces samples were analysed at

the Parasitology Laboratory, ICDDR,B, by an experienced laboratory technician.

Biochemical analyses of serum samples

Serum retinol concentrations were measured by using HPLC as described in detail previously[6]. For quality control, a pooled human serum sample was calibrated against standard reference material (SRM; fat-soluble vitamins, carotenoids and cholesterol in human serum, 968c; National Institute of Standards and Technology, Gaithersburg, MD, USA). Three aliquots of the serum pool were analysed with each set of samples, and retinol concentrations were calculated based on the known concentration of retinol in the serum pool. Within-day and between-day CV for retinol in the serum pool were 1·4 and 1·5 %, respectively.

Serum RBP concentrations were quantified by a radial immunodiffusion technique using a commercial kit and quality-control materials (The Binding Site Ltd, Birmingham, UK). The accuracy was verified with each radial immunodiffusion plate by analysing the quality control of RBP (58·5–71·5 mg/l). The within-day and between-day CV were 1·1 and 1·3 %, respectively. The recovery of serum RBP was 95–105 %.

Serum Zn concentrations were measured using flame atomic absorption spectrophotometry as described in detail previously[21], using a standard curve prepared from a commercial zinc nitrate standard solution (BDH Laboratory Supplies, Poole, Dorset, UK). The accuracy was verified with each set of samples using commercial serum Zn quality controls (Utak Laboratories Inc., Valerncia, CA, USA) of normal (12–18 μmol/l) and high (34–52 μmol/l) levels as well as a pooled human serum sample in triplicate. For normal and high levels of Zn quality controls, the within-day CV were 2·6 and 1·0 %, respectively, and the between-day CV were 3·4 and 1·8 %, respectively.

Serum CRP and transthyretin concentrations were measured by immunoturbidimetric assay (Hitachi 902 Automatic Analyser; Boehringer Mannheim, Mannheim, Germany) using commercial kits and quality-control materials (Roche Diagnostics GmbH, Mannheim, Germany). The accuracy was verified with each run by analysing quality controls of serum CRP of low (13·9–22·9 mg/l) and high (35·6–58·4 mg/l) levels and serum transthyretin of low (188–308 mg/l) and high (299–485 mg/l) levels. For low and high levels of controls of serum CRP, the within-day CV were 2·5 and 1·0 %, respectively, and the between-day CV 3·4 and 1·2 %, respectively. The recovery of serum CRP was 95–105 %. For low and high levels of controls of serum transthyretin, the within-day CV were 1·9 and 1·5 %, respectively, and the between-day CV 2·2 and 2·0 %, respectively.

Serum concentrations of retinol and RBP were determined from 196 and 182 (not analysed for children without endpoint blood collection, n 12; insufficient serum, n 2) children, respectively, at screening and 184 children at endpoint. In the RDR test, serum retinol concentrations at 0 and 5 h were determined from forty-five children at baseline, and forty-six at endpoint (endpoint: refused to give blood, n 1; blood was haemolysed at 5 h, n 1). Serum concentrations of Zn and transthyretin at screening were determined from 196

children, and serum CRP at screening from 195 children (insufficient serum, n 1).

Dish sample collection and chemical analyses

For each of the three fish dishes, sampling was done every week (n 9), whereas for the rice (n 5) and vegetable curry (n 5), sampling was done biweekly. Representative quadruple samples of each of the dishes were taken on randomly selected week days (immediately before the children's portions were weighed), placed in labelled polyethylene containers which were placed in freezer plastic bags and stored immediately at −20°C. For the rui curry with added retinyl palmitate, a sample corresponded to a child's serving and the retinyl palmitate was added to the rui curry in the container, just before serving (at the same time of adding retinyl palmitate to the children's portions). Sampling was done by two of the nutritionists and one of the cooks, after training by K. K. who supervised and monitored the collection, handling and storage of the samples each time.

All-trans-retinol, DM, energy, N and fat were analysed in the samples in duplicate at ICDDR,B. For the analyses of vitamin A compounds (retinol and 3,4-dehydroretinol isomers and β-carotene) in the fish curries, the samples was transported on dry ice to Denmark and analysed in single at the National Food Institute, Technical University of Denmark, Søborg. For the analyses of ash and minerals (total Fe, non-haem Fe, Zn and Ca), samples were collected in acid-washed containers, freeze-dried, transported to Denmark, and analysed in duplicate at the Faculty of Agricultural Sciences, University of Aarhus, Tjele, Denmark.

For all-trans-retinol, subsamples (n 9; 20–50 mg) were saponified with methanolic pyrogallol (25 % (w/v), 100 μl), methanol (1000 μl) and methanolic KOH (40 % (w/v), 200 μl), incubated for 1 h at 70°C, cooled and extracted twice with n-hexane (1000 μl). The n-hexane layers were pooled and evaporated under N_2 gas. The residue was reconstituted in 500–1000 μl methanol and water (95:5, v/v) as mobile phase, and 25 μl were injected on a Shimadzu HPLC instrument (Shimadzu Corporation, Kyoto, Japan) using a Supelco discovery C_{18} column (5 μm; 4·6 × 250 mm) (Supelco, Bellefonte, PA, USA) and a Shimadzu SPD-10A UV-VIS detector at 325 nm, as well as a Shimadzu Liquid Chromatograph LC-10AT pump (flow rate 0·8 ml/min) and a Shimadzu Chromatopac C-R8A data processor (Shimadzu Corporation, Kyoto, Japan). The accuracy was verified with each run of samples by analysing the SRM (baby food composite 2383; National Institute of Standards and Technology, Gaithersburg, MD, USA). Within-day and between-day CV were 6·3 and 9·6 %, respectively. The other vitamin A compounds (13-cis-retinol, all-trans-3,4-dehydroretinol, 13-cis-3,4-dehydroretinol and β-carotene) as well as all-trans-retinol were analysed in mola curry (n 8; one missing sample as it was used for another analysis) by HPLC as described by Leth & Jacobsen[31] and Roos et al.[12]. The rui curry was not expected to contain any vitamin A compounds; however, this was verified using one pooled sample of n 9. Vitamin A compounds in the rui curry with added retinyl palmitate (n 2; missing samples as they were used for other analyses, n 7) were also verified. Accuracy of all-trans-retinol analysis was assured by participation in the Food Examination Performance

Assessment Scheme (FAPAS®; Central Science Laboratory, York, UK) and the between-day CV was 4·4 %. For quality control, all-*trans*-retinol was analysed at both the ICDDR,B, Bangladesh, and National Food Institute, Technical University of Denmark, Søborg, in duplicate mola curry samples (*n* 8), and the values obtained were 325 (SD 99) and 320 (SD 54) μg all-*trans*-retinol/100 g mola curry, respectively.

Before analysing for DM, energy, N and fat, the rice and vegetable curry samples were homogenised, using a blender. DM was determined by drying the sample in a hot air oven at 103 (SD 15)°C until constant weight was obtained[32]. The accuracy was verified with each run of samples by analysing the SRM. The SRM value for DM was 37·19 (SD 0·46) g/100 g and the analysed value was 37·03 (SD 0·05) g/100 g (*n* 6). Gross energy was determined by using an automatic adiabatic bomb calorimeter (Autobomb CBA-305; Gallenkamp, Loughborough, Leics, UK), according to the procedure given by the manufacturer. The accuracy was verified with each run of samples by analysing the SRM and the British Chemical Standard-Certified Reference Material (benzoic acid no. 190r; Bureau of Analysed Samples Ltd, Middlesbrough, UK). The SRM value for energy was 696·6 (SD 14·6) kJ (166·5 (SD 3·5) kcal)/100 g and the analysed value was 697·5 (SD 4·6) kJ (166·7 (SD 1·1) kcal)/100 g (*n* 6), and the British Chemical Standard-Certified Reference Material value was 26·435 (SD 0·004) kJ (6·318 (SD 0·001) kcal)/g and analysed value was 26·443 (SD 0·059) (6·320 (SD 0·014) kcal)/g (*n* 6). N was determined by the micro-Kjeldahl method[33] (Labconco 60 300; Kansas City, MO, USA). A factor of 6·25 was used to calculate protein from N values. The accuracy was verified with each run of samples by analysing the SRM and an internal control, ammonium sulfate (BDH Laboratory Supplies). The SRM value for protein was 3·89 (SD 0·17) g/100 g and the analysed value was 3·86 (SD 0·08) g/100 g (*n* 6), and the CV for the internal control was 1·2 % (*n* 6). Fat was determined using the method developed by van de Kamer[34]. The accuracy was verified with each run of samples by analysing the SRM and an internal control, steric acid (Sigma Chemical Co., St Louis, MO, USA) in 1 % ethanol (w/v). The SRM value for fat was 4·67 (SD 0·26) g/100 g and the analysed value was 4·69 (SD 0·11) g/100 g (*n* 6), and the CV for the internal control was 1·6 % (*n* 6).

Before analysing for minerals, the freeze-dried rice and vegetable curry samples were homogenised, using a metal-free blender equipped with titanium blades. All equipment used for mineral analyses was acid washed. Total Fe, Zn and Ca were determined by atomic absorption spectrophotometry (PU 9400 X; Philips Scientific, Cambridge, UK) after dry-ashing as described by Larsen & Sandström[35]. Non-haem Fe was determined spectrophotometrically (Spectronic UV-1201; Milton Roy, Rochester, New York, USA) by the Ferrozine method[36], using a standard curve prepared from Fe (II) chloride tetrahydrate (Fe(II)Cl$_2$.4H$_2$O) (no. 103860; Merck, Darmstadt, Germany). The accuracy and precision CV were: 3·0 and 1·2 %, respectively, for total Fe; 2·7 and 1·9 %, respectively, for Zn; 1·6 and 1·8 %, respectively, for Ca.

Statistical analyses

Data were entered twice and cleaned in Fox Pro (Microsoft, Redmond, WA, USA) and analysed using SPSS for Windows (version 12.0; SPSS Inc., Chicago, IL, USA). Normal probability plots were used to assess whether continuous variables were normally distributed. Data were analysed on an intention-to-treat basis, regardless of compliance; missing values were not imputed. Differences in means of dish intakes between groups were tested with the Kruskal–Wallis test. Differences in proportions between groups were tested with the Pearson χ^2 test.

The paired *t* test or Wilcoxon signed rank test was used to test for differences between screening and endpoint values of serum retinol and RBP within each group. Comparisons of the outcome variables – endpoint serum retinol and RBP – between groups were done using the negative control group as the reference group. Between-group comparisons of the outcome variables were done using one-way ANOVA with Tukey's multiple comparison *post hoc* test, as well as analysis of covariance, adjusting for the screening values of the outcome variable[37] and screening serum CRP concentrations – after $\log_{10}$(CRP concentration +1) transformation – to adjust for the effect of the acute-phase response[6,38]. In addition, in the analysis of covariance, the influence of selected potential confounding screening and baseline variables – the biochemical indicators at screening, serum concentrations of Zn, transthyretin and RBP[39,40], expected to be strong predictors of the outcomes, as well as sex, age at baseline, and *A. lumbricoides* and *T. trichiura* infections at screening – was assessed by backward selection, and covariates statistically significantly related to the outcomes were retained and adjusted for in the multiple linear regression model, in order to reduce some of the random error[41]. The covariates included in the reduced model are mentioned in Table 6. Biochemical indicators and age were continuous variables, whereas helminthic infections and sex were binary variables.

Values of $P < 0.05$ were considered statistically significant. Residual analysis including normal distribution and homogeneity of variance of standardised residuals was carried out by investigating normal probability plots and plotting standardised residuals against predicted values.

Results

Characteristics of the children

Of the 196 children who were randomised, informed consent was obtained from 192 and these children started the intervention at baseline (Fig. 1). Subsequently, eight children (experimental group, *n* 4; positive control group, *n* 1; negative control group, *n* 3) (4·2 %) dropped out before blood collection at endpoint (*n* 184). The simple randomisation used resulted in relatively well-balanced treatment groups at screening and baseline (Table 1; Table 2). The children did not have hookworm and very few had trophozoites of *G. intestinalis* (*n* 2) and *E. histolytica* (*n* 2) at screening.

Analysed nutrient composition of the dishes

The three fish dishes were balanced with respect to the contents of macronutrients (Table 3). The all-*trans*-retinol content in a serving of the mola curry was about one-quarter of that of the rui curry with added retinyl palmitate, but mola

Table 1. Characteristics of the 196 children (with serum retinol concentrations of 0·36–0·75 μmol/l at screening) by treatment group at screening and baseline*

	Treatment group...											
	Experimental (n 66)				Positive control (n 65)				Negative control (n 65)			
	Mean	SD	%	n	Mean	SD	%	n	Mean	SD	%	n
Age (years) at baseline	4·8	1·4			5·0	1·3			4·9	1·4		
Boys at baseline			56·1	37			53·8	35			61·5	40
Anthropometric status at baseline†												
Height (cm)	101·3	10·2			103·0	11·1			101·6	12·1		
Weight (kg)	14·6	2·6			15·1	3·5			14·8	3·2		
Mid-upper arm circumference (mm)	153	11			152	13			152	10		
Weight-for-height Z-score < −2			15·4	10			10·8	7			11·3	7
Weight-for-age Z-score < −2			49·2	32			49·2	32			58·1	36
Height-for-age Z-score < −2			56·9	37			47·7	31			54·8	34
Educational level of mother (years of schooling)‡												
0			75·4	46			81·0	51			60·3	38
0–5			16·4	10			17·5	11			30·2	19
6 +			8·2	5			1·5	1			9·5	6
Helminths at screening§												
Ascaris lumbricoides												
Prevalence			54·5	36			58·5	38			61·5	40
Intensity (eggs/g faeces)‖	874 (446–2227)				522 (207–2368)				1074 (533–2924)			
Trichuris trichiura												
Prevalence			60·6	40			72·3	47			64·6	42
Intensity (eggs/g faeces)‖	256 (105–668)				302 (155–622)				278 (128–438)			
Reported morbidity at screening¶												
Diarrhoea			13·6	9			12·3	8			10·8	7
Nasal discharge			25·8	17			18·5	12			24·6	16

* Values are mean ± SD or % (n) unless stated otherwise. Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry.
† In the experimental and negative control group, anthropometric status was obtained for n 65 and n 62, respectively.
‡ Educational level of mother: experimental group, n 61; positive control group, n 63; negative control group, n 63.
§ Assessed before anthelmintic treatment was done.
‖ Values are median of eggs/g faeces in infected children (interquartile range).
¶ Reported morbidity during the previous 2 weeks from blood collection at screening.

curry had a high content of 3,4-dehydroretinol isomers and the two dishes were balanced with respect to the total retinol activity equivalents (Table 3). In the mola curry, all-*trans*-3,4-dehydroretinol and 13-*cis*-3,4-dehydroretinol contributed approximately 61 and 23 %, respectively, of the total amount of vitamin A compounds (1255 μg) (Table 3). The mola curry was more mineral-dense than rui curry as shown by the higher contents of ash, Fe, Zn and Ca. In the mola curry and rui curry, 59·3 and 34·4 %, respectively, of the total Fe was present as haem Fe (Table 3).

A serving (50 g) of the mola curry or the rui curry contributed the following percentages of the US daily Dietary Reference Intakes for children aged 4–8 years[3]: Fe, 14·0 or 3·2 %, respectively; Zn, 18·0 or 7·0 %, respectively; Ca, 36·0 or 1·4 %, respectively.

The amounts of absorbable Fe in the fish curries were calculated using a haem Fe absorption of 25 % and a non-haem Fe absorption of 16·8 %, whereas for absorbable Zn, 30 % was used[3]. A serving of mola curry (0·30 mg absorbable Fe) or rui curry (0·063 mg absorbable Fe) contributed 44·7 or 9·2 %, respectively, of the daily requirement for absorbed Fe for children aged 3·5–7·5 years[3]. A serving of mola curry (0·27 mg absorbable Zn) or rui curry (0·11 mg absorbable Zn) contributed 22·5 or 8·8 %, respectively, of the daily requirement for absorbed Zn for children aged 4–8 years[3].

Dish intake, compliance rate and attendance rate

There was no statistically significant difference between the three treatment groups in the amount of fish curry consumed during the feeding trial (Table 4). Similarly, the amounts of rice and vegetable curry consumed did not differ significantly by treatment group (Table 5). The proportion of children in all groups who received ≥90 % of the treatment given (Table 4), i.e. the total amount served of mola curry in the experimental group, retinyl palmitate in the positive control group, and rui curry in the negative control group, was 88·6 % (n 163 out of 184) (there was no statistically significant difference in proportions between groups; P = 0·53).

In all groups (n 184), the median total fish curry intake during the feeding trial was 2650 (interquartile range 2555–2700) g/child per 54 d out of the total amount of fish curry served, 2700 g; or 49·1 (interquartile range 47·3–50·0) g/child per d out of the daily serving of 50 g (Table 4). Using these values, the median compliance rate for the fish curries was 98 %. Non-compliance was mostly due to days absent from feeding rather than not consuming the amount of fish curry served. The attendance rates of children who completed the feeding trial in the three groups (defined as the total number of days of attendance, expressed as a percentage of the total number of days of

Table 2. Biochemical indicators of the 196 children (with serum retinol concentrations of 0·36−0·75 μmol/l at screening) by treatment group at screening and baseline*

| | Treatment group… | | | | | | | | | | | |
| | Experimental (*n* 66) | | | | Positive control (*n* 65) | | | | Negative control (*n* 65) | | | |
	Mean	SD	%	*n*	Mean	SD	%	*n*	Mean	SD	%	*n*
Biochemical indicators at screening												
Serum retinol (μmol/l)	0·59	0·11			0·60	0·10			0·60	0·11		
Serum RBP (μmol/l)†	0·74	0·15			0·72	0·13			0·76	0·16		
Serum Zn (μmol/l)	9·2	1·0			9·3	1·1			9·4	0·9		
Serum transthyretin (μmol/l)	2·1	0·8			1·9	0·4			1·9	0·4		
Serum CRP (mg/l)‡§			2·7 (1·5−6·2)				3·1 (1·3−8·2)				3·6 (2·1−5·3)	
2 to <10‖			50·0	33			40·6	26			70·8	46
≥10‖			15·2	10			18·8	12			7·7	5
Serum retinol of 0·36−0·75 μmol/l, corrected for the effect of serum CRP§¶			56·1	37			59·4	38			52·3	34
Serum Zn < 9·9 μmol/l**			80·3	53			76·9	50			72·3	47
Serum Zn < 9·9 μmol/l, corrected for the effect of serum CRP**§††			63·6	42			65·6	42			61·5	40
RDR test (random subsample in each group; *n* 15) at baseline												
RDR test value ≥20 %			60·0	9			60·0	9			53·3	8
Serum retinol (μmol/l)	0·60	0·21			0·64	0·22			0·68	0·21		
Serum CRP (mg/l)‡			1·1 (0·1−4·4)				1·6 (0·7−6·0)				1·4 (0·4−5·5)	
2 to <10‖			26·7	4			33·3	5			20·0	3
≥10‖			6·7	1			6·7	1			13·3	2

RBP, retinol-binding protein; CRP, C-reactive protein; RDR, relative dose−response

* Values are mean ± SD or % (*n*) unless stated otherwise. Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry.

† Serum RBP: experimental group, *n* 61; positive control group, *n* 62; negative control group, *n* 59.

‡ Values are median (interquartile range).

§ In the positive control group, serum CRP was measured in *n* 64.

‖ Serum CRP ≥2 mg/l were considered elevated. Based on data of the children screened (*n* 577), the two CRP categories were statistically significantly associated with a depression in serum retinol concentration, higher in the category ≥10 mg CRP/l serum[6].

¶ The individual serum retinol concentrations were increased by the estimated depression due to the effect of serum CRP before categorisation. For serum CRP levels 1 to <2, 2 to <5, 5 to <10 and ≥10 mg/l, serum retinol values of 0·04, 0·12, 0·16 and 0·32 μmol/l, respectively, were added[6].

** Serum Zn concentrations < 9·9 μmol/l were considered low[66].

†† The individual serum Zn concentrations were increased by the estimated depression due to the effect of serum CRP before categorisation. For serum CRP levels 1 to <2, 2 to <5, 5 to <10 and ≥10 mg/l, serum Zn values of 0·08, 0·33, 0·73 and 0·89 μmol/l, respectively, were added[21].

feeding trial in the group) were: experimental, 97·8 % (*n* 3221 out of *n* 3294); positive control, 96·9 % (*n* 3350 out of *n* 3456); negative control, 97·7 % (*n* 3112 out of *n* 3186). The attendance rate for all groups was 97·4 %, and the major reason for children not attending the feeding was 'not at home or travelling'.

Serum retinol concentration

The experimental and negative control groups did not show a statistically significant within-group increase in serum retinol concentration from screening to endpoint, but the positive control group did (Table 6). In the negative control group, a very weak trend towards an increased serum retinol was seen. In the covariate-adjusted analysis of endpoint serum retinol concentrations, there was no statistically significant difference between the experimental and negative control group (Table 6). In contrast, serum retinol concentration increased significantly by an estimated 0·20 μmol/l more on average in the positive control group than in the negative control group (Table 6). The covariate-adjusted analysis of endpoint serum retinol did not alter the conclusions compared with the unadjusted analysis; however, the precision (95 % CI) of the estimated treatment differences was improved somewhat (Table 6).

Not adjusting for screening serum CRP in the covariate-adjusted analysis did not change the conclusions and the estimated treatment effects in the experimental and positive control groups were virtually unchanged (results not shown). The statistical analysis was by intention-to-treat; however, when children who had received < 90 % of the treatment given were excluded from the analysis, the estimated treatment effects in the experimental and positive control groups, expressed as regression coefficients, were 0·014 (95 % CI −0·06, 0·09) μmol/l; *P*=0·69; *n* 55) and 0·23 (95 % CI 0·16, 0·30) μmol/l; *P*<0·001; *n* 56), respectively, and the conclusions (Table 6) did not change.

Serum retinol-binding protein

In all three groups, serum RBP concentration increased significantly within group from screening to endpoint (Table 6), although the increase within the experimental group was statistically weak, bordering of being non-significant. The increase within the positive control group was marked, much more than in both the experimental and negative control groups. In the covariate-adjusted analysis of endpoint serum RBP concentrations, the experimental group was not statistically significantly different from the negative control group (Table 6). In contrast, serum RBP concentration increased significantly by an estimated 0·18 μmol/l more on average in the positive control group than in the negative control group (Table 6). The covariate-adjusted analysis of endpoint serum RBP did not alter the

Table 3. Analysed nutrient composition of the dishes served during the feeding trial (9 weeks, 6 d/week)

	Per 50 g fish curry, served in one test meal per child per d						Per 100 g dish, served *ad libitum* in one test meal per child per d			
	Treatment group...						All treatment groups			
	Experimental		Positive control		Negative control					
	Mola curry (*n* 9)		Rui curry with added retinyl palmitate (*n* 9)		Rui curry (*n* 9)		Rice (*n* 5)		Non-leafy vegetable curry (*n* 5)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Moisture (g)*	32·4	0·5	33·2	0·2	33·3	0·1	69·6	0·5	83·4	0·3
Ash (g)†	1·9	0·05	0·99	0·09	0·99	0·09	0·16	0·008	1·5	0·02
Energy and macronutrients										
Gross energy (kJ)‡	487·3	12·6	467·4	2·2	461·0	2·2	497·9	2·8	333·9	1·4
Metabolisable energy (kJ)§	454·4	13·1	445·3	4·3	438·8	2·7	520·5	8·7	351·0	7·6
Protein (g)†	5·0	0·1	5·9	0·08	5·9	0·08	2·75	0·04	2·28	0·02
Protein (% of metabolisable energy)§	18·7	0·3	22·6	0·4	23·0	0·3	9·0	0·3	11·0	0·2
Fat (g)	8·9	0·3	8·4	0·09	8·2	0·1	0·28	0·02	4·5	0·1
Fat (% of metabolisable energy)§	74·3	0·1	71·7	0·7	70·7	0·7	2·0	0·1	48·5	0·7
Carbohydrate (g)‖	1·9	0·1	1·5	0·3	1·6	0·2	27·3	0·5	8·4	0·3
Carbohydrate (% of metabolisable energy)§	7·0	0·3	5·7	1·0	6·3	0·9	89·0	0·2	40·5	0·8
Vitamin A¶										
All-*trans*-retinol (μg)	161	46	601	65	ND		–		–	
13-*cis*-retinol (μg)	35	9	ND		ND		–		–	
All-*trans*-3,4-dehydroretinol (μg)	770	129	ND		ND		–		–	
13-*cis*-3,4-dehydroretinol (μg)	287	55	ND		ND		–		–	
β-carotene (μg)**	2·3	0·6	1	0	0·5		0		4·5	
Total vitamin A (RAE)††	612	94	601	65	0		0		0·4	
Minerals†										
Fe										
Non-haem Fe (mg)	0·58	0·17	0·21	0·03	0·21	0·03	–		–	
Haem Fe (mg)‡‡	0·83	0·37	0·11	0·07	0·11	0·07	–		–	
Total Fe (mg)	1·4	0·4	0·32	0·08	0·32	0·08	0·22	0·06	0·68	0·15
Zn (mg)	0·90	0·08	0·35	0·03	0·35	0·03	0·52	0·07	0·4	0·04
Ca (mg)	287·7	10·5	11·0	1·3	11·0	1·3	3·9	0·6	13·3	0·7

RAE, retinol activity equivalents; –, not analysed; ND, not detected.

* Moisture content per 100 g dish was calculated by subtracting the DM content (g) from 100 g dish.

† In the rui curry with added retinyl palmitate, ash, protein, Fe, Zn and Ca were not analysed and the values presented are the same as for the analysed rui curry.

‡ Gross energy (kJ) was calculated using the conversion factor of 4·184 kJ/kcal.

§ Metabolisable energy (kJ) content was calculated by summation of the metabolisable energy in protein, fat and carbohydrate, using the conversion factors 17 kJ/g protein, 38 kJ/g fat and 17 kJ/g carbohydrate.

‖ Carbohydrate content per 100 g dish was calculated by subtracting the contents (g) of moisture, protein, fat and ash from 100 g dish. In the rui curry with added retinyl palmitate, protein and ash were not analysed and the values used were the same as for the analysed rui curry.

¶ Vitamin A compounds, except all-*trans*-retinol, were analysed in eight samples of mola curry, two samples of rui curry with added retinyl palmitate and one pooled sample (*n* 9) of rui curry.

** The values presented for the content of β-carotene in the non-leafy vegetable curry and rice were calculated based on values from food composition tables[67] and a previously analysed value for roasted chilli seeds (4 μg β-carotene/100 g).

†† Total vitamin A (RAE) = all-*trans*-retinol + 13-*cis*-retinol + all-*trans*-3,4-dehydroretinol + 13-*cis*-3,4-dehydroretinol + β-carotene. For conversion of the amounts of the vitamin A compounds in μg to RAE, biological activities in relation to all-*trans*-retinol used were: 75 % for 13-*cis*-retinol[68]; 40 % for all-*trans*-3,4-dehydroretinol and 13-*cis*-3,4-dehydroretinol[25]; 8·3 % for β-carotene[3].

‡‡ Haem Fe content was calculated by subtracting non-haem Fe from total Fe.

conclusions compared with the unadjusted analysis; however, the precision of the estimated treatment differences was improved somewhat (Table 6).

Not adjusting for screening serum CRP in the covariate-adjusted analysis did not affect the results (results not shown). Excluding children who had received < 90 % of the treatment given from the analysis did not change the conclusions (Table 6), and the estimated treatment effects in the experimental and positive control groups, expressed as regression coefficients, were 0·02 (95 % CI −0·08, 0·11) μmol/l; $P=0·73$; *n* 55) and 0·19 (95 % CI 0·10, 0·28) μmol/l; $P<0·001$; *n* 56), respectively.

The relative dose–response test

At baseline, 57·8 % (*n* 26 out of *n* 45) of all children had positive RDR test values (Table 2). At endpoint, the proportions of children with a positive RDR test value were almost twice as high in the experimental and negative control groups as the positive control group, although the difference among groups was not statistically significant (Table 7). Excluding those who had received < 90 % of the treatment given from the analysis did not alter the conclusions (results not shown).

Table 4. Amount of fish curry consumed from the test meals during the feeding trial (9 weeks, 6 d/week) by treatment group*

	Treatment groups...					
	Experimental (n 61)		Positive control (n 64)		Negative control (n 59)	
	%	n	%	n	%	n
Total fish curry intake (g/child per 54 d)†‡§	2650 (2596–2700)		2650 (2538–2700)		2631 (2529–2689)	
Daily fish curry intake (g/child per d)‡§	49·1 (48·1–50·0)		49·1 (47·0–50·0)		48·7 (46·8–49·8)	
Total fish curry intake (g) per child during the 54 d by category and percentage compliance‖¶						
2565–2700 g (≥95 % compliance)	78·7	48	71·9	46	69·5	41
2430–2564 g (90–94 % compliance)	11·5	7	15·5	10	15·2	9
2295–2429 g (85–89 % compliance)	6·6	4	4·7	3	8·5	5
2160–2294 g (80–84 % compliance)	0	0	1·6	1	5·1	3
2025–2159 g (75–79 % compliance)	1.6	1	3·1	2	1·7	1
1890–2024 g (70–74 % compliance)	0	0	1·6	1	0	0
1755–1889 g (65–69 % compliance)	1·6	1	1·6	1	0	0

* Values are % (*n*) unless stated otherwise. Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry. Each child was served one test meal per d consisting of 50 g fish curry and the same rice and non-leafy vegetable curry *ad libitum* during the feeding trial.
† The total amount of the fish curry served in the test meals during the feeding trial per child was 2700 g, computed by multiplying the total number of days of the feeding trial (54 d) and the daily amount of fish curry (50 g) served per child.
‡ The days on which children were absent from feeding were included. There were no between-group differences (*P*=0·19; Kruskal–Wallis test).
§ Values are median (interquartile range).
‖ Percentage compliance is defined as the total amount of fish curry consumed by the child, expressed as a percentage of the total amount served (2700 g) during the feeding trial.
¶ For retinyl palmitate intake in the positive control group, the percentage retinyl palmitate compliance was defined as the total amount of retinyl palmitate consumed by the child, expressed as a percentage of the total amount served during the feeding trial. The percentage compliance categories with proportions of children were: ≥95 % compliance, 81·3% (*n* 52); 90–94 % compliance, 9·4 % (*n* 6); 85–89 % compliance, 3·1 % (*n* 2); 80–84 % compliance, 1·6 % (*n* 1); 75–79 % compliance, 3·1 % (*n* 2); 70–74 % compliance, 0 % (*n* 0); 65–69 % compliance, 1·6 % (*n* 1).

Discussion

Biochemical indicators of vitamin A status measured

In the present randomised, controlled efficacy study, conducted among Bangladeshi slum children with serum retinol concentrations of 0·36–0·75 μmol/l, the changes in the biochemical indicators of vitamin A status (serum concentrations of retinol and RBP, and results of the RDR test) in the group fed mola curry were similar to those in the group fed rui curry (negative control), after 9 weeks. However, in the group fed rui curry with added retinyl palmitate (positive control), the changes in serum concentrations of retinol and RBP were statistically significantly higher than those in the negative control group, and although the results of the RDR test subsamples at endpoint were not statistically significantly different among groups, the proportion of children with inadequate liver vitamin A stores in the positive control group was half of those in the other two groups.

The number of children used for the RDR test may, however, have been too small to show a significant difference, as the proportion of children with inadequate liver vitamin A stores in the negative control group was less than assumed.

In the present study, equal amounts of vitamin A in terms of RE were served daily to the group fed mola curry or rui curry with added retinyl palmitate. The vitamin A content supplied by a daily serving of mola curry, i.e. 500 RE, was estimated using a 40 % biological activity for 3,4-dehydroretinol isomers in relation to all-*trans*-retinol, based on the growth response found in rats[25]. It was assumed that this vitamin A intake would result in an increase in serum retinol of at least 0·15 μmol/l in the group fed mola curry. However, the results indicated that in the mola curry served, the amount of all-*trans*-retinol was insufficient, and/or the large amount of 3,4-dehydroretinol isomers was not converted to retinol or converted in an insufficient amount to exert an improvement in the serum retinol concentration or the

Table 5. Amounts of non-leafy vegetable curry and rice consumed daily from the test meals during the feeding trial (9 weeks, 6 d/week) by treatment group*

	Treatment groups...					
	Experimental (n 61)		Positive control (n 64)		Negative control (n 59)	
	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range
Rice†						
Daily intake (g/child per d)‡	132·1	95·4–168·0	138·6	107·3–194·2	123·3	92·0–154·4
Non-leafy vegetable curry§						
Daily intake (g/child per d)‡	98·4	64·9–117·5	89·2	59·6–120·1	85·4	48·8–107·6

* Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry. Each child was served one test meal per d consisting of 50 g fish curry and the same rice and non-leafy vegetable curry *ad libitum* during the feeding trial.
† There were no between-group differences (*P*=0·07; Kruskal–Wallis test).
‡ The days on which children were absent from feeding were included.
§ There were no between-group differences (*P*=0·50; Kruskal–Wallis test).

Table 6. Serum concentrations of retinol and retinol-binding protein (RBP) (μmol/l) at screening and endpoint (the day after the last day of the feeding trial, 9 weeks, 6 d/week) in 184 children by treatment group*

| | | Screening | | Endpoint | | P value for within-group change† | Differences in endpoint outcome in relation to the negative control group | | | | | |
| | | | | | | | Unadjusted‡ | | | Adjusted§ | | |
	n	Mean	SD	Mean	SD		Mean	95% CI	P	Mean	95% CI	P
Serum retinol (μmol/l)												
Experimental	61	0·59	0·11	0·60	0·18	0·71	−0·06	−0·15, 0·03	0·27	−0·02	−0·09, 0·05	0·52
Positive control	64	0·59	0·10	0·84	0·20	<0·001	0·18	0·09, 0·27	<0·001	0·20	0·13, 0·27	<0·001
Negative control	59	0·60	0·11	0·66	0·25	0·13	0	0	–	0	0	–
Serum RBP (μmol/l)												
Experimental	61	0·74	0·15	0·80	0·25	0·049	−0·06	−0·18, 0·05	0·42	−0·01	−0·10, 0·08	0·81
Positive control	62||	0·72	0·13	1·02	0·24	<0·001	0·16	0·04, 0·27	0·004	0·18	0·09, 0·27	<0·001
Negative control	59	0·76	0·16	0·86	0·31	0·009	0	0	–	0	0	–

* Treatment groups: experimental, mola curry; positive control, nui curry with added retinyl palmitate; negative control, rui curry. Each child was served one meal per d consisting of 50 g fish curry and the same rice and non-leafy vegetable curry *ad libitum* during the feeding trial.
† P value for within-group change from screening to endpoint by paired *t* test (experimental group) or Wilcoxon signed rank test (positive and negative control groups).
‡ Unadjusted differences in mean endpoint value between the experimental or positive control group and the negative control group (ANOVA).
§ Adjusted differences between the experimental or positive control group and the negative control group. Analysis of covariance with endpoint serum retinol concentration as the outcome, treatment group as the main effect and screening serum concentrations of retinol, C-reactive protein (CRP) (after $\log_{10}$(CRP concentration + 1) transformation), transthyretin and RBP, and *Ascaris lumbricoides* infection as the covariates. Analysis of covariance with endpoint serum RBP concentration as the outcome, treatment group as the main effect and screening serum concentrations of RBP, CRP (after $\log_{10}$(CRP concentration + 1) transformation) and transthyretin as the covariates. For the positive control group, *n* 61 (due to insufficient serum at screening for CRP, *n* 1 and RBP, *n* 2).
|| Endpoint serum RBP in the positive control group, *n* 64.

other measured biochemical indicators of vitamin A status. In the present study, in which serum retinol was the primary outcome, using a 40 % conversion of 3,4-dehydroretinol isomers to all-*trans*-retinol may not have been appropriate. Also, it may be speculated that the degree of absorption of these vitamin A compounds in the group fed mola curry was inadequate. The results indicated, however, that the retinyl palmitate was efficacious in improving serum concentrations of retinol and RBP.

Studies that can be related to the present study have been conducted in rats. The results of the present study are consistent with those from recent studies in vitamin A-deficient rats suggesting that 3,4-dehydroretinol is not converted to retinol, based on a lack of a detectable change in serum retinol concentration[42] and no conversion in tissues of visual pigment extract from the eyes, fetuses or testes[43]. However, this is in contradiction with older studies in vitamin A-deficient rats indicating that 3,4-dehydroretinol is converted to retinol in the eyes[44–46].

In the present study, serum 3,4-dehydroretinol concentration was unfortunately not measured. However, 3,4-dehydroretinol is found in the serum in rats after administration of 3,4-didehydroretinyl acetate[42,47] as well as in humans, bound to holo-RBP[48–50]. At the same time, 3,4-dehydroretinol has been found in the liver of rats[19,42,47,51]. The results of the present study indicated that the 3,4-dehyroretinol isomers, assuming they were absorbed, may not have been bound to RBP in serum in a sufficient degree to exert an improvement in serum RBP in the group fed mola curry, suggesting a low affinity of 3,4-dehydroretinol to holo-RBP[49].

Studies in vitamin A-deficient rats have shown that 3,4-dehyroretinol can replace retinol with respect to physiological functions such as growth[25,47,52], reproduction[43,53] and vision[43,51,53,54]. Some evidence in both humans[54] and rats[43,51,53] suggests that the aldehyde, dehydroretinal, can replace retinal in the visual role of vitamin A by combining with opsin to form a new visual pigment, porphyropsin instead of rhodopsin in the retina[43]. In a study among rural women in Bangladesh about the health and nutritional benefits of eating small fish, 45 % of women considered mola as being 'good for/protecting the eyes'[17]; a perception that may have originated from indigenous knowledge that night blindness can be cured by eating mola[12]. Thus, we hypothesise that the 3,4-dehydroretinol in mola may exhibit some important physiological functions in humans.

The usefulness of biochemical indicators to evaluate the efficacy and effectiveness of micronutrient interventions in populations may be limited; therefore, functional outcomes as true indicators of their effects should be assessed in studies at the endpoint[55]. In light of the large amount of 3,4-dehydroretinol isomers in mola and some other commonly consumed freshwater small fish species in Bangladesh[12], it may by worthwhile to investigate the effects these fish have on functional outcomes in humans – i.e. the functional bioefficacy, defined as the proportion of an ingested nutrient which carries a given metabolic function[56] – such as vision, growth, cognitive and psychomotor function, immune function, morbidity from infections and reproduction.

In the present study, collection of blood was done at screening but not again at baseline, with the intention of not compromising the acceptability of the study among the families and reducing the discomfort in the children in connection with repeated blood collections, thus ensuring the compliance. For the same reasons, different subsamples of children were used for the RDR test at baseline and endpoint.

Table 7. Percentage of children from a random subsample at endpoint (the day after the last day of the feeding trial, 9 weeks, 6 d/week) with positive relative dose−response (RDR) test value by treatment group*

	Treatment group…											
	Experimental (*n* 15)				Positive control (*n* 15)				Negative control (*n* 16)			
	Mean	SD	%	*n*	Mean	SD	%	*n*	Mean	SD	%	*n*
RDR test value ≥20 %†‡			46·7	7			26·7	4			50·0	8
Serum retinol (μmol/l)	0·55	0·16			0·87	0·23			0·67	0·23		

* Treatment groups: experimental, mola curry; positive control, rui curry with added retinyl palmitate; negative control, rui curry. Each child was served one meal per d consisting of 50 g fish curry and the same rice and non-leafy vegetable curry *ad libitum* during the feeding trial.
† A RDR test value ≥20 % was considered positive and indicative of inadequate liver vitamin A stores.
‡ There were no between-group differences ($P=0.37$; χ^2 test).

The unintentional trend toward lower serum retinol and RBP concentrations in the experimental group, at endpoint, in relation to the negative control group may be due to random errors. Some factors, which were not controlled for in the statistical analyses, may have influenced the outcomes of the present study, such as natural variability, technical error, intake of vitamin A supplement (perhaps due to awareness of the treatments), vitamin A from foods eaten at home and infections. Food intake and vitamin and mineral supplementation at home were not recorded. On the other hand, infectious episodes such as diarrhoea and reinfection with intestinal helminths (data not shown) before and during the feeding trial might have diminished the vitamin A stores[39,57–59]. Serum retinol and RBP are both transiently depressed during subclinical infection, induced by the acute-phase response[6,38,58,60]. It could not be ruled out that serum CRP, like helminthic infections, at endpoint (data not shown) was affected by the treatments, and therefore, it was not included as a covariate in the statistical analyses of the outcomes.

Contribution of multiple nutrients from mola curry

Mola is an important animal-source food of other nutrients besides vitamin A. Because multiple nutrient deficiencies often coexist in populations[61], as in the present study population, there is a need for multi-nutrient instead of single-nutrient interventions.

In the present study, the daily serving of mola curry contained moderate amounts of haem Fe and Zn, and a large amount of Ca, contributing considerably to the recommended daily intakes of these nutrients, often limited in plant-based diets. In contrast, rui curry contributed to very small amounts of these nutrients. Children can easily consume the amount of fish curry served in the present study and even larger amounts in a single meal (own observations). Studies in both humans and rats have shown that the bioavailability of Ca in mola (whole with bone) is as high as that from milk[13,14]. Moreover, beside providing easily absorbable haem Fe, fish protein has been shown to have a possible enhancing effect on non-haem Fe and Zn absorption from the diet in humans[62,63]. Thus, the small fish, mola has the potential to provide several nutrients in considerable amounts, some of which are not found in the large fish, rui.

Mola as a food-based approach

In Bangladesh, the production of carp species, including rui, has increased tremendously, over the last two decades[10]. Very recently, however, some attention has also been given to increasing the production of nutrient-dense, commonly consumed, indigenous small fish – in particular mola[10,64]. Based on a successfully developed technology of producing mola in small, seasonal ponds together with carps[65], the Ministry of Fisheries and Livestock in Bangladesh has issued a directive to project directors in the fisheries extension services to implement carp and mola pond polyculture throughout rural Bangladesh. At the same time, this production technology is being implemented by non-governmental organisations working with poor, rural households in Bangladesh, and recently also in West Bengal, India. Furthermore, studies have shown that in poor, rural households practising this technology, most of the carps is sold, while most of the mola is consumed[10,11].

Thus, steps have been taken towards increasing the mola production aimed at enhancing the availability and accessibility of mola for the rural poor in Bangladesh; however, further actions may be needed to reach the urban poor. To promote an increased consumption of mola by populations at risk in Bangladesh, including children, however, nutrition and health education and social marketing are also required[7].

This food-based approach, using nutrient-dense, indigenous small fish, can also be applicable in other developing countries with inland water resources and habitual small fish consumption, for example, regions in the Mekong delta, Asia and Lake Victoria, Africa.

Conclusions

The results of the present randomised, controlled efficacy study showed no changes in the measured biochemical indicators of vitamin A status (serum concentrations of retinol and RBP, and results of the RDR test) after 9 weeks' intake of the freshwater small fish, mola (eaten whole with bone, head and eyes, and excluding the viscera) in a daily meal by Bangladeshi slum children with serum retinol concentrations of 0·36–0·75 μmol/l. The present results do not suggest conversion of the large amount of 3,4-dehydroretinol isomers found in mola curry to retinol. Further research is needed to assess the functional bioefficacy of mola in humans, such as the ability to reverse impaired dark-adaptation, improve growth and cognitive and psychomotor development, and reduce morbidity from infections such as diarrhoea. In addition, commonly consumed, small fish species should be screened for all-*trans*-retinol, and if identified with high contents, investigated further. Focusing on several nutrients instead of one is important for combating and preventing multiple nutrient deficiencies. Mola consumed whole with bone is a nutrient-dense animal-source

food, rich in haem Fe, Zn and bioavailable Ca. Thus, the consumption of mola in Bangladesh should continue to be encouraged. The use of culturally acceptable, nutrient-dense small fish, such as mola, in food-based approaches should be considered in developing countries with inland water resources and habitual small fish consumption.

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British Journal of Nutrition

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